# CHAPTER I

# INTRODUCTION

## 1. Rationale and Background

Inflammation is a complex phenomenon involving numerous mediators caused by tissue injuries or cell death eliminations (Balkwill, Coussens, 2004), It is an essential process to restore homeostasis (Radogna et al., 2010) but the inflammatory process causes many cellular responses such as vasodilation, increased vaso-permeability, blood flow and increased leukocyte extravasation and other homeostasis changes to protect tissue and eliminate any pathogens. Sign from inflammation response are pain (dolor), heat (calor), redness (rubor), swelling (tumor), and loss of function (functio laesa) caused from inflammatory mediators or pro-inflammatory cytokines such as prostaglandins (PGs), prostacyclin (PGI), nitric oxide (NO) and tumor necrosis factor (TNF) (Kiefer, Dannhardt, 2002).

Eicosanoids (PGI, PGs, tromboxane A2 ($TXA_2$) and leukotriene) are mostly derived from the arachidonic acid by the action of PG synthase in a two-step conversion via cyclooxygenase enzyme (COX) and specific synthase enzyme, respectively. PGs and PGI have important roles for inducing of the process of inflammation, such as enhanced vasodilation and increased vaso-permeability (Mollace et al., 2005).

The cyclooxygenase (COX) enzyme have two isoforms, COX-1, the constitutive isoform, and COX-2 the inducible isoform. Both enzymes have a molecular weight of 70 to 71 kDa, respectively. COX-1 is ubiquitous and has clear physiological functions. Its activation leads, for instance, to the production of prostacyclin $I_2$ ($PGI_2$) which, when released into blood vessels, produces vasodilation and antithrombogenic activity and is cytoprotective. COX-2 has low levels of mRNA but up-regulation in inflammatory process. COX-2 is expressed in many cells (e.g., macrophages, monocytes, fibroblasts, synoviocytes, etc.) and accounts for the synthesis of prostanoids involved in pathological processes (Vane et al., 1998).

NO is a free radical molecule, produced from activated macrophages and endothelial cells which was discovered to be a potent vasodilator. High amounts of

NO can react with concomitantly produced superoxide anions, which thereby generate highly toxic compounds, such as peroxynitrite and hydroxyl radicals. Nitric oxide synthases (NOSs) are important enzymes for generating NO. There are three isoforms of nitric oxide synthase enzymes: neuronal nitric oxide synthases (nNOS), endothelial nitric oxide synthases (eNOS) that are constitutive enzymes, and inducible nitric oxide synthases (iNOS) that are inducible enzymes that are induced from inflammation or viral or bacterial endotoxins (Mollace et al., 2005).

Inflammation is a complex process involved immune system that can up-regulate of other proinflammatory cytokines and chemokines, immunoglobulins, as well as increase the expression of many cellular adhesion molecules (CAMs). The phagocytosis of bacteria or foreign particles is associated with an increase in oxygen uptake by neutrophils, during which large amounts of reactive oxygen species (ROS) are generated, ROS can damage or cause complete degradation of essential complex molecules in the cells, including lipid molecules, proteins, and DNA, leading to many diseases including cancer. Thus if we can inhibit the inflammatory process or inflammatory cytokines, signs and symptoms of inflammation will be decreased, and inhibition of inflammatory processes will also decrease free radical generation (Wu, Cederbaum, 2003).

Melatonin (*N*-acetyl-5-methoxytryptamine) is a natural hormone mainly secreted by the pineal gland in mammals during the dark phase (Mayo et al., 2005). The important role of melatonin is circadian and circannual rhythm regulation for physiologic, adjustments of animals to seasonal environmental changes (Reiter, 1991). In addition to the pineal gland, melatonin is also produced by the other organ such as Harderian glands, gut, ovary, testes, bone marrow, retina and lens (Menendez-Pelaez et al., 1987). Melatonin has been reported for its potent antioxidant activity on survival radical species in both *in vivo* and *in vitro* model (Allegra et al., 2003; Bonnefont-Rousselot, Collin, 2010; Eskiocak et al., 2007; Fagali, Catalá, 2007), and plays a significant role in modulating of the immunological system (Esquifino et al., 2004; Radogna et al. 2010). Moreover, melatonin has also been shown to have anti-inflammatory (Radogna et al., 2010, Crespo et al., 1999) and anti-nociceptive activities in animal models (Perissin et al., 2004; Wilhelmsen et al., 2011). It has been demonstrated that melatonin decreases the level of NO and PGs and regulates

expression of inducible iNOS and COX-2 genes. However, melatonin has short half-life and poor oral bioavailability (Marot et al., 1998). Therefore, many efforts have been reported in the past decades to modify its lead structure for more favorable pharmacokinetics properties and also therapeutic effects (Ahmed et al., 2010; Fee et al., 2010; Lira-Rocha et al., 2002). The melatonin structure, indoleamine, functions as an electron donor for antioxidant properties. The *o*-methyl and *N*-acetyl residues of melatonin make melatonin an amphiphilic compound (both hydrophilic and lipophilic) allowing it to permeate into all subcellular compartments.

The indole ring is the core structure which is required for anti-inflammatory effect of Non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin (Olgen et al., 2001). Therefore, in this study, we performed structural modification of a core structure at indole rings of melatonin (Figure 1.1) and determined the antinociceptive effects of melatonin derivatives via tail flick and writhing test, and also investigated the anti-inflammatory effects by using croton oil induced ear edema test and cotton pellet induced granuloma method.

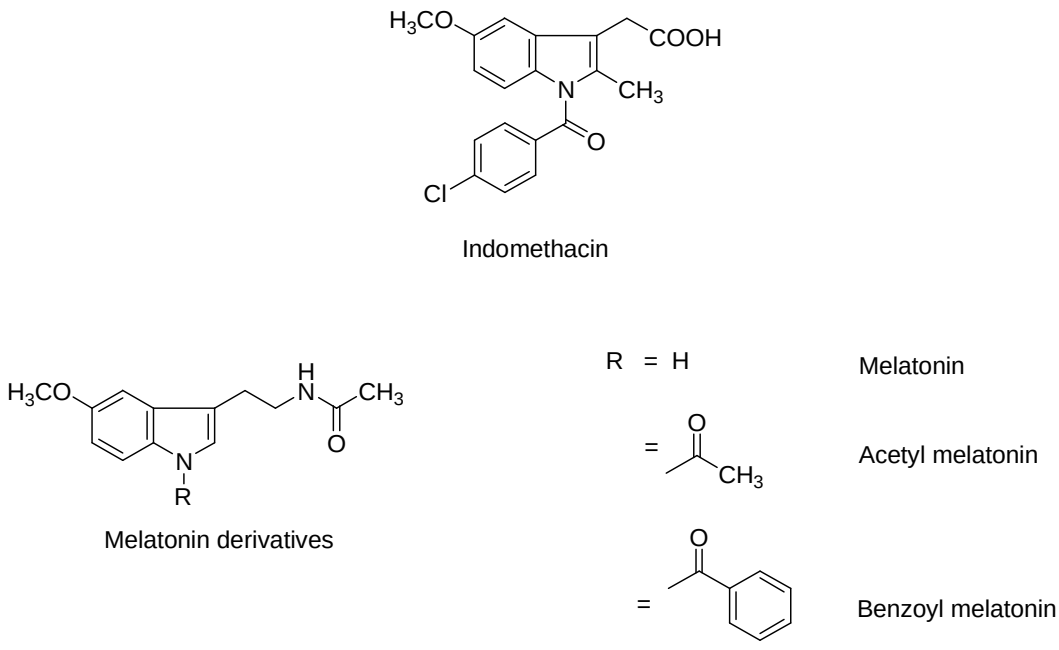



**Figure 1.1** Structure of indomethacin and melatonin derivatives

## 2. Objectives of the Study

2.1 To synthesize acetyl melatonin and benzoyl melatonin derivatives

2.2 To study the analgesic activities of melatonin and its derivatives in ICR mice with tail flick test and acetic acid induced writhing tests

2.3 To study the anti-inflammatory activities of melatonin and its derivatives in ICR mice with croton oil induced ear edema and cotton pellet induced granuloma methods

## 3. Scope and Limitation of the Study

The experiments can be divided into three parts which are:

3.1 Synthesis of melatonin derivatives; acetyl melatonin and benzoyl melatonin

3.2 Evaluation of the anti-inflammatory effects in animal models; acetic acid induced writhing test, tail flick test, croton oil induced ear edema and cotton pellet induced granuloma method

## 4. Anticipated Outcomes

4.1 Obtain melatonin derivatives that have higher anti-inflammatory effect than the parent compound.

4.2 Obtain information of the anti-inflammatory properties of melatonin and its derivatives.

4.3 Publish the results in a national or international journal.

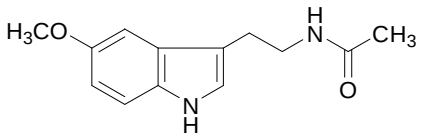
# CHAPTER II

# LITERATURE REVIEWS

## 1. Melatonin

Melatonin (*N*-acetyl-5-methoxytryptamine) is a natural hormone mainly secreted by pineal gland during dark phase in mammals (Reiter, 1991a). The structure of melatonin is shown in Figure 2.1. In general, the blood concentration of melatonin in mammals varies with age and time of day. The amount of melatonin circulating in the blood has been shown to rise and fall during a 24 hour photoperiod. Melatonin begins to be produced since 21.00-22.00 PM and increases until the highest peak of melatonin occurs during 02.00-04.00 AM (60-70 pg/ml), after that it declines to a lowest level of around 10 pg/ml (Figure 2.2). New borns produce minimal melatonin but the level increases until 5-6 years old when it will show the highest peak level (Piyarat, 2002). After that the melatonin level will decline until it is unable to measured in some older people. The amount of melatonin sharply decreases at teenager period, and may be related to the onset of puberty as shown in Figure 2.3.

Many biological activities of melatonin have been reported (Russel, 2003), the most important role being circadian and circannual rhythm regulation for physiologic, adjustments of animals to seasonal environmental changes and other numerous physiological processes such as blood pressure regulation, oncogenesis, retinal physiology, seasonal reproduction, ovarian physiology and immune function via melatonin receptors (Altun, Ugur-Altun, 2007). Also, melatonin, its metabolites, and other indole derivatives have been shown good scavengers of free radicals (Allegra et al., 2003) because of indoleamine moeity has an electron-rich aromatic ring to increase electron/proton donor properties. Moreover, because of its O-methyl and N-acetyl residues, melatonin is an amphiphilic compound. These properties have been suggested as the molecular basis for the widely documented antioxidant protection afforded by melatonin at the levels of various subcellular compartments (Reiter, 1997).




