

Novel bromomelatonin derivatives as potentially effective drugs to treat bone diseases

メタデータ	言語: eng 出版者: 公開日: 2017-10-05 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属:
URL	http://hdl.handle.net/2297/11839

Mini-review

Novel bromomelatonin derivatives as potentially effective drugs to treat bone diseases

Nobuo Suzuki^{1*}, Masanori Somei², Azusa Seki³, Russel J. Reiter⁴, and Atsuhiko Hattori^{5*}

¹Noto Marine Laboratory, Institute of Nature and Environmental Technology, Kanazawa University, Housu-gun, Ishikawa, Japan; ²Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa, Ishikawa, Japan; ³HAMRI Co. Ltd., Koga, Ibaraki, Japan; ⁴Department of Cellular and Structural Biology, The University of Texas, Health Science Center at San Antonio, San Antonio TX, USA; ⁵Department of Biology, College of Liberal Arts and Sciences, Tokyo Medical and Dental University, Ichikawa, Chiba, Japan

Running title: Novel indole derivatives as drugs for bone diseases

Key words: bromomelatonin derivatives; osteoblasts; osteoclasts; scales; bone diseases; ovariectomized rats; low-calcium diet rats

* Corresponding authors:

Nobuo Suzuki

Noto Marine Laboratory, Institute of Nature and Environmental Technology, Kanazawa University, Housu-gun, Ishikawa 927-0553, Japan

Tel: +81-768-74-1151; Fax: +81-768-74-1644;

E-mail: nobuo@kenroku.kanazawa-u.ac.jp

Atsuhiko Hattori

Department of Biology, College of Liberal Arts and Sciences, Tokyo Medical and Dental University, Ichikawa, Chiba 272-0827, Japan

Tel/Fax: +81-47-300-7126

E-mail: ahattori.las@tmd.ac.jp

Abstract

Several reports indicate that melatonin is involved in the regulation of bone metabolism. To examine the direct effect of melatonin on osteoclasts and osteoblasts, we developed an *in vitro* assay using fish scales that contain osteoclasts, osteoblasts, and bone matrix, all of which are similar to those found in mammalian membrane bone. Using the assay, we demonstrated that melatonin suppressed osteoclastic and osteoblastic activities. These findings are in agreement with the reports from *in vivo* studies in mice and rats. In an attempt to develop molecules that increase bone mass, novel bromomelatonin derivatives were synthesized, and the effects of these chemicals on osteoclasts and osteoblasts using the scale assay were examined. As a result, novel bromomelatonin derivatives with the ability to possibly increase bone formation were identified. In scale osteoclasts, particularly, 1-benzyl-2,4,6-tribromomelatonin had a more potent activity than melatonin. In reference to osteoblasts, this agent (10^{-9} to 10^{-6} M) significantly activated osteoblasts. The effect of 1-benzyl-2,4,6-tribromomelatonin on bone formation was confirmed in ovariectomized rats. Thus, the oral administration of 1-benzyl-2,4,6-tribromomelatonin augmented the total bone mineral density of the femoral metaphysis of ovariectomized rats. The stress-strain index of the diaphysis in 1-benzyl-2,4,6-tribromomelatonin-treated rats significantly increased in comparison with that in ovariectomized rats. In rats fed a low-calcium diet, the total bone mineral density of the femoral metaphysis significantly increased following the oral administration of 1-benzyl-2,4,6-tribromomelatonin. These studies identified a melatonin derivative that may have potential use in the treatment of bone diseases, such as osteoporosis.

Introduction

Melatonin, N-acetyl-5-methoxytryptamine, is the principal secretory product of the vertebrate pineal gland and is synthesized almost exclusively during darkness; as a result, blood levels exhibit a conspicuous circadian rhythm [1, 2]. In mammals, including man, nighttime melatonin levels progressively drop throughout life [3, 4]; therefore, nocturnal melatonin levels in older individuals are much lower than in younger ones [5].

Melatonin prevents phototherapy-induced hypocalcemia in newborn rats [6]. Thus, the regulation of calcium homeostasis and bone metabolism by melatonin is suggested. Subsequently, the direct effects of melatonin on osteoclasts and osteoblasts were examined [7] using an *in vitro* assay system [8] with fish scales containing osteoclasts, osteoblasts, and bone matrix. Using this system, evidence was provided that melatonin suppressed osteoclastic and osteoblastic activities [7]. Thereafter, we synthesized novel melatonin derivatives and demonstrated that some of these agents inhibited osteoclastic activity but increased osteoblastic activity in the scale assay system [9]. Preliminary studies also indicated that, in ovariectomized rats, the melatonin derivative significantly increased the stress-strain index of the diaphysis and significantly augmented the total bone mineral density in the femoral metaphysis in rats fed a low-calcium diet.

Novel indole derivatives assayed using the *in vitro* scale assay system indicate that a melatonin derivative may have potential benefits for the treatment of bone diseases, such as osteoporosis. In the present review, we discuss the implications of melatonin and melatonin derivatives for bone disease therapy.

