2.4 Non Clinical Overview

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2.4.1 OVERVIEW OF THE NONCLINICAL TESTING STRATEGY

2.4.1.1 PHARMACOLOGICAL CLASS

Melatonin, also known as N-acetyl-5-methoxytryptamine, is a small lipid and water soluble hormone of natural origin produced mainly by the pineal gland which is located behind the third ventricle in the brain. It plays an important role in the regulation of circadian rhythms. In humans the most important circadian rhythms is the sleep-wake cycle.

Melatonin is synthesized in the pineal gland during the dark phase of the light/dark cycle and is rapidly delivered to the body via the systemic circulation. In addition to the pineal gland, melatonin is synthesized in several other structures (retina, Harderiangland, gut) where the genetic expression and biochemical activity of the melatonin-synthesizing enzymes have been detected. Melatonin is produced from tryptophan which is converted to serotonin (5-hydroxytryptamine), then acetylated (N-acetylserotonin) and with a final conversion to melatonin, which is chemically an indole (N-acetyl-5 methoxytryptamine).

The sleep-wake cycle may be pathologically affected in different ways. Furthermore, the sleep may also be disturbed by various processes. The disturbances of the sleep-wake cycle are called circadian rhythms disorders and include the jet lag (tome zone change) syndrome, shift work sleep disorder, advanced sleep phase syndrome, non-24h sleep-wake syndrome. In all these insomnia might appear as a symptom.

Normally, melatonin production begins in the evening and is rapidly released into the blood and the cerebrospinal fluid. Melatonin can advance or delay sleep onset through G-protein coupled receptors (MT2). This explains why this indole is considered to have chronobiotic properties. Melatonin also has a sleep promoting effect through the more numerous MT1 receptors, as it can induce, maintain, and consolidate fragmented sleep patterns. Functional magnetic resonance imaging shows that both endogenous and exogenous melatonin similarly alter brain activity and induce sleep [1].

The pineal gland can only store Melatonin for a short time and the half-life of this hormone is 30 to 53 minutes. It is metabolized in the liver to its hydroxylated 6-sulfatoxymelatonin form, which is then excreted in the urine.

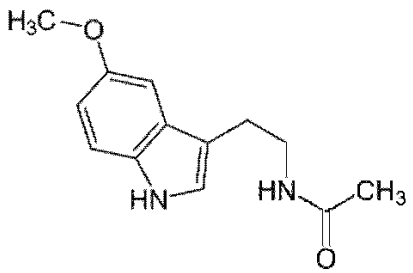


Figure 1 Chemical structure of Melatonin

2.4.1.2 SCIENTIFIC BACKGROUND

This application has been made under Article 10(a) of Directive 2001/83/EC Well Established Use. Melatonin [redacted] has [redacted] been widely available as both a nutritional supplement and an approved medicine for more than 10 years. Melatonin has been

a medicinal product, and therefore as such the dossier can refer to the reference product, in this case Bio-Melatonin, Hungary. [redacted]

2.4.1.3 NON CLINICAL DEVELOPMENT PROGRAM

As mentioned above, medicinal products containing melatonin have been registered as medicinal products within the European Union and have been marketed worldwide for more than 10 years. In addition, melatonin has been marketed as a food supplement in many countries and has been subject to regulatory opinion as such. 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.

The present application does not include nonclinical trials as they are not required for products submitted under Article 10(a) of Directive 2001/83/EC.

The applicant performed one clinical study to determine the bioavailability of the proposed product Melatonin 1 mg/ml Oral Solution [redacted] and demonstrate a link between the proposed product and the products described in the literature. [redacted]

2.4.1.4 SEARCH STRATEGY

[redacted]

2.4.2 PHARMACOLOGY

2.4.2.1 PRIMARY PHARMACODYNAMICS

There is substantial data contained within the literature as to melatonin's involvement within the whole circadian system and its influence upon 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 nuclei of the hypothalamus (SCN) which have melatonin receptors [2]. In addition to the pineal gland, melatonin is synthesized in several other structures (retina, Harderiangland, gut) where the genetic expression and biochemical activity of the melatonin-synthesizing 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 and have demonstrated 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 [3-5].

2.4.2.1.1 Mechanism of action / in vitro studies

Mechanism of action of melatonin is presented in Figure 2. Melatonin shows its affects by four mechanisms:

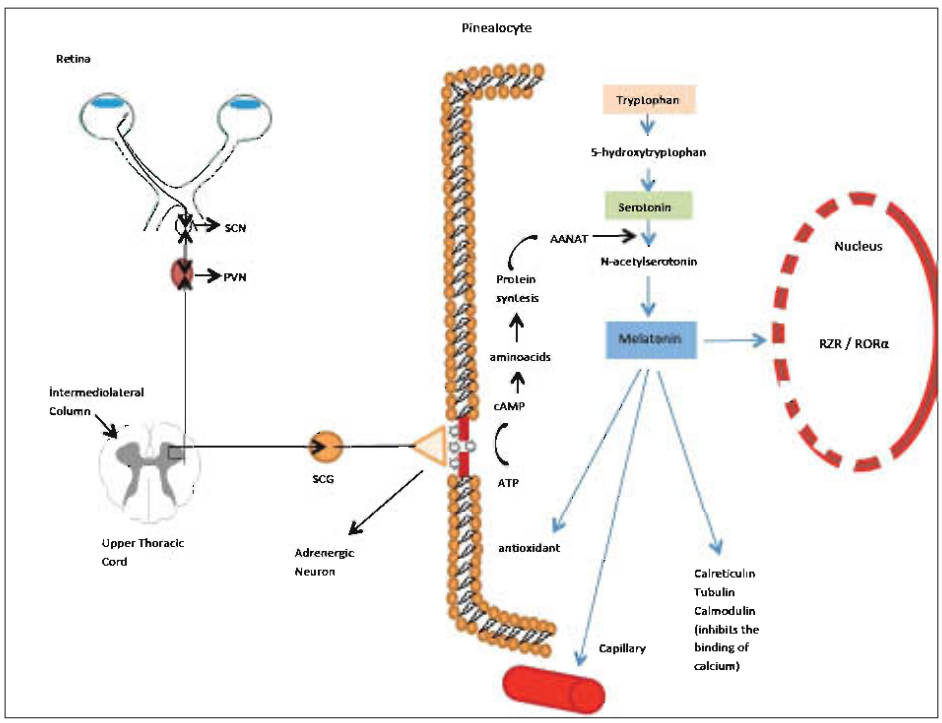


Figure 2 The neurologic pathway from the eyes through the pineal gland. SCN: Suprachiasmatic Nucleus, PVN: Paraventricular Nucleus of the Hypothalamus, SCG: Superior Cervical Ganglion, RZR/RORa: Retinoid-related Orphan Nuclear Hormone Receptor.

Melatonin shows its affects by four mechanisms in mammals:

1. Binding to melatonin receptors in plasma membrane
2. Binding to intracellular proteins such as calmoduline
3. Binding to Orphan nuclear receptors
4. Antioxidant effect [6]

Melatonin interacts with intracellular proteins named calmoduline, calreticulin and tubulin [7]. Calmoduline is an intracellular secondary messenger. Melatonin directly antagonizes binding of calcium to calmoduline [6, 7] (Figure 2). The anti-proliferative 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. IL-2 and IL-6 are produced in mononuclear cells by this mechanism [6].

There are three different membrane receptors and one nuclear receptor:

1. *Melatonin receptor type 1a:* Mel 1a, ML1a, ML1, MT1, MTNR1A

It is encoded in human chromosome #4 and consists of 351 amino acids [8]. MT1 receptor constitutes adenylate cyclase inhibition by binding to various G-proteins [9]. MT1 receptors are commonly found in human skin [7]. During aging process and Alzheimer's disease, the expression of MT1 receptor in suprachiasmatic nucleus (SCN) and cortex decreases [7]. MT1 receptors reduce the neuronal discharge rate in SCN and suppress prolactin secretion [10].

2. *Melatonin receptor type 1b:* Mel 1b, ML1b, MT2, MTNR1B

It is encoded in human chromosome 11 and consists of 363 amino acids [8]. MT2 receptor creates adenylate cyclase inhibition by binding to various G-proteins. Additionally, it inhibits the soluble guanylyl cyclase pathway [9]. Through melatonin receptor activation, adenylate cyclase inhibition occurs and the production of cyclic AMP (cAMP) is reduced [11, 12].

In the skin, MT2 receptors are located within normal and malign melanocytes and eccrine sweat glands [7]. MT2 receptors inhibit GABA-A receptor-related functions in the hippocampus in rats [10].

In Alzheimer's disease, MT2 receptor expression is reduced. MT2 receptors are involved in antidepressant activity. MT2 receptors contribute to the pathophysiology and pharmacology of sleep disorders, anxiety, depression, Alzheimer's disease and pain [9]. MT2 receptors may be the new target for development of hypnotic agents. MT2 receptors are responsible for anxiolytic effects of melatonin.

Pharmacological studies have revealed that MT2 receptors regulate sleep, particularly NREMS. MT2 receptor ligands have more powerful hypnotic properties when compared to non-selective MT1/MT2 ligands [9].

Mel1c, MTNR1C: It is not present in humans. It is found in fish, amphibians and birds [8]. In chicken, the rhythm of MTNR1C receptor is the opposite of MT1 and MT2. Its level is highest at daytime and lowest at night-time [8, 13].

MT3, ML2= NQO2= Quinone reductase 2 enzyme= QR2. This enzyme belongs to the reductase group, which are involved in prevention from oxidative stress by inhibiting the electron transfer reactions of quinones [7]. This enzyme (or MT3 receptor) is located in liver, kidney, heart, lung, intestine, muscle

and brown fat tissue. It is a detoxification enzyme [6]. There is evidence for its involvement in regulation of intra-ocular pressure [6].

3. *RZR/RORα:* Retinoid-related Orphan nuclear hormone receptor. With this receptor, melatonin binds to the transcription factors in nucleus which belong to retinoic acid receptor super-family. The following are described for retinoic acid receptor super-family variants RORα (retinoic acid receptor-related Orphan receptor-α; human gene ID: 6095): RORα isoform a (aka RORα1), RORα isoform b (aka RORα2) and RORα isoform d (also known as RZRα), and the product of another gene, RORβ (aka RZRβ; human gene ID: 6096) [7].
4. *GPR50: H9, ML1X:* Melatonin-related Orphan receptor. 'X linked Orphan G-protein coupled' (It is an X-linked inherited receptor, binding to G-protein. It is the orthologue of MEL1c, which is found in non-mammalian living creatures [14]. Its gene is located on the X chromosome (Xq28) and consists of 618 amino acids [8]. It is present in all mammalians including humans. It does not have the characteristics of binding to melatonin [6]; however, it is effective in binding of melatonin to MT1 [15]. GPR50 is not present in birds and fish [8, 16]. 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 [17]. GPR50 has no affinity to melatonin; however, when it dimerizes with MT1, it inhibits the melatonin signal [12, 18].

GPR50 has other functions apart from melatonin [8]. GPR50 interacts with neurite outgrow inhibitor (NOGO-A) [8, 19] and TIP60 (glucocorticoid receptor signal coactivator and histone acetyltransferase) [8, 20].

Interaction with melatonin MT1 and MT2 receptor subtypes seem to be involved in the action. MT1 receptors are located mainly in cells of the pituitary pars tuberalis (PT), controlling seasonal prolactin variations in ruminants, whereas there is no evidence to suggest that MT2 receptors are present in the PT. By contrast, both MT1 and MT2 receptors are located in the suprachiasmatic nucleus (SCN). The molecule ^{125}I-melatonin has been used in binding and autoradiographic studies and has enabled detection of melatonin binding sites expressed at low density in most tissues in which effect of melatonin have been reported.

The transduction pathways mediated by these melatonin receptors remain an unsolved and complex issue. The MT1 receptor couples to different G protein, one of which mediates inhibition of adenylcyclase and the other activates phopsholipase Cβ. The MT2 receptor couples to phosphoinositide production, the inhibition of adenylcyclase and the inhibition of the soluble guanylyl cyclase pathway. The MT2 receptor mRNA present in human retina and brain is responsible for entrainment of circadian rhythms in the SCN. MT1 and MT2 polymorphisms have been found in humans and may be associated with sleep disorders.

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 melatonin receptors. These receptors have been identified on macrophages, B cells and T cells [21, 22]. Melatonin can modulate proliferation and cytokine secretion via these receptors on immune cells [23, 24]. In animals, melatonin can inhibit chemically induced tumours, which is increase by pineal suppression (long light phases) or pinealectomy [25]. Pinealectomy stimulates and/or melatonin inhibits the growth and sometimes the metastasis of experimental cancers of the lung, liver, ovary, pituitary, prostate as well as melanoma and leukaemia. [redacted]

[redacted] the impact of the melatonin on other pharmacodynamics pathways is important relating to safety of the product for its intended use.

2.4.2.1.2 Mechanism of action / in vivo studies

In mammals melatonin is mainly synthesised in the pineal gland from serotonin but it is also formed in the gut and retina. The production is circadian and it is stimulated by photic stimulus arising after the onset of darkness. 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.

A limitation of studies in nocturnal laboratory animals 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 has no analogue in humans; 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 (i.e. 2 – 20 mg/kg) produce pharmacological circulating levels, several orders of magnitude greater than what is observed naturally, so like many of the human studies these may not reflect the endogenous physiological role of the hormone.

A study has been conducted in diurnal macaques to explore the nature of sleep-promoting effects of melatonin [26]. 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:

1. 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;
2. Similar temporal patterns of melatonin production, occurring during habitual night-time sleep period;
3. 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.

In all three species of diurnal macaques studied, the sleep process showed high sensitivity to daytime melatonin administration. Sleep initiation was significantly promoted by a wide range of melatonin doses used and, as in humans, showed a lack of dose dependence of the effect, once the dose (5 – 20 µg/kg, orally) was sufficient to induce physiologic circulating levels of the hormone (above 50 pg/ml). Lower doses failed to promote sleep in the macaques studied.

The effect of melatonin on hexobarbital (75 mg/kg, i.p.)-induced narcosis was investigated in mice using 20 mg/kg* melatonin i.p. (low dose) and 100 mg/kg* melatonin i.p. (high dose). The onset time for hypnosis and the duration of the sleeping period were measured in all groups. The results are exposed in the Table I.

Table I Effect of Melatonin on hexobarbital induced narcosis in mice

Groups	Hypnotic onset time (min.)	Sleeping time (min).
Control	2.18 ± 0.74	28.8 ± 13.22
20 mg/Kg, ip	5.08 ± 2.09*	43.94 ± 12.52
100 mg/Kg, ip	2.47 ± 1.46	78.51 ± 19.46**

*P < 0.05; **P < 0.01

The results show that melatonin, at the dose of 20 mg/Kg delayed the hypnosis induced by hexobarbital and increased the sleeping time of the animals. Furthermore the animals showed excitation and body rotation before following asleep. For the animals treated with the dose of 100 mg/Kg the duration of the sleeping period increased and the onset time for hypnosis was similar (slightly higher) to the one from controls. The results seem to suggest that melatonin potentiated the sleeping effect induced by hexobarbital, but increased the onset time for hypnosis (vs controls) for which a plausible explanation was not provided [27].