Melatonin

**Figure 2.1** The structure of melatonin

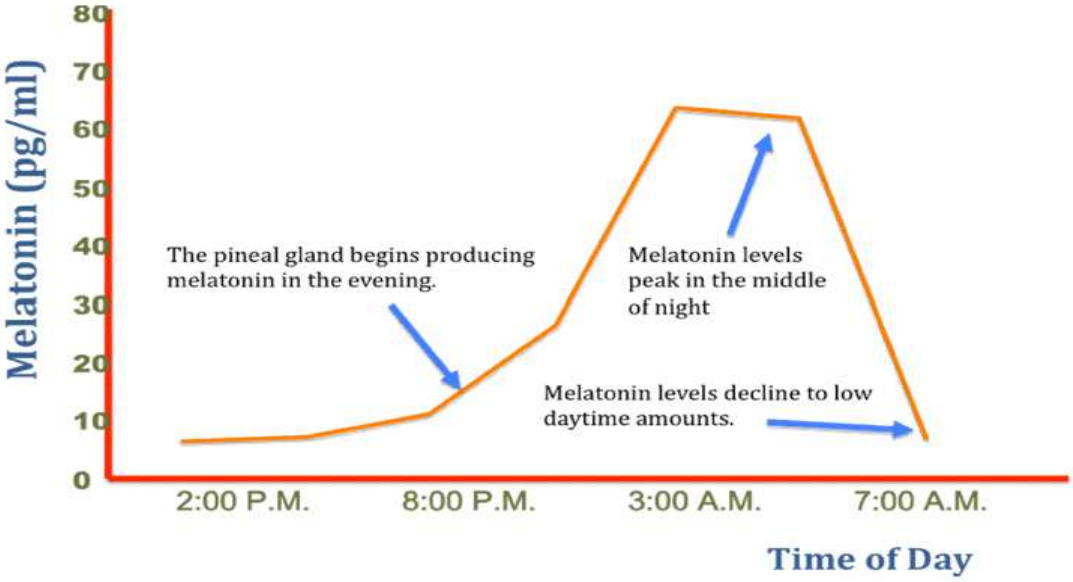



**Figure 2.2** The 24-hour cycle of melatonin production in human (Piyarat, 2002)

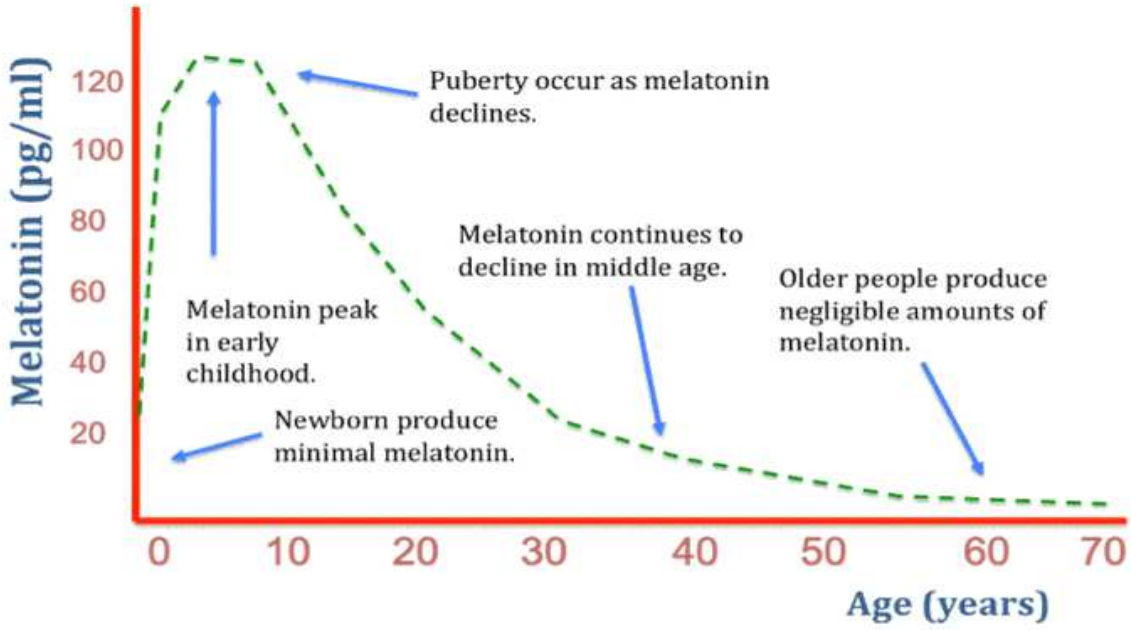



**Figure 2.3** The amount of melatonin secretion in human pineal gland in various ages (Piyarat, 2002)

### 1.1 Biosynthesis of melatonin

Melatonin is primarily synthesized and secreted by the pineal gland in the human brain. Moreover, melatonin is synthesized and secreted in a circadian manner with high levels occurring in all species at night. In mammals, the melatonin rhythm is generated by an endogenous circadian clock in the suprachiasmatic nucleus (SCN) of the hypothalamus.

The process of biosynthesis uses L-tryptophan as precursor (Reiter, 1991b), which is taken up from the cerebral vessels and turned into 5-hydroxytryptophan (5-HTP) when oxidized by tryptophan-5-hydroxylase enzyme. In the next step, 5-hydroxytryptophan is cleaved at the carboxylic group via 5-HTP-decarboxylase enzyme and converted to serotonin (5-hydroxytryptamine, 5-HT). Serotonin is subsequently metabolized by the rate limiting step by serotonin *N*-acetyltransferase (NAT) to *N*-acetylserotonin. The final step of the synthesis pathway is the conversion of *N*-acetylserotonin to melatonin by hydroxyindole-o-methyltransferase (HIOMT) as shown in Figure 2.4

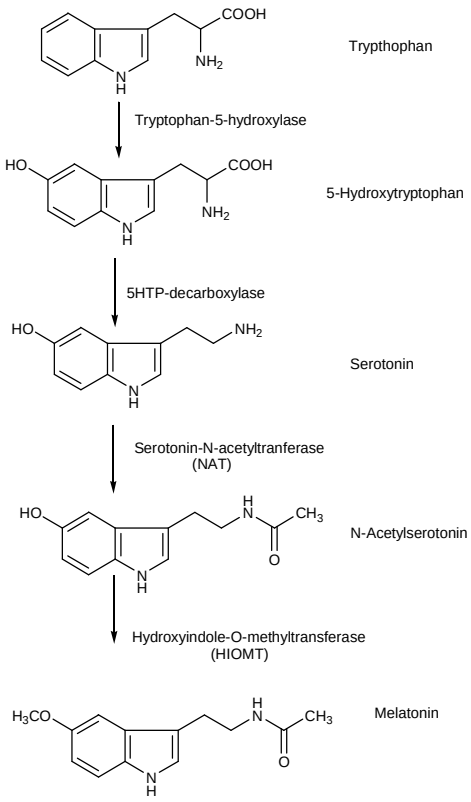



**Figure 2.4** Biosynthesis pathway of melatonin

## 1.2 Melatonin receptor

There are three types of melatonin receptor, namely melatonin receptor type 1 ($MT_1$), type 2 ($MT_2$) and melatonin receptor type 3 ($MT_3$), but only melatonin receptors type 1 ($MT_1$) and type 2 ($MT_2$) can be found in humans, while $MT_3$ is found only in some animals. Two classes of human melatonin receptors, $MT_1$ and $MT_2$, have been identified in a wide variety of tissues with different expression profiles as summary in Table 2.1 (Ekmekcioglu, 2006).

### 1.2.1 Melatonin receptor type 1

The human $MT_1$ receptor contains 350 amino acid proteins presenting a sequence homology of 60% with the 362 amino acid $MT_2$ (Farce et al., 1998). It is a protein that immerses in the seven transmembrane and binding with G-protein (Gi) to inhibit adenylatecyclase enzyme which will change ATP to be camp. The $MT_1$ receptor has been shown to couple Gi and although it has a high binding affinity for melatonin, the $MT_1$ receptor maintains some constitutive activity (Ram et al., 2002).

$MT_1$ can be tested by radioligand binding by using 2-[$^{125}$I]-iodomelatonin with a competitive $MT_1$ substance. Results have shown that 2-iodomelatonin has a higher affinity to bind at $MT_1$ than melatonin, 6-chloromelatonin, N-acetylserotonin and prazosin respectively. The $IC_{50}$ equilibrium binding concentrations are shown in units of picomolar.

### 1.2.2 Melatonin receptor type 2

The human $MT_2$ receptor contains 362 amino acid. $MT_2$ can also be studied by radioligand binding with results for binding in nanomolar concentration region. The mechanism of action occur by binding is with Gi protein, the same as for $MT_1$

The binding site of melatonin in $MT_1$ and $MT_2$ are different. In $MT_1$, the methoxy group binds hydrogen bonding with histidine 195 and amide side chain will form hydrogen bond with serine 110 and 114. However, in $MT_2$ receptor, amide side chain form interaction with asparagine and methoxy group form hydrogen bond with histidine at 208 as shown in Figure 2.5

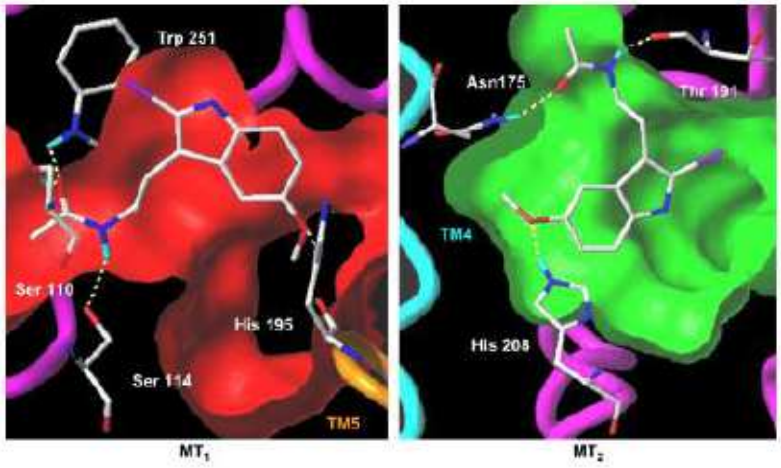



**Figure 2.5** The docking of melatonin on $MT_1$ and $MT_2$ receptor (Farce et al., 2008)

**Table 2.1** The expression of melatonin receptor and its function

| Location | $MT_1$ | $MT_2$ | Function |
|---|---|---|---|
| Adipocytes | ✓ | ✓ | Lowering of GLUT4 mRNA levels and glucose uptake |
| Aorta | ✓ | ✓ | Vasodilatation? |
| Breast cancer cell | ✓ | ✓ | Inhibition of proliferation in ERα-positive cells |
| Central dopaminergic system | ✓ | | - Modulation of dopamine synthesis and Release<br>- Activation of dopamine receptors |
| Cerebellum | ✓ | ✓ | Interactions with glutamatergic synapses? |
| Choriocarcinoma | ✓ | ✓ | Antiproliferative effects |
| Coronary arteries | ✓ | ✓ | - Vasodilatation via $MT_2$<br>- Vasoconstriction via $MT_1$ |
| Duodenal enterocyte | ✓ | ✓ | Stimulation of $HCO_3$– secretion via neural stimulation |
| Gallbladder epithelial | ✓ | ✓ | Modulation of absorptive and/or secretory processes |
| Granulosa cell | ✓ | ✓ | - Increase in LH receptor mRNA levels<br>- Decrease in GnRH-(receptor) levels |
| Hippocampus | ✓ | ✓ | - Memory, excitation and inhibition of neuronal activity<br>- Enhancement of seizure threshold |
| Immune system | ✓ | | Different immunostimulatory effects |
| Myometrium | ✓ | ✓ | Modulation of uterine contraction |
| Retina cell | ✓ | ✓ | Inhibition of stimulation evoked release of dopamine |
| Suprachiasmatic nucleus | ✓ | | Modulation (entrainment) of circadian rhythms |
| Skin | ✓ | ✓ | Antiproliferative effects on cutaneous melanoma cells and normal cell |
| Spermatozoa | ✓ | ✓ | Inhibition of sperm motility? |
| Hypothalamus, pituitary | ✓ | ✓ | Inhibition of LH-secretion and ovulation |

(Ekmekcioglu, 2006)

### 1.2.3 Structure Activity Relationship (SAR) of the melatonin receptor

According to various studies of melatonin and MT receptor binding (Mor et al., 1998; Rivara et al., 2006; Lira-Rocha et al., 2002), the SAR of melatonin derivatives for binding affinity with melatonin receptor can be described as follows (Figure 2.6).

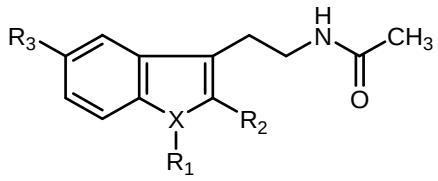



**Figure 2.6** Size of substation via melatonin receptor binding studies

**$R_1$:**

Alkylation at the nuclear nitrogen leads to a slight decrease in affinity with a small methyl group, and to a more pronounced one with bulkier groups. Which means that the affinity when $R_1$ = H > methyl > $CH_2C_6H_5$ > $C_6H_5$

**$R_2$:**

If $CH_2C_6H_5$ is at position 2, it shows lower affinity.

If $R_2$ = Br, I, Cl they show higher affinity.

If $R_2$= methyl it shows slight increase, but for a longer chain it shows decreased affinity.

**$R_3$:**

The absence of the 5-methoxy group gave a general drop in affinity, with being 1000 times less potent than melatonin.

The introduction of a halogen atom led to a loss of affinity, compared to melatonin.

A methyl group or the bulkier group caused a greater loss of affinity.

Moving the position of methoxy from 5 to another position displayed significantly decrease in affinity.

When more than one methoxy group is present, the binding of the 5-group seems prevalent, such as the 5, 7 dimethoxy derivative have a higher affinity than the 7-methoxy derivative.

**X:**

If replace N with O or S, the result showed decreasing in affinity.

**Acetylaminoethyl side chain**

If the 3-acetylaminoethyl side chain is moved from position 3 to 4, it shows lower in binding affinity.

If change 3-acetylaminoethyl into 3-acetylaminopropyl group lead to a slight increase affinity.

From this information, important parts of the structure for receptor binding of melatonin are seen to be the 5-methoyl group and the 3-acetylaminoethyl; the positions of melatonin that it best suited for modification are the *N*-atom position 1 and C-atom position 2.

