EFFECTS OF Erythroxylum Cuneatum (Miq.) Kurz ON CELLULAR AND SYNAPTIC ADAPTATION OF CHRONIC MORPHINE-ADDICTED HUMAN NEUROBLASTOMA CELL LINE AT PROTEIN LEVEL

NOOR AZUIN BINTI SULIMAN

FPSK(P) 2017 10
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By

NOOR AZUIN BINTI SULIMAN

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

March 2017
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NOOR AZUIN BINTI SULIMAN

March 2017

Chair: Mohamad Aris Bin Mohd Moklas, PhD
Faculty: Medicine and Health Sciences

Erythroxylum cuneatum (E. cuneatum) is a tropical flowering plant listed under Erythroxylaceae family. E. cuneatum is widely distributed within Southeast Asia. Uses of E. cuneatum in alternative medicines or remedies are limited. Indigenous traditional healer claimed that the plant was used in treating drug withdrawal. However, there is no scientific data to support the claim. Thus, the study was designed to evaluate the potential of anti-withdrawal properties of alkaloid extract of the plant on chronic morphine-addicted cell. An alkaloid extract of E. cuneatum (designated as ECAI) was extracted for all the tests. The human neuroblastoma cell line, SK-N-SH, was used throughout the study. The effects of ECAI on the chronic morphine-addicted cell were observed in two different groups, the co- and pre-treatments of morphine. Throughout the study, ECAI (0.1, 0.5, and 1.0 µg/mL) was compared to morphine and methadone. The receptor involved for the effects of the plant was determined using antagonists. The expressions of Cyclic adenosine 3', 5'-monophosphate (cAMP), intracellular calcium ion ([Ca^{2+}]), and α-synuclein were studied. At the beginning of the study, withdrawal markers [α-synuclein and calmodulin] were observed, followed by the receptor trafficking [Vesicle-associated membrane protein 2 (VAMP 2) and synaptotagmin 1], desensitisation or internalisation of the receptor [G protein-coupled receptor kinases (GRK) 2, β-arrestin 1/2, and clathrin], and cellular adaptation [mitogen-activated protein (MAP)/extracellular signal-regulated (ERK) kinase (MEK) 1/2, ERK 2, cAMP-
dependent protein kinase (PKA), and protein kinase C (PKC)] affected by the ECAI. Through the receptor affinity studies, ECAI bound to µ-opioid receptor, similar to methadone and morphine. Present data showed that ECAI possesses anti-withdrawal properties. ECAI was observed to enhance the receptor trafficking and cause the internalisation of the receptor. The cellular and synaptic adaptations modulated by ECAI were consistent throughout all study and parallel with the effects of the methadone. The administration of ECAI at the optimal doses was postulated to minimise the withdrawal, dependence, and tolerance against morphine-addicted cell. The alkaloid extract of the plant has a potential in opioid substitution therapy.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN-KESAN TERHADAP ADAPTASI SEL DAN SINAPS OLEH ERYTHROXYLUM CUNEATUM

Oleh

NOOR AZUIN BINTI SULIMAN

Mac 2017

Pengerusi: Mohamad Aris Bin Mohd Moklas, PhD
Fakulti: Perubatan dan Sains Kesihatan