2.4.2.2 SECONDARY PHARMACODYNAMICS

Studies have been conducted in a variety of species (including mice, rats, hamsters and baboons) to investigate metabolic/behavioural response to melatonin, effects on the immune systems, nervous system, endocrine and reproductive systems and cardiovascular system. These studies showed no special hazard for humans based on conventional safety pharmacology studies. The studies relating to the secondary pharmacodynamics of concern (immune systems, reproductive and endocrine systems) are further discussed:

2.4.2.2.1 Immune System

There is substantial evidence that melatonin exerts some of its effects as an immunomodulatory compound, though there is little understanding how melatonin actually regulates the immune system. Some references suggest that melatonin acts as an immunostimulant, whilst other studies suggest that the molecule exerts anti-inflammatory properties. Some theories suggest that Melatonin acts as an "immune buffer", acting as a stimulant under basal or immunosuppressive conditions, or as an anti-inflammatory compound in the presence of exacerbated immune responses such as acute inflammation.

It has been established that the pineal gland, the primary source of melatonin, is an immune target [28] 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 colony-stimulating factor (GM-CSF) stimulated the synthesis of melatonin both in vivo and in vitro [29].

Lipopolysaccharide (LPS) treatment not only reduced the production of nocturnal melatonin in rats but also enhanced endothelial cell adherence, which was normalized after melatonin administration [30]. LPS was shown to induce TNF-α production in the rat pineal gland through activating toll-like receptor 4 (TLR-4) [31]. Subsequently, the production of TNF-α by pineal gland microglia was found to act on tumour necrosis factor receptor 1 (TNFR1), driving the nuclear translocation of NF-κB, which represses Aa-nat transcription and in turn suppresses melatonin synthesis [32]. Suppression of increased nocturnal melatonin in human mothers with mastitis was highly correlated with increased TNF-α

production [33]. Likewise, an increase in TNF-α levels after Caesarean section resulted in the suppression of serum melatonin nocturnal levels [34].

Melatonin and/or its biosynthetic machinery have been located in a variety of immune tissues, organs and cells, such as rat, mouse and human thymus [35, 36] spleen, bone marrow and circulating leukocytes [37] mast cells, natural killer cells and eosinophils [38] and in several immune cell lines [39-42]. Rat peritoneal macrophages also produce melatonin in vitro after incubation with tryptophan [43]. It has been found that in vitro-cultured human lymphocytes not only actively synthesize and release substantial amounts of melatonin [44], but that this melatonin modulates the IL-2/IL-2 receptor (IL-2R) system via receptor-mediated intra-, auto- and/or paracrine actions [45].

A large amount of evidence has demonstrated the immunomodulatory capacity of melatonin administration in both in vivo and in vitro models [44]. Some studies have shown that melatonin treatment promotes an increase in the weight of immune organs, both under basal and immunosuppressed conditions [46-49]. Conversely, the anti-proliferative effects of melatonin have been observed in vitro in PHA-stimulated human lymphocytes [50]. Melatonin also modulates both the innate and specific immune responses through regulation of immunocompetent cell proliferation [51, 52] and secretion of immune mediators, such as cytokines [28].

[redacted] reconstitution of the night-time plasma melatonin peak completely abrogated the humoral and cellular responses in propranolol-immunosuppressed mice [53]. Mice immunosuppressed by lead recovered splenic $CD4^+$ cell numbers and functions after melatonin treatment [54]. Melatonin also averted age-induced immunosuppression in rats by increasing IgG1 and IgM levels [55]. Furthermore, melatonin significantly restored both dexamethasone- and aging-induced immunosuppression in squirrels [49, 56]. Melatonin also increased B cell proliferation and the Th1 response (IL-2 and IFN-γ production) and decreased Th2 cytokines such as IL-10 in old mice [57].

Early in vitro studies suggested that melatonin has pro-Th1 effects [24]. Sub-stimulated PBMCs displayed enhanced production of Th1 cytokines, such as IFN-γ and IL-2, after in vitro melatonin treatment. The diurnal rhythmicity of human cytokine production indicated that the IFN-γ/IL-10 peak occurs during the early morning; this peak positively correlated with plasma melatonin [58], suggesting a melatonin/Th1 causality. Splenocyte proliferation in response to the T cell mitogen concanavalin A, was also enhanced by the addition of melatonin in vitro [59].

Conversely, melatonin significantly reduced the splenic $CD19^+$ B-cell population in mice with experimental membranous nephropathy and diminished the overexpression of TNF-α, IL-1β and IFN-γ [60]. Further in vivo studies have shown the capacity of melatonin to promote a Th2 response in several models. The first report demonstrated that high doses of melatonin enhanced the production of the hallmark Th2 cytokine IL-4 in bone marrow lymphocytes c Early nocturnal sleep induced a shift in the Th1/Th2 cytokine balance towards increased Th1 activity, whereas the Th2 response dominated during late sleep. A robust decrease in TNF-α-producing $CD8^+$ cells was also observed during sleep [61], suggesting a correlation between melatonin and the Th2 response.

Likewise, the absence of melatonin due to pinealectomy polarized rat thymic Th1/Th2 cells towards a Th1 response by increasing the production of IFN-γ and reducing IL-10 levels, implying that melatonin skews the immune response towards Th2 dominance [62]. Chronic administration of melatonin to antigen-primed mice increased the production of IL-10 and decreased the secretion of TNF-α, suggesting a Th2 response [63]. Melatonin inhibited the Th1 response by suppressing IFN-γ and IL-12 in mice with contact hypersensitivity [64]. Furthermore, melatonin protected against experimental

reflux esophagitis by suppressing the Th1-mediated immune response [65]. Melatonin also acted as an immunosuppressive agent and reduced Th1 cytokine levels in an experimental model of ovarian transplant in mice, permitting prolonged graft survival [66].

From the extensive research on the impact of endogenous and exogenous melatonin on the immune response pathways, it has been reported that melatonin possesses an important role in the treatment of a number of different clinical conditions, including as an antiviral, antibiotic and anti-parasitic molecule [67, 68].

The impact of melatonin has been investigated in auto-immune conditions such as Rheumatoid Arthritis (RA), where several models have suggested deleterious actions for both endogenous and exogenous melatonin. Fibroblasts from synovial membranes collected from RA patients also show impaired circadian expression of timekeeping genes and pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 [69]. When the in vitro data is correlated to human RA patients with active disease, who are administered daily melatonin, it was reported that low antioxidant profiles were observed along with increased neopterin concentrations and erythrocyte sedimentation rates (inflammation indicators) and no changes in pro-inflammatory cytokine levels (TNF-α, IL-1β and IL-6), but these effects were not associated with any changes in clinical symptoms [70].

Other clinical conditions that have been investigated both in vitro and in vitro that involve auto-immune conditions are multiple sclerosis (MS), Systemic Lupus Erythematosus (SLE), Type 1 Diabetes (T1D), Irritable Bowel Syndrome/Inflammatory Bowel Disease (IBS/IBD), Breast Cancer, AIDS. The correlation of the in-vitro data has not been yet shown to have any significant positive impact on clinical outcomes with humans.

In addition, to the above clinical conditions, melatonin has been extensively studied within the ageing processes and immunosenescence. The immunomodulatory effects of melatonin in aging are evident in the central nervous system (CNS), as dietary melatonin was shown to selectively reverse the lack of response to an inflammatory stimulus in the brains of aged mice [71].

2.4.2.2.2 Endocrine and Reproductive Systems

Melatonin regulates pubertal development in some juvenile mammals. In seasonal breeders, melatonin seems to act as either pro-gonadotrophic or anti-gonadotrophic according to 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 LH and prolactin, corticosteroids, thyroid hormones and insulin.

In adult female rats, it was observed that a single intravenous dose of melatonin (12.8 mg/kg) increased serum prolactin levels [72]. In adult males, SC infusion of melatonin decreased serum prolactin levels and (at ~ 4.8 mg/kg) caused a decrease in testes weight and testicular degenerative changes [73]

Special studies in juvenile rats have been conducted to investigate the role of melatonin in sexual development. In male juvenile rats, an initial study [redacted] investigated the influence of daily subcutaneous administration of melatonin (5 - 100 µg/day) on sexual development in prepubertal and pubertal male rats. This study did show that melatonin administration could inhibit or delay sexual development, but importantly a subsequent study demonstrated that any effects were reversible [75]. The latter study confirmed that melatonin (100 µg/day) delays sexual maturation in young male rats

when administered daily in the afternoon. It was demonstrated that 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. The reversibility of the effects were also confirmed in the study by Olivares [76].

A study has shown that melatonin can also delay sexual maturation in female juvenile rats [77]. This study confirmed that chronic melatonin administration (100 µg /day) delays sexual maturation of female rats, probably by retarding maturation of hypothalamic GnRH-producing cells. Thus, melatonin could modify basal GnRH secretion of pulsatile release. This study suggested that pituitary and ovarian responsiveness do not seem to be affected since proestrous surges of 17β-estradiol, LH, and FSH occur, albeit at reduced frequency.

Various other effects on hormone levels have also been seen following administration to animals. A study in adult female hamsters has demonstrated that administration of melatonin (25 µg SC for 8 or 11 weeks) inhibited blood levels of thyroxine, triiodothyronine and thyrotropin [78]. Studies in male rats have demonstrated that administration of melatonin at 30 mg/kg SC for 10 days decreases adrenal gland and serum corticosterone levels, and at 8 mg/kg SC for 30 days decreases uptake of [3H]-Testosterone by the prostate [79, 80]. A further study in 10-week old, hypothyroid male hamsters demonstrated that melatonin administration (25 µg SC for 10 weeks) led to a decrease in pituitary and serum prolactin, TSH and LH content and decrease in serum thyroxine and triiodothyronine [81].

2.4.2.2.3 Cardiovascular and Respiratory Systems

Melatonin receptors were identified on the anterior cerebral and caudal arteries of rats and on the coronary and pulmonary arteries of pigs.

In rats, a dose-related fall of mean arterial pressure, heart rate and also of brain serotonin release were observed in consequence of 30 - 60 mg/Kg melatonin i.v. Bradycardia was abolished by pre-treatment with bilateral vagotomy thus suggesting that it may be mediated through a parasympathetic action [82]. Also studies in porcine and coronary arteries suggest the potential for melatonin to have tensive effects [83, 84]. In baboons, 0.3 to 0.4 mg/Kg melatonin, i.v. caused a statistically significant increase of the cardiac output and ventricular ejection associated to a reduction in heart rate [85]

2.4.2.2.4 Central Nervous System

In mice, the Irwin test showed that at doses > 8 mg/kg melatonin had no behavioural effects. At 16 mg/kg a slight sedation was observed. Such sedation was also reported in the repeated dose studies conducted by the Company in rats. At doses of 64, 128 and 256 mg/kg decreased fear, reactivity, muscle tone and hypothermia were observed with dose-dependent intensity and duration. At 128 mg/kg it also showed analgesic activity in the four-plate test [86].

Daily administration of 2.5 – 10 mg/kg melatonin prior to the swimming test significantly reversed the increased immobility period that was observed on chronic exposure to swimming test. This effect was reported to be comparable with that of GABA-benzodiazepine (BZ) receptor agonists, appearing to involve GABA-benzodiazepine receptors [87]. In other studies, acute administration of melatonin did not reveal antidepressant activity.

The anticonvulsant effects of melatonin have been the subject of a number of reports and considerable conflicting data have been presented. The effect of melatonin in these tests has been compared to its neurotoxicity and acute toxicity. From the results of a study conducted in mice it does appear that melatonin has anticonvulsant activity in some of the tests used to screen clinically important anticonvulsants. However, the doses needed to produce an anticonvulsant effect (significant effect vs pentylenetetrazole at 200 mg/kg; ED_{50} vs 3-MPA, 115 mg/kg; ED_{50} vs ECS, 159 mg/kg) are similar to those which produce signs of motor incoordination in the rotorod test at this pre-dose interval. Thus, the authors suggest that the anticonvulsant action of melatonin may not represent a specific neuropharmacological action but rather an inability of the animal to make the appropriate motor response [88].

2.4.2.3 SAFETY PHARMACOLOGY

All data for safety of melatonin are summarized in Table II.

It has been reported that 100 mg/kg dose of melatonin slight decreases the heart rate and blood pressure, although The Q-T interval of the ECG and the respiratory rate were not changed.

Table II Safety Pharmacology of Melatonin

Study type	Species & strain	Route of administration	Duration of dosing	Melatonin dose	Ref.
Events on Nervous System	Mouse, male (MFI)	IP	Single dose	50 - 400 mg/kg	[88]
	Mouse, male (NMRI)	IP	Single dose	128, 256 mg/kg	[86]
	Mouse (Balb/c, C57BL/6J)	IP	6 days	2.5 - 10 mg/kg	[87]
	Rat (SD)	Oral, IV, IP	Single dose	To determine ED_{50}	[88]
Effects on endocrine and reproductive systems (adult animals)	Rat, female (4- 5 months old)	IV	Single dose	12.8 mg/kg	[72]
	Rat, male (Wistar)	SC	10 days	1, 5, 15, 30 mg/kg	[79]
	Rat, male & female(SD, 8-9 weeks old)	SC	28 days	Male: 0.05, 0.5, 4.8 ng/kg Female: 0.07, 0.75, 7.3 mg/kg	[73]
	Rat, male (Wistar)	SC	30 days	0.8, 2.4, 4.8, 8 mg/kg	[80]
	Hamster, female (Syrian)	SC implant	8 or 11 weeks	25 µg, 2.5 mg, 1 mg sc	[78]
	Hamster, male (10 week old, hypothyroid)	SC	10 weeks	25 µg	[81]
Effects on endocrine and reproductive systems (juvenile animals)	Rat, male (Wistar)	SC	Pre-pubertal: 100 µg/d from 5 - 20 days old Pubertal: 5 – 100 µg/d from 20 - 45 days old Adult: 100 µg/day from 70 – 90 days old		[74]
	Rat, male (Wistar)	SC	200 µg/d sc from various ages between 20-up to 115 days of age		[75]
	Rat, male (Wistar)	SC	0.1 mg from age 20 to up to 39 days		[76]
	Rat, female (45 months old)	SC	100 µg/d from age 15 days up to and beyond opening of vagina to follow estrous cycles		[77]
Effects on cardiovascular and respiratory systems	Rat (SD)	IV	Single dose	30 - 60 mg /kg	[82]
	Rat (SHR, spont hypertensive)	IP	5 days	20 mg/kg/d	[89]
	Baboon	IV	Single dose	0.3 to 0.4 mg/kg	[85]

2.4.2.3.1 Central nervous system

Melatonin in physiological doses causes vasoconstriction and also constricts cerebral arteries" in rats [90]. Experiments have been carried out [redacted] on the potential effects of melatonin on the nervous system. Although many compounds which prolong hypnotic activity can lower body temperature, melatonin (25 mg/kg) had no hypothermic action in the rabbit. Melatonin at doses of 10 mg/kg produced no change in post-synaptic spike potentials in the cat superior cervical ganglion and no change in the response in the nictitating membrane. In mice, 30 mg/kg of melatonin given

intraperitoneally every 3 hours for 18 hours caused no change in gross behaviour or in the amount of noradrenaline in the brain or heart [91].