Melatonin, calcium metabolism and spinal deformities

Csaba and collaborators [10-12] were the first to report a relationship between the pineal and calcium-regulating endocrine glands, such as the parathyroid gland and parafollicular cells of the thyroid gland. This was shown in experiments using pinealectomized rats. In the hamster,

the ultrastructure of the parathyroid gland was also affected by melatonin treatment [13, 14]. As both melatonin and pineal extract reduced the serum calcium levels in rats [15], it is surmised that melatonin may be a calcemic hormone.

Spinal malformations have been reported in chickens [16-20], rats [21], and fish [22] after surgical ablation of the pineal gland. The experimental scoliosis observed in pinealectomized chickens was similar to that in human idiopathic scoliosis. However, the role of melatonin in the development of spinal deformities in pinealectomized chickens is less clear. Machida et al. [16] reported that pinealectomy in young chickens followed by transplantation of the pineal gland led to recovery of the serum melatonin levels and a significant reduction in pre-existing spinal deformities. Conversely, Turgut et al. [23] claimed that pineal transplantation following pinealectomy in young chickens had no significant effect on the development of scoliosis, even though serum melatonin returned to their normal levels. It is possible that the effects of pinealectomy on the spinal column may be an indirect effect related to the pathophysiological consequences of disrupted biorhythms, rather than to a direct effect of melatonin [20].

Melatonin and bone metabolism in osteoblast cell lines

High concentrations of melatonin and the activities of the enzymes (arylalkylamine *N*-acetyltransferase and hydroxyindole-*O*-methoxyltransferase) which synthesize melatonin from serotonin have been identified in bone marrow [24, 25]. To investigate the potential pineal origin of bone marrow melatonin, long-term (8 months) pinealectomized rats were used. The authors found that the bone marrow in the pinealectomized rats still exhibited high levels of melatonin even though the pineal gland had been removed 8 months earlier [24]. These results suggest that the melatonin in bone metabolism is not of pineal origin.

Roth et al. [26] examined the direct effect of melatonin on osteoblasts using two cell lines: MC3T3-E1 pre-osteoblasts and rat osteoblast-like osteosarcoma 17/2.8 cells. Bone sialoprotein is an acidic, tyrosine-sulfated, extracellular bone matrix protein that is expressed

during osteoblastic cell differentiation and is required for mineralization. Melatonin increased the expression of bone sialoprotein as well as several other essential bone marker proteins, including alkaline phosphatase (ALP) and osteocalcin. In addition, melatonin stimulated both osteoblast differentiation and mineralization [26].

In another study, melatonin acted directly on human bone cells (HOB-M) and an osteoblast cell line (SV-HFO) and dose-dependently increased the proliferation in both cell types, with a maximal effect being achieved at a concentration of 50 μ M [27]. Type 1 collagen synthesis also was elevated in both cell types [27]. In a more recent study, reverse transcription-polymerase chain reaction and Western blot analysis showed that human osteoblasts express the melatonin 1a receptor and that its expression levels decrease gradually with the age of the host [28]. Collectively, these findings using osteoblast cell lines indicate that melatonin has a promotional action on osteoblasts.

Melatonin therapy for bone diseases such as osteoporosis

Sack et al. [4] reported that melatonin secretion decreased sharply during menopause. This reduction may contribute to post-menopausal osteoporosis. In the ovariectomized rat model of post-menopausal osteoporosis, the serum melatonin levels negatively correlated with the biochemical markers of bone resorption (cross-linked carboxyterminal telopeptide of type I collagen in serum, hydroxyproline in urine, and total calcium in urine) [29]. A negative correlation between the salivary melatonin levels and the biochemical markers of bone resorption was also found in post-menopausal obese women [30].

Ladizesky et al. [31] tested the effect of melatonin in ovariectomized rats. Urinary deoxypyridinoline (bone resorption marker) increased significantly after ovariectomy by 51% (30 days after surgery) and by 47% (60 days after surgery). Following melatonin administration (25 μ g/ml water), the increase in urinary deoxypyridinoline found at 30 days after ovariectomy was not observed. However, the bone mineral density and content as well

as bone area did not change at 60 days after surgery. To augment the bone area and bone mineral content in rats that had been ovariectomized, melatonin required small amounts of estrogen (10 µg/kg BW, 5 days/week) [32]. In intact mice, pharmacological doses (5 or 50 mg/kg BW/day) of melatonin elevated the bone mineral density [33]. This treatment significantly reduced the bone resorption parameters, including the osteoclastic surface and osteoclastic number, but did not increase the histomorphometric bone formation parameters (bone formation rate, mineral apposition rate, and osteoid volume) [33]. The skeletal effects of melatonin are, presumably, a result of the inhibition of osteoclast activity. In addition, a pharmacological dose (10 or 30 mg/kg BW/day) of melatonin elevated the trabecular thickness and trabecular area of the vertebra and femur and the cortical thickness of the femur in ovariectomized rats [34]. Based on currently available data, physiological doses of melatonin supplementation with estrogen or pharmacological doses of melatonin are required to increase the bone mineral content or bone mineral density, respectively, in the ovariectomized rat model of post-menopausal osteoporosis.

