Neuroprotective and Neuritogenic Activities of Melatonin and N-acetyl Substituent Derivative
Panyada Panyatip1,2, Ploenthip Puthongking2,3*, Sarin Tadtong4
IJPS, March 2015; 11(Supplement) : 14-19

Introduction: Melatonin (MLT) is the pineal hormone, which has many pharmacological activities as following antioxidant, anticancer, and immune enhancement. Due to its antioxidant ability, MLT was investigated the neuroprotective effect on neurons. However, the poor pharmacokinetic profiles such as bioavailability and rapid metabolism were its limit for pharmacological study. Therefore, the structure development is needed for improving pharmacological activity. Materials and Method: N-acetyl melatonin (AcO-MLT) was produced from the reaction of MLT and acetic anhydride by using 4,4-dimethyl aminopyridine (DMAP) as catalyst. After the reaction completed, liquid-liquid extraction and chromatographic purification were used to obtain AcO-MLT. Then, MLT and N-acetyl melatonin AcO-MLT were evaluated neuroprotective ability by serum deprivation method and neuritogenic effect in P19-derived neurons. Cell viability was measured by XTT.

Abstract
Neuroprotective and Neuritogenic Activities of Melatonin and N-acetyl Substituent Derivative
Panyada Panyatip1,2, Ploenthip Puthongking2,3*, Sarin Tadtong4
IJPS, March 2015; 11(Supplement) : 14-19

Introduction: Melatonin (MLT) is the pineal hormone, which has many pharmacological activities as following antioxidant, anticancer, and immune enhancement. Due to its antioxidant ability, MLT was investigated the neuroprotective effect on neurons. However, the poor pharmacokinetic profiles such as bioavailability and rapid metabolism were its limit for pharmacological study. Therefore, the structure development is needed for improving pharmacological activity. Materials and Method: N-acetyl melatonin (AcO-MLT) was produced from the reaction of MLT and acetic anhydride by using 4,4-dimethyl aminopyridine (DMAP) as catalyst. After the reaction completed, liquid-liquid extraction and chromatographic purification were used to obtain AcO-MLT. Then, MLT and N-acetyl melatonin AcO-MLT were evaluated neuroprotective ability by serum deprivation method and neuritogenic effect in P19-derived neurons. Cell viability was measured by XTT.
reduction assay. **Results:** Both of MLT and its derivative, AcO-MLT, at 1 nM significantly protected neuron cells from toxicity of oxidative stress. From XTT reduction assay showed cell viability treated with MLT and AcO-MLT compared with oxidative stress condition group was 98.0% ± 44.3, 74.3% ± 7.4, and 15.7% ± 5.2, respectively. MLT and AcO-MLT also increased the number of neurites compared with the control (3.4 ± 1.1, 4.0 ± 1.7, and 2.0 ± 1.1, respectively). **Conclusion:** MLT and AcO-MLT significantly protected neuronal cell death from oxidative stress. For neuritogenic ability, MLT and AcO-MLT could promote the neurite outgrowth but could not increase the neurite length. From the result can be summarized that MLT and its N-acetylated derivative may have potential to further study and develop as a neuroprotective agent.

**Keywords:** Melatonin, Melatonin derivative, Neuroprotective effect, Neuritogenic effect

---

**Introduction**

Melatonin (N-acetyl-5-methoxytryptamine, MLT) is the hormone which is secreted from the pineal gland in the brain (Lerner et al., 1958). This hormone synchronizes the biological clock of the body in sleep-wake cycle regulation (Arendt, 2000). Moreover, numerous pharmacological studies show that melatonin possess many activities such as antioxidant, anticancer, and immune enhancement (Cos and Blask, 1990; Reiter, 1995; Epstein and Brzezinski, 1997; Srinivasan et al., 2005).

