MOLECULAR MECHANISMS OF MITRAGYNINE INHIBITION ON HERG1A/1B CHANNEL

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UNIVERSITI SAINS MALAYSIA

2018

MOLECULAR MECHANISMS OF MITRAGYNINE INHIBITION ON HERG1A/1B CHANNEL

by

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Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy

April 2018

ACKNOWLEDGEMENT

First and foremost, I would like to express my deepest gratitude to my supervisor, Assoc. Prof. Dr. Tan Mei Lan for her limitless dedication, continuous support, encouragement and patience in guiding me towards the completion of my Ph.D study. Over the years, I could always count on her valuable advices and feedback on my work.

In addition, I would like to express my warmest appreciation to my co-supervisors, Prof. Habibah A. Wahab and Dr. Tan Jun Jie for their time, knowledge and guidance on *in silico* and cell culture work.

I gratefully acknowledge Universiti Sains Malaysia for the financial support under USM Fellowship Scheme. This work was supported by ScienceFund from the Ministry of Science, Technology and Innovation Malaysia, and Fundamental Research Grant Scheme (FRGS) from the Ministry of Higher Education Malaysia as well as the Research University Individual Grant (RUI) from Universiti Sains Malaysia.

I would like to extend my sincere thanks to all members of the lab (Asyraf, Ee Lin, Heng Kean, Rina, Yanti, Yi Fan and Yoong Min), not only for their useful suggestions but also for all the great time, laughter and fun that we had shared.

Last but not least, my heartfelt thanks go to my family members for their unconditional love, understanding and moral support. They selflessly encouraged me to explore new directions in life and seek my own destiny. This journey would not have been possible if not for them, and I dedicate this milestone to them.

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LIST OF ABBREVIATIONS

τ_{fast}	Fast time constant
2D	Two-dimensional
A_{230}	Absorbance at 230 nm
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
ACTB	Beta-actin
ANOVA	Analysis of variance
APS	Ammonium persulfate
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
cg	Core-glycosylated
CO_2	Carbon dioxide
COPII	Coat protein complex II
C_q	Quantification cycle
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DI H_2O	Deionized water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DSP	Dithiobis(succinimidylpropionate)
E	Efficiency
ECL	Enhanced chemiluminescence
EGTA	Ethylene-bis(oxyethylenenitrilo)tetraacetic acid

exp	Exponential
FBS	Fetal bovine serum
FEB	Free energy of binding
fg	Fully-glycosylated
FP	Forward primer
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI_{50}	Half maximal growth inhibitory concentration
h	Hill coefficient
HCl	Hydrochloric acid
HEK293	Human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hERG1	Human ether-a-go-go related gene 1
HRP	Horseradish peroxidase
Hsp	Heat shocked protein
IC_{50}	Half maximal inhibitory concentration
IgG	Immunoglobulin G
I_{max}	Maximum peak tail current
I_{tail}	Peak tail current
I-V	Current-voltage
k	Slope factor
KCl	Potassium chloride
K-Fluoride	Potassium fluoride
K_i	Estimated inhibition constant
KOH	Potassium hydroxide
LB	Luria broth
LC_{50}	Half maximal lethal concentration
MEM	Minimum Essential Medium

$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
Na^+	Sodium ion
NaCl	Sodium chloride
NaOH	Sodium hydroxide
p	Probability
PBS	Phosphate buffered saline
PBST	0.1% (v/v) Tween-20-phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PG	Percentage growth
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride
QSAR	Quantitative structure-activity relationship
R^2	Coefficient of determination
RIPA	Radioimmunoprecipitation assay
RMSD	Root-mean-square deviation
RNA	Ribonucleic acid
RP	Reverse primer
rRNA	Ribosomal ribonucleic acid
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TBE	Tris/Borate/EDTA
TE	Tris/EDTA
TEMED	Tetramethylethylenediamine

TGI	Total growth inhibition
t_{zero}	Time zero
UV	Ultraviolet
v/v	Volume per volume
$V_{1/2}$	Half maximal activation potential
w/v	Weight per volume

LIST OF SYMBOLS

~	Approximately
*	Asterisk
β	Beta
>	Greater than
≥	Greater than or equal to
<	Less than
≤	Less than or equal to
π	Pi
±	Plus or minus
®	Registered trademark
τ	Tau
™	Trademark

LIST OF UNITS

%	Percentage
°C	Degree Celsius
µg	Microgram
µg/ml	Microgram per milliliter
µl	Microliter
µm	Micrometer
µM	Micromole
A	Ampere
Å	Ångström
cells/ml	Cells per milliliter
cm	Centimeter
cm^2	Square centimeter
G	Gram
GΩ	Giga Ohm
h	Hour
kb	Kilobase pair
kPa	Kilopascal
M	Mole
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimole
mOsmol	Milliosmole
ms	Millisecond

mV	Millivolt
MΩ	Mega Ohm
N	Normal
ng	Nanogram
ng/µl	Nanogram per microliter
nm	Nanometer
nM	Nanomole
rpm	Revolutions per minute
s	Second
U/µl	Unit per microliter
V	Volt
x *g*	Times gravity