2.4.2.3.2 Cardiovascular system

Depending on the concentrations of melatonin or the preparation used, melatonin can exert either a vasoconstrictory effect at physiological concentrations (nanomolar) or a vasodilatory effect at higher concentrations (micromolar or millimolar), suggesting a biphasic pharmacology of melatonin. The subcellular mechanism of such an activity is as yet unknown despite the fact that melatonin receptors have been identified in different structures (arteries). Melatonin and its main target, the SCN, are able to modify cardiovascular rhythms (e.g., blood pressure, heart rate). Taken together, these data, among others, show that melatonin could modulate the rhythmicity of the cardiovascular system. Again, alterations of the circadian rhythmicity of melatonin could be deleterious from a long-term effect point of view [92].

The effects of melatonin on the circulatory system were investigated [redacted]. The results of experiments on the blood pressure of cats indicate that the administration of 10 mg/kg of melatonin did not alter the blood pressure. Melatonin (10 mg/kg) also failed to alter the contractile force or electrocardiogram of the dog. The inotropic and chronotropic actions of the isolated guinea pig and rat heart were unaltered when melatonin was perfused at a concentration of 10^{-4} moles/l [91].

2.4.2.4 PHARMACODYNAMIC DRUG INTERACTIONS

Melatonin has been shown to enhance tamoxifen's effects [93].

Additionally, the potential synergistic effect of melatonin on a classical drug, imipramine was evaluated [redacted]. To test this hypothesis, porsolt swim test, a test predictive of antidepressant-like action, was conducted in mice. Imipramine at doses of 20 and 40 mg/kg caused no alteration and statistically significant reduction in the duration of immobility in forced swim test, respectively. While 5 mg/kg melatonin had no effect, 10 mg/kg melatonin slightly reduced the duration of immobility. When sub-effective 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 [94].

2.4.3 PHARMACOKINETICS

2.4.3.1 ABSORPTION

In order to evaluate and to bridge to the published data, the Applicant performed an in-vitro study to assess the bi-directional permeability of the test compound to clarify the rate and extent of absorption. Using standardized Caco-2 cells it was determined that the permeability coefficient (P_{app}) was 40.3 and 39.9×10^{-6} cms^{-1} for the A2B and B2A directions respectively. The mean percentage recovery A2B was

99.4 % and for the B2A direction it was 97.6 %. The calculated efflux ratio was 0.990 thereby indicating that Melatonin is not subject to active efflux. It therefore is expected that melatonin is completely absorbed in vivo. [redacted]

These calculated permeability values are also supported by the literature (Table III).

Table III Reported permeability values of Melatonin

Reference	Dose of Melatonin	$P_{app\ A-B}$	$P_{app\ B-A}$	Efflux ratio ($P_{app\ B-A}/P_{ap\ A-B}$)
[redacted]	5 µM	11.56 ± 2.00 × 10^{-6} cm/s	11.58 ± 1.01 × 10^{-6} cm/s	1.0
	6.5 µM	12.5 × 10^{-6} cm/s	-	-
	10 µM	40.3 ±4.74 × 10^{-6} cm/s	39.9 ± 1.30 × 10^{-6} cm/s	0.99
	50 µM	~ 11.0 × 10^{-6} cm/s	-	-

Usually, molecules with permeability > 10 nm/s in the Caco-2 system (1 × 10^{-6} cm/s) are classified as highly permeable and have intestinal absorption >90 % [98]. Other authors suggest a cut-off value of 100 nm/s (or 10 × 10^{-6} cm/s) [99]. It is reported that the overall ranking of compounds with P_{app} < 1 × 10^{-6} cm/sec, between 1 - 10 ×10^{-6} cm/sec and > 10 ×10^{-6} cm/sec can be classified as poorly (0 – 20 %), moderately (20 – 70 %) and well (70 – 100 %) absorbed compounds, respectively [99]. According to the table above (Table III) all reported values from literature are > 10 × 10^{-6} cm/sec for Melatonin indicating that the proposed product has high permeability.

Ascending doses of oral melatonin was studied in two dogs. Each dog received 10, 20, 40 and 80 mg/kg body weight of melatonin given at 2-hour intervals. The authors state that, melatonin concentrations in serum increased proportionally with increasing dose, however no exposure parameter (C_{max} or AUC) values were reported. The mean peak concentration after 80 mg/kg was approximately 100 µM.

In addition, 4 dogs were given a single melatonin dose of 40 mg/kg. Melatonin was rapidly absorbed and reached a peak value in serum (circa 5 µM) between 20 to 30 min following its administration. The distribution phase was 3 - 4 hours and the elimination half time ($t_{1/2}$) was approximately 5 hours. In one dog urinary excretion of melatonin was also investigated. The total excreted amount of immunoreactive melatonin during the five hours after its administration was 0.25 % of the dose. The authors commented that the endogenous serum levels of melatonin were low as compared to those obtained after oral administration of melatonin, which gave 104 to 106 times higher levels [100].

In another study, [96] the oral bioavailability of a 10 mg/kg dose of melatonin in rats was 53.5 %, while in dogs and monkeys, it was > 100 %. Also, in rats the bioavailability of a 10 mg/kg dose of melatonin administered intraperitoneally was found to be 74 %. Since the oral dose used in dogs and monkeys

(10 mg/kg) was three-fold higher than the intravenous dose (3 mg/kg), a bioavailability value in excess of 100 % may be indicative of non-linearity and hence dose dependency in the pharmacokinetics of melatonin. To probe the issue of nonlinear pharmacokinetics, oral bioavailability of a 1 mg/kg dose of melatonin was studied in dogs. The results indicate significant dose dependency in the pharmacokinetics, with the plasma AUC and oral bioavailability of the 1 mg/kg dose being disproportionately lower than that of the 10 mg/kg dose (Table IV).

Table IV Summary of pharmacokinetic parameters of melatonin in rat, dog and monkey (mean value obtained from two animals in each case)

Parameter	SD Rat	Beagle dog		Cyno monkey
Intravenous dosing				
Dose (mg/kg)	5.00	2.95		2.98
AUC (mg.hr/L)	2.38	0.81		1.78
Clearance (L/hr/kg)	2.11	3.84		1.68
Half-life (hr)	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	1030	10.00
AUC (mg.hr/L)	2.49	0.05	3.44	8.85
Dose adjusted F (%)	53.5	16.9	>100	>100

The bioavailability after nasal application of 1.5 mg of melatonin in rabbits was found about 60 % and C_{max}, T_{max} and $t_{1/2}$ were found 160 ng/ml, 5 min and 10 min respectively [101].

2.4.3.2 DISTRIBUTION

Melatonin readily penetrates biological membranes and appears in tissues or body fluids in concentration on the same order of magnitude as plasma.

The steady state volume of distribution of melatonin in different species (SD rat, Beagle dog and Cyno monkey) ranged from 1.05 to 1.48 L/kg , as reported by Yeleswaram et al, indicating moderate tissue distribution of melatonin in these animals [96]. In humans, [redacted] a steady state volume of distribution of 0.55 L/Kg, suggesting a significantly reduced distribution of melatonin in humans than in the animal models [102].

Melatonin has been shown to cross the placenta in rats, sheep and rhesus monkeys and can be transferred to rat pups in maternal milk. Subcutaneous administration of ^{3}H-acetyl-melatonin to Sprague-Dawley (SD) rats on day 18 of gestation resulted in detection of radioactivity in whole fetuses and fetal tissues (brain, liver, heart, viscera, skin, muscle, and bone), with highest concentrations in fetal liver and lowest concentrations in fetal brain.

Melatonin seems to distribute fast through tissues in the rat after systemic injections, rapidly penetrates into brain and cerebrospinal fluid [103].

2.4.3.3 METABOLISM

From the bibliography, it is generally accepted that melatonin is primarily metabolised by CYP1A1 and CYP1A2. [redacted], urinary metabolites were determined from the

chromatographic analysis, after intraperitoneally administration of radio-labelled melatonin in rats and three distinct peaks were identified. Two of these identified 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 5 %. The unidentified metabolite corresponded to 12 % of radioactivity [104, 105].

[redacted] when ^{14}C-melatonin was injected intracisternally into rats, 2.35 % of the total radioactivity in the urine was recovered as compound II (N-acetyl-formyl-5-methoxykynurenamine). In the case of intravenous administrations of melatonin, 15 % of the total radioactivity was recovered as compound (II). In either case, 65 % of the radioactivity administered was recovered in the urine within 24 hours. These results taken together strongly indicate that the conversion of melatonin to compound II via compound I (after melatonin degradation) represents one of the major metabolic pathways of melatonin in the mammalian brain. Leone et al demonstrated that melatonin has two principal metabolites, N-Acetyl-Serotonin as well as 6-Ha-melatonin after administration of various doses of melatonin in rats [107]. The authors concluded that the conversion of melatonin to 6-Ha-melatonin and NAS resulted from two independent metabolic pathways.

From in vitro metabolism studies using liver microsomes it is suggested that 6-hydroxylation of melatonin is the primary metabolic route. In addition, 5-methoxyindoleacetic acid appears to be formed by de-acetylation of melatonin followed by de-amination [108, 109].

Following administration of different doses of melatonin, plasma hydroxymelatonin and melatonin concentrations increased in a dose-dependent manner (R = 0.99). Plasma 6- hydroxymelatonin always represented approximately 1 % of plasma melatonin, irrespectively of the dose of melatonin administered [110].

2.4.3.4 ELIMINATION

The disposition of exogenous melatonin in the rat, dog, and monkey following intravenous and oral administrations was studied [96]. Following the intravenous administration of a 5 mg/kg dose, the apparent elimination half-life of melatonin in rats was 19.8 minutes. It was reported within this study that the half-life seen in other studies were similar even though the doses employed were significantly lower than the 5mg/kg within this study (1 - 100 µg). A similar half-life estimate was obtained in dogs (18.6 minutes), while it was longer (33.9 minutes) in monkeys. A half-life of 30 minutes has been reported in the rhesus monkey [96]. The calculated clearance values in this study indicate that the beagle dog (CL = 3.84 L/hr/kg) clears melatonin faster than the rat (2.11 L/hr/kg) and the monkey (1.68 L/hr/kg)

The main excretion route of the melatonin metabolites is renal. In rats and rabbits administered labelled melatonin by intraperitoneal injection or stomach tubes, 70 and 20 % of the activity was excreted in urine and faeces respectively [104].

2.4.3.5 PHARMACOKINETIC DRUG INTERACTIONS

Cytochrome P450 1A2 (CYP1A2) accounts for about 10 to 15 % of the total CYP content of human liver and is the major enzyme involved in the metabolism of imipramine, propranolol, clozapine, theophylline, and caffeine. It is also involved in the conversion of heterocyclic amines to their proximal carcinogenic and mutagenic forms, as well as in the metabolism of endogenous substances, including

17 beta-estradiol and uroporphyrinogen III. Fluvoxamine is a potent inhibitor of CYP1A2, and there is potential for interaction with drugs that are metabolised by this isoenzyme. [redacted] the biotransformation of melatonin and the effects of fluvoxamine on the metabolism of melatonin in vitro using human liver microsomes and recombinant human CYP isoenzymes [111]. Melatonin was found to be almost exclusively metabolized 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 the other antidepressants, only paroxetine was able to affect the metabolism of melatonin at supratherapeutic concentrations of 20 μM, which did not reach by far the magnitude of the inhibitory potency of fluvoxamine.

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 [112]. 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 interacting drug, and the production of 6-sulphatoxymelatonin monitored using a radioimmunoassay procedure. Of the drugs screened, only the potent CYP1A2 inhibitor 5-methoxypsoralen impaired the 6-melatonin hydroxylation at pharmacologically relevant concentrations, and is likely to lead to clinical interactions; diazepam, tamoxifen and acetaminophen (paracetamol) did not impair the metabolic conversion of melatonin to 6-sulphatoxymelatonin at concentrations attained following therapeutic administration. 17-Ethinhyloestradiol 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 the inhibition of melatonin metabolism in human and rat hepatic post-mitochondrial preparations were evident implying that the rat may not be an appropriate surrogate of human in such studies.

As Melatonin's metabolism is mainly mediated by the CYP1A enzymes, there are theoretical interactions that could be possible between melatonin and other active substances as a consequence of their effect on CYP1A enzymes. As melatonin does not induce the CYP1A enzymes in vitro at supra-therapeutic concentrations it is unlikely that these interactions would be seen to be significant. Caution should be advised with the concomitant administration with cimetidine, a known CYP2D inhibitor, fluvoxamine, oestrogens, quinolones all potentially increasing melatonin levels. CYP1A2 Inducers such as carbamazepine and rifampicin theoretically could reduce the plasma concentrations of melatonin.

2.4.3.6 OTHER PHARMACOKINETIC STUDIES

It has been shown that the distribution and metabolism of exogenous melatonin in neonatal rats is similar to that in adult rats. Neonatal rats showed rapid absorption (~ 90 %) of total dose within 45 minutes) and metabolism (~ 60 % of total dose within 60 minutes) following incubation of ^{3}H-Melatonin. General tissue distribution was similar to that found in adult rats and the urinary metabolites were primarily the sulphate and glucuronide conjugates of 6-hydroxymelatonin.

[redacted] reported that the oral transmucosal route demonstrated higher C_{max} values of melatonin with similar T_{max} values compared to oral melatonin [113]. On the other hand, the possibility of direct transport of melatonin from nasal cavity into the cerebrospinal fluid (CSF) after nasal administration

(40 µg/rat) in rats has been also investigated. Melatonin quickly absorbed in plasma (T_{max} = 2.5 min) and showed a delayed uptake into the CSF (T_{max} = 15 min) after nasal administration. The melatonin concentration-time profiles in plasma and CSF were comparable to those after intravenous delivery. The AUC_{CSF}/AUC_{plasma} ratio after nasal delivery (32.7 ± 6.3 %) did not differ from the one after intravenous injection (46.0 ± 10.4 %), which indicates that melatonin enters the CSF via the blood circulation across the blood-brain barrier. That result demonstrated that there is no additional transport via the nose-CSF-pathway [114].

Additionally, melatonin supplementation in diabetes and acute exercise significantly changes the element metabolism of the liver tissue in adult male rats. Prevention of the decrease in liver zinc in diabetes by melatonin supplementation in particular suggests that melatonin treatment can be beneficial in diabetes [redacted].

The effect of melatonin on cholesterol absorption in rats has been investigated [redacted] Melatonin suspension (10 mg/kg) was administered to Sprague Dawley rats. Treatment with melatonin inhibited cholesterol absorption in intestine of rats fed on high cholesterol diet and consequently positively modified lipoprotein cholesterol profile in plasma and the content of lipids (Cholesterol, TG) in the liver [116].

The study [redacted] demonstrated that prolonged constant light exposure modified the distribution (reduced V_{ss}) and elimination (reduced CL_s) of a bolus injection of 1 mg/kg melatonin in rats, without modifying its elimination half-life [117]. 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.