Erythroxylum cuneatum (E. cuneatum) adalah tumbuhan berbunga tropika yang disenaraikan di bawah keluarga Erythroxylaceae. E. cuneatum tumbuh secara meluas di Asia Tenggara. Penggunaan E. cuneatum sebagai ubat-ubatan atau rawatan alternatif adalah terhad. Terdapat dakwaan oleh pengamal perubatan tradisional dikalangan orang asli mengenai penggunaan tumbuhan ini dalam merawat ketagihan dadah. Walau bagaimanapun, tiada data saintifik untuk menyokong dakwaan tersebut. Oleh itu, kajian ini bertujuan untuk mengkaji kewujudan ciri-ciri anti-ketagihan dalam ekstrak alkaloid tumbuhan ke atas sel yang terawat dengan morfin secara kronik. Ekstrak alkaloid E. cuneatum (dinyatakan sebagai ECAl) telah diekstrak untuk semua ujian. Sel neuroblastoma manusia, SK-N-SH, telah digunakan untuk kajian ini. Kesaran ECAI pada kronik morfin diperhatikan dalam dua kumpulan yang berbeza iaitu rawatan bersama dan pra-rawatan morfin. Sepanjang kajian ini, ECAI (0.1, 0.5, dan 1.0 μg/mL) dibandingkan dengan morfin dan metadon. Reseptor yang terlibat bagi kesan tumbuhan itu telah ditentukan dengan menggunakan antagonis. Ekspresi cyclic adenosine 3',5'-monophosphate (cAMP), kalsium ion intrasel ([Ca2+]), dan α-synuclein telah dikaji. Di awal kajian, protein sebagai indikasi penarikan (α-synuclein dan calmodulin) telah diperhatikan, diikuti dengan kitaran reseptor [vesicle-associated membrane protein 2 (VAMP 2) dan synaptotagmin 1], penyahpekaan atau internalisasi reseptor [G protein-coupled receptor kinases (GRK) 2, β-arrestin 1/2, dan clathrin], dan adaptasi sel [mitogen-activated protein (MAP)/extracellular signal-regulated (ERK) kinase (MEK) 1/2, ERK 2, cAMP-dependent protein kinase (PKA), dan protein kinase C (PKC)] dipengaruhi oleh ECAI. Melalui kajian terhadap afiniti reseptor, ECAI terikat untuk μ-opioid reseptor, sama seperti metadon dan morfin. Data kajian
ACKNOWLEDGEMENTS

“In the name of Allah S.W.T, the most Benevolent and Most Merciful”

Praise to Allah for granting me grace and strength to persevere throughout my study and to overcome all the challenges that I have gone through during the project.

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APPROVAL

I certify that a Thesis Examination Committee has met on (date of viva voce) to conduct that final examination of Noor Azuin Binti Suliman on her thesis entitled “Exploration of Erythroxylum Cuneatum on Cellular and Synaptic Adaptation” 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 with (insert the name of relevant degree).

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<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine neurotransmitter</td>
</tr>
<tr>
<td>AFDX-116</td>
<td>11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-on</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-2</td>
<td>Adaptor protein 2</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>Ca(^{2+})-CaM</td>
<td>Ca(^{2+})-calmodulin</td>
</tr>
<tr>
<td>CAMKII</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine 3', 5'-monophosphate</td>
</tr>
<tr>
<td>cGMP/PKG</td>
<td>Cyclic guanosine 3',5'-monophosphate / protein kinase G</td>
</tr>
<tr>
<td>CME</td>
<td>Clathrin-mediated endocytosis</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPP</td>
<td>Conditioned place preference</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAO</td>
<td>Diamineoxide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECAI</td>
<td>Alkaloid extract of <em>Erythroxylum cuneatum</em></td>
</tr>
<tr>
<td>EGR1</td>
<td>Early growth response 1</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FRIM</td>
<td>Forest Research Institution of Malaysia</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GIRK</td>
<td>G protein-linked inwardly rectifying K⁺ channels</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein-coupled receptor kinases</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloride acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxide</td>
</tr>
<tr>
<td>Hr(s)</td>
<td>Hour (s)</td>
</tr>
<tr>
<td>IBMX</td>
<td>Isobutylmethylxanthine</td>
</tr>
<tr>
<td>IDV</td>
<td>Integrated density values</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IH</td>
<td>Voltage-dependent current</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>JNK</td>
<td>C-Jun N-terminal kinase</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium</td>
</tr>
<tr>
<td>K&lt;sub&gt;ATP&lt;/sub&gt;</td>
<td>Adenosine triphosphate (ATP)-sensitive K⁺</td>
</tr>
<tr>
<td>LC</td>
<td>Locus coeruleus</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potential</td>
</tr>
<tr>
<td>M</td>
<td>Muscarinic receptor</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein (MAP) kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein (MAP)/extracellular signal-regulated (ERK) kinase</td>
</tr>
<tr>
<td>Min (s)</td>
<td>Minute (s)</td>
</tr>
<tr>
<td>MRI</td>
<td>Mean relative intensity</td>
</tr>
<tr>
<td>MTT</td>
<td>Thiazolyl blue tetrazolium bromideme</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate receptors</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OST</td>
<td>Opioid substitution therapy</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
</tbody>
</table>