MEKANISME MOLEKULAR MITRAGYNINE ATAS PERENCATAN SALURAN HERG1A/1B

ABSTRAK

Daun *Mitragyna speciosa* Korth (*M. speciosa*), pada umumnya dikenali sebagai daun ketum di Malaysia, telah digunakan secara meluas untuk merawat penyakit yang lazim seperti jangkitan usus, sakit otot, batuk, cirit-birit, kencing manis, darah tinggi dan untuk memperbaiki peredaran darah. Walau bagaimanapun, keadaan keselamatan dan potensi penyalahgunaan tumbuhan ini yang mempamerkan kesan ketagihan semakin membimbangkan. Profil keselamatan tumbuhan ini tidak dijelaskan sepenuhnya. *Ether-a-go-go-related* gen manusia 1 (hERG1) mengekod subunit yang mendasari pembentukan liang protein saluran lambat kalium (I_{Kr}). Sekatan farmakologi atas I_{Kr} boleh menyebabkan sindrom perolehan QT panjang yang bakal mengakibatkan aritmia jantung yang boleh membawa maut. Justeru, kajian ini bertujuan untuk menyelidik kesan mitragynine, sebatian bioaktif utama dalam ekstrak daun ketum, atas perencatan saluran hERG1a/1b dengan menggunakan kaedah *in silico* dan *in vitro*. Simulasi dok telah dijalankan dengan menggunakan model homologi hERG1 dan interaksi serta affiniti pengikatan antara molekul telah dianalisis. Sel rekombinan HEK293-hERG1a/1b telah dihasilkan untuk menilai kesan mitragynine atas arus dan ungkapan mRNA hERG1a/1b masing-masing dengan menggunakan analisis sistem patch-clamp seluruh sel dan RT-qPCR. Di samping itu, ungkapan protein saluran hERG1a/1b telah dikaji dengan menggunakan analisis Western blot dan imuno pendarfluor. Simulasi dok mencadangkan mitragynine berkemungkinan merupakan perencat hERG1 dengan membentuk interaksi π dengan

asid amino yang penting bagi saluran hERG1, iaitu Tyr652 and Phe656. Sistem patch-clamp seluruh sel yang menggunakan sel rekombinan HEK293-hERG1a/1b telah berjaya dihasilkan dan disahkan dengan beberapa penyekat hERG1 yang diketahui. Sertindole, terfenadine, propafenone dan fluoxetine masing-masing mendedahkan nilai IC_{50} 17.44 nM (h = 0.76), 66.44 nM (h = 0.84), 361.8 nM (h = 1.56) dan 640.5 nM (h = 1.19). Mitragynine didapati menyekat arus hERG1a/1b dengan IC_{50} 332.7 nM (h = 0.61). Namun, mitragynine tidak menunjukkan kesan penyekatan yang nyata atas ungkapan mRNA hERG1a/1b (p > 0.05). Di samping itu, ungkapan protein hERG1a/1b tidak terjejas pada kepekatan yang rendah (p > 0.05). Sebaliknya, analisis Western blot dan imuno pendarfluor menunjukkan pengurangan yang nyata dalam ungkapan protein hERG1a pada 10 µM (p = 0.0217 dan 0.0028). Akan tetapi, ungkapan protein hERG1b tidak terjejas (p = 0.4677 dan 0.2667). Analisis Western blot telah menunjukkan peningkatan yang nyata dalam ungkapan protein hERG1a yang tidak berglikolisis penuh (cg-hERG1a) (p = 0.0188), dan pengurangan yang nyata dalam ungkapan protein hERG1a yang berglikolisis penuh (fg-hERG1a) (p = 0.0217). Pemerhatian ini mencadangkan aliran trafik hERG1a telah terjejas. Namun, mitragynine tidak menyekat interaksi antara cg-hERG1a dengan chaperone sitoplasma, Hsp70 dan Hsp90. Sebaliknya, mitragynine meningkatkan interaksi antara cg-hERG1a dengan Hsp90 (p = 0.0101). Pemerhatian ini mencadangkan bahawa mitragynine menyekat aliran trafik hERG1a dengan menggunakan mekanisme yang lain. Kesimpulannya, mitragynine merencat arus hERG1a/1b dengan penyekatan terus saluran hERG1a/1b pada kepekatan yang rendah serta pengurangan ungkapan protein hERG1a/1b pada kepekatan yang tinggi. Kajian ini mencadangkan alkaloid ini berkemungkinan menyebabkan ketoksikan terhadap jantung.

MOLECULAR MECHANISMS OF MITRAGYNINE INHIBITION ON HERG1A/1B CHANNEL

ABSTRACT

The leaves of *Mitragyna speciosa* Korth (*M. speciosa*), commonly known as ketum in Malaysia, have been widely used to treat common illnesses such as intestinal infections, muscle pain, cough, diarrhea, diabetes, hypertension and to improve blood circulation. Nevertheless, there are growing concerns about the safety and abuse or misuse potentials of the plant which exhibits addictive effects. The safety profile of the plant is not fully elucidated. The *human ether-a-go-go-related gene* 1 (hERG1) encodes the pore-forming subunit underlying cardiac rapidly delayed rectifier potassium current (I_{Kr}). Pharmacological blockade of the I_{Kr} can cause acquired long QT syndrome, which may lead to lethal cardiac arrhythmias. Hence, this study aims to investigate the effects of mitragynine, the major bioactive compound derived from the leave extracts of ketum, on hERG1a/1b channel inhibition, using both *in silico* and *in vitro* approaches. Docking simulations were performed using a hERG1 homology model and their molecular interactions and binding affinities were analyzed. A recombinant HEK293-hERG1a/1b cell line was established and subsequently used to evaluate the effects of mitragynine on hERG1a/1b current and mRNA expression using whole cell patch clamp and RT-qPCR analyses respectively. In addition, the protein expression of hERG1a/1b channels was studied using both Western blot and immunofluorescence analyses. *In* silico study shows that mitragynine is likely a hERG1 inhibitor by forming π interactions with the important residues, Tyr652 and Phe656, of the hERG1 channel. Whole cell patch clamp system using the recombinant

HEK293-hERG1a/1b cell line was successfully established and verified using several known hERG1 blockers. Sertindole, terfenadine, propafenone and fluoxetine revealed IC_{50} values of 17.44 nM (h = 0.76), 66.44 nM (h = 0.84), 361.8 nM (h = 1.56) and 640.5 nM (h = 1.19) respectively. On the other hand, mitragynine was found to inhibit the current with an IC_{50} of 332.7 nM (h = 0.61). However, mitragynine did not show significant inhibitory effect ($p > 0.05$) on the hERG1a/1b mRNA expression. At lower concentration, the hERG1a/1b protein expression was not affected ($p > 0.05$). Interestingly, Western blot and immunofluorescence analyses showed a significant reduction in the hERG1a protein expression at 10 µM (p = 0.0217 and 0.0028 respectively), but not hERG1b (p = 0.4677 and 0.2667 respectively). Western blot analysis showed an increase in protein expression of core-glycosylated (cg) hERG1a (p = 0.0188) but a reduction in expression of fully-glycosylated (fg) hERG1a (p = 0.0217), suggesting an impaired hERG1a trafficking. Nevertheless, mitragynine did not inhibit the interactions between cg-hERG1a and cytosolic chaperones, Hsp70 and Hsp90. On the other hand, mitragynine treated cells have up-regulated the hERG1a-Hsp90 complexes (p = 0.0101), indicating a different trafficking inhibition pathway by mitragynine. In conclusion, mitragynine inhibits hERG1a/1b current by direct channel blockade at lower concentration and reduces hERG1a/1b channel protein expression at higher concentration, suggesting the possibility of the alkaloid to cause cardiac toxicity.