Tissue distribution f ^{125}I-thyroxine (T_4) and ^{3}H-melatonin and the effect of each hormone on the tissue content of the other were studied [redacted]. Late pre- to prometamorphic Rana catesbeiana tadpoles on an 18 light: 6 dark cycle were used for injection of hormones in vivo or to supply tissues for in vitro hormone administration. Labeled melatonin uptake was highest in intestine, ventral skin and pituitary; lowest in thyroid and brain and intermediate in hindlimb, tail and gills. The tissue content of labeled T_4 was distributed in nearly the same way, except that the thyroid level was relatively higher, and pituitary lower, than that of labeled melatonin. The pineal, studied only in the tracer T_4, experiments, had the highest content of labeled T4 of all tissues. Simultaneous injection of either 0.007 or 0.2 pg T_4, increased ^{3}H-melatonin uptake into peripheral tissues that undergo major metamorphic changes but not into neural or endocrine organs. In contrast, 0.033, 3.75 or 15 pg melatonin had no significant influence on the content of ^{125}I-T_4 in any tissue studied in viva. Results of in vitro labeling of selected tissues were generally in agreement with the in vivo work except that the ^{124}I-T_4 content of intestinal segments from late prometamorphic larvae was lower in melatonin-treated than in control groups. The results suggest that peripheral tissues are a major site for T_4-melatonin interactions and that T_4, may modulate its own action through influencing melatonin levels in target tissues and perhaps in the thyroid. Because melatonin had no effect on tissue T_4 content in young tadpoles, retardation of metamorphic events by melatonin does not seem to involve modulation of T_4 availability to the tissues.

2.4.4 TOXICOLOGY

[redacted]

2.4 Nonclinical overview

Table V Toxicity studies in animals

Study Type	Species & Strains	Route of administration	Duration of doses	Melatonin Dose	Ref
TOXICOLOGY					
Single Dose	Mouse , male (MFI)	Oral, IV, IP, SC	Single dose	To determine LD_{50}	[88]
	Rat, male (SD)	Oral, IV, IP, SC	Single dose	To determine LD_{50}	[88]
Repeat-Dose	Rat (Fischer 244 and Long Evans)	Oral	14 or 90 days	0.005, 0.050, 5, 50 and 200 mg/kg	
	Rat, male (SD)	IV	6 days	5 or 15 mg/kg	[119]
	Rat, male & female (SD)	SC, infusion	28 days	Males: 0.05, 0.5, 4.8 mg/kg/d Female: 0.07, 0.75, 7.3 mg/kg/d	[73]
Genotoxicity	In vitro: reduced Ames test: 3 strains S. typhimurium (TA97,TA98, TA100)				[120]
	In vitro modulatory: modulatory effect of melatonin on genotoxic response of 12 reference mutagens in the Ames test and comet assay				[121]
	In vitro: Ames test, comet assay and effect against NMU				[122]
	In vitro: genotoxicity testing on formation of DNA adducts				[123]
	In vitro, mouse (ICR)	IP	2 mg/kg- 30 min prior to ip injections of paraquat (2 × 15 mg/kg given 24 h apart) then at 6h intervals until 72 h		[124]
	In vivo, mouse, male (Swiss)	IP	10 mg/kg- 30 min prior to ip injections of paraquat (20 mg/kg × 2 given 48 h apart) then at 6h intervals until 72 h		[125]
Carcinogenicity	Mouse, female (hemizygous TG.NK with MMTV/c-neuoncogene)	Oral	20 weeks	50, 100, 200g/kg/d	[126]
	Mouse, female (CBA)	Oral	20 mg/L in drinking water-5 consecutive days/month from age 6 months until natural death		[127]
	Mouse, female (Swiss-derived SHR)	Oral	2 or 20 mg/L in drinking water-5 consecutive days/month from age 3 months until natural death		[128]
	Mouse, female (HER-2/neu)	Oral	20 mg/L in drinking water-5 times monthly (interrupted treatment) or constantly from age 2 months to natural death		[129]
	Rat, female (2 months old, LOI)	Oral	20 mg/L in water-2 days before and 1 day after NMU induced (50 mg/kg) carcinogenesis		[122]
	Rat, female (Wistar:Han)	Oral	100 µg/ml in drinking water from beginning of irradiation dosing until 26 weeks after (28 weeks total)		[130]
Reproductive & Developmental: Fertility & Early	Mouse, female, CD-1	IP	19 days prior to cohabit	3 - 4 mg/kg/d	[131]
	Rat, male (Wistar)	SC	30 days at 1700 h	0.8, 2.4, 4.8, 8 mg/kg/d	[80]

Study Type	Species & Strains	Route of administration	Duration of doses	Melatonin Dose	Ref
TOXICOLOGY					
Embryonic Development	Rat, male (pinealectomized Wistar rats, 5-weeks old)	SC	30 days at 1700	3, 8 mg/kg/d	[80]
Reproductive & Developmental: embryofetal development	Rat, female (SDCD, pregnant)	Oral	Gestational days 6 - 19	50, 100, 200 mg/kg/d	[132]
Reproductive & Developmental: prenatal & postnatal development	Rat, female (Wistar)	SC	2.5 mg/kg/d throughout gestation		[133]
	Rat, female (Wistar)	Injection	2.5 mg/kg/d throughout gestation at end of light phase		[134]

2.4.4.1 SINGLE-DOSE TOXICITY

The acute toxicity of melatonin was studied after different routes of administration and in mice and rats, and the LD_{50} of melatonin has been determined in both species not only by the clinical route of administration (oral), but also by intravenous, intraperitoneal and subcutaneous administration. At high doses (400 mg/kg), vasodilatation of the extremities indicated by a reddening of the ears and feet, piloerection and ptosis were common. In addition, muscle relaxation, a marked lack of motor activity and ataxia were evident. At higher doses an impairment of the righting, placing and hind limb ipsilateral flexor reflexes, a marked reduction in body temperature and slow, labored respiration preceded 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 the mouse.

Importantly, the LD_{50} by the oral route was shown to be approximately 1250 mg/kg in mice and > 3200 mg/kg in rats, which is greatly in excess of the maximum envisaged daily dose 6mg in adults. The main effects observed within these two species at high doses were sedation, lethargy, and vasodilatation. The higher doses led to impairment of righting, placing and flexor reflexes, marked reduction in body temperature and respiratory distress preceding death [88].

In the table below (Table VI) the LD_{50} values of melatonin by different routes of administration in mice and rats are compared.

Table VI Acute toxicity (LD_{50}) of Melatonin in animals (mg/kg/body weight) [88]

Organism	Test Type	Route	Reported Dose (Normalized Dose)	Source
Mouse	LD_{50}	Intraperitoneal	1375 mg/kg (1375 mg/kg)	
Mouse	LD_{50}	Intravenous	180 mg/kg (180 mg/kg)	
Mouse	LD_{50}	Oral	1250 mg/kg (1250 mg/kg)	[88]
Mouse	LD_{50}	Subcutaneous	> 1600 mg/kg (1600 mg/kg)	[88]
Rat	LD_{50}	Intraperitoneal	1131 mg/kg (1131 mg/kg)	[88]
Rat	LD_{50}	Intravenous	356 mg/kg (356 mg/kg)	[88]
Rat	LD_{50}	Oral	> 3200 mg/kg (3200 mg/kg)	[88]
Rat	LD_{50}	Subcutaneous	> 1600 mg/kg (1600 mg/kg)	[88]

Melatonin was also found to produce considerable motor incoordination in mice at high doses in the same study [88]. By both routes (p.o. and i.p.) melatonin was most potent 15 to 30 minutes after dosing. A rapid decline in potency was seen after this presumably due to the rapid metabolism of melatonin.

Table VII effect of Melatonin on forced coordinated motor ability [88]

Time after drug administration	ED_{50} (mg/kg)	
	i.p.	p.o.
Time (minutes)		
15	210 ± 45	450 ± 95
30	186 ± 48	650 ± 42
60	380 ± 50	620 ± 20
120	560 ± 75	790 ± 62
240	650 ± 65	990 ± 66

2.4.4.2 REPEAT DOSE TOXICITY

Melatonin has been studied in repeat-dose toxicity tests mainly in within the Rat species through oral, intravenous and subcutaneous administration. These studies have included significant doses of up to 200 mg/kg/d (orally) for 90 days, 15 mg/kg/d (intravenously) for 6 days [119] and ~ 5 mg/kg/d(male) or ~ 7 mg/kg/d (female) (subcutaneously) for 28 days [73].

Melatonin was administered by gavage to Long-Evans and Fischer 344 rats in a 90-day toxicity study. The dose levels administered were 0, 0.005, 0.05, 5.0, 50 or 200 mg/kg bw/day. There was a Special Study Group comprising of Fischer 344 rats alongside two primary Core Groups, one composed of Long-Evans rats and the other composed of Fischer 344 rats. Doses were administered daily for 90 days, excluding weekends and holidays, for a total of 17 dosing days and 68 dosing days, for the Special Study Group and Core Groups, respectively. Body weights were recorded weekly for all 3 groups and, clinical observations weekly from the 2 Core Groups only. Sperm morphology and vaginal cytology evaluations were conducted in the Core male and female rats in the 0, 5, 50 and 200 mg/kg treatment groups.

Dark-coloured faeces were observed in the two highest dosage groups (50 and 200 mg/kg bw/day). No treatment-related individual organ weight changes were observed during the study. However, mean weight gains over the entire study in all the female Long-Evans Melatonin treated groups were 7 to 10 % less than their control. Also in the Fischer rats, a reduction in body weight gain was observed,

though only in dosages starting from 5 mg/kg bw/day. As far as clinical biochemistry is concerned, increases in T_3 and T_4 were observed at dosages starting from 0.05 mg/kg/day, but these measurements have been declared as not clinically significant, since no concurrent effects on thyroid histopathology were observed. Cystic uterine endometrial hyperplasia was observed in a number of treated Long-Evans female rats, but also in their respective control group. Finally, one treatment-related finding in a 50 mg/kg bw/day treated Long-Evans female was a dilated uterus at necropsy.

[redacted] it was shown that there was no change in blood pressure, heart rate and body temperature. At intravenous doses of 5 mg/kg complete blood counts were not affected, but there was a significant increase in total protein and AST ($P < 0.05$). At 15 mg/kg there was a significant increase in polymorphonuclear cells, a significant decrease in lymphocytes, mononuclear cells and platelets with a significant increase in creatinine, AST and LDH. It was also noticed that there was a significant decrease in body weight over both doses of approximately 5.5 %. There was no evidence of organ toxicity (brain, kidney, liver and spleen) [119].

In a 28-day toxicity study, Sprague-Dawley rats received subcutaneously by an osmotic pump 60 µl/day of vehicle (PEG 400) containing 0.03 %, 0.3 % or 3 % Melatonin, continuously for 28 days. The dose of Melatonin delivered based on weekly group mean body weights (n = 10) was approximately 0.050, 0.50 and 4.8 mg/kg bw/day for the males and 0.074, 0.75 and 7.3 mg/kg bw/day for the females. An additional group (19/sex) underwent surgery, but no osmotic pumps were implanted (sham control) [73]. No deaths or changes in clinical observations occurred. No substance-related effect was noted in body weights, haematology, clinical chemistry, urinalyses or gross pathology. A dose-related trend of increasing serum Melatonin concentrations occurred in males and females. In males, there was a trend toward decreasing serum prolactin concentrations with time at all levels of Melatonin treatment. No difference in serum follicle-stimulating hormone (FSH) concentrations occurred between treated groups. Most of the samples were at the limit of detection for the serum luteinizing hormone (LH) assay (0.157 ng/ml). A dose- related increase occurred in urine 6-sulphatoxymelatonin (the primary metabolite) concentrations in Melatonin-treated male and female groups. No treatment-related organ weight or histopathology changes were noted in rats infused with 0.03 % or 0.3 % Melatonin. Two of 10 males administered 3.0 % Melatonin had decreased testes weights and testicular degenerative changes composed of reduced or absent spermatogenesis, spermatidic giant cells and oedema.

In rats, the toxicological profile of melatonin after 90-day period of administration was low but very low doses were used in the study (0.3, 1.2 and 6 mg/kg/day). Data showed that 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.

A combined 13-week study in rat with a 4-week recovery period coupled to a 26-week toxicity and a 104-weeks carcinogenicity phase was conducted. The oral dose levels used in this study were 0, 15, 75 and 150 mg/kg/day. In the 13-weeks and the 26-weeks studies increased haemoglobin concentration and platelet counts were observed at 75 and 150 mg/kg/day treated animals. Increased liver weights with minor centrilobular hepatocytic hypertrophy were observed. Increased testes, prostate and epididymides weights were seen in mid and high dosed males. At 26 weeks, macroscopically dark thyroid was also recorded in several high dose animals. Microscopically, minor liver hypertrophy was seen in some high dose animals but reported as less obvious than in the 13 weeks treated group.

In the 6-months study in dogs, 0.4, 1.5 and 8 mg/kg of melatonin was administered and 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 that ca be reached in humans.

2.4.4.3 GENOTOXICITY

2.4.4.3.1 Genotoxicity studies for Melatonin

The mutagenicity of melatonin and its major metabolite 6-hydroxymelatonin was evaluated by the in vitro tests: Ames test (S. tyhimurium), Single Cell Gel Eletrophoresis (COMET assays) or chromosomal aberrations test (human lymphocytes). Results in the in vivo mouse micronucleus test were also negative.

[redacted] a reduced Ames test, a bacterial reverse mutation test, using three strains of Salmonella typhimurium--TA 97, TA 98, and TA 100. Neither compound exhibited mutagenicity whether in the presence or absence of an activation system derived from rats induced with Aroclor 1254. Positive controls were employed throughout and gave the expected response. It was concluded that melatonin, 6-hydroxymelatonin, and their microsomal metabolites are not mutagenic in the Ames test [120]. At concentrations of up to 5 mg/plate melatonin showed no mutagenicity in either the presence or absence of an S9 activation system in any of the bacterial strains used. No doubling of the spontaneous reversion rate was seen at any of the concentrations used. Similarly, 6-hydroxymelatonin exhibited no mutagenicity at the concentrations studied, a maximum of 100 µg/plate, either in the presence or absence of the activation system in three bacterial strains.

