ฤทธิ์ปกป้องเซลล์ประสาทและเพิ่มแขนงประสาทของเมลาโทนินและอนุพันธ์

ปัญญดา ปัญญาทิพย์[1,2], เพลินทิพย์ ภูทองกิ่ง[2,3*], สริน ทัดทอง[4]

บทคัดย่อ

ฤทธิ์ปกป้องเซลล์ประสาทและเพิ่มแขนงประสาทของเมลาโทนินและอนุพันธ์

ปัญญดา ปัญญาทิพย์[1,2] เพลินทิพย์ ภูทองกิ่ง[2,3*],สริน ทัดทอง[4]

ว. เภสัชศาสตร์อีสาน, มีนาคม 2558; 11(ฉบับพิเศษ) : 14-19

บทนำ: เมลาโทนินเป็นฮอร์โมนจากต่อมไพเนียลในสมอง จากรายงานการศึกษาต่างๆ พบว่าเมลาโทนินมีฤทธิ์ทางเภสัชวิทยามากมาย เช่น ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านมะเร็ง และ ฤทธิ์กระตุ้นระบบภูมิคุ้มกัน เป็นต้น นอกจากนี้ยังมีการรายงานการศึกษาฤทธิ์ปกป้องเซลล์ประสาทของเมลาโทนินในเซลล์เพาะเลี้ยงชนิดต่างๆ อีกด้วย แต่เนื่องจากข้อจำกัดทางเภสัช-จลนศาสตร์ของเมลาโทนิน เช่น การดูดซึมเข้าสู่ร่างกายได้น้อย และ การถูกทำลายในร่างกายเกิดขึ้นอย่างรวดเร็ว จึงทำให้ต้องมีการพัฒนาอนุพันธ์ของเมลาโทนิน เพื่อให้ได้อนุพันธ์ที่มีคุณสมบัติทางเภสัชจลนศาสตร์และฤทธิ์ทางเภสัชวิทยาที่ดีขึ้นกว่าสารต้นแบบคือ เมลาโทนิน **วิธีการดำเนินการวิจัย:** อนุพันธ์ อะซิติลเมลาโทนิน ได้มาจากการทำปฏิกิริยาระหว่างเมลาโทนินและ กรดอะซิติก โดยใช้ 4,4-dimethyl aminopyridine (DMAP) เป็นตัวเร่งปฏิกิริยา หลังจากปฏิกิริยาเกิดขึ้นอย่างสมบูรณ์แล้ว จะทำการหยุดปฏิกิริยาด้วยวิธี liquid-liquid extraction จากนั้นแยกสารให้บริสุทธิ์ด้วยเทคนิคทางโครมาโทกราฟี จากนั้นนำสารที่ได้มาทดสอบฤทธิ์ในการปกป้องเซลล์ประสาทและฤทธิ์ในการเพิ่มแขนงประสาทในเซลล์ประสาท P19 ตรวจวัดอัตราการมีชีวิตรอดของเซลล์ด้วยวิธี XTT reduction assay **ผลการศึกษาวิจัย:** ในการทดสอบฤทธิ์ปกป้องเซลล์ประสาทจากสภาวะเครียดออกซิเดชัน ในเซลล์ประสาท P19 ที่เลี้ยงในอาหารเลี้ยงเชื้อที่ปราศจากซีรัม พบว่าทั้งเมลาโทนิน และ อนุพันธ์ AcO-MLT เมื่อเทียบกับกลุ่มที่เพาะเลี้ยงในสภาวะเครียดออกซิเดชัน สามารถลดอัตราการตายของเซลล์ประสาทได้ โดยเซลล์ประสาทมีอัตราการรอดชีวิตเท่ากับ 98.0% ± 44.3, 74.3% ± 7.4 และ 15.7% ± 5.2 ตามลำดับ และสำหรับฤทธิ์เพิ่มแขนงประสาท พบว่าเมลาโทนิน และ อนุพันธ์ สามารถเพิ่มจำนวนแขนงประสาทได้มากกว่ากลุ่มควบคุม (3.4 ± 1.4, 4.0 ± 1.7, และ 2.0 ± 1.1 ตามลำดับ) **สรุปผลการวิจัย:** จากการทดลองพบว่าเมลาโทนินและอนุพันธ์อะซิติล สามารถปกป้องเซลล์ประสาท P19 และยังสามารถกระตุ้นให้เซลล์ประสาทมีการเพิ่มจำนวนของแขนงประสาทได้จริง จึงสามารถสรุปในเบื้องต้นว่าเมลาโทนินและอนุพันธ์อะซิติลเมลาโทนิน มีศักยภาพที่จะสามารถนำไปพัฒนาเพื่อใช้เป็นยาสำหรับปกป้องเซลล์ประสาทต่อไป