A novel assay system using fish scales

The teleost scale is a calcified tissue (Fig.1A) that contains osteoclasts (Fig.1B) and osteoblasts (Fig.1C) [8, 35-38]; these cells are similar to those found in avian and mammalian membrane bones. In the scale as well as in mammalian bone, the bone matrix includes type I collagen [39], bone γ -carboxyglutamic acid protein [40], and osteonectin [41]. Hydroxyapatite also exists in the scale [42]. In addition, multi-nucleated osteoclasts (the active type of osteoclasts) have been detected in the goldfish scale using *in situ* hybridization of cathepsin K [43]. The scales of some teleosts contain as much as 20% of the total body calcium and are, thus, a better potential internal calcium reservoir than vertebral bone during periods of increased calcium demand, such as sexual maturation and starvation [35-37, 44, 45]. Teleost scales are a simple and suitable model for bone. Considering these facts, we developed an *in*

in vitro assay system using the teleost scale to study bone metabolism [7, 8]. In this system, tartrate-resistant acid phosphatase (TRAP) and ALP were used as respective markers of osteoclasts and osteoblasts. It is known that these enzymes can be utilized as markers for these cells in mammals [46, 47]. In the fish scale, TRAP (Fig.1B) and ALP (Fig.1C) are also detected in these cells. The respective enzyme activity from a single scale can be detected after directly incubating the scale with the appropriate substrate. From one fish, roughly 100 scales having similar cell activity can be obtained.

Using this *in vitro* assay system, we found that melatonin suppressed both osteoclastic and osteoblastic activities [7]. This was the first report related to the function of melatonin in osteoclasts and the inhibitory effect of melatonin in osteoblasts in any vertebrate species. Melatonin also suppressed osteoblastic activity in an *in vivo* experiment using ovariectomized rats [32] as well as in an *in vitro* co-culture scale system of osteoclasts and osteoblasts. Furthermore, high endogenous levels of melatonin correlated with low levels of bone-forming markers (i.e., ALP and carboxyterminal propeptide of type I procollagen) in male rats [48]. Thus, a co-culture of osteoclasts and osteoblasts should be used to evaluate the effect of melatonin on bone metabolism. An interaction between osteoclasts and osteoblasts was also recently noted in mammals, and both actions must be considered when examining bone metabolism [49, 50]. The receptor activator of NF- κ B (RANK) and the receptor activator of the NF- κ B ligand (RANKL) have been identified in osteoclasts and osteoblasts, respectively [51]. Multi-nucleated osteoclasts (an active type of osteoclasts) are activated by the binding of RANKL to RANK [51]. A report showing that melatonin reduces the mRNA levels for RANKL in the osteoblast cell line [33] is consistent with our hypothesis that melatonin inhibits osteoblastic activity.

Bromomelatonin derivatives: Effects on osteoclastic and osteoblastic activities

The structures of the derivatives examined are illustrated in Figure 2. The details of the

studies are described in Suzuki et al. [9]. Melatonin (10^{-8} , 10^{-6} , and 10^{-4} M) suppressed the osteoclastic activity. For 2-bromomelatonin and 2,4,6,8-tetrabromomelatonin, the inhibitory action was less than that of melatonin, at least under the *in vitro* conditions using fish scales. The strength of the suppression of osteoclastic activity by tribromomelatonin derivatives (2,4,6-tribromomelatonin, 1-allyl-2,4,6-tribromomelatonin, 1-propargyl-2,4,6-tribromomelatonin, and 1-benzyl-2,4,6-tribromomelatonin) was similar to that of melatonin. While melatonin inhibited the osteoblastic activity, all bromomelatonin derivatives shown in Figure 2 had a promotional action of osteoblasts. In particular, 1-benzyl-2,4,6-tribromomelatonin (benzyl-tribromomelatonin) possessed the strongest stimulatory activity for osteoblasts. We subsequently analyzed the detailed actions of this derivative on scale osteoclasts and osteoblasts and compared them with those of melatonin.

Benzyl-tribromomelatonin had a stronger activity in osteoclasts than did melatonin. This inhibitory action of the derivative remains effective at 10^{-10} M after 6 h of incubation. In addition, benzyl-tribromomelatonin activated osteoblasts (10^{-9} to 10^{-6} M) after 6 h of incubation, while melatonin (10^{-8} to 10^{-6} M) suppressed osteoblastic activity. The action of these agents on osteoclasts and osteoblasts after 18 h incubation was similar to that at 6 h of incubation. In addition, estrogen receptor mRNA expression (an osteoblastic marker) was increased in benzyl-tribromomelatonin (10^{-7} M)-treated scales. A different receptor for bromomelatonin may exist in osteoblasts. Bromomelatonin derivatives theoretically bind to this receptor and activate osteoblastic activity. Certainly, different actions of melatonin and melatonin-related substances have been reported in a variety of studies [52-54]. Plans are underway to examine the characterization of binding for melatonin and bromomelatonin derivatives using both goldfish scale and mammalian osteoblastic cell lines.