## 2. Inflammation

Inflammation is a complex process initiated by several factors such as bacterial infection and chemical injury including other reasons that result in cell injury or death. The sign of inflammation is pain, redness, swelling, heat, and loss of function that results from dilation of the blood vessels leading to an increased blood supply, and from increased intercellular spaces resulting in the movement of leukocytes, protein and fluids into the site of inflammation (Iwalewa et al., 2007; Nakamura et al., 2003). Tissue injury induced by this trauma results in the release of inflammatory mediators including the cytokines and tumor necrosis factor (TNF-α), interleukin-1 (IL-1) from leukocytes, monocytes and macrophages, this result also up-regulation of other pro-inflammatory cytokines and chemokines, immunoglobulins, as well as increase the expression of many cellular adhesion molecules (CAMs). In other settings the phagocytosis of bacteria or foreign particles is associated with an increase in oxygen uptake by neutrophils, during which large amounts of reactive oxygen species (ROS) are produced (Colin, Monteil, 2003). The main inflammatory cytokine

such as nitric oxide (NO), prostaglandins (PGs), leukotrienes (LK) , tumor necrosis factor (TNF) (Esch, Stefano, 2002) and vasoactive amines (histamine and serotonin). Moreover, many diseases also related to inflammatory cytokine such as Acquired Immunodeficiency Syndrome (AIDS), asthma, cancer, congestive heart failure and neurological diseases (Iwalewa et al., 2007).

Nuclear factor-κB (NF-κB) is one of the main inducible transcription factors that regulates expression of pro-inflammatory cytokines such as interleukins (IL) and TNF. In alterations causing inflammation it results in the an activation of cells, such as macrophages, which release cytokines (e.g. TNF-α), as well as ROS. The ROS can contribute to the appearance of oxidative stress, mainly in those cases in which there is an imbalance with enzymatic (e.g. glutathione, superoxide dismutase, catalyse) and nonenzymatic (urate, vitamin etc.) antioxidant defenses. In situations that cause oxidative stress, this can be an important stimulus for the activation of NF-κB, which appears in latent form in the cytoplasm of nonstimulated cells, forming a complex with its inhibitors, the IκBs (IκBα and IκBβ). When the cell is stimulated, NF-κB factor is activated by means of the phosphorylation and degradation of the IκB proteins and migrates to the nucleus (Figure 2.7), stimulating the expression of its target genes. The degradation of IκBα results in rapid changes in the induction of NF-κB, whereas the degradation of IκBβ is associated with a prolonged activation of NF-κB. NF-κB can stimulate the expression of iNOS and COX-2, with an increase in the nitric oxide and PGs formation (González-Gallego et al., 2007).

PGs and related compounds are some of the most prevalent autacoids detected in all of tissue and body fluid except for the red blood cells. They mostly derive from the 20-carbon fatty acid that is arachidonic acid by the action of the PG synthase in a two-step conversion (Figure 2.8). First, arachidonic acid is converted into a cyclic and unsTable endoperoxide ($PGG_2$) by PG endoperoxide synthase (PGHS) or COX, followed by a peroxidase that cleaves the peroxide to yield the endoperoxide ($PGH_2$). These unsTable intermediate products of arachidonic acid metabolism are then rapidly converted to the prostanoids ($PGD_2$, $PGF_2$, $PGE_2$, thromboxane $A_2$, $PGI_2$) by specific isomerase enzymes (Flower, Vane, 1972; Needleman, Johnson, 1986). In mammals, there are 2 isoform of COX. COX-1 is ubiquitous and has clear physiological functions. Its activation leads, for instance, to the production of prostacyclin ($PGI_2$)

which, when released into blood vessels, produces vasodilation and antithrombogenic activity and is cytoprotective when released by the gastric mucosa and maintenance of kidney function (Whittle et al., 1978). COX-2 on the other hand, is undetectable in most normal tissues, but is an inducible enzyme (Harris et al., 1994), that can increase on expression becoming abundant in activated macrophages and other cells at sites of inflammation or other pathology and on release of $PGE_2$ and $PGI_2$.

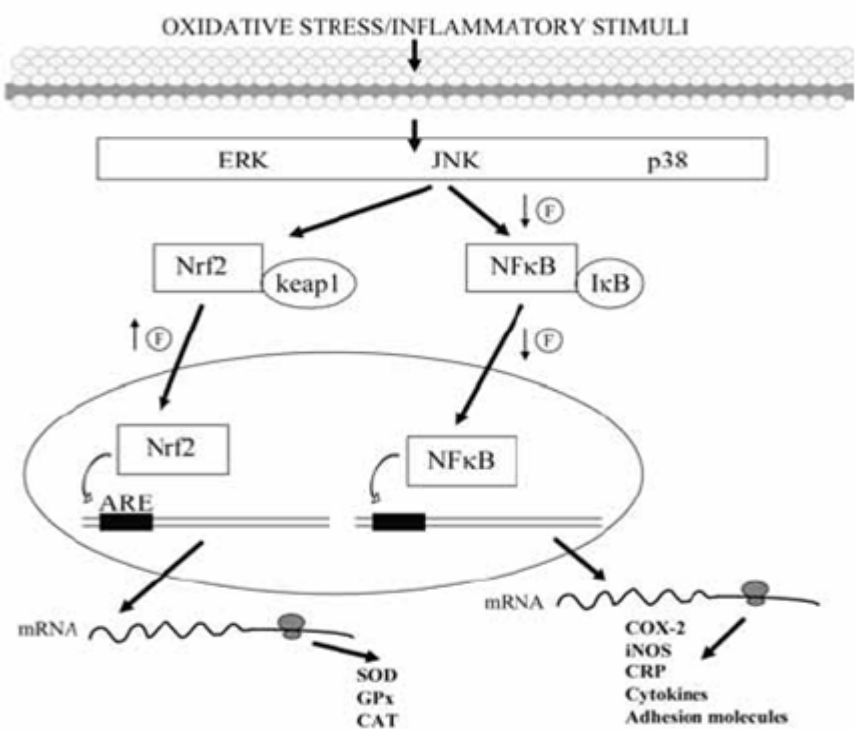



**Figure 2.7** Activation of NF-κb pathways (González-Gallego et al., 2007)

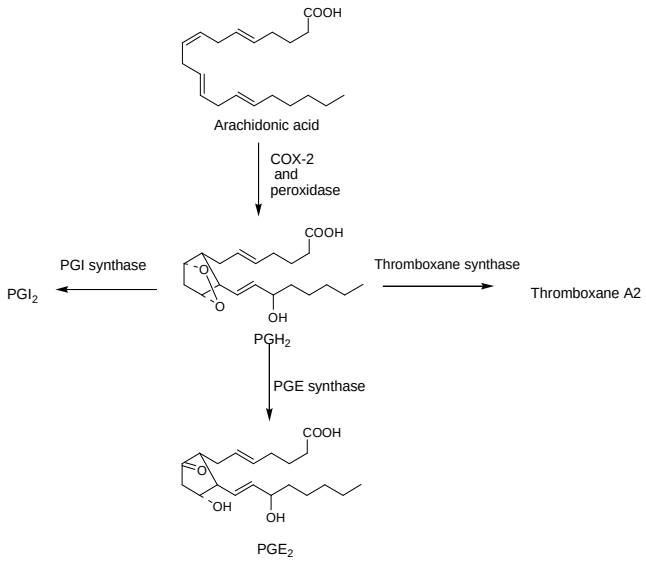



**Figure 2.8** Biosynthesis pathway of $PGE_2$ (Mollace et al., 2005)

PGs are important mediators in acute and chronic peripheral inflammation. Level of PGs are relate to expression of COX-2. When $PGE_2$ and $PGI_2$ released, activating the cAMP and protein kinase A pathway, PGs inhibit voltage-dependent potassium currents, and increase voltage-dependent calcium inflow in nociceptive afferents. These events decrease pain threshold (Vanegas, Schaible, 1991).

NO is a free radical molecule, which acting as a potent vasodilator (Vallance et al., 1989). At the physiological level, NO appears to mediate endothelium-dependent relaxation of vascular smooth muscle. NO had high level in many disease, such as inflammatory bowel disease, Crohn's disease and Alzheimer's disease (Clancy, Abramson, 1995) that have high expression of iNOS. The role of NO in normal physiologic homeostasis are regulates blood pressure and vasodilating stimulated. However, role of NO also different from each kind of NOS as follows

1. Endothelial Nitric-Oxide Synthase (eNOS). Vascular eNOS is a calcium/calmodulin-dependent enzyme with a monomeric molecular weight of 133 kDa NO derived from eNOS has numerous effects on the vessel wall, including vasodilation, inhibition of platelet aggregation, inhibition of the production of monocyte chemoattractant protein-1 and macrophage-colony stimulating factor (De Caterina et al., 1995; Nadaud et al., 2000)

2. Neuronal Nitric-Oxide Synthase (nNOS). The NOS isoform present in the central and peripheral nervous system (nNOS) is a $Ca^{2+}$ -calmodulin-dependent enzyme with a molecular weight of 166 kDa that is present in dimeric form. The important roles of this NO such as pain, sleep, feeding behavior or thermo-regulation, and regulation of microcirculation (Szabo, 1996).

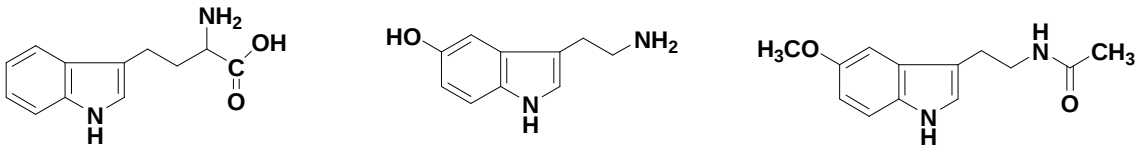
3. Inducible Nitric-Oxide Synthase (iNOS). Inducible NOS is active as a homodimer of approximately 260 kDa, and only the dimeric forms exhibit catalytic activity. The expression of iNOS is regulated at the level of transcription and at the level of iNOS mRNA stability. Compared with constitutive NOS isozymes, it is iNOS which is thought to mediate the vast majority of pathophysiological effects attributed to NO. Consequently, this isoform is believed to be of fundamental importance to the inflammatory process.

Lipophilicity is a one of important factors for anti-inflammatory drugs. It's very important for describe permeation property of compound across biological membrane. In general lipophilicity is express in $\log_{10}$ of the partition of compound in *n*-octanol and water (LogP). Singh and Roberts (1994) were study about effect of nonsteroidal anti-inflammatory drugs (NSAIDs) that is salicylic acid, diethylamine salicylate, indomethacin, naproxen, diclofenac and piroxicam in topical formulation. They found parabolic relationship between LogP and tissue concentration that optimum LogP was 3.

## 3. Melatonin and anti-inflammatory studies

Melatonin is the main product of the pineal gland with well documented antioxidant and immuno-modulatory effects. Deng et al. (2006) studies was shown melatonin (0.1 and 1 mM) can suppresses the expression of pro-inflammatory genes such as COX-2, iNOS but not for tryptophan or serotonin (1 mM) in lipopolysaccharide (LPS) or IFN-α activated RAW 264.7 cells. From this result, if we consider structure of melatonin from Figure 2.9, tryptophan and serotonin, they have different at position 3 at indole ring. 3-acetylaminoethyl moiety of melatonin is a important part of melatonin for binding MT receptor, this result can suggest that mechanism of melatonin for suppressed pro-inflammatory genes may be work though specific receptor. Moreover, Mayo et al. (2005) were test the effect of melatonin,

*N*(1)-acetyl-N(2)-formyl-5-methoxykynuramine (AFMK) and *N*1-acetyl-5-methoxykynuramine (AMK) on the activities of COX-2 and inducible nitric oxide synthase (iNOS), using lipopolysaccharide (LPS)-activated RAW 264.7 macrophages as a model. The result shown melatonin and both of its metabolite can prevent COX-2 activation without affect COX-1 protein.