CHAPTER 1

INTRODUCTION

1.1 Problem statement

In recent years, there has been an increased demand for herbal medicines all over the world, including Malaysia. According to World Health Organization (WHO), herbal medicines consist of herbs, herbal materials, herbal preparations and finished herbal products, which contain parts of plants, other plant materials, or combinations, as active ingredients (WHO, 2017). Herbal medicines are widely used by the public for general health care, for the prevention or treatment of minor ailments, as well as for chronic illnesses (Aziz and Tey, 2009; Sofowora *et al.*, 2013).

A survey conducted in 2009 indicated that 88.9% and 87.3% of the Malaysian population utilized biologically-based remedy such as supplements, vitamins and herbs for health problem and health maintenance respectively (Siti *et al.*, 2009). One of the key reasons for the public to rely on herbal medicines is that these products are relatively inexpensive. In addition, majority of the public believe that herbal medicines do not contain harmful chemicals and are free of side effects comparing to commercially available pharmaceutical drugs (Law and Soon, 2013). This is not always true, especially when herbs are consumed with prescription drugs, over-the-counter medications, or other herbs, in which drug-herb interactions are very commonly reported (Wachtel-Galor and Benzie, 2011; Singh and Zhao, 2017).

The widespread use of herbal medicines by the Malaysian population is of special concern, particularly when herbal medicines are not strictly controlled by the Malaysian Drug Control Authority (DCA). The safety and quality assessment of herbal medicines by the DCA is restricted to regulation on the content of specified

contaminants and adulterants such as microorganisms and heavy metals (Aziz and Tey, 2009; Chandra Siri *et al.*, 2013). Lack of knowledge and scientific evidence on the safety of herbal medicines may pose additional risk to the public.

Mitragyna speciosa Korth from the Rubiaceae (coffee) family is a medicinal plant which is native to countries in South-East Asia, especially in Thailand, Malaysia, Indonesia and Papua New Guinea. It is commonly known as "ketum" or "Biak-Biak" in Malaysia or "kratom" in Thailand (Ahmad and Aziz, 2012; Hassan *et al.*, 2013). In Malaysia, the plant is predominantly found in the northern states, especially Perlis and Kedah, and it is commonly sold as beverages and teas (Vicknasingam *et al.*, 2010; Ahmad and Aziz, 2012). Ketum leaves have been used by natives to treat common illnesses, for instance, intestinal infections, cough, diarrhea, muscle pain, diabetes and hypertension, as well as to improve blood circulation (Adkins *et al.*, 2011; Ahmad and Aziz, 2012; Hassan *et al.*, 2013). Moreover, the leaves are popular for their energizing and pain alleviating effects that are known to possess psychostimulant- and addictive-like properties, depending on the dosage taken (Singh *et al.*, 2014).

Apart from traditional uses, ketum is often misused for recreational purposes. A survey conducted in two northern states of Malaysia (Kedah and Penang) revealed that 90% of the subjects relied on ketum as an inexpensive alternative to the more expensive opiates, and 84% used it to alleviate their opioid withdrawal symptoms (Vicknasingam *et al.*, 2010). Another recent study indicated that more than half of the regular ketum users developed severe ketum dependence problems, whereas 45% showed a moderate ketum dependence (Singh *et al.*, 2014).

Usage of ketum is no longer restricted to its native country. In recent years, ketum containing preparations, which are in the form of dried leaf, resin or crude

extracts, are widely accessible on the internet and users can conveniently buy them online (Schmidt *et al.*, 2011; Cinosi *et al.*, 2015). Ketum is generally consumed deliberately to gain the desirable effects of euphoria, to manage opiate withdrawal, or to ease chronic pain. Most users prefer self-treat to avoid the stigma of being classified as drug-dependent (Ramanathan and Mansor, 2014). Several poisoning and fatal cases related to ketum and mitragynine (the main alkaloid in the leave extracts of ketum) have been reported. However, the underlying causes remain ambiguous (Boyer *et al.*, 2008; Nelsen *et al.*, 2010; Kronstrand *et al.*, 2011; Neerman *et al.*, 2013).

Cardiac toxicity is one of the major reasons responsible for the suspension of preclinical or clinical drug discovery programs, for severe adverse drug reactions, and for withdrawal of licensed drugs (Schramm *et al.*, 2014). Between 1957 and 2007, there were 47 cases of post-marketing withdrawal of drugs, in which 45% of these were due to concerns about cardiac toxicity (Redfern *et al.*, 2010). Likewise, 27% of the potential new drug molecules failed in the pre-clinical stage since 1990s due to cardiac toxicity (Turner *et al.*, 2014). The risk of developing Torsade de Pointes (TdP), a lethal cardiac arrhythmia which is portrayed by long QT interval in electrocardiogram, has been the main reason for the removal of approximately 26% of post-marketed drugs between 1990 and 2005 (Valentin, 2010; Kothari *et al.*, 2015).

