2.4 Non Clinical Overview

Melatonin 1 mg/ml Oral Solution

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This document has been prepared according to NTA, Vol. 2B-CTD and ICH M4S Safety guideline.

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2.4.1 OVERVIEW OF THE NON CLINICAL TESTING STRATEGY

The current Non Clinical Overview refers to a medicinal product containing **Melatonin** as active substance **in the form of Oral Solution and at the strength of 1 mg/ml.** The product is designated for oral use and the intended claimed indications include: **(i) the short-term treatment of jet lag in adults, (ii) sleep disorders in children and adolescents aged 6-17 years with attention-deficit/hyperactivity disorder (ADHD), where sleep hygiene measures have been insufficient and (iii) the management of shift-work disorder in adults.** The formulation of oral solution offers an alternative option for paediatric and adult patients who are not capable of shallowing tablets or capsules, thus, achieving an easier method of administration and improved treatment compliance. The relevant Marketing Authorisation Application (MAA) is submitted under Article 10.a of Directive 2001/83/EC, as amended, namely Well-Established Use (WEU) Application. According to Article 10.a of Directive 2001/83/EC as amended, '*the Applicant shall not be required to provide the results of pre-clinical tests or clinical trials if he can demonstrate that the active substance(s) of the medicinal product have been in well-established medicinal use within the Community for at least ten years, with recognised efficacy and an acceptable level of safety*'. Therefore, the test and trial results are replaced by appropriate scientific literature.

According to Annex I of Directive 2001/83/EC as amended, where it is stated that for the purpose of demonstrating that the constituent(s) of a medicinal product has a well-established use, the following criteria should be taken into account:

a) the time over which a substance has been used with regular application in patients,

b) quantitative aspects of the use of the substance, taking into account the extent to which the substance has been used in practice, the extent of use on a geographical basis and the extent to which the use of the substance has been monitored by pharmacovigilance or other methods,

c) the degree of scientific interest in the use of the substance (reflected in the published scientific literature) and the coherence of scientific assessments.

Therefore, a careful assessment of all these aspects has been performed by the Applicant, along with all the non-clinical data found in the published literature related to the pharmacology, pharmacokinetics (PKs) and toxicology of melatonin in general but also more specifically, for the oral route of administration.

Melatonin is a member of the class of acetamides and a member of tryptamines; it is an acetamide in which one of the hydrogens attached to the nitrogen atom is replaced by a 2-(5-methoxy-1*H*-indol-3-yl)ethyl group. Melatonin is a ubiquitous molecule present in almost every live being from bacteria to humans. In vertebrates, besides being produced in peripheral tissues and acting as an autocrine and paracrine signal, melatonin is centrally synthetised by a neuroendocrine organ, the pineal gland. Independently of the considered species, pineal hormone melatonin is always produced during the night and its production and secretory episode duration are directly dependent on the length of the night. As its production is tightly linked to the light/dark cycle, melatonin main hormonal systemic integrative action is to coordinate behavioural and physiological adaptations to the environmental geophysical day and season. The circadian signal is dependent on its daily production regularity, on the contrast between day and night concentrations, and on specially developed ways of action. During its daily secretory episode, melatonin coordinates the night adaptive physiology through immediate effects and primes the day adaptive responses through prospective effects that will only appear at daytime, when melatonin is absent. Similarly, the annual history of the daily

melatonin secretory episode duration primes the central nervous (CNS)/endocrine system to the seasons to come. Remarkably, maternal melatonin programs the fetuses' behaviour and physiology to cope with the environmental light/dark cycle and season after birth. These unique ways of action turn melatonin into a biological time-domain–acting molecule. Melatonin is also dependent on beta-adrenergic receptor function [redacted] Melatonin is involved in numerous biological functions including synchronising circadian rhythms, including sleep-wake timing and blood pressure (BP) regulation, the stress response, aging, immunity, and in control of seasonal rhythmicity including reproduction, fattening, moulting and hibernation. Many of its effects are through activation of the melatonin receptors (MTs), while others are due to its role as an antioxidant [redacted]

Melatonin or *N*-[2-(5-methoxy-1H-indol-3-yl)ethyl]acetamide (IUPAC) has the molecular formula $C_{13}H_{16}N_2O_2$ and a molecular weight of 232.28 g/mol. In its pure form, it appears as an off-white odouress crystalline powder that does not appear to exhibit polymorphism. It is very slightly soluble in water (0.01%) and diluted hydrochloric acid (0.08%) [redacted] Melatonin has obtained the ATC Code N05CH01 for being a nervous system, psycholeptic, hypnotic and sedative, melatonin-receptor agonist for oral use *(WHOCC ATC/DDD Melatonin)*.

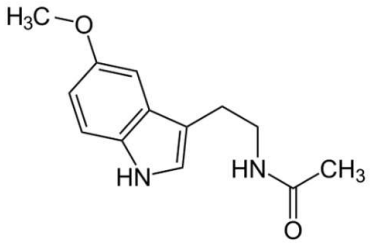

Figure 1. Chemical structure of Melatonin.

Melatonin was first identified by Dr. Aaron Lerner and his team at Yale University in the late 1950s, as the pineal substance responsible for bleaching in frog skin. It was found to be the 5-methoxy-*N*-acetylated derivative of serotonin or 5-hydroxytryptamin. For many years, it was considered to be only a hormone of the pineal gland. As soon as highly sensitive antibodies to indolealkylamines became available, melatonin was identified not only in pineal gland, but also in extrapineal tissues. These included the retina, Harderian gland, gut mucosa, cerebellum, airway epithelium, liver, kidney, adrenals, thymus, thyroid, pancreas, ovary, carotid body, placenta and endometrium. It has also been localised in non-neuroendocrine cells such as mast cells, natural killer (NK) cells, eosinophilic leukocytes, platelets and endothelial cells. This list of cells indicates that melatonin has a unique position among the hormones of the diffuse neuroendocrine system. It is found in practically all organ systems. Functionally, melatonin-producing cells are part and parcel of the diffuse neuroendocrine system as a universal system of response, control and organism protection [redacted]

Melatonin is a well-studied human hormone, the pharmacological and toxicological profiles of which being well-known. The role of melatonin as a therapeutic agent is well-established within the European the Union (EU)/United Kingdom (UK), being tested in numerous large clinical trials through the past decades. Melatonin as immediate release (IR) formulations has been used both as medicinal product as well as food supplement since many decades in the management of various sleep disorders in adults and children, namely in adults for short-term treatment of jet lag, as well as other indicatons relative to its circadian effects, such as insomnia

related to neurological disorders and shift work, as it resets disturbed circadian rhythms and promotes sleep [redacted] It is an approved medicine in the EU/UK for more than 10 years. A prolonged-release formulation containing 2 mg melatonin, Circadin®, has been registered in Europe since 2007 for the short-term treatment of primary insomnia characterised by poor quality of sleep, in patients aged ≥55 years [redacted] IR products in solid formulations have been also authorised for sleep disorders in EU Member States and marketed since many years, with the first medicinal products being authorised back in early 00's , namely, Bio-Melatonin 3 mg Tablets [redacted] and Melatonin-LEK-AM 1 mg, 3 mg and 5 mg Tablets [redacted] As far as the liquid dosage forms of oral solution is concerned, it has to be mentioned that similar oral formulations are already registed in the UK, namely Melatonin Colonis 1 mg/ml oral solution [redacted] and Melatonin Consilient Health 1 mg/kg oral solution [redacted] Other melatonin oral solution products, e.g., Melatonin Unimedic Pharma 1 mg/ml oral solution [redacted] Melatonin Orifarm 1 mg/ml oral solution [redacted] are also authorised within EU. Most of the IR products have been approved based on bibliographic applications.

In addition, melatonin has been marketed as a food supplement in many countries and has been subject to regulatory opinion as such. Indeed, following a request from the European Commission, the Panel on Dietetic Products, Nutrition and Allergies (NDA) of the European Food Safety Authority (EFSA) was asked to provide a scientific substantiation of a health claim in relation to melatonin and alleviation of subjective feelings of jet lag [redacted] Due to the endogenous role of melatonin within the body, it has been extensively researched both *in vitro* and *in vivo* across a multitude of species including humans. Furthermore, the extended use of melatonin for over 10 years to numerous patients and heterogeneous populations along with the degree of the scientific interest provide evidence of their well-known pharmacological and toxicological profile. Additionally, the indications and recommended posology of the proposed product are similar to those investigated in the scientific literature and recommended for the proposed indications for EU/UK marketed IR products.

Overall, melatonin meets the criteria for classification as a well-established drug substance for which the Applicant is entitled to replace the results of non clinical studies and clinical trials by appropriate scientific literature and appropriate bridging documentation, since:

- Medicinal products containing melatonin have been used extensively in the EU/UK and worldwide, for several decades, with demonstrated efficacy and safety. Approved medicinal products for the treatment of jet lag disorder, insomnia in paediatric populations with ADHD as well as management of shift work disorder are already present in European markets since the early 00's and numerous food supplements are also marketed. Safety and efficacy of melatonin in the proposed indications have been also demonstrated in various clinical trials and can be considered as being well-established within the Community as well as worldwide. These are confirmed by extended data from the scientific literature and information of approved products' Summary of Product Characteristics (SmPCs).

- The use of melatonin formulations to numerous patients and heterogeneous populations, the degree of scientific interest, the well-known pharmacological and toxicological profile of the active substance as well as the pharmaceutical form intended for authorisation prove the WEU.

- The indications and recommended posology of the proposed product formulation for oral use are similar to those investigated in the scientific literature claimed by already authorised products and recommended in clinical practice worldwide. Further clinical trials have involved the use of melatonin also for the treatment of other sleep disorders in adults and paediatric populations worldwide. The clinical aspects will be explained in detail within the Clinical Overview. The findings of the animal studies are also in agreement with the human results.

- In addition to the literature data, appropriate 'bridging data' are also included in the submission application in order to show the relevance of the literature used to demonstrate the safety and efficacy with regards to the actual product concerned; these will be discussed in detail within the Clinical Overview. Additionally, the selected excipients are well-known for their use in the manufacture of pharmaceutical products and are not expected to modify the biopharmaceutics*, in vivo* performance, safety or efficacy profile of the active substance.

Considering the type of the present application as a WEU application according to Article 10.a of Directive 2001/83/EC as amended, concerning Melatonin 1 mg/ml Oral Solution, the Applicant is not required to provide new (own) non clinical studies. Therefore, this Non Clinical Overview reviews the current state of scientific knowledge available on the non-clinical properties of the active substance and it mainly refers to sound scientific data presented in published literature. Extensive preclinical tests concerning pharmacology and toxicology have been described in the literature. The Applicant selected the most relevant studies/reviews which are used in the Non clinical Overview to support this Application.

Further to the above, the qualitative formulation of the product contains, apart from the active substance, i.e., melatonin, also sorbitol 70%, propylene glycol, xanthan gum, citric acid anhydrous, paraben methyl sodium, orange IFF 3912, sodium citrate dihydrate, saccharin sodium and purified water. The excipients included in the proposed formulation are commonly used ingredients for the formulation of oral dosage forms, including oral solutions, and are compliant with Pharmacopoeial requirements where applicable. They exhibit a good safety profile and have been used extensively in other commercially available formulations, including other melatonin-containing oral solutions. All the amounts of excipients used are well below the reported toxic doses.

For the impurities present in batches of the drug substance as well as those of the final product, please refer to the relevant parts of Module 3. Impurities in the active substance and in the finished product do not pose any special concern from a toxicological point of view and are in compliance with the European requirements. It can be recognised that the product under approval, as applied, has a comparable pharmaceutical quality as the similar products already authorised in Europe.

As a result, the extensive knowledge of pharmacology, PKs and toxicology of the substance as well as its excellent efficacy and safety profile can be confirmed and are in full agreement with the well-known biochemical properties of the active substance (melatonin) and the authorised products throughout the world containing melatonin as active substance. For a detailed scientific bibliography to address the Non Clinical characteristics, please refer to Module 4.

2.4.1.1 SEARCH STRATEGY

In order to compile the Non Clinical Overview, a literature review was performed aiming to properly describe the relevant aspects regarding the pharmacology, PKs and toxicology of the drug. A detailed search was conducted within the biomedical databases; mainly in PubMed (http://www.ncbi.nlm.nih.gov/pubmed) standard database, in IPCS INCHEM (www.inchem.org) database and also in the new locations of the former ToxNet (http://toxnet.nlm.nih.gov/) database. PubMed comprises more than 35 million citations for biomedical literature from MEDLINE, life science journals, and online books. Citations may include links to full-text content from PubMed Central and publisher web sites. The former ToxNet tracked numerous databases within the US National Library of Medicine (NLM), related to toxicology, hazardous chemicals, environmental health and safety information (such as Pubchem, ChemIDplus, LactMed, LiverTox). IPCS INCHEM is a valuable tool for those concerned with chemical safety and the sound management of chemicals. Other literature of pharmacological and toxicological interest has been searched by using the current web engines, including sources such as EMA (European Medicines Agency), US-FDA (Food and Drug Administration), Classification by the monograph of IARC (monograph on the evaluation of carcinogenic risk to human), the US NTP (National Toxicology Programme), US OSHA (Occupational Safety and Health Administration), EFSA (European Food Safety Authority) DART (Developmental and Reproductive Database), Safety and Toxicity of Excipients for Paediatrics (STEP) database, World Health Organisation (WHO), International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), and Joint FAO/WHO Expert Committee on Food Additives (JECFA). The literature search included biomedical journals, medical literature and textbooks of recognised scientific relevance. Pre- and postmarketing studies were taken into consideration and special emphasis was put to include robust published literature. At the first level, the search terms included the keyword ‘*’melatonin’’*. In a second round, more specific keywords, such as ‘*’pharmacology’’, ‘’pharmacodynamics’’, ‘’pharmacokinetics’’, ‘’toxicology’’* and others, were searched in relation to the above-mentioned drug. The search was restricted to the English language, even though papers written in another language but with an abstract available in English were also considered. Both favourable and unfavourable documentation is being presented.

The search followed these steps:

- Performance of the literature search based on the above search terms and identification of the relevant studies from data sources, like PubMed and others.
- A primary screen through titles, and then abstracts for the search criteria above.
- Article selection based on their relevance to the topic.
- Interpretation of the findings.
- Based on findings, performance of additional and specific literature search aiming to complete the overall understanding, when necessary.

The exploration reported here, which chronologically covers from the early 1960s to present day, reveals that a number of pre-clinical studies exists defining the pharmacology, PKs, and toxicity profile of melatonin, involving both *in vitro* tests and several studies in various animal species. Although many of the studies reported in publications mentioned in this report do not contain statements of compliance with Good Laboratory Practice (GLP) regulations, in view of the amount of cumulated knowledge (as per February 2023, the word ‘melatonin’ retrieved in database PubMed over 30,900 results), the overall reported findings were considered valid to support the Non Clinical aspects of melatonin.

2.4.2 PHARMACOLOGY

2.4.2.1 PRIMARY PHARMACODYNAMICS

Melatonin is involved within the whole circadian system and influences the induction of sleep. The pineal gland located behind the third ventricle in the brain with daily and seasonal rhythms mainly under the control of the circadian oscillator located in the suprachiasmatic nucleus (SCN) where MTs are present [redacted] Melatonin is synthesised in several other structures (retina, Harderian gland, gut) as well where the genetic expression and biochemical activity of the melatonin-synthesising enzymes have been detected. It has been proposed that melatonin plays an auto/paracrine role in these structures. Extensive studies have been performed to understand the mechanisms of action of melatonin in the regulation of some seasonal and circadian functions, demonstrating that the dynamic pattern of melatonin secretion is fundamental for its time-giving function. The rhythmic pattern of melatonin secretion is important because it provides information to the host about the concept and sense of time which in turn allows them to adapt some of their physiological functions to the daily and seasonal variations of their environment [redacted]. As for its amphiphilicity, melatonin is able to cross the cell, organelles, and nuclear membranes, and directly interact with intracellular molecules in the so-called non–receptor-mediated actions. In addition to that, melatonin also presents receptor-mediated actions that result from the interaction of this hormone with both membrane and nuclear receptors [redacted]

As mentioned, melatonin is implicated in numerous physiological processes, including circadian rhythms, stress and reproduction, many of which are mediated by the hypothalamus and pituitary. The physiological actions of melatonin are mainly mediated by MTs. MT1 is mainly distributed in the hypothalamus and pituitary. MT1 immunoreactivity showed a widespread pattern in the hypothalamus. In addition to the area of the SCN, a number of novel sites, including the paraventricular nucleus (PVN), periventricular nucleus, supraoptic nucleus (SON), sexually dimorphic nucleus, the diagonal band of Broca, the nucleus basalis of Meynert, infundibular nucleus, ventromedial and dorsomedial nucleus, tuberomamillary nucleus, mamillary body and paraventricular thalamic nucleus have been observed to have neuronal MT1 receptor expression. The MT1 was colocalised with some vasopressin (AVP) neurons in the SCN, with some parvocellular and magnocellular AVP and oxytocine neurons in the PVN and SON and some parvocellular corticotropin-releasing hormone (CRH) neurons in the PVN. In the pituitary, strong MT1 expression has been observed in the pars tuberalis, while a weak staining was found in the posterior and anterior pituitary. These findings provide a neurobiological basis for the participation of melatonin in the regulation of various hypothalamic and pituitary functions. The colocalisation of MT1 and CRH suggests that melatonin might directly modulate the hypothalamus-pituitary-adrenal (HPA) axis in the PVN, which may have implications for stress conditions, like depression [redacted]

2.4.2.1.1 Mechanism of action

Melatonin is a derivative of tryptophan a natural indoleamine hormone produced (from serotonin) by the pineal gland of humans and other mammals; chemically, it is not related to the steroid or peptide hormones. Tryptophan is first 5-hydroxylated (by tryptophan hydroxylase) and then decarboxylated (by aromatic L-amino acid decarboxylase) to form 5-hydroxytryptamine or serotonin. During daylight hours, the serotonin in pinealocytes tends to be stored, and is unavailable to enzymes (monoamine oxidase [MAO] and the melatonin-forming enzymes) that would otherwise act on it. With the onset of darkness, post-ganglionic sympathetic outflow to the pineal increases, and the consequent release of norepinephrine onto

pinealocytes causes stored serotonin to become accessible for intracellular metabolism [redacted]

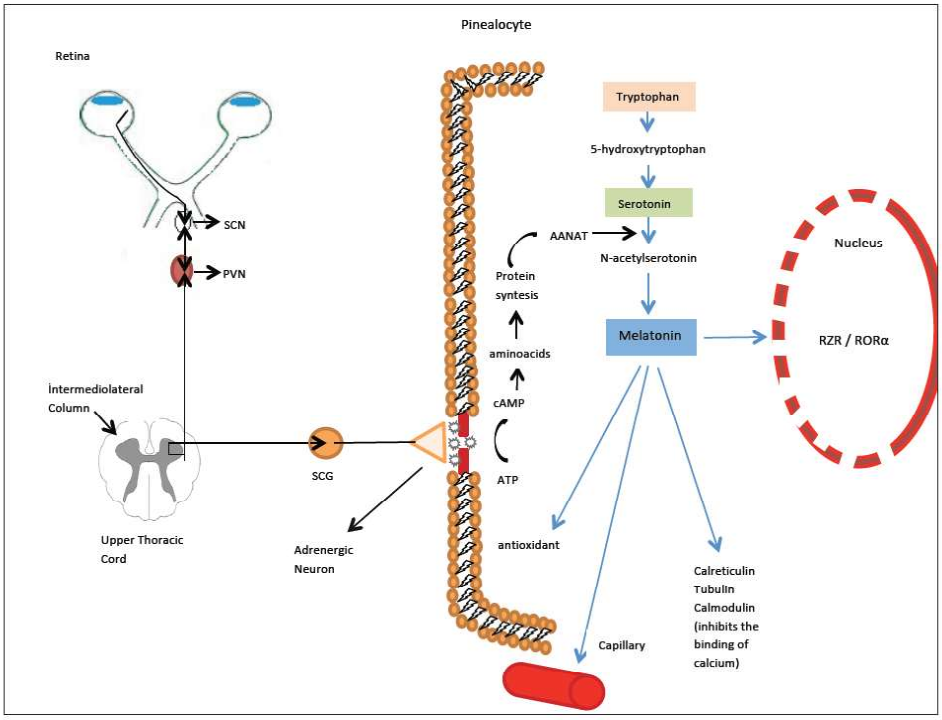

Figure 1. Mechanism of action of melatonin: the neurologic pathway from the eyes through the pineal gland (Figure adopted from [redacted] **Abbreviations:** PVN, paraventricular nucleus of the hypothalamus; RZR/RORα, retinoid-related orphan nuclear hormone receptor; SCG, superior cervical ganglion; SCN, suprachiasmatic nucleus.

At the same time, norepinephrine activates the enzymes that convert serotonin to melatonin, especially serotonin-*N*-acetyltransferase and hydroxyindole-*O*-methyltransferase. Consequently, pineal melatonin levels rise manifold. Melatonin then diffuses out of the pineal gland into the blood stream and cerebrospinal fluid (CSF), rapidly raising human plasma melatonin levels from about 2-10 to 100-200 pg/ml. Melatonin binds to MT type 1A, which then acts on adenylate cylcase and the inhibition of a cyclic adenosine monophosphate (cAMP) signal transduction pathway. Melatonin not only inhibits adenylate cyclase, but it also activates phospholipase C; this potentiates the release of arachidonate. By binding to MTs 1 and 2, the downstream signallling cascades have various effects in the body. The MTs are G protein-coupled receptors (GPCRs) and are expressed in various tissues of the body. There are two subtypes of the receptor in humans, the MT1 and MT2. Melatonin and MT agonists, on market or in clinical trials, all bind to and activate both receptor types. The binding of the agonists to the receptors has been investigated for over two decades. It is somewhat known, but still not fully understood. When MT agonists bind to and activate their receptors, this causes numerous physiological processes. MT1 receptors are expressed in many regions of the CNS, i.e., SCN, hippocampus, substantia nigra, cerebellum, central dopaminergic pathways, ventral tegmental area and nucleus accumbens. MT1 is also expressed in the retina, ovary, testis, mammary gland, coronary circulation and aorta, gallbladder, liver, kidney, skin and the immune system. MT2 receptors are expressed mainly in the CNS, also in the lung, cardiac, coronary and aortic tissue, myometrium and granulosa cells, immune cells, duodenum and adipocytes. The binding of melatonin to MTs activates a few signaling pathways. MT1 receptor activation inhibits the

adenylyl cyclase, and its inhibition causes a rippling effect of non activation; starting with decreasing formation of cAMP, and then progressing to less protein kinase A activity, which in turn hinders the phosphorilation of cAMP responsive element-binding protein (CREB binding protein) into P-CREB. MT1 receptors also activate phospholipase C, affect ion channels and regulate ion flux inside the cell. The binding of melatonin to MT2 receptor inhibits adenylyl cyclase which decreases the formation of cAMP. As well it hinders guanylyl cyclase and therefore, the forming of cyclic guanosine monophosphate (cGMP). Binding to MT2 receptors probably affects phospholipase C which increases PKC activity. Activation of the receptor can lead to ion flux inside the cell [redacted]

2.4.2.1.2 Animal Pharmacology

Extensive studies have been performed to understand the mechanisms of action of melatonin in the regulation of some seasonal and circadian functions and have demonstrated that the dynamic pattern of melatonin secretion is fundamental for its time-giving function. In mammals, the rhythmic secretion of melatonin from the pineal gland is driven by the circadian clock in the SCN of the hypothalamus. The robust nightly peak of melatonin secretion is an output signal of the circadian clock and is supposed to deliver the circadian message to the whole of the organism. Since the circadian system regulates many behavioral and physiological processes, its disruption by external (shift work or jet lag) or internal desynchronisation (blindness, aging) causes many different health problems. Externally administered melatonin is used in humans as a chronobiotic drug to treat desynchronisation and circadian disorders, and the success of these treatments does, at first glance, underline the supposed pivotal role of melatonin in the synchronisation of the circadian system. On the other hand, pinealectomy in experimental animals and humans does not abolish their rhythms of rest and activity. Furthermore, mice with deficient melatoninergic systems neither display overt defects in their rhythmic behaviour nor do they show obvious signs of disease susceptibility, let alone premature mortality. During the last years, several mouse stains with intact or compromised internal melatonin signaling systems as well as other animal models have been investigated, for better understanding of the physiological role of the melatoninergic system. These investigations confirm the synchronising effect of endogenous melatonin and the melatoninergic system. However, melatonin does not appear as the master of internal synchronisation, but as one component in a cocktail of synchronising agents [redacted]

In vitro studies

Melatonin acts by 4 mechanisms in mammals, i.e., (i) binding to MTs in plasma membrane; (ii) binding to intracellular proteins such as calmoduline; (iii) binding to orphan nuclear receptors; (iv) antioxidant effect [redacted]

Melatonin interacts with intracellular proteins named calmoduline, calreticulin and tubulin. Calmoduline is an intracellular secondary messenger. Melatonin directly antagonises binding of calcium to calmoduline [redacted] (Figure 2). The antiproliferative effect in cancer may be related to this. Retinoid-related Orphan nuclear hormone receptor family (RZR/ROR) is responsible for the immunomodulatory effects of melatonin. Interleukin (IL)-2 and IL-6 are produced in mononuclear cells by this mechanism [redacted] There are 3 different membrane receptors and 1 nuclear receptor. In humans, MTs are also detected in several organs, including

brain and retina, cardiovascular system, liver and gallbladder, intestine, kidney, immune cells, adipocytes, prostate and breast epithelial cells, ovary/granulosa cells, myometrium and skin [redacted]

- *Melatonin receptor type 1a: MT1 (or else Mel1a, ML1a, MT1, MTNR1A)*

It is encoded in human chromosome #4 and consists of 351 amino acids [redacted] MT1 constitutes adenylate cyclase inhibition by binding to various G-proteins [redacted] MT1 receptors are commonly found in human skin. During aging process and Alzheimer's disease, the expression of MT1 in SCN and cortex decreases [redacted] MT1 receptors reduce the neuronal discharge rate in SCN and suppress prolactin secretion [redacted]

- *Melatonin receptor type 1b: MT2 (or Mel 1b, ML1b, MT2, MTNR1B)*

It is encoded in human chromosome 11 and consists of 363 amino acids [redacted] MT2 creates adenylate cyclase inhibition by binding to various G-proteins. Additionally, it inhibits the soluble guanylyl cyclase pathway [redacted] Through MT activation, adenylate cyclase inhibition occurs, and the production of cAMP is reduced [redacted]

In the skin, MT2s are located within normal and malign melanocytes and eccrine sweat glands [redacted] They inhibit gamma-aminobutyric acid (GABA) A receptor-related functions in the hippocampus in rats [redacted] In Alzheimer's disease, MT2 expression is reduced. MT2 receptors contribute to the pathophysiology and pharmacology of sleep disorders, anxiety, depression, Alzheimer's disease and pain, and are also involved in antidepressant activity. MT2 receptors are responsible for anxiolytic effects of melatonin. Pharmacological studies have revealed that MT2 regulates sleep, particularly non-rapid eye movement sleep (NREMS). MT2 ligands have more powerful hypnotic properties when compared to non-selective MT1/MT2 ligands [redacted]

The MT3 subtype (or else Mel1c, MTNR1C) is not present in humans, but it is found in fish, amphibians and birds [redacted] In chicken, the rhythm of MT3 is the opposite of MT1 and MT2. Its level is highest at daytime and lowest at night-time [redacted]. MT3 or else the enzyme quinone reductase 2 (QR2) belongs to the reductase group, involved in prevention from oxidative stress by inhibiting the electron transfer reactions of quinones [redacted] There is additional evidence for its involvement in regulation of intraocular pressure. MT3 is located in the liver, kidney, heart, lung, intestine, muscle and brown fat tissue [redacted]

- *Retinoid-related orphan nuclear hormone receptor family (RZR/RORα):*

Via this receptor, melatonin binds to the transcription factors in nucleus which belong to retinoic acid receptor super-family [redacted]

- *Melatonin-related orphan receptor; 'X linked Orphan G-protein coupled' (GPR50: H9, ML1X)*

It is an X-linked inherited receptor, binding to G-protein. It is the orthologue of MT3, which is found in non-mammalian living creatures [redacted] This recepror's gene is located on the X chromosome (Xq28) and consists of 618 amino acids [redacted] It is present in all mammalians including humans. It does not have the characteristics of binding to melatonin [redacted] however, it is effective in binding of melatonin to MT1

[redacted] GPR50 is not present in birds and fish [redacted] It is located in the brain and periphery. Its natural ligand has not been defined yet. It was reported that a deletion mutant in GPR50 might have been associated with bipolar disorder and major depression [redacted] GPR50 has no affinity to melatonin; however, when it dimerises with MT1, it inhibits the melatonin signal [redacted] It also posseses other functions apart from melatonin; it interacts with neurite outgrow inhibitor and the glucocorticoid receptor signal co-activator and histone acetyltransferase [redacted]

In vivo studies

In mammals, melatonin is mainly synthesised in the pineal gland from serotonin, but it is also formed in the gut and the retina. The production is circadian and is stimulated by photic stimulus arising after the onset of darknes. Peak melatonin levels are reached in the middle of the night (between 2-4 a.m.) and decrease to low levels in the second half of the night. The role of melatonin as a chronobiotic and resynchronisator has been demonstrated in different nocturnal animals. Animal models representative of sleep disorders seen in humans, such as jet lag, shift work or advanced sleep phase syndrome, non-24h sleep-wake disorder and irregular sleep-wake disorders, have been developed in rodents. In most of these models, melatonin, like other melatoninergic agonists, has shown a resynchronising activity at doses below 1 mg/kg, given subcutaneously (SC) [redacted]

Jet lag desynchronises the internal sleep-wakefulness cycle with the environmental light/dark cycle. Advance (but not delay) of light onset is known to abolish pineal *N*-acetyltransferase activity and urine excretion of 6-sulphatoxymelatonin. Measurements of pineal serotonin, the substrate of melatonin biosynthesis; *N*-acetylserotonin, the immediate melatonin precursor; and melatonin in the animal (rat) model of jet lag revealed that prolonged delay of dark-phase onset disrupted the rhytms in comparable ways as the advance of light-phase onset. Advance of dark phase onset resulted in less severe disturbances of rhythms as compared with the advance of light phase onset. Melatonin, but not *N*-acetylserotonin, injections at the beginning of a new dark period accelerated recovery of *N*-acetylserotonin and melatonin, but not serotonin, rhythms. Spontaneously hypertensive rats were more sensitive to advance of light onset and less responsive to melatonin injections than normotensive rats. *N*-acetylserotonin and methylene blue attenuated light-induced disruption of *N*-acetylserotonin but not melatonin rhythms [redacted]*)*.