Tryptophan Serotonin Melatonin

**Figure 2.9** Structure of tryptophan, serotonin and melatonin

There are various *in vitro* studies of melatonin on analgesic and anti-inflammatory activities. Yu et al. (2000) studied effect of luzindole (a MT melatonin receptor antagonist) and prazosin (a selective $MT_3$ melatonin receptor antagonist) on melatonin-induced antinociception via tail flick test on hot water 50 °C. Melatonin (30, 60, 120 mg/kg) resulted in a dose-dependent antinociception and luzindole (50, 100 mg) administered significantly the antinociceptive effect induced by i.p. melatonin (120 mg/kg), whereas prazosin (50 mg) did not. Those result suggested that melatonin-induced antinociception is mediated through the $MT_1$ or $MT_2$ melatonin receptor. For melatonin derivatives studies, Elmegeed et al. (2006) was synthesis pyrrolo[1,2-a]indole derivatives and pyrido[1,2-a]indole derivatives as shown in Figure 2.10 of melatonin for studies anti-inflammatory and anti-nociceptive activities compare to melatonin. Anti-inflammatory study via inhibited the paw edema shown Compound 2 has the strongest anti-inflammatory activity with exceeds of melatonin followed by compounds 3 and 4. Anti-nociceptive of melatonin derivatives via acetic acid-induced writhing and gastric lesions caused by indomethacin shown Compounds 2 and 4 were more potent as anti-nociceptive. Those results shown that some derivatives which substitution at position 1 and 2 of melatonin have potential to increase anti-inflammatory activity.

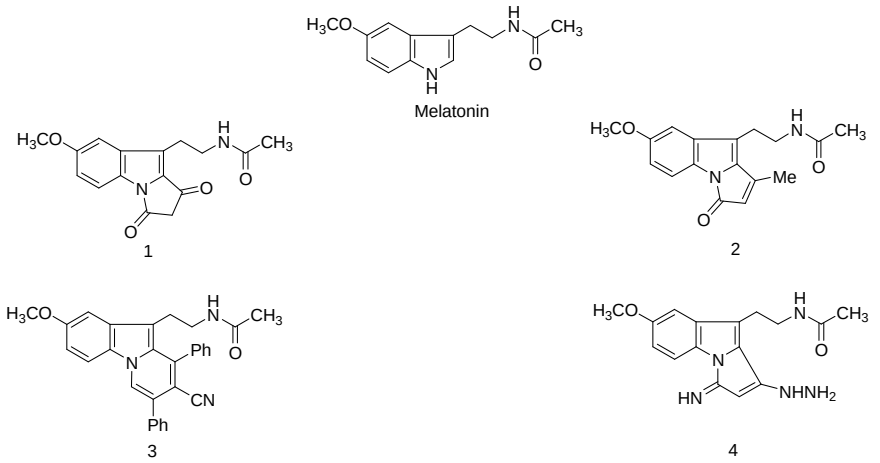



**Figure 2.10** Melatonin derivatives in Elmegeed (2006) studies

The study of melatonin related structure. Chao et al. (2005) the methyl ester, ethyl ester, and γ-lactone derivatives of indomethacin were synthesised (Figure 2.10), the indole core structure anti-inflammatory drugs. They studied the inhibition of $PGE_2$ and NO production via LPS-Stimulated RAW 264.7cells and found that the methyl ester derivative of indomethacin (M-IN) had the highest potency to inhibit $PGE_2$ and NO. Moreover, M-IN also had higher efficacy for inhibiting the expression of iNOS and COX-2 than indomethacin or the other derivatives. If we consider the structure of the indomethacin derivative, M-IN is the most similar to melatonin compared to each others. Thus it is possible to develop melatonin derivatives as anti-inflammatory agents

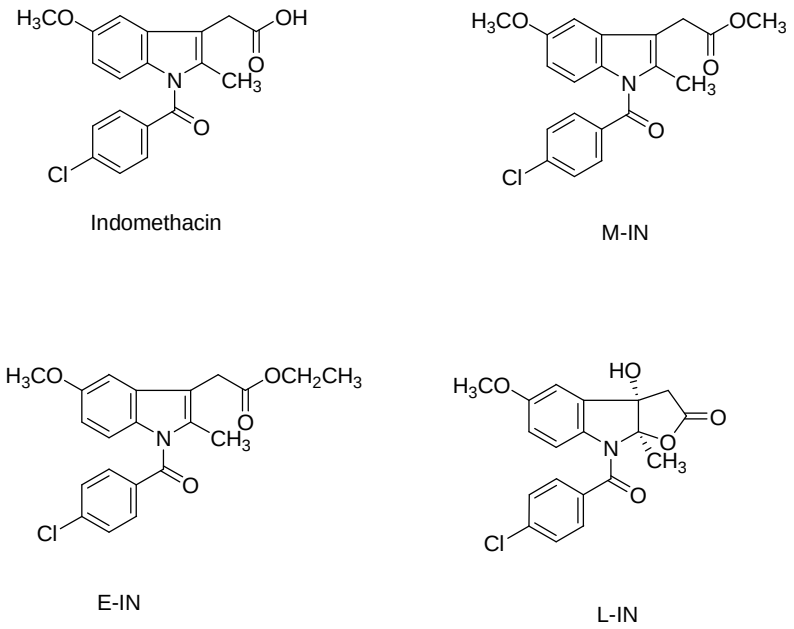



**Figure 2.11** Structure of indomethacin and derivatives (Chao et al., 2005)

## 4. Anti-inflammatory models

Inflammation was characterized two thousand years ago by Celsus using the four Latin words: rubor, calor, tumor and dolor. Inflammation has different phases: the first phase is caused by an increase of vascular permeability resulting in exudation of fluid from the blood into the interstitial space, the second one by infiltration of leukocytes from the blood into the tissues and the third one by granuloma formation. Accordingly, anti-inflammatory tests have to be divided into those measuring acute inflammation, subacute inflammation and chronic repair processes. In some cases, the screening is directed to test compounds for local application. Predominantly, however, many these studies are aimed to find new drugs against diseases such as polyarthritis and other rheumatic diseases. Since the etiology of polyarthritis is considered to be largely immunological, special tests have been developed to investigate various immunological and allergy factors.

For *in vitro* studies, an array of physiological substances or autacoids are involved in the process of inflammation and repair. These include histamine, serotonin, bradykinin, substance P, the group of eicosanoids, the platelet-activating factor (PAF) as well as cytokines and lymphokines. Their discovery makes the use of *in vitro* studies possible. The influence of non-steroidalanti-inflammatory agents on

the eicosanoid pathway has given rise to numerous studies. *In vivo* studies of the inflammatory process involves a series of events that can be elicited by numerous stimuli. The response is accompanied by the clinical signs of erythema, edema, hyperalgesia and pain. Inflammatory responses occur in three distinct phases, each apparently mediated by different mechanisms. An acute or transient phase is characterized by local vasodilatation and increased capillary permeability. A subacute phase is characterized by infiltration of leukocytes and phagocytic cells and a chronic proliferative phase, in which tissue degeneration and fibrosis occur.

Methods for testing acute and subacute inflammation are UV-erythema in guinea pigs, vascular permeability, oxazolone-induced ear edema in mice, croton-oil ear edema in rats and mice, paw edema in rats (various modifications and various irritants), pleurisy tests, granuloma pouch technique (various modificationsand various irritants). The proliferative phase is measured by methods for testing granuloma formation, such as cotton wool granuloma, glass rod granuloma, PVC sponge granuloma.

For analgesic activity, the differentiation between central and peripheral analgesic drugs is nowadays of more or less historical value. Most of the so called peripheral analgesics possess anti-inflammatory properties and in some cases also antipyretic activity besides analgesia. For many of them the mode of action has been elucidated as an inhibition of cyclooxygenase in the prostaglandin pathway. Nevertheless, new peripheral analgesics have to be tested not only for their *in vitro* activity on cyclooxygenase but also for their *in vivo* activity.

Pain is a symptom of many diseases requiring treatment with analgesics. Medicines are divided into two types of analgesic drugs: central and peripheral analgesic drugs. Several methods are available for testing central analgesic activity, such as Haffner's tail clip method in mice, tail flick or other radiant heat methods, tail immersion tests, hot plate methods in mice or rats, electrical stimulation (grid shock, stimulation of tooth pulp or tail), monkey shock titration, formalin test in rats. For *in vivo* studies of peripheral analgesic activity, the most commonly used methods are the writhing tests in mice and the Randall-Selitto test in rats. All of the limitation of anti-inflammatory and analgesic models are summarized in Table 2.2 and Table 2.3 respectively (Vogel, 2007).

**Table 2.2** Limitation of *in vivo* studies anti-inflammatory models

| Method | Critical assessment or Limitation |
|---|---|
| **Acute Inflammatory** | |
| Ultraviolet erythema in guinea pigs | The test has the advantage of simplicity but need straining of the investigators. Attempts to use reflection photometers in order to eliminate subjective scoring were unsuccessful. Corticosteroids after systemic application are rather ineffective in this test, however, can be evaluated after topical administration. The test is not particularly useful to study the duration of the anti-inflammatory effect |
| Vascular permeability | Compounds with sympathomimetic activity have an effect this test cann't be regarded as a primary screening test for anti-inflammatory products. |
| Inhibition of leukocyte adhesion to rat mesenteric venules | - |
| Oxazolone-induced ear edema | The method is suiTable for both steroidal and nonsteroidal compounds as well as for the evaluation of various topical formulations. |
| croton-oil ear edema in rats and mice | The method is useful for evaluation of anti-inflammatory topical steroids especially in the modification when thymus weight is determined simultaneously. The method also can be used for topically applied nonsteroidal antiphlogistics. |

**Table 2.2** Limitation of *in vivo* studies anti-inflammatory models (Cont.)

| Method | Critical assessment or Limitation |
|---|---|
| Paw edema | The paw edema method has been used by many investigators and has been proven to be suiTable for screening purposes as well as for more in depth evaluations. Dependent on the irritant steroidal and nonsteroidal anti-inflammatory drugs, antihistaminics and also, to a lesser degree, serotonin antagonists are active in the paw edema tests. Since so many different irritants have been used by the various investigators the results are often difficult to compare. |
| Granuloma pouch technique | The method has been very useful to estimate the potency of anti-inflammatory corticosteroids both after local and after systemic application. By injection of a depot-preparation and induction of the granuloma pouch after various time intervals up to 4 weeks the duration of action can also be determined. |
| **Chronic Inflammation** | |
| Cotton wool granuloma | The method has been useful for evaluation of steroidal and nonsteroidal anti-inflammatory drugs. For testing corticosteroids, the test can be performed in adrenalectomized rats. |
| Sponge implantation technique | The sponge implantation technique has been proven to be a versatile method which was used and modified by many investigators. |

(Vogel HG, 2007)

**Table 2.3** Limitation of *in vivo* studies analgesic models

| Method | Critical assessment or Limitation |
|---|---|
| **Central Analgesic Activity** | |
| Haffner's tail clip method | The test does not need any sophisticated equipment but a skilled, preferably "blind", observer. Peripheral analgesics of the salicylate type are not detected by this test. |
| Radiant heat method | The radiant heat test on the tail of mice is very effective to estimate the efficacy and potency of central acting analgesic drugs. Compounds like acetylsalicylic acid and phenyl-acetic acids show only slight effects. |
| Hot plate method | The hot plate test has suiTable for evaluation of centrally but not of peripherally acting analgesics. Mice as well as rats have been used. The method has the drawback that sedatives and muscle relaxants or psychotomimetics cause false positives |
| Tail immersion test | The test is useful to differentiate central opioid likeanalgesics from peripheral analgesics. |
| Electrical stimulation of the tail | The test is useful to differentiate central opioid likeanalgesics from peripheral analgesics. |

**Table 2.3** Limitation of *in vivo* studies analgesic models (Cont.)

| Method | Critical assessment or Limitation |
|---|---|
| Tail immersion test | The effect of central analgesics can be clearly demonstrated, however also the activity of peripheral analgesics given at higher doses can be detected. |
| Tooth pulp stimulation | Central analgesics, , have been found to be very active in this test. Compared with other tests for central analgesic activity, like the hot plate test in mice. In addition, non-opiate analgesics like ketamine and peripheral analgesics like pyrazolone derivatives gave a positive response. |
| Formalin test in rats | The formalin test identifies mainly centrally active drugs, whereas peripherally acting analgesics are almost ineffective. Therefore, the formalin test may allow a dissociation between inflammatory and non-inflammatory pain, a rough classification of analgesics according to their site and their mechanism of action. |
| **Peripheral Analgesic Activity** | |
| Writhing tests | In this test both central and peripheral analgesics are detected. The test recommended as a simple screening method. However, it has to be mentioned that other drugs such as clonidine and haloperidol also show a pronounced activity in this test. Because of the lack of specificity. |

**Table 2.3** Limitation of *in vivo* studies analgesic models (Cont.)

| Method | Critical assessment or Limitation |
|---|---|
| **Peripheral Analgesic Activity**<br>Pain in inflamed tissue<br>(Randall-Selitto Test) | The method originally described by Randall and Selitto has been used by many investigators and has been proven to detect central analgesics as well as peripheral analgesics. Peripherally acting analgesics such as the nonsteroidal anti-inflammatory drugs increase only the threshold of the inflamed paw, whereas opiate analgesics increase also the threshold of the intact paw. |

(Vogel HG, 2007)

# CHAPTER III
# RESEARCH METHODOLOGY

## 1 Materials and reagents

### 1.1 Materials and reagents

Chemicals used for melatonin derivatives synthesis were melatonin (Shanghai Chemical Co. Ltd., PR China), acetic anhydride, benzoyl chloride, 4-dimethylaminopyridine (DMAP) from Sigma-Aldrich (U.S.A.), pyridine, hexane, dichloromethane, methanol, ethyl acetate (Fluka, Japan.) and silica gel GE0049 (0.06 - 0.2 mm).