Neurodegenerative disorders are the result when neuronal cells and their functions are damaged (Checkoway et al., 2011). Because these disorders are usually found in elderly population, more people are suffering from neuronal diseases from the rapid increment of aged people in society (Gallego et al., 2011). Furthermore, the degeneration of neuronal systems can lead to many brain diseases for example Alzheimer’s disease, Parkinson’s disease and amyotrophic lateral sclerosis (ALS) (Beal, 1996). Although the evidence that indicates the cause of neurodegenerative disorders is still unclear, free radicals are believed to be involved in these disorders (Beal et al., 2005). MLT has been investigated for neuroprotective activity due to the powerful antioxidant effect and ability to cross the blood brain barrier easily (Cardinali and Pévet, 1998). Many researches have reported that melatonin can prevent neuronal cell death via oxidative stress and neurotoxic substances such as 1 -methyl-4 -phenyl-1 ,2 , 3 ,6 -tetrahydropyridine (MPTP), kainic acid and methamphetamine (Stull et al., 2002; Chung and Han, 2003; Parameyong et al., 2013).

However, MLT has some disadvantages in pharmacokinetic properties such as low bioavailability and short half-life (Di et al., 1997). Development of derivatives is needed for improving pharmacokinetic properties and pharmacological activities. Therefore, this research aims to study the neuroprotective and neuritogenic effect of the N-substituted derivative acetyl-melatonin (AcO-MLT) compared with parent compound MLT. Data from this research will establish further structure activity relationships (SAR) help to find new neuroprotective agents.

---

**Figure 1** Structure of (1) MLT and (2) AcO-MLT
Materials and Method

Chemicals and materials

Chemicals used for MLT derivative synthesis were MLT (Shanghai Chemical Co. Ltd., Shanghai, China), acetic anhydride, 4,4-dimethyl aminopyridine (DMAP), pyridine (Fluka Chemika, Buchs, Switzerland), silica gel GE0049 (0.06–0.2 mm) (Sigma-Aldrich, St. Louis, USA) and TLC silica gel 60 F254 plates (Merck, Darmstadt, Germany). Solvents (commercial grade) including hexane, dichloromethane, methanol, and ethyl acetate were distilled prior to use. Nuclear magnetic resonance (1H-NMR and 13C-NMR) were measured in CDCl3 on an NMR Spectrometer (Varian NMR-400 MHz). Chemical shifts were expressed in δ (ppm) relative to TMS as reference standard and coupling constants (J) in Hertz (Hz).

Cell culture

Murine embryonal carcinoma cells, P19 ATCC CRL-1857 (the American Type Culture Collection, USA) were incubated in alpha minimal essential medium (α-MEM) with 7.5% newborn calf serum (NCS), 2.5% fetal bovine serum (FBS), and 1% antibiotics-antimycotic solution in 5% CO2 humidified atmosphere and at temperature 37°C. To maintain in exponential growth, the monolayer cell cultures were sub-cultured every 2 days.

Acetylated melatonin derivative synthesis

In a 50 mL round bottom flask covered with a guard column filled with CaCl2, MLT (232 mg, 1 mmol) was dissolved by pyridine (1 mL) and stirred at room temperature under nitrogen gas and using DMAP (61 mg, 0.5 mmol) as catalyst. Then, the temperature was cooled down to 5 °C in the ice bath for 10 min. Next, acetic anhydride (470 μL, 5 mmol) was slowly added into reaction mixture over 15 min and temperature was maintained at 5 °C for 10 minutes. After that, the temperature was raised to room temperature and stirring was continued for 24 hr. The reaction was confirmed by thin layer chromatography (TLC) technique. After chemical reaction was completed, 50 mL of water was added to stop the reaction. Then, the mixture was extracted with 50 mL of dichloromethane three times. Following this, the organic layer was collected and dried over anhydrous Na2SO4. The solution was filtered and concentrated by rotary evaporator to obtain the crude extract. Column chromatography (CC) was used to purify the crude by gradient of solvent system which was started from 100% of hexane with gradually increased ratio of ethyl acetate to 100% of ethyl acetate. After the purification process, a white solid product was obtained (125 mg, 46%). The structure of isolated product was confirmed by using 1H-NMR and 13C-NMR methods.

