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The anti-oxidant effects of melatonin derivatives on human gingival fibroblasts

Chawapon Phiphatwatcharaded[a,b], Ploenthip Puthongking[a,b], Ponlatham Chaiyarit[c,d], Nutjaree Pratheepawanit Johns[a,b], Sumon Sakolchai[a], Pramote Mahakunakorn[a,b,*]

[a] Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand
[b] Melatonin Research Group, Khon Kaen University, Khon Kaen 40002, Thailand
[c] Research Group of Chronic Inflammatory Oral Diseases and Systemic Diseases Associated with Oral Health, Khon Kaen University, Khon Kaen, 40002, Thailand
[d] Department of Oral Diagnosis, Faculty of Dentistry, Khon Kaen University, Khon Kaen, 40002, Thailand

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ABSTRACT

Objectives: Aim of this *in vitro* study was to evaluate the anti-oxidant activity of indole ring modified melatonin derivatives as compared with melatonin in primary human gingival fibroblast (HGF) cells.
Methods: Anti-oxidant activity of melatonin (MLT), acetyl-melatonin (AMLT) and benzoyl-melatonin (BMLT) was evaluated by5 standard methods as follows: 2, 2-diphenyl-1-picrylhydrazyl (DPPH); ferric ion reducing antioxidant power (FRAP); superoxide anion scavenging; nitric oxide (NO) scavenging; and thiobarbituric acid reactive substances (TBARs).Evaluation of cellular antioxidant activity (CAA) and protectivity against H_2O_2 induced cellular damage was performed via MTT assay in HGF cells.
Results: According to the standard anti-oxidant assays, the antioxidant power of AMLT and BMLT were slightly less than MLT in FRAP and superoxide scavenging assays. In the NO scavenging and TBARs assays, BMLT and AMLT were more potent than MLT, whereas DPPH assays demonstrated that MLT was more potent than others. BMLT and AMLT had more potent anti-oxidant and protective activities against H_2O_2in HGF cells as compared with MLT.
Conclusions: MLT derivatives demonstrated different anti-oxidant activities as compared with MLT, depending upon assays. These findings imply that *N*-indole substitution of MLT may help to improve hydrogen atom transfer to free radicals but electron transfer property is slightly decreased. Anti-oxidant and protective effects of melatonin derivatives (AMLT and BMLT) on human gingival fibroblasts imply the potential use of these molecules as alternative therapeutics for chronic inflammatory oral diseases.

1. Introduction

Most oral diseases are associated with inflammatory response such as periodontitis, dental pulpitis, oral mucositis or recurrent aphthous stomatitis (Kim and Amar, 2006; Mehta & Kaur, 2014; Preeti, Magesh, Rajkumar, & Karthik, 2011; Prieto-Prieto and Calvo, 2004). During inflammation, the immune cells such as macrophages, neutrophils and other phagocytic cells are aggregated into the site of tissue damage and release inflammatory cytokines. Moreover, many oxidant compounds are also produced leading to oxidative stress. It is well known that inflammatory diseases are related with an increase in free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) at the site of inflammation (Emelyanov et al., 2001). Self-defense mechanisms generate ROS and RNS at the site of inflamed tissues to kill pathogenic microorganisms. The inflammatory processes involve enzymes that produce free radicals such as NADPH oxidase which is capable of generating $O^{\bullet-}_2$ by enzymatically dismutating H_2O_2. These free radicals can disrupt the cellular functions by peroxidation. The imbalance between oxidants and antioxidants has been considered as a risk factor for chronic oral inflammatory diseases (Bagan et al., 2014; Kesarwala, Krishna, & Mitchell, 2016). It would be beneficial if we could produce the compounds containing anti-inflammatory and anti-oxidant activities for prevention or treatment of chronic inflammatory oral diseases.

Melatonin (*N*-acetyl-5-methoxytryptamine, MLT) is a neurohormone mainly secreted by the pineal gland during the dark

* Corresponding author at: Melatonin Research Group, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand.
E-mail addresses: pipat_cp@hotmail.com (C. Phiphatwatcharaded), pploenthip@kku.ac.th (P. Puthongking), cponla@kku.ac.th (P. Chaiyarit), pnutja@kku.ac.th (N.P. Johns), sumon@kku.ac.th (S. Sakolchai), pramah@kku.ac.th (P. Mahakunakorn).