Chemicals for animal models were aspirin, croton oil, Tramadol and predisolone from Sigma (USA), ethyl ether from RCL Labscan (Thailand) and pentobarbital from Ceva Animal Health (Thailand)

### 1.2 Instruments

Equipments and instruments used were rotary evaporator, autopipettes (10, 100 and 1000 µl), pH meter, vortex mixer, centrifuge, small animal surgical instruments, and digital vernier caliper.

## 2. Methods

### 2.1 Synthesis of melatonin derivatives

#### 2.1.1 Acetyl melatonin

Melatonin (232 mg, 1 mmol) and DMAP (122 mg, 1 eq.) were placed in a round bottom flask, then pyridine 2 ml added and stirred on a magnetic stirrer until a clear solution appeared. Afterwards, acetic anhydride (0.5 ml) was slowly added and the mixture stirred at room temperature for 24 hours. TLC was used to check the reaction at 1, 2, 4, 6 and 24 hours to make sure the reaction was completed. Then the solution was evaporated by rotary evaporator at 45 °C to acquire a crude extract of 293 mg and this crude extract was purified by column chromatography (column diameter 2 cm, silica gel 15g) using various elutions of a

gradient of hexane-ethyl acetate 8:2 to 0:10. The eluent gave a yellowish powder product (122 mg, 44% yield).

### 2.1.2 Benzoyl melatonin

Melatonin (232 mg, 1 mmol) and DMAP (122 mg, 1 eq.) were placed in a round bottom flask, then pyridine 2 ml added and stirred on magnetic stirrer until a clear solution appeared. Afterwards benzoyl chloride (0.3 ml) was slowly added and stirred at room temperature for 24 hours. TLC was used to check the reaction at 1, 2, 4, 6 and 24 hours to make sure the reaction was completed. Then the solution was evaporated by rotary evaporator at 45 °C, to acquire a crude extract of 613 mg and this crude extract was purified by column chromatography (column diameter 2 cm, silica gel 15g) by using various elutions of a gradient of hexane-ethyl acetate 10:0 to 0:10. The eluent gave a yellowish crystalline product (75 mg, 35% yield).

## 2.2 Anti-inflammatory activities in animal model

### 2.2.1 Preparation of animal study

Antinociceptive and anti-inflammatory activity was performed on four-weeks old male ICR mice weighing 20-30g obtained from National Laboratory Animal Center, Mahidol University, Thailand. The animal were acclimatized in the laboratory in a ventilated room at the ambient temperature of 25 °C on a control light-dark cycle (12:12 hour) for at least one week prior to the experiments. For all experiments each animal was used only once. All of the experiments divided mice into 11 groups as summary in Table 3.1.

### 2.2.2 Preparation of samples

Melatonin and derivative doses of 1, 2.5 and 5 mg/ml were prepared as suspensions in 15% tween, except for croton oil induced ear edema test that used acetone as vehicle.

**Table 3.1** Summary grouping in animal models

| **Group (n = 6)** | **Compound (dose)** | | |
|---|---|---|---|
| | **Tail flick test** | **Acetic acid induced writhing test** | **Cotton oil induced ear edema** |
| 1 | 15 % Tween - Negative control | 15 % Tween - Negative control | Acetone - Negative control |
| 2 | Tramadol (50 mg/kg) - Positive control | Aspirin (300 mg/kg) - Positive control | Aspirin (600 µg/ear) - Positive control |
| 3 | Melatonin (10 mg/kg) | Melatonin (10 mg/kg) | Melatonin (10 µg/ear) |
| 4 | Melatonin (25 mg/kg) | Melatonin (25 mg/kg) | Melatonin (25 µg/ear) |
| 5 | Melatonin (50 mg/kg) | Melatonin (50 mg/kg) | Melatonin (50 µg/ear) |
| 6 | Acetyl melatonin (10 mg/kg) | Acetyl melatonin (10 mg/kg) | Acetyl melatonin (10 µg/ear) |
| 7 | Acetyl melatonin (25 mg/kg) | Acetyl melatonin (25 mg/kg) | Acetyl melatonin (25 µg/ear) |
| 8 | Acetyl melatonin (50 mg/kg) | Acetyl melatonin (50 mg/kg) | Acetyl melatonin (50 µg/ear) |
| 9 | Benzoyl melatonin (10 mg/kg) | Benzoyl melatonin (10 mg/kg) | Benzoyl melatonin (10 µg/ear) |
| 10 | Benzoyl melatonin (25 mg/kg) | Benzoyl melatonin (25 mg/kg) | Benzoyl melatonin (25 µg/ear) |
| 11 | Benzoyl melatonin (50 mg/kg) | Benzoyl melatonin (50 mg/kg) | Benzoyl melatonin (50 µg/ear) |

### 2.2.3 Tail flick test

Melatonin and derivatives dose at 50 mg/kg, vehicle (negative control) or tramadol 50 mg/kg (positive control) were given subcutaneous (s.c.). Then every 10 minutes until 1 hour, mice were placed with the distal part of the tail (5.5 cm) in a beaker, containing hot water maintained at the temperature of 50±1 °C. The time was recorded for mice to withdraw their tail from the water. If the mice

could tolerance more than 10 seconds, the mice were removed from the water immediately and 10 seconds was recorded as the result. The appropriate time for peak effectiveness of melatonin and derivatives was thus determined. The dose dependent study used melatonin and derivatives dose 10, 25, 50 mg/kg respectively and control group same as described above but the tail flick time was measured at 30 and 60 minutes after the samples were administered.

### 2.2.4 Acetic acid induced writhing test

Melatonin and derivatives at doses of 10, 25, 50 mg/kg respectively, or vehicle (negative control) or aspirin 300 mg/kg (positive control) were given s.c. before i.p. injection of 0.7% acetic acid (10 ml/kg) in mice. The interval between sample and acetic acid administration was 0.5 or 1 hour. The number of writhes (constriction of abdomen, twisting of trunk and extension of hind legs) was counted after 5 minutes after acetic acid injected, and the observation periods were 20 minutes for each sample.

### 2.2.5 Croton oil induced ear edema test

10 µl of melatonin and derivatives at concentrations of 1, 2.5 and 5 mg/ml in acetone respectively, or vehicle (negative control) or aspirin 60 mg/ml (positive control) were applied to the outer surface of the right ear of mice for 1 hour before treatment of the inner surface of the right ear with 10 µl of freshly prepared croton oil (2.5% in acetone). Measurement of the thickness of right ear after croton oil was apply for 3 hours compared to the left ear (control) was made by digital caliper vernier.

### 2.2.6 Cotton pellet induced granuloma test

Mice were anesthetized with pentobarbital sodium 30 mg/kg at route i.p., the abdomen shaved and washed with 70% alcohol. Sterile cotton pallets weighing 10±0.5mg were implanted subcutaneously in the ventral abdominal area. On the following day mice were treated with melatonin and derivatives doses 10, 25, 50 mg/kg respectively, vehicle (negative control) or prednisolone 5 mg/kg (positive control) in s.c. route administered. After 7 days of therapy, mice were sacrificed with

ether. The cotton pellets along with the surrounding inflammatory tissue were carefully dissected from the surrounding tissue. The pellets were dried overnight at 60°C before weighing.

### 2.2.7 Statistical analysis

All of experimental data were expressed as the mean±SE. Analysis of variance (ANOVA) was used to analyze group differences via software SigmaStat for Windows version 3.11. A probability (*P*) level lower than 0.05 indicated significance.

# CHAPTER IV
# RESULTS AND DISCUSSION

## 1. Chemistry

The acetyl melatonin and benzoyl melatonin were prepared by acylation reaction between melatonin with acetic anhydride or benzoyl chloride, respectively. This reaction was used DMAP as catalyst and pyridine as solvent. The reaction mixture was stirred under room temperature for 24 hours. The predicted mechanisms of reaction are shown in Figure 4.1 and Figure 4.2. After reaction completed, the crude extracts of the reaction were purified by column chromatography and the results are shown in Table 4.1

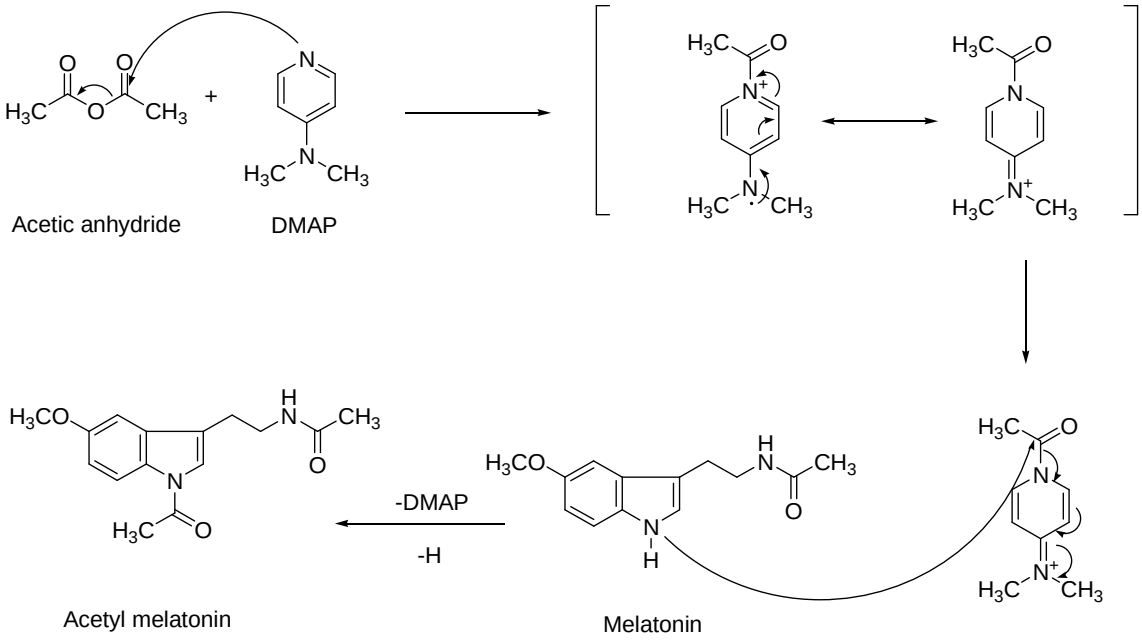



**Figure 4.1** Reaction of acetyl melatonin synthesis

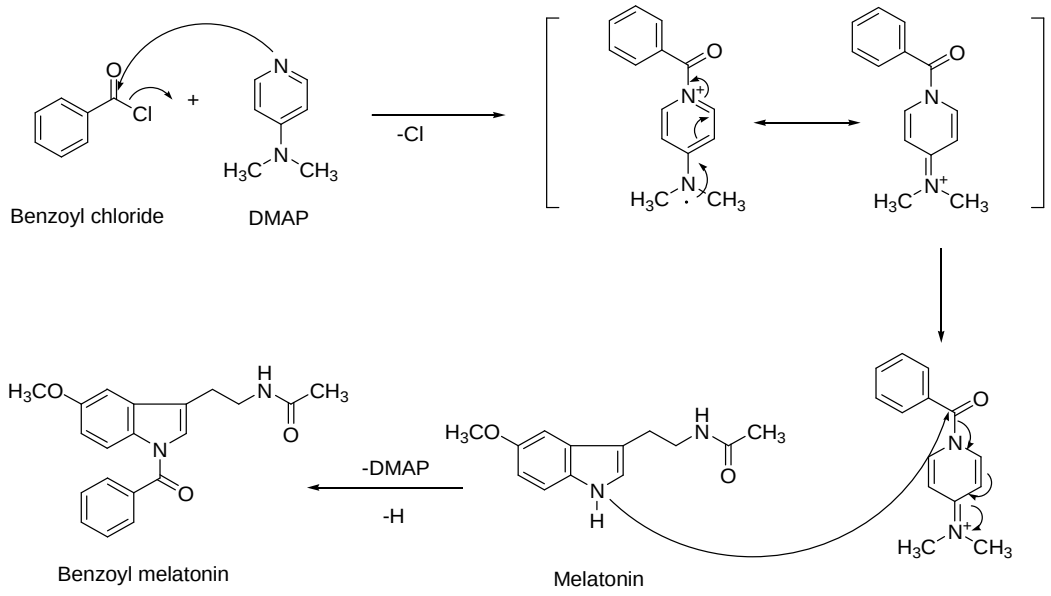



**Figure 4.2** Reaction of benzoyl melatonin synthesis

**Table 4.1** Condition for synthesis melatonin derivatives

| Condition | Compounds | |
|---|---|---|
| | **Acetyl melatonin** | **Benzoyl melatonin** |
| Melatonin (mmol) | 1.0 | 1.0 |
| Acid chloride (mmol) | 5.0 | 3.0 |
| Pyridine (ml) | 2.0 | 2.0 |
| DMAP (mmol) | 0.5 | 0.5 |
| Temperature | Room temperature | Room temperature |
| Reaction time (hour) | 24 | 24 |
| Product weight (mg) | 122 | 75 |
| Yield (%) | 44 | 35 |