Sedative hypnotic activity of melatonin has also been demonstrated, by the potentiation of barbiturate-induced sleep, in rodents with IP-administered doses ranging from 0.05-40 mg/kg. Average median effective dose (ED_{50}) values for the duration of the loss of righting reflex also ranged from 6-110 mg/kg IP, oral or intravelously (IV) in mice and rats. These values were within a similar magnitude of the doses employed in the rat toxicity studies (0.3-200 mg/kg orally), suggesting that pharmacologically active doses were employed in these studies. Additional limited literature data also suggested a direct sedative effect of melatonin in 4-day-old chicks (2.5 mg; jugular vein or IP) and cats (25-30 µg injection, not specified) [redacted].

In rats with free-running circadian rhythms, oral and SC administration of melatonin resulted in a stable diurnal rhythm. A limitation of studies in nocturnal laboratory animals, however, is that melatonin is often administered during the light phase, when it is not endogenously produced but the animals are most likely asleep. Nevertheless, rats display intermittent periods of sleep and wakefulness in both light and dark phases rather than a single consolidated sleep

period such as observed in humans. This situation clearly does not correlate to human conditions; therefore, the conclusions drawn from laboratory studies in rats may be of limited value when extrapolated to other species. In addition, the doses typically employed in rats, namely 2-20 mg/kg, produce pharmacological circulating levels, several orders of magnitude greater than what is observed naturally. Hence, like many of the human studies, these may not reflect the endogenous physiological role of the hormone [redacted]

Sleep-promoting amounts of astaxanthin (ranging from about 0.1 to 60 mg/kg) and melatonin (0.1-40 mg/kg) were administered concurrently to promote sleep in cats and dogs, improving their quality of life. The pharmaceutical composition further comprised of an amount of zinc ranging from about 10 to 100 mg/kg. Studies on 48 dogs, recording total sleep and wake minutes during the dark and light phases, demonstrated that the combined administration of melatonin, astaxanthin and zinc provided beneficial effects in sleep cycle [redacted]

Another study conducted in diurnal macaques has explored the nature of sleep-promoting effects of melatonin with favorable results, as shown in Table 1 [redacted] In addition to the phylogenetic proximity, there are several important similarities between humans and diurnal non-human primates, favouring the use of these animals to model normal and pathological sleep-related processes. These include (i) similar temporal patterns of activation of the major circadian pacemaker, the SCN, relative to the rest-activity cycle in both species, i.e., high activity of the SCN neurons during the day correlates with these species' daytime activity, in contrast to nocturnal animals whose SCN is active during their daytime rest period; similar temporal patterns of melatonin production, occurring during habitual night-time sleep period; a consolidated nocturnal sleep episode, with similar sleep architecture, in contrast to the majority of nocturnal or diurnal species which tend to have a polyphasic sleep pattern.

A literature study in monkeys has shown promotion of sleep onset at endogenous melatonin levels prior to dark onset. In pigtail macaques given daily oral melatonin for 1 week, 2 h before lights-off, a minimum effective dose of melatonin promoting sleep onset compared with placebo of 5 μg/kg was established. This dose produced mean circulating plasma melatonin levels of 54 pg/ml, which was within the range of normal endogenous peak levels for this species. While no information about the PK profile of melatonin in this species was provided to validate its use as a surrogate human model, this study suggested that physiological melatonin levels induced by exogenous melatonin facilitate sleep induction in similarly diurnal species [redacted]

Table 1. Representative *in vivo* pharmacodynamic (PD)/pharmacology studies performed with melatonin in mice and non-human primates.

Specie	Melatonin tested doses	Route	Effect	Reference
Mouse (C57BL/6J mice)	Melatonin 500 pmol/50 nl/ side, at dark onset	IV	Melatonin infusion into the PFH, at dark onset, site-specifically and significantly increased NREM sleep (43.7%, *P*=0.003) and reduced wakefulness (12.3%, *P*=0.013). Local melatonin infusion at dark onset inhibited orexin neurons as evident by a significant reduction (66%, *P*=0.0004) in the number of orexin neurons expressing c-Fos. Melatonin may act via the MT1 receptors to inhibit orexin neurons and promote sleep.	[redacted]
Mouse	20 and 100 mg/kg	IP	At 20 mg/kg, melatonin delayed the hypnosis induced by hexobarbital and increased the sleeping time of the animals. The animals showed excitation and body rotation before falling asleep. The 100-mg/kg group had an increased duration of sleeping period with the onset time for hypnosis similar (slightly higher) to the one from controls.	[redacted]
Mouse (adult C57Bl6 mice)	8 mg/kg; photoperiod alterations were applied	IP	It was found that jet lag simulation reduced hippocampal neural precursor cell proliferation by 24% and impaired spatial memory performance in the water maze indicated by a prolonged swim path to the target (~23%). While melatonin	[redacted]

Specie	Melatonin tested doses	Route	Effect	Reference
	to simulate jet lag		prevented both the cellular (~1%) as well as the cognitive deficits (~5%), environmental enrichment only preserved precursor cell proliferation (~12%).	
Macaques	5-20 µg/kg	Oral	The sleep process showed high sensitivity to daytime melatonin dosing. Sleep initiation was significantly promoted by a wide range of melatonin doses used showed a lack of dose dependence of the effect, once the dose (5-20 µg/kg) was sufficient t induce physiologic circulating levels of the hormone (>50 µg/ml). Lower doses failed to promote sleep in the macaque studies.	[redacted]
2 Pigtail macaques (*Macaca Nemestrina*)	0.05 mg	Oral	Melatonin given at different times of the day significantly decreased motor activity and promoted earlier sleep onset, as measured actigraphically. The decline in the animals' motor activity occurred within 25-40 min post-dose. The duration of motor inhibition was dose-dependent. Melatonin administration induced serum melatonin levels comparable to the peak levels reported in untreated humans and the non- physiologic human primates.	[redacted]

Abbreviations: IP, intraperitoneal; IV, intravenous; MT, melatonin receptor; NREM, non-rapid eye movement; PFH, perifornical lateral hypothalamus.

2.4.2.2 SECONDARY PHARMACODYNAMICS

Studies have been conducted in various species (including mice, rats, hamsters and baboons) to investigate metabolic/behavioural response to melatonin, effects on the immune system, nervous system, endocrine, reproductive and cardiovascular systems, revealing no special hazard for humans based on conventional safety pharmacology studies. Extensive bibliography is available establishing the link between melatonin and the immune system. The evidence suggests that melatonin can influence immune cells through nuclear and membrane MTs. These receptors have been identified on macrophages, B and T cells [redacted] Melatonin can modulate proliferation and cytokine secretion via these receptors on immune cells [redacted] It can inhibit chemically induced tumours in animals; this is increased by pineal suppression (long light-phases) or pinealectomy [redacted] Indeed, pinealectomy stimulates and/or melatonin inhibits the growth and sometimes the metastasis of experimental cancers of the lung, liver, ovary, pituitary, and prostate, as well as melanoma and leukaemia. The studies relating to the secondary PDs (immune systems, reproductive and endocrine systems) are discussed in this Section.

Immune system

Melatonin is involved in many immunoregulatory functions. Studies have shown that the mouse and human bone marrow, in which immunocompetent cells are generated, and bone marrow cells are capable of *de novo* synthesis of melatonin, which may have intracellular and paracrine functions [redacted] Melatonin modulates both the innate and specific immune responses through regulation of immunocompetent cell proliferation [redacted] and secretion of immune mediators, such as cytokines [redacted] It has been established that the pineal gland, the primary source of melatonin, is an immune target [redacted] Interferon-gamma (IFN-γ) was shown to increase the production of melatonin from *in vitro* cultured rat pineal glands. Administration of recombinant IL-1β inhibited serum melatonin levels in rats through a receptor-mediated mechanism, whereas granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage CSF (GM-CSF) stimulated the synthesis of melatonin both *in vivo* and *in vitro* [redacted] From further extensive research on the impact of endogenous and exogenous melatonin on the immune response pathways, it has been reported that melatonin has also antiviral, antibiotic and antiparasitic properties [redacted].

Melatonin has been shown to activate human T helper 1 (Th1) lymphocytes by increasing the production of IL-2 and IFN-γ *in vitro*. Th2 cells appear not to be affected by melatonin, since IL-4, which is mostly produced by Th2 cells, is not modified by the hormone. Melatonin also enhances IL-6 production by peripheral blood mononuclear cells (PBMCs). The activation of IL-6 production by melatonin is apparently related to the presence of monocytes, rather than to Th2 cells, in the cell preparation, since PBMCs depleted of monocytes ($CD14^+$ cells) were not activated. Activation of PBMCs by melatonin was dependent on the dose and, measured by cytokine production, was observed only when cells were either not activated or only slightly activated by low concentrations of phytohemagglutinin (PHA) or when cell activation was achieved by incubating the cells with previously irradiated cells. Using a different approach to identify what type of cells among the PBMC subsets was activated by melatonin, the expression of CD69, a marker of cell activation, has been studied. Melatonin increased the percentage of cells expressing the CD69 Ag in $CD4^+$ but not in $CD8^+$ cells. Production of IL-2 and IL-6 was enhanced using a specific ligand of the putative nuclear melatonin receptor RZR/ROR, raising the possibility of direct effects of melatonin on gene regulation in both Th1 cells and monocytes. Melatonin may be involved in the regulation of human immune functions by modulating the activity of Th1 cells and monocytes via nuclear receptor-mediated transcriptional control [redacted]

In preclinical studies, lipopolysaccharide (LPS) treatment has been shown to induce tumour necrosis factor-alpha (TNF-α) production in the rat pineal gland through activating toll-like receptor 4 (TLR-4). Subsequently, the production of TNF-α by pineal gland microglia was found to act on TNF receptor 1 (TNFR1), driving the nuclear translocation of NF-κB, which represses Aa-nat transcription and in turn suppresses melatonin synthesis [redacted] Melatonin has also been shown to possess both *in vitro* and *in vivo* important antioxidant activities as well as to inhibit the activation of poly (adenosine diphosphate [ADP] ribose) synthetase. A number of experimental studies have documented that melatonin exerts important anti-inflammatory actions [redacted]

Endocrine and reproductive systems

Melatonin regulates pubertal development in some juvenile mammals. In seasonal breeders, melatonin seems to act as either pro- or anti-gonadotrophic agent based on the period of the year (autumn-winter/short days or spring-summer/long days, respectively). Melatonin has also been shown to influence secretion of several hormones in animals and in humans in some situations, namely the luteinising hormone (LH) and prolactin, corticosteroids, thyroid hormones and insulin [redacted]. Administration of melatonin in male rats between the 45^{th} and 84^{th} days of life not only failed to stimulate but provided an additional inhibitory effect on reproductive system [redacted]

The mammalian pineal gland exerts an inhibitory effect on female gonad weight and function; these effects, as well as the morphology of pineal gland, are modified by exposure to light. Melatonin, localised in the pineal gland of mammals, is also responsible for the action of the pineal gland on gonads. The repeated daily administration of small amounts of melatonin to immature rats has been shown to delay the vaginal opening and produce a highly significant inhibition of oestrus and a decrease in ovary weight. Melatonin acutely reverses the persistent oestrus induced by exposure to constant light. Circulating tritiated melatonin has been found to be preferentially concentrated in the pineal gland and the ovary, as well as in other endocrine organs and nervous tissues. Chronic exposure to light reduced the uptake of circulating melatonin in the ovary and the pineal gland. Since the enzyme for synthesis of melatonin has

been found only in the pineal gland, the previously established presence of melatonin in peripheral tissues suggests that it is secreted by the pineal gland into the blood; this secretion may be dependent on light. The selective uptake of melatonin by the ovary and the oestrus-inhibitory effects of the pure substance suggest a direct physiologic effect, but also indirect effects cannot be ruled out [redacted]

Cancer

Short-term studies in mice administered 10 µg of melatonin topically for 14 days [redacted] and rats given 20 µg/ml in drinking water for 3 days [redacted] or 100 µg/ml in water for 28 weeks [redacted] have shown evidence of the protective effect of melatonin against known carcinogens.

A major mechanism through which melatonin reduces the development of breast cancer is based on its anti-oestrogenic actions by interfering at different levels with the oestrogen-signalling pathways. Melatonin inhibits both aromatase activity and expression *in vitro* (oestrogen-responsive MCF-7 cells) as well as *in vivo*, thus, behaving as a selective eostrogen enzyme modulator. Transfection of the MT1 in MCF-7 cells significantly decreased aromatase activity of the cells and MT1-transfected cells showed a level of aromatase activity that was 50% of vector-transfected MCF-7 cells. Proliferation of MCF-7 cells in an oestradiol-free media, but in the presence of testosterone (an indirect measure of aromatase activity), was strongly inhibited by melatonin in those cells overexpressing the MT1. This inhibitory effect of melatonin on cell growth was higher on MT1 transfected cells than in vector transfected ones. In MT1-transfected cells, aromatase activity (tritiated water release assay) was inhibited by melatonin (20% at 1 nM; 40% at 10 µM). The same concentrations of melatonin did not significantly influence the aromatase activity of vector-transfected cells. MT1 melatonin receptor transfection also induced a significant 55% inhibition of aromatase steady-state mRNA expression in comparison to vector-transfected MCF-7 cells (P<0.001). In addition, in MT1-transfected cells, melatonin treatment inhibited aromatase mRNA expression and 1 nM melatonin induced a higher and significant down-regulation of aromatase mRNA expression (P<0.05) than in vector-transfected cells. The findings presented herein point to the importance of MT1 in mediating the oncostatic action of melatonin in MCF-7 human breast cancer cells and confirm MT1 as a major mediator in the melatonin signalling pathway in breast cancer [redacted]

Table 2. Representative *in vitro* secondary pharmacology studies performed in various animal/human cell lines treated with melatonin.

***In vitro* model**	**Tested melatonin levels**	**Effect**	**Reference**
Cancer			
Thoracic mammary glands of 21-day-old mice	For 6 days, 0 (control), 10^{-6}, 10^{-9} or 10^{-12} M	Relative to controls, 10^{-12} M melatonin increased and 10^{-6} M melatonin decreased mammary DNA and uptake of [methyl-^{3}H]-thymidine.	[redacted]
Normal human lymphocytes, leukaemic K562 and HeLa cancer cells	50 µM	Melatonin protected both normal and cancer cells against genotoxic treatment and apoptosis induced by idarubicin.	[redacted]
Immune system			
PHA-stimulated human lymphocyte cultures	10^{-3} to 10^{-12} M; incubation for 24, 48 and 72 h	Melatonin does not increase T and LAK cell responses; in fact, a reduction in the transcription of all the considered genes was observed.	[redacted]
PBMC cultures stimulated with PHA	Incubation for 72 h, without or with melatonin (10^{-3} M) and PCPA (10^{-3} M)	Human lymphocyte-synthesised melatonin modulates IL-2/IL-2 receptor system because when blocking melatonin biosynthesis by the tryptophan hydroxylase inhibitor, PCPA, both IL-2 and IL-2 receptor levels fell, restoring them by adding exogenous melatonin. Endogenous melatonin interfered with exogenous melatonin effect on IL-2 production.	[redacted]

***In vitro* model**	**Tested melatonin levels**	**Effect**	**Reference**
Splenocytes from female prairie voles (rodents) held in long or short days	0 or 500 pg/ml	Splenocyte proliferation in response to the T-cell mitogen concanavalin A was enhanced by melatonin, as compared to cultures receiving no melatonin. Body mass increased in short-day housed prairie voles, indicating that the animals were responsive to photoperiod. Photoperiod did not affect splenocyte proliferation.	[redacted]
Primary cultured human FLSs	10 and 100 μM; incubation for 24 h	Melatonin inhibited RA-FLS proliferation dose-dependently and reduced proliferation of passage 2 FLSs by 25% at 10 μM and by ~40% at 100 μM. The inhibitory effect of melatonin on RA-FLS proliferation was observed in passages 4 and 6. Melatonin upregulated the expression levels of P21 and P27 dose-dependently (24 h), induced the phosphorylation of ERK time-dependently (10 μM) but did not affect phosphorylation of P38 in RA-FLSs.	[redacted]
Abbreviations: ERK, extracellular signal-regulated protein kinase; FLS, fibroblast-like synoviocyte; IL, interleukin; LAK, lymphokine activated killer; PBMC, peripheral blood mononuclear cell; PCPA, parachlorophenylalanine; PHA, phytohemagglutinin; RA, rheumatoid arthritis.			

Table 3. Tabulated *in vivo* secondary pharmacology studies performed in various animal models with melatonin administration via different routes.

Specie	**Melatonin doses**	**Route**	**Effect**	**Reference**
Immune system				
Mouse (old [16.5 months] female C57BL/6 mice)	8 young and 4 old mice/group: (A): controls; (B) DHEA-diet + water; (C): melatonin 49.8 μg/day for 12 weeks in drinking water; and (D) DHEA-diet and melatonin, for 12 weeks	Oral (in drinking water)	As expected, splenocytes were significantly (P<0.05) higher in old than in young mice. DHEA, melatonin and DHEA+melatonin significantly (P<0.005) increased B cell proliferation in young mice. However, only melatonin and DHEA+melatonin significantly (P<0.05) increased B cell proliferation in old mice. DHEA, melatonin and DHEA + melatonin help to regulate immune function in aged female C57BL/6 mice by significantly (P<0.05) increasing Th1 cytokines, IL-2 and IFN-γ or significantly (P<0.05) decreasing Th2 cytokines, IL-6 and IL-10, thus regulating cytokine production. DHEA and melatonin effectively modulate suppressed Th1 cytokine and elevated Th2 cytokine production, but their combination produced a limited additive effect.	[redacted]
Mouse (OVX mice with vitrified-thawed whole ovaries of newborn mice)	0, 20, 50, 100 and 200 mg/kg/day, for 32 days	Oral (gavage)	Administration of melatonin did not disturb the circadian rhythm of melatonin concentration. The ovarian graft lifespan was prolonged at 200 mg/kg/day melatonin (P<0.001). However, at doses >20 mg/kg/day, the proportion of healthy follicles and ovary size decreased. Th1 cytokines levels were reduced dose dependently. However, the effect of melatonin on Th2 cytokines was not pronounced. IgM and IgG_{2a} decreased in recipients receiving 200 mg/kg/day in comparison with non-treated group (P<0.001), while these variables were significantly increased at 50 mg/kg/day (P<0.001).	[redacted]
Mouse (antigen-primed mice)	0, 10 and 20 mg/kg/day	SC	Chronic (5-day) melatonin dosing increased the production of pro-inflammatory cytokine IL-10 but decreased the secretion of anti-inflammatory cytokine TNF-α. The Th2 cell response induced by melatonin in antigen-sensitised mice neither dependent on endogenous opioid system nor is modulated through the central or peripheral benzodiazepine receptors.	[redacted]
Mouse (with membranous nephropathy)	20 mg/kg/day	SC	Melatonin caused a significant reduction in proteinuria and a marked amelioration of glomerular lesions, with attenuated immunocomplex deposition. The subpopulations of T cells were not altered; the $CD19^+$ B-cell subpopulation was significantly reduced in the mice treated with melatonin. The expression of cytokine mRNAs in splenocytes indicated that melatonin reduced the expression of proinflammatory and increased the expression of anti-inflammatory cytokines. The production of ROS and TUNEL-positive apoptotic cells in the kidney were significantly reduced in melatonin-treated mice. Melatonin upregulated HO1 and ameliorated membranous nephropathy.	[redacted]
Rat (aged and young male Wistar rats)	10 mg/kg/day, for 7 days	SC	18 aged (28 months old; 6 in control and 12 in melatonin group) and 25 young (9 months old; 10 in control and 15 in melatonin group) rats were given 4×10^8 sheep erythrocytes IP, to evoke humoural immune responses. Melatonin was found to increase IgG_1 and IgM responses of aged vs control rats (P=0.049 and 0.007), respectively. In the young rats, the IgG1 levels of controls were significantly higher than that of the melatonin group (P=0.021); IgM levels were not significantly different (P=0.563). Exogenous melatonin may augment the depressed humoral immune responses in aged rats.	[redacted]
Indian palm squirrel (*Funambulus pennanti*)	250 μg/kg/day, for 60 consecutive days, at 17.30-18.00 h to adult male squirrels, during May-June	SC	Daily melatonin significantly increased the lymphocyte count of blood and bone marrow and the blastogenic response-to-%stimulation ratio of spleen and thymus. Histologically, densely packed thymocytes and splenocytes were found. During this period, peripheral testosterone level was high; melatonin was low establishing an inverse relationship as noted earlier for this squirrel. In pinealectomised squirrels, decreased total and percentage leukocyte count in peripheral blood and bone marrow, along with a decreased cell density in spleen and thymus was noted histologically. Melatonin resulted in restoration of the immune parameters in line with a normal control level.	[redacted]

Specie	Melatonin doses	Route	Effect	Reference
Endocrine and reproductive system				
Rat (females)	12.8 mg/kg in the morning of pro-oestrus	IV	Melatonin significantly increased serum prolactin over control levels by 1 and 2 h after injection, in hypophysectomised, pituitary-grafted female rats.	[redacted]
Rat (males)	Exp. 1: in castrated rats, testosterone propionate (300, 600 or 1,200 µg/day) in sham-PINX; PINX; PINX + melatonin 50 or 200 µg/day, for 10 days Exp. 2: sham-PINX, PINX and PINX + melatonin 50, 200 or 800 µg/day, for 20 days	SC	In castrated prepubertal rats, pinealectomy enhanced the testosterone-induced growth response of the seminal vesicles and melatonin inhibited this effect dose-dependently. In entire animals, the serum LH level was increased after pinealectomy with no significant changes in other parameters. Administration of melatonin to intact, PINX rats did not affect the serum concentrations of LH or testosterone but caused a dose-related decrease in the weight of the seminal vesicles. The highest dose of melatonin tested reduced the weight of the ventral prostate gland and the uptake of radioactivity by both the ventral prostate gland and the testes after injection of [5-^{3}H]uridine.	[redacted]
Rat (males)	5-100 µg/day	SC	When melatonin was injected daily into young animals from day 20 of age, dose-dependent reductions in plasma testosterone, testis and seminal vesicles weights, plasma FSH and LH levels and pituitary GnRH receptor number were seen at day 40 or 45 of age (sacrifice). Prepubertal rats (5-20 days old) showed no significant responses to similar treatment with melatonin, whereas in adult animals (70-90 days old), melatonin elicited only a small decrease in plasma testosterone level. Chronic melatonin dosing from 20-50 days of life did not alter the occurrence of the nocturnal rise of circulating plasma melatonin but did enhance its amplitude. Overall, exogenous melatonin can inhibit or delay sexual maturation in the male rat if administered between 20 and 40 days of age; this inhibitory action is exerted at the hypothalamic and/or pituitary level.	[redacted]
Rat (10-12 male rats/group)	1-100 µg/day, in the afternoon, for 20 days	SC	Melatonin inhibited the neuroendocrine-reproductive axis during sexual maturation, when injected in the afternoon, but had no effect when injected in the morning. Daily injections of 1 µg/day from 20 to 40 days of age significantly lowered weights of seminal vesicles. With 5 µg/day, values for plasma levels of testosterone and LH, weights of testes and seminal vesicles and pituitary contents of FSH were the lowest measured and values for plasma levels of FSH and pituitary contents of GnRH receptors and LH were near the lowest measured. All parameters were at their lowest in rats treated at 16:00 h with 100 µg/day. The inhibitory action of melatonin is most critical between 20 and 30 days of life and is reversible regardless of whether melatonin administration is continued/discontinued after 45 days of life. The suppression of the pubertal peaks of pituitary GnRH receptor number and pituitary and plasma FSH concentrations in treated rats suggests that melatonin interferes with the pubertal increase in GnRH secretion.	[redacted]
Rat	Daily injections from 20 up to 25, 30, 35 or 40 days of age	SC	Rats treated with melatonin for 15 or 20 days presented at the end of the juvenile period, abnormal progression of spermatogenesis and decreased ability of their Leydig cells to produce testosterone both *in vivo* and *in vitro*. This was associated with a lower number of binding sites for hCG and diminished production of testosterone in response to receptor and post-receptor-mediated stimulation of steroidogenesis. Melatonin caused a marked decrease in LH serum levels. The diminished LH supply to the testis probably interfered with differentiation or impaired the functional ability of Leydig cells. As a consequence, testicular testosterone production was insufficient to support normal spermatogenic progression and growth and development of the sexual accessory organs.	[redacted]
Rat (immature females)	100 µg/day, starting on day 15 of age, in rats housed in 12 h of light, 12 h of darkness or 16 h of light, 8 h of darkness	SC	Melatonin given 9-11 h after the onset of light in both lighting regimens resulted in a 10-day delay of vaginal opening, a dissociation of the relation between vaginal opening and first pro-oestrus and a disruption of the initial oestrous cycles. The same dose of melatonin given at other times during the photoperiod had no effect on sexual maturation. GnRH secretion in melatonin-treated animals was decreased, as judged by 30% lower pituitary GnRH receptor number in animals killed after opening of the vagina. During the di-oestrous phases, plasma levels of LH, FSH and 17β-oestradiol were similar to those in control rats, but during pro-oestrus, the surge of FSH was higher, and the peak of oestradiol was higher and of a longer duration.	[redacted]
Rat (males)	1, 5, 15 or 30 mg/kg/day, for 10 days, injected at 17:00 h	SC	The weight of adrenal gland was decreased by the highest melatonin dose (30 mg/kg) given once daily for 10 days. Corticosterone levels in adrenal gland and in serum were significantly decreased. Lower doses (1, 5 and 15 mg/kg) had no effect. The increase of corticosterone levels in adrenal gland by successive treatments with ACTH was suppressed by simultaneous melatonin injections. After dexamethasone, the increase in corticosterone levels induced by ACTH were also suppressed by melatonin treatment.	[redacted]
Rat (23-day-old intact males)	1, 50, 100 or 500 µg, after surgical blinding and anosmia (pinealectomy)	SC implant	Only the highest dose of melatonin (1 mg) restored the testicular and accessory sex organ weights to those of the intact controls. As little as 1 µg of melatonin restored plasma and pituitary LH levels to those of the intact controls. However, none of the melatonin doses reversed plasma PRL levels to those of the controls.	[redacted]

Specie	Melatonin doses	Route	Effect	Reference
			The decrease in pituitary PRL induced by blinding and anosmia was reversed by pinealectomy or by melatonin 1, 50 or 100 µg.	
Hamster (females)	2.5 or 25 µg/day, late in the light period, for 8 or 11 weeks, or implants of 1 mg of melatonin	SC	The free T_4 index and the free T_3 index were significantly inhibited by melatonin. Decreasing the photoperiod under which the hamsters were kept, from 14 h light/10 h dark to 10 h light/14 h day, resulted in decreased blood levels of these hormones. Melatonin at 2.5 µg/day did not significantly inhibit blood levels of T_3, T_4 or TSH; injection of this dose every afternoon into hamsters in long photoperiod significantly augmented the blood levels of T_4. Continuously available melatonin in the form of SC implants of 1 mg melatonin in beeswax did not inhibit blood levels of thyroid hormones; such implants prevented the inhibitory effects of 25-µg melatonin injections.	[redacted]
Hamster (females)	25 µg/day, for 10 weeks	SC	Controls responded to daily evening melatonin injections with a decrease in serum T_4. Serum T_3 levels were decreased by thiourea and increased by T_4 replacement. The pituitary PRL content was significantly reduced below control values in hamsters receiving melatonin; the sensitivity to melatonin was inhibited by thiourea and restored by T_4 replacement. Melatonin-induced testicular involution was attenuated by thiourea, which was reversed by T_4 replacement. Similarly, a melatonin-induced decrease in serum LH was prevented by thiourea and restored by T_4 replacement.	[redacted]
Hamster (males)	melatonin-beeswax (1:24 mg) pellets, either once per week, per 2, 3, 4, 6 weeks, or only one pellet during the 12-week experiment (doses: 1 mg, 500, 100, 50 or 1 µg melatonin)	SC implant	1st exp: The melatonin-beeswax pellets, regardless of the frequency of implantation, overcame completely the inhibitory effects of blinding on reproduction and nearly completely the depressant action of light deprivation on pituitary PRL levels. 2nd exp: melatonin-beeswax pellets were implanted SC into blind hamsters every 2 weeks. The pellets contained either 1 mg, 500, 100, 50 or 1 µg melatonin. With the exception of the 1-µg dosage, melatonin again negated almost totally the inhibitory action of darkness on the gonads and accessory organs and, for the most part, prevented the drop in pituitary PRL levels. Based on these studies, when melatonin is chronically administered SC in a beeswax pellet the minimal dosage of melatonin required to counteract the inhibitory effect of darkness on reproduction seems to be <3.6 µg/day. The effects of chronic melatonin treatment are similar to those of pinealectomy.	[redacted]
Protective role on carcinogenicity				
Mouse (female C3H/Jax mice)	25 µg/day from 21 to 44 days of age and 50 µg/day from day 45 onwards	Oral	By the age of 12 months, 62.5% of controls developed tumours as opposed to 23.1% in the melatonin-treated group (P<0.02). In both control and treated mice, the thoracic pairs of mammary glands were obviously more susceptible to spontaneous mammary tumour development, as at least 50% of the total tumours developed in this region. Reduction in submaxillary and pituitary gland weights of treated animals was seen at necropsy (P<0.001). Decreased serum 17β-oestradiol levels in melatonin-treated mice (P<0.05) and a marked reduction in [^{3}H]-thymidine incorporation into DNA of melatonin-treated mammary glands (P<0.02) positively correlated with the sparse mammary gland development seen in these mice.	[redacted]
Mouse (4-week-old hemizygous TG.NK female mice with MMTV/c-neu oncogene)	0.05-0.2 ml of flaxseed oil or **melatonin at 50-200 mg/kg**, 0.10 ml flaxseed oil + 50 mg/kg melatonin, for 30 weeks.	Oral (gavage)	At the high dose (0.2 ml) of flaxseed oil, when the ω-6:ω-3 PUFA ratio was closer to 1, there was some delay in the growth of mammary tumours. Melatonin delayed the appearance of palpable tumours and the growth of the tumours with a dose-related statistically significant negative trend for the incidence of tumours. The combination of flaxseed oil and melatonin caused a significant decrease in the number of tumours and tumour weight per mouse compared to the control and to flaxseed oil but not to melatonin alone.	[redacted]
Mouse (female CBA mice)	From 6 months of age until natural deaths: melatonin 0 or 20 µg/ml for 5 consecutive days/month.	Oral (in drinking water)	Melatonin did not significantly influence food consumption, nor the physical strength or the presence of fatigue; it increased the body weight of older mice; it decreased locomotor activity and body temperature; it inhibited free radical processes in serum, brain and liver; it slowed down the age-related switching-off of oestrous function; it increased life span.	[redacted]
Mouse (54 female Swiss-derived SHR mice/ group)	From the age of 3 months until natural death: melatonin 0, 2 or 20 µg/ml for 5 consecutive days/ month.	Oral (in drinking water)	Treatment with melatonin did not significantly influence food consumption; administration at lower doses decreased body weight of mice; it slowed down the age-related switching-off of oestrous function; it did not influence the frequency of chromosome aberrations in bone marrow cells nor the mean life span; it increased life span of the last 10% of the survivors vs controls. Low-dose melatonin significantly decreased spontaneous tumour incidence (by 1,9-fold), mainly mammary carcinomas, in mice whereas higher doses (20 µg/ml) failed to influence tumour incidence as compared to controls. The geroprotective effect of melatonin is dose-dependent.	[redacted]
Mouse (female HER-2/neu mice)	Melatonin 0 or 20 µg/ml during night-time, 5 times/month (interrupted) or constantly, from the age of 2 months to natural death	Oral (in drinking water)	Treatment with melatonin slowed down age-related disturbances in oestrous function most in the group exposed to interrupted treatment. Constant treatment decreased incidence and size of mammary adenocarcinomas and incidence of lung metastases, compared to controls. The number of mice bearing ≥4 tumours was reduced in the constant-treatment group. Interrupted treatment promote mammary carcinogenesis in HER-2/neu transgenic mice.	[redacted]
Rat	2.5 mg/kg/day (in the afternoon), for	SC	Melatonin significantly reduced the incidence of mammary tumours from 79% (control) to 20% (treated) (P<0.002). Rats pinealectomised at 20 days of age	[redacted]