Table VIII The mutagenicity of Melatonin in the Ames Test [120]

Compound (µg/plate)	TA 97	TA 98	TA 100
	Without activation		
Spontaneous reversion rate	132 ± 11	22 ± 2	88 ± 7
Melatonin 5	139 ± 40	22 ± 6	107 ± 16
Melatonin 50	133 ± 8	20 ± 8	85 ± 6
Melatonin 500	140 ± 24	28 ± 3	98 ± 12
Melatonin 5000	138 ± 6	21 ± 3	93 ± 19
MNNG	-	-	7777 ± 836
2-Nitrofluorene 2	-	302 ± 15	-
9-Aminoacridine 20	368 ± 96	-	-
With activation			
Spontaneous reversion rate	143 ± 14	26 ± 1	90 ± 14
Melatonin 5	151 ± 12	22 ± 3	103 ± 2
Melatonin 50	151 ± 8	25 ± 3	89 ± 18
Melatonin 500	143 ± 22	22 ± 5	83 ± 14
Melatonin 5000	139 ± 10	17 ± 2	87 ± 16
2-Aminoanthracene 5	1999 ± 100	2789 ± 508	1807 ± 172

2.4.4.3.2 Genotoxicity studies and protective role of Melatonin

In the study of Musatov, the effect of melatonin on the initiation of N-nitroso-N-methylurea (NMU)-induced carcinogenesis in rats and mutagenesis was investigated, in vitro. Within the in vitro tests performed for the mutagenesis studies an Ames test was conducted using strains TA 100 and TA 102 of Salmonella typhimurium. 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 [122]. A second in vitro test was performed alongside the Ames test. A Single Cell Gel Electrophoresis assay (SCGE assay or COMET assay) was performed on CHOK1 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} - 10^{-7} M) was observed. This therefore indicates that melatonin may act as an anti-initiating hormone in NMU-induced carcinogenesis and possess anticlastogenic activity towards NMU in CHOK1 cells.

The protective role of Melatonin on radiation-induced DNA damage in human lymphocytes was investigated by studying the chromosomal rearrangement on metaphases stained with the fluorescence plus Giemsa technique. Cells in human peripheral blood were treated in vitro with increasing concentrations of melatonin (0.5 or 1.0 or 2.0 mM) for 20 min at 37 ± 1 ° C and then exposed to 150 cGy gamma-radiation from a 137Cs source. The lymphocytes which were pre-treated with melatonin 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 pre-treatment. The extent of the reduction in radiation-induced chromosome damage observed with 2.0 mM melatonin was similar to that found in lymphocytes pre-treated with 1.0 M dimethyl sulfoxide, 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 dimethyl sulfoxide at 1.0 M [135]. It has

been concluded that Melatonin by itself was not found to be clastogenic. The experiment performed 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 chromosomal aberration assay performed indicates the protective effect of melatonin on gamma radiation-induced damage in human lymphocytes. The study was not designed to test Melatonin itself for a clastogenic potential, due to the non-conformity with the guideline for the chromosomal aberration test (no mitotic index, dose selection, harvest time, etc.).

In an in vivo micronucleus test [136], the ability of Melatonin to influence lipopolysaccharide (LPS)-induced genotoxicity was tested using micronuclei as an index in both bone marrow and peripheral blood cells of rats. Melatonin (5 mg/kg bw) was injected prior to a single dose of 10 mg/kg bw LPS and thereafter at 6-hours intervals up to 72 hours. The number of micronucleated polychromatic erythrocytes increased significantly after LPS administration both in cells from peripheral blood and bone marrow. Melatonin administration to LPS- treated rats highly significantly reduced micronuclei formation in both peripheral blood and bone marrow cells beginning at 24 h after LPS administration and continuing to the end of the study (72 hours). In blood, the increase in micronuclei formation was time-dependent in LPS-treated rats with peak values being reached at 36 – 48 hours. According to the authors, the ability of Melatonin to reduce LPS-related genotoxicity is likely related to its antioxidant activity.

In a further in vivo micronucleus test in mice [124], the protection afforded by Melatonin against paraquat-induced genotoxicity in both bone marrow and peripheral blood cells was tested using micronuclei as an index of induced chromosomal damage. Melatonin (2 mg/kg bw) or an equal volume of saline were injected intraperitoneally (i.p.) into mice 30 min prior to the i.p. administration of paraquat (2 injections of 15 mg/kg bw; the paraquat injections were given with a 24-h interval) and thereafter at 6-h intervals to the end of the study (72 h). Using fluorescence microscopy, the number of micronuclei (MN) in polychromatic erythrocytes (PCE) 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 hours, 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 more than 50 % at 48 and 72 hours. The proposed mechanism of action of Melatonin is its free radical scavenging ability.

The ability of melatonin to influence paraquat-induced genotoxicity was tested using micronucleated polychromatic erythrocytes as an index of damage in both bone marrow and peripheral blood cells of mice [125]. Melatonin (10 mg/kg) or an equal volume of saline were administered intraperitoneally (ip) to mice 30 min prior to an i.p. injection of paraquat (2 × 20 mg/kg), and thereafter at 6-hours intervals until the conclusion of the study (72 h). The number of the micronucleated polychromatic erythrocytes increased after paraquat administration both in peripheral blood and bone marrow cells. Melatonin administration to paraquat-treated mice significantly reduced micronuclei formation in both peripheral blood and bone marrow cells; these differences were apparent at 24, 48 and 72 hours after paraquat administration. The induction of micronuclei was time-dependent with peak values occurring at 24 and 48 hours. The reduction in paraquat-related genotoxicity by melatonin is likely due in part to the antioxidant activity of the indole. We did not observe effects of melatonin over paraquat in paraquat + melatonin groups incubated at 0, 60 and 120 minutes. Mitomycin C, which was used as a positive control, also caused the expected large rises in micronuclei in both bone marrow and peripheral blood cells at 24, 48 and 72 hours after its administration.

2.4.4.4 CARCINOGENICITY

2.4.4.4.1 Carcinogenicity studies for Melatonin

Toxicity studies in different species and with varying treatment durations have not demonstrated any carcinogenic potential of melatonin.

2.4.4.4.2 Protective role of Melatonin on carcinogenicity

Melatonin was administered orally in doses of up to 200 mg/kg/d for 30 weeks (from 4 weeks of age) [126]. Fibre-rich non-purified diet (NTP-2000) and some retinoid analogues have been shown to significantly delay the development of mammary cancer in the TG.NK model. Four-week-old hemizygous TG.NK female mice with MMTV/c-neu oncogene fed NTP-2000 diet were gavaged with 0.05 - 0.2 ml of flaxseed oil as the source of omega-3 rich PUFA, or melatonin at 50 - 200 mg/kg or a combination of 0.10 ml flaxseed oil and 50 mg/kg melatonin in a gavage volume of 0.2 ml per mouse with corn oil as the vehicle for 30 weeks. 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] the effect of various regimens of treatment with melatonin on the development of mammary tumours in HER2/neu transgenic mice was investigated. Female HER-2/neu mice starting from the age of 2 months were kept under standard light/dark regimen and as given melatonin with tap water (20 mg/l) during the night time 5 times monthly (interrupted treatments) or constantly to natural death. Intact mice served as controls. Treatment with melatonin slowed down age-related disturbances in estrous function most in the group exposed to interrupted treatment with the hormone. Constant treatment with melatonin decreased incidence and size of mammary adenocarcinomas, and incidence of lung metastases, compared to controls. The number of mice bearing 4 and more tumours was reduced in the group with constant melatonin treatment. Interrupted treatment with melatonin promote mammary carcinogenesis in HER-2/neu transgenic mice. The data demonstrate the regimen-dependent inhibitory effect of melatonin on the development of spontaneous mammary tumours in HER-2/neu mice but not on overall survival with implication about the likely cause of the effect [128].

In a second study [redacted], female Swiss-derived SHR mice were given melatonin with their drinking water (2 or 20 mg/L) for 5 consecutive days every month, from the age of 3 months until their natural death. Intact mice served as controls. The results of this study show that the treatment of melatonin did not influence the frequency of chromosome aberrations in bone marrow cells; it did not influence mean life span; and it increased life span of the last 10 % of the survivors in comparison to controls. It was also found that treatment with low dose melatonin (2 mg/L) significantly decreased spontaneous tumour incidence (by 1.9-fold), mainly mammary carcinomas, in mice whereas higher doses (20 mg/L) failed to influence tumour incidence as compared to controls. For this reason, it was concluded that the effect of melatonin as a geroprotector is dose-dependent [129].

Spontaneous mammary tumour incidence in C3H/Jax mice was studied, an animal model for human breast cancer, following prolonged oral melatonin administration [137]. A group of 39 mice received melatonin (dissolved in ethanol) in drinking water around the clock (25 µg/mouse/day from day 21 to day 44; 50 µg/mouse/day from day 45 to sacrifice at 1 year). They reported that melatonin modulated

the degree of development of mammary epithelium and significantly reduced spontaneous mammary tumour incidence; 62.5 % of control animals developed tumours vs. 23.1 % in the melatonin treated group ($P < 0.02$).

[redacted] investigated the effect of melatonin administration on the incidence of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary adenocarcinoma in Sprague-Dawley rats. They reported that, when a control group and a treatment group of 30 - 50-day old rats given a 15 mg dose of DMBA by intragastric intubation were put on a regimen of daily i.p injections of 500 µg melatonin for the next consecutive 90 days, delayed onset and reduced incidence of tumours occurred. The animals were observed for 50 days after discontinuation of melatonin (140 days after dosing with DMBA), at which point 79 % of the control animals, but only 20 % of the melatonin treated animals had developed breast tumours [138].

[redacted] investigated the effect of melatonin on oestrogen-responsive rat mammary carcinogenesis caused by the direct acting DNA-alkylating agent, N-nitroso-N-methylurea, a mammary tumorigen in which the successive stages of initiation and promotion are well delineated. When female Sprague-Dawley rats received daily subcutaneous injections of melatonin (500 µg) only during the initiation phase of NMU mammary tumorigenesis (melatonin from age 37 days to 60 days and 2 doses of NMU administered on day 50 and day 60), the hormone was ineffective in altering tumour incidence or number over a 20-week observation period. When melatonin administration was delayed for 4 weeks after NMU injection and then continued throughout the remainder of the promotion phase, only tumour number was significantly lower than controls. However, when melatonin was administered during the entire promotion phase, both the incidence and numbers of tumours were significantly lower than controls. It was concluded that melatonin inhibits of NMU-induced rat mammary tumorigenesis by acting the promotion rather than the initiation phase and that melatonin appears to have antiestrogenic properties [139].

Further short-term studies in mice (10 µg topical administration for 14 days [140] and rats (20 mg/L in water for 3 days [122]; 100 µg/mL in water for 28 weeks [130] showed further evidence of the protective effect of melatonin against known carcinogens.

2.4.4.5 REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

2.4.4.5.1 Fertility and Early Embryonic Development

In a study in females, CD-1 mice (16/group) were injected with melatonin (100 µg [~3 - 4 mg/kg] i.p.) for 19 days prior to cohabtitation. Melatonin–treated mice showed disruption of the normal estrous cycle (longer cycles), primarily due to the greater number of days spent in diestrous. During cohabitation, the daily injection of females continued until mating was confirmed or until 2 weeks had elapsed, whichever occurred first. The proportion of mated females delivering was decreased for melatonin-treated mice (7/16 versus 13/16 for controls) but litter size from fertile matings was not affected [131].

A study in male Wistar rats administered melatonin 0.8, 2.4, 4.8, or 8.0 mg/kg (subcutaneously) for 30 days (at 1700 hours) has suggested that melatonin may have an inhibitory action on the male rat prostate but only at the high dose of 8mg/kg. Melatonin (8 mg/kg) caused a decreased prostate weight but not testes or other reproductive organs. Lower doses (0.8, 2.4 and 4.8 mg/kg) had no effect. Successive treatment with melatonin (8 mg/kg) produced no effect on testosterone levels in testes and serum nor on the conversion rate of [3H]Testosterone to [3H]dihydrotestosterone in prostate but

caused a significant decrease in activity of acid phosphatase and uptake of [3H]Testosterone by the prostate [80]. A further study in male Wistar rats has suggested that melatonin inhibits the reproductive behaviour of male rats following melatonin treatment (3.0 or 8.0 mg/kg SC for 30 days at 1700 h) in comparison to vehicle-treated and untreated pinealectomized rats. 5/12 rats dosed at 8 mg/kg melatonin did not copulate (compared to 2/12, 1/12 and 0/12 in the 3 mg/kg, vehicle control and untreated pinealectomized groups, respectively) [80].

2.4.4.5.2 Embryofetal Development

The developmental toxicity potential for repeated oral doses of melatonin was evaluated [redacted]. Melatonin was administered to Sprague-Dawley derived (CD) rats on gestation days 6 - 19. Melatonin treated groups received 1, 10, 100, 150, or 200 mg/kg body weight/day in the screening study and 50, 100, or 200 mg/kg/day in the definite study. At termination, maternal liver and gravid uterine weights, number of ovarian corpora lutea, conceptus survival, fetal sex and fetal body weight were evaluated. Fetal morphological examination included external structures, as well as visceral and skeletal structures. No maternal morbidity/mortality was found in either study. Melatonin had no effect on prenatal survival, fetal body weight, or incidences of fetal malformations/variations. Thus, in the definitive study, the maternal toxicity NOAEL and LOAEL were 100 and 200 mg/kg/day, respectively and the developmental toxicity NOAEL was ≥ 200 mg/kg/day [141].

In a US National Toxicology Programme (NTP) rat study, melatonin was administered by gavage to 25 timed-mated Sprague Dawley (CD) female rats on gestation day 6 to 19, at doses of 50, 100 and 200 mg/kg/day. No maternal deaths were observed and the clinical signs reported were classified as minimal. Transient reduction of the body weight gain and relative decreased food intake were observed at the high dose group. Increased relative maternal liver weight was also observed in the animals from mid and high dose. Absolute liver and gravid uterine weights were not affected. The endpoints related to embryo/foetal growth, viability or morphological development were not modified by melatonin treatment. Based on the lack of embryo/foetal toxicity, the developmental toxicity NOAEL of melatonin was considered as 200 mg/kg/day. Based on the slight maternal toxicity reported at 200 mg/kg/day treated animals, the maternal toxicity NOAEL was considered as 100 mg/kg/day [142].

A study of embryo-foetal development in NZ rabbits has been performed with oral administration of melatonin at 0 (control), 15, 50 and 150 mg/kg/day from days 7 to 19 of gestation. There were no dose-related maternal effects at any dose. No effects were observed on pre or post- implantation loss and mean number of foetuses/female. Foetal, 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 24000 to 45000 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 [143].

The effect of exogenous melatonin on embryo viability in undernourished ewes was investigated [redacted]. Their data demonstrated that the treatment with melatonin implants at lambing improves the viability of embryos of undernourished ewes during the reproductive season, although the effect of melatonin seems not to be mediated at the oocyte competence level. Moreover, melatonin induces changes in the endometrial sensitivity of steroids in undernourished ewes. Neither nutrition and melatonin nor their interaction had a significant effect on the in vitro oocyte development. Melatonin treatment tended to increase the percentage of 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). Thus, the use of melatonin implants at parturition, even during the breeding season could be helpful tool, particularly when embryo development is affected by negative factors as undernutrition or the post-partum period. [144].

2.4.4.5.3 Prenatal and Postnatal Development

A study in female Wistar rats (19 - 20/group) administered melatonin (2.5 mg/kg/d SC) throughout gestation has shown altered reproductive maturation of female offspring. Injections of melatonin were given 2 hours prior to the end of the light phase under a constant photoperiod (12:12, lights off at 1200). At birth, litters were standardized to 12 offspring per litter. Vaginal opening was significantly delayed in female offspring of melatonin-treated vs saline control rats (mean of 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 bodyweight, melatonin levels, organ weights (absolute or relative for ovary, pineal, and pituitary), or % off-spring in each phase of the oestrous cycle [133].