Effect of benzyl-tribromomelatonin on bone tissues in ovariectomized rats and low-calcium diet rats

We have confirmed the effect of benzyl-tribromomelatonin on bone using the two common animal models of bone diseases (ovariectomized rats and low-calcium diet rats).

Oral administration of benzyl-tribromomelatonin (0.6 mg/ animal/ day) led to increase the total bone mineral density of the femoral metaphysis in ovariectomized rats (Table 1). Using the stress-strain index of the diaphysis, a significant difference was recorded between the ovariectomized animals that received 1-benzyl-2,4,6-tribromomelatonin and those that did not (Table 1). In addition, compression analysis of the femoral metaphysis indicated that the values of stiffness, bone fracture force, fracture energy, and maximum load tended to increase after 1-benzyl-2,4,6-tribromomelatonin treatment.

In rats fed a low-calcium diet, total bone mineral density in the femoral metaphysis significantly increased due to oral administration of 1-benzyl-2,4,6-tribromomelatonin (0.3 or 3 mg/ animal/ day) (Table 2). In the compression test, the values of stiffness, fracture energy, and maximum load in 1-benzyl-2,4,6-tribromomelatonin-treated rats were higher than those for the control animals.

Thus, the results using the scale assay system were reproduced in mammalian models of bone diseases. Additional studies will be required to examine the detailed mechanisms of the bromomelatonin derivatives in rat models of bone diseases. In addition, the actions of some bromomelatonin derivatives seem to resemble, in some respects, those of the osteoporosis-fighting drugs, i.e., bisphosphonates. Given that these latter drugs occasionally cause osteonecrosis of the jaw, this may be a potential side effect of melatonin derivatives to which attention must be given [55].

Conclusion

Melatonin directly influences bone metabolism. Judging from *in vivo* studies, the skeletal effects of melatonin probably are a result of its inhibition of osteoclasts. In osteoblasts, a suppressive action of melatonin has been detected *in vivo*, while a promotional effect was

recorded *in vitro*.

In ovariectomized rats as a model of post-menopausal osteoporosis, the administration of physiological doses of melatonin with adequate estrogen or pharmacological doses of melatonin is required to increase the bone mineral content or bone mineral density, respectively.

Using the teleost scale assay system, physiological doses of melatonin were found to suppress both osteoclasts and osteoblasts. These findings are consistent with the results of *in vivo* studies in mammals. Thus, the scale assay system has utility in the development of new drugs for bone diseases, such as osteoporosis.

A novel bromomelatonin derivative, 1-benzyl-2,4,6-tribromomelatonin, increases bone strength in ovariectomized rats and increases bone mineral density in rats fed a low-calcium diet. This melatonin derivative may have potential for use as a beneficial drug for bone diseases, such as osteoporosis.

Acknowledgments

This study was supported in part by grants to N.S. [Grant-in-Aid for Scientific Research (C) No. 18500375 by JSPS] and to A.H. [Grant-in-Aid for Scientific Research (C) No.18570055 by JSPS].

References

1. REITER RJ. Melatonin: the chemical expression of darkness. *Mol Cell Endocrinol* 1991; **79**:C153-C158.
2. REITER RJ. The melatonin rhythm: both a clock and a calendar. *Experientia* 1993; **49**:654-664.
3. REITER RJ, CRAFT CM, JOHNSON JE JR et al. Age-associated reduction in nocturnal pineal melatonin levels in female rats. *Endocrinology* 1981; **109**:

- 1295-1297.
4. SACK RL, LEWY AJ, ERB DL et al. Human melatonin production decreases with age. *J Pineal Res* 1986; **3**:379-388.
 5. REITER RJ. The ageing pineal gland and its physiological consequences. *BioEssays* 1992; **14**:169-175.
 6. HAKANSON DO, BERGSTROM WH. Phototherapy-induced hypocalcemia in newborn rats: prevention by melatonin. *Science* 1981; **214**:807-809.
 7. SUZUKI N, HATTORI A. Melatonin suppresses osteoclastic and osteoblastic activities in the scales of goldfish. *J Pineal Res* 2002; **33**:253-258.
 8. SUZUKI N, SUZUKI T, KUROKAWA T. Suppression of osteoclastic activities by calcitonin in the scales of goldfish (freshwater teleost) and nibbler fish (seawater teleost). *Peptides* 2000; **21**:115-124.
 9. SUZUKI N, SOMEI M, KITAMURA K, et al. Novel bromomelatonin derivatives suppress osteoclastic activity and increase osteoblastic activity: implications for the treatment of bone diseases. *J Pineal Res* 2008; **44**:326-334.
 10. KISS J, BANHEGYI D, CSABA G. Endocrine regulation of blood calcium level. II. Relationship between the pineal body and the parathyroid glands. *Acta Med Acad Sci Hung* 1969; **26**:363-370.
 11. CSABA G, BARATH P. The effect of pinealectomy on the parafollicular cells of the rat thyroid gland. *Acta Anat (Basel)* 1974; **88**:137-146.
 12. CSABA G, BARATH P. Morphological changes of thymus and the thyroid gland after postnatal extirpation of pineal body. *Endocrinol Exp* 1975; **9**:59-67.
 13. CHEN H, SHOUMURA S, EMURA S et al. Effects of melatonin on the ultrastructure of the golden hamster parathyroid glands. *Histol Histopathol* 1991; **6**:1-7.
 14. SHOUMURA S, CHEN H, EMURA S et al. An in vitro study on the effects of melatonin on the ultrastructure of the hamster parathyroid gland. *Histol Histopath*