## 2. Structure elucidation

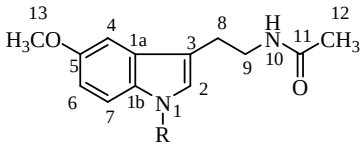
### 2.1 Acetyl melatonin structure elucidation

Acetyl melatonin was dissolved with $CDCl_3$ for structure elucidation by $^{1}$H-NMR and $^{13}$C-NMR. The $^{1}$H-NMR spectrum of the product shows an extra methyl group at chemical shift 2.52 as singlet pattern that suggests to methyl on the acetyl moiety, the chemical shifts of H-2 and H-7 have downfield shifted from 6.98 to 7.20 ppm and 7.24 to 8.26 ppm from the electron withdrawing effect of the amide functional group. Moreover, the $^{13}$C-NMR spectrum shows two extra peaks of carbon at chemical shift 23.33 and 168.04 ppm that suggests methyl and carbonyl groups respectively. In addition, the quaternary carbon that is located near N-atom position 1 also downfield shifted from 127.65 to 131.55 ppm. This result from NMR confirms the success of acetylation on melatonin, and the $\delta_H$ and $\delta_C$ of acetyl melatonin are summarized in Table 4.2 and 4.3, respectively. The IR spectrum also shows a peak of N-H stretch of amine or amide (3304 $cm^{-1}$), C=H stretch of double bond in indole ring (3071 $cm^{-1}$), C-H stretch of alkenes (2927 $cm^{-1}$) and C=O stretch of amides group (1694 $cm^{-1}$). The melting point of acetyl melatonin is 67-69 °C and close range of temperature indicates the good purity of the compound.

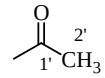
### 2.2 Benzoyl melatonin structure elucidation

The benzoyl melatonin product structure was confirmed via $^{1}$H-NMR and $^{13}$C-NMR using $CDCl_3$ as solvent. The five extra proton signals at chemical shift 7.28 to 7.45 ppm suggest to the protons on the benzoyl group. In addition, the $^{13}$C-NMR spectrum shows five extra carbon signals. The first one is a quaternary carbon at chemical shift 173.59 that is the carbonyl carbon and the other carbons on the benzene ring show at 132.01, 128.50, 128.15 and 127.74 ppm as summarized in Table 4.3. The $\delta_H$ and $\delta_C$ of acetyl melatonin are summarized in Table 4.2 and 4.3, respectively. The IR spectrum also shows peaks of N-H stretch of amine or amide (3333 $cm^{-1}$), C=H stretch of double bond in indole ring (3031 $cm^{-1}$), C-H stretch of alkenes (2927 $cm^{-1}$) and C=O stretch of amides group (1696 $cm^{-1}$). Melting point of benzoyl melatonin was 187-189 °C.

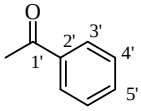
**Table 4.2** Summary $^{1}H$ –NMR of melatonin, acetyl melatonin and benzoyl melatonin in $CDCl_3$




R = H Melatonin




Acetyl melatonin




Benzoyl melatonin

| **Position** | **Melatonin reference (Pavlát et al., 1999)** | **$^{1}H$ NMR our data (ppm, pattern, coupling constant)** | | |
|---|---|---|---|---|
| | | **Melatonin** | **Acetyl melatonin** | **Benzoyl melatonin** |
| 1 | 7.89 | 8.16 s | - | - |
| 2 | 6.74 | 6.98 s | 7.2 s | 6.77-6.81 m |
| 4 | 6.77 | 7.01 d, 2.2 Hz | 6.96 d,2.2 Hz | 6.89 d, 2.2 Hz |
| 6 | 6.61 | 6.85 dd, 8.8 and 2.3 Hz | 6.92 dd,8.9 and 2.4 Hz | 6.77-6.81 m |
| 7 | 7.00 | 7.24 d, 8.8 Hz | 8.26 | 7.18 d, 9.5 Hz |
| 8 | 2.68 | 2.92 t, 6.7 Hz | 2.86 t,6.9 HZ | 3.02 t, 7.4 |
| 9 | 3.31 | 3.56 q, 6.5 Hz | 3.57 q, 6.6 Hz | 4.05 t, 7.4 Hz |
| 10 | 5.50 | 5.62 s | 5.76 s | 7.94 s |
| 12 | 1.66 | 1.91 s | 1.94 s | 2.18 s |
| 13 | 3.60 | 3.84 s | 3.83 s | 3.73 s |
| 2’ | - | - | 2.92 s | - |
| 3’ | - | - | - | 7.28-7.36 m |
| 4’ | - | - | - | 7.28-7.36 m |
| 5’ | - | - | - | 7.45 m |

**Table 4.3** Summary $^{13}C$ –NMR of melatonin, acetyl melatonin and benzoyl melatonin in $CDCl_3$

| Position | Melatonin reference (Pavlát et al., 1999) | $^{13}C$ NMR from our data (ppm) | | |
|---|---|---|---|---|
| | | Melatonin | Acetyl melatonin | Benzoyl melatonin |
| 2 | 123.19 | 122.90 | 123.17 | 123.41 |
| 3 | 112.96 | 112.90 | 117.50 | 112.06 |
| 4 | 112.77 | 112.48 | 113.49 | 111.79 |
| 5 | 154.40 | 154.04 | 156.51 | 154.17 |
| 6 | 112.40 | 112.29 | 119.41 | 112.06 |
| 7 | 100.77 | 100.52 | 101.82 | 100.27 |
| 8 | 25.63 | 25.15 | 25.28 | 24.89 |
| 9 | 40.10 | 39.82 | 38.94 | 47.01 |
| 11 | 170.52 | 170.28 | 170.46 | 174.54 |
| 12 | 23.67 | 23.30 | 23.62 | 26.11 |
| 13 | 56.28 | 55.77 | 55.71 | 55.80 |
| 1a | 131.91 | 131.65 | 131.55 | 131.33 |
| 1b | 128.07 | 127.65 | 130.78 | 135.45 |
| 1’ | - | - | 168.04 | 173.59 |
| 2’ | - | - | 23.33 | 127.74 |
| 3’ | - | - | - | 128.50 |
| 4’ | - | - | - | 128.15 |
| 5’ | - | - | - | 132.01 |

### 3. Effect of melatonin and derivatives on tail flick latency

Painful stimuli consist of direct stimulation of the efferent sensory nerves or stimulation of pain receptors by various means such as heat or pressure, and are also elicited by inflammation. Progress has been made in elucidating the role of various endogenous substances such as prostaglandins and peptides in the inflammatory process. To determine analgesic activity via *in vivo* study, the tail flick or tail immersion method has been used for selective opiate compounds or central nervous system analgesic compounds. The time for tail flick reflects the analgesic activity and this test is useful to differentiate central opioid like analgesics from peripheral analgesics. Therefore we measured duration of tail flick for different time intervals as shown in Figure 4.3. Tramadol, an opioids derivative was the positive control of this study that has increase time for tail flick of mice to 6-10 second compare to vehicle. The effect of melatonin 50 mg/kg s.c. on analgesic activity increased until 30 minutes

after administration and highest effect for increase tail flick latency was up to 6.4 seconds (Figure 4.3). After that the activity decreased every 10 minute, and this result have related to half-life of melatonin (Marot et al. 1998). For s.c. acetyl melatonin, 50 mg/kg effect was highest at 30 minute the same as melatonin and peak time was 5.7 seconds, then slightly decreasing after compound intake 30 minutes. Benzoyl melatonin s.c. 50 mg/kg had high potency, early onset and prolonged activity compare to melatonin and acetyl melatonin, with peak efficacy at 50 minutes when tail flick latency was 7.8 seconds, as shown in Table 4.4.

This tail flick study to determine analgesic activity of melatonin and its derivatives at 30 and 60 minutes after the compound was administrated by S.C. route show that melatonin, acetyl melatonin and benzoyl melatonin have increased tail flick latency time in a dose-dependent manner at both of 30 and 60 minutes, but the activity of melatonin and acetyl melatonin at 60 minutes have decreased from 30 minutes after compound administrated. The activity of benzoyl melatonin was constant between 30 to 60 minutes after compound administrated as shown in Figure 4.4, suggesting benzoyl melatonin has longer half-life and prolong acting compared to melatonin and acetyl melatonin. However, this result shown that acetyl melatonin and benzoyl melatonin also have the same CNS analgesic activity as melatonin.

Yu et al. (2000), who studied antinociceptive effect of melatonin via tail flick test in male Sprague-Dawley rats, also showed that melatonin has a dose dependent response at 30, 60 and 120 mg/kg respectively, with peak time of melatonin is 30 minute. This study also showed melatonin-induced antinociception via tail flick model is probably mediated through the $MT_2$ receptor subtype by using MT receptor antagonist (luzindole) and $MT_3$ antagonist (prazosin) and shown that luzindole significantly reduced the antinociceptive effect induced by i.p. melatonin. Compared to our result, we did not prove that antinociceptive of melatonin derivatives mediated through MT receptors the same as melatonin or not, but another study of many derivatives of melatonin including benzoyl melatonin also shown that benzoyl melatonin bound to MT receptor but at lower affinity than melatonin (Lira-Rocha et al., 2002).

**Table 4.4** Analgesic activity of the melatonin and derivatives in tail flick model with varies time s.c. after compound administration (n = 6 for each group)

| **Group** | **Drug/ compound** | **Dose (mg/kg)** | **Tail flick latency (s) at varies time after compound administrated (min.) (Mean ± SE)** | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 10 | 20 | 30 | 40 | 50 | 60 |
| 1 | Tween 15% | - | 3.1± 0.4 | 2.9± 0.2 | 2.8± 0.1 | 2.8± 0.2 | 3.2± 0.1 | 3.3± 0.1 |
| 2 | Tramadol | 50 | 5.4± 0.5* | 8.2± 0.6* | 7.8± 0.4* | 8.0± 0.6* | 9.0± 0.5* | 9.3± 0.4* |
| 3 | Melatonin | 50 | 3.7± 0.2 | 4.7± 0.2 | 6.4± 0.3* | 5.8± 0.2* | 5.3± 0.3* | 4.8± 0.1* |
| 4 | Acetyl melatonin | 50 | 3.9± 0.2 | 5.3± 0.3* | 5.7± 0.5* | 5.4± 0.2* | 5.3± 0.4* | 5.0± 0.2* |
| 5 | Benzoyl melatonin | 50 | 5.9± 0.5* | 6.9± 0.3* | 6.7± 0.4* | 6.6± 0.4* | 7.8± 0.4* | 7.7± 0.4* |

* = $P<0.05$ when compared to control group (15% tween).