xxi
pERK  Phosphorylated ERK1/2
PIP2  Phosphatidylinositol 4,5-bisphosphate
PKA  cAMP-dependent protein kinase
PKC  Protein kinases C
PKCε  Phosphokinase C
PLA2  Phospholipase A₂
PLC  Phospholipase C
PMT  Putrescine N-methyltransferase
PNS  Peripheral nervous system
PSD  Post-synaptic density
PVDF  Polyvinylidene difluoride
RA  Retinoid acid
RIPA  Radioimmunoprecipitation assay
RNA  Ribonucleic acid
RNApol  Ribonucleic acid polymerase
RSK  Ribosomol S6 Kinase
SAPK  Stress-activated protein kinases
SDS  Sodium dodecyl sulfate
SFK  Src family kinase
SNARE  Soluble N-ethylmaleimide-sensitive factor activating protein receptor
t-SNARE  Target soluble N-ethylmaleimide-sensitive factor activating protein receptor
TBST  Tris-buffered saline and tween 20
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TR-I</td>
<td>Tropinone reductase I</td>
</tr>
<tr>
<td>TR-II</td>
<td>Tropinone reductase II</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>UPM</td>
<td>Universiti Putra Malaysia</td>
</tr>
<tr>
<td>USA</td>
<td>United State of America</td>
</tr>
<tr>
<td>v-SNARE</td>
<td>Vesicular soluble N-ethylmaleimide-sensitive factor activating protein receptor</td>
</tr>
<tr>
<td>VAMP 2</td>
<td>Vesicle-associated membrane protein 2</td>
</tr>
<tr>
<td>VDCC</td>
<td>Voltage-gated Ca(^{2+}) channel</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Background of study

Three classes of medication that responsible for misuse liability are prescription opioids, stimulants, and the central nervous system (CNS) depressants. There are the number of factors that contribute to such phenomenon which includes great distribution of medications, aggressive marketing by pharmaceutical companies, and easy accessibility to public users. These contributing factors escalate the number of non-medical usage of opioid analgesic and overdose deaths (Mack, 2013).

In 2013, more than 11 million people mistreated with heroin or prescription pain reliever were recorded ("SAMHSA Releases Behavioral Health, United States, 2012", 2013). There is a long list of adverse effects of the opioid abuse. One of the major problems of opioid abuse is the chronic pain experienced by more than 62% of opioid substitution therapy (OST) patients (Voon et al., 2015). Meanwhile, 30.7% of general population grieved the pain (Johannes et al., 2010). The chronic pain is observed from the exhibition of opioid-induced hyperalgesia and super-sensitivity to pain (Williams et al., 2001).

Repeated intake of opioids causes the inhibition of production of endogenous opioids such as endorphins and encephalins. The cellular adaptation is responsible for the withdrawal, tolerance, and dependence signs. These addictive symptoms in future will trigger the drug addicts to increase their uptake of the drug to obtain desired effects. This subsequential event will lead to overdose, a new problem of drug usage (Williams et al., 2001).

Opioids are effective for acute severe pain following trauma, extensive burns, or surgery. They also are used for painful terminal diseases such as cancer (Pasternak, 2011). Edlund et al. (2007) claimed that the rare addiction incident occurred when opioid analgesics are used appropriately. At the same time, Ballantyne and LaForge (2007) suggested that chronic opioid on chronic pain patient has increased the addiction and opioid abuse. Thus, it is consistent with Kalso et al. (2004) proclaimed the effective analgesia in chronic pain patient treated with the acute opioid. To what extent is a prescription of painkillers to create an epidemic abuse? The answer is not simple (Fields, 2011). Fields (2011)
suggested that more than 70% of the opioid abusers got the drug unlawfully while less than 20% got the drugs through a prescription from a doctor. Some users overdose or wind up dead from respiratory depression (Edlund et al., 2007).