- 1992; **7**:715-718.
15. CSABA G, BOKAY J. The effect of melatonin and corpus pineal extract on serum electrolytes in the rat. *Acta Biol Acad Sci Hung* 1977; **28**:143-144.
 16. MACHIDA M, DUBOUSSET J, IMAMURA Y et al. An experimental study in chickens for the pathogenesis of idiopathic scoliosis. *Spine* 1993; **18**:1609-1615
 17. BAGNALL K, RASO VJ, MOREAU M et al. The effects of melatonin therapy on the development of scoliosis after pinealectomy in the chicken. *J Bone Joint Surg Am* 1999; **81**:191-199.
 18. SADAT-ALI M, AL-HABDAN I, AL-OTHMAN A. Adolescent idiopathic scoliosis. Is low melatonin a cause ? *J Bone Spine* 2000; **67**:62-64.
 19. MACHIDA M, DUBOUSSENT J, SATOH T et al. Pathologic mechanism of experimental scoliosis in pinealectomized chickens. *Spine* 2001; **26**:E385-E391.
 20. TURGUT M, KAPLAN S, TURGUT AT et al. Morphological, sterological and radiological changes in pinealectomized chicken cervical vertebrae. *J Pineal Res* 2005; **39**:392-399.
 21. MACHIDA M, MURAI I, MIYASHITA Y et al. Pathogenesis of idiopathic scoliosis: experimental study in rats. *Spine* 1999; **24**:1985-1989.
 22. FJELLDAL PG, GROTMOL S, KRYVI H et al. Pinealectomy induces malformation of the spinal and reduces the mechanical strength of the vertebrae in Atlantic salmon, *Salmo salar*. *J Pineal Res* 2004; **36**:132-139.
 23. TURGUT M, YENISEY C, UYSAL A et al. The effects of pineal gland transplantation on the production of spinal deformity and serum melatonin level following pinealectomy in the chicken. *Eur Spine J* 2003; **12**:487-494.
 24. TAN DX, MANCHESTER LC, REITER RL et al. Identification of highly elevated levels of melatonin in bone marrow: its origin and significance. *Biochim Biophys*

- Acta 1999; **1472**:206-214.
25. CONTI A, CONCONI S, HERTENS E et al. Evidence for melatonin synthesis in mouse and human bone marrow cells. *J Pineal Res* 2000; **28**:193-202.
 26. ROTH JA, KIM B-G, LIN W-L, CHO M-I. Melatonin promotes osteoblast differentiation and bone formation. *J Biol Chem* 1999; **274**:22041-22047.
 27. NAKADE O, KOYAMA H, ARIJI H et al. Melatonin stimulates proliferation and type I collagen synthesis in human bone cells in vitro. *J Pineal Res* 1999; **27**: 106-110.
 28. SATOMURA K, TOBIUME S, TOKUYAMA R et al. Melatonin at pharmacological doses enhances human osteoblastic differentiation in vitro and promotes mouse cortical bone formation in vivo. *J Pineal Res* 2007; **42**:231-239.
 29. OSTROWSKA Z, KOS-KUDLA B, SWIETOCHOWSKA E et al. Assessment of the relationship between dynamic pattern of nighttime levels of melatonin and chosen biochemical markers of bone metabolism in a rat model of postmenopausal osteoporosis. *Neuroendocrinol Lett* 2001; **22**:129-136.
 30. OSTROWSKA Z, KOS-KUDLA B, MAREK B et al. Assessment of the relationship between circadian variations of salivary melatonin levels and type I collagen metabolism in postmenopausal obese women. *Neuroendocrinol Lett* 2001; **22**:121-127.
 31. LADIZESKY MG, CUTRERA RA, BOGGIO V et al. Effect of melatonin on bone metabolism in ovariectomized rats. *Life Sci* 2001; **70**:557-565.
 32. LADIZESKY MG, BOGGIO V, ALBORNOZ LE et al. Melatonin increases oestradiol-induced bone formation in ovariectomized rats. *J Pineal Res* 2003; **34**: 143-151.
 33. KOYAMA H, NAKADE O, TAKADA Y et al. Melatonin at pharmacologic doses increases bone mass by suppressing resorption through down-regulation of the