คำสำคัญ: เมลาโทนิน, อนุพันธ์เมลาโทนิน, ฤทธิ์ปกป้องเซลล์ประสาท, ฤทธิ์เพิ่มแขนงประสาท

Abstract

Neuroprotective and Neuritogenic Activities of Melatonin and *N*-acetyl Substituent Derivative

Panyada Panyatip[1,2], Ploenthip Puthongking[2,3*], Sarin Tadtong[4]

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

[1] Graduate School, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand
[2] Melatonin Research Group, Khon Kaen University, Khon Kaen, 40002, Thailand
[3] Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand
[4] Faculty of Pharmacy, Srinakharinwirot University, Nakhon-nayok, 26120, Thailand
*** Corresponding author:** Faculty of Pharmaceutical Sciences, Khon Kaen University, KhonKaen, Thailand 42000 Tel/Fax 043-203149
e-mail: pploenthip@kku.ac.th

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.

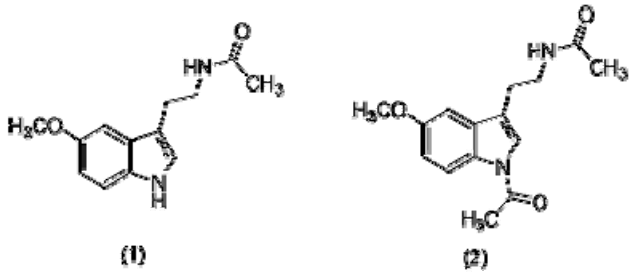

Figure 1 Structure of (1) MLT and (2) AcO-MLT

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 F_{254} plates (Merck, Darmstadt, Germany). Solvents (commercial grade) including hexane, dichloromethane, methanol, and ethyl acetate were distilled prior to use. Nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) were measured in $CDCl_3$ 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% CO_2 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 $CaCl_2$, 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 Na_2SO_4. 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 ^{1}H-NMR and ^{13}C-NMR methods.

^{1}H-NMR (400 MHz, $CDCl_3$): 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)

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.44, 168.08, 156.55, 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 (2×10^6 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 7×10^4 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-1-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 at1, 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% CO_2 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.

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 IC_{50} 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).

Table 1 Neuroprotective ability of MLT and AcO-MLT

Compounds	Cell Viability (% ± SD)
Control	100.0 ± 0.0
Control without FBS	15.7 ± 5.2**
MLT	138.7 ± 23.7**
MLT without FBS	98.0 ± 44.3*
AcO-MLT	129.3 ± 16.6**
AcO-MLT without FBS	74.3 ± 7.4*,**

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

** $p < 0.05$ compared with control group

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.

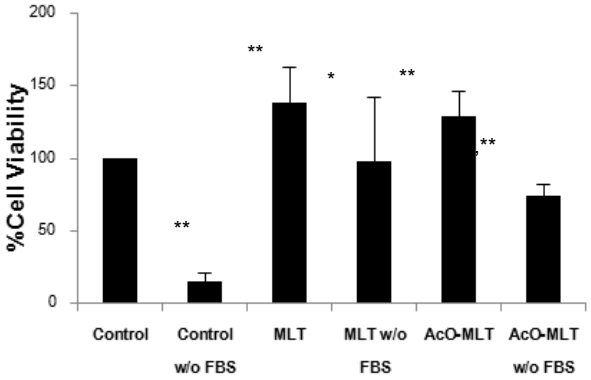

Figure 2 The viability of P19-derived neurons in oxidative stress condition treated MLT and AcO-MLT.

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 3 Neuritogenicity of MLT and AcO-MLT

Compounds	Average length (μm ± SD)	Average number of neurites ± SD
Control	88.4 ± 43.5	2.0 ± 1.1
MLT	109.6 ± 61.0	3.4 ± 1.4*
AcO-MLT	87.0 ± 37.8	4.0 ± 1.7*