1.2 Problem statement

Opioids, such as morphine, heroin, and oxycodone, act as an agonist of the µ-opioid receptor to produce analgesia effect. Though, drugs activating µ-opioid receptor are most commonly abused (Koob and Le Moal, 2005). Opioid addiction becomes an epidemic problems. One death in every 19 minutes was recorded in the United States only (Centers for Disease Control and Prevention (CDC), 2012).

There are numbers of therapies or commercialised drugs that are used to treat opioid addiction, withdrawal, tolerance, or overdoses such as buprenorphine, methadone, and clonidine (Doyon et al., 2004). However, these drugs are classified as opioid and widely known to cause abuse (Bailey et al., 2009). Prescribed opioid addiction or morphine addiction patients in the US are enrolled in methadone maintenance treatments programs (Rosenblum et al., 2007).

Methadone is an opioid agonist that is effective for treating severe pain. It has potential advantages against another opioid including low cost, high bioavailability, long half-life, and lack of active metabolites. According to the National Institutes of Health, the government of United States spent over $180 billion for illicit drug abuse just in 2008. The costs include the medical expenses and unlawful activity, social welfare, secondary medical issues, and efficiency losses. The misuse of methadone and opioid contributes to noticeable economic burden to civilisation (Scavone et al., 2013). Furthermore, methadone substitution as a treatment for opioid addiction has been criticised widely. It is claimed that the methadone is not effective to restraint addiction (Bennett, 2011).

The focus of the management is to confront the negative impact of the abuse on health and mortality while preserving the role of the opioid in managing pain. The use of alternative medicines is one of the options to deal with opioid misusage. The local Malaysian folks claim the use of Chinta Mula on treating morphine craving. Chinta Mula or scientifically known as Erythroxylum
*Erythroxylum cuneatum* (EC) can be found in Southeast Asia, especially in Malaysia, Philippine, and Indonesia (Chung, 2006). EC belongs to the family of Erythroxylaceae, *Erythroxylum spp.* and contains cocaine as one of its psychoactive alkaloid that proclaimed to influence the CNS (Plowman and Rivier, 1983).

### 1.3 Significance of the study

The output from this study will provide a new approach in regards to managing the opioid misuse. Instead of using a drug to treat misuse of the drug, alternative medicines such as plant will be a better approach. As compared to commercialised drugs, alternative medicines are cheaper and comparatively safer.

### 1.4 Hypothesis

Following the problem statements, the hypothesis of the study is that *Erythroxylum cuneatum* (EC) is mimicking the effects of methadone against the induction of morphine. EC is hypothesised to express anti-addiction properties; anti-dependence, anti-tolerance, and anti-withdrawal, against chronic morphine. EC is expected to minimise the addiction symptoms on the morphine-treated cell line observed by increasing fusion machinery at the pre-synaptic terminal internalising the involved receptor and influencing the cellular adaptation processes.

### 1.5 Objectives of the study

**General Objective**

- To observe the anti-withdrawal properties of alkaloid extract of EC (designated as ECAI) by comparing to methadone in morphine-induced addicted cell line.

**Specific Objectives**

1. To determine the ideal dosage of ECAI on treating the morphine-treated cell on different time duration.
2. To predict the involvement of receptor on withdrawal properties of ECAI.
3. To examine the role of ECAI in the fusion machinery at the pre-synaptic terminal against chronic morphine.
4. To predict the effects of ECAI on desensitisation/internalisation of receptor on the morphine-treated cell line.
5. To study the cellular adaptation induced by the prolonged morphine and counteracting of ECAI.
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phospholipase C, [Ca\textsuperscript{2+}]\textsubscript{i} and adenylyl cyclase. *British Journal of Pharmacology, 120*(6): 1165-1171.


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