- RANKL-mediated osteoclast formation and activation. *J Bone Miner Res* 2002; **17**:1219-1229.
34. USLU S, UYSAL A, ÖKTEM G et al. Constructive effect exogenous melatonin against osteoporosis after ovariectomy in rats. *Anal Quant Cytol Histol* 2007; **29**: 317-325.
 35. YAMADA J. Studies on the structure and growth of the scales in the goldfish. *Mem Fac Fish Hokkaido Univ* 1961; **9**:181-226.
 36. YAMADA J. A fine structural aspect of the development of scales in the chum salmon fry. *Bull Jap Soc Sci Fish* 1971; **37**:18-29.
 37. BERITER-HAHN J, ZYLBERBERG L. Regeneration of teleost fish scale. *Comp Biochem Physiol* 1993; **105A**:625-641.
 38. YOSHIKUBO H, SUZUKI N, TAKEMURA K et al. Osteoblastic activity and estrogenic response in the regenerating scale of goldfish, a good model of osteogenesis. *Life Sci* 2005; **76**:2699-2709.
 39. ZYLBERBERG L, BONAVENTURE J, COHEN-SOLAL L et al. Organization and characterization of fibrillar collagens in fish scales in situ and in vitro. *J Cell Sci* 1992; **103**:273-285.
 40. NISHIMOTO SK, ARAKI N, ROBINSON FD, WAITE JH. Discovery of bone γ -carboxyglutamic acid protein in mineralized scales. *J Biol Chem* 1992; **267**: 11600-11605.
 41. LEHANE DB, MCKIE N, RUSSELL RGG, HENDERSON IW. Cloning of a fragment of the osteonectin gene from goldfish, *Carassius auratus*: its expression and potential regulation by estrogen. *Gen Comp Endocrinol* 1999; **114**:80-87.
 42. ONOZATO H, WATABE N. Studies on fish scale formation and resorption. III. Fine structure and calcification of the fibrillary plates of the scales in *Carassius auratus* (Cypriniformes: Cyprinidae). *Cell Tissue Res* 1979; **201**:409-422.

43. AZUMA K, KOBAYASHI M, NAKAMURA M et al. Two osteoclastic markers expressed in multinucleate osteoclasts of goldfish scales. *Biochem Biophys Res Commun* 2007; **362**: 594-600.
44. BERG A. Studies on the metabolism of calcium and strontium in freshwater fish. I. Relative contribution of direct and intestinal absorption. *Mem Ist Ital Idrobiol* 1968; **23**:161-196.
45. MUGIYA Y, WATABE N. Studies on fish scale formation and resorption II: effect of estradiol on calcium homeostasis and skeletal tissue resorption in the goldfish, *Carassius auratus*, and the killifish, *Fundulus heteroclitus*. *Comp Biochem Physiol* 1977; **57A**:197-202
46. VAES G. Cellular biology and biochemical mechanism of bone resorption. *Clin Orthop* 1988; **231**:239-271.
47. DIMAI HP, LINKHART TA, LINKHART SG, et al. Alkaline phosphatase levels and osteoprogenitor cell numbers suggest bone formation may contribute to peak bone density differences between two inbred strains of mice. *Bone* 1998; **22**:211-216.
48. OSTROWSKA Z, KOS-KUDLA B, MAREK B, KAJDANIUK D. Influence of lighting conditions on daily rhythm of bone metabolism in rats and possible involvement of melatonin and other hormones in this process. *Endocr Regul* 2003; **37**: 163-174.
49. SUDA T, TAKAHASHI N, UDAGAWA N et al. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr Rev* 1999; **20**:345-357.
50. MANOLAGAS SC. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr Rev* 2000; **21**:115-137.

51. TEITELBAUM SL. Bone resorption by osteoclasts. *Science* 2000; **289**:1504-1508.
52. ZISAPEL N, EGOZI Y, LAUDON M. Circadian variations in the inhibition of dopamine release from adult and newborn rat hypothalamus by melatonin. *Neuroendocrinology* 1985; **40**:102-108.
53. SEEGER H, MUECK AO, LIPPERT TH. Effect of melatonin and metabolites on copper-mediated oxidation of low density lipoprotein. *Br J Clin Pharmacol* 1997; **44**:283-284.
54. WILLIS GL, ROBERTSON AD. Recovery of experimental Parkinson's disease with the melatonin analogues ML-23 and S-20928 in a chronic, bilateral 6-OHDA model: a new mechanism involving antagonism of the melatonin receptor. *Pharmacol Biochem Behav* 2004; **79**:413-429.
55. MARX RE, CILLO JE JR, ULLOA JJ. Oral bisphosphonate-induced osteonecrosis: risk factors, prediction of risk using serum CTX testing, prevention, and treatment. *J Oral Maxillofac Surg* 2007; **65**:2397-2410.