1H-NMR (400 MHz, CDCl3): δ 7.25 (s, 1H), 6.99 (d, 1H, J = 2.1), 6.97 (d, 1H, J = 2.3), 5.62 (s, 1H), 6.95 (d, 1H, J = 2.4), 3.87 (s, 3H), 3.61 (q, 2H, J = 6.7), 2.90 (t, 2H, J = 6.9), 2.58 (s, 3H), 1.97 (s, 3H)

13C-NMR (100 MHz, CDCl3): δ 170.44, 168.08, 156.5, 131.56, 130.62, 123.26, 119.58, 117.45, 113.44, 101.95, 55.75, 38.98, 25.28, 23.60, 23.28

Differentiation of P19 cells into P19-derived neurons

Exponentially growth cell cultures were trypsinized and dissociated into single cells. P19 cells (2 × 106 cells/mL) were suspended in 10 mL of α-MEM supplemented with 5% FBS, 1% antibiotics-antimycotic solution (P19IM) and 0.5 μM of trans-retinoic acid (RA), and seeded to a 100-mm bacteriological culture dish. The cells formed large aggregates in suspension. After 4 days of treatment, aggregates were dissociated by glass measuring pipette, re-plated on poly-L-lysine-pre-coated multi-well plates (multi-well plates were coated with 50 μg/mL poly-L-lysine dissolved in PBS overnight and sterilized under UV light for 30 min) at 7 × 104 cells/mL in P19SM (α-MEM supplemented with 10% FBS, 1% antibiotics-antimycotic solution) and incubated for 24 hr. After plating for 1 day, 10 μM cytosine-β-D-arabinoside (Ara-C) was added and the medium was changed every 2-3 days. The differentiated neuronal cells, P19 derived-neurons, were used after 14 days of the differentiation process (Jones-Villeneuve et al., 1982; MacPherson and McBurney, 1995).

Neuronal viability assay

The assay was carried out on P19-derived neurons cultured in a 96-well plate. The assay was performed in triplicate. After 14 days of differentiation process, P19SM was removed and DMSO solutions of MLT diluted with P19SM in the presence of 10 μM Ara-C were
added to give final concentrations at 1, 10, 100, 1,000, and 10,000 nM. DMSO was added to the cultures at 0.5% as solvent control. P19SM with 10 μM Ara-C was used as the control which representing 100% cell viability. P19-derived neurons were incubated for 18 hr at 37°C. Then, 150 μL of the medium was removed and 50 μL of XTT solution (1 mg/mL XTT in α-MEM and 25 μM phenazine methosulfate) was added. After incubation at 37°C for 4 hr, 100 μL of PBS was added. The OD value was determined on a microplate reader at 450 nm. The data were expressed as the mean ± SD. (Tadtong et al., 2013).

Neuroprotective assay
The serum deprivation method was modified from Iacovitti et al. (1997) and López-Maderuelo et al. (2001) methods. The assay was carried out on P19-derived neurons cultured in a 96-well plate and performed in triplicate. Following this, P19SM was removed and the DMSO solution of MLT or acetyl-MLT diluted with P19SM and 10 μM Ara-C and α-MEM supplemented with 10 μM Ara-C, and 1% antibiotics-antimycotic solution without FBS were added to give a final concentration of test compounds that enhanced survival of cultured neurons more than the control. Cell cultures were incubated in medium without FBS to generate oxidative stress condition in 5% CO₂ humidified atmosphere at 37°C for 18 hr. DMSO was added to the cultures at 0.5% as control solvent. P19SM with 10 μM Ara-C was used as the control which representing 100% cell viability. Cell viability was measured by the XTT reduction method. The data were expressed as the mean ± SD.

Neuritogenicity assay
As described by Tadtong et al. (2013), the assay was carried out with P19-derived neurons cultured in a 6-well plate. Geldanamycin at concentration 1 nM was used as positive control. The neuron morphology was observed under a phase-contrast microscope. The appearance of P19-derived neurons were compared with the control and measured for the length and number of neurites. Thirty neurons from the assay were measured for average length and number of neurites. The data were expressed as the mean ± SD.