A subsequent study has investigated reproductive development in both male and female offspring following gestational exposure to melatonin in rats entrained for 3 weeks to a 12:12 light:dark cycle with lights on at 2400 hours. Female Wistar rats (34 - 38/group) were injected (route not specified) with 2.5 mg/kg/d melatonin throughout gestation at the end of the light phase and allowed to deliver naturally. Melatonin exposure was associated with a significantly shorter gestational period (mean 20.9 vs 21.5 days for controls), but did not affect maternal weight gain, litter size, or male/female ratios per litter. Offspring were evaluated at 5 (neonatal), 15 (infantile), 25 and 30 (juvenile), or 55 (pubertal) days of postnatal age to evaluate developmental patterns for reproductive hormones. Plasma levels of LH and prolactin but not FSH were affected in female offspring. In male offspring, developmental patterns for all 3 hormones were affected [134].

In a pre- and post-natal developmental study in rats, 24 pre-mated females were treated with 0, 15, 55 and 200 mg/kg/day of melatonin from Day 6 of gestation to Day 21 post-partum, inclusive. 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 F1 development was not modified. Therefore, melatonin intake during lactation should be avoided [143].

Although melatonin may exert inhibitory effects on puberty, its continuous administration is only capable to delay of 20 to 30 days, but not to block pubertal development [75, 145].

2.4.4.5.4 Other studies

It has been reported that melatonin acts on the hypothalamus to inhibit LHRH secretion and on the pituitary to suppress the stimulatory effect of LHRH 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 [146].

2.4.4.5.5 Pregnancy and Lactation

Published reports indicate that melatonin is a safe drug with low toxicity in experimental studies, where melatonin has been given in doses of 200 mg/kg to pregnant rats throughout pregnancy or 800 mg/kg body weight in mice, no toxicity or death was observed [91, 141, 147].

Exogenous administration of melatonin has no specific use during breastfeeding and no data exist on the safety of maternal use of melatonin during breastfeeding. However, doses higher than those expected in breastmilk after maternal supplementation have been used safely in infants [148]. It is unlikely that short-term use of usual doses of melatonin in the evening by a nursing mother would adversely affect her breastfed infant, although some authors recommend against its use in breastfeeding because of the lack of data and a relatively long half-life in preterm neonates [149].

2.4.4.6 LOCAL TOLERANCE

Not applicable since oral administration applies for Melatonin Oral Solution.

2.4.4.7 OTHER TOXICITY STUDIES

2.4.4.7.1 Excipients

The product under assessment contains the following excipients:

- Propylene Glycol
- Sorbitol Liquid (non crystalling)
- Sucralose
- Strawberry flavour
- Hydrochloric acid

The pharmaceutical excipients are well known and commonly used in the pharmaceutical industry and fulfil the requirements of Ph. Eur or BP.

2.4.4.7.1.1 Propylene Glycol

Propylene glycol is used in a wide variety of pharmaceutical formulations and is generally regarded as a relatively nontoxic material. It is also used extensively in foods and cosmetics. Probably as a consequence of its metabolism and excretion, propylene glycol is less toxic than other glycols. Propylene glycol is rapidly absorbed from the gastrointestinal tract; there is also evidence that it is absorbed topically when applied to damaged skin. It is extensively metabolized in the liver, mainly to lactic and pyruvic acids, and is also excreted unchanged in the urine [150-152].

Propylene glycol is estimated to be one-third as intoxicating as ethanol, with administration of large volumes being associated with adverse effects most commonly on the central nervous system, especially in neonates and children [153-155]. Other adverse reactions reported, though generally isolated, include: ototoxicity; cardiovascular effects; seizures; and hyperosmolarity and lactic acidosis, both of which occur most frequently in patients with renal impairment [156]. Adverse effects are more likely to occur following consumption of large quantities of propylene glycol or on administration to neonates, children under 4 years of age, pregnant women, and patients with hepatic or renal failure. Adverse events may also occur in patients treated with disulfiram or metronidazole.

On the basis of metabolic and toxicological data, the WHO has set an acceptable daily intake of propylene glycol at up to 25 mg/kg body-weight. Formulations containing 35 % propylene glycol can cause hemolysis in humans [157].

EMA has recently reviewed the available safety data of propylene glycol with the intent to update the labelling of selected excipients [158, 159]. The following limits, expressed in terms of maximum daily dose, are considered to be safe whatever the duration and the route of administration, with the exception of inhalation.

Table IX Maximum daily dose of propylene glycol

	Neonates up to 28 days (or 44 weeks post menstrual age for pre-terms)	**1 month (29 days) up to 4 years**	**5 years up to 17 years and adults**
Safety limits	1 mg/kg	50 mg/kg	500 mg/kg

In animal studies, there has been no evidence that propylene glycol is teratogenic or mutagenic. Rats can tolerate a repeated oral daily dose of up to 30 mL/kg body-weight in the diet over 6 months, while the dog is unaffected by a repeated oral daily dose of 2 g/kg in the diet for 2 years.

Reported LD_{50} values [160]:

- LD_{50} (mouse, IP): 9.72 g/kg
- LD_{50} (mouse, IV): 6.63 g/kg
- LD_{50} (mouse, oral): 22.0 g/kg
- LD_{50} (mouse, SC): 17.34 g/kg
- LD_{50} (rat, IM): 0.01 g/kg
- LD_{50} (rat, IP): 6.66 g/kg
- LD_{50} (rat, IV): 6.42 g/kg
- LD_{50} (rat, oral): 0.02 g/kg
- LD_{50} (rat, SC): 22.5 g/kg

2.4.4.7.1.2 Sorbitol

Sorbitol is widely used in a number of pharmaceutical products and occurs naturally in many edible fruits and berries. It is absorbed more slowly from the gastrointestinal tract than sucrose and is metabolized in the liver to fructose and glucose. Its caloric value is approximately 16.7 J/g (4 cal/g). Sorbitol is better tolerated by diabetics than sucrose and is widely used in many sugar-free liquid vehicles. However, it is not considered to be unconditionally safe for diabetics [161].

Reports of adverse reactions to sorbitol are largely due to its action as an osmotic laxative when ingested orally, which may be exploited therapeutically. Ingestion of large quantities of sorbitol (> 20 g/day in adults) should therefore be avoided [161, 162].

Sorbitol is not readily fermented by oral microorganisms and has little effect on dental plaque pH; hence, it is generally considered to be noncariogenic [161, 163].

Sorbitol is generally considered to be more irritating than mannitol [161].

Reported LD_{50} values [164]:

- TDLo (human, oral): 1.7 g/kg
- LD_{50} (mouse, IV): 9.48 g/kg
- LD_{50} (mouse, oral): 17.8 g/kg
- LDLo (mouse, IP): 15 g/kg
- LD_{50} (rat, IV): 7.1 g/kg
- LD_{50} (rat, SC): 29.6 g/kg

2.4.4.7.1.3 Sucralose

Sucralose is generally regarded as a nontoxic and non-irritant material and is approved, in a number of countries, for use in food products. Following oral consumption, sucralose is mainly unabsorbed and is excreted in the feces [165-167].

The WHO has set an acceptable daily intake for sucralose of up to 15 mg/kg body-weight [168].

Reported LD_{50} values:

- LD_{50} (mouse, oral): > 16 g/kg
- LD_{50} (rat, oral): > 10 g/kg

2.4.4.7.1.4 Hydrochloric acid

Hydrochloric acid is widely used as an acidifying agent, in a variety of pharmaceutical and food preparations. It may also be used to prepare dilute hydrochloric acid, which in addition to its use as an excipient has some therapeutic use, intravenously in the management of metabolic alkalosis, and orally for the treatment of achlorhydria.

When used diluted, at low concentration, hydrochloric acid is not usually associated with any adverse effects. However, the concentrated solution is corrosive and can cause severe damage on contact with the eyes and skin, or if ingested [169].

Reported LD_{50} values [170]:

- LD_{50} (mouse, IP): 1.4 g/kg
- LD_{50} (rabbit, oral): 0.9 g/kg

2.4.4.7.1.5 Strawberry flavour

Strawberry flavour is widely used in a number of pharmaceutical products.

According to qualitative composition of strawberry flavour, [redacted]

[redacted]

2.4.4.7.2 Impurities

The specifications of melatonin impurities are shown in the Table below (Table X).

Table X Proposed impurity limits for the drug product – shelf life limit

[redacted]

[redacted]

The limit set for any unknown impurities is based on the ICH guideline; the maximum recommended dose of Melatonin is 6 mg daily. [redacted]

[redacted]

2.4.5 INTEGRATED OVERVIEW AND CONCLUSIONS

Melatonin, as a neurohormone that is primarily produced in the pineal gland, can acutely attenuate the activity of the SCN. This melatonin action is likely to support a normal decline in the activity of the SCN at night, further promoting melatonin secretion and contributing to an overall increase in the amplitude of circadian body rhythms. Melatonin has a proven effectiveness in sleep-wake disorders and especially in jet lag disorder and shift work disorder. The planed medicinal product will be available as oral solution with concentration 1 mg/ml.

In vitro, melatonin is described in the literature as acting at the central nervous system level, modulating the synchronisation of the biological clock and promoting sleep through stabilisation and phase-shifting effects on the suprachiasmatic nucleus of the hypothalamus possibly involving interaction with melatonin MT1 and MT2 receptor subtypes. In vivo, studies in animals looked essentially at sleep induction effects, but the results are difficult to interpret and extrapolate to humans. However, sleep initiation was significantly promoted by a wide range of melatonin doses in diurnal macaques and, as in humans, showed a lack of dose dependence of the effect, once the dose (5 – 20 µg/kg, orally) was sufficient to induce physiologic circulating levels of the hormone (above 50 pg/ml). Lower doses failed to promote sleep in the macaques studied.

The mean oral bioavailability varies from 17 % to 100 % depending on the dose and the animal species. Melatonin is promptly distributed in tissues and rapidly metabolised in the liver mainly by CYP1A enzymes. The main excretion route is renal.

The toxicological studies performed in animals prove that the LD_{50} values are considerably higher than the therapeutic dose range in humans. The data indicate a low potential of acute and chronic toxicity. Melatonin has not been proved to be mutagenic, carcinogenic or significantly affect the reproduction in various animal models studied. In repeat-dose toxicity in rats, the few effects seen were observed at exposure in large excess of the intended human exposure at the therapeutic dose.

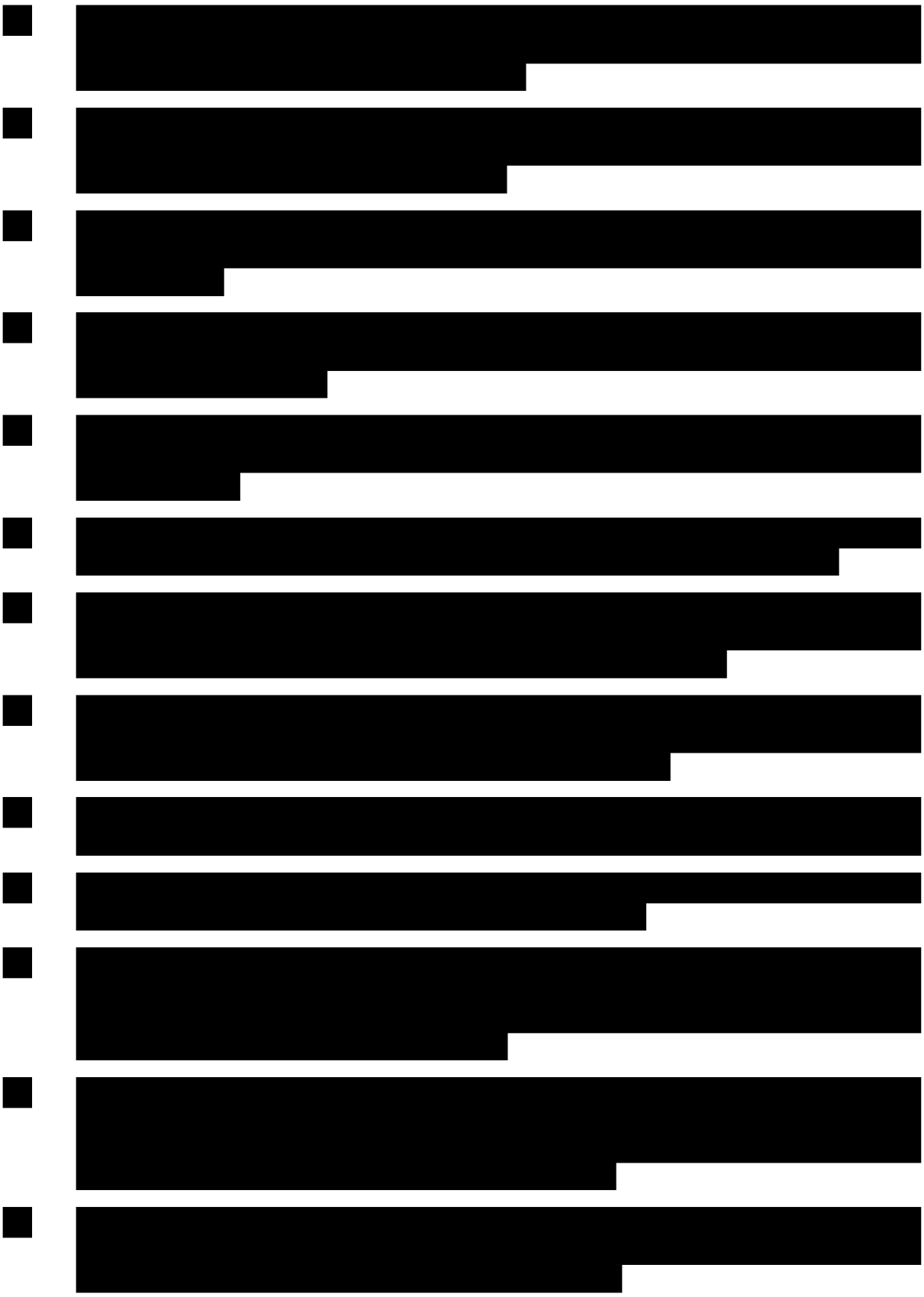
No genotoxic or carcinogenic properties have been identified for melatonin. Studies showed that continual melatonin treatment is not carcinogenic and may inhibit tumour formation, although interrupted treatment may increase tumour formation.