Figure Legends

Fig. 1. Diagram of a transverse section of teleost skin with scales (A), typical multi-nucleated osteoclasts (TRAP staining) (B) and osteoblasts (ALP staining) (C) in goldfish scales. Each arrowhead indicates multi-nucleated osteoclasts and osteoblasts.

Fig. 2. Chemical structures of the melatonin and novel bromomelatonin derivatives.

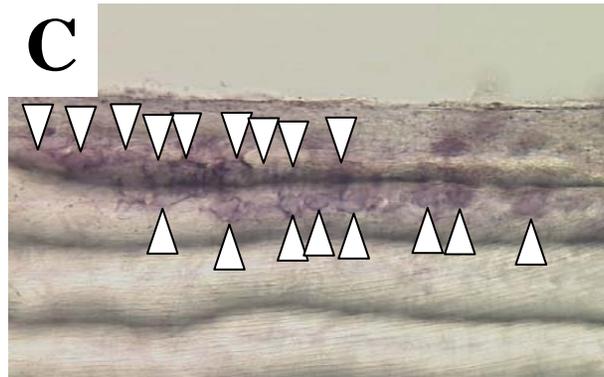
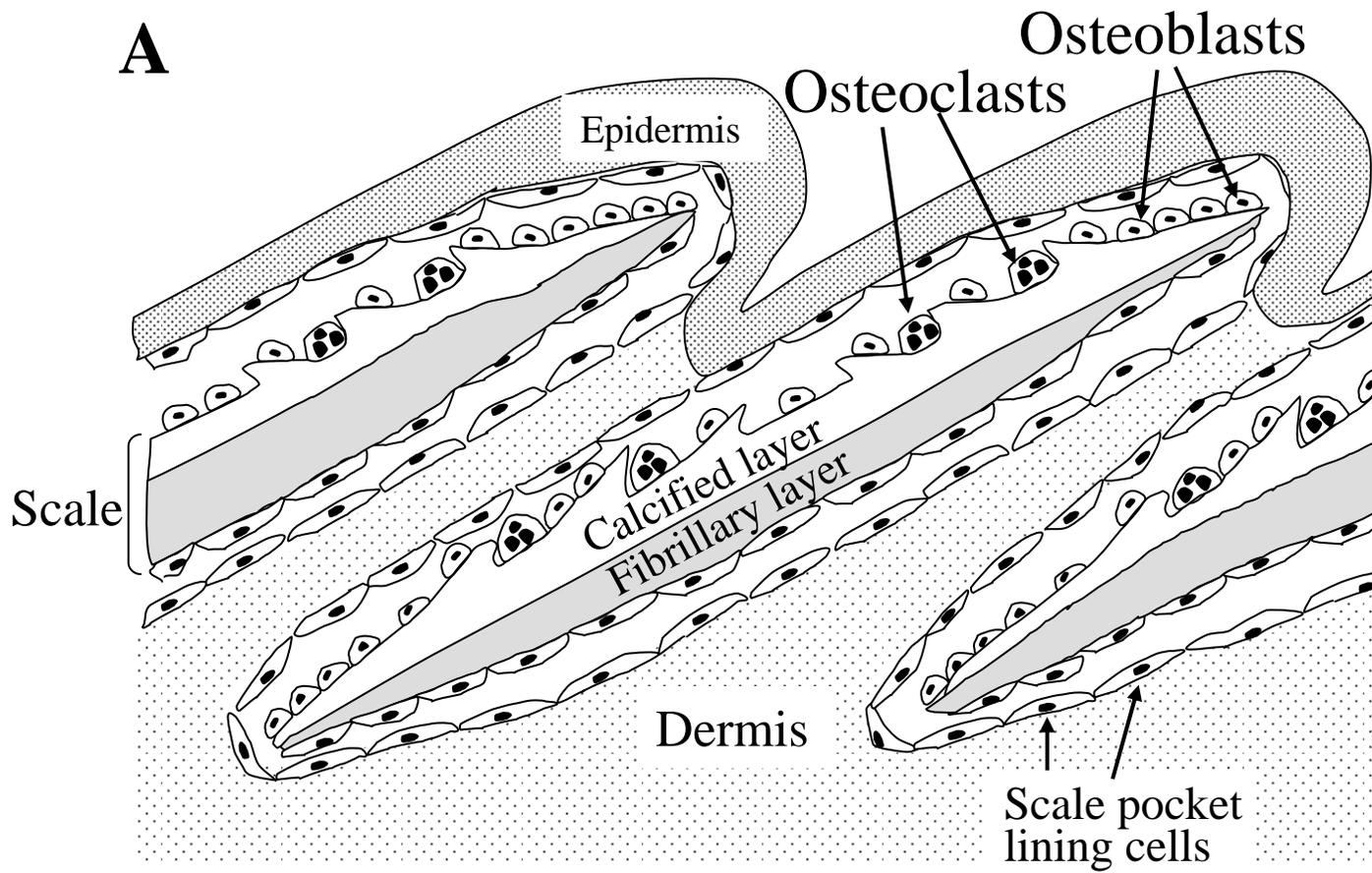
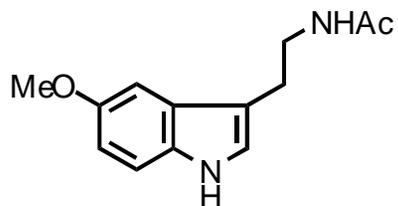
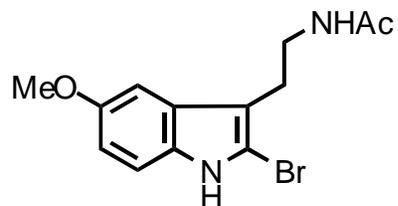