Table 1  Neuroprotective ability of MLT and AcO-MLT

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Cell Viability (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>Control without FBS</td>
<td>15.7 ± 5.2**</td>
</tr>
<tr>
<td>MLT</td>
<td>138.7 ± 23.7**</td>
</tr>
<tr>
<td>MLT without FBS</td>
<td>98.0 ± 44.3</td>
</tr>
<tr>
<td>AcO-MLT</td>
<td>129.3 ± 16.6**</td>
</tr>
<tr>
<td>AcO-MLT without FBS</td>
<td>74.3 ± 7.4**</td>
</tr>
</tbody>
</table>

* p < 0.05 compared with control group without FBS
** p < 0.05 compared with control group

Statistical analysis
Average viability of the neurons was statistical analyzed by Student’s t-test to compare the statistical significance between either the control or oxidative stress conditions and experimental groups. Average length and branching number of the neurites were statistical analyzed by Student’s t-test to compare the statistical significance between either the control or positive control and experimental groups. Differences were considered significant only when the p-value was less than 0.05.

Results
Neuronal viability
The neurotoxicity test of MLT to P19-derived cultures by XTT reduction method exhibited that IC50 of MLT was more than 10 μM. At the concentration of 1 nM, the lowest concentration can promote viability of the neuron which further used for neuroprotective and neuritogenic tests.

Neuroprotective activity
The neuroprotective ability of MLT and AcO-MLT were evaluated under serum deprivation model. Interestingly, both of MLT and its derivative significantly protected neuron cells from toxicity of reactive oxygen species occurring from serum deprivation induced oxidative stress. From XTT reduction assay showed cell viability treated with MLT and AcO-MLT was 98.0% ± 44.3 and 74.3% ± 7.37, respectively, whereas, the oxidative stress control group exhibited the viability as 15.7% ± 5.2 (Table 1 and Figure 2).
Neuritogenic activity

The phase-contrast micrographs illustrated that both of MLT and AcO-MLT promoted the neurite outgrowth as shown in Figure 3. MLT and AcO-MLT significantly increased the number of neurite branching compared with the control ($p < 0.05$). However, both of MLT and its derivative could not increase the length of neurite.

Figure 2 The viability of P19-derived neurons in oxidative stress condition treated MLT and AcO-MLT.

Conclusion

The neuronal viability test by XTT reduction assay indicated that IC$_{50}$ of MLT was more than 10 μM. At 1 nM, the lowest concentration, MLT also promoted cell growth. In following studies, the concentration at 1 nM was applied.

In oxidative stress conditions due to serum deprivation, MLT and AcO-MLT at 1 nM significantly protected neuronal cell death compared with the cell treated in serum deprivation condition (control without FBS). For neuritogenic ability, MLT and AcO-MLT significantly enhanced the neuron to form more number of the neurites than the control. Although MLT showed ability to increase the length of neurites but statistical significance was not found. In conclusion, MLT and AcO-MLT could promote the neurite outgrowth but could not increase the neurite length.

The result from this preliminary study indicated that MLT and its N-acetylated derivative may have potential to further study and develop as a drug used to treat or prevent neurological diseases.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Average length (μm ± SD)</th>
<th>Average number of neurites ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>88.4 ± 43.5</td>
<td>2.0 ± 1.1</td>
</tr>
<tr>
<td>MLT</td>
<td>109.6 ± 61.0</td>
<td>3.4 ± 1.4*</td>
</tr>
<tr>
<td>AcO-MLT</td>
<td>87.0 ± 37.8</td>
<td>4.0 ± 1.7*</td>
</tr>
</tbody>
</table>

* $p < 0.05$ compared with control

Table 3 Neuritogenicity of MLT and AcO-MLT

Figure 3 Neuritogenicity of MLT and AcO-MLT on P19-derived neurons culture. (a) Control, (b) 1 nM MLT, and (c) 1 nM AcO-MLT (Bar = 10 μm, at 100x)
Acknowledgements

Financial supports for this work were provided by the Melatonin Research Group, Khon Kaen University and the National Research Council of Thailand (fiscal year 2011 and 2012).

References


Iacovitti L, Stull ND, Johnston K. Melatonin rescue dopamine neurons from cell death in tissue culture models of oxidative stress, Brain Research 1997; 768: 317-326.