Specie	Melatonin doses	Route	Effect	Reference
(DMBA-treared Sprague-Dawley rats)	90 days, starting on the same day as DMBA (5 mg) treatment		and treated with 7 mg DMBA at 50 days of age had a higher incidence of tumours (88%) than controls (22%). Melatonin only partially reversed the effects of pinealectomy, reducing the incidence from 87% (pinealectomy alone) to 63% (pinealectomy+melatonin); however, the tumour incidence was still lower (27%) in non-pinealectomised melatonin-treated animals. Assessment of plasma PRL, LH, FSH, oestradiol and cortisol in DMBA-treated tumour-free and tumour-bearing rats revealed a significantly lower plasma PRL level in melatonin- than vehicle-treated rats. Plasma PRL level was less in melatonin-treated, pinealectomised rats than in vehicle-treated, pinealectomised rats. Other hormones were not affected by melatonin.	
Rat	Melatonin 20, 100 or 500 µg/day	SC	Daily late-afternoon injections of melatonine 500 µg. restricted to the initiation phase of NMU mammary tumourigenesis, were ineffective in altering tumour growth over a 20-week period. When melatonin treatment was delayed for 4 weeks after NMU and then continued through the remainder of the promotion phase, only tumour number was significantly lower than in controls. However, when melatonin injections encompassed the entire promotion phase, both tumour incidence and number were significantly lower than in controls. Although elimination of the endogenous melatonin signal via pinealectomy promoted tumour growth, the effect was not statistically significant. Serum levels of oestradiol and tumour oestrogen receptor content were unaltered by either melatonin or pinealectomy. While melatonin treatment failed to affect circulating PRL levels, pinealectomy caused a 2-fold increase in serum PRL. The oestradiol-stimulated recrudescence of tumours following ovariectomy was completely blocked by all melatonin doses.	[redacted]
Neuroprotective effects (CNS)				
Mouse (4-month-old male and female transgenic mice for AD; n=10/ group)	Melatonin 0 or 10 mg/kg/day, for 4 months	Oral (gavage)	An increase in the levels of brain TBARS and a decrease in GSH content, as well as accelerated upregulation of the apoptotic-related factors, such as Bax, caspase-3 and Par-4 in transgenic mice, but not in WT littermates. Significantly, the increase in TBARS levels, reduction in SOD activity and GSH content were reinstated by melatonin. Transgenic mice given melatonin showed a significant reduction in upregulated expression of Bax, caspase-3 and Par-4, indicating inhibited triggering of neuronal apoptosis.	[redacted]
Mouse	Melatonin (128 or 256 mg/kg IP) + diazepam (0.5 mg/kg orally) or either monotherapy	IP	The test models used were the four plates test and the tail suspension test. In the former, anxiolytics increase the number of punished crossings and in the latter increase the duration of immobility of mice suspended by the tail. In the four plates test, combined treatment with melatonin (128 and 256 mg/kg) and diazepam (0.5 mg/kg) caused a significant increase in the number of punished crossings, whereas each treatment alone was without effect. Similarly, in the tail suspension test, a clear increase in the duration of immobility was observed after combined treatment (256 mg/kg IP melatonin + 0.5 mg/kg oral diazepam), whereas no effects were observed with the individual treatments alone. Melatonin can enhance the anxiolytic actions of diazepam.	[redacted]
Mouse (BALB/c and C57BL/6J mice)	Melatonin 10-1,000 µg/kg; 2.5-10 mg/kg (acute administration or daily doses). The duration of immobility period during a 6-min swim test was measured at noon (11:00-12:00 h), early dark (20:00-21:00 h) and at midnight (1:00-2:00 h).	IP	The circadian time cycle did not alter the duration of immobility in either strain of mice. Similarly, exogenous melatonin at doses that could act on high affinity melatonin receptors (10-1,000 µg/kg) did not modify the duration of immobility period at any of the time intervals studied. Hence, neither circadian variation influenced the duration of immobility period of mice nor at physiological doses melatonin showed any antidepressant action. Acute administration of higher doses of melatonin (2.5-10 mg/kg) failed to induce any antidepressant activity in mice subjected to forced swimming test for the first time. However, daily dosing (2.5-10 mg/kg) prior to swimming test significantly reversed the increase in immobility period that was observed on chronic exposure to swimming test. This effect was comparable with the effect of GABA-benzodiazepine receptor agonists.	[redacted]
Rat (adult male Wistar rats with LPS-induced ON)	Sham or SC pellet of 20 mg melatonin at 24 h before vehicle or LPS injection	SC	Melatonin completely prevented the decrease in VEPs and pupil light reflex and preserved anterograde transport of cholera toxin β-subunit from the retina to superior colliculus. It prevented microglial reactivity (P<0.01), astrocytosis (P<0.05), demyelination (P<0.01) and axon (P<0.01) and retinal ganglion cell (P<0.01) loss induced by LPS. Melatonin completely prevented the increase in NOS-2, COX-2 and TNF-α levels and partly prevented experimentally induced ON. When implanted at 4 days post-LPS, melatonin completely reversed the decrease in VEPs and pupil light reflex.	[redacted]
Cardiovascular, pulmonary vascular and renoprotective effects				
Rat (young and adult SHR; n=10/ group)	Group 1 (control): no treatment; Group 2: L-NAME 80 mg/L; Group 3: L-NAME 80 mg/L+ 0.01% melatonin; young	Oral (in drinking water)	L-NAME exacerbated the elevation of blood pressure, renal dysfunction and glomerular sclerosis in young SHRs and induced an increase of ADMA and a decrease of arginine-to-ADMA ratio in the SHR kidney. Melatonin prevented L-NAME-exacerbated hypertension and nephrosclerosis in young SHRs. It also restored L-NAME-induced reduction of dimethylarginine dimethylaminohydrolase activity in SHR kidney. Melatonin decreased renal ADMA concentrations, increased renal arginine-to-ADMA ratio and restored	[redacted]

Specie	Melatonin doses	Route	Effect	Reference
	mice took L-NAME 80 mg/L		NO production in L-NAME-treated young. It also reduced the degree of oxidative damaged DNA product in L-NAME-treated SHR kidney.	
Lamb (n=10 newborn sheep [*Ovis aries*])	Melatonin 0 (n=5) or 1 mg/kg/day (n=5), for the first 3 weeks of life (PNDs: 4-21); after 1 week of treatment	Oral	Melatonin decreased pulmonary arterial pressure the first 4 treatment days. At 1 month of age, it decreased the contractile response to the vasoconstrictors K^+, TX2 and ET-1 and increased the endothelium-dependent and muscle-dependent vasodilation of SPA. Melatonin decreased pulmonary oxidative stress by inducing antioxidant enzymes and diminishing pro-oxidant sources. Overall, it improved vascular reactivity and oxidative stress at the pulmonary level in PAHN lambs gestated and born in chronic hypoxia.	[redacted]
Other effects				
Rat (40 healthy adult male albino rats)	For 6 weeks, Group-A: control; B: STZ 37 mg/kg, IP; C: melatonin 10 mg/100 ml; D: only melatonin.	Oral (in drinking water)	STZ significantly increased serum glucose and decreased weight in group B animals, whereas in group C, melatonin significantly restored serum glucose but could not restore body weights reduced by STZ. There was a significant reduction in body weight in melatonin-treated group D.	[redacted]

Abbreviations: ACTH, adrenocorticotropic hormone; AD, Alzheimer's disease; ADMA, asymmetric dimethylarginine; COX, cyclooxygenase; DHEA, dehydroepiandrosterone; DMBA, 7,12-dimethylbenz(α)-anthracene; ET-1 endothelin-1; FSH, follicle-stimulating hormone; GABA, gamma-amino-butyric acid; GnRH, gonadotropin-releasing hormone; GSH, glutathione; hCG, human chorionic gonadotropin; HO, haeme oxygenase; Ig, immunoglobulin; IL, interleukin; INF-γ, interferon-gamma; IP, intraperitoneal; IV, intravenous; K^+, potassium cations; LH, luteinising hormones; L-NAME, N^G-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; NMU, *N*-methyl-*N*-nitrosourea; NOS, nitric oxide synthase; NO, nitric oxide; ON, optic neuritis; OVX, ovariectomised; PAHN, pulmonary arterial hypertension of the neonate; Par-4, prostate apoptosis response-4; PINX, pinealectomised; PND, postnatal day; PRL, prolactin; PUFA, purified fatty acids; ROS, reactive oxygen species; SC, subcutaneous; SHR, spontaneously hypertensive rat; SOD, superoxide dismutase; SPA, small pulmonary arteries; STZ, streptozotocin; T_3, triiodothyronine; T_4, thyroxine; TBARS, thiobarbituric acid-reactive substances; Th, T helper; TNF-α, tumour necrosis factor-alpha; TSH, thyrotropin; TX, thromboxane; VEP, visual evoked potential; WT, wild type.

Cardiovascular system

MTs have been identified at the anterior cerebral and caudal arteries of rats and at the coronary and pulmonary arteries of pigs. In rats, a dose-related fall of mean arterial pressure, heart rate and brain serotonin release were observed after 30-60 mg of melatonin/kg (IV). Bradycardia was abolished by pretreatment with bilateral vagotomy, thus, suggesting that it may be mediated through a parasympathetic action [redacted] In rats, melatonin not only prevented increases in BP during the developing stage of stress-induced hypertension (SIH) but could also reduce the BP of rats that had already developed SIH. The antihypertensive effects of melatonin may be mediated by $GABA_A$ receptors through inhibition of plasma angiotensin II levels [redacted] Studies in porcine and coronary arteries suggest the potential for melatonin to have tensive effects [redacted]). In baboons, IV melatonin doses of 0.3-0.4 mg/kg caused a statistically significant increase of the cardiac output and ventricular ejection associated with a reduction in heart rate [redacted]

A study by [redacted] demonstrated that increased nitric oxide synthase (NOS) activity and endothelial NOS (eNOS) upregulation in particular brain regions may contribute partially to decrease in BP in spontaneously hypertensive rats with metabolic syndrome after oral 3-week treatment with melatonin (10 mg/kg/day). Participation of MT1 receptors in this melatonin action may be supposed [redacted]

CNS

In laboratory animals, relatively high doses of melatonin had to be administered in order to produce pharmacological such as sedation, analgesia and anticonvulsant activity; in humans, melatonin had short-acting mild sedative properties. It also had endocrine effects; a No-Observed-Effect-Level (NOEL) of 0.04 mg/kg/day for this endpoint was established in a study in which adult males received repeated oral doses for up to 2 months [redacted] Melatonin has high cell permeability and is able to cross the blood-brain

barrier. Apart from that, there are no reported adverse events (AEs) associated with long-term usage of melatonin at both physiological and pharmacological doses. [redacted] has summarised the pharmacological effects of melatonin as neuroprotectant in CNS injury, ischaemic-reperfusion injury, optic nerve injury, peripheral nerve injury, neurotmesis, axonotmesis, scar formation, cell degeneration and apoptosis in rodent models.

The effect of melatonin in established tests of sedative/hypnotic, anticonvulsant and analgesic activities in mice and rats has been compared to its neurotoxicity and acute toxicity. In the mouse, a low dose of melatonin (20 mg/kg, IP) potentiated pentobarbitone- and barbitone-induced sleep. Melatonin also potentiated pentobarbitone-induced sleep in the rat. Higher IP doses (≥200 mg/kg) antagonised experimentally induced convulsions in mice and had analgesic activity in both hot-plate and writhing tests. The presence of motor incoordination, indicated by the rotorod test, after administration of melatonin doses ≥200 mg/kg (IP) suggests that the anticonvulsant and analgesic activities of melatonin may not represent specific neuropharmacological actions. A sedative dose of melatonin (20 mg/kg, IP) did not alter whole brain 5-hydroxytryptamine or 5-hydroxyindole acetic acid concentrations, suggesting that the hypothesis that the sedative action of melatonin is due to an interaction with serotoninergic neurons may need to be re-examined [redacted]

2.4.2.3 SAFETY PHARMACOLOGY

In this Section, the most important *in vivo* safety pharmacology studies involving melatonin administration are summarised. Depending on the concentrations of melatonin or the preparation used, melatonin can exert either a vasoconstrictory effect at physiological concentrations (nM) or a vasodilatory effect at higher concentrations (μM or mM), suggesting a biphasic pharmacology of the hormone. Melatonin and its main target, the SCN, can modify cardiovascular rhythms, e.g., BP, heart rate. Taken together, these data, among others, show that melatonin could modulate the rhythmicity of the cardiovascular system. Also, alterations of the circadian rhythmicity of melatonin could be deleterious from a long-term effect point of view [redacted] Melatonin in physiological doses causes vasoconstriction and constricts cerebral arteries in rats. The inotropic and chronotropic actions of the isolated guinea pig and rat heart were unaltered when melatonin was perfused at 10^{-4} M [redacted]

In vivo safety pharmacology studies investigating the effects of melatonin administration in various animal species on different systems, such as the CNS, the cardiovascular and respiratory systems, are presented below.

Table 4. Safety pharmacology studies with melatonin in various animal models.

Specie	Dose of melatonin	Route	Effect	Reference
Cardiovascular system				
Rat (HCD-fed rats)	Melatonin 10 mg/kg, cholestyramine (230 mg/kg) or ezetimibe (145 μg/kg), for 30 days	Oral	HCD induced a remarkable increase in hepatic and plasma TC, plasma VLDL-C and LDL-C, a decrease in HDL-C and an elevation in TG levels in plasma and the liver. Melatonin significantly reduced cholesterol absorption in rats fed on HCD and caused significant decreases in TC, TG, VLDL-C and LDL-C in plasma and contents of cholesterol and TG in the liver. HDL-C levels were significantly increased by melatonin.	[redacted]
Rat	Melatonin 30-60 mg/kg or vehicle (10% alcohol)	IV	Administration of melatonin produced a dose-related fall in mean arterial pressure, heart rate or serotonin release in both the corpus striatum and hypothalamus. It also had an insignificant effect on either Pa_{CO2}, Pa_{O2} or pH.	[redacted]
Baboon (n=6)	Melatonin 0.3-0.4 mg/kg or DMSO (solvent medium)	IV	The only statistically significant change due to melatonin administration was the increase in the cardiac output and left ventricular ejection fraction. With the reduced heart rate, the increase in cardiac output implies a positive inotropic action on the heart by melatonin. There are indications that DMSO possibly suppresses cardiovascular actions of melatonin.	[redacted]

Specie	Dose of melatonin	Route	Effect	Reference
Rat (23-week-old male SHR)	6 mg/day for 5 days	IP	Melatonin IP dose of 6 mg/day for 5 days produced a significant reduction of blood pressure and a slight but significant decrease of heart rate in the conscious and unrestrained state. These cardiovascular effects of melatonin developed gradually. Plasma renin concentration tended to decrease after melatonin treatment. These results demonstrate that melatonin has an antihypertensive action.	[redacted]
Dog	10 mg/kg	Not referred	Melatonin failed to alter the contractile force or electrocardiogram.	[redacted]
Cat			Administration of 10 mg/kg of melatonin did not alter the blood pressure.	
Respiratory system				
Dog	Melatonin 60×10^{-6} moles/kg	IV	The effect of melatonin was examined on the pulmonary mechanics of intact dogs before and after vagotomy. The following results were obtained: (i) a decrease in total lung resistance; (ii) an increase in lung compliance; (iii) inhibition of 5-hydroxytryptamine-induced bronchoconstriction; (iv) the bronchodilation and inhibition are most probably caused by local and indirect action of melatonin on the bronchial smooth muscle.	[redacted]
Endocrine and Reproductive system				
Rat (21-day Long-Evans females)	0, 10 or 100 µg/day, for 2 or 4 weeks	SC	Daily melatonin during days 21-35 had no influence on ovarian growth or on anterior pituitary weight and LH concentration. During days 35-49, an increase in ovarian weight (in controls) was inhibited to a significant degree by 100 µg melatonin/day. This dose delayed the mean age at which vaginal opening occurred in one experiment by 5 days but not in a 2nd experiment. The pituitaries of untreated controls increased in size during days 35-49 and LH level decreased markedly during that period of growth in which ovaries hypertrophied and vaginal opening occurred. In animals given 100 µg/day on days 35-49, pituitaries were smaller and contained a higher level of LH, in keeping with the decreased ovarian weight and delayed vaginal opening. In two experiments, LH levels in pituitaries of ovariectomised rats were not significantly affected by daily 100-µg melatonin.	[redacted]
Rat (immature and adult albino rats)	30 or 150 µg/day, during 21 to 29 days	SC	Daily melatonin during 21-29 days in immature and adult rats exposed to normal light conditions (daily dose: 30 or 150 µg) or to continuous light (daily dose: 150 µg) did not affect gonadal weight or the oestrous cycle. After the same treatment, the pineal parenchymal cells seemed morphologically normal, and the size of their nuclei was not changed. Under normal day/night conditions, 30 or 150 µg melatonin/day did not alter the lipid content of pineal parenchymal cells. Under continuous light, a daily dose of 150 µg reversed to a slight extent the light-induced lipid depletion.	[redacted]
Rat (Sprague-Dawley-Rolfsmeyer strains of rats at 55 and 85 days of age)	50 µg melatonin/rat at 55 days and 75 µg/rat at 85 days but not by 100 µg/day at 115 days of age, for 12 days	SC	TSR estimation of the 55- and 85-day-old rats was found to be highly significantly reduced by 22.8% and 14.8%, respectively, with of 50-µg melatonin at 55 days and 75 µg at 85 days, but not by 100 µg/day at 115 days of age. These results indicate that melatonin depresses TSR, but the effect appears to be reduced with advaancing age. Feed consumption was reduced 8.76%, a non-significant reduction, in 115-day-old rats given 100 µg/day, for 12 days. The effect may be cumulative due to increasing doses of melatonin during each TSR estimation at monthly intervals. At the end of the experiment, the mean ovarian weight sholwed a highly significant reduction of 30.1% and the pituitary weight of 34.6% vs controls. The mean adrenal weight showed a highly significant increase of 22.7%.	[redacted]
Rat (25- to 26-day-old males)	Single, daily afternoon (17:00-18:00 h) injections of 50 µg of melatonin, from the day following the operations and for 6 weeks; an additional group of intact controls received injections of the diluent.	SC	The rats were either olfactory bulbectomised (rendering them anosmic), bulbectomised+PINX, or left intact. At the end the treatment period, body, anterior pituitary, testicular and seminal vesicle weights were significantly reduced in intact-melatonin-treated animals. Anosmic melatonin-treated rats showed a further, highly significant, 65%, 90% and 85% depression in testicular, seminal vesicle and ventral prostate weights, respectively, vs intact control and melatonin-treated rats. Both body and anterior pituitary weights were significantly decreased in melatonin-treated, anosmic rats. Anosmic-PINX rats treated with melatonin had organ and body weights that were intermediate between those of intact- and anosmic-melatonin-treated ones. Pituitary and serum PRL levels were significantly lower in anosmic- than in intact-melatonin-treated rats. Similarly, PRL levels were depressed in the anosmic-PINX rats treated with melatonin; however, serum PRL was not statistically lower than in intact or intact-melatonin-treated animals.	[redacted]
Rat	Melatonin 100 µg/day, for different periods of life	SC	Melatonin given to young male rats from 20-30 days of life had the same inhibitory effect on sexual maturation at 40 days as melatonin given on 20-40 days. Administration of melatonin from 30-40 days only slightly decreased plasma testosterone level, weight of seminal vesicles, and pituitary GnRH receptor content. Administration from 38-40 days had no effect. Daily melatonin dosing from 20-45 days of age was followed by resumption of sexual maturation, as observed at 70 days. The recovery was complete by 80 days of age when all parameters studied reflected complete sexual maturation. In rats treated continuously with melatonin from days 20 until 115, sexual maturation occurred but was delayed by ~20-30 days. Beginning of sexual development was noted at 60 days of life and full development was attained only at 100 days.	[redacted]

Specie	Dose of melatonin	Route	Effect	Reference
Hamster	Melatonin 25 µg/day	SC	Cyclic female hamsters were rendered anovulatory by daily SC melatonin in 29 days or by transfer to a short light cycle, L:D 6:18 in 33 days. Oestrous cyclicity was reinitiated in these animals in 44 or 45 days, after cessation of melatonin or transfer to long light-cycles (LD 14:10), respectively. Exposure of both groups to LD 6:18 after reinitiation of oestrous cyclicity caused a second cessation of ovulation in 75 (melatonin group) or 61 (short light cycle group) days. Thus, although both treatments disrupted oestrous cyclicity for nearly 6 weeks, this was not sufficient to induce photorefractoriness (failure to respond to short light cycles with continued estrous cyclicity). Rather, every animal responded to LD 6:18 and ceased ovulating. Melatonin-induced anovulatory hamsters showed daily gonadotropin release patterns identical to those reported in hamsters in other anovulatory states (lactating, prepubertal, and photoinduced anovulatory hamsters); that is, peak LH and FSH release at 17:00 h daily.	[redacted]
Chick (55 males and females)	Melatonin 500 and 1,000 µg/kg/day	SC	The mean TSR of a cortrol and melatonin groups determined starting at 7 weeks were approximately equal, indicating that melatonin had no effect upon TSR of immature chickens. At 10 weeks of age, melatonin significantly reduced the weights of testes (94%, P<0.001) and ovaries (67%, P<0.025) at the higher level as well as the adrenal and thymus glands. The pituitary gland weights of females were progressively increased by melatonin, while males were unaffected. The pineal glands were increased in both sexes at 500 µg/kg. At 1,000 µg/kg, the males showed an increase but the females showed a slight decrease.	[redacted]
CNS effects				
Mouse	30 mg/kg, every 3 h, for 18 h	IP	No change in gross behaviour or in the amount of noradrenaline in the brain or heart.	[redacted]
Rabbit	25 mg/kg	Not referred	Melatonin (25 mg/kg) had no hypothermic action in the rabbit.	
Cat	10 mg/kg		No change in postsynaptic spike potentials in the cat superior cervical ganglion and in the response in the nictitating membrane.	
Haemopoietic system				
Dog (n=40 healthy dogs)	0, 3 mg (for dogs weighing <15 kg), or 6 mg (weight ≥15 kg), every 12 h, for 28 days	Oral	No AEs or sedation were noted in any dog. The placebo group had a statistically significant transient increase in both plateletcrit and mean platelet volume on day 7 relative to baseline, which was not observed in the melatonin group. Oral melatonin did not appear to have a direct thrombopoietic effect in normal healthy dogs.	[redacted]

Abbreviations: AEs, adverse effects; CNS, central nervous system; DMSO, deimethylsulfoxide; HCD, high cholesterol diet; HDL-C, high-density lipoprotein cholesterol; IP, intraperitoneal; IV, intravenous; LDL-C, low-density lipoprotein cholesterol; LH, luteinising hormone; PINX, pinealectomised; PRL, prolactin; SC, subcutaneous; SHR, spontaneously hypertensive rat; TC, total cholesterol; TG, triglycerides; TSR, thyroid hormone secretion rate; VLDL-C, very low-density lipoprotein cholesterol.

No specific studies investigating the potential gastrointestinal (GI) effects of melatonin have been provided. While no remarkable GI effects were reported in chronic toxicity studies of melatonin in rats and dogs, no specific assessments of GI function were made in these studies. It is noted that gastroprotective effects of melatonin have been reported in rats (30 mg/kg IP) and pigs (5 mg/kg in food) [redacted]

2.4.2.4 PHARMACODYNAMIC DRUG INTERACTIONS

Because relevant human data are available for drug interactions with melatonin, limited preclinical drug-drug interaction studies have been conducted involving melatonin administration to animals.

Antiepileptic drugs: Melatonin (50 mg/kg; 60 min before the test) significantly raised the electroconvulsive threshold in mice. At the subconvulsive dose of 25 mg/kg, melatonin potentiated the anticonvulsive activity of carbamazepine and phenobarbital; the ED_{50}s were significantly decreased from 12.1 to 8.3 and from 18.9 to 11.8 mg/kg, respectively. No potentiation was observed in the case of valproate and diphenylhydantoin, the ED_{50}s of which were changed from 253 to 249 and from 10.3 to 9.7 mg/kg, respectively. Since melatonin did not influence the plasma or brain levels of the studied antiepileptics, a PK interaction is not probable. Melatonin (25 mg/kg) alone and combined with carbamazepine or phenobarbital, providing a 50% protection against maximal electroshock, were devoid of significant motor AEs, but caused strong long-term memory deficit. Such results were also observed in studies with phenytoin dosing in the same animal model [redacted]

Alcohol: Four groups of male Sprague-Dawley rats were divided in untreated controls, those that received a daily single SC injection of melatonin (10 mg/kg, in the afternoon), and those given only ethanol (in the evening) for 30 consecutive days. Another group of rats was given both melatonin and ethanol with melatonin preceding ethanol by 30 min. Products of lipid peroxidation, such as MDA and 4-hydroxyalkenals (4-HDA), were measured in the brain, heart, liver, lung and testes. At the end of the study, MDA+4-HDA levels were significantly increased in brains, hearts, lungs and testes, but not livers, of alcohol-treated compared with control rats. The % increases in lipid peroxidation products were 21.8%, 28.8%, 35.9% and 45.3% for the brain, heart, lung and testes, respectively. In animals given melatonin 30 min before ethanol, the increases in MDA+4-HDA levels were significantly reduced in all organs investigated, with levels not different from those in control rats [redacted]

Anticoagulants: Case reports have reported that patients treated with melatonin and warfarin received concurrent changes in INR and prothrombin time. The combination of warfarin or other vitamin K antagonists with melatonin may require dose adjustment of the anticoagulant drugs and should be avoided [redacted]

Benzodiazepines: As both melatonin and benzodiazepines bind $GABA_A$ receptors, there is a potential for PD interaction with benzodiazepines. An *in vitro* study using rat brain tissue found that melatonin inhibited binding of diazepam to purified rat brain synaptosomal. The combination of melatonin plus diazepam, but not either monotherapy, was found to increase the duration of immobility in the mouse tail suspension test *in vivo*. A rat study has also demonstrated that although mostly devoid of anxiolytic-like action *per se*, melatonin may potentiate the anxiolytic effects of diazepam [redacted]

Zaleplon, zolpidem, zopiclone: Melatonin may enhance the sedative properties of benzodiazepine and non-benzodiazepine hypnotics, such as zaleplon, zolpidem and zopiclone. In a clinical study, there was clear evidence of a transient PD interaction between melatonin prolonged-release tablet and zolpidem 1 h after concomitant dosing. Concomitant administration led to an increased reduction in attention, memory and coordination compared to zolpidem alone [redacted]

Nifedipine: Melatonin may reduce the hypotensive effect of nifedipine, so caution should be exercised in this combination and dose adjustment of nifedipine may be needed [redacted]

Clomiphene: The effect of melatonin on the LH-release response after the administration of synthetic LH-releasing hormone (RH) and clomiphene citrate was investigated in adult male rats. The SC administration of melatonin (1 mg/day) for 6 days produced a significant decrease in serum LH levels and in seminal vesicles and ventral prostate weights. On the other hand, the daily clomiphene citrate injection of 0.1 mg/kg/day for 6 days significantly stimulated LH levels and the weights of the accessory sex glands. Simultaneous treatment with melatonin and clomiphene produced an inhibitory effect similar to that obtained with melatonin alone. Neither pretreatment with melatonin (1 mg/day, SC for 6 days) nor its simultaneous IV administration (500 μg) with 75 ng of LH-RH modified the LH-release in response to the hypothalamic hormone [redacted]

Imipramine: Imipramine at IP doses of 20 and 40 mg/kg to mice caused no alteration and statistically significant reduction in the duration of immobility in forced swim test, respectively. While 5 mg/kg (IP) melatonin had no effect, 10 mg/kg melatonin slightly reduced

the duration of immobility. When subeffective doses of imipramine and melatonin (20 and 5 mg/kg, respectively) were co-administered, there was no alteration in responses compared with those of each drug alone. Likewise, the effective dose of melatonin (10 mg/kg) did not cause any increase in responses to 20 mg/kg imipramine. Although combination of imipramine (40 mg/kg) and melatonin (5 mg/kg) did not exert an antidepressant effect above that of imipramine alone, co-administration of the effective doses (10 and 40 mg/kg for melatonin and imipramine, respectively) displayed an additive effect. There were no significant differences between groups in relation with locomotor activity test. The results show that co-administration of imipramine and melatonin exhibits an additive effect and that there seems to be no interaction between the drugs [redacted]

Morphine: In a mouse model of pentylenetetrazole (PTZ)-induced clonic seizures melatonin exerted anticonvulsant effect with doses as high as 40-80 mg/kg, but at 10 mg/kg, it potentiated both the anticonvulsant and proconvulsant effects of morphine. Possible PK interaction of melatonin and morphine cannot be ruled out in the enhancement of two opposing effects of morphine on seizure threshold [redacted]

Adrenergic agonists/antagonists, opiate agonists/antagonists, antidepressants, prostaglandin inhibitors, tryptophan and alcohol affect the endogenous secretion of melatonin in the epiphysis. Whether these interactions are of clinical significance is unknown [redacted].