The human ether-a-go-go related gene (hERG1) channel is the currently most important target for cardiac safety evaluation (Gintant *et al.*, 2016; Grandi *et al.*, 2017). Inhibition of hERG1 by a wide range of drug molecules can prolong repolarization of the cardiac action potential and eventually lead to serious complication, such as TdP and sudden cardiac death (Kallergis *et al.*, 2012). To reduce cardiac risks following drug development, both the pharmaceutical industry and U.S. Food and Drug Administration (FDA) have mandated *in vitro* cardiac toxicity screening early in new

chemical entity (NCE) development (FDA, 2005a; Liang *et al.*, 2013). Nevertheless, little is known about the hERG1 inhibitory potential of herbal medicines (Schramm *et al.*, 2014). To date, several natural compounds under the class of flavonoids (e.g., acacetin and naringenin) and alkaloids (e.g., changrolin, dauricine, and lobeline) are known as hERG1 blockers *in vitro* (Zitron *et al.*, 2005; Schramm *et al.*, 2014; De Mieri *et al.*, 2015). Furthermore, it was revealed that drinking 1 L of pink grapefruit juice, which contains large amounts of naringenin, led to significant QT prolongation in healthy volunteers (Zitron *et al.*, 2005). However, the cardiac toxicity of ketum or mitragynine is poorly understood. Hence, there is a vital need to evaluate comprehensively the potential risks of ketum or mitragynine in hERG1 channel inhibition.

1.2 Objectives of study

1.2.1 General objective

The general objective of the present work is to determine the molecular mechanism of mitragynine inhibition on the hERG1a/1b channel.

1.2.2 Specific objectives

The specific objectives of the present work are:

1. To predict interaction of hERG1 with mitragynine and its analogues using an *in silico* approach.
2. To develop a whole-cell patch-clamp assay using a recombinant HEK293 cell line expressing human ether-a-go-go related gene 1a/1b (hERG1a/1b) channel.
3. To determine the effects of mitragynine on hERG1a/1b current.

4. To determine the effects of mitragynine on hERG1a/1b mRNA and protein expression.
5. To determine the effects of mitragynine on the interactions between hERG1a protein and cytosolic chaperones Hsp70 and Hsp90.

CHAPTER 2

LITERATURE REVIEW

2.1 Mitragynine and its analogues

Mitragynine is a major bioactive compound isolated from the leave extracts of *Mitragyna speciosa* Korth, which is commonly known as "ketum" in Malaysia. The plant is a tall leafy plant which can grow to a normal height of 4 – 9 m and 5 m wide and it grows mainly in tropical and sub-tropical regions of South-East Asia and Africa. (Hassan *et al.*, 2013). The leaves of the plant are either made into drink, chewed when freshly picked, or smoked when dried (Ahmad and Aziz, 2012).

Ketum is well-known for its addictive properties. The usage of ketum in Malaya (Peninsula Malaysia in olden days) was first reported by Burkill in 1935 as a wound poultice, remedy for fever and an alternative for opium (Burkill, 1935; Ahmad and Aziz, 2012). Traditionally, ketum has been used by the natives to disengage addicts from heroin addiction, to cure diarrhea, to treat diabetes as well as to improve blood circulation (Chan *et al.*, 2005; Singh *et al.*, 2017). In addition, the leaves of the plant have long been consumed by male manual laborers to enhance physical endurance and as a means to overcome stress (Vicknasingam *et al.*, 2010; Singh *et al.*, 2016). Opiate addicts rely on ketum to reduce consumption of more expensive opiates, to alleviate opium withdrawal symptoms and to treat chronic pain (Boyer *et al.*, 2008; Vicknasingam *et al.*, 2010; Cinosi *et al.*, 2015). Nevertheless, long-term consumption of ketum can lead to misuse, abuse, dependence and addiction (Boyer *et al.*, 2008; Trakulsrichai *et al.*, 2013; Galbis-Reig, 2016). Typical withdrawal symptoms reported include aggression, hostility, excessive tearing, inability to work and jerky movements of limbs, whereas long-term usage of ketum has been known to cause symptoms such as insomnia, anorexia and weight loss (Suwanlert, 1975; Ahmad and Aziz, 2012).

Extensive phytochemical studies carried out since the 1960s have revealed the natural occurrence of alkaloids, terpenoids and flavonoids in the plant material of the genus *Mitragyna* (Brown *et al.*, 2017). The major phytochemicals isolated and characterized from the plant *M. speciosa* (ketum) are the alkaloid compounds (summarized in Table 2.1) (Beckett *et al.*, 1966a; Beckett *et al.*, 1966b; Shellard *et al.*, 1978a; Shellard *et al.*, 1978b; Houghton and Said, 1986; Houghton *et al.*, 1991; Takayama *et al.*, 1998; Takayama *et al.*, 1999; Takayama *et al.*, 2000; Takayama, 2004; Kitajima *et al.*, 2006; Hassan *et al.*, 2013; Ali *et al.*, 2014; Brown *et al.*, 2017). The main pharmacologically active alkaloids derived from the ketum leaves are the indole alkaloids, mitragynine and 7-hydroxymitragynine (7-HMG) (Warner *et al.*, 2016). Mitragynine, accounting for approximately 12-66% of the total alkaloid mixture, appears to be the dominant active alkaloid and is exclusive to the plant whereas 7-HMG accounts for only up to 2% (Hassan *et al.*, 2013; Warner *et al.*, 2016).

The alkaloid content varies depending on stage of maturity or ecotype of the plant (Shellard *et al.*, 1978b; Takayama *et al.*, 1998; Leon *et al.*, 2009; Brown *et al.*, 2017). Depending on ecotype, mitragynine makes up approximately 66% of the total alkaloid content in plants of Thai origin, whereas it constitutes only 12% in plants of Malaysian origin (Ponglux *et al.*, 1994; Takayama *et al.*, 1998; Brown *et al.*, 2017). Furthermore, some of the alkaloids were only found in the Malaysian sample, for instance, mitragynaline, 3,4,5,6-tetradehydromitragynine, mitralactonal and mitrasulgynine (Takayama *et al.*, 1998; Hassan *et al.*, 2013). On the other hand, the major alkaloid derived from the leaves of the plant grown in the United States at the University of Mississippi is the oxindole-type mitraphylline, instead of mitragynine (Leon *et al.*, 2009; Gogineni *et al.*, 2015). In addition, mitragynine and paynantheine were found abundantly in the mature leaves of ketum, whereas speciogynine (Thai

Table 2.1 Alkaloid profile of *Mitragyna speciosa* Korth.