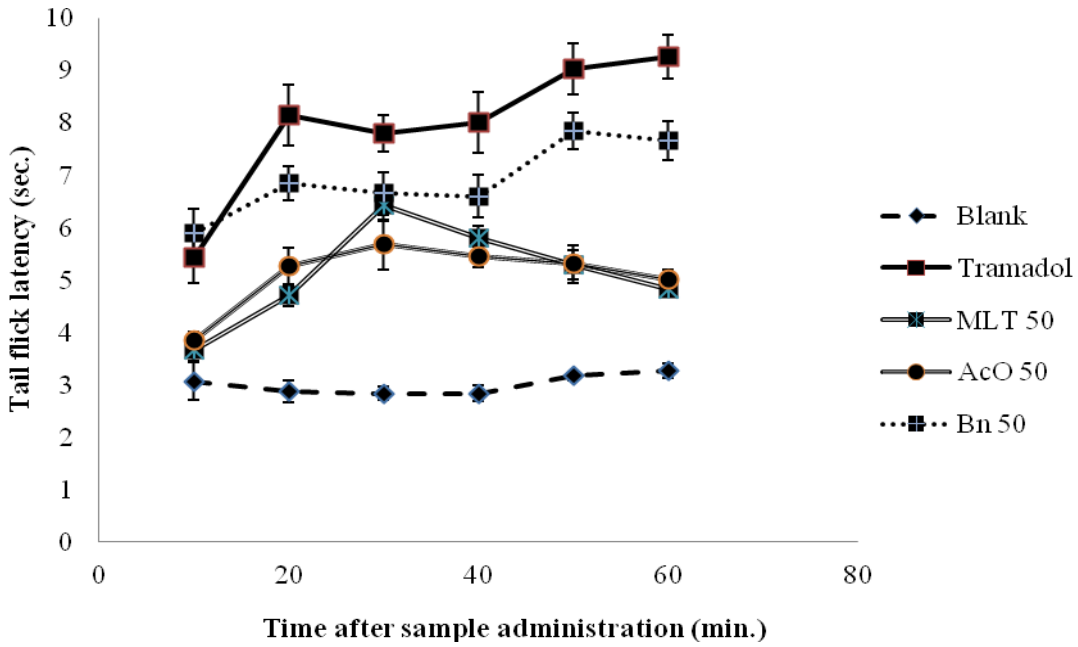



**Figure 4.3** Time-course effect of melatonin (MLT), acetyl melatonin (AcO) and benzoyl melatonin (Bn) derivatives on tail flick induced by warm water 50°C. Mice were treated by subcutaneous route with melatonin and its derivatives (50 mg/kg), Tramadol (50 mg/kg) or vehicle. The result was shown a duration before mice withdrawal or immersed tail as means ± SE (n = 6 for each group).

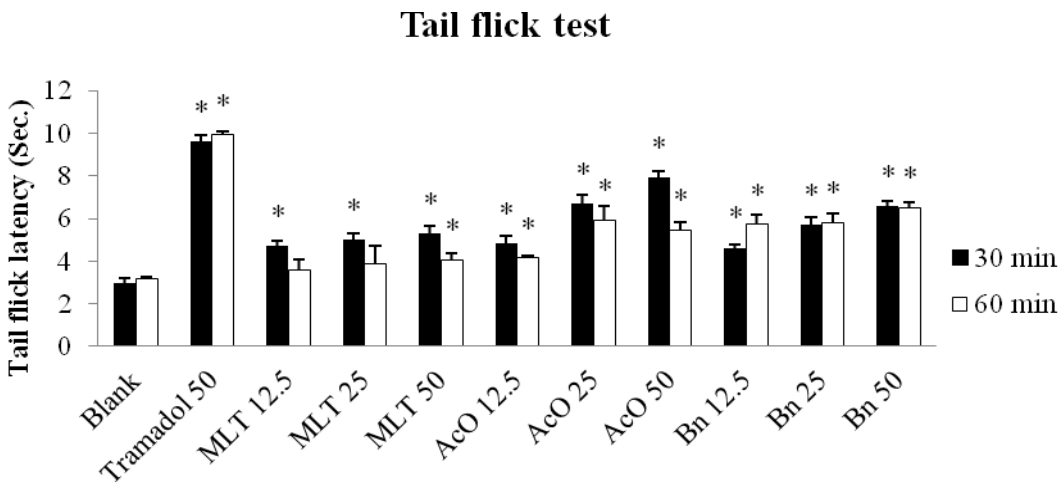



**Figure 4.4** Effect of melatonin (MLT) and its acetyl (AcO), benzoyl (Bn) derivatives on tail flick latency time. Mice were treated by subcutaneous route with melatonin and its derivatives (10-50 mg/kg), Tramadol (50 mg/kg) or vehicle at 30 or 60 min prior to experiment. The result was shown duration before mice withdrawal or immersed tail, values are expressed as the means ± SE, * = $P$<0.05 when compared to vehicle treatment. (n = 6 for each group)

**4. Effect of melatonin and derivatives on acetic acid-induced writhing**

For the acetic acid induced writhing test, acetic acid-induced represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from the tissue phospholipid (Kaushik et al., 2012)**.** In this experiment, we induced pain by injection of an irritant compound (acetic acid) into the peritoneum. The animals react with a characteristic stretching behavior which is called writhing. Aspirin, the positive control, can reduce writhing in mice about 80% both at 30 and 60 minutes for compound administrated before induced writhing as shown in Table 4.5. Melatonin, acetyl melatonin and benzoyl melatonin at the s.c. concentrations of 10, 25 and 50 mg/kg significantly reduced the number of abdominal writhes induced by i.p. injection of acetic acid in mice at 30 minutes in a dose dependent manner, and all of them have comparably potency, This result was same at 60 minutes for compound intake before induced writhing by acetic acid, but at low dose (10 mg/kg), no significant inhibition of writhing was seen, 60

minutes, the number for writhing of melatonin, acetyl melatonin and benzoyl melatonin at 25 and 50 mg/kg was close to counts at 30 minutes after compound administrated (Figure 4.5), and not shown significant when compare result between 30 and 60 minutes after compound administrated as shown in table 4.5.

There was another study of acetic acid induced writhing test on melatonin derivatives in Swiss albino mice. Derivatives structures were fused with the pyrrolo and pyrido ring at *N*-atom position 1 and C-atom position 2 as described in chapter 2 (Elmegeed et al., 2007). Many pyrrolo derivatives had greater effects for inhibiting writhing than melatonin. If we consider the structure, those structures are similar to our acetyl and benzoyl derivatives because if we open ring at C-atom position 2, side chain of N-atom position it will show as an amide structure.

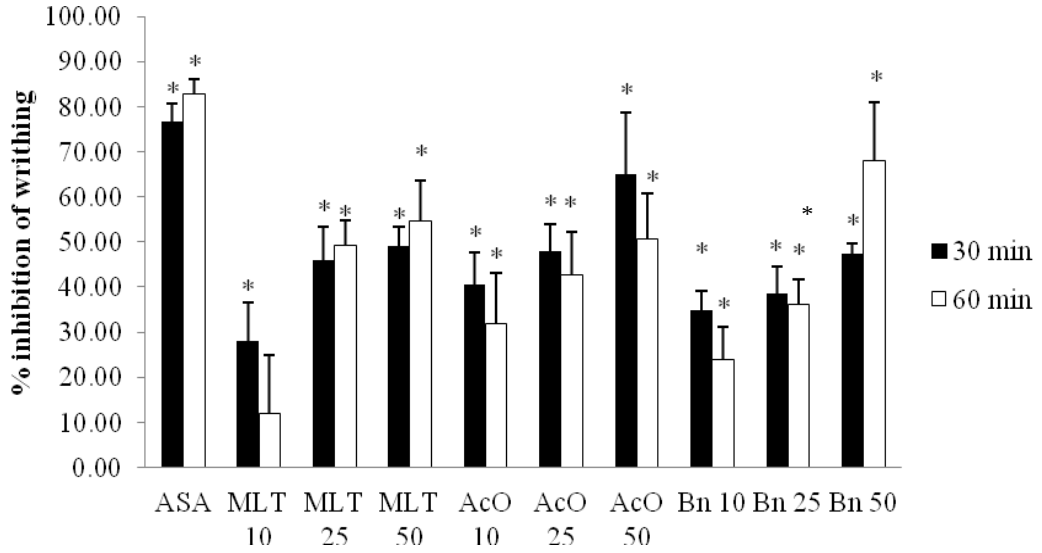



**Figure 4.5** Effect of melatonin (MLT), acetyl melatonin (AcO), benzoyl melatonin (Bn) and aspirin (ASA) on acetic acid induced writhing. Mice were treated by subcutaneous route with melatonin and its derivatives (10-50 mg/kg), aspirin (300 mg/kg) or vehicle at 30 or 60 min prior to acetic acid injection. The result shows the total number of abdomen writhing. Values are expressed as the means ± SE, * = $P$<0.05 when compared to vehicle treatment (n = 6 for each group).

**Table 4.5** Summary results of analgesic activities of melatonin and derivatives in mice

| **Group** | **Increase of tail flick duration Mean ± SE** | | **%Inhibition of writhing Mean ± SE** | |
|---|---|---|---|---|
| | **30 minutes after compound intake** | **60 minutes after compound intake** | **30 minute after compound intake** | **60 minute after compound intake** |
| Positive control | 6.7± 0.3* | 6.8±0.2* | 76.7 ±3.9* | 82.7±3.4* |
| Melatonin 10 mg/kg | 1.8±0.2 | 0.5±0.5*† | 28.1±8.4* | 12.0±12.8† |
| Melatonin 25 mg/kg | 2.1±0.3 | 0.8± 0.8* | 45.8 ±7.4* | 49.3±5.6* |
| Melatonin 50 mg/kg | 2.3±0.4* | 0.9±0.3*† | 49.0±4.3* | 54.7±8.8* |
| Acetyl melatonin 10 mg/kg | 1.9± 0.3* | 1.0±0.1* | 40.6 ±7.1* | 32.0±11.0* |
| Acetyl melatonin 25 mg/kg | 3.7±0.4* | 2.8±0.6* | 47.9 ±6.0* | 42.7±9.6* |
| Acetyl melatonin 50 mg/kg | 5.0± 0.3* | 2.3±0.4*† | 65.0±13.8* | 50.7±10.1* |
| Benzoyl melatonin 10 mg/kg | 1.7±0.2* | 2.6±0.5* | 34.7 ±4.3* | 24.0±7.0* |
| Benzoyl melatonin 25 mg/kg | 2.8±0.3* | 2.7± 0.4* | 38.5±6.0* | 36.0±5.8* |
| Benzoyl melatonin 50 mg/kg | 3.6±0.2* | 3.4±0.3* | 47.5±2.1* | 68.0±12.8* |

* = $P<0.05$ when compared to vehicle treatment.

† = $P<0.05$ when compared effect to 30 minutes of same dose

## 5. Effect of melatonin and derivatives on croton oil induced ear edema

The inflammatory process involves a series of events that can be elicited by numerous stimuli, e.g., infectious agents, ischemia, antigen-antibody interactions, chemical, thermal or mechanical injury. Croton oil-induced ear edema model is a model that chosen for testing activity of local anti-inflammatory effects and used for testing acute and sub-acute inflammation. Croton oil contains 12-o-tetracanoilphorbol-13-acetate (TPA) and other phorbol esters as main irritant agents. TPA is able to activate protein kinase C (PKC), which activates other enzymatic cascades in turn, such as mitogen activated protein kinases (MAPK), and phospholipase $A_2$ ($PLA_2$), leading to release of platelet activation factor (PAF) and arachidonic acid (Patel et al. 2012). From experiment, we applied melatonin and each derivative for 1 hour, and after that croton was applied for another 3 hours. The results are summarized in table 4.6. Aspirin (600 µg/ear), the positive control showed inhibition of ear edema after application of croton oil (about 35% compare to vehicle treatment). Melatonin, acetyl melatonin and benzoyl melatonin have significant ability to inhibit thickness of the ear after application of croton oil (2.5%v/v) at all of dose, and in as a dose dependent manner. Benzoyl melatonin have highest potency in this model (Figure 4.6). Previous study about optimum partition coefficients (log P) on anti-inflammatory drugs after topical application showed that optimum values of logP should be near 3 (Singh, Roberts, 1994). Calculated logP from Marvin View Version 5.12.2 showed log P of melatonin is 1.15, acetyl melatonin is 0.47 and benzoyl melatonin is 2.31, by this effect, benzoyl melatonin should show greater penetration into skin than melatonin or acetyl derivatives. However, acetyl melatonin has lower logP than melatonin but the activity are quite similar. This result may be related to others mechanism of anti-inflammation or transport across the skin.