2.4.3 PHARMACOKINETICS

2.4.3.1 ABSORPTION

The gastrointestinal absorption of oral melatonin is almost complete in adult humans. The overall bioavailability is however low, ranging at 10-35% due to the extensive first-pass metabolism of melatonin. Maximum concentration (C_{max}) of orally administered melatonin occurs after 15-90 min (median time to maximum concentration [T_{max}]=52 min). Plasma melatonin C_{max} and area under the concentration-time curve (AUC) increase in a directly proportional, linear manner for oral doses of immediate-release melatonin in the range of 0.1 mg to at least 5 mg, whereas T_{max} remains constant [redacted]

The PKs of oral and IV melatonin has been studied in rats, dogs and monkeys. The dose-normalised oral bioavailability of melatonin after a 10-mg/kg oral dose was 53.5% in rats and in excess of 100% in dogs and monkeys; after a 10-mg/kg IP dose in rats, it was 74.0%, suggesting the lack of substantial first-pass hepatic extraction of melatonin in rats. However, oral bioavailability of melatonin in dogs decreased to 16.9% following a 1-mg/kg oral dose, indicating dose-dependent bioavailability in this specie. Since the oral dose given to dogs and monkeys (10 mg/kg) was 3-fold higher than the IV dose (3 mg/kg), a bioavailability value in excess of 100% may be indicative of non-linearity and hence, dose dependency in melatonin PKs. To probe the issue of non-linear PKs observed in this case, oral bioavailability of a 1-mg/kg melatonin dose was studied in dogs. The results of this study indicated significant dose-dependency in the PKs of these animals with the plasma AUC and oral bioavailability of the 1-mg/kg dose being dose proportionately lower than that of the 10-mg/kg dose (Table 5) [redacted]

Table 5. Summary of PK parameters of exogenous melatonin in rat, dog, and monkey (Table adapted from [redacted] .

Parameter	SD Rat	Beagle dog		Cynomolgus monkey
IV dosing				
Dose (mg/kg)	5.00	2.95		2.98
AUC (mg×h/L)	2.38	0.81		1.78
Clearance (L/h/kg)	2.11	3.84		1.68
Half-life (h)	0.33	0.31		0.57
Vd_{ss} (L/kg)	1.05	1.48		1.20
Oral dosing				
Dose (mg/kg)	10.00	0.98	10.30	10.00
AUC (mg.hr/L)	2.49	0.05	3.44	8.85
Dose adjusted F (%)	53.5	16.9	>100	>100

In another study, 2 dogs received orally melatonin at doses of 10, 20, 40 and 80 mg/kg, given at 2-h intervals. In contrast to the previous study results, melatonin concentrations in serum increased proportionally with increasing dose; however, no exposure parameter (i.e., C_{max} or AUC) values were reported. The mean C_{max} after 80 mg/kg was ~100 µM. Four dogs were given a single melatonin dose of 40 mg/kg. Melatonin was rapidly absorbed, reaching a C_{max} in serum (~5 µM) at 20-30 min (T_{max}) post-dose. The endogenous serum levels of melatonin were low as compared to those obtained after oral administration of the hormone, which gave 104-106 times higher levels [redacted]

Melatonin was administered orally to chickens using different doses and various ways of applying melatonin to the feed; for SC injections, it was suspended in propylene glycol or grape seed oil. The hormone always appeared in the first blood samples taken within 1 h of administration. When melatonin was absorbed into feed pellets or whole wheat, a high initial plasma concentration was reached, followed by a rapid decrease over the ensuing 2-3 h, but was still detectable as long as 24 h post-dose. For example, doses of 300 µg/kg produced 15 nM, which is more than 10 times higher than the nocturnal C_{max}. When melatonin was absorbed into cracked wheat grains that were subsequently washed with ethanol, the initial transitory peak was eliminated, levels in plasma were sustained for at least 12 h in the normal nocturnal range (750 pM) and no melatonin (<60 pM) was present 18 h later. When injected (2 µg), the C_{max} (610 pM) was reached within 30 min and decreased rapidly over the next 2-3 h ([redacted]).

In a PK-safety animal study, after a single IV injection of 5 or 15 mg melatonin/kg to adult male Sprague-Dawley rats, plasma concentrations of melatonin increased to 39 and 199 µg/ml at 2 min and 128 and 772 ng/ml at 120 min [redacted] . In sheep, melatonin was well-absorbed after SC injection but was more slowly absorbed after oral administration. It was well-distributed and ~60% of melatonin in blood was loosely bound to albumin [redacted]). Oral doses of ~2 mg proved adequate to raise the normal daytime plasma levels in both sheep and goats to levels within the normal night-time range ([redacted]

The bioavailability after nasal application of 1.5-mg melatonin in rabbits has been about 60%; C_{max}, T_{max} and half-life (t½) were 160 ng/ml, 5 min and 10 min, respectively ([redacted]).

2.4.3.2 DISTRIBUTION

Plasma protein binding of melatonin *in vitro* is approximately 60%. The mean volume of distribution (V_d) is 1.2 and 1.8 L/kg, corresponding to 84 and 126 L for a 70-kg human, for subjects receiving 10 mg and 0.5 µg/kg IV doses of melatonin, respectively ([redacted]

Melatonin readily penetrates biological membranes and thus, appears in tissues or body fluids in concentrations of the same order of magnitude as plasma. It also seems to distribute fast through tissues in the rat after systemic injections, and rapidly penetrate into brain and CSF. In one study, the steady-state volume of distribution (V_{dss}) of melatonin in different species (Sprague-Dawley rat, beagle dog and cynomolgous monkey) ranged from 1.05 to 1.48 L/kg, indicating moderate tissue distribution of melatonin in these animal species. In humans, authors have reported a $V_{d,ss}$ of 0.55 L/kg, suggesting a significantly reduced distribution of melatonin in humans than in animal models. Most circulating melatonin has been shown to bind to albumin in rat and human plasma in concentrations up to 1.5 mM. Melatonin was also shown to bind to human plasma proteins (albumin > α_1-acid glycoprotein > high-density lipoprotein with weak binding to other proteins) over the concentration range 0.2-2 nM. Protein binding was not examined in any other species [redacted]

The use of melatonin in sheep increased the residues of melatonin in most tissues, over the endogenous hormone concentrations. However, in many cases, the residues have been of a similar order of magnitude to the endogenous concentrations arising during the peak production time, during the night. Melatonin has been shown to cross the placenta in rats, sheep and rhesus monkeys and can be transferred to rat pups in maternal milk [redacted] [^{3}H]-Melatonin was found to be rapidly transferred from the maternal circulation into lactating mammary tissue, and the stomach of each suckling rat was found to contain [^{3}H]-melatonin [redacted]). SC administration of [^{3}H]-acetylmelatonin to Sprague-Dawley rats on gestational day (GD) 18 resulted in detection of radioactivity in whole fetuses (brain, liver, heart, viscera, skin, muscle, and bone), with highest concentrations in fetal liver and lowest levels in fetal brain ([redacted]).

Following IV injection of [^{14}C]-melatonin (10 µCi, 370 nmol) to 5 rats, analysis of its plasma kinetics showed a 3-compartment model with t½s of 0.21±0.05, 5.97±1.11 and 47.52±8.86 min. The volume of distribution (V_d) and the clearance were 1,736±349 ml/kg and 25.1±1.7 ml/min/kg, respectively. The entry of [^{14}C]-melatonin into the CSF was rapid and reached a maximum at 5 min. The decay followed a two-compartment model with t½s of 16.5±2.9 and 47.3±8.6 min. The CSF/plasma concentration ratio was 0.38 at the steady state (30 min). At 2 min, the [^{14}C]-melatonin level in the brain was 3.8 higher than in the CSF. Representative autoradiograms revealed a heterogeneous localisation of labelled melatonin in the grey matter. The highest regional values were found in cortex, thalamic nuclei, medial geniculate nucleus, anterior pretectal area, paraventricular nucleus of the hypothalamus, choroid plexuses and bulb-pons. After 30 min, [^{14}C]-melatonin was still detected in most of the analysed brain regions. These results point to a low but rapid penetration of circulating melatine into the brain and CSF. The heterogeneous distribution and the partial retention of [^{14}C]-melatonin in the brain are compatible with the hypothesis of a central action of this hormone mediated via binding sites ([redacted]

After intracerebroventricular administration of [^{3}H]-melatonin to rats, the rate of its disappearance from the brain was found to be multiphasic. At 48 h post-injection, radioactivity was still present in the brain, which may account for long-term effects of melatonin on brain function. Nonlinear regression data analysis confirmed a very rapid t½ component (3.04 min) and a slower one (36 min); a much slower component (t½=24 h) was also found. Considerable metabolism of melatonin was detected since only 36.5% of the administered radioactivity remained as melatonin at 45 min. The subcellular distribution of the radioactivity present in the brain at all tested times revealed that a major proportion of the radioactivity remained in the cytosol and respectively decreasing proportions in the 900g pellet, mitochondrial pellet and the microsomes ([redacted]).

Dynamics of radioactive accumulation in rat greater salivary gland following systemic (IV) administration of [^{3}H]-melatonin was studied to determine a possible action of the hormone in the gland. Progressive decline of [^{3}H]-melatonin concentrations was found in serum, lung, skeletal muscle, liver, kidney and salivary gland during 60 min post-dose. On the contrary, there was a progressive accumulation of radioactive substance other than [^{3}H]-melatonin in the salivary gland but not in the other tissues mentioned. The radioactivity was progressively and preferentially localised in the nuclear fraction of the gland cells. Hence, a possible direct action of melatonin in the rat salivary gland is suggested ([redacted]).

Eight rats were given melatonin both intranasally in one nostril (40 μg/rat) and IV by bolus injection (40 μg/rat). Melatonin was quickly absorbed in plasma, with a T_{max} of 2.5 min, and showed a delayed uptake into CSF (T_{max}=15 min) after nasal administration. The melatonin AUC profiles in plasma and CSF were comparable to those after IV delivery; the AUC_{CSF}/AUC_{plasma} ratio after nasal delivery (32.7±6.3%) did not differ from the one after IV injection (46.0±10.4%), indicating that melatonin enters the CSF via the blood circulation across the blood-brain barrier. This demonstrates that there is no additional transport via the nose-CSF pathway ([redacted]

2.4.3.3 METABOLISM

Most of the melatonin in the circulation is inactivated in the liver (primarily metabolised by CYP1A1 and CYP1A2) where it is first oxidised to 6-hydroxymelatonin by a P450-dependent microsomal oxidase and then largely conjugated to sulphate or glucuronide before being excreted into the urine or the faeces ([redacted]). In rats, sheeps and humans, as well as in *in vitro* metabolism studies using liver microsomes, melatonin is metabolised chiefly to 6-hydroxymelatin followed by conjugation with sulphate or glucuronide and excretion predominantly in the urine. In addition, 5-methoxyindoleacetic acid appears to be formed by de-acetylation of melatonin followed by de-amination ([redacted]). Following administration of different melatonin doses, plasma hydroxymelatonin and melatonin levels increased in a dose-dependent manner. Plasma 6-hydroxymelatonin always represented ~1 % of plasma melatonin, irrespectively of the administered dose of melatonin ([redacted]). *In vitro* metabolism studies with fresh liver slices from rats and human donors compared the initial rates of melatonin metabolism between the two species, suggesting that the intrinsic clearance of melatonin in humans may be lower than that in rats ([redacted]).

Melatonin is metabolised enzymatically or nonenzymaucally, yielding to the formation of various bioactive compounds such as *N*-acetylserotonin, 5-methoxytryptamine, *N,N*-dimethyl-

5-methoxytryptamine, 5-methoxytryptophol, cyclic 2-hydroxymelatonin, pinoline and 5-methoxylated kynuramines, which then result in a spectrum of effects, which exceed substantially those transduced by membrane receptors. Apart from enzymatic metabolism, non-enzymatic reactions with free radicals, particularly the superoxide anion and the hydroxyl radical, represent a significant aspect of the biological role of melatonin. Melatonin represents the most potent physiological scavenger of hydroxyl radicals, which suggests an essential role of this indoleamine for protection from hydroxyl radical-induced carcinogenesis and neurodegeneration ( ).

In an early study, following IP administration of radiolabelled melatonin in rats, 2 of the 3 identified distinct peaks corresponded to the glucoronic and sulphate conjugates of 6-hydroxymelatonin and the third compound was not completely characterised. It was further determined that the major metabolite accounting for 70-80% of the radioactivity was the sulphate conjugate of 6-hydroxymelatonin whereas the glucoronic acid conjugate represented only 5%. The unidentified metabolite corresponded to 12% of radioactivity When [^{14}C]-melatonin was injected intracisternally into rats, 2.35% of the total radioactivity in the urine was recovered as *N*-acetyl-formyl-5-methoxykynurenamine (II). In the case of IV melatonin administration, 15% of the total radioactivity was recovered as compound (II). In either case, 65% of the administered radioactivity was recovered in urine within 24 h. These results strongly indicate that the conversion of melatonin to compound II via N^1-acetyl-N^2-formyl-5-methoxykynurenamine (after melatonin degradation) represents one of the major metabolic pathways of melatonin in the mammalian brain (

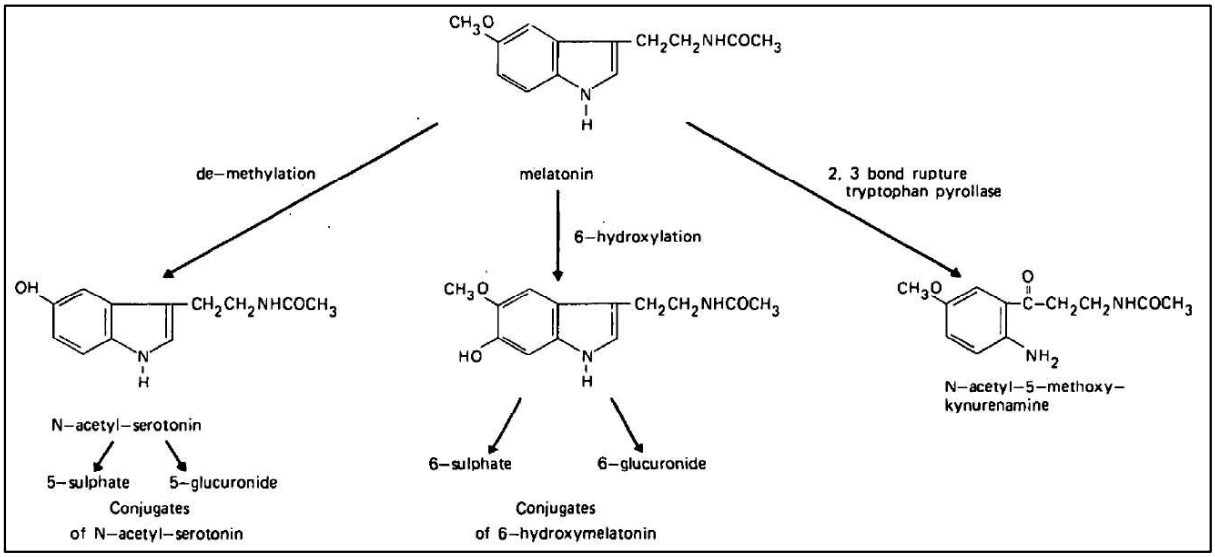

Figure 3. The three pathways of melatonin metabolism (Figure adopted from).

As mentioned, in the metabolic pathways of melatonin, 6-hydroxymelatonin and *N*-acetylserotonin sulphates are the most abundant metabolites accounting for over 90% of total melatonin metabolites in humans, indicating that sulfation plays an important role in reflecting the functions and clearance of melatonin *in vivo*. A study by) characterised melatonin sulphation using various human organ cytosols (liver, lung, kidney, small intestine, and brain), liver cytosols from 5 different animal species, and cDNA-expressed human sulfotransferase (SULT) for the first time. The results demonstrated that the liver, lung, kidney

and small intestine in humans had high catalytic efficiency for the sulphation melatonin; though, brain contained a very low reaction rate. Interestingly, organ cytosols prepared from females exhibited higher sulphation activity than those of males. SULT isoforms 1A1, 1A2, 1A3, 1B1 and 1E1 exhibited metabolic activities toward melatonin. SULT1A1 has been determined as the major enzyme responsible for melatonin sulphation. Furthermore, considerable species differences in melatonin sulphation have been observed; the total intrinsic clearance rate of this melatonin metabolic pathway was as follows: monkey> rat> dog> human> pig> mouse (

After SC implantation of melatonin for 5 months in sheeps, plasma levels of 6-hydroxymelatonin sulphate were also insignificant. On the other hand, both a single oral dose of melatonin (3 mg) and daily oral dosing in the same animal model increased the circulating levels of 6-hydroxymelatonin sulphate in the range of 150-1,500 pg/ml for at least 18 h. The profiles seen after 180-day treatment were similar to those seen after a single dose, indicating that this route of melatonin metabolism is not induced by chronic administration. IV injection of melatonin (200 or 20 μg) increased the detectable levels of the metabolite in the plasma. Therefore, the quantitative aspects of melatonin metabolism in sheep differ according to the route of administration (

2.4.3.4 EXCRETION

The main excretion route of melatonin metabolites is renal. In rats and rabbits administered labelled melatonin by IP injection or stomach tubes, 70% and 20% of the radioactivity was excreted in urine and the faeces, respectively (*)*. After oral administration of doses ranging within 10-80 mg/kg to dogs, melatonin was rapidly absorbed with a distribution phase of 3.5 h and t½β of 5 h. The fraction excreted in urine was 0.25% of the administrated dose during the first 5 h (. After oral and IV administration of melatonin in rats, dogs and monkeys, the apparent t½β of melatonin after an IV dose of 3 mg/kg (5 mg/kg in rats) was 19.8, 18.6, and 34.2 min, respectively, in rats, dogs and monkeys (*)*. The t½s seen in other studies were similar even though the doses employed were significantly lower than the 5-mg/kg within this study (1-100 μg). The calculated clearance values in this study indicate that the beable dog (3.84 L/h/kg) clears melatonin faster than the rat (2.11 L/h/kg) and the monkey (1.68 L/h/kg) (*)*. In another study, tritiated (32-80 μg; n=4 rats) or unlabelled melatonin (0.8-2.0 μg; n=4 rats) was infused intra-arterially into unanesthetised male Sprague-Dawley rats, data revealing t½s values of 23 and 17 min, respectively (

Results from *in vitro* brain microdialysis studies have also confirmed the rapid accumulation and clearance of extracellular melatonin in the vicinity of its putative target tissues (*)*.

2.4.3.5 PHARMACOKINETIC DRUG INTERACTIONS

Possible interactions of melatonin with concurrently administered drugs were investigated in *in vitro* studies utilising human hepatic post-mitochondrial preparations; similar studies were conducted with rat preparations to ascertain whether rat is a suitable surrogate for human. Drugs were selected based not only on the knowledge that the 6-hydroxylation of exogenous melatonin, its principal pathway of metabolism, is mainly mediated by hepatic CYP1A2, but also on the likelihood of the drug being concurrently administered with melatonin. Hepatic preparations were incubated with either melatonin or 6-hydroxymelatonin in the presence and

absence of a range of concentrations of the interacting drug, and the production of 6-sulphatoxymelatonin was monitored using a radioimmunoassay procedure. Of the drugs screened, only the potent CYP1A2 inhibitor 5-MOP impaired the 6-melatonin hydroxylation at pharmacologically relevant concentrations and is likely to lead to clinically significant interactions; diazepam, tamoxifen and acetaminophen did not impair the metabolic conversion of melatonin to 6-sulphatoxymelatonin at concentrations attained following therapeutic administration. 17-Ethinylestradiol appeared not to suppress the 6-hydroxylation of melatonin but inhibited the sulphation of 6-hydroxymelatonin, but this is unlikely to result in an interaction following therapeutic intake of the steroid. Species differences in inhibition of melatonin metabolism in human and rat hepatic postmitochondrial preparations were evident implying that the rat may not be an appropriate surrogate of human in such studies [redacted].

Antidepressants: Fluvoxamine is an inhibitor of several CYP isoenzymes. The effects of fluvoxamine on the metabolism of melatonin were studied *in vitro* using human liver microsomes and recombinant human CYP isoenzymes. Melatonin was found to be almost exclusively metabolised by CYP1A2 to 6-hydroxymelatonin and *N*-acetylserotonin with a minimal contribution of CYP2C19. Both reactions were potently inhibited by fluvoxamine, with a K_i of 0.02 μM for the formation of 6-hydroxymelatonin and 0.05 μM for the formation of *N*-acetylserotonin. Other than fluvoxamine, fluoxetine, paroxetine, citalopram, imipramine and desipramine were also tested at 2 and 20 μM. Among them, only paroxetine affected the metabolism of melatonin at supratherapeutic concentrations of 20 μM, which did not reach by far the magnitude of the inhibitory potency of fluvoxamine ([redacted]). In man, fluvoxamine has been shown to increase serum concentrations of orally administered melatonin (17-fold higher AUC and 12-fold higher C_{max}) ([redacted]). Hence, the combination should be avoided.

Caffeine: Concomitant consumption of caffeine whose metabolism is principally catalysed by CYP1A2, more than doubled plasma levels and increased the bioavailability of melatonin, by impairing its presystemic metabolism ([redacted]

Chloropromazine: Sprague-Dawley rats were injected IV with [^{3}H]-melatonin in doses ranging from 8 to 63.6 μCi; 30 min prior to melatonin injection, rats were treated with chloropromazine 20 mg/kg. Chloropromazine markedly enhanced the accumulation of tritiated melatonin by all the organs studied; the brain, ovary and heart each contained more than 3 times as much hormone as the organs of control animals, 30 min post-injection ([redacted]).

Cigarette smoking: Exogenous serum melatonin levels were suppressed by smoking, especially when the levels of the hormone were high. Polycyclic aromatic hydrocarbons, a class of carcinogenic compounds present in tobacco, up-regulate CYP1A2 expression leading to accelerated melatonin metabolism ([redacted]

CYP substrates: Increased CYP1A2 activity will lead to lower plasma levels and *vice versa*. For example, plasma melatonin levels were increased following fluvoxamine (a potent inhibitor of CYP1A2 and to a lesser extent of CYP2C19) administration, presumably by impairing its CYP-mediated metabolism. Moreover, drugs that are CYP2C19 substrates, such as fluvoxamine, quinolones, cimetidine, 5- and 8-MOP, omeprazole, lansoprazole and citalopram, increased the urinary excretion of 6-sulphatoxymelatonin in individuals receiving exogenous melatonin; presumably these compounds decrease the CYP219-mediated metabolism of melatonin to acetylserotonin [redacted] Co-administration of melatonin with CYP1A2 inducers, such as

carbamazepine, rifampicin and phenytoin, may result in reduced melatonin exposure through an increase in melatonin metabolism ([redacted]).

5-Methoxypsoralen (5-MOP): PK studies involving exogenous melatonin (1 or 100 µg) administration to 5-MOP-treated rats showed that MOP treatement induced an increase in the t½ of melatonin from 22 to 67 min. This delayed degradation of melatonin was the consequence of 5-MOP-induced inhibition of the 6-hydroxylation of melatonin. Under *in vitro* experimental conditions, 5-MOP did not affect melatonin synthesis and release ([redacted]). Previously, the effect of 5-MOP on plasma melatonin concentrations alone or in combination with isoproterenol (10 mg/kg, IP) or propranolol (20 mg/kg, SC) was studied in the rat. An increase in plasma melatonin concentrations was observed 1 h after psoralen administration and lasted 8 h. The stimulatory effect of 5-MOP on circulating melatonin concentrations was dose-dependent above 5 mg/kg. 5-MOP also potentiated the isoproterenol-induced rise in plasma melatonin levels. Furthermore, propranolol did not block the effect of 5-MOP, indicating that psoralen acts independently of the noradrenergic system controlling pineal melatonin synthesis. No increase in plasma melatonin levels was noted in pinealectomised animals after injection of 5-MOP (20 mg/kg) or 8-MOP (20 mg/kg) ([redacted]

Oestrogens: Melatonin downregulates the circulating levels of gonadal oestrogens and acts as an antioestrogen with mechanisms of action different to those of the commercially available anti-oestrogens and inhibits aromatase expression in human breast cancer cells. The metabolism by CYP1A1 isoenzymes is inhibited and CYP1A2 increases melatonin levels [redacted] . Caution should be exercised in patients on oestrogens (e.g., contraceptive or hormone replacement therapy), which increase melatonin levels by inhibiting its metabolism by CYP1A1 and CYP1A2 enzymes ([redacted] .

2.4.3.6 SPECIFIC PHARMACOKINETIC STUDIES

Age: Age-related changes in levels of melatonin and 6-hydroxymelatonin sulphate and effects of dietary melatonin on their levels in different tissues were determined in mice. Levels of melatonin were highest in the serum followed by liver, kidney, cerebral cortex and heart. Serum melatonin levels decreased with age and were reduced by 80% in 27- relative to 12-month-old mice. The levels of the melatonin metabolite 6-hydroxymelatonin sulphate were significantly higher than free melatonin in all tissues tested and highest in the cerebral cortex followed by the serum, heart, kidney and liver. In 12-month-old mice, the concentration of 6-hydroxymelatonin sulphate was approximately 1,000-fold greater than that of melatonin in the cerebral cortex and was only 3-fold greater than melatonin levels in the serum. Thus, only 0.1% of total melatonin in the brain was present in the free and unconjugated form, but the corresponding value for serum was 27.4%. The cerebral cortex had the highest levels of combined melatonin and 6-hydroxymelatonin sulphate than other tissue tested in control mice. There was no significant change in 6-hydroxymelatonin sulphate levels between young and old mice ([redacted]

Gestation: [redacted] measured plasma melatonin concentrations in pregnant ewes and fetal sheeps during 24-h periods between GDs 114 and 142. There was a clear diurnal rhythm in the plasma melatonin concentrations in both the ewe and fetus from GD 114. There was no gestational age trend in maternal or fetal day time plasma melatonin concentrations during late pregnancy. To establish whether there was transplacental transfer of melatonin, pregnant ewes were injected with [^{3}H]-melatonin and total radioactivity was measured in maternal and fetal arterial plasma and amniotic fluid collected before and for 1 h post-dose. Two minutes after [^{3}H]-melatonin injection, radioactivity was detected in both maternal and

fetal sheep plasma. The amount of [^{3}H]-melatonin in fetal plasma accounted for 48.0±7.2% of total radioactivity at 2 min post-injection. In one pregnant ewe infused with unlabelled melatonin (0.3 µg/ml/min for 20 min), maternal and fetal plasma melatonin levels increased within 6 min after initiation of infusion.

Lactation: In 4 Ayrshire cows at the beginning of the lactation period, the nocturnal rise in milk melatonin was moderate (from 7±2 pg/ml at noon to 15±1 pg/ml at night; mean±standard error of the mean [SEM]) and did not correlate well with the melatonin level in serum (from 7±2 to 27±7 pg/ml, respectively). On the other hand, 6 cows in a later phase of lactation showed a clear long-lasting nocturnal melatonin increase both in serum (from 9±1 pg/ml at noon to 26±3 pg/ml at night) and in milk (from 12±5 pg/ml to 26±7 pg/ml, respectively). Melatonin kinetics during lactation was further studied in 4 Ayrshire cows and 4 dairy goats by injecting an IV bolus of melatonin, using a 3-compartment model with melatonin elimination from the central compartment. The values (mean±standard deviation [SD]) for the cows and the goats were: $t_{½\beta}$=27±4 and 27±1 min, mean residence time (MRT) of 24±4 and 18±4 min, V_{ss} of 1.0±0.3 L/kg and 0.6±0.1 L/kg (P<0.05) and plasma clearance 0.044±0.004 and 0.035±0.011 L/kg/min, respectively. Following injection, melatonin concentration in milk increased rapidly and exceeded the corresponding serum level 15-30 min later, remaining thereafter above the serum level. Therefore, milk melatonin levels reflect blood concentrations of melatonin with a short delay ([redacted] .

Mid-light and mid-dark conditions: The distribution and disappearance of melatonin 10 µg or [^{3}H]-melatonin (76 ng) after IV injection were studied at mid-light and mid-dark, in male rats adapted under a photoperiod of 12-h light/12-h dark cycle for a minimum of 1 week. The distribution of melatonin followed a 2-compartment model, showing an initial distribution phase followed by an elimination phase. Its biological t½s at mid-light and mid-dark were similar but the V_d and metabolic clearance rate were greater at mid-dark than at mid-light. The estimated melatonin secretory rate at mid-dark was 5-fold greater than that at mid-light. When [^{3}H]-melatonin was injected, the t½ was significantly longer ([redacted]).

PK-PD study: C57BL/6 mice were given orally a supra-pharmacological dose of 250 mg/kg and a tissue distribution study was conducted in vital organs. PK analysis of melatonin revealed a T_{max} at 5 min with closely spaced another distinct C_{max} at 20 min. Plasma total antioxidant capacity (TAC) of melatonin showed similar peaks at 5 min and 45 min, with the highest TAC at 45 min. Survival following a lethal radiation dose was 20% and 40% after 5 and 45 min of melatonin administration, respectively. The drug radiation gap period (DRGP) for melatonin was thus 45 min, while optimal oral dose ranged from 125 to 250 mg/kg. PK parameters at 250 mg/kg dose were qualitatively similar to low dose of melatonin, thus, preventing chances of unexpected toxicity ([redacted]).

Rats with circadian system suppression: [redacted] demonstrated that prolonged constant light exposure modified the distribution (reduced V_{ss}) and elimination (reduced clearances) of a bolus injection of 1 mg/kg melatonin in rats, without modifying its $t_{½\beta}$. Only the administration of low doses (0.01 mg/kg/day) resulted in both a circadian pattern for 6-sulfatoxymelatonin excretion and normal physiological values during the infusion-free intervals.

2.4.4 TOXICOLOGY

Non-clinical data with melatonin reveal no special hazard for humans based on conventional studies of safety pharmacology, single- and repeated-dose toxicity, mutagenicity, genotoxicity and carcinogenic potential. A number of toxicology studies has been identified in the literature, including acute toxicity and single-dose toxicity data (in the rat, mouse and hamster), studies documenting repeated-dose toxicity in rats (3 months) and dogs (6 months), a combined toxicity/carcinogenicity study in rats (3-24 months), *in vitro* and *in vivo* genotoxicity/mutagenicity and reproductive toxicity in rats and rabbits. Effects have been observed only at exposures considered sufficiently in excess of the maximum human exposure indicating little relevance to clinical use. Overall data on melatonin-induced toxicity in various animal species are presented in the following Sections.

2.4.4.1 SINGLE-DOSE TOXICITY

The acute toxicity of melatonin has been studied following administration via different routes in mice and rats; the median lethal dose (LD_{50}) values of melatonin have been determined in both animal species for the oral and parenteral (IV, IP and SC) routes of administration. At high melatonin doses (>400 mg/kg), the most common adverse reactions observed elicited predominantly CNS (including piloerection, marked hypoactivity, ataxia) and cardiovascular (vasodilation of the extremities manifested as reddening of the ears and feet and muscle relation), with dose-related severity. Higher doses caused impairment of the righting, placing and hind limb ipsilateral flexor reflexes, a marked reduction in body temperature and slow, laboured respiration preceding death. Values were similar for both species except that oral administration of melatonin had less behavioural effect and was considerably less toxic in the rat than in the mouse. Importantly, the LD_{50} by the oral route was approximately 1,250 mg/kg in mice and >3,200 mg/kg in rats, which is greatly in excess of the maximum recommended daily dose of 6 mg in adults in proposed uses of melatonin. The main effects observed within these two species at high doses were sedation, lethargy and vasodilatation. Melatonin has been also found to induce considerable motor incoordination in mice at high doses. Orally and IP administered melatonin was most potent at 15 or 30 min post-dose. A subsequent rapid decline in potecy was noted due to the rapid metabolism of melatonin ([redacted]

Table 6. LD_{50} values for rodents after administration of melatonin via various routes.