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phase in mammals (Bonnefont-Rousselot and Collin, 2010). MLT is also found in oral compartments such as the salivary glands, saliva, and oral mucosa (Reiter et al., 2014). It was reported that decreased levels of salivary MLT were found in patients with periodontal diseases (Abdolsamadi et al., 2014; Reiter et al., 2014).MLT has anti-inflammatory and anti-oxidant activities in both *in vitro* and *in vivo* models (Cuesta et al., 2011; Guney et al., 2007; Radogna, Diederich, & Ghibelli, 2010). Production of nitric oxide (NO), prostaglandin (PGE) and inflammatory cytokines was inhibited by MLT and its metabolites in macrophage cell line (Elmegeed, Baiuomy, & Abdel-Salam, 2007; Mayo et al., 2005; Zhang, Li, Gao, & Wei, 2004). Although, melatonin has low toxicity, it also has limitation in pharmacokinetic issues such as short half-life and oral/tissue bioavailability. In our previous studies (Phiphatwatcharaded, Topark-Ngarm, Puthongking, & Mahakunakorn, 2014), we synthesized MLT derivatives including acetyl melatonin (AMLT) and benzoyl melatonin (BMLT) (Fig. 1). These derivatives were able to reduce the production of inflammatory mediators such as NO and PGE in macrophage cells and reduced pain in rat with prolong duration of action (Rivara, Pala, Bedini, & Spadoni, 2015). Moreover, these MLT derivatives have been reported to act as a prodrug that can be cleaved to MLT by systemic enzymes (Thoại and Nam, 2013). However, no studies have investigated the anti-oxidant and protective activities of these derivatives in human gingival fibroblasts (HGF). Thus, the present study compared the efficacy of MLT derivatives with MLT using five standard anti-oxidant assays and one assay for evaluating protective effect of these MLT derivatives in a primary HGF cell model.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals for antioxidant assay; 2,2-Diphenyl-1-picrylhydrazyl (DPPH), fluorescein, sulfanilamide, *N*-(1-Naphthyl) ethylenediamine dihydrochloride (NED), phosphoric acid, phenazine methosulfate (PMS), sodium phosphate dibasic (Na_2HPO_4), dipotassium phosphate (K_2HPO_4), 2-thiobarbituric acid were purchased from Sigma–Aldrich Ltd. (USA). Nitro blue tetrazolium (NTB), nicotinamide adenine dinucleotide (NADH), 2,4,6-Tripyridyl-*s*-Triazine (TPTZ), iron(III) chloride hexahydrate ($FeCl3{\cdot}_6H_2O$), sodium acetate trihydrate, dichloro-dihydro-fluorescein diacetate (DCFH-DA) and H_2O_2 were purchased from Fluka Chemika (Buchs, Switzerland) and 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) was purchased from Calbiochem (USA).

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Glutamax®, Dulbecco's Phosphate-Buffered Saline (DPBS), Antibiotic-Antimycotic (100×) and Hank's balanced salts solution (HBSS) were purchased from Gibco Inc. (USA). 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Fluka Chemika (Buchs, Switzerland).

Melatonin derivatives were synthesized and characterizationsin a previous study of Phiphatwatcharaded et al. (2014).

2.2. Cell culture

Human gingival fibroblast (HGF) cells were kindly provided by Assist Prof. Dr. Doodsadee Homdee from Research Group of Chronic Inflammatory Oral Diseases and Systemic Diseases Associated with Oral Health, Khon Kaen University. These primary cells were cultured in DMEM with 10% FBS, 1% Glutamax® and 1% (v/v) penicillin–streptomycin at 37 °C and 5% CO_2. Cells were grown to 80–90% confluence in a 10 cm cell culture dish before the experiments. Cells were counting by hemocytometer with Trypan blue staining and calculated cell number per wells before seeding into wells palte.

2.3. DPPH radical scavenging assay

The procedure for DPPH assay was carried out according to Nicklisch and Waite (Nicklisch and Waite, 2014). Briefly, DPPH was prepared to be used in final concentration 200 μM in ethanol. The various concentrations of MLT, AMLT and BMLT (200, 600 and 1000 μM) were dissolved in DMSO and added into DPPH solution with equal volume in 96 well plates. Absorbance was recorded at 550 nm after reaction started for 30 min. The results are expressed as % of DPPH scavenging. DPPH solution with vehicle DMSO used as negative control, DMSO without DPPH was used as a blank for background subtraction.

2.4. Reduction of the superoxide anion radical

The reduction of superoxide anion was tested by reduction of NTB, as described by Liu and Ng (Liu and Ng, 2000). The reaction mixture contained 78 μM of NTB, 234 μM of NADH and 8 μM of PMS in phosphate buffered saline (PBS) at pH 7.4 with various concentrations of MLT, AMLT and BMLT. The reduction of NBT was measured at absorbance 550 nm after 10 min of the reaction development. The activity of the MLT, AMLT and BMLT was expressed as % of superoxide scavenging in comparison with the negative control that contained only the reaction mixture without compounds tested. The reaction mixture without NTB was used as blank for background subtraction.

2.5. The ferric ion reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the protocol previously reported by Benzie and Strain (Benzie and Strain, 1996). The FRAP reagent consists of acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in HCl 40 mM) and $FeCl_3{\cdot}6H_2O$ (20 mM) in ratio 10:1:1. For the measurement, FRAP reagent was mixed with various concentration of MLT, AMLT and BMLT or vehicle (ethanol, negative control) as ratio 10:1. Absorbance was measured 595 nm after 10 min of the reaction development. An aqueous solution of $FeSO_4$ (1–100 μM) was used for calibration. The results were expressed as mol of Fe^{2+}.

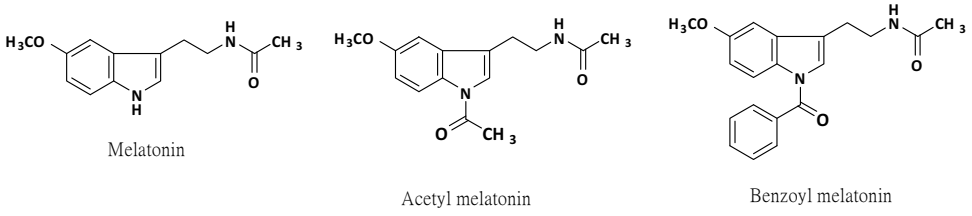

Fig. 1. Structure of melatonin (MLT), acetyl melatonin (AMLT) and benzoyl melatonin (BMLT).