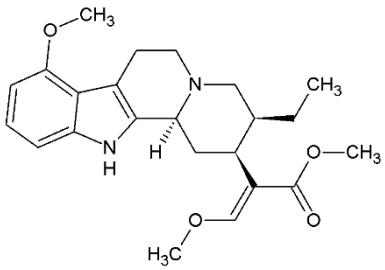
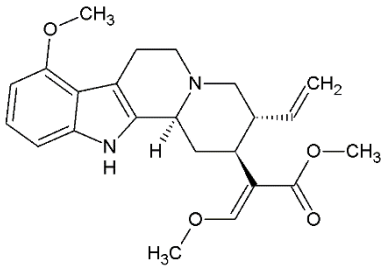
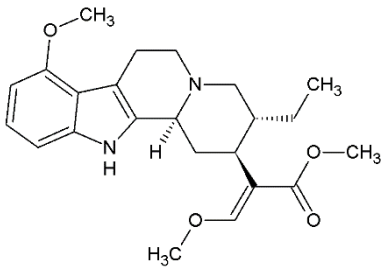
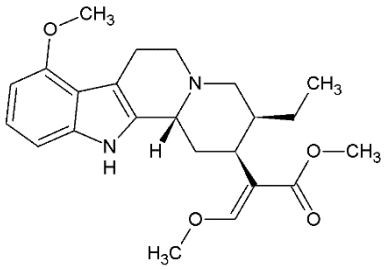
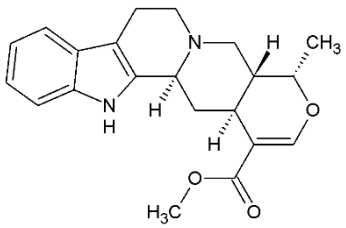
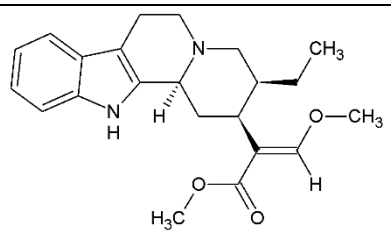
Alkaloid	Chemical structure	Reference(s)
Indole alkaloids		
Mitragynine		Beckett *et al.*, 1966b; Leon *et al.*, 2009; Ali *et al.*, 2014; Lesiak *et al.*, 2014; Wang *et al.*, 2014; Avula *et al.*, 2015; Lesiak and Musah, 2016
Paynantheine		Beckett *et al.*, 1966b; Leon *et al.*, 2009; Ali *et al.*, 2014; Lesiak *et al.*, 2014; Wang *et al.*, 2014; Avula *et al.*, 2015; Lesiak and Musah, 2016
Speciogynine		Beckett *et al.*, 1966b; Ali *et al.*, 2014; Lesiak *et al.*, 2014; Wang *et al.*, 2014; Avula *et al.*, 2015
Speciociliatine		Beckett *et al.*, 1966b; Ali *et al.*, 2014; Lesiak *et al.*, 2014; Wang *et al.*, 2014; Avula *et al.*, 2015
Ajmalicine		Beckett *et al.*, 1966b; Leon *et al.*, 2009; Lesiak *et al.*, 2014; Lesiak and Musah, 2016
Corynantheidine		Beckett *et al.*, 1966b; Leon *et al.*, 2009

Table 2.1 Continued

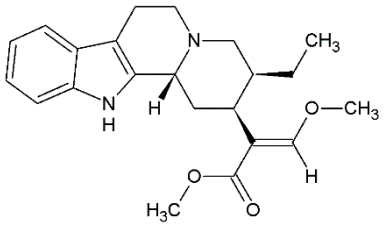
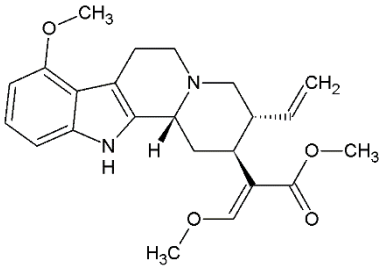
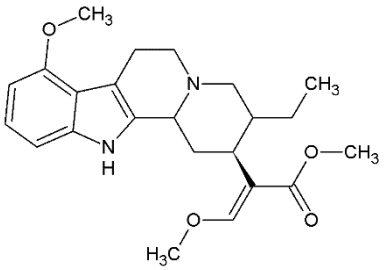
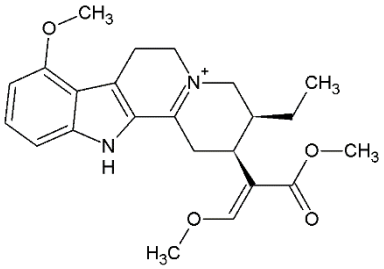
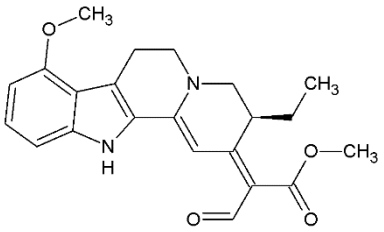
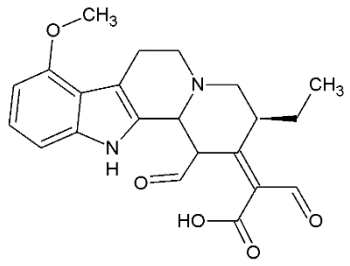
Alkaloid	Chemical structure	Reference(s)
Isocorynantheidine		Shellard *et al.*, 1978b; Leon *et al.*, 2009
3-isopaynantheine		Shellard *et al.*, 1978b; Ali *et al.*, 2014; Wang *et al.*, 2014; Avula *et al.*, 2015
Mitraciliatine		Shellard *et al.*, 1978b Lesiak *et al.*, 2014;
3-dehydromitragynine		Houghton and Said, 1986
Mitragynaline		Houghton *et al.*, 1991; Takayama *et al.*, 2001
Mitragynalinic acid		Houghton *et al.*, 1991