Specie	Route	LD_{50} Value	Reference
Mouse	Oral	1,250 mg/kg	[redacted]
Mouse	IV	180 mg/kg	
Mouse	IV	472 mg/kg	
Mouse	IP	1,375 mg/kg	
Mouse	IP	>800 mg/kg	
Mouse	IP	1,168 mg/kg	
Mouse	SC	>1,600 mg/kg	
Mouse	SC (20W-I)	Lowest toxic dose=4,200 mg/kg	
Rat	Oral	>3,200 mg/kg	
Rat	Oral	Lowest toxic dose= 27 mg/kg	
Rat	IV	356 mg/kg	
Rat	IP	1,131 mg/kg	
Rat	SC	>1,600 mg/kg	
Hamster	SC (60D male)	Lowest toxic dose=12 mg/kg	

Abbreviations: IP, intraperitoneal; IV, intravenous; LD_{50}, medial lethal dose; SC, subcutaneous.

It has been shown that melatonin and the pineal gland peptides (epithalamine and epitalon) exert a correcting influence on the diurnal dynamics of norepinephrine in the medial preoptic area (MPA) and dopamine in the median eminence with arcuate nuclei (ME-Arc) disturbed by

single administration of the neurotoxic xenobiotic 1,2-dimethylhydrazine (DMH) in female rats. Experiments with DMH administration can be used as an animal model of female reproductive system premature aging. The investigation of epithalamine (a polypeptide preparation from the bovine pineal gland) effect on circadian rhythms disturbed by the neurotoxic compound DMH has shown a recovery of the diurnal dynamics of norepinephrine in MPA. In addition, norepinephrine was found to decrease from 9:30 till 11:00, circadian time, which was typical of control animals. Epitalon (Ala-Glu-Asp-Gly) proved to be more effective in ME-Arc. This peptide prevents the xenobiotic caused disturbance of DA diurnal rhythm, keeping this metabolite low at 5:00 circadian time with it having increased by 11:00 circadian time. The data suggest that the pineal gland is important for the circadian signal normalisation needed for gonadoliberin surge on the day of pro-oestrus. Melatonin and peptides of the pineal gland can be considered as effective protectors of female reproductive system from xenobiotics and premature aging ([redacted]).

In a PK-safety study, melatonin was evaluated in adult male Sprague-Dawley rats. Following a single IV injection at 5 or 15 mg/kg, plasma concentrations of melatonin increased to 39 and 199 μg/ml at 2 min and 128 and 772 ng/ml at 120 min. Within 60 min of injection, the BP, heart rate and body temperature remained unaffected. Melatonin at 5 mg/kg did not influence the complete blood counts at 60 min, but at 15 mg/kg, it had some effects on the differential white cell and platelet counts. Both melatonin doses slightly elevated some liver enzymes (aspartate aminotransferase [AST]) and total protein (P<0.05) at 60 min post-dose, while at 15 mg of melatonin/day, there was a significant increase in polymorphonuclear cells, a significant decrease in lymphocytes, mononuclear cells and platelets with a significant increase in plasma creatinine, AST and lactate dehydrogenase levels. At 24 h after completion of 6 daily injections of melatonin, there was a 5.5% reduction in body weight (observed with both melatonin doses). Gross post-mortem examination and histological examination of the brain, kidney, liver and spleen did not reveal any evidence of toxicity [redacted]

Photoirritation/ photosensitisation

In a photoirritation-photosensitisation study, the cells in two parallel plates were treated for 1 h with 0.032, 0.10, 0.32, 1.0, 3.2, 10, 32 and 100 μg of melatonin/ml. One of the parallel samples was treated for 50 min in absence, the other in presence of a non-toxic dose of ultraviolet (UV) A light. One day after treatment, cytotoxicity was analysed as a measure of reduction of neutral red uptake and compared to the untreated negative controls. The BALB/3T3 mouse fibroblast cell line treated with the test item did not show significant cytotoxic effects either in absence (–UVA) or in presence (+UVA) of UVA light. Relative cell viability in the –UVA experiment was 92%, in the +UVA experiment 79% compared to the untreated negative controls. Thus, no median effective concentration (EC_{50}) values could be determined and only a formal photoirritation factor (PIF) of 1 was calculated. The controls confirmed the validity of the study. The negative controls of the +UVA experiment showed a viability of 83% of the untreated negative controls. Overall, melatonin showed no phototoxic potential under the conditions of this study [redacted]

2.4.4.2 REPEAT-DOSE TOXICITY

Subchronic and chronic toxicity

In repeated-dose toxicity studies, melatonin was well-tolerated in the target species tested, such as sheep, rats (up to 24 months), dogs (6 months) and rabbits (2 weeks, pregnant and non-pregnant) ([redacted]). Mild treatment-related effects were observed in rats and dogs including clinical signs (salivation, forelimb paddling, raised tail, rooting in

cage bedding) and/or changes in body weight and food consumption at oral doses ranging from 50-200 mg/kg/day and at 8 mg/kg/day, respectively. Based on body surface area (BSA) and AUC (dogs only), these doses represented more than 100-900 times the exposure anticipated at the proposed clinical dose. Clinical pathology, urinalysis, ophthalmic and/or indirect cardiovascular measurements were generally unremarkable and inconsistent following 3 and/or 6 months dosing in both species. Chronic toxicity studies identified the liver, thyroid and kidney as potential target organs (Table 7).

Repeated-dose toxicity studies involving melatonin administration have been conducted mainly in rats via the oral, IV and SC routes. These studies have included significant doses of up to 200 mg/kg/day (orally) for 90 days, 15 mg/kg/day (IV) for 6 days ([redacted] and ~5 mg/kg/day (males) or ~7 mg/kg/day (females) (SC) for 28 days ([redacted] Although high parenterally administered melatonin doses (10-450 mg/kg) have sometimes elicited antioxidant effects in experimental animals *in vivo*, neither their long-term safety nor their effects on blood melatonin levels of the the animals have been characterised. In humans, if not in nocturnally active lab rodents, such high doses might ultimately impair sleep or various circadian rhythms, perhaps by downregulating melatonin receptors ([redacted]).

The duration of the pivotal toxicity studies (6-24 months) was adequate and employed appropriate animal numbers (10-50/sex/group in rats and 4/sex/group in dogs, 19-20/group in rabbits; reproductive toxicity) to support the long-term use in humans. Dose-selection, although not always clearly justified, was based on achieving exposures "significantly higher than that targeted for use in humans for sleep" and/or "minimal toxic effects" at the highest doses. However, the absence of any severe dose-limiting toxicity or dosing feasibility constraints in rats, dogs and rabbits suggests that higher doses could have been employed in these studies. Nonetheless, high doses employed in the pivotal toxicity studies were adequate based on toxicity (minimal toxic effects at high dose) and/or PK endpoints, where applicable (≥25 times ratio of animal/human plasma AUC). Large intersubject variability was evident in toxicokinetic data obtained for all species, including humans, however, this is unlikely to significantly diminish the large clinical exposure multiples determined in these studies based on AUC ([redacted]

Table 7. Repeated-dose toxicity studies in the mouse, rat and the dog with melatonin.

Duration	Specie	Route	Melatonin doses tested	Value	Effect	Reference
-	Mouse (aging BALB/c females, i.e., 15 months of age; New Zealand Black females at 5 months; C57BL/6 males at 19 months)	Oral (in drinking water)	Dark-cycle, night administration of 10 µg (dissolved to 0.01% in ethanol)/ml of tap water	-	Melatonin prolonged survival of BALB/c females from 23.8 to 28.1 months and preserved aspects of their youthful state. Similar results were seen in New Zealand Black females beginning at 5 months and C57BL/6 males beginning at 19 months. As melatonin is produced in circadian fashion from the pineal, pineals from young 3- to 4-month-old donors were grafted into the thymus of 20-month-old syngeneic C57BL/6 male recipients and a 12% increase in survival was noted. Prolongation of survival was seen on pineal transplant to the thymus in C57BL/6, BALB/cJ and hybrid females at 16, 19 and 22 months. The endogenous pineal of grafted mice was left *in situ*. Pineal grafted aged mice displayed a remarkable maintenance of thymic structure and cellularity. Preservation of T-cell-mediated function, despite age, is seen.	[redacted]
90 days	Rat (10 Long-Evans and Fischer 344/sex/ dose-group)	Oral (gavage)	0, 0.005, 0.05, 5.0, 50 or 200 mg/kg/day, for a total of 17 and 68 dosing days, for the Special Study Group and Core Groups, respectively	NOAEL=5 mg/kg/day (based upon the significant treatment-related AEs, being the coloured faces and the dilated uterus)	Dark-coloured faeces were seen at 50 and 200 mg/kg/day. No treatment-related individual organ weight changes were observed during the study. In the Fischer rats, a reduction in body weight gain was noted, though only in dosages ≥5 mg/kg/day. Clinical biochemistry: increases in T_3 and T_4 were observed at dosages ≥0.05 mg/kg/day (but not clinically significant as no concurrent effects on thyroid histopathology were noted). Cystic uterine endometrial hyperplasia was noted in a number of treated Long-Evans	[redacted]

Duration	Specie	Route	Melatonin doses tested	Value	Effect	Reference
					females, but also in their respective control group. Finally, one treatment-related finding in a 50 mg/kg/day treated Long-Evans female was a dilated uterus at necropsy.	
Combined 13-week study with a 4-week recovery period coupled to a 26-week toxicity+ 104-week carcinogenicity phase	Rat	Oral	0, 15, 75 and 150 mg/kg/day	NOAEL=15 mg/kg/day LOAEL=75 µg/kg/day	*13- and 26-week studies:* increased haemoglobin level and platelet counts were seen at 75 and 150 mg/kg/day treated animals; increased testes, prostate and epididymides weights in mid- and high-dosed males. *At 26 weeks:* macroscopically dark thyroid in several high-dose animals. Hepatic effects: Liver weights were slightly increased at 75 and 150 mg/kg/day in both males (10-13%) and females (8-10%) after 13-week dosing (reversible after the 4-week recovery period). To a lesser extent, liver weight increase was also observed in males (7%) but not females at 150 mg/kg/day after 26-week dosing. No remarkable changes in liver enzymes or macroscopic liver changes were seen at 13, 26 or 104 weeks. Minor centrilobular hypertrophy was detected in 9/10 (0/10 control) and to a lesser magnitude, 9/19 (0/20 control) males given 150 mg/kg/day melatonin after 13- and 26-week dosing, respectively. Liver hypertrophy was not seen in any treated females or male recovery animals, indicating reversibility; no microscopic liver changes were observed after 104 weeks dosing in either gender. Thyroid effects: 26-week treatment with melatonin was associated with dark thyroid at 75 (3/20 males, 1/20 females) and 150 (9/19 males, 7/20 females) mg/kg/day, correlated with agonal congestion/haemorrhage microscopically (findings not seen in controls). Increased dark and/or large thyroid was also observed in rats treated for 104 weeks at 75 and 150 mg/kg/day. Microscopically, treatment-related increases in both incidence and severity of thyroid pigment and thyroid follicular cell hypertrophy were evident at 75 and 150 mg/kg/day vs control animals. A slightly increased incidence of thyroid follicular cell tumours was also evident in male, but not female rats given 150 mg/kg/day for 104 weeks (7/50 = 14% vs 3/50 = 6% in both control groups). Renal effects: Kidney weights were marginally increased at 75 and 150 mg/kg/day in males (5-7%) and 150 mg/kg/day in females (7%) after 13-week dosing (reversible after 4-week recovery). A marginal kidney weight increase was noted in males (7%) only at 150 mg/kg/day after 26-week dosing. Organ weights were not assessed after 104 weeks. Macroscopic renal findings were limited to kidney pelvic dilatation in 2 female rats given 150 mg/kg/day for 13 weeks. Microscopically, a treatment-related increase in renal pigment incidence and a marginal increase in the severity of chronic nephropathy was seen in rats given 150 mg/kg/day for 104 weeks. The kidney was identified as a potential organ of long-term physiologic or metabolic adaptive change, at least in rats.	[redacted]
14 days	Rat (n=10/ sex/dose group)	Oral	0.0, 0.005, 0.050, 5.00, 50 and 200 mg/kg/day	NOAEL= 200 mg/kg/day	No melatonin-related clinical signs, no early deaths, no drug-related body-weight changes, no organ-weight changes, no gross lesions or histopathological findings were noted. Administration of melatonin did not result in dose-response related differences in retinal outer nuclear layer thickness means in any sex, strain and lighting treatment groups up to the highest tested dose.	[redacted]
16 months	Rat (adult CD rats, 11-13 months old)	Oral (in drinking water)	4 mg/L water (n=15) or vehicle (n=16); sacrifice at 29 months of age	-	Melatonin supplementation markedly increased the number of rats which survived to the age of 27-29 months (87%) while survival of the control group was in agreement with the life expectancy of the species (43%).	[redacted]
28 days	Rat (19 Sprague-Dawley rats/sex/ dose-group)	SC	0.050, 0.50 and 4.8 mg/kg/day for the males and 0.074, 0.75 and 7.3 mg/kg/day for the females (n=10/sex); and a sham control group (n=19 rats/sex)	NOAEL= 0.5 mg/kg/day on decreased testes weights and testicular degenerative changes	No deaths or changes in clinical observations; no drug-related effect in body weights, haematology, clinical chemistry, urinalyses or gross pathology. A dose-related trend of increasing serum melatonin levels occurred in both sexes. In males, there was a trend toward decreasing serum PRL levels with time at all melatonin doses. No difference in serum FSH levels occurred between treated groups. Most of the samples were at the limit of detection for the serum LH assay. A dose-related increase occurred in urine 6-sulphatoxymelatonin (the primary metabolite) levels in melatonin-treated male and female groups. No treatment-related organ weight or histopathology changes were noted in rats infused with the low- and mid-dose melatonin. 2/10	[redacted]

Duration	Specie	Route	Melatonin doses tested	Value	Effect	Reference
					males at high-dose melatonin had decreased testes weights and testicular degenerative changes composed of reduced or absent spermatogenesis, spermatidic giant cells, oedema.	
90 days	Rat	Not referred	0.3, 1.2 and 6 mg/kg/day	NOAEL=0.3 mg/kg/day LOAEL=1.2 mg/kg/day	Plasma concentrations were up to 40 pg/ml, which are lower than those expected to be reached in humans, but the time of sampling is not specified. The only melatonin-related effect reported was a decreased body-weight gain of the animals at mid (males) and high doses (males and females). Also, decreased testis and increased kidney relative weights were observed at high dose.	[redacted]
7 or 12 weeks	Hamster (adult male and female Syrian hamsters)	SC	(In the afternoon) melatonin 25 µg/day, i.e., equal to 257.2 µg/kg/day (body weight of hamster=97.2±11.7 g; Canadian Council on Animal Care), for 7 or 12 weeks	LOAEL= 257.2 µg/kg/day	Melatonin-treated animals exhibited splenic hypertrophy and extramedullary haematopoiesis in addition to a marked regression in testicular weight. The testicular regression, and the changes in spleen weight and histology could be prevented if the animals in short photoperiod were either pinealectomised or implanted SC with a pellet of 1 mg melatonin (once every 2 weeks). Female Syrian hamsters given afternoon injections of melatonin for 7 or 12 weeks had ovaries devoid of corpora lutea and reduced relative spleen weights compared to controls.	[redacted]
6 months	Dog	Oral	0.4, 1.5 and 8 mg/kg/day	A NOAEL for possible liver changes in dogs could not be clearly established.	Increased serum glucose levels were observed at some time-points of the study. Microscopic examination revealed pituitary gland and parathyroid cysts, adenomyosis of the uterus, capsular fibrosiderosis of the spleen and cytoplasmatic rarefaction of hepatocytes consistent with the presence of glycogen. Based on toxicokinetic data the C_{max} values obtained with the mid and high doses were high compared to the levels reached in humans. Doses up to 8 mg/kg/day had no remarkable effect on blood pressure or heart rate at 15-20 min post-dose at weeks 13 and 25. Melatonin exposure at 8 mg/kg/day was >120 times that expected at the proposed clinical dose. Hepatic effects: Liver weights were slightly increased with melatonin 8 mg/kg/day in males (11%) and dose-independently in 1.5 and 8 mg/kg/day in females (7-16%) after 6 months dosing. No remarkable macroscopic liver changes were noted, while chronic liver inflammation was observed in 4/8 dogs from all dose groups compared to 1/8 controls (for both genders combined). The no clear treatment-relationship in this species was possibly confounded by the small number of dogs and hence greater intra-group variability in parameters examined and the greater duration of exposure. Liver may be an organ of histopathological adaptive change, with a reduced activity at this organ with repeated dosing potentially linked to the induction of enzymes. Thyroid hormones: thyroid weights were slightly increased at melatonin 8 mg/kg/day in males (8%) and dose-independently at all doses (0.4-8 mg/kg/day) in females (5-19%) after 6 months of treatment, without remarkable macroscopic or microscopic thyroid changes.	[redacted]

Abbreviations: AEs, adverse effects; C_{max}, peak concentration; FSH, follicle-stimulating hormone; LH, luteinising hormone; LOAEL, Lowest-Observed-Adverse-Effect-Level; NOAEL, No-Observed-Adverse-Effect-Level; PRL, prolactin; SC, subcutaneous; T_3, triiodothyronine; T_4, thyroxine.

2.4.4.3 GENOTOXICITY

A full battery of genotoxicity tests has been performed with melatonin. Although the individual studies were generally not conducted specifically according to International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines and the GLP status was not reported, they consistently found no evidence of melatonin genotoxicity (neither mutagenicity or clastogenicity) [redacted]). The mutagenicity of melatonin and its major metabolite 6-hydroxymelatonin was evaluated by the Ames test (*Salmonella tyhimurium*), Single Cell Gel Eletrophoresis (COMET assays) or chromosomal aberrations (CAs) test (human lymphocytes). Results in the *in vivo* mouse micronucleus (MN) test were also negative ([redacted]). No other mutagenicity studies were carried out [redacted] . Many studies have shown that melatonin has an **antigenotoxic** effect in various tissues and cell lines. In the Comet assay, melatonin was

found to inhibit clastogenic effects when applied together with mutagenic substances [redacted]).

Bacterial reverse mutation test

Melatonin, at doses of 5, 50, 500 and 5,000 µg/plate, was reported negative in an Ames assay in *Salmonella typhimurium* strains TA97, TA98 and TA100 both with and without metabolic activation with rat liver S9 ([redacted]). The same study has also demonstrated that the major metabolite of melatonin, i.e., 6-hydroxymelatonin, as well as their microsomal metabolites are not mutagenic in the Ames test. In addition, melatonin was reported negative at doses up to 500 µg/plate in an Ames assay in *Salmonella typhimurium* strain TA100 without metabolic activation. The test was repeated after treatment with nitrite under acidic conditions to evaluate whether melatonin showed any potential to be nitrosated to a mutagen; after nitrite treatment, melatonin showed low mutagenic activity at doses of 100, 200 and 500 µg/plate in strain TA100 without metabolic activation ([redacted] . Within further *in vitro* mutagenicity tests conducted by [redacted] , including an Ames test with *Salmonella typhimurium* strains TA100 and TA102, melatonin (0.25-2 µmol/plate) itself revealed no genotoxic effect.

The so-called "WP2 Mutoxitest" is an assay under development that could be an analogue of the Ames test, involving the tester strain IC 203, deficient in OxyR, together with its $OxyR^+$ parent WP2 uvrA/pKM101 (denoted IC188). Using this assay, melatonin was tested at the concentration of 5,000 µg/plate with negative results ([redacted]). As melatonin was only tested without metabolic activation, the results can only be considered as indicative ([redacted]

In vitro and in vivo DNA damage

[redacted] evaluated the effects of chronic melatonin consumption on genotoxic and mutagenic parameters in 3-month-old Swiss albino male mice (n=240). The animals ere were divided into 8 groups and subdivided into 2 experiments; 1st (3 groups): natural ageing experiment; 2nd (5 groups): animals that started water or melatonin supplementation at different ages (3, 6, 12 and 18 months) until 21 months. After 21 months, the animals from the 2nd experiment were euthanised to perform the comet assay, MN test and western blot analysis. The results demonstrated that melatonin prolonged the lifespan of the animals. Relative to genomic instability, melatonin was effective in reducing DNA damage caused by ageing, presenting antigenotoxic and antimutagenic activities, independently of initiation age. The group receiving melatonin for 18 months had high levels of APE1 and OGG1 repair enzymes. Conclusively, melatonin presents an efficient antioxidant mechanism aiding in modulating genetic and physiological alterations due to ageing.

In another study, cells in human peripheral blood were treated *in vitro* with increasing concentrations of (0.5 or 1.0 or 2.0 mM) for 20 min at 37±1°C and then exposed to γ-radiation. The melatonin-pretreated lymphocytes exhibited a significant and concentration-dependent decrease in the frequency of radiation-induced chromosome damage as compared with the irradiated cells which did not receive the pretreatment ([redacted]examined the potential radioprotective properties of pharmacological melatonin doses in whole-body irradiated (150 cGy of ^{137}Cs γ-rays) CD2-F1 male mice. Whole-body irradiation resulted in a dramatic increase in the incidence of MN formation (~10-fold) in both tissues. Melatonin treatment significantly and dose-dependently attenuated this increase by less than 20%. These findings were asssociated with an antioxidative effect of melatonin that

protects "the genetic material of haematopoietic cells" from the damaging effects of acute whole-body irradiation.

In an *in vivo* study evaluating the of B16F10 melanoma cells and the effects of melatonin supplementation on genotoxic parameters in murine melanoma models, 32 male C57Bl/6 mice were treated as follows: PBS+vehicle (n=6), melanoma+vehicle (n=10), PBS+melatonin (n=6) and melanoma+melatonin (n=10). The melanoma groups received a B16F10 cell injection and melatonin was administered for 60 days. B16F10 cells effectively induced DNA damage in all tissues, whereas melatonin supplementation decreased DNA damage in the blood, liver, cortex and spinal cord. Melatonin exerts its anti-tumour activity via its antiproliferative, antioxidative and proapoptotic effects. As this result was not observed within the lungs, it was hypothesised that melatonin can induce apoptosis in cancer cells and this was not evaluated by comet assay ([redacted]).

Sister chromatid exchange (SCE)

Cells from human peripheral blood were cultured *in vitro* in the presence of 0.05-1.00 mM melatonin, 10^{-7} M mitomycin C (positive control) and 0.5% ethanol (solvent control) for 72 h at 37±1°C. Lymphocytes were examined for mitotic indices (MIs) and proliferation indices and for the incidence of SCE. The results indicate that the lymphocytes which were cultured in the presence of melatonin at concentrations of ≥0.20 mM exhibited a significant and concentration-dependent decrease in MI and alteration in proliferation kinetics. This was demonstrated by an increase in the frequency of lymphocytes in their first division, with a concomitant decrease in the 2nd and 3rd or later division cells. The incidence of SCE was similar in the lymphocytes exposed to 0.05-1.00 mM of melatonin and of untreated controls. Exposure of the cells to ethanol did not alter either the MIs or proliferation indices or the frequency of SCE. Lymphocytes treated with mitomycin C showed the expected decrease in MIs and proliferation indices, and an increased incidence of SCE ([redacted]

In another study, normal human peripheral lymphocytes were treated with 50 and 200 μM of melatonin *in vitro*. In the *in vivo* study, 20 volunteers were administered a single oral melatonin dose of 3 mg/day, for 2 weeks. After sufficient time for its clearance, 1.5 mg melatonin/day was given to the same volunteers for the same period. Both *in vitro* and *in vivo* results demonstrated the antigenotoxic effect of melatonin in human blood lymphocyte. *In vitro*, hypoxia increased the SCE frequency compared to the control and both doses of melatonin significantly decreased the SCE frequency in the hypoxic cells (P<0.001). *In vivo*, oral 3-mg melatonin administration significantly increased the frequency of SCE, yet a small increase of SCE by hypoxia was found. Oral 1.5-mg melatonin dosing caused no DNA damage but had an antigenotoxic effect ([redacted]

Genotoxicity studies and the protective role of melatonin

Bacterial reverese mutation assays

The effect of melatonin on 7,12-dimethylbenz[a]anthracene (DMBA)-induced mutagenicity was investigated in a bacterial reverse mutation test with preincubation, using the strains *Salmonella tiphymurium* TA100 and TA102, with and without metabolic activation. Melatonin was tested at the levels of 0.25-2 μmol/plate and inhibited DMBA mutagenicity in a dose-dependent manner. Melatonin did not show mutagenic activity by itself but a protective effect against the DMBA-induced lesions ([redacted]

[redacted]) investigated the effect of melatonin on the initiation of *N*-nitroso-*N*-methylurea (NMU)-induced carcinogenesis in rats and mutagenesis. Within the *in vitro* mutagenicity tests, an Ames test conducted using *Salmonella typhimurium* strains TA100 and TA102, melatonin itself revealed no genotoxic effect. No protective action of melatonin (at doses of up to 2 µmol/plate) towards NMU was found in the Ames test. Another *in vitro* test, the Single Cell Gel Electrophoresis assay (SCGE or COMET assay) was performed on Chinese hamster ovary (CHO-K1) cells. Melatonin itself revealed no genotoxic effect from this test. The SCGE assay showed a slight, but statistically significant (P<0.001), dose-related anticlastogenic effect of melatonin (10^{-10} to 10^{-7} M) was observed. Thus, melatonin may act as an anti-initiating hormone in NMU-induced carcinogenesis and possess anticlastogenic activity towards NMU in CHO-K1 cells.

In vivo MN assay

In an *in vivo* MN test, melatonin (5 mg/kg) was injected prior to a single LPS dose and thereafter at 6-h intervals up to 72 h. The number of MN polychromatic erythrocytes (PCEs) increased significantly after LPS administration both in cells from peripheral blood and bone marrow. Melatonin administration to LPS-treated rats highly significantly reduced MN formation in both peripheral blood and bone marrow cells beginning at 24 h after LPS administration and continuing to the end of the study. In blood, the increase in MN formation was time-dependent in the LPS-treated rats with peak values being reached at 36-48 h. It was assumed that the ability of melatonin to reduce LPS-related genotoxicity may be related to its antioxidant activity ([redacted]

Another *in vivo* MN test in mice ([redacted]) examined the protection by melatonin against paraquat-induced genotoxicity in both bone marrow and peripheral blood cells, using MN as an index of induced chromosomal damage. Melatonin (2 mg/kg) or saline were injected IP into mice 30 min prior to IP injections of paraquat (2×15 mg/kg, given with a 24-h interval) and thereafter at 6-h intervals to the end of the study (72 h). The number of MN in PCEs per 2,000 PCEs (1,000 PCEs/slide) per mouse was counted both in blood and bone marrow, and the ratio of PCEs to normochromatic erythrocytes (NCE) (PCE/NCE) was calculated. Paraquat treatment increased the number of MN-PCE at 24, 48 and 72 h, both in peripheral blood and bone marrow cells, while no differences were observed in the PCE/NCE ratio. Melatonin inhibited the paraquat-induced increase in MN-PCE by >50 % at 48 and 72 h. The free radical scavenging ability of melatonin was proposed as the most likely mechanism of action. In a subsequent MN-PCE assay, melatonin (10 mg/kg) or saline were administered IP to mice 30 min prior to an IP injection of paraquat (2 × 20 mg/kg), and at 6-h intervals until the conclusion of the study (72 h). The number of the MN-PCEs increased after paraquat administration both in peripheral blood and bone marrow cells. Melatonin dosing to paraquat-treated mice significantly reduced MN formation in both peripheral blood and bone marrow cells; these differences were apparent at 24, 48 and 72 h after paraquat dosing. The induction of MN was time-dependent with peak values occurring at 24 and 48 h. The melatonin-induced reduction in paraquat-related genotoxicity is likely due in part to the antioxidant activity of the indole structure. No effects of melatonin over paraquat in paraquat+melatonin groups incubated at 0, 60 and 120 min were noted ([redacted]).

In another study, blood samples collected from 5 volunteers were incubated with melatonin at different concentrations (100, 200, 300 and 400 µM) for 1 h and were then incubated with 750 µM diazinon (DZN) for 1 h. Subsequently, the lymphocytes were cultured with a mitogenic stimulant to evaluate MN formation in cytokinesis-blocked binucleated cells. The incubation of lymphocytes with DZN induces additional genotoxicity. Pretreatment with melatonin at these doses significantly reduced the MN frequency in cultured lymphocytes (P<0.05 to

<0.0001). The maximum decrease in the frequency of MN was observed at 400 μM of melatonin, which caused a reduction of 87%. Melatonin also exhibited an excellent and dose-dependent radical-scavenging activity against 1,1-diphenyl-2-picrylhydrazyl free radicals ([redacted]).

Another study evaluated the protective effects of exogenous melatonin in rats and their offspring on the genotoxic response induced by chronic alcohol consumption during pregnancy. Twenty-five pregnant rats were divided into: NC, negative control; ET, rats receiving ethanol (3 g/kg/day); ET+10 M, rats receiving ethanol (3 g/kg/day) and melatonin (10 mg/kg/day); ET+15 M, rats receiving ethanol (3 g/kg/day) and melatonin (15 mg/kg/day); and PC, positive control (40 mg/kg cyclophosphamide). The dams and 10 pups (n=5/sex) from each group were used to evaluate the frequency of DNA damage by the comet assay and the MN test. The results demonstrated a significant increase in DNA damage in the blood and liver cells of dams receiving ethanol and their offspring as well as in the brain of these neonates. Treatments with melatonin (10 and 15 mg/kg/day) significantly reduced the genotoxicity caused by ethanol in the blood of dams and neonates (both sexes), liver of dams and male offsprings, and in the brain of female offsprings. It was shown that only the female offspring exposed to maternal alcohol consumption showed a higher frequency of MN in PCEs ([redacted]

In vitro CAs and DNA damage

The protective role of melatonin on radiation-induced DNA damage in human lymphocytes has been investigated by studying the chromosomal rearrangement on metaphases stained with the fluorescence plus Giemsa technique. Human peripheral blood lymphocytes, treated *in vitro* with increasing concentrations (0.5, 1.0 or 2.0 mM) for 20 min at 37±1°C and then exposed to *γ*-radiation, exhibited a significant and concentration-dependent decrease in the frequency of radiation-induced chromosome damage as compared with the irradiated cells which did not receive the pretreatment. The extent of the reduction in radiation-induced chromosome damage observed with 2.0 mM melatonin was similar to that found in lymphocytes pretreated with 1.0 M dimethylsulfoxide (DMSO), i.e., a known free radical scavenger. Melatonin at 2.0 mM (a 500× lower concentration) was as effective in decreasing the radiation-induced chromosome damage as DMSO at 1.0 M ([redacted] melatonin itself was not found to be clastogenic. This study did not allow detecting whether the mechanism of action of melatonin involved scavenging free radicals or activating repair enzymes, neither did it provide information on the primary DNA damage. The CA assay indicated the protective effect of melatonin on *γ*-radiation-induced damage in human lymphocytes.