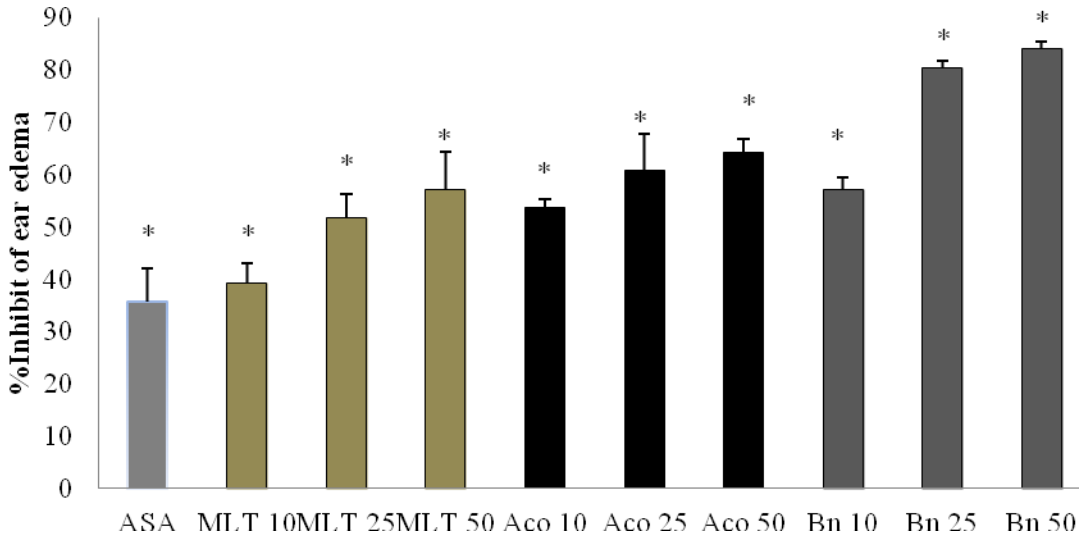



**Figure 4.6** Effect of melatonin (MLT) and its acetyl (AcO), benzoyl (Bn) derivatives on croton oil induce ear edema. Melatonin and its derivatives (10-50 µg/ear) or aspirin (600 µg/ear) was applied to the ear 1 hr before croton oil. The result shown is relative %inhibition of ear thickness. Values are expressed as the means ± SE, * = $P$<0.05 when compared to vehicle treatment. (n =6 for each group)

**Table 4.6** Summary results of croton oil induced ear edema of melatonin and derivatives in mice

| **Group** | **% Inhibition ear edema (Mean±SE) (n = 6)** |
|---|---|
| Aspirin 600 mg/kg | 35.7±6.3* |
| Melatonin 10 mg/kg | 39.3 ±3.8* |
| Melatonin 25 mg/kg | 51.8 ±4.4* |
| Melatonin 50 mg/kg | 57.1 ±7.1* |
| Acetyl melatonin 10 mg/kg | 53.6 ±1.7* |
| Acetyl melatonin 25 mg/kg | 60.7±6.9* |
| Acetyl melatonin 50 mg/kg | 64.3 ±2.4* |
| Benzoyl melatonin 10 mg/kg | 57.1 ±2.4* |
| Benzoyl melatonin 25 mg/kg | 80.4 ±1.5* |
| Benzoyl melatonin 50 mg/kg | 83.9 ±1.5* |

* = $P<0.05$ when compared to vehicle treatment.

**6. Effect of melatonin and derivatives on cotton pellet induced granuloma**

The effect of melatonin and derivatives on cotton pellets induced granuloma is summarized in Table 4.7. Prednisolone 5 mg/kg the positive control showed significant reduction in the amount of granuloma formation (26.9%) compared to vehicle treated group. For melatonin and derivatives, the result showed that the reduction in granuloma formation wasn't significant when treated with melatonin 10 mg/kg and 25 mg/kg, acetyl melatonin 10 mg/kg or benzoyl melatonin 10 mg/kg and 25 mg/kg but at higher doses all of them showed significance for inhibiting granuloma formation dose- dependently as shown in Figure 4.7. Acetyl melatonin and benzoyl melatonin had higher potency for inhibiting granuloma formation than melatonin.

Bianchine and Eade (1967) study the effect of 5-hydroxytryptamine (serotonin) that is precursor of melatonin on the cotton pellet induced granuloma in the rat. Their results showed that serotonin 10 mg/kg can increase the weight of granuloma formed after 7 days of therapy. This result showed serotonin can enhances the inflammatory

reponse. Comparing to our result of melatonin and derivatives that can be inhibited granuloma formation, the difference between melatonin and serotonin is 5-methoxy and n-acetyl position in structure which important to MT receptor binding. Suggest that melatonin receptor have role for anti-inflammation

The cotton pellet-induced granuloma thst has been used as a model to evaluate the chronic anti-inflammatory effects. Lipid peroxide, phospholipase A2 and increase of TNF-α that predict to promote immune cells in exudate granuloma. Compounds that decrease weight of dry granuloma are suggested to decreased the amount of phospholipase A2 and accumulated of immune cells (Sadique 1987). Compare to our result that melatonin and derivatives showed effect to inhibited granuloma formation but not for serotonin that is melatonin precursor. That mean 5-methoxy and *N*-acetyl functional group of melatonin is important role for anti-inflammation effect.

In cotton pellet induced granuloma study, the potency of benzoyl melatonin is quite similar to acetyl melatonin, suggesting there are more complex mechanisms than inhibited production of inflammatory cytokine for the cotton pellet induced granuloma.

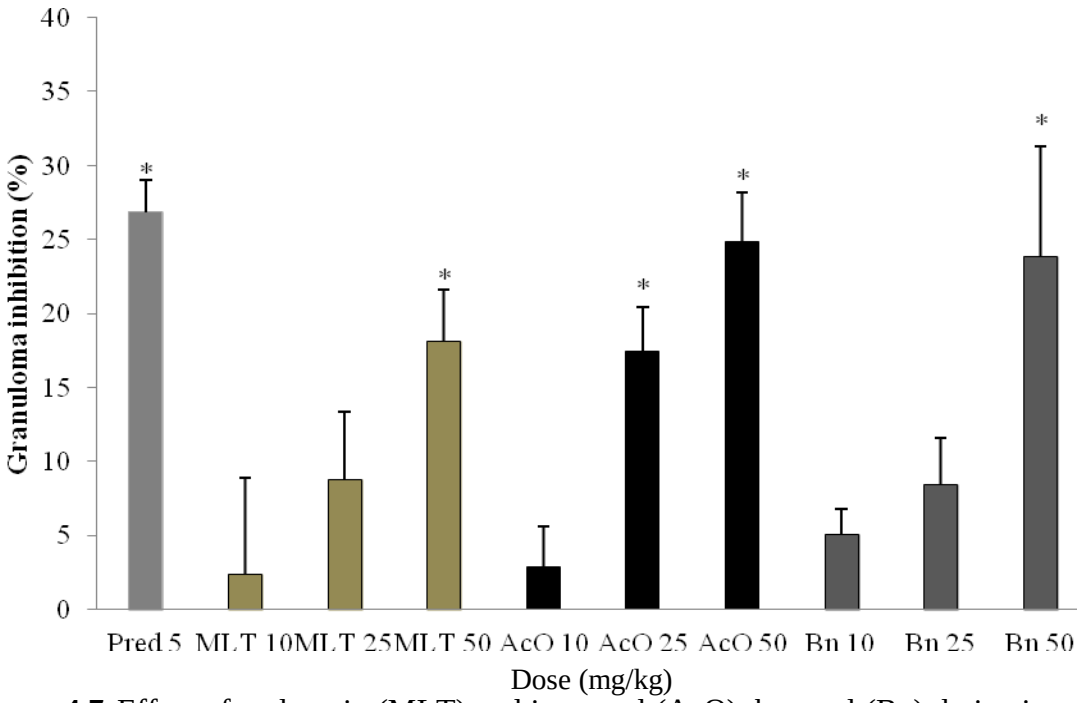



**Figure 4.7** Effect of melatonin (MLT) and its acetyl (AcO), benzoyl (Bn) derivatives and prednisolone (Pred) on granuloma tissue formation in mice. Mice were treated by subcutaneous route with melatonin and its derivatives (10-50 mg/kg), prednisolone (5 mg/kg) or vehicle daily for 7 days. Result shows as a granuloma weight. Values are expressed as the means ± SE, * = $P<0.05$ when compared to vehicle treatment.

**Table 4.9** Summary result of chronic anti-inflammatory activities of melatonin and derivatives in mice

| **Group** | **% Inhibition of granulation (Mean±SE) (n=6)** |
|---|---|
| Prednisolone 5 mg/kg | 26.9±2.2* |
| Melatonin 10 mg/kg | 2.4± 6.5 |
| Melatonin 25 mg/kg | 8.8± 4.6 |
| Melatonin 50 mg/kg | 18.1±3.5* |
| Acetyl melatonin 10 mg/kg | 2.9± 2.8 |
| Acetyl melatonin 25 mg/kg | 17.5 ±3.0* |
| Acetyl melatonin 50 mg/kg | 24.8 ±3.3* |
| Benzoyl melatonin 10 mg/kg | 5.0± 1.8 |
| Benzoyl melatonin 25 mg/kg | 8.4± 3.2 |
| Benzoyl melatonin 50 mg/kg | 23.8 ±7.5* |

# CHAPTER V
# CONCLUSIONS AND SUGGESTION

Our research involved synthesis of melatonin derivatives via N-acetylation and N-benzoylation reaction of melatonin to obtain acetyl melatonin and benzoyl melatonin with 30-40 %yield. The data from structure elucidation by IR showed that our melatonin derivatives have a carbonyl functional group, and from NMR spectrum also showed extra hydrogens and a carbon atom of an acetyl or benzoyl group, proving the successful of synthesized.

Considering the melatonin derivatives structures, *N*-acetyl substitution and *N*-benzoyl substitution have more efficacy than melatonin in ours studies; changing property at *N*-atom in the indole ring from amine to amide appears to have benefits to anti-inflammation. The larger substitution group, benzoyl, had more efficacy than the smaller acetyl group, but cytotoxicity also increased. The main change of physiochemical property of melatonin derivatives is increasing of lipophilicity (logP), and this effect undoubtedly increased permeability of the compound into target cells (Cross et al., 2005).

In analgesic activity studies, melatonin and derivatives gave positive result in the tail flick test that is used for screening analgesics acting in the CNS. Melatonin and acetyl melatonin had high analgesic effects at 30 minutes after administrated but the efficacy decreased when compound intake after 30 minutes, probably related to the half-life of melatonin. The activity of benzoyl melatonin was constant at 30 and 60 minutes after administrated. $MT_2$ receptor agonists like melatonin can have analgesic effects in the CNS (Yu et al., 2000), and our results showed that acetyl melatonin and benzoyl melatonin also had analgesic activity in the CNS. The predicted analgesic mechanism of melatonin derivatives is mediated via the $MT_2$ receptor as same as melatonin. In mouse writhing models, melatonin and derivatives had an analgesic effect at both 30 and 60 minutes after test compound administration before induced writhing by acetic acid, and the most potent compound in this model was benzoyl melatonin followed by acetyl melatonin and melatonin, respectively.

Benzoyl melatonin also the most potency in local anti-inflammation activity from croton oil induced ear edema, but melatonin and acetyl melatonin seem to be of equal activity. The mechanism of croton oils induced inflammation is contact dermatitis and induced by stimulated COX-2 activity. However for local topical anti-inflammation drugs, partial coefficient or logP values are important and the optimized logP for topical application is 3, close to the calculated logP of benzoyl melatonin. Furthermore, in a chronic anti-inflammatory model, cotton pellet induced granuloma, benzoyl melatonin and acetyl melatonin both had higher activities than melatonin and this model indicates that these compounds may be inhibit the function of $PLA_2$ and aggregation of immune cells.

These results show that melatonin and derivatives have analgesic and anti-inflammatory activities. Almost all experiments showed that the benzoyl derivative had highest efficacy compared to the others follow by acetyl melatonin and melatonin, respectively. These results suggest that new synthesized melatonin derivative, acetyl melatonin and benzoyl melatonin have anti-inflammatory effect more potent than their parent compound, melatonin, and their anti-inflammatory mechanism may be partially associated with blocking of pro-inflammatory mediator release.

Further studies should investigate the effect of electron donating and electron withdrawing substitution such as nitro-benzoyl melatonin or methoxy-benzoyl melatonin at vary positions o- (ortho), m- (meta), and p- (para) substitution to obtain SAR data from acyl derivatives of melatonin. However, there need to confirm another mechanisms of action, such as MT-receptor binding, and effect on NF-κB expression, iNOS and COX-2 expression. This would require measurement or modeling of binding to melatonin receptors and COX-2 enzyme, and direct measurement of expression of COX-2, NF-κB and i-NOS *in vitro* and *in vivo*. Pharmacokinetics and toxicity of our melatonin derivatives also needs consideration for the potential to develop melatonin derivatives as novel drugs.