Table 2.1 Continued

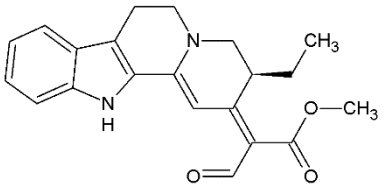
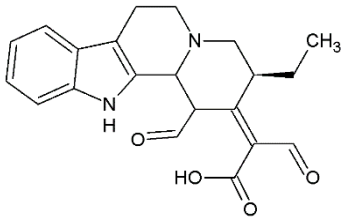
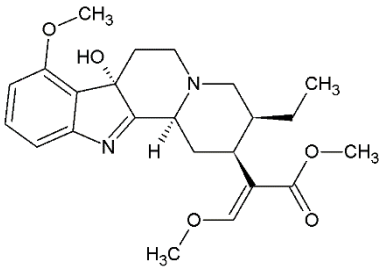
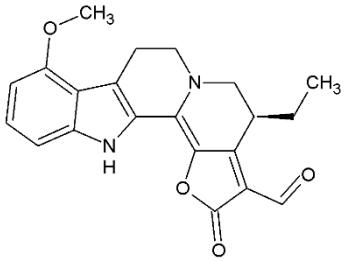
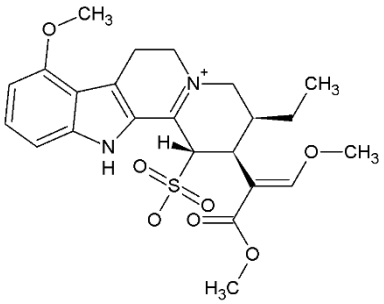
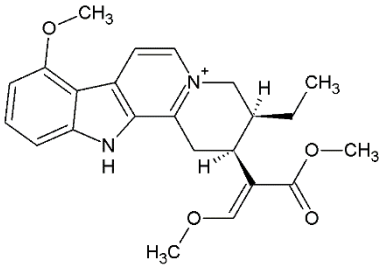
Alkaloid	Chemical structure	Reference(s)
Corynantheidaline		Houghton *et al.*, 1991; Takayama *et al.*, 2001
Corynantheidinalinic acid		Houghton *et al.*, 1991
7-hydroxymitragynine		Ponglux *et al.*, 1994; Leon *et al.*, 2009; Lesiak *et al.*, 2014; Avula *et al.*, 2015; Lesiak and Musah, 2016
Mitralactonal		Takayama *et al.*, 1998
Mitrasulgynine		Takayama *et al.*, 1998
3,4,5,6-tetradehydromitragynine		Takayama *et al.*, 1998

Table 2.1 Continued

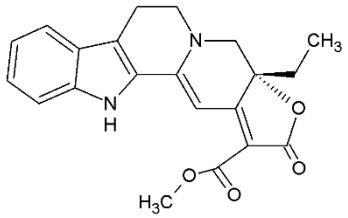
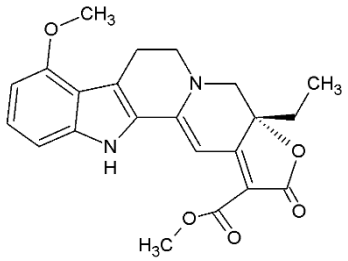
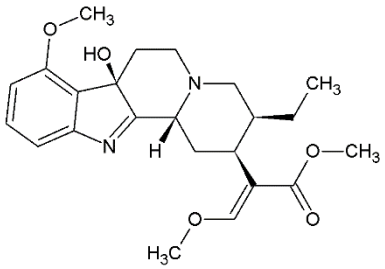
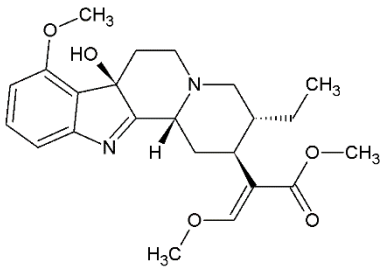
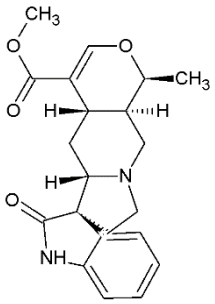
Alkaloid	Chemical structure	Reference(s)
Mitralactonine		Takayama *et al.*, 1999
9-methoxymitralactonine		Takayama *et al.*, 2000
7-hydroxyspeciociliatine		Kitajima *et al.*, 2006
7β-hydroxy-7H-mitraciliatine		Ali *et al.*, 2014; Avula *et al.*, 2015
Oxindole alkaloids		
Mitraphylline		Beckett *et al.*, 1966a; Leon *et al.*, 2009; Lesiak *et al.*, 2014; Lesiak and Musah, 2016

Table 2.1 Continued

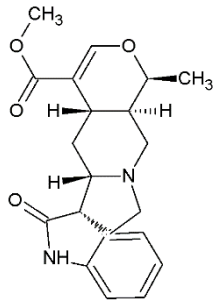
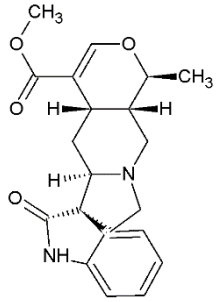
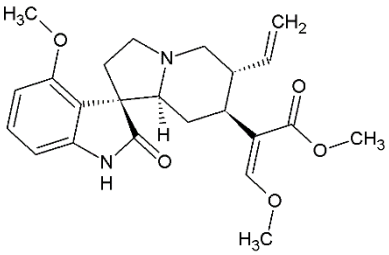
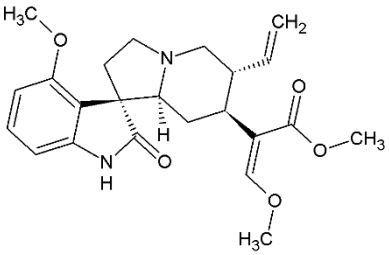
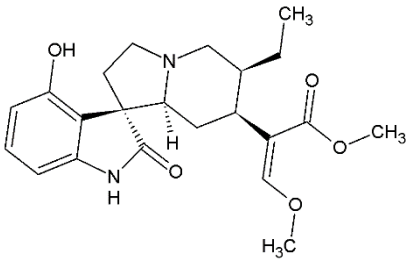
Alkaloid	Chemical structure	Reference(s)
Isomitraphylline		Beckett *et al.*, 1966a; Leon *et al.*, 2009
Speciophylline		Beckett *et al.*, 1966a
Specionoxeine		Trager *et al.*, 1968; Shellard *et al.*, 1978b
Isospecionoxeine		Trager *et al.*, 1968; Shellard *et al.*, 1978b
Mitrafoline		Hemingway *et al.*, 1975; Shellard *et al.*, 1978a