A significant dose-dependent increase in CA and SCEs was shown in human lymphocyte cultures treated with carbamazepine (4-12 μg/ml). The MI and proliferation indices were also found to be decreased but only significantly in case of high doses of carbamazepine (12 μg/ml). Pretreatment of human lymphocytes with melatonin (0.5 mM) exhibited a significant decrease in the frequencies of carbamazepine-induced CA and SCEs as compared with non-treated cultures. The depressed mitotic and proliferation indices were also found to be improved in melatonin-pretreated cultures ([redacted]

Normal human cultured peripheral lymphocytes were treated with melatonin (100, 200 and 400 μM) and melphalan (330, 490 and 650 nM) and incubated for 72 h prior harvesting. The results demonstrated the protective effect of melatonin on cells treated with melphalan *in vitro*. The greatest protective effect of melatonin at 100 and 400 μM was illustrated against 330 nM of melphalan in comparison with all other doses of the latter[redacted]

Lead exposure at doses of 50 and 100 mg/kg/day without melatonin caused high levels of DNA damage, induced apoptosis and altered DNA repair. Melatonin (10 mg/kg/day) co-treatment did not attenuate the effects of lead at 100 mg/kg/day, indicating that the effect of melatonin on GSH reduction is not sufficient to reduce the genotoxic effects of lead at this high dose. The protective action of melatonin against lead toxicity is dependent on the dose of lead [redacted]

[redacted] evaluated the effect of melatonin on the genotoxic activity of irinotecan in healthy human lymphocytes and a lung cancer cell line (A549) and a colorectal adenocarcinoma cell line (HT29) *in vitro*. Irinotecan, as a single agent, was shown to induce DNA damage in all types of analysed cells. The combination of melatonin at 50 μM with increasing doses of irinotecan (7.5, 15, 30 and 60 μM) resulted in an increase in the amount of DNA damage in A549 and HT29 cancer cells but was not effective in inducing DNA damage in healthy human lymphocytes. Analysis of the efficacy of DNA repair, performed after 60 and 120 min, post-incubation, showed the gradual decrease of DNA percentage in comet tails during repair postincubation in all experimental samples.

HepG2 cells were co-treated by melatonin as a genoprotective and silver nanoparticles (NPs) as a carrier against genotoxicity of mitoxantrone (anticancer drug). The results were analysed based on the Comet assay method and the genoprotective effect of melatonin was investigated in presence and absence of mirrors against the genotoxicity of mitoxantrone. Additionally, the autooptic effect was investigated in presence of Ag NPs. The results indicated that Ag NPs with lower concentrations of melatonin made more protection as genoprotective agent, and the same results were obtained by increasing cells' access to drug [redacted]

Laser irradiation-induced phototoxicity has been intensively applied in clinical photodynamic therapy for the treatment of a variety of tumours. It has been shown that, similar to vitamin E, the antioxidant melatonin (100 μM) was able to largely attenuate laser irradiation-induced mitochondrial reactive oxygen species formation and to prevent apoptosis when applied 30 min before irradiation [redacted]

2.4.4.4 CARCINOGENICITY

Relevant information suggests lack of genotoxicity of melatonin, following testing in the required non-clinical systems, no clear evidence of carcinogenicity in animal studies and lack of evidence of carcinogenicity in epidemiological data. Toxicity studies in different species and with varying treatment durations have not demonstrated any carcinogenic potential of melatonin. In addition, it is considered relevant that numerous *in vitro*, *in vivo* non-clinical and *ex vivo* clinical studies have found melatonin to reduce the potency of known mutagens and carcinogens [redacted] Such representative *in vitro* and *in vivo* studies supporting the protective effects of melatonin have been summarised in Tables 2 and 3, in the subsection *'2.4.2.2 Secondary pharmacodynamics'*. Also, short-term studies in mice (10 μg topical application for 14 days) and rats (20 mg/L in drinking water for 3 days; 100 mg/L in drinking water for 28 days) showed further evidence of the protective effect of melatonin against known carcinogens [redacted] However, an earlier study reported a slight increase (26%) compared with 18% in controls) in the frequency of transplacental neoplasms in the offspring of C57BL/6 mice injected SC with melatonin [redacted].

Spontaneous tumour incidence following melatonin administration was studied in an animal model to human breast cancer, C3H/Jax mice. A group of 39 mice received melatonin in drinking water around the clock (25 μg/day from day 21 to 44; 50 μg/day from day 45 to

sacrifice at 1 year). Melatonin was reported to modulate the degree of development of mammry epithelium and significantly reduced spontaneous mammary tumour incidence; 62.5% of controls developed tumours vs 23.1% in the melatonin-treated mice ($P<0.02$) [redacted]

In a short-term study conducted by the US-NTP, the effect of oral melatonin administration (50-200 mg/kg) on mammary tumourigenesis in transgenic female mice with the c-neu breast cancer oncogene was examined. However, the ability of melatonin itself to induce neoplasms in any organs or tissues was not investigated in this study; also, animal numbers (6/group) and treatment duration (6 months) were limited. Parameters examined were confined to body weight, food consumption, survival and effects on mammary tumour growth and development. Body weights were significantly lower in the melatonin groups given 100-200 mg/kg/day, with mortality considerably higher in the melatonin group given 200 mg/kg/day (8/16 survivors compared with 12/16 controls). Melatonin delayed the appearance of palpable tumours and inhibited the growth of mammary tumours dose-dependently. However, it should be noted that the reduced survival in the 200-mg/kg group may have influenced tumour development if younger animals were included in this evaluation [redacted]

In a pivotal toxicity/carcinogenicity study in rats (Table 7), the slightly elevated incidence of pituitary adenomas and thyroid follicular cell adenomas observed in male rats given 150 mg/kg/day oral melatonin for 2 years, would appear to be of limited clinical concern, given their increase in a single gender only, common occurrence at levels within available historical control data (pituitary adenomas) or lack of statistical significance and plausible mechanism of action (thyroid adenomas). Given the tumour incidence was only examined in the high dose (150 mg/kg/day) and the control groups, a NOAEL could not be clearly established. Nonetheless, this dose represented a 700-times margin over the proposed clinical dose, based on BSA. In addition, plasma samples were analysed for thyroid-stimulating hormone (TSH), T_3 and T_4 in the day 1, week 13, 78 and 104 for the control, the 15-, 75- and 150-mg/kg groups. Overall, there appeared to be no remarkable difference in TSH, T_3 and T_4 levels in animals dosed with up to 150 mg/kg/day compared with control values at the various sampling time-points. However, the sponsor noted an almost 2-time increase in mean TSH levels for day 91 (week 13; 2,243 pg/m,) compared with day 1 (1,132 pg/m,) values for the three male rats sampled at the 150 mg/kg dose. These males also had "minimal centrilobular hypertrophy with minimal to slight inflammatory cell foci". It was stated that "the available TSH data, although limited in terms of animal numbers, clearly supports the mechanism of action: liver enzyme induction leading to accelerated metabolic elimination of T_4 and consequent TSH release." However, "no result" was available for female rat TSH levels at day 1 and 91 for comparison. Moreover, there was no increase in T_3 or T_4 values at any dose, nor were any consistent increases in TSH at the lower (15 and 75 mg/kg) doses observed between day 1 and 91 ([redacted]).

2.4.4.5 REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Fertility: Possible implications of melatonin in reproduction have been reviewed. Several rat studies showed that sexual maturation was delayed, however not prevented, in young female and male rats after SC administration of melatonin for a treatment duration ranging from several days to several weeks. In humans, sexual maturation and the reproductive cycle are not dependent on season (photoperiod), which makes it unlikely that melatonin plays a significant role in the development of sexual maturity in humans. No AE of melatonin was found on sexual behaviour in two rat studies. Embryonic *in vitro* studies in the rat, the mouse and the pig did

not indicate any AE of melatonin on *in vitr*o fertilisation and early embryonic development ([redacted]

Melatonin can not be considered a pro- or anti-gonadotrophic hormone, since its action differs among species. Even within the same animal specie, the effect of melatonin may be different depending on the time, the length and in a lesser extent, the quantity of its secretion or administration. Melatonin probably can not be easily assimilated to other classical hormones. Its action is very likely widespread and does not necessarily require the presence of receptors. Probably, melatonin can be considered as a fine tuner of all endogenous biological functions, including those relevant to reproduction. Its circadian rhythm of secretion probably creates an ideal synchrony among functions involved in reproductive processes. Departure from this optimality, consequent to modifications of the melatonin message, induced by seasons or physiopathological states, may create the conditions for less favourable reproductive situations. Therapeutic applications of melatonin to animal or human reproduction should probably focus into the adaptive and optimising capabilities of this hormone rather than in trying to translate to a specie the effect exerted by melatonin in another specie ([redacted]).

Table 8. Dose, frequency and route of administration of exogenous melatonin in female and male cats and associated reproductive outcomes (Table adopted from [redacted]

Sample size (n)	Dose and route of administration	Cycle stage at initiation of treatment	Outcome
16 females	5 mg q48h SC	Not stated	Suppressed ovarian activity even under a 24 h light photoperiod for 60 days
6 females	30 mg q24h PO 3 h before lights off for 35 days	Not stated	Returned to estrus 33 ± 2.8 days (range 21–40 days) after treatment ended
4 females	60 mg (five 12 mg implants)	Not stated	Suppressed estrus in 75% (duration of estrus suppression not given)
9 females	18 mg (single implant)	During interestrus	Within 3–9 days of implant insertion, 33% had superficial cells present on their vaginal cytology and estrous behavior for 2 days followed by estrus suppression for 4 months
9 females	18 mg (single implant)	During estrus	Within 9–11 days of implant insertion, 78% had superficial cells present on vaginal cytology and estrous behavior for 2–3 days followed by estrus suppression for 2 months
12 females	4 mg/cat q24h PO until onset of estrus	During interestrus	Prolonged interestrus (interval between onset of treatment and first estrous cycle was 50 ± 6.1 days)
17 females	18 mg (single implant)	During interestrus	Prolonged interestrus (interval between onset of treatment and first estrous cycle was 51 ± 4.7 days)
4 males	18 mg (single implant)	Not applicable	100% significantly decreased their sperm quality until 120 ± 15 days after implant insertion
12 females	18 mg (single implant)	Prepubertally	Did not delay puberty
10 females	4 mg/cat q24h PO until onset of estrus	Prepubertally	Did not delay puberty

SC = subcutaneous, PO = oral

Reproduction, pregnancy and embryo/fetal-neonatal development: Several studies in mice and rats have shown that melatonin had no toxic effect on embryo/fetal development. No maternal deaths occurred. None of the rodent studies found any effect of melatonin on the morphological development of embryos and fetuses *in utero*. A study in pregnant rats did not show direct or indirect harmful effects with respect to pregnancy, fetal survival or fetal development. After IP administration of a single large melatonin dose to pregnant mice, fetal body weight and length tended to be lower, possibly due to maternal toxicity. Delay in sexual maturation in male and female offspring of the rat and ground squirrel occurred upon exposure to melatonin during pregnancy and postpartum (Table 9). These data indicate that exogenous melatonin crosses the placenta and is secreted in milk and may influence the ontogeny and activation of the hypothalamic-pituitary-gonadal axis. As the rat and ground squirrel are

seasonal breeders, the implications of these findings for humans uncertain ([redacted] . Information on melatonin use in sheep during pregnancy indicated that it was not teratogenic in this species ([redacted]).

As already mentioned, the reproductive system is a notable target for melatonin as it actively participates on reproductive physiology and regulates the hypothalamus-pituitary-gonads axis, influencing gonadotropins and sexual hormones synthesis and release. For its antioxidant properties, melatonin is also vital for the quality and viability of the oocytes and spermatozoa and for the blastocyst development. Maternal pineal melatonin blood levels increase during pregnancy and triggers the maternal physiological alterations in energy metabolism both during pregnancy and lactation to cope with the energy demands of both periods and to promote adequate mammary gland development. Moreover, maternal melatonin freely crosses the placenta and is the only source of this hormone to the fetus. It importantly times the conceptus physiology and influences its development and programing of several functions that depend on neural and brain development, ultimately priming adult behaviour and energy and glucose metabolism ([redacted]).

Pre- and postnatal development studies: Published data on potential effects of melatonin on pre- and postnatal development are limited and the implications of these findings for humans are uncertain ([redacted]

Table 9. Fertility, reproduction and developmental toxicity studies with melatonin in various animal species via different routes of administration.

Specie	Route	Melatonin doses	Endpoint/ Value	Effects	Reference
Fertility impairment					
Mouse (16 CD-1 females/ group)	IP	100 µg, i.e., ~3-4 mg/kg/day, for 19 days before and during cohabitation, until mating or until 2 weeks had elapsed	LOAEL=3 mg/kg/day	Disruption of the normal oestrous cycle (longer cycles), primarily due to the greater number of days spent in dioestrous. The proportion of mated females delivering was decreased for melatonin-treated mice (7/16 vs 13/16 for controls) but litter size from fertile matings was not affected.	[redacted]
Mouse (C3H/HeN) and Rat (Sprague-Dawley)	IP	20 µg/day to immature rats, for 28 days; 10 µg/day to adult rats, for 4-8 weeks; 1 or 10 µg/day for 2-3 weeks, in PINX rats and in mice	LOAEL=1 µg/rat/day	Microgram doses of melatonin decrease the incidence of vaginal smears that demonstrate oestrous phases in Sprague-Dawley rats and C3H/HeN mice. The minimum effective dose of melatonin is decreased when daily melatonin injections are started prior to gonadal maturation. Pinealectomy is followed by an increase in the incidence of oestrus; this increase is inhibited by melatonin treatment.	[redacted]
Rat (Wistar males)	SC	0.8, 2.4, 4.8 or 8.0 mg/kg/day, for 30 days	NOAEL=4.8 mg/kg/day LOAEL=8 mg/kg/day	Melatonin may have an inhibitory action on rat prostate only at 8 mg/kg; decreased prostate weight but not testes or other reproductive organs; no effect on testosterone levels in testes and serum nor on the conversion rate of [^{3}H]-testosterone to [^{3}H]-dihydrotestosterone in prostate; a significant decrease in activity of acid phosphatase and uptake of [^{3}H]-testosterone by the prostate.	[redacted]
Rat (Wistar males)	SC	3.0 or 8.0 mg/kg, for 30 days	NOAEL=3.0 mg/kg/day LOAEL=8.0 mg/kg/day	Melatonin inhibited the reproductive behaviour of male rats following melatonin treatment compared with vehicle-treated and untreated PINX rats. 5/12 rats given 8 mg/kg melatonin did not copulate (compared to 2/12, 1/12 and 0/12 in 3 mg/kg, vehicle control and untreated PINX groups, respectively).	[redacted]
Rat	Intracardial injection	0.3, 0.625, 1.25, 2.5 or 5.0 mg during the critical period of pro-oestrus (2-4 p.m.)	LOAEL=0.625 mg/day	Melatonin dosing of 1.25-5 mg during the critical period of pro-oestrus to rats resulted in complete inhibition of ovulation during presumptive oestrus. All animals given the vehicle shed an average of 10 ova. The inhibition can be overcome by IV injection of 10 µg LH, thereby suggesting that melatonin is not acting at the ovarian level. Results of radioimmunoassay of serum LH levels in melatonin- and vehicle-treated animals indicate that multiple injections of melatonin prevented the release of LH and inhibited ovulation. However, the same doses	[redacted]

Specie	Route	Melatonin doses	Endpoint/ Value	Effects	Reference
				given before or after the critical period on the day of pro-oestrus did not affect the incidence of ovulation.	
Hamster (Syrian hamsters of both sexes)	SC	Hamsters were maintained on a long photoperiod (14L:10D) and given melatonin 10-25 µg/day, i.e., equal to 102.9-257.2 µg/kg/day (body weight of hamster=97.2±11.7 g; Canadian Council on Animl Care)	LOAEL=102.9 µg/kg/day	Males that received melatonin in the afternoon showed regressed testes and decreased levels of serum LH and FSH after several weeks of treatment. Injections of the oil vehicle or melatonin given in the morning had no detectable effect on testicular size or on serum gonadotropins. Females which received melatonin during the afternoon became acyclic after several weeks of treatment and showed a diurnal pattern of LH secretion. The acyclic females required 4-6 weeks to resume oestrous cyclicity after termination of the melatonin injections. Melatonin effects on gonadal function and on serum gonadotropin levels in both sexes were similar to the previously observed effects of prolonged exposure to short photoperiods.	[redacted]
Golden hamster (females; 8-9 weeks of age)	SC	Single injections of melatonin 0 or 25 µg every 4th day (or else 64.3 µg/kg/day; body weight of hamster= 97.2±11.7 g; *Canadian Council on Animl Care*), given 15 min before lights-out (14L:10D), during either the early di-oestrous (day 1) or pro-oestrous (day 4) phase of oestrous cycle	LOAEL=64.3 µg/kg/day	Hamsters which received melatonin only on the evening of pro-oestrus became anovulatory by 3 weeks of treatment, while those that were injected with melatonin during di-oestrus, or oil on either day 1 or 4, continued to exhibit normal oestrous cycles. These results indicate that quartan injections of melatonin can suppress reproductive function in female hamsters, and that the effectiveness of the injections may be dependent upon the stage of the oestrous cycle at which they are administered.	[redacted]
Deer (Red deer stags [*Cervus elaphus*])	SC implant	Exp. 1 (n=24 stags): 2 groups treated with 3 implants (18 mg melatonin in a 4-mm^3 coated pellet implant)/stag each month from 8 November to 5 February (EM) or 9 December to 5 February (LM), 1 untreated group of control stags remained with the melatonin-treated stags and the other untreated control group remained isolated. Exp. 2 (n=30 stags): 4 groups treated with 3 implants/stag at monthly intervals for 6 months from 22 June (J), 4 August (A), 16 September (S) and 23 October (O), a further group of stags was treated in the same manner for 12 months from 22 June (Y), and the remaining group was untreated (C).	-	**Exp. 1:** Melatonin treatment advanced the seasonal changes in scrotal circumference, liveweight, antler state and coat type compared with control stags. The extent of advancement was greater in EM than LM stags. In EM and LM stags, size of testes regressed rapidly, and antlers were cast shortly after melatonin implants became exhausted in March. This was followed by an additional antler cycle and reproductive development and decline from June to November. EM and LM stags became synchronised with control stags 14-15 months after melatonin treatment began. The extra cycle of seasonal changes was more pronounced in EM than in LM stags. **Exp. 2:** Compared to controls, testicular regression and antler casting was delayed in Groups J, A and Y. These events occurred at the same time as in control stags in Groups S and O. Subsequent reproductive development was advanced in Groups S and O and delayed in Groups J, A and Y. The results demonstrated that treatment with melatonin implants in November or December advanced reproductive development. When stags were treated with melatonin implants from June to August, reproductive development was delayed, indicating a change in response to melatonin treatment during the year. The change in response to melatonin treatment between late winter and early spring was interpreted as a resetting of an endogenous circannual rhythm caused by a photoperiodic cue responsible for initiating the final stages of reproductive regression.	[redacted]
Deer (23 adult females red deer [*Cervus elaphus*])	SC implant	Treatment 1 (n=6): 2 SC melatonin implants at monthly intervals from 2 October (i.e., about 80 days before predicted parturition) to 1 February (about 150-day treatment). Treatment 2 (n = 6): 2 melatonin implants/month from 2 November (i.e., ~40 days before predicted parturition) to 1 February (about 120-day treatment); Treatment 3 (n=5): 2 implants/month from parturition (early December) until 1 February (about 90-day treatment); Treatment 4 (n = 6): controls. The stag received the same treatment protocol as hinds in treatment 1. The implantation schedule was	Not established	Calving occurred between 28 November and 24 December, with no significant differences among treatments (P>0.10). Hinds in treatment 1 exhibited significant retardation of mammary gland development and live-weight gain leading up to parturition (P<0.01). Furthermore, sex-adjusted calf birth weights were on average 3 kg lighter for treatment 1 (P<0.05), with all calves either removed for bottle-rearing or having died within a few hours of birth. Failure of lactogenesis in treatment 1 was characterised by the presence of underdeveloped, hard mammary tissue devoid of expressible milk. Hinds in treatments 2-4 all exhibited full lactation and successfully reared their calves, and there were no significant differences in calf weaning weight and growth rates. Likewise, there were no significant differences in mean live-weight or lactation score profiles. Mean plasma PRL concentrations varied significantly between treatments (P<0.05), and control hinds exhibited a marked seasonal pattern of secretion	[redacted]

Specie	Route	Melatonin doses	Endpoint/ Value	Effects	Reference
		based on previous studies on red deer that demonstrated effective delivery of physiological (night-time) plasma melatonin levels (50-200 pg/ml) for periods of 30-40 days.		which reached a peak at calving. However, hinds in treatments 1 and 2 failed to show any discernible seasonal increase in mean plasma PRL concentrations, whereas there was a marked increase in mean PRL levels in hinds in treatment 3 up to parturition, but concentrations decreased rapidly thereafter relative to those of control hinds. Melatonin treatment significantly advanced the date of first oestrus and decreased the postpartum-oestrus interval (P<0.05).	
Reproductive/ Developmental (fetotoxicity) studies					
Rat (time-mated, Sprague-Dawley-derived [CD] rats; GLP study)	Oral (gavage)	Screening study: 1, 10, 100, 150 or 200 mg/kg/day (15 rats/group), through GDs 6-19; sacrifice on GD20	Maternal toxicity LOAEL=100 mg/kg/day; Developmental toxicity NOAEL=200 mg/kg/day	Maternal toxicity: no maternal morbidity/mortality; aversion to treatment (≥100 mg/kg/day) and reduced maternal weight gain (≥150 mg/kg/day) were noted. Fetotoxicity: reproductive/endocrine parameters and fetal development were not affected.	
		Definite study: 50, 100 or 200 mg/kg/day (25 rats/group), on GDs 6-19; sacrifice on GD20	Maternal toxicity NOAEL=100 mg/kg/day and LOAEL=200 mg/kg/day; Developmental toxicity NOAEL ≥200 mg/kg/day	Maternal toxicity: no maternal morbidity/mortality; aversion to treatment at ≥50 mg/kg/day; mild sedation, reduced maternal food intake and body weight gain during initial treatment with 200 mg/kg/day. Fetotoxicity-teratogenicity: no effect on prenatal survival, fetal body weight nor incidences of fetal malformations/variations.	[redacted]
Rat (n=1-20 female Wistar rats/group)	SC	0 or 2.5 mg/kg/day throughout gestation, 2 h before the end of the light phase under a constant photoperiod (12:12, lights off at 12:00)	LOAEL=2.5 mg/kg/day	Melatonin has shown altered reproductive maturation of female offspring. At birth, litters were standardized to 12 offspring per litter. Vaginal opening was significantly delayed in female offspring of melatonin-treated vs control rats (mean: 40.63 vs 37.25 days, respectively). On the day of vaginal opening, lower LH levels were observed in the melatonin group, but no effects were noted for birth weight, melatonin levels, organ weights (absolute or relative for ovary, pineal, and pituitary) or % offspring in each phase of the oestrous cycle.	[redacted]
Rat (Wistar)	SC	Groups: (A) control, (B) melatonin treated (250 µg/100 g body weight/day or else 2.5 mg/kg/day, 2 h before lights off) throughout pregnancy and (C) PINX rats	LOAEL=2.5 mg/kg/day (vaginal opening)	The female offspring were observed until the onset of puberty. The offspring of melatonin-treated rats showed later vaginal opening than did those of groups A and C (P<0.05 vs control and P<0.01 vs PINX offspring), accompanied by a lower LH level, 0.42±0.05 ng LH/ml, showing statistically significant differences with control levels [1.00±0.22 ng LH/ml (P<0.05)] and levels of the PINX group [1.16±0.22 ng LH/ml (P<0.05)]. The % of rats in pro-oestrus was higher in the offspring of PINX rats (78.6%; P<0.01) vs control offspring (30%) and offspring of melatonin-treated rats (11.8%). Melatonin levels, 5.5 h after darkness, were not significantly different among groups. No differences were noted in the body, ovarian and pineal weights of offspring. The pituitary weight was significantly lower (P<0.05) in the offspring of PINX rats than in the other two groups.	[redacted]
Rat (19-20 Wistar females/ group)	SC	Control, PINX or melatonin (250 µg/100 g body weight/day or else **2.5 mg/kg/day**)-treated mother rats during pregnancy. Newborns were studied at the following phases of sexual development: neonate (5 days old), infantile (15 days old), juvenile (25 and 30 days old) and pubertal phase (55 days).	LOAEL=2.5 mg/kg/day	In female offspring, melatonin treatment during pregnancy significantly increased plasma LH in 15- and 25-day-old rats; however, at the end of the prepubertal period (30 days), LH level in plasma decreased significantly as compared to control rats. This hormonal pattern was different from that observed in offspring of control and PINX rats, which had low LH levels at 25 days and higher LH levels at 30 days of age. FSH did not vary significantly among the 3 groups. Plasma PRL levels were affected by PINX of the mother (showing significantly higher levels in the 5-day-old offspring than in controls) and also by maternal melatonin treatment, producing hyperprolactinaemia in the 30-day-old female offspring. In male offspring, sexual development in control male rats progressed rapidly with significantly increased LH and FSH levels at 25 and 30 days compared to those measured during the neonatal and infantile periods. Pinealectomy of the mother induced the following modifications: in 5-, 15- and 30-day-old male rats, decreased LH levels were measured relative to the other two groups studied in 5- and 25-day-old rats, significantly lower FSH levels than in the control rats	[redacted]

Specie	Route	Melatonin doses	Endpoint/ Value	Effects	Reference
				were recorded. However, in 5- and 15-day-old rats, significantly higher PRL levels than in control rats were measured. Melatonin injections during pregnancy decreased FSH levels at 5, 25, 30 and 55 days as compared to the control males. Also, melatonin increased LH levels in 25-day-old rats and significantly decreased prolactin levels in 15- and 55-day-old rats as compared to the other two groups.	
Hamster	SC	50 ng given over 6 or 8 h per 24-h period to PINX dams for at least 3 days prior to birth	Not referred	Maternal plnealectomy eliminated the influence of prenatal photoperlod on testicular and body weights of male pups, suggesting that a product from the maternal pineal gland communicates day-length to the fetus. Infusion of melatonin into PINX dams for various durations during gestation mimicked the effect of varying the prenatal photoperlod on both testicular and body weights.	[redacted]
Rabbit	Oral	0 (control), 15, 50 and 150 mg/kg/day from GD 7 to 19	NOAEL=150 mg/kg/day (maternal and developmental toxicity)	Maternal toxicity: no dose-related maternal effects at any dose; no effects on pre- or post-implantation loss and mean number of fetuses/females. Fetotoxicity/teratogenicity: fetal, litter and placental weighs were not affected by treatment. Visceral and skeletal malformations and/or variations were observed in all groups including controls. Some of such malformations/variations showed a trend or a significant increase in the treated groups, such as absence of lung or iliac alignment/caudal shift of vertebrae at high dose corresponding to an approximate AUC of 24,000-45,000 ng×h/ml. When compared to the AUC values to be achieved in man (<4 ng×h/ml), very high exposure ratios were reached in this study.	[redacted]
Heifer (3 groups of 4 primiparous Holstein-Friesian heifers)	Oral (in diet)	Throughout pregnancy either a control diet or that diet supplemented with either 5-6 g/day of rumen-protected intestinally available methionine or **25 mg melatonin**; they were euthanased 3 days after calving.	-	The dietary supplements had no effect on the impression hardness or the concentrations of cysteine and methionine in samples of claw horn collected from a range of sites, or on the areas of erosion in the sole and heel. Significant differences were recorded for the hardness of the horn in the order wall >sole >heel and were associated with higher concentrations of cysteine and lower levels of methionine in samples of horn from the dorsal wall than in samples from the prebulbar region of the sole. There were no significant differences attributable to the dietary supplements in the soft tissue anatomy of the solear dermis and epidermis.	[redacted]
Developmental toxicity effects / Postnatal studies					
Rat (24 pre-mated females)		Melatonin 0, 15, 55 and 200 mg/kg/day from GD 6 to day 21 postpartum, inclusive	Not established	The treatment had no effect on parturition and outcome of pregnancy, but the subsequent growth and viability of the high-dose offspring was slightly reduced during lactation. At weaning, a slight reduction of offspring maturity was observed in all dose groups, but the subsequent F_1 development was not modified. Thus, melatonin intake during lactation should be avoided.	[redacted]
Rat (45 male Sprague-Dawley neonates)	SC implant	Treatment groups: I (n=9): untreated controls; II (n=6): TP-treated rats; III (n=11): TP-treated, blinded rats; IV (n=10): TP-treated, blinded PINX rats; V (n=9): TP-treated, blinded rats given **melatonin 1 mg** in 24-mg beeswax pellet	Not established	The testes and accessory sex organs of testosterone-treated and blinded rats grew significantly slower than those of intact control rats. Either treatment of animals with melatonin or pinealectomy partially restored growth of the reproductive organs. In that, the effects of melatonin treatment were similar to those of pinealectomy in this experiment model, it is speculated that melatonin may not be the principal pineal antigonadotrophic substance in the male rat.	[redacted]
Rat (pregnant rats and into 2-, 6-, and 13-day-old female rats)	SC	Pregnant rats: vehicle, 500 µg melatonin or 1 µg TP, or their combination on GDs 17-20; neonatal rats: (single injection) vehicle, 100 µg TP or 250 µg melatonin or combination	LOAEL=1,515 µg/kg/day (body weight of pregnant rat= 33 g; *EMA ICH Q3C R8, 2022*)	Melatonin had an inhibitory effect on vaginal introitus and decreased the incidence of oestrous smears, when injected during the early postnatal period. Melatonin combined with TP reduced the incidence of such persistent vaginal cstrus and established irregular vaginal cycling.	[redacted]
Rat (male and female juvenile marsh rice rat *Oryzomys palustris*)	SC implant	Melatonin implants	Not established	Juvenile rice rats of both sexes were left gonadally intact (control group) or unilaterally castrated and housed on 12L:12D, 14L:10D, or 16L:8D. Within a photoperiod (14L:10D and 16L:8D, but not 12L:12D), growth of the remaining testis, but not the remaining ovary, as well as several additional organs in both sexes were significantly affected, suggesting that the compensatory hypertrophy of the testis is photoperiod dependent. There was no effect	[redacted]

Specie	Route	Melatonin doses	Endpoint/ Value	Effects	Reference
				of testis asymmetry on CGH as unilaterally castrated of either testis in the rats housed on 14L:10D resulted in a comparable increase of CGH. Melatonin implants in rice rats maintained on 16L:8D had little to no effect (CGH included) on most parameters examined. Both melatonin implants and pinealectomy (separate experiments) in rice rats transferred to 12L:12D prevented short photoperiod-induced effects on CGH, the growth of the reproductive organs and the Harderian glands. Evening melatonin injections had a significant inhibitory effect on the growth of the remaining testis (no CGH was observed) and all other parameters measured. Lastly, unilaterally castrated did not alter the percentage of males which successfully mated compared to intact animals. Taken together, these data suggest that photoperiod, melatonin, and the pineal gland can affect and regulate reproductive (e.g., CGH in some cases) and non-reproductive growth during postnatal development in the marsh rice rat.	
Abbreviations: AUC, area under the concentration-time curve; CGH, compensatory gonadal hypertrophy; FSH, follicle-stimulating hormone; GD, gestation day; GLP, Good Laboratory Practice; IP, intraperitoneal; IV, intravenous; LH, luteinising hormone; LOAEL, Lowest-Observed-Adverse-Effect-Level; NOAEL, No-Observed-Adverse-Effect-Level; PINX, pinealectomised; PRL, prolactin; SC, subcutaneous; TP, testosterone propionate.					