2.4 Nonclinical overview

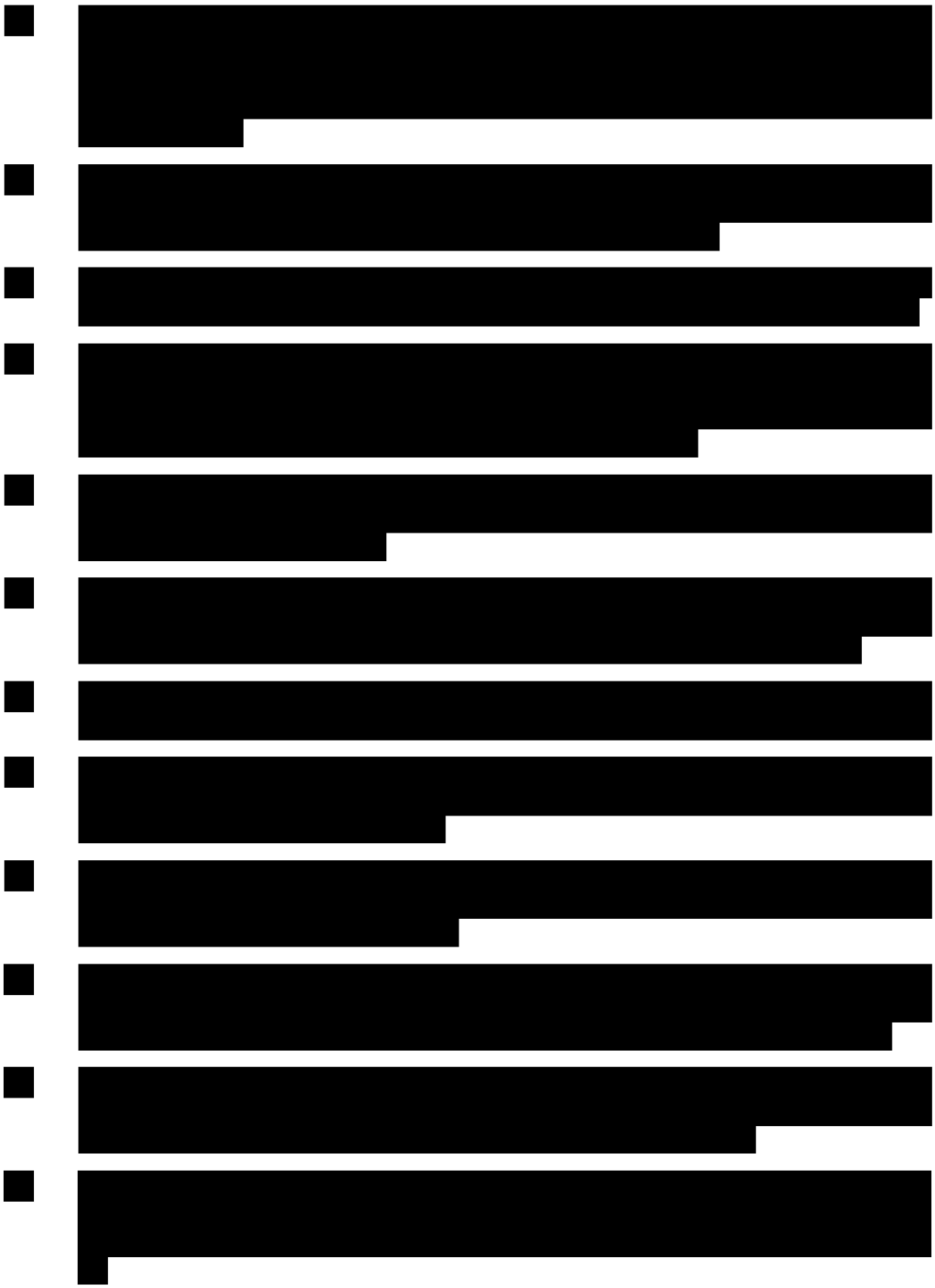
In reproductive studies, melatonin induced some toxicological effects on the fertility and embryo-foetal development in mice or rats and on postnatal development on rats. However, all exposures were in large excess of anticipated clinical doses.

Based on the extensive analysis of literature data, it can be stated 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 the use of Melatonin represents any significant risk and further toxicological studies are not deemed necessary.

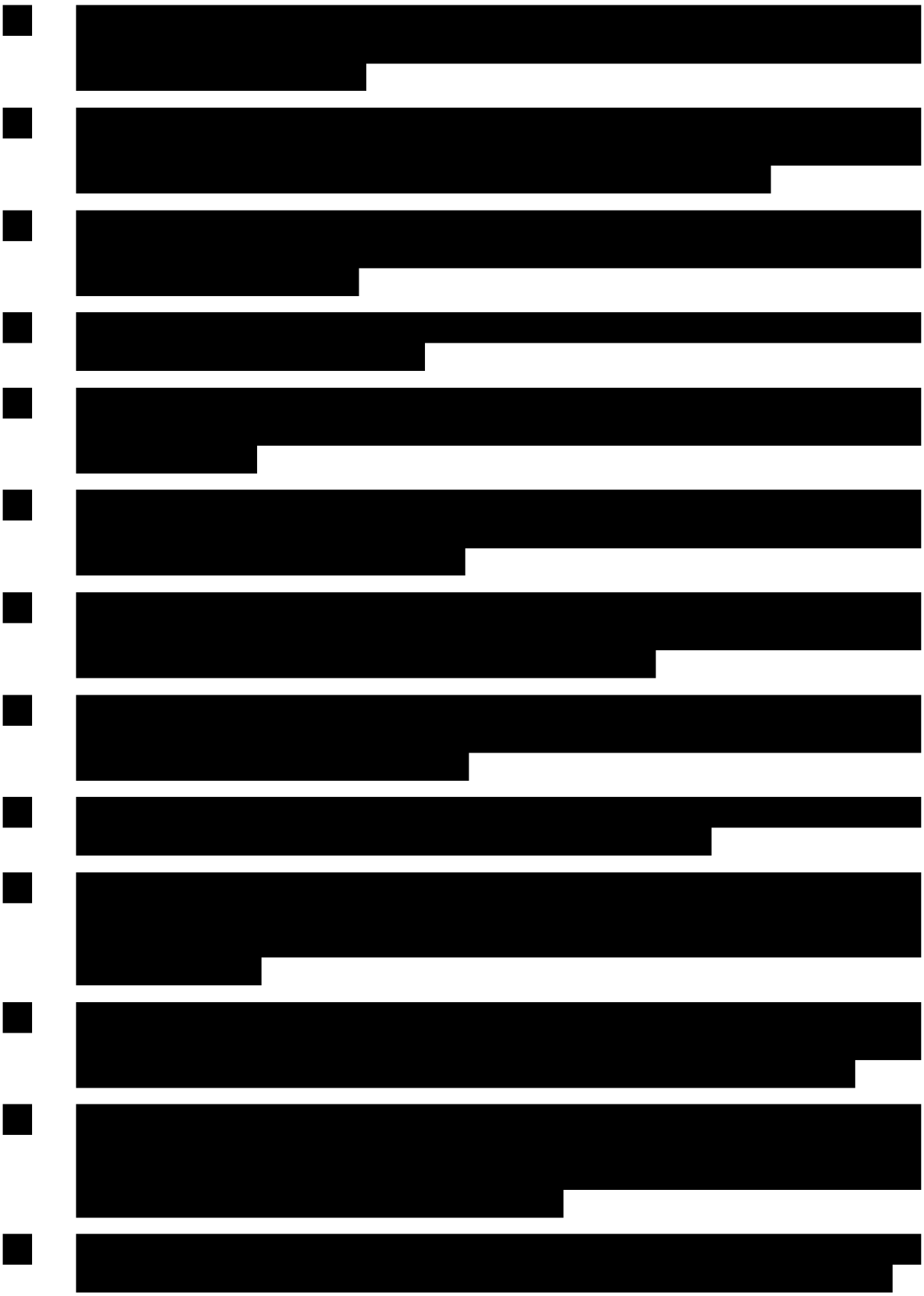
2.4 Nonclinical overview



2.4 Nonclinical overview



2.4 Nonclinical overview



2.4 Nonclinical overview

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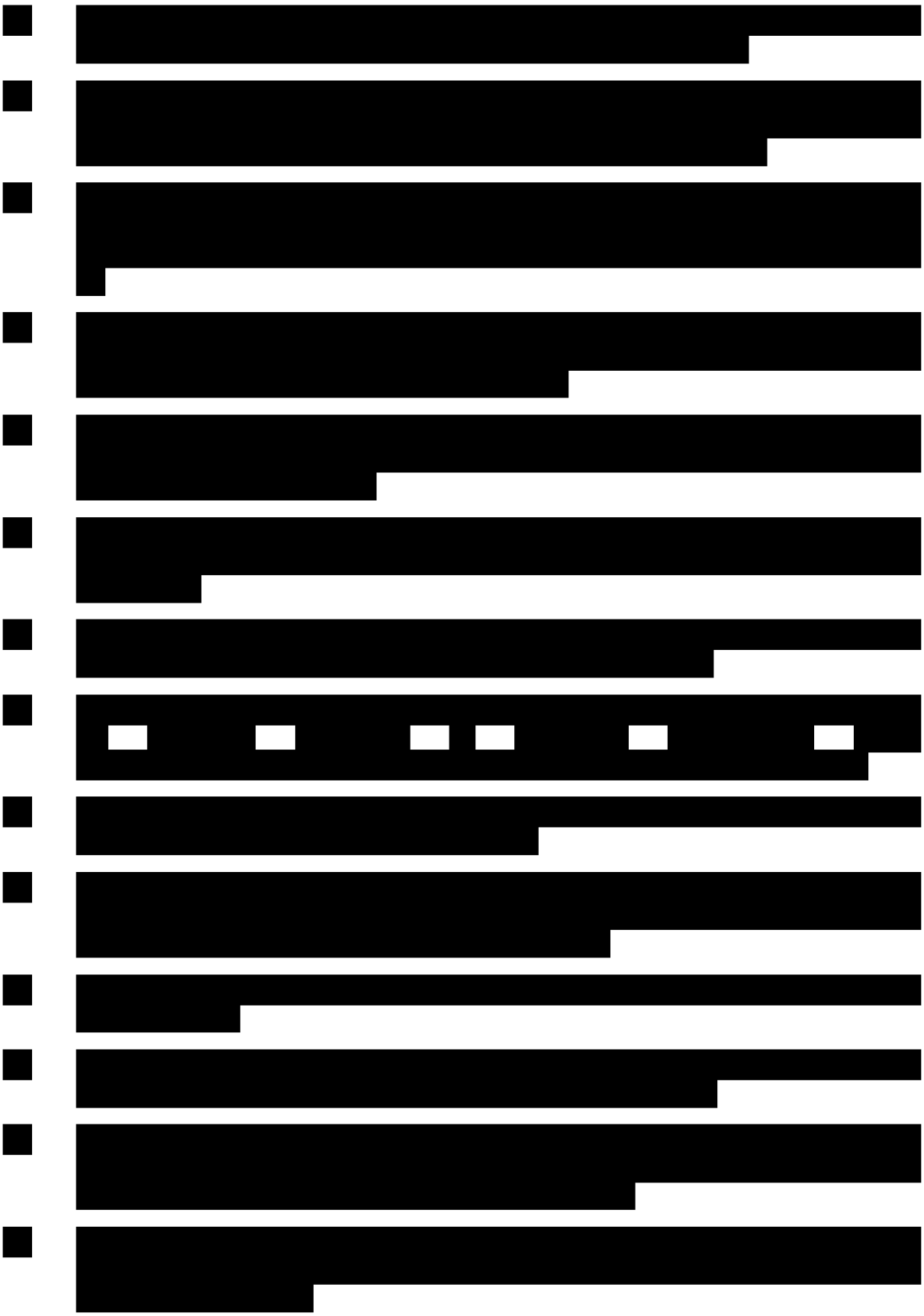

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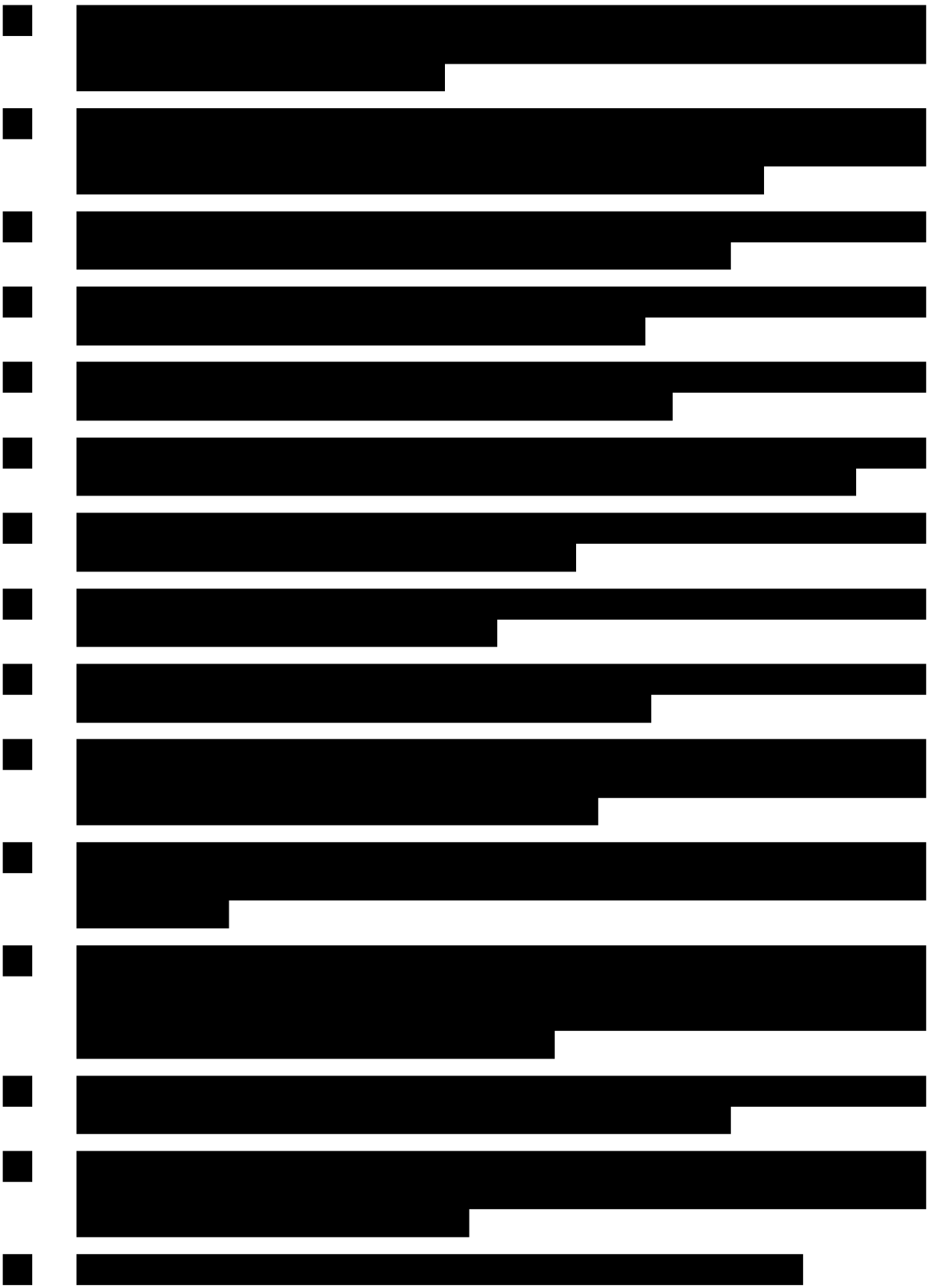