Table 2.1 Continued

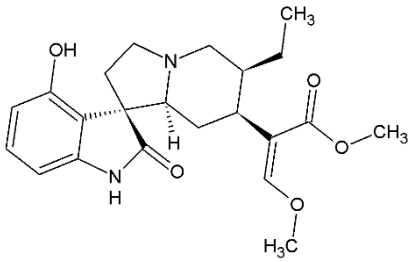
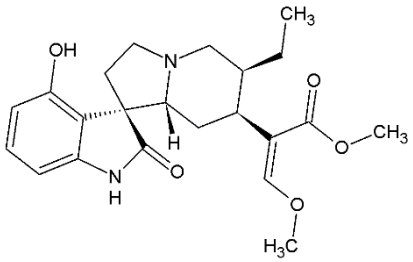
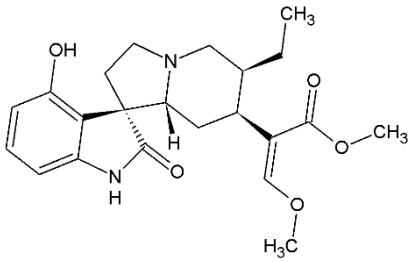
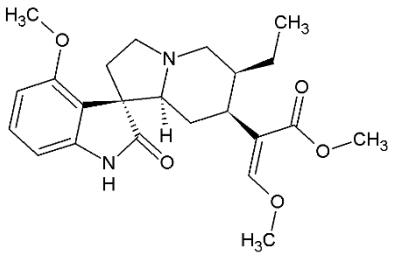
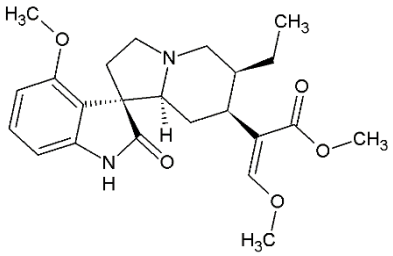
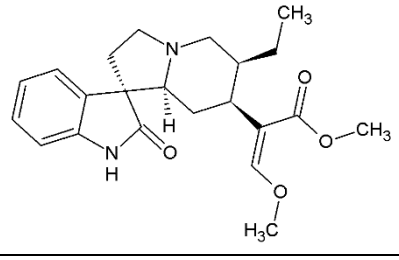
Alkaloid	Chemical structure	Reference(s)
Isomitrafoline		Hemingway *et al.*, 1975; Shellard *et al.*, 1978a
Speciofoline		Hemingway *et al.*, 1975; Shellard *et al.*, 1978a
Isospeciofoline		Hemingway *et al.*, 1975; Shellard *et al.*, 1978a; Ali *et al.*, 2014; Avula *et al.*, 2015
Mitragynine oxindole A		Shellard *et al.*, 1978a
Mitragynine oxindole B		Shellard *et al.*, 1978a
Corynoxine A		Shellard *et al.*, 1978a; Ali *et al.*, 2014; Wang *et al.*, 2014; Avula *et al.*, 2015

Table 2.1 Continued

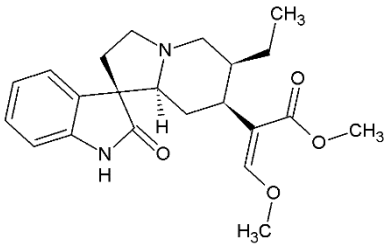
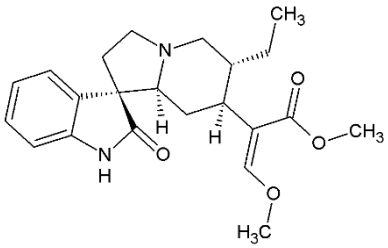
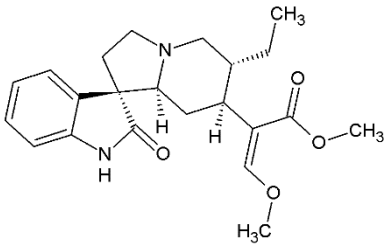
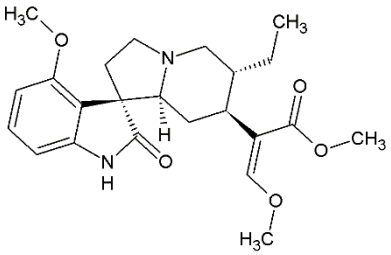
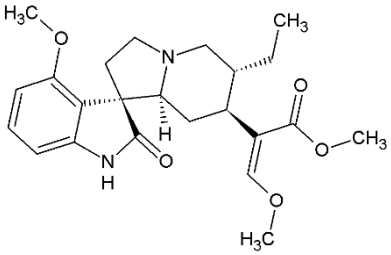
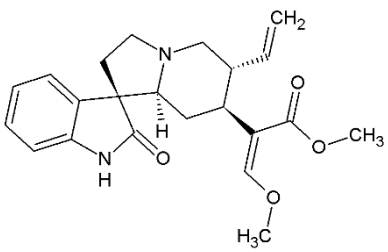
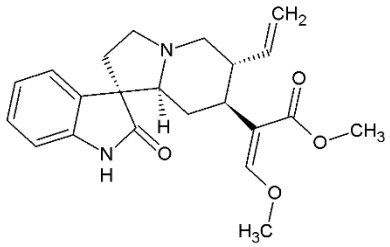
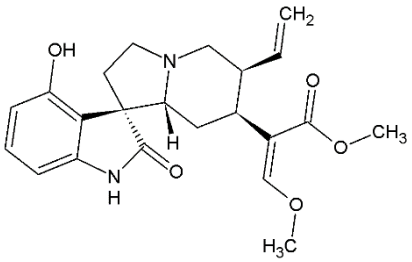
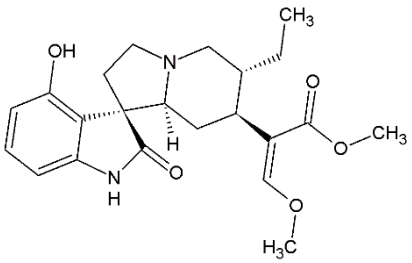
Alkaloid	Chemical structure	Reference(s)
Corynoxine B		Shellard *et al.*, 1978a; Ali *et al.*, 2014; Wang *et al.*, 2014; Avula *et al.*, 2015
Rhynchophylline		Shellard *et al.*, 1978a; Lesiak *et al.*, 2014; Lesiak and Musah, 2016
Isorhynchophylline		Shellard *et al.*, 1978a
Rhyncociline		Shellard *et al.*, 1978a
Ciliaphylline		Shellard *et al.*, 1978a
Corynoxeine		Shellard *et al.*, 1978a; Kitajima *et al.*, 2006; Lesiak *et al.*, 2014; Lesiak and Musah, 2016