Additional (limited) reproductive studies of melatonin in mice reported no embryofetal effect of a single 40-mg/kg SC dose given on gestational day (GD) 8.5 or two SC doses of 120 mg/kg administered on GDs 7.5 and 8.5 when examined on GDs 10.5 and 17.5, respectively. However, this is not unexpected given the limited fetal exposure during embryogenesis. *In vitro* studies using cultured GD-8.5 mouse embryos showed that 100-200 µg/ml melatonin increased the number of abnormal mouse embryos, however the clinical significance of this finding is unknown. Reproductive studies of melatonin in rats were somewhat at odds. In one study, administration of 2.5 mg/kg SC during "pregnancy" produced no remarkable effect on weight gain, litter size, gender composition or pregnancy duration. In contrast, SC administration of 2.5 mg/kg from GD 0 through to parturition in a second study marginally (≤10%) reduced ovarian and pineal gland weights, significantly reduced pituitary gland weight, delayed vaginal opening and lowered LH levels in the offspring. Overall, based on the limited data provided, poor study design and/or study inconsistencies, these additional literature-based studies have very limited value-adding effect on reproductive toxicity assessments of melatonin [redacted].

Developmental toxicity

The effect of exogenous melatonin on embryo viability has been investigated in undernourished ewes; in particular, at lambing, 24 ewes were treated (+) or not (–) with a melatonin implant. After 45 days, both groups were fed to provide 1.5 (Control, C) or 0.5 (Low, L) times daily maintenance requirements, so that experimental groups were: C–melatonin, C+melatonin, L–melatonin and L+melatonin. Ewes were mated (day 0) and on day 5, embryos were recovered and classified according to their developmental stage and morphology. Ovaries were used for *in vitro* fertilisation and uterine horns were processed to study progesterone and oestrogen receptor (PR and ERα) expression. After 21 days, groups L–melatonin and L+melatonin had an average weight loss of 10kg (P<0.001). The number of viable embryos per CL from L+melatonin (0.50±0.2) was higher than from other groups (P<0.05). Overall, the melatonin effect was particularly evident in undernourished ewes, increasing both viability (L+melatonin: 65%; L–melatonin: 25%; P<0.05) and pregnancy rates (L+melatonin: 66.6%; L-melatonin: 16.6%; P<0.05). Neither nutrition and melatonin nor their interaction had a significant effect on the *in vitro* oocyte development. Melatonin treatment tended to increase the %positive cells to PR in deep glandular epithelium, independently of diet (P=0.09) and the greatest staining intensity of PR was observed in the luminal and superficial glandular epithelia (P<0.0001) ([redacted]).

Maternal-fetal transfer of melatonin

Melatonin has been detected in the circulation of the near-term rhesus monkey (*Macaca mulatta*) and baboon (*Papio papio*) fetus; [redacted] determined whether the source could be the mother by studying placental transfer of melatonin in the rhesus monkey. When [^{3}H]-melatonin was administered IV to the mother it promptly appeared in the fetal circulation; the rates of disappearance of [^{3}H]-melatonin in the maternal and fetal circulations were parallel. The rapid decrease in circulating [^{3}H]-melatonin was associated with a rapid accumulation of [^{3}H]-melatonin-metabolites in the maternal and fetal circulations. Although the pattern of appearance of metabolites was similar in both circulations, relatively less [^{3}H]-melatonin-metabolites appeared in the fetal circulation. Acute changes in total maternal plasma melatonin, experimentally produced by giving a 20-min infusion of melatonin, were rapidly reflected in the fetus. This suggests that a daily rhythm in maternal melatonin would generate a similar rhythm in the fetus. The fetal monkey pineal was found to have the two enzymes necessary for the conversion of serotonin to melatonin. It is, however, not known whether fetal melatonin synthesis is rhythmic or the extent to which it could contribute to circulating melatonin levels at this or earlier stages of gestation.

The previous study showed that melatonin in the maternal circulation can cross the placenta and is the major source of melatonin in the fetal circulation. Melatonin has been postulated to act as a prostaglandin (PG) synthetase inhibitor in the uterus; it is also known that PG synthetase inhibitors decrease myometrial contractility. To assess transplacental passage of melatonin and potential influences of melatonin on uterine contractility, melatonin doses of 0, 150, 600, 2,400 and 5,600 μg/h were infused continuously into the maternal jugular vein in 7 pregnant sheep at GDs 138-142 (term=47 GDs) at 3 infusion rates for successive 1-h periods during the late morning to late afternoon. There was no change in the total time during which the myometrium was active, as indicated by myometrial electromyographic activity or the myometrial contracture frequency during the 3 h before and after melatonin infusions and for each hour of the infusions. The metabolic clearance rate for melatonin in the ewe was 4,128±410 ml/min (mean ± standard error [SE]; n=7). The resting maternal-to-fetal melatonin concentration ratio was 0.8; it was maintained at 2.28 during melatonin infusion to the ewe at a wide range of maternal melatonin levels. Melatonin levels in the range of 3-200 times normal had no effect on the maternal plasma $PGF_{2\alpha}$ metabolite level but caused a 40.4% fall in fetal plasma PGE_2 (P<0.05) [redacted]

Other effects

It has been reported that melatonin acts on the hypothalamus to inhibit LH-RH secretion and on the pituitary to suppress the stimulatory effect of LH-RH on LH release. Melatonin was able to further depress the weight of testes and ventral prostates in rats after hypophysectomy. Melatonin inhibited testosterone production by rat testicular tissue *in vitro* but exerted no effect on cAMP level. Guanylate cyclase activity and cGMP level, on the other hand, increased [redacted]

2.4.4.6 LOCAL TOLERANCE

No specific studies on melatonin's local tolerance were identified in the public domain. The current product is intended for the oral route of administration and is well-tolerated in terms of gastrointestinal safety in the general toxicology studies.

2.4.4.7 OTHER TOXICITY STUDIES

2.4.4.7.1 Excipients

The safety assessment of the excipients included in the formulation is also a very important apsect. The drug product formulation under the current submission, namely Melatonin 1 mg/ml oral solution, containing melatonin as the active substance, also contains the following excipients: sorbitol 70%, propylene glycol, xanthan gum, citric acid anhydrous, paraben methyl sodium, orange IFF 3912, sodium citrate dihydrate, saccharin sodium and purified water (Table 10).

Table 10. Qualitative and quantitative (quantities per 1 ml) composition of the proposed product Melatonin 1 mg/ml oral solution.

≠	Name	Function	Reference	[redacted]
		Drug substances		
1	Melatonin	Active ingredient	Ph. Eur.	
		Excipients		
1	Sodium methyl parahydroxybenzoate (E219)	Antimicrobial preservative	Ph. Eur.	
2	Sorbitol, liquid (non-crystallising) (E420)	Solvent	Ph. Eur.	
3	Propylene glycol (E1520)	Solvent	Ph. Eur.	
4	Xanthan gum	Thickening agent	Ph. Eur.	
5	Citric acid	Buffering agent	Ph. Eur.	
6	Orange flavour	Flavouring agent	In-House	
7	Sodium citrate	Buffering agent	Ph. Eur.	
8	Saccharin sodium	Sweetener	Ph. Eur.	
9	Water, purified	Solvent	Ph. Eur.	

Since the medicinal product under submission is also intended for paediatric use, namely in children and adolescents aged 6-17 years, the *EMA Guideline on pharmaceutical development of medicines for paediatric use* [redacted] was taken into consideration as well [redacted] According to this EMA Guideline, the choice of suitable excipients in a paediatric medicinal product is one of the key elements of its pharmaceutical development. Although the basic considerations regarding the use of a specific excipient are similar for adult and paediatric preparations, the inclusion of any excipient in paediatric preparations, even those which are normally accepted for use in medicines for adults or those which are present in authorised paediatric medicines, requires special safety considerations. The intake of an excipient may result in a different exposure in children to that in adults or in children of different ages. Also, the excipient may have a different effect on developing organ systems. A conservative approach should be followed in

case of limited safety data relevant to the use of an excipient in a specific age group. As per the recommendations of this EMA Guideline, overall, the following aspects are to be considered when selecting an appropriate excipient for inclusion in a paediatric medicinal product: (i) the function of the excipient in the formulation and potential alternatives; (ii) the safety profile of the excipient for children in the target age group(s) on the basis of single and daily exposure (and not the concentration or strength of the preparation); (iii) the expected duration of the treatment, i.e. short- (single dose/few days) vs long-term (weeks, months, chronic); (iv) the severity of the condition to be treated (e.g., life-threatening disease) and the therapeutic alternatives; (v) the patient acceptability including palatability (e.g. local pain, taste); and (vi) allergies and sensitisation [redacted]

The excipients used in the proposed oral solution are commonly used ingredients for the formulation of medicinal products, including also oral solutions, and are all comprehensively described in European Pharmacopoeia apart from the flavouring agent that does not have a monograph in pharmacopoeias. They demonstrate a good safety profile also in paediatrics and have been in other commercially available formulations indicated for children use. Moreover, from a quantitative point of view, all relevant guidelines and mostly, requirements based on the revised European Commission (EC) Guideline on excipients in the label and package leaflet of medicinal products for human use [redacted] and its current Annex [redacted] have been taken into consideration in order not to overpass the safety thresholds stated by the Authorities, were reported.

In this Section, an overview of the safety profile, including non-clinical and clinical safety data, of the contained excipients is presented (in alphabetical order) and discussed. For all contained excipients, the administered amounts are well below the reported toxic doses.

2.4.4.6.1.1 Citric acid anhydrous and Sodium citrate dihydrate

Citric acid is a weak organic acid that is widely used (as either the monohydrate or anhydrous material) in pharmaceutical formulations, including effervescent granules, tablets and oral solutions, cosmetics and food products. It is found naturally in citrus fruit, such as lemons and limes, and is used as a natural preservative. Sodium citrate is the sodium salt of citric acid. Citrates or citric acid is often used as the buffering agents to control pH of oral liquid formulations.

A large body of toxicological data exists for citric acid and citrates, many of which are relatively old. Based on wide spectrum of data relating to experimental animals and on human experience, citric acid has a low acute toxicity potential; only one case of near fatal human intoxication was found. Following oral administration of citric acid, the LD_{50} values were in the range of 3,000-12,000 mg/kg for the rat and 5,400 mg/kg for the mouse. General clinical effects comprised physiological disturbances, namely acidosis and calcium deficiency, while “high” doses caused nervous system effects as well as severe damage to the stomach mucosa. In a repeated-dose rat study, a Non-Observed-Adverse-Effect-Level (NOAEL) of 1,200 mg/kg/day and a Lowest-Obserevd-Adverse-Effect-Level (LOAEL) of 2,000 mg/kg/day have been determined. Subchronic and chronic toxicity studies, involving oral dosing in rodents (rats, guinea pigs, rabbits) and dogs revealed no advers events (AEs) nor histopathological changes in growth or survival. The major subchronic and chronic toxic effects seem to be limited to changes in blood chemistry, respectively, metal absorption and excretion kinetics, even at high doses. Based on several studies, citric acid is not suspected of being a carcinogen. Reproductive-developmental toxicity studies have shown no indications of teratogenicity or other AEs in rats. Genotoxicity studies revealed no mutagenic potential both *in vitro* and *in*

vivo. Judging from the few reports on local tolerance, also, the sensiting potential of citric acid is considered to be low [redacted]

Citric acid is a natural substance that also appears as an intermediate in the basic physiological citric acid or Krebs cycle in every eukaryote cell. It is found naturally in the body, mainly in the bones, and is commonly consumed as part of normal diet. Orally ingested citric acid is absorbed and is generally regarded as a nontoxic materal when used as an excipient. It is a powerful chelating agent and there is evidence that dietary citric acid may reduce the biological availability of iron and calcium. Hence, therapeutically, citric acid-containing preparations have been used to dissolve renal calculi since citric acid, being a urinary acidifier, inhibits formation and allows dissolution of calcium phosphate, calcium carbonate and magnesium ammonium phosphate [redacted]

Citric acid/citrates enhance intestinal aluminum absorption in haemodialysis patients, resulting in increased harmful aluminum levels in serum. Thus, it has been suggested patients with renal failure taking aluminum compounds to control phosphate absorption should not be prescribed citric acid-/citrate-containing products [redacted]. Tooth erosion through dissolution of the enamel due to the acid effect in aqueous solution as well as exposure to citric acid fumes has been reported as a possible adverse consequence of long-term over-exposure to citric acid [redacted]. Ingestion of a single 25-g dose of citric acid by a woman (corresponding to approximately 417 mg/kg) caused vomiting and nearly death in one reported case. Volunteers given oral doses of potassium or magnesium citrate corresponding to approximately 4.7 g of citric acid did not suffer any overt GI effects. Injection of large volumes of citrated blood during transfusion may lead to hypocalcaemia and changes in blood composition with concomitant nausea, muscle weakness, breathing difficulties and even cardiac arrest [redacted]. After ingestion, sodium citrate is absorbed and metabolised to bicarbonate. Although it is generally regarded as a nontoxic and nonirritant excipient, excessive consumption may cause GI discomfort or diarrhoea. Therapeutically, in adults, up to 15 g of socium citrate daily may be administered orally in divided doses as an aqueous solution to relieve the painful irritation caused by cystitis [redacted] To our knowledge, no specific data regarding the safety of citric acid and sodium citrate as excipients in paediatric patients are available. Nevertheless, this excipient is used in various oral formulations also intended for paediatric use.

Citric acid and citrate salts are listed as Generally-Recognised-As-Safe (GRAS) ingredients and are included in FDA-Inactive Ingredients Database (IID). It is also accepted as a food additive in Europe. The average daily intake of citric acid from natural sources in the diet and food additives has been estimated at ~40 mg/kg for women, 130 mg/kg for infants and 400 mg/kg for individuals on slimming diets; hence, maximum daily intake is reported to reach levels of 500 mg/kg. No formal ADI value has been specified for citric acid and its common salts by the JECFA nor the EC Scientific Committee for Food (SCF). More specifically, in 1973, the Joint FAO/WHO Expert Committtee on Food Additives (JECFA) established a 'Group ADI' for citric acid and its calcium, potassium, sodium and ammonium salts of 'not limited', concluding that since these substances are natural constituents of the diet and their intake from food additives is likely to be insufficient compared to intake from natural sources, there are no specific safety aspects [redacted] In 1998, the EC SCF performed a re-evaluation of sodium citrate and considered that its use is acceptable up to 2 g/L in infant formulae and follow-on formulae for infants and young children in good health and in foods for special medical purposes [redacted]

The melatonin 1 mg/ml oral solution product formulation under submission contains [redacted] citric acid anhydrous and [redacted] sodium citrate dihydrate per ml of oral solution. Since the MRDD is 6 mg melatonin (6 ml) in all target population groups (paediatric and adult patients) and intended indications, the maximum daily ingested amounts of these excipients will be [redacted] and [redacted] respectively. Taking also into consideration the fact that no ADI value has been specified for citric acid and its citrate salts, the proposed content for both excipients in the melatonin oral solution is safe for use in the intended population groups.

2.4.4.6.1.2 Flavouring agent (Orange IFF 3912)

Flavouring agents play an important role in patient acceptability especially for oral liquid formulations intended for paediatric use. The selection of flavourants should be clearly described and justified as some have toxic effects especially in paediatric patients. The safety concerns must be recognised including the risk of allergies and sensitisation [redacted] In the proposed product formulation, Orange flavour IFF 3912 is used as a flavouring and is contained at an amount of [redacted] per ml of oral solution. Taking into consideration the MRDD of 6 mg, the corresponding maximum daily ingested amount of this excipient will be [redacted] based on the maximum administered volume of 6 ml of solution. The Applicant has taken all the EMA requirements on the selection of the appropriate flavouring into account (including quality/composition characterisstics), hence, no safety issues are expected.

2.4.4.6.1.3 Paraben methyl sodium

Methylparaben sodium is the sodium salt of the methyl ester of *p*-hydroxybenzoic acid. Parabens are some of the oldest preservatives and have been used since the 1920's. They occur naturally in some fruits and vegetables, including blueberries. Parabens are effective over a wide pH range and have a broad spectrum of antimicrobial activity, although they are most effective against yeasts and moulds. It is considered that they act by disrupting membrane transport processes ([redacted]) or by inhibiting synthesis of DNA and RNA [redacted]) or some key enzymes, e.g., ATPases and phosphotransferases, in some bacterial species ([redacted]). Since most of the studies on the mechanism of action of parabens suggest that their antibacterial action is linked to the membrane, it is possible that its greater lipid solubility disrupts the lipid bilayer, thereby interfering with bacterial membrane transport processes and perhaps causing the leakage of intracellular constituents ([redacted]). Methylparamen exhibits antimicrobial activity in a pH range of 4-8. Preservative efficacy decreases with increasing pH owing to the formation of the phenolate anion. Antimicrobial activity increases as the chain length of the alkyl moiety is increased, but aqueous solubility decreases.

Parabens are widely used as antimicrobial preservatives in cosmetics, oral and topical pharmaceutical formulations to prevent microbial proliferation arising under in use conditions. These properties are due to certain chemical groups which are usually harmful to living cells and might therefore be associated with certain risks when used in humans. Thus, inclusion of antimicrobial preservatives or antioxidants in a medicinal product needs special justification. Wherever possible the use of these substances should be avoided, particularly in case of paediatric formulations. The concentration used should be at the lowest feasible level ([redacted]

Allergic, hypersensitivity reactions and contact dermatitis have been reported after topical application of parabens. However, given the widespread use of parabens as preservatives, such

reactions are relatively uncommon; the classification of parabens in some sources as high-rate sensitizers may be overstated. Concern has been expressed over the use of methylparaben in infant parenteral products because bilirubin binding may be affected, which is potentially hazardous in hyperbilirubinemic neonates ( ).

In 2015, the EMA has published a reflection paper on the use of methyl- and propylparaben as excipients in human medicinal products for oral use. Parabens are included in the current *EC Guideline on excipients in the label and package leaflet of medicinal products for human use* and its *Annex* indicating a threshold 'zero' for their allergenic (possibly delayed) potential. Further safety concerns were raised as a consequence of possible endocrine-disrupting effects, an effect that was explicitly addressed by the Agency.

After a careful evaluation of all available data, methylparaben has not been associated with AEs on the male and female reproductive organs in juvenile rats or in embryo/fetal development studies. This allowed concluding that the use of methylparaben in oral formulations up to 0.2% of the product, which is also within the recommended effective concentrations as a preservative, is not a concern for humans including the paediatric population whatever the age group. This limit corresponds to a maximal intake of ~140 mg/day (2.8 mg/kg/day based on an individual body weight of 50 kg) ( )

Risk assessments on parabens have also been performed by several European expert panels including the (EFSA and the Scientific Committee on Consumer Safety (SCCS). EFSA established a full-group acceptable daily intake (ADI) of 0-10 mg/kg body weight for the sum of methylparaben, ethylparaben and propylparaben present in a formulation. Similarly, the WHO has set an estimated total ADI for methyl-, ethyl- and propylparabens at up to 10 mg/kg body weight ( ). Methylparaben is a GRAS-listed excipient and is included in FDA-IID for various dosage forms.

Melatonin oral solution (1 mg/ml) contains methylparaben at the concentration range of 1.6 mg/ml. This amount is considered acceptable according to the published acceptable daily exposure values for this excipient, even for the worst-case scenario, namely administration of the maximum proposed daily dose of 6 mg of melatonin (6 ml of solution) that leads to a maximum daily intake of 9.6 mg methylparaben. Taking all the above data into consideration, it can be concluded that the quantity of methyl paraben in the melatonin product formulation under current submission is safe, as being far below the threshold established by EU Authorities.

2.4.4.6.1.4 Propylene glycol

In pharmaceutical industry, propylene glycol (PPG) is used as a solvent and/or co-solvent in oral solutions at a usual concentration range of 10%-25% ( ).

In preclinical *in vivo* studies, PPG was not acutely toxic. The lowest oral LD_{50} values ranges between 18 and 23.9 mg/kg in 5 different species and the reported dermal LD_{50} is 20.8 mg/kg. It is essentially nonirritating to the skin and mildly irritating to the eyes. PPG glycol (in drinking water or diet) did not result in AEs at levels up to 10% in water (~10 g/kg/day) or 5% in feed (2.5 g/kg/day) for periods up to 2 years. In cats, two studies of at least 90-day duration have demonstrated species-specific effect of increased Heinz bodies (NOAEL=80 mg/kg/day; LOAEL=443 mg/kg/day), with other haematological effects, namely decrease in number of erythrocytes and erythrocyte survival, reported at higher doses (6-12% in diet or else 3.7-10.1 g/cat/day). PPG did not cause fetal or developmental toxicity in rats, mice, rabbits, or hamsters

(NOAEL=1.2-1.6 g/kg/day). No reproductive effects were found when PPG was administered at up to 5% in the drinking water (10.1 g/kg/day) of mice. However, results of recent studies have demonstrated that PPG could produce *in vitro* DNA damage, in the presence and absence of metabolic activation, leading to chromosomal mutations in CHO cells; high cytotoxic concentrations of PPG were tested, i.e., 50-150 mg/ml, for the evaluation of its potential effects when used as a cryoprotectant in oocyte vitrification. PPG was not a genetic toxicant as demonstrated by a battery of both *in vivo* (MN, dominant lethal, CA) and *in vitro* (bacterial and mammalian cells and cultures) studies. No increase in tumours was found in all tissues examined when it was administered via the diet in rats (2.5 g/kg/day for 2 years) or applied on the skin of female rats (100% PPG; total dose not reported) for 14 months or mice (~2 g/kg/week) for a lifetime. These data support a lack of carcinogenicity for this agent ([redacted].

Absorption of PPG by the oral route is rapid and nearly complete. PK parameters are generally considered similar whenever PPG is administered by the oral, IV or IP route. During oral administration after rapid absorption from the GI tract, it is extensively metabolised in the liver mainly to lactic and pyruvic acids and is excreted unchanged in urine. Regarding paediatric populations, it has been demonstrated that the PK parameters of PPG in neonates differ significantly from adult values leading to its accumulation after repeated dosing, due to longer $t_{1/2\beta}$, limited renal and metabolic clearances, or when administered in combination with another substrate of alcohol dehydrogenase (limiting step of metabolism) such as ethanol (e.g., toxicity of some antiviral treatments in neonates) ([redacted]

Clinically, the use of PPG as an excipient in marketed products is generally well-tolerated. However, AEs have been described in the literature in association with intoxications due to consumption of PPG-containing products or medicines containing PPG when administered as a prolonged treatment and/or at very high doses in patients. PPG toxicity profile was established mainly on the basis of the effects induced by IV infusion of lorazepam, benzodiazepine or etomidate in both critically ill adults and children. Various AEs attributed to PPG have been reported, such as hyperosmolality, lactic acidosis, renal dysfunction (acute tubular necrosis), acute renal failure (ARF), cardiotoxicity (arrhythmia, hypotension), central nervous system (depression, coma, seizures), respiratory depression, dyspnoea, liver dysfunction, haemolytic reaction (intravascular haemolysis) and haemoglobinuria or multisystem organ dysfunction ([redacted]

As mentioned, intake of PPG is generally critical in paediatric populations, especially in neonates and young children. Theoretically liver and/or renal impairment are susceptible to induce accumulation and increased risk of toxicity that is particularly critical in the case of neonates and even more importantly in case of competitive inhibition of metabolic clearance (e.g., when another aldehyde dehydrogenase substrate is coadministered) in neonates with very low renal excretion due to renal immaturity [redacted] collected data from 69 neonates given paracetamol formulated in PPG and compared them to data from patients given paracetamol formulated with mannitol (n=149, historical controls). No short-term biochemical impact was detected during or after a median PPG exposure of 34 mg/kg/24 h (range: 14-252). Exposure to PPG seemed well-tolerated and did not affect normal postnatal maturational changes in renal, metabolic and hepatic functions. A PK model showed that for these commonly used dosing regimens, the population mean PPG C_{max} and trough (C_{min}) concentration range between 3.3-14.4 and 2.8-21.8 mg/dl (C_{max}) and 1.9-10.9 and 0.6-11.2 mg/dl (C_{min}) for paracetamol and

phenobarbital formulations, respectively, depending on birth weight and age of the neonates ([redacted]

PPG is accepted as a food additive in Europe. It is also a GRAS-listed excipient and is included in the FDA-IID for various dosage forms. In the basis of metabolic and toxicological data, the WHO has set an ADI value of 25 mg/kg/day ([redacted]). It has to be mentioned that Agenerase®, an antiretrovial medicinal product containing amprenavir, received a contraindication for infants and children below 4 years of age due to its content in PPG exceeding the recommended WHO limit of 25 mg/kg/day ([redacted]

Published reviews by [redacted]) indicated that PPG doses up to 500 mg/kg/day could be administered safely to adult patients even for long-term periods. In children below 5 years of age, a dose limit of 50 mg/kg for which no effects are expected is being proposed based upon the data of the study by [redacted] , demonstrating that no short-term biochemical impact was detected during or following a median PPG exposure of 34 mg/kg/24 h. Exposure to PPG seemed well-tolerated, without affecting normal postnatal maturational changes in renal, metabolic and hepatic functions. In addition, the human equivalent dose to the NOAEL of 1,000 mg/kg in the juvenile mouse was calculated to be 192 mg/kg for a neonate (3.5 kg), 150 mg/kg for a 1-year-old child (9 kg) and 126 mg/kg for a 4-year-old child (15 kg). The proposed dose limit of 50 mg/kg is still 2.5 times lower. Also, model-based simulated concentration-time profiles of PPG in a term neonate (birth weight 3,500 g) after the administration of 34 mg of PPG/kg/day in paracetamol did not show accumulation, namely no increase in PPG serum concentration after repeated dosing. Therefore, the risk of accumulation may be considered limited above the age of 1 month in children administered 50 mg PPG/kg with non-impaired liver and/or renal functions. This is confirmed by data from [redacted]

Nevertheless, this is not applicable for children below 1 month of age. In (pre)term neonates, it has been demonstrated that total body clearance is very low ([redacted]) compared to the adult clearance ([redacted] but also that the contribution of renal clearance to the total body clearance is very low. The results of this study ([redacted] may indicate that due to maturational changes, some drug/drug metabolic interactions are more relevant for this specific population. This may explain the toxicities observed in neonates given Kaletra® (an antiretrovial fixed combination medicinal product containing lopinavir/ritonavir) that contains 356.3 mg ethanol/ml and 152.7 mg PPG/ml.

Nevertheless, clinical data showed that in children from the age of 5 years and adult patients, up to 500 mg/kg/day of PPG could generally be considered safe. In the absence of compelling data, this safety threshold is decreased to 50 mg/kg/day in children <5 years of age and even to 1 mg/kg/day in preterm and term neonates due to known immaturity of both metabolic and renal clearances of PPG in these populations. Considering also the data produced by [redacted] as well as other publications showing the multiple sources of PPG and ethanol in neonatology units, it is proposed to restrict the safety limit to 1 mg/kg in preterm neonates or below 1-month postnatal age for term neonates [redacted]

The substance is therefore included in the current *EC Guideline on excipients in the label and package leaflet of medicinal products for human use* ([redacted] and its *Annex* [redacted]), indicating that PPG content should be defined and presented in a clear and meaningful way in the product's labeling, with a lowest safety threshold of 1 mg/kg/day for a newborn and up to a conservative

threshold of 50 mg/kg/day for all other age groups (infants older than 1 month up to adults), mostly concerning safety issues in case of co-administration with other medicines containing ethanol or use in pregnancy and lactation. Hence, for a 6-year-old-child weighing ~20 kg, based on the weight-for-age charts according to WHO ([redacted]), and a 60-kg adult person person, the corresponding acceptable doses for PPG will be 1 g and 3 g, respectively.

Table 11. Information on the package leaflet for PPG for medicinal products administered either orally or parenterally.

Threshold	Information for the Package Leaflet	Comments
1 mg/kg/day	This medicine contains x mg propylene glycol in each <dosage unit><unit volume> <which is equivalent to x mg/<weight><volume>>.	
1 mg/kg/day	If your baby is less than 4 weeks old, talk to your doctor or pharmacist before giving them this medicine, in particular if the baby is given other medicines that contain propylene glycol or alcohol.	Co-administration with any substrate for alcohol dehydrogenase such as ethanol may induce serious adverse effects in neonates.
50 mg/kg/day	If your child is less than 5 years old, talk to your doctor or pharmacist before giving them this medicine, in particular if they use other medicines that contain propylene glycol or alcohol.	Co-administration with any substrate for alcohol dehydrogenase such as ethanol may induce adverse effects in children less than 5 years old.
50 mg/kg/day	If you are pregnant or breast-feeding, do not take this medicine unless recommended by your doctor. Your doctor may carry out extra checks while you are taking this medicine.	While propylene glycol has not been shown to cause reproductive or developmental toxicity in animals or humans, it may reach the foetus and was found in milk. As a consequence, administration of propylene glycol to pregnant or lactating patients should be considered on a case-by-case basis.
50 mg/kg/day	If you suffer from a liver or kidney disease, do not take this medicine unless recommended by your doctor. Your doctor may carry out extra checks while you are taking this medicine.	Medical monitoring is required in patients with impaired renal or hepatic functions because various adverse events attributed to propylene glycol have been reported such as renal dysfunction (acute tubular necrosis), acute renal failure and liver dysfunction.
500 mg/kg/day	Propylene glycol in this medicine can have the same effects as drinking alcohol and increase the likelihood of side effects. Do not use this medicine in children less than 5 years old. Use this medicine only if recommended by a doctor. Your doctor may carry out extra checks while you are taking this medicine.	Various adverse events, such as hyperosmolality, lactic acidosis; renal dysfunction (acute tubular necrosis), acute renal failure; cardiotoxicity (arrhythmia, hypotension); central nervous system disorders (depression, coma, seizures); respiratory depression, dyspnoea; liver dysfunction; haemolytic reaction (intravascular haemolysis) and haemoglobinuria; or multisystem organ dysfunction, have been reported with high doses or prolonged use of propylene glycol. Therefore, doses higher than 500 mg/kg/day may be administered in children >5 years old but will have to be considered case by case. Adverse events usually reverse following weaning off of propylene glycol, and in more severe cases following haemodialysis. Medical monitoring is required.