* p < 0.05 compared with control

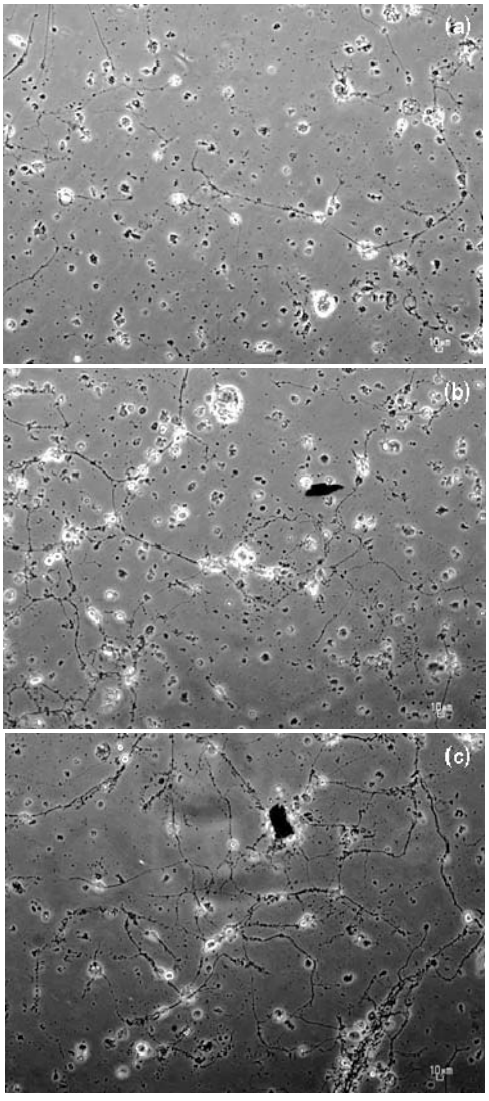

Figure 3 Neuritogenicity of MLT and AcO-MLT on P19-derived neurons culture. (a) Control, (b) 1 nM MLT, and (c) 1 nM AcO-MLT (Bar = 10 μm, at 100x)

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

Arendt J. Melatonin, circadian rhythms, and sleep. *N Engl J Med* 2000; 343(15): 1114–1116.

Beal MF. Mitochondria, free radicals, and neurodegeneration. *Curr Opin Neurobiol* 1996; 6(5): 661–666.

Beal MF, Lang AE, Ludolph AC. *Neurodegenerative diseases: neurobiology, pathogenesis and therapeutics*. Cambridge: Cambridge University Press; 2005.

Cardinali DP, Pévet P. Basic aspects of melatonin action. *Sleep Med Rev* 1998; 2(3): 175–190.

Checkoway H, Lundin JI, Kelada SN. Neurodegenerative diseases, *IARC Sci Publ* 2011; (163): 407–419.

Chung SY, Han SH. Melatonin attenuates kainic acid-induced hippocampal neurodegeneration and oxidative stress through microglial inhibition, *J Pineal Res* 2003; 34(2): 95-102.

Cos S, Blask DE. Effects of the pineal hormone melatonin on the anchorage-independent growth of human breast cancer cells (MCF-7) in a clonogenic culture system. *Cancer Lett* 199; 50(2): 115–119.

Di WL, Kadva A, Johnston A, Silman R. Variable bioavailability of oral melatonin, *N Engl J Med* 1997; 336: 1028-1029.

Epstein FH, Brzezinski A. Melatonin in humans. *N Engl J Med* 1997; 336(3): 186–195.

Gallego D, Rojas M, Orozco C. Chapter 7: Free Radicals, Neuronal Death and Neuroprotection. In: Chang RCC*, editor. Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring*. Shanghai: InTech; 2011. 165–198.

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

Jones-Villeneuve EM, McBurney MW, Rogers KA, Kalnins VI. Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells, *J Cell Biol* 1982; 94(2): 253–262.

Lerner AB, Case JD, Takahashi Y, Lee TH, Mori W. Isolation of melatonin, the pineal gland factor that lightens melanocytes. *J Am Chem Soc* 1958; 80(10): 2587.

López-Maderuelo MD, Fernández-Renart M, Moratilla C, Renart J. Opposite effects of the Hsp90 inhibitor geldanamycin: induction of apoptosis in PC12, and differentiation in N2A cells. *FEBS Letters* 2001; 490: 23-27.

MacPherson PA, McBurney MW. P19 Embryonal carcinoma cells: a source of cultured neurons amenable to genetic manipulation. *Methods* 1995; 7(3): 238–252.

Parameyong A, Charngkaew K, Govitrapong P, Chetsawang B. Melatonin attenuates methamphetamine-induced disturbances in mitochondrial dynamics and degeneration in neuroblastoma SH-SY5Y cells. *J Pineal Res* 2013; 55(3): 313-323.

Reiter RJ. Functional pleiotropy of the neurohormone melatonin: antioxidant protection and neuroendocrine regulation. *Front Neuroendocrinol* 1995; 16(4): 383–415.

Srinivasan V, Maestroni GJM, Cardinali DP, Esquifino AI, Perumal SP, Miller SC. Melatonin, immune function and aging. *Immun Ageing* 2005; 2(1): 17.

Stull ND, Polan DP, Iacovitti L. Antioxidant compounds protect dopamine neurons from death due to oxidative stress in vitro, Brain Research 2002; 931(2): 181-185.

Tadtong S, Kanlayavattanakul M, Lourith N. Neuritogenic and neuroprotective activities of fruit residues. *Nat Prod Commun* 2013; 8(11): 1583-1586.