Table 2.1 Continued

Alkaloid	Chemical structure	Reference(s)
Isocorynoxeine		Kitajima *et al.*, 2006
Isospeciofoleine		Ali *et al.*, 2014; Avula *et al.*, 2015
Isorotundifoline		Ali *et al.*, 2014; Avula *et al.*, 2015

samples) as well as mitragynaline and corynantheidaline (Malaysian samples) appeared to be the dominant alkaloids in the very young leaves of ketum, suggesting the variation of alkaloid contents in plants of different maturity (Shellard *et al.*, 1978a; Shellard *et al.*, 1978b; Houghton *et al.*, 1991; Brown *et al.*, 2017).

The usage of ketum has gained increasing popularity in the Western countries since the herbal products can be easily accessible over the internet with minimal regulation of their sale (Adkins *et al.*, 2011; Singh *et al.*, 2016). People consume them primarily for recreational purposes, to ease dependence on alcohol, and to cure anxiety and depression (McWhirter and Morris, 2010; Cinosi *et al.*, 2015). Due to the concerns over addiction, possessing or selling ketum leaves or preparations in any form are prohibited in Malaysia, Thailand, Australia and Myanmar, and are listed as controlled drugs in many European Union countries, such as Denmark, Poland and Sweden (Hassan *et al.*, 2013). Despite the tree and its leaves being illegal, there is still a widespread misuse of ketum by the locals in Malaysia and Thailand (Adkins *et al.*, 2011; Singh *et al.*, 2016). In August 2016, the US Drug Enforcement Administration (DEA) announced its plans to list ketum, mitragynine and 7-HMG as controlled substances. However, the plan was temporary put on hold due to public outcry (Prozialeck, 2016). Currently, ketum and ketum-derived drugs are legal in the US, except in certain states like Alabama, Arkansas, Indiana, Tennessee, Vermont and Wisconsin (Prozialeck, 2016). Meanwhile, ketum is legally cultivated in Indonesia and exported on large scale to Asia, Europe and North America, making it harder for the authorities to control the access of ketum (Hassan *et al.*, 2013).

In spite of the wide range of pharmacological effects, reports of toxicity related to ketum use are scarce. Most of the *in vivo* studies involved the acute oral toxicity of ketum extracts in animal models. In addition, clinical toxicological investigations were

limited to a few human case-reports concerning the toxic effect of ketum extracts following short or long term consumption (Sabetghadam *et al.*, 2013a). Ketum and its related products have been linked to severe drug toxicity. Cases of ketum use associated with seizures have been reported after co-administering the herb with modafinil (Boyer *et al.*, 2008) and *Datura stramonium* (Nelsen *et al.*, 2010). Majority, but not all, of the cases linked to death involved those who had abused other substances along with ketum or had histories of alcohol dependence or heroin abuse (Singh *et al.*, 2016). Ketum has been shown to cause serious adverse effects, such as elevated blood pressure, nephrotoxic effects, impaired cognition and behavior, dependence potential, and hepatic failure (Apryani *et al.*, 2010; Harizal *et al.*, 2010; Kapp *et al.*, 2011; Cinosi *et al.*, 2015; Yusoff *et al.*, 2016). However, the cardiac toxicity of ketum and its analogues is not clearly known (Lu *et al.*, 2014).

Lu and co-workers (2014) reported an *in vitro* cardiac toxicity study of mitragynine and its analogues using recombinant HEK293 cell expressing human ether-a-go-go-related gene 1a channel (HEK293-hERG1a) and human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). They found that 10 µM mitragynine significantly reduced the rapid outward delayed rectifier potassium current (I_{Kr}) current in HEK293-hERG1a. In addition, mitragynine, paynantheine, speciogynine and speciociliatine dosage-dependently (0.1 – 100 µM) suppressed I_{Kr} in hiPSC-CMs by 67% - 84% with IC_{50} values ranging from 0.91 to 2.47 µM. Furthermore, mitragynine at 10 µM was found to significantly prolong the action potential duration (APD) at 50 and 90% repolarization respectively (APD50 and APD90). They concluded that these alkaloids may trigger TdP via inhibition of I_{Kr} in human cardiomyocytes (Lu *et al.*, 2014). Nevertheless, the effects of mitragynine on channel trafficking remained unknown. Apart from acute hERG1 inhibition via

channel blockade, hERG1 channel trafficking defect represents the alternative yet more chronic cellular mechanism of hERG1 inhibition (Steele *et al.*, 2007; Nogawa and Kawai, 2014).

2.2 The cardiac action potential

A body surface electrocardiogram (ECG) demonstrates the electrophysiological events taking place during impulse generation and conduction in the heart. Each heartbeat starts as an electrical stimulus generated in the sinoatrial (SA) node and is rapidly spreading out through the atria. The impulse from the SA node eventually passes through the atrioventricular (AV) node and rapidly propagates to both ventricles via the His-Purkinje system. The 'P' wave on the ECG measurement reflects the combined electrical activity of action potential depolarization in the atria. The QRS complex represents the action potential depolarization occurring in the ventricles, whereas the T wave