In the proposed melatonin oral solution, the amount of contained PPG is 150 mg per 1 ml of solution. Taking into consideration the MRDD of 6 mg of melatonin (6 ml of oral solution), the corresponding maximum daily ingested amount of this excipient will be 900 mg, which is below the safety threshold for all age population groups intended to use the proposed product formulation. Hence, no safety issues are expected.

2.4.4.6.1.5 Saccharin sodium

Saccharin sodium is an artificial sweetener with effectively no nutritional value, being about 300-600 times as sweet as sucrose but having a bitter or metallic aftertaste, especially at high concentrations. It is used as an intense sweetening agent in beverages, food products, table-top sweeteners and pharmaceutical formulations, e.g., tablets, powders, medicated confectionery, gels, suspensions, liquids and mouthwashes, as well as in vitamin preparations. Saccharin sodium is considerably more soluble in water than saccharin, thus, being used more frequently in pharmaceutical formulations to enhance flavour systems and mask some unpleasant taste characteristics. In oral solution formulations, concentrations of 0.075-0.6% are usually selected [redacted]

There has been considerable controversy concerning the safety of saccharin. The published literature has reported extensive studies since the mid-1970s, as studied in rats receiving 5%-7.5% total saccharin, i.e., equivalent to 175 g/day in humans, suggested that the incidence of bladder tumours was significantly greater in saccharin-treated males than in control. However, an investigation of saccharin performed by the American Medical Association in 1985 concluded that bladder changes were species-specific, were confined to the second generation of male rats and occurred in association with large doses that were equivalent to several hundred cans of diet soft drink per day. The No-Observed-Effect-Level (NOEL) was equivalent to 500 mg/kg/day [redacted] Since then, several animal studies have provided information on the mechanisms behind this carcinogenic response in male rats, as well as demonstrating no carcinogenic effect of saccharin in other species. Furthermore, extensive research on human populations has established no association between saccharin and cancer. Hence, from the overall available data, it appears that the development of tumours is a sex-, species- and organ-specific phenomenon [redacted]. Sub-chronic and chronic rat (*in utero*, neonatal and juveline animals) studies with saccharin sodium have revealed an acceptable safety profile [redacted].

Regarding carcinogenicity, IARC reviewed human and animal carcinogenicity data together with the available information on mutagenicity and genotoxicity of saccharin and its salts [redacted]. Based upon this analysis and review of all available studies, IARC reached the following overall conclusions: (i) there is inadequate evidence in humans for the carcinogenicity of saccharin salts used as sweeteners; (ii) there is inadequate evidence in experimental animals for the carcinogenicity of saccharin (acid form) and calcium saccharin; (iii) there is sufficient evidence in experimental animals for the carcinogenicity of sodium saccharin. IARC concluded that *"sodium saccharin produces urothelial bladder tumours in rats by a non-DNA-reactive mechanism that involves the formation of a urinary calcium phosphate-containing precipitate, cytotoxicity and enhanced cell proliferation. This mechanism is not relevant to humans because of critical interspecies differences in urine composition"* [redacted]

Saccharin was negative for mutagenicity/genotoxicity in the Ames test, the highest ineffective dose tested in any *Salmonella typhimurium* strain being 10 mg/plate [redacted] but was found to induce greater DNA damage in the bone marrow assay in Swiss albino mice following oral administration (50, 100 and 200 mg/kg) than aspartame, although not acting as a potential mutagen [redacted]. Further tests on somatic cells, induction of CAs in bone marrow cells and MN-PCEs, as well as tests on germ cells, the spermatocyte test

on treated males and the dominant lethality test yielded all negative results [redacted]. Highly purified preparations of saccharin caused a significant, dose-related increase in CAs in CHO cells in the presence of liver homogenate. Chromatid breaks and gaps were also induced in CHO-k1 cells treated with sodium saccharin. Aberrations have been induced by saccharin and its sodium salt also in other CHO cell lines. No clastogenicity was revealed when saccharin (5 or 50 mg/kg/day) was given to C57Bl/6 mice for 5 days. Animal reproductive, developmental toxicity and teratogenicity studies with saccharin (sodium) revealed no toxic effects [redacted]

Saccharin has been found to change the composition of the gut microbiota, related to alterations in the metabolic pathways linked to glucose tolerance and dysbiosis in human subjects, especially with the ingestion of saccharin (data reviewed by [redacted]. It does not undergo detectable metabolism in either humans or other animals ([redacted] and is excreted through urine. It can cross the placenta and be transferred through breast milk. Its consumption is not recommended during pregnancy or lactation [redacted]

Anecdotal reports of an accidental ovedose in an adult and a child discussed reactions of generalised oedema, oliguria and persistent albuminuria [redacted] Adverse reactions to saccharin, although few in relation to its widespread use, include urticaria with pruritus and photosensitisation reactions. In a series of 42 patients with AEs related to the ingestion of saccharin in pharmaceutical agents, pruritus and urticaria were the most common reactions, followed by eczema, photosensitivity and prurigo. Other reactions include wheezing, nausea, diarrhoea, tachycardia or headache [redacted]). Other systemic reactions have been however reported: irritability, insomnia, opisthotonos and strabismus in children assuming saccharin-containing feed formulas ([redacted] . Due to the lack of data on the toxicity of saccharin in children, the American Medical Association has recommended limiting intake of saccharin in young children and pregnant women [redacted] However, no specific EMA Guideline or recommendation is in place regarding any saccharin upper threshold for adult or paediatric use; indeed, saccharin is not concerned as an excipient requiring specific labelling warning since it is not included in the Annex provided in the EC revised Guideline on Excipients in the label and package leaflet of medicinal products for human use [redacted] Also, saccharin is not included in CHMP Excipients Drafting Group (ExcpDG) specific excipient monograph documents developed so far for excipients under safety evaluation from the EMA.

Saccharin sodium is accepted for use as a food additive in Europe; 'E954' is applied to both saccharin and saccharin salts. It is also included in the FDA-IID and in nonparenteral medicines licensed in the UK. Saccharin and its sodium salt are quite often used in paediatric formulations worldwide ([redacted] and its use is not restricted in children; indeed, the use of saccharin in paediatric formulations is reported also in the *EMA Reflection paper on the formulations of choice for the paediatric population* [redacted]). They were first evaluated by the SCF in 1977 when a temporary ADI of 0-2.5 mg/kg was allocated. The Committee reviewed saccharin again in 1985 and decided to maintain the temorary ADI set in 1977. The JECFA has set an ADI value range of 0-5 mg/kg for saccharin and its calcium, potassium and sodium salts ([redacted]). The same ADI of saccharin and saccharin sodium has been set to 5 mg/kg (including young children) by the EFSA (2007) [redacted] , as well as by the EC (2001) ([redacted]). Hence, for a 6-year-old-child weighing ~20 kg [redacted]), the acceptable dose of saccharin is 100 mg per day, while for a 60-kg adult person, the corresponding daily dose will be 300 mg.

In the proposed melatonin oral solution, the amount of contained sasccharin sodium is [redacted] per ml of solution. Taking into consideration the MRDD of 6 mg (6 ml) for the intended target aged population groups, the corresponding maximum daily ingested amount of the excipient will be [redacted] which if far below the established ADI value. Hence, no safety issues are expected.

2.4.4.6.1.6 Sodium content

Increasing the level of sodium in the body causes an expansion of the extracellular fluid which increases BP. High intake of table salt in food is associated with hypertension (high BP) and stroke in adults. Maintaining steady sodium levels is principally achieved through regulation of renal excretion. The capacity for renal excretion is lower in the very young, including neonates, and the elderly. Regarding paediatric populations, as young babies have lower capacity for removing sodium from the body, high sodium intakes from any source can result in dangerously raised sodium levels in the blood (hypernatraemia). This can result most commonly in events including listlessness, serious dehydration and seizures. In children, chronic high dietary sodium intake can raise BP which increases the risk of hypertension and cardiovascular disease in adulthood [redacted] .

Elevation of BP and the risk for progression to hypertension is of increasing concern in children and adolescents. Indeed, it is increasingly recognised that target organ injury may begin with even low levels of BP elevation. Sodium intake has long been recognised as a modifiable risk factor for hypertension. While it seems clear that sodium impacts BP in children, its effects may be enhanced by other factors including obesity and increasing age. Evidence from animal and human studies indicates that sodium may have adverse consequences on the cardiovascular system independent of hypertension. Thus, moderation of sodium intake over a lifetime may reduce risk for cardiovascular morbidity in adulthood. Considering that eating habits in childhood have been shown to track into adulthood, modest sodium intake should be advocated as part of a healthy lifestyle [redacted]

The WHO recommends that adults consume less than 5 g of sodium chloride (table salt) per day, i.e., equivalent to less than 2 g (or 87 mmol) sodium per day. For children, the WHO advises that recommended maximum daily intakes should be proportional to adults and based on energy requirements [redacted]. Evidently, sodium content in medicinal products has to be carefully considered, as high levels of sodium in some medicines may not be appreciated by patients or healthcare professionals. Sodium is one of the excipients firstly included in the EC Guideline; a threshold of 1 mmol of sodium (23 mg) per dose is proposed to declare a product as ‘’sodium-free’’, intended for either oral or parenteral administration.

The current *EC Guideline on excipients in the label and package leaflet of medicinal products for human use* [redacted] and its [redacted] indicates that sodium content should be defined and presented in a clear and meaningful way in the product labeling. Thus, the sodium content in the maximum daily dose recommended for a medicine is presented as a proportion of the WHO maximum recommended daily dietary intake for sodium. The Annex also introduces an additional threshold to define levels of sodium in medicines considered to be ‘high’. There is no evidence to suggest what level of sodium in medicines is acceptable and this will vary upon individual. However, it is proposed that any product where the maximum daily dose contains ≥17 mmol (391 mg) sodium (~20% of the WHO recommended maximum daily intake for sodium), should be considered as having a ‘high’ sodium content. 1 mmol of sodium (Na) = 23 mg Na = 58.4 mg

salt (NaCl). Information required to be included in the labelling for sodium, depending on its concentration is tabulated below (Table 12).

Table 12. Information on the package leaflet for sodium for medicinal products administered either orally or parenterally.

Threshold	Information for the Package Leaflet	Comments
Less than 1 mmol (23 mg) per dose	This medicine contains less than 1 mmol sodium (23 mg) per <dosage unit><unit volume>, that is to say essentially 'sodium-free'.	Information relates to a threshold based on the total amount of sodium in the medicinal product. It is especially relevant to products used in children or in patients on a low sodium diet, to provide information to prescribers and reassurance to parents or patients concerning the low level of sodium in the product.
1 mmol (23 mg) per dose	This medicine contains x mg sodium (main component of cooking/table salt) in each <dosage unit><unit volume>. This is equivalent to y% of the recommended maximum daily dietary intake of sodium for an adult.	"This medicinal product contains x mg sodium per <dosage unit>, equivalent to y% of the WHO recommended maximum daily intake of 2 g sodium for an adult."
17 mmol (391 mg) in the maximum daily dose	Talk to your doctor or pharmacist if you need <Z> or more <dosage units> daily for a prolonged period, especially if you have been advised to follow a low salt (sodium) diet.	This applies only to products for which the labelled posology allows the product to be taken on a daily basis for > 1 month or repeated use for more than 2 days every week. 17 mmol (391 mg) is approximately 20% of the WHO adult recommended maximum daily dietary intake of 2 g sodium and is considered to represent 'high' sodium. This is also relevant for children, where the maximum daily intake is considered to be proportional to adults and based on energy requirements. <Z doses> reflects the lowest number of dosage units for which the threshold of 17 mmol (391 mg) of sodium is reached/ exceeded. Round down to the nearest whole number. For SmPC wording please refer to PRAC recommendation: "1.3. Sodium-containing effervescent, dispersible, and soluble medicines – Cardiovascular events" (EMA/PRAC/234960/2015).

The proposed melatonin 1 mg/ml oral solution contains [redacted] of sodium per ml of solution. Taking into consideration the MRDD of 6 mg melatonin (6 ml of solution) in all target population groups (paediatric and adult populations) and intended indications, the maximum daily ingested amount of this excipient will be [redacted] Since this medicine contains less than 1 mmol sodium (23 mg) per ml of solution, it is considered as 'sodium-free'.

2.4.4.6.1.7 Sorbitol

Sorbitol is a hexahydric alcohol (polyol) that occurs naturally in many edible fruits and berries. It is widely used as an excipient in pharmaceutical formulations, i.e., as a sweetener in oral solutions. Authorised medicinal products for oral use may contain sorbitol in the range of 2%-90% per dosing unit ([redacted]

The oral LD_{50} values for sorbitol in mice and rats were 17.8 and 15.9 g/kg, respectively. In chronic toxicity/carcinogenicity studies, rats fed a diet enriched in sorbitol exhibited a readily detectable decrease of liver hexokinase. At the same time the glycogen content of liver increased ([redacted]). Developmental/reproductive toxicity studies revealed no abnormalities of the adrenal medulla in any generation or microscopically in the high-dose and control rats. Thus, it was concluded that sorbitol administered in diet to 3 successive generations of rats at levels up to 10% had no AE on growth or reproductive performance in either sex ([redacted]

Sorbitol is incompletely absorbed at a rate much slower than that of fructose, its absorption being dose- and concentration-dependent. When sorbitol is given as a single oral dose, the intestinal absorptive capacity has been estimated to be no more than 2-10 g. It has also been suggested that, when given together, sorbitol and fructose compete for absorption [redacted]

Sorbitol accumulation plays an important role in diabetic modulations involving the kidney, nerves, retina, lens and cardiac muscle as weel as the thyroid hormone seems to influence the sorbitol pathway in diabetic mellitus euthyroid rats. Thyroid hormone reduced the aldose reductase activities in the kidney, liver and sciatic nerve of the diabetic mellitus rats, and similarly reduced AR in the kidney and liver, but not in the sciatic nerve, of the non-diabetic rats. The sorbitol dehydrogenase (SDH) activities were decreased by thyroid hormone in the kidney and liver, but not the sciatic nerve of diabetic rats. In the non-diabetic rats, this enzyme activity was decreased in liver, but not in kidney or sciatic nerve. A positive correlation between sorbitol concentration and aldose reductase activity has been observed in the kidney and liver but not in the sciatic nerve from control, diabetic and diabetic euthyroid rats. A negative correlation was observed between sorbitol levels and SDH activities in the same organs [redacted]

Reports of adverse reactions to sorbitol are largely due to its action as an osmotic laxative when ingested orally, which may be exploited therapeutically. Its activity on the GI tract is well-known to result in GI AEs, such as gas, bloating, abdominal discomfort and osmotic diarrhoea. These effects are promoted mainly after ingestion of large quantities of sorbitol (>20 g/day in adults) that should be avoided [redacted]. It should be noted that these effects are also noted in infants receiving doses exceeding 20 g/day, when it was corrected for weight [redacted]. The currently established threshold of 10 g of sorbitol triggering a warning on potential laxative effect is considered correct but a weight adjusted threshold of 140 mg/kg (equivalent of 10 g/70 kg) is considered the most appropriate for all patients [redacted]

The current EC Guideline on excipients in the label and package leaflet of medicinal products for human use [redacted] and its Annex [redacted] has included sorbitol among the excipients requiring reference in labelling, with a threshold ‘’zero’’ triggering a warning about the risk in case of intolerance to some sugars. This warning mostly relates to patients with the rare genetic disorder of hereditary fructose intolerance (HFI). Patients with HFI develop a natural defense mechanism against fructose and sorbitol by vomiting any food containing either of the two substances. In addition, it should not be disregarded that symptomatic carbohydrate malabsorption is a type of idiosyncratic syndrome which is unpredictable [redacted].

It is acknowledged that malabsorption of carbohydrates (e.g., fructose, lactose, sorbitol) can often be detected among patients suffering from the so-called nonspecific abdominal complaints [redacted]. Most patients with symptomatic carbohydrate malabsorption complain of flatulence, distension, diffuse abdominal pain with colics, sometimes with concomitant diarrhoea. The abdominal symptoms are considered to be caused by an increased osmotic load of the malabsorbed sugar remaining in the small intestine with increased intraluminal water and consecutive accelerated transit. In other words, GI disorders from sorbitol are largely due to its action as an osmotic laxative when ingested orally [redacted]. The threshold value of sorbitol ingestion is ~10 g in adults to trigger these effects [redacted] Similar findings also exist for children. Limited data are available for sorbitol malabsorption in case of children [redacted] Besides,

extrapolating data from fructose malabsorption, one can consider that malabsorption rates for children can nearly be identical to those of adults. As already mentioned above, even the total administered daily dose of sorbitol is much lower than the >10 g values responsible for triggering the GI discomfort. In addition, the symptoms of GI disorders are easily detectable by the children.

Sorbitol is a GRAS-listed excipient and is included in the FDA-IID for various dosage forms. It is also accepted as a food additive in Europe. Information on sorbitol required to be placed on labeling of medicinal products is tabulated below [redacted].

Table 13. Information on the package leaflet for sorbitol (following oral administration).

Threshold	Information for the Package Leaflet	Comments
Zero	This medicine contains x mg sorbitol in each <dosage unit><unit volume> <which is equivalent to x mg/<weight><volume>>.	The additive effect of concomitantly administered products containing sorbitol (or fructose) and dietary intake of sorbitol (or fructose) should be taken into account. The content of sorbitol in medicinal products for oral use may affect the bioavailability of other medicinal products for oral use administered concomitantly.
5 mg/kg/day	Sorbitol is a source of fructose. If your doctor has told you that you (or your child) have an intolerance to some sugars or if you have been diagnosed with hereditary fructose intolerance (HFI), a rare genetic disorder in which a person cannot break down fructose, talk to your doctor before you (or your child) take or receive this medicine.	Patients with hereditary fructose intolerance (HFI) should not take/be given this medicinal product
140 mg/kg/day	Sorbitol may cause gastrointestinal discomfort and mild laxative effect	

The proposed formulation product under submission contains 140 mg of sorbitol per ml of solution. Therefore, regarding the proposed formulation, it is estimated that when the MRDD of 6 mg of melatonin is administered, i.e., 6 ml of oral solution, the patient will receive 840 mg. Hence, for a 6-year-old-child weighing ~20 kg [redacted] and an adolescent or adult weighing 60 kg, the safety threshold of sorbitol would be 140 mg/kg×20 kg = 2,800 mg (2.8 g) and 140 mg/kg×60 kg = 8,400 mg (8.4 g), respectively, which is far below the maximum daily amount that a person (child aged >6 years, adolescent or adult) would receive from the proposed product. In case of patients with sugar intolerance, the safety threshold would be 5 mg/kg×20 kg = 100 mg in the case of a 6-year-old-child weighing ~20 kg [redacted] and 5 mg/kg×60 kg = 300 mg for an adolescent/adult; as a result, the proposed formulation is not considered appropriate for patients with such disorders and labeling information as above are to be included, accordingly.

2.4.4.6.1.8 Water, purified

Water is the most commonly used excipient in medicinal products, the minimum quality of water selected depending on the intended use of the product, according to a risk-based approach to be applied as part of an overall control strategy. Purified water is water used for the preparation of medicines other than those that are required to be both sterile and apyrogenic, unless otherwise justified and authorised [redacted] Obviously, the nature of this excipient does not pose any safety issues.

2.4.4.6.1.9 Xanthan gum

Xanthan gum is widely used in oral and topical pharmaceutical formulations, cosmetics and foods as a suspending and stabilising agent.

No eye or skin irritation has been observed in rabbits and no skin allergy has been observed in guinea pigs following skin exposure. No AEs were observed in long-term feeding studies with rats and dogs up to 1,000 mg/kg/day and in a 3-generation reproduction study with rats administered daily doses up to 500 mg/kg. No AEs were reported at the highest doses tested in chronic and carcinogenicity studies and there is no concern with respect to the genotoxicity [redacted]

Based on the reported use levels, a refined exposure of up to 64 mg/kg/day in children for the general population, 38 mg/kg/day for children consuming only of food supplements at the high-level exposure and 115 mg/kg/day for infants consuming foods for special medical purposes and special formulae (FSMPs), were estimated by the EFSA Panel. Xanthan gum is unlikely to be absorbed intact and is expected to be fermented by intestinal microbiota. Repeated oral intake by adults of xanthan gum up to 214 mg/kg/day for 10 days was well-tolerated, but some individuals experienced abdominal discomfort, an undesirable but not AE. The Panel concluded that there is no need for a numerical ADI for xanthan gum and no safety concern for the general population. Considering the outcome of clinical studies and post-marketing surveillance, the Panel concluded that there is no safety concern from the use of xanthan gum in infants and young children at concentrations reported by the food industry [redacted]

Xanthan gum is generally regarded as nontoxic and nonirritant at the levels employed as a pharmaceutical excipient. The estimated ADI value for xanthan gum has been set by the WHO at up to 10 mg/kg body weight. Xanthan gum is a GRAS-listed excipient and is accepted for use as a food additive in Europe. It is also included in the FDA-IID and nonparenteral medicines licensed in the UK.

In the melatonin 1 mg/ml oral solution, xanthan gum is contained in the amount of [redacted] of solution. Taking into consideration the MRDD of 6 mg melatonin (6 ml of solution) in all target population groups (paediatric and adult populations) and intended indications, the maximum daily ingested amount of this excipient will be [redacted] The established ADI value of 10 mg/kg corresponds to daily intakes of 600 mg and 200 mg based on a 60-kg-adult person basis and 20-kg-child body weight basis (aged 6 years), respectively. In any case, no safety concerns are excepted for the target age population groups.

2.4.4.7.2 Impurities

Generally, impurities in the drug substance and in the final product are not of special concern from a toxicological point of view. The specification limits have been set based on the relevant API manufacturer info, the maximum daily dose mentioned in the SmPC, the ICH Guideline Q3B R2 ‘Impurities in New Drug Products’ and the corresponding Ph. Eur. monograph, related to ‘melatonin’ please see Module 3).

2.4.5 INTEGRATED OVERVIEW AND CONCLUSIONS

The current Non Clinical Overview refers to a medicinal product containing melatonin as active substance in the form of an IR oral solution at the strength of 1 mg/ml. The product is intended for use in (i) the short-term treatment of jet lag in adults, (ii) sleep disorders in children and adolescents aged 6-17 years with ADHD, where sleep hygiene measures have been insufficient and (iii) the management of shift-work disorder in adults. The relevant Marketing Authorisation Application is submitted under Article 10.a of Directive 2001/83/EC, as amended (WEU Application). According to Article 10.a of Directive 2001/83/EC as amended, '*the Applicant shall not be required to provide the results of pre-clinical tests or clinical trials if he can demonstrate that the active substance(s) of the medicinal product have been in well-established medicinal use within the Community for at least ten years, with recognised efficacy and an acceptable level of safety*'. Therefore, the test and trial results are replaced by appropriate scientific literature.

Melatonin is a member of the class of acetamides and a member of tryptamines. In vertebrates, besides being produced in peripheral tissues and acting as an autocrine and paracrine signal, melatonin is centrally synthetised by a neuroendocrine organ, the pineal gland. Independently of the considered species, pineal hormone melatonin is always produced during the night and its production and secretory episode duration are directly dependent on the length of the night. As its production is tightly linked to the light/dark cycle, melatonin main hormonal systemic integrative action is to coordinate behavioural and physiological adaptations to the environmental geophysical day and season. The circadian signal is dependent on its daily production regularity, on the contrast between day and night concentrations, and on specially developed ways of action. During its daily secretory episode, melatonin coordinates the night adaptive physiology through immediate effects and primes the day adaptive responses through prospective effects that will only appear at daytime, when melatonin is absent. Similarly, the annual history of the daily melatonin secretory episode duration primes the CNS/endocrine system to the seasons to come. Remarkably, maternal melatonin programs the fetuses' behaviour and physiology to cope with the environmental light/dark cycle and season after birth. These unique ways of action turn melatonin into a biological time-domain–acting molecule. Melatonin is also dependent on beta-adrenergic receptor function.

Melatonin is involved in numerous biological functions including synchronising circadian rhythms, including sleep-wake timing and BP regulation, the stress response, aging, immunity, and in control of seasonal rhythmicity including reproduction, fattening, moulting and hibernation. Many of its effects are through activation of the MTs, while others are due to its role as an antioxidant. The therapeutic role of melatonin is clinically significant and well-established within the EU/UK, being approved as medicinal product as well as food supplement. Also, large clinical trials proving the efficacy of melatonin-containing products are present in published literature. Its pharmacology, toxicology and PKs have been fully characterised through systemic routes of administration since decades.

Non-clinical pharmacology and PKs of melatonin have been studied in experimental animals since several decades and are well-acknowledged. This Non Clinical Overview contains dedicated sections for primary and secondary PDs, safety pharmacology, PKs and PD/PK drug interactions. The potential toxicity of the hormone has also been evaluated in a series of studies in animal models. Single-dose toxicity studies have been adequately addressed and discussion on repeated-dose toxicity in various animal species administered melatonin via different routes of administration for various treatment periods has been provide.

A thorough presentation and discussion of the data on the potential for genotoxicity of melatonin concluding that melatonin is not mutagenic or clastogenic has been provided. The available carcinogenicity data from corresponding animal studie did not indicate carcinogenic effect but rather a protective effect against tumours. The studies presented in the current Non Clinical Overview indicated that melatonin may have some effects on the reproductive behaviour and sexual maturation.

All safety aspects of the active substance and the proposed formulation have been carefully evaluated. Given the long-standing use and the available non-clinical and clinical data of melatonin and the excipients chosen for the proposed formulation, it is suggested that no safety concerns are raised. The requirements of the legal basis selected for submission include (within robust literature data) string evidence of the well-established use of oral melatonin formulations within the Community for the intended indication in adults for more than 10 years. Additionally, the safety of the excipients that are present in the formulation is also a very important issue that is well investigated in accordance with EMA relevant Guidelines.

Based on the extensive analysis of literature data, it can be suggested that the pharmacology and toxicity of melatonin are well known with the non-clinical safety profile being acceptable for the proposed indications. It is unlikely that its use represents any significant risk and further toxicological studies are not deemed necessary. Overall, the safety and efficacy of oral melatonin use has been established over many decades of clinical use of many oral formulations for different indications, including the currently claimed, within the EU/UK and worldwide. The bibliographical data and the justifications presented in this Non Clinical Overview provide a good justification for the approval of melatonin in the form of oral solution for the proposed indication and at the strength of 1 mg/ml.

2.4.6 LIST OF LITERATURE REFERENCES

[redacted]

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[redacted]

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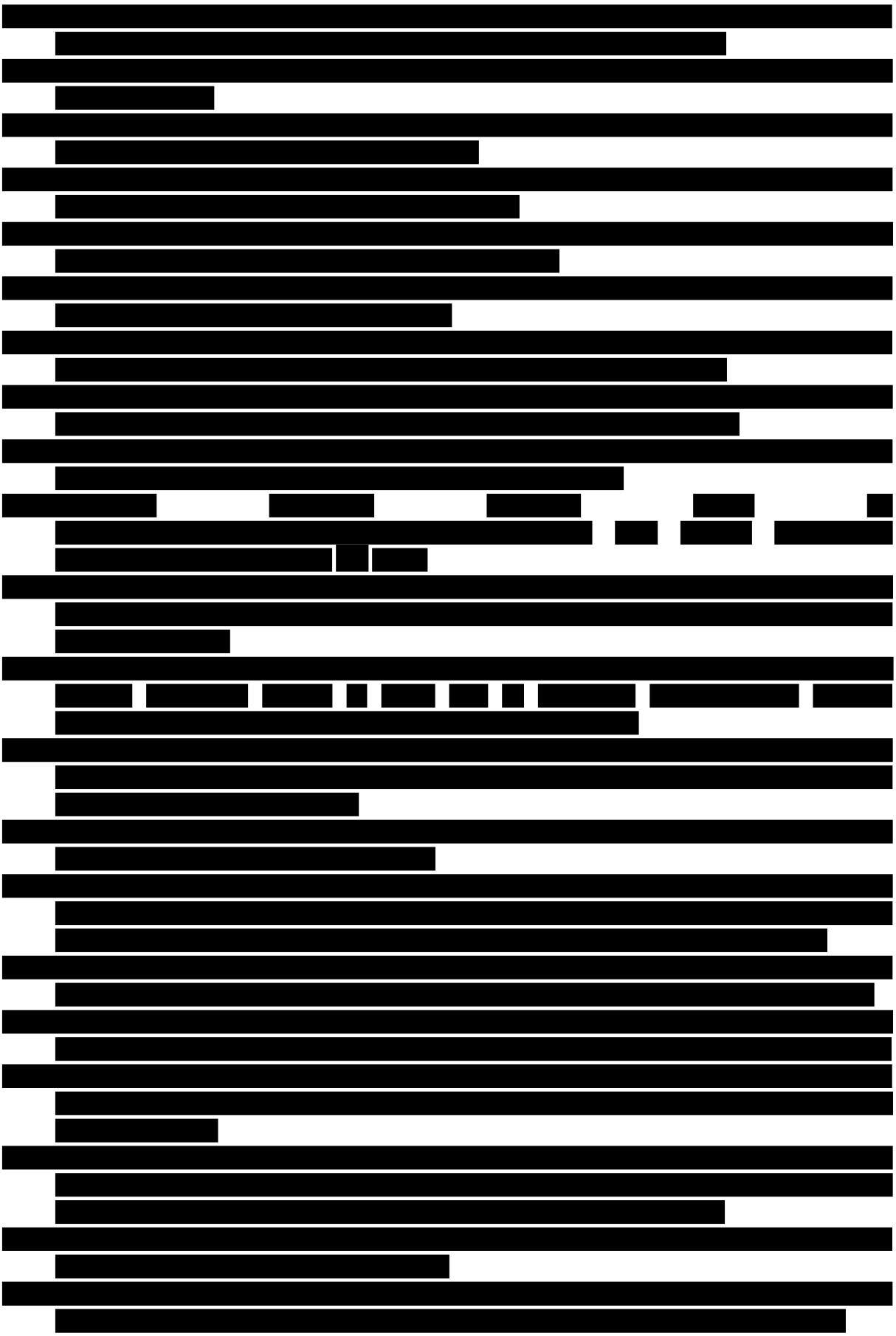

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