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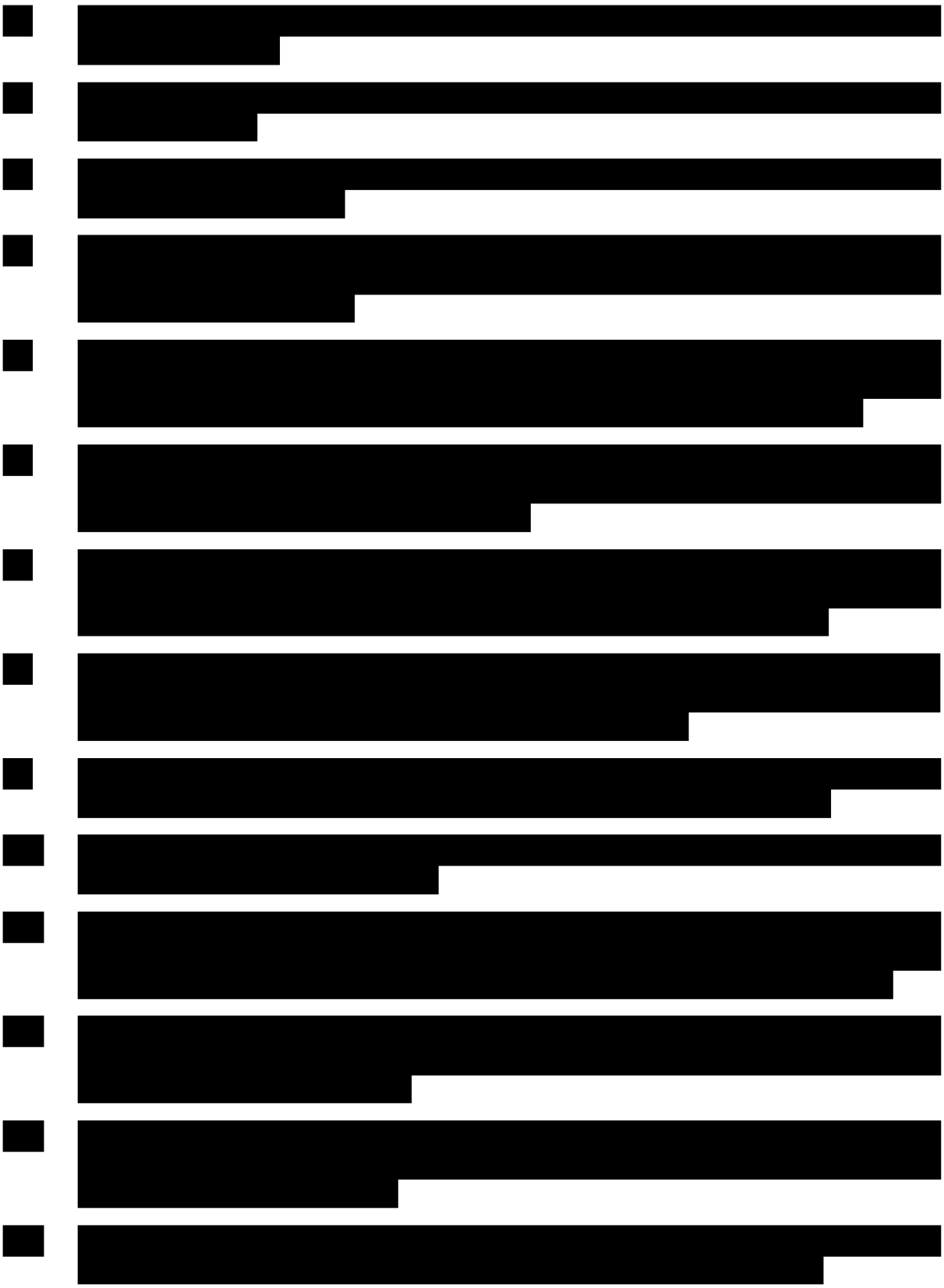
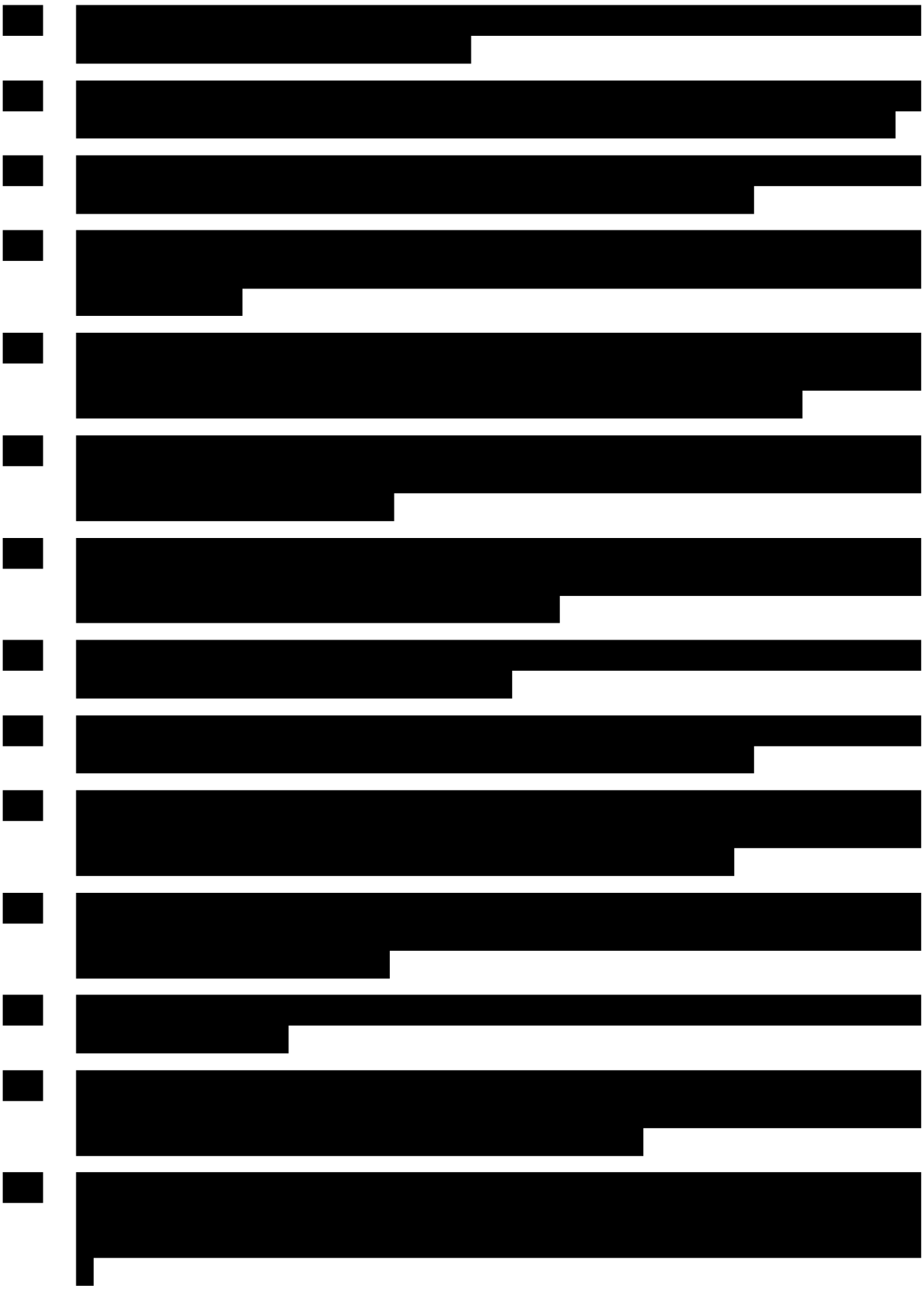


2.4 Nonclinical overview



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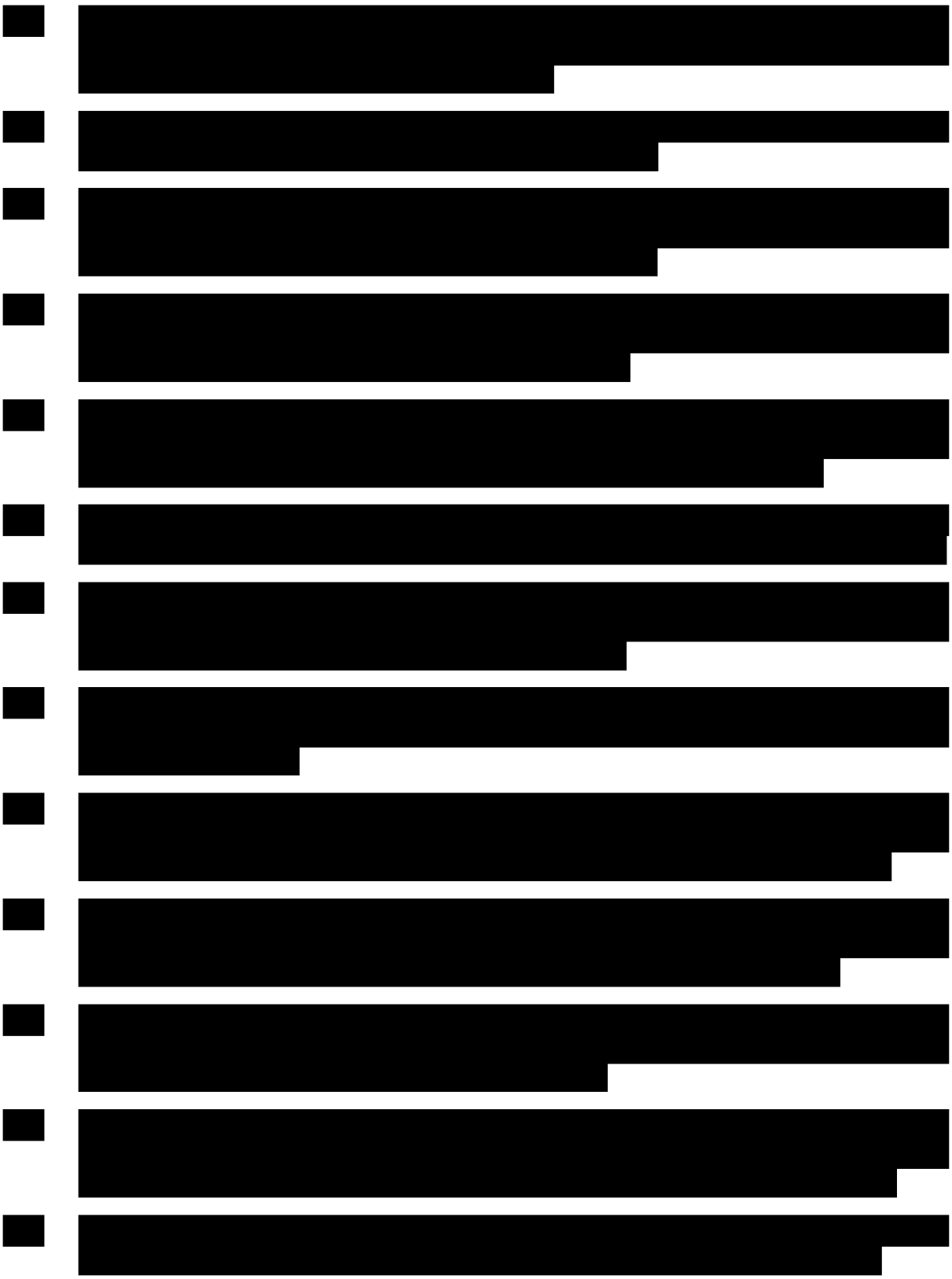

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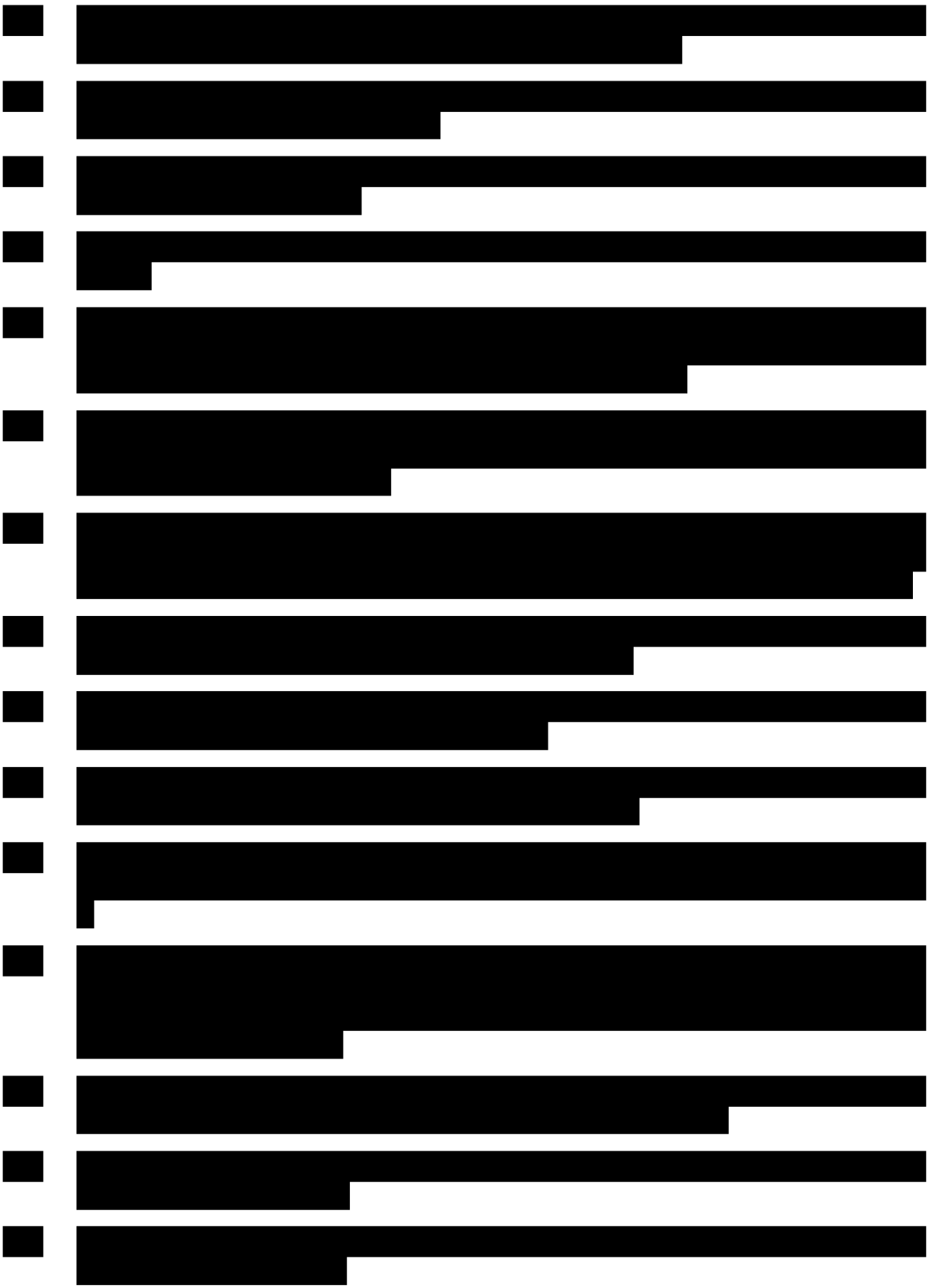

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