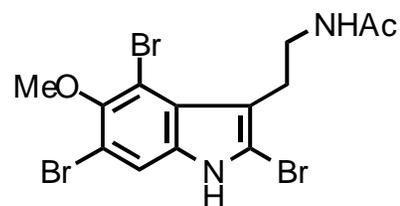
Figure 1 Suzuki et al.



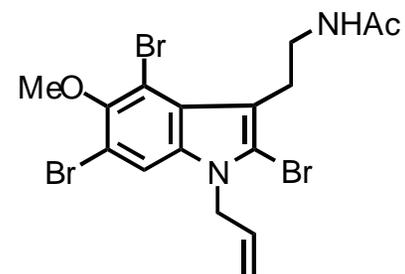
melatonin



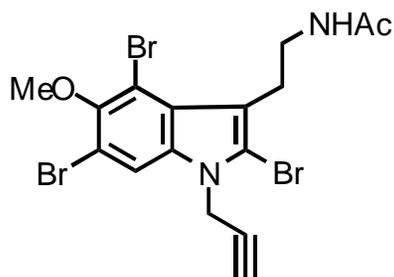
2-bromomelatonin



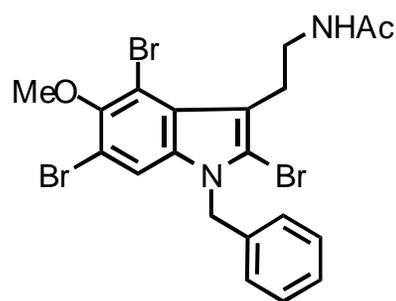
2,4,6-tribromomelatonin



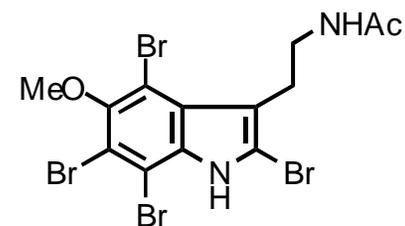
1-allyl-2,4,6-tribromo-melatonin



1-propargyl-2,4,6-tribromomelatonin



1-benzyl-2,4,6-tribromomelatonin



2,4,6,7-tetrabromomelatonin

Table 1 Analysis of bone mineral density and stress-strain index in femoral metaphysis and diaphysis of ovariectomized (OVX) rats

	N	Bone mineral density (mg/cm ³)			Stress-strain index	
		Metaphysis		Diaphysis		
		Total	Subcortical + Cortical	Trabecular		Cortical
SHAM	5	672.3 ± 21.3	733.5 ± 12.5	280.9 ± 12.8	1278.0 ± 5.1	6.34 ± 0.30
OVX	8	501.5 ± 11.9	680.8 ± 10.7	204.4 ± 6.9	1273.7 ± 4.9	6.60 ± 0.11
OVX + Br-MEL	6	519.3 ± 14.3	672.7 ± 23.0	212.6 ± 14.5	1274.0 ± 6.1	7.15 ± 0.18 *

Data as mean ± SE

*: significantly different (P<0.05) from OVX rats and OVX + bromomelatonin (Br-MEL)-treated rats.

Table 2 Analysis of bone mineral density and stress-strain index in femoral metaphysis and diaphysis of rats fed a low-calcium diet

	N	Bone mineral density (mg/cm ³)			Stress-strain index	
		Metaphysis		Diaphysis		
		Total	Subcortical + Cortical	Trabecular		Cortical
Control	6	330.5 ± 8.0	614.2 ± 16.6	160.4 ± 4.7	1219.9 ± 7.8	5.33 ± 0.17
Low dose Br-MEL (0.3 mg/ animal)	7	375.1 ± 7.6**	613.8 ± 13.9	179.5 ± 8.2	1224.0 ± 11.3	5.58 ± 0.22
High dose Br-MEL (3 mg/ animal)	6	364.6 ± 11.1*	645.3 ± 21.4	179.3 ± 11.9	1224.1 ± 7.6	5.49 ± 0.36

Data as mean ± SE

* and **: significantly different (P<0.05 and P<0.01, respectively) from control rats and bromomelatonin (Br-MEL)-treated rats.