UNIVERSITI PUTRA MALAYSIA

ANTIHYPERALGESIC AND ANTI-ALLODYNIC PROPERTIES OF CARDAMONIN IN MICE MODEL OF NEUROPATHIC PAIN

YOGESVARI SAMBASEVAM

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ANTIHYPERALGESIC AND ANTI-ALLODYNIC PROPERTIES OF CARDAMONIN IN MICE MODEL OF NEUROPATHIC PAIN

By

YOGESVARI SAMBASEVAM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

Every challenging work needs own great efforts and guidance, love as well as support from people who are very close to our heart.

I would like to dedicate my humble effort to my

Father & Mother
Mr Sambasevam Mukan & Mrs Santhi Muniaindy

Husband
Mr Sharvieen Jaganathan

Brothers
Mr Balachandran Sambasevam, Mr ManiRajan Sambasevam & Mr Kurubaraaneish Sambasevam
Neuropathic pain is a chronic pain state caused by injury in the nervous system and often characterised by symptoms such as spontaneous pain, allodynia and hyperalgesia. Neuropathic pain is debilitating and highly resistant to current treatments such as nonsteroidal anti-inflammatory drugs (NSAIDS), anticonvulsants, antidepressants and opioids analgesics. Cardamonin is a naturally occurring chalcone. Studies have shown that cardamonin exhibited promising therapeutic effects such as antinociceptive and anti-inflammatory. Importantly, cardamonin is able to reduce the production of inflammatory mediators that are also involved in the pathophysiology of neuropathic pain. The study was aimed to investigate the antihyperalgesic and anti-allodynic properties of cardamonin in CCI-induced neuropathic pain in mice and its possible mechanism of actions. Male ICR mice were used throughout the project. CCI-induced neuropathic pain model was performed. A small incision was made to expose the sciatic nerve on the left hind leg and loose ligatures were placed around the nerve. The neuropathic pain response was measured quantitatively by using Hargreaves Plantar test, dynamic plantar aesthesiometer test, cold plate test and Randall-Selitto test. Ligations study consisted of 5 groups of animals; sham-operated, 1-Ligation, 2-Ligations, 3-Ligations and 4-Ligations. Behavioural assessments were carried out for 12 weeks. Investigation of antihyperalgesic and anti-allodynic properties of cardamonin were carried out by treating animals exposed to CCI with vehicle (DMSO, Tween20, & distilled water), Amitriptyline (20 mg/kg) or cardamonin (3, 10 & 30 mg/kg) via intraperitoneal route. All treatments were administered for 7 days consecutively from day 15 till day 21 after surgery. Behavioural assessments were carried out on day 0, 14 (before treatment & after treatment) and 21. Mechanisms of actions (MOA) that were investigated in this project were the involvement of opioid receptors, L-arginine-nitric oxide/cGMP/ATP-sensitive K⁺ channel pathway and potassium channels. Animals exposed with CCI were pre-treated with antagonists before the administration of cardamonin or vehicle. Behavioural tests were conducted after the administration of their respective treatments. Brain samples were collected to study the expression of opioid receptors via Western blotting. All
data were collected and expressed as mean ± SEM and were statistically analysed by using one-way Analysis of Variance (ANOVA), followed by Tukey’s post-hoc test. The results were considered significant at $p<0.05$. Cardamonin (3, 10 & 30 mg/kg) exhibited antihyperalgesic and anti-allodynic activities on CCI-induced neuropathic pain model in mice. Cardamonin elicited its analgesic effects by activating L-arginine/cGMP/K⁺-ATP channel pathway, opening the potassium channels as well as modulating pain signal via activation of delta- and kappa-opioid receptors. Modulation by these pathways and ion channels suppressed the neuronal hyperexcitability that arose due to peripheral nerve injury, hence producing analgesic effects. Taken together, cardamonin has the potential to be developed as a drug candidate for management of pain.
CIRI-CIRI ANTIHIPERALGESIK DAN ANTI-ALODINIK CARDAMONIN PADA MODEL KESAKITAN NEUROPATIK MENCIT

Oleh

YOGESVARI SAMBASEVAM

Mei 2018

Pengerusi: Enoch Kumar Perimal, PhD
Fakulti : Perubatan dan Sains Kesihatan

dikumpulkan dan dinyatakan sebagai purata ± SEM dan dianalisis secara statistik dengan menggunakan Analisa Variasi satu arah (ANOVA), diikuti dengan ujian perbandingan pelbagai Tukey. Hasilnya dianggap signifikan pada p <0.05. Cardamonin (3, 10 & 30 mg / kg) mempamerkan aktiviti antihiperalgesik dan antialodinik pada model sakit neuropatik yang disebabkan oleh CCI pada tikus. Cardamonin menimbulkan kesan analgesiknya dengan mengaktifkan saluran L-arginine / cGMP / K⁺-ATP, membuka saluran kalium serta memodulasi isyarat sakit melalui pengaktifan reseptor delta dan kappa-opiod. Modulasi oleh saluran dan saluran ion ini akan memulihkan saraf yang terangsang secara luar biasa akibat kecederaan saraf periferal, dengan itu menghasilkan kesan analgesik. Kesimpulannya, cardamonin berpotensi untuk menjadi calon ubat yang efektif untuk pengurusan sakit kronik seperti neuropatik.
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Last but not least, I am deeply grateful and thankful to my family members who have given me encouragements and motivations which never fail me to finish my study until the end. They have been understanding and support me with all the challenges faced throughout the year.
I certify that a Thesis Examination Committee has met on 17 May 2018 to conduct the final examination of Yogesvari a/p Sambasevam on her thesis entitled "Antihyperalgesic and Antiallodynic Properties of Cardamonin in Mice Model of Neuropathic Pain" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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Summary of symptoms in neuropathic pain

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CCI-induced mechanical allodynia in mice ipsilateral paw

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>TRPV</td>
<td>Transient receptor potential vanilloid</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin-gene related peptide</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal gray</td>
</tr>
<tr>
<td>RVM</td>
<td>Rostral ventral medulla</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricyclic antidepressants</td>
</tr>
<tr>
<td>SNI</td>
<td>Spared nerve injury</td>
</tr>
<tr>
<td>PSNL</td>
<td>Partial sciatic nerve ligation</td>
</tr>
<tr>
<td>SNL</td>
<td>Spinal nerve ligation</td>
</tr>
<tr>
<td>CCI</td>
<td>Chronic constriction injury</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ion</td>
</tr>
</tbody>
</table>

xxi
$K_v$ Voltage-gated potassium channel
$Ca^{2+}$ Calcium ion
$BK_{Ca}$ Large conductance calcium-activated potassium channel
$SK_{Ca}$ Small conductance calcium-activated potassium channel
NRM Nuclues raphe magnus
COX-2 Cyclooxygenase-2
TNF-α Tumor necrosis factor α
IL-1β Interleukin 1β
IL-6 Interleukin 6
NF-κB Nuclear factor kappa B
$PGE_2$ Prostaglandin E2
sGC Soluble guanylyl cyclase
CHAPTER 1

INTRODUCTION

Chronic pain is a critical health issues and it is a great challenge of currently available medicines to provide complete pain relief to the patients in clinical settings (Nguelefack et al., 2010). Chronic pain can be grouped as nociceptive pain, sensory hypersensitivity and neuropathic pain, in relation to their respective underlying pathobiology (McCarberg et al., 2017). Nociceptive pain does not involve damage or dysfunction of the nervous system. However, it is concomitant with tissue damage due to trauma or inflammation (IASP, 2012). Sensory hypersensitivity is the amplification of sensory signal and lowering of pain threshold, due to prolong dysfunction of nervous system and does not involve any tissue or nerve damage (Petersel et al., 2011; Woolf, 2011). In contrast, neuropathic pain is the pain that arises due to direct impairment that takes place in the nervous system (Treede et al., 2008).

Millions of people worldwide are suffering from neuropathic pain that could give distressing impact to their quality of life as well as engaging in daily routines (Hall et al., 2006). Signs and symptoms such as hyperalgesia, allodynia are strongly characterised with neuropathic pain (Bridges et al., 2001). The prevalence of neuropathic pain has been evaluated to be in the range of 7-10% according to the studies carried out on the general population (Bouhassira et al., 2008; Van Hecke et al., 2014). In Malaysia, Hospital Selayang pain clinic reported that 38.8% of patients had neuropathic pain (Othman et al., 2011). In addition, a primary care clinic located at University Malaya Medical Center found that 54.8% patients are present with chronic pain (Ambigapathy, 2010). Nationwide, the prevalence of chronic persistent pain was found to be 7.1% among 33,733 adults (Cardosa et al., 2008). Generally, patients suffering from diabetic neuropathy, HIV infection, stroke and amputations are closely associated with neuropathic pain conditions (Colloca et al., 2017).

Neuropathic pain is unfavourable and unmanageable due to its wide-ranging and complex mechanisms (Baron et al., 2010). Available drugs such as antidepressants like amitriptyline, nortriptyline, anticonvulsants such as gabapentin, carbamazepine and opioids such as morphine and tramadol have limited therapeutic capability in the management of the debilitating chronic pain (Baron et al., 2010; O’Connor et al., 2009). Besides, administration of these drugs often produces side effects such as nausea, constipation and addiction to the patients and this limits the usage for neuropathic pain management (Attal et al., 2010). Thus, this study is conducted to explore on a novel potential lead compound, which is able to exhibit potent analgesic effects towards chronic pain as well as producing lesser or no side effects upon consumption.
The use of natural products has been closely related to the application of traditional medicines since thousands years ago. The natural products consist of medicinal plants are commonly used for treating, curing and preventing from various diseases, is the primitive form of medical practice in mankind (Li et al., 2009). In addition, aspirin and morphine are the pronounced example of drugs derived from plants, which are being incorporated in chemical, pharmacological and clinical studies (Newman et al., 2012). Natural products and active components with fewer adverse effects are emerging as beneficial medicament resources for the evolution of new drugs in the pain management procedures. In fact, neuropathic pain conditions are still contributing to their fair share in the process of developing novel medicines (Butler, 2004; Li et al., 2011).

An enormous number of studies have been focused on chalcones, an aromatic enones belonging to the flavonoids family and often responsible for yellow pigmentation of the plants (K Sahu et al., 2012). Cardamonin, a name thought to be originated from cardamom spices, is one of the examples of naturally occurring chalcone found in various plant species (Krishna et al., 1973). Researchers claim that cardamonin is highly potential in exhibiting various medicinal properties (Gonçalves et al., 2014). Most importantly, it has been shown to exhibit anti-inflammatory (Israf et al., 2007) and anti-nociceptive (Park et al., 2014) properties in in vivo as well as in vitro models. It is known that inflammatory and nociception involves the peripheral and central sensitization, thus it is relevant to study the effect of cardamonin on neuropathic pain since it shares similar pathophysiology.

Problem statement and justification of present study

The present study is carried out to establish a safer and effective drug in pursuit of better neuropathic pain management. Currently available drugs in clinical settings prescribed for patients diagnosed with neuropathic pain symptoms, are often associated with adverse effects and ineffectiveness. An evidence-based approach and specific recommendation has been shown by randomized controlled trials carried out for treatment of neuropathic pain for the use of various mode of drugs such as anticonvulsants, tricyclic antidepressants, topical lidocaine, serotonin–norepinephrine reuptake inhibitors as well as opioids such as tramadol. Among these drugs, anti-convulsants such as gabapentin and opioids like morphine have been chosen as two of several first-line treatments options for neuropathic pain (Dworkin et al., 2007). However, the adverse effects associated with opioids are distressing. Patients treated with opioid analgesics tend to develop dependence, withdrawal, abuse and immunologic changes (Vallejo et al., 2004). Besides, it was found that the response towards these drugs is often insufficient.

Therefore, a reliable novel substitute is needed to solve this worldwide issue while adding value to the drug development process. This study was commenced to provide a preclinical data on investigating the potential of cardamonin as antihyperalgesic and anti-allodynic agent. Cardamonin has been extensively studied and has been proved to exhibit various medicinal benefits, importantly,
antinociceptive and anti-inflammatory activities. Thus, it is admissible that cardamonin could be one of the candidate for the treatment of neuropathic pain symptoms.

**Hypothesis**

Cardamonin may potentially exhibit antihyperalgesic and anti-allodynic effects in chronic constriction injury-induced neuropathic pain in mice via activation of L-arginine-cGMP-K⁺-ATP channel pathway, other potassium channels and opioidergic system.

**Objectives of the Study**

The general objective of the study is to investigate the antihyperalgesic and anti-allodynic properties of cardamonin in chronic constriction injury (CCI)-induced neuropathic pain model of mice and its possible mechanism of actions.

The specific objectives were to:

i. compare the effects of different number of ligations in CCI model of neuropathic pain in mice
ii. investigate the effects of cardamonin in attenuating hyperalgesia and alldynia in chronic constriction injury model of neuropathic pain in mice
iii. investigate the involvement of L-arginine-nitric oxide-cGMP pathway in cardamonin-induced antihyperalgesic and anti-allodynic properties.
iv. investigate the involvement of potassium channels in cardamonin-induced antihyperalgesic and anti-allodynic properties
v. elucidate the involvement of opioidergic system in cardamonin-induced antihyperalgesic and anti-allodynic properties
REFERENCES


Xp11. 23 cause incomplete X-linked congenital stationary night blindness. *Nature Genetics*, 19(3), 264-267.


International Association for the Study of Pain Taxonomy: Neuropathic Pain. IASP. Available at: [http://www.iasp-pain.org/Taxonomy#Neuropathicpain](http://www.iasp-pain.org/Taxonomy#Neuropathicpain) [Last accessed 16 November 2017]

International Association for the Study of Pain Taxonomy: Nociceptive Pain. IASP. Available at: [http://www.iasp-pain.org/Taxonomy#Nociceptivepain](http://www.iasp-pain.org/Taxonomy#Nociceptivepain) [Last accessed 16 November 2017]


Nockemann, D., Rouault, M., Labuz, D., Hublitz, P., McKnelly, K., Reis, F. C., Stein, C., & Heppenstall, P. A. (2013). The K\(^+\) channel GIRK2 is both necessary and sufficient for peripheral opioid-mediated analgesia. *EMBO Molecular Medicine, 5*(8), 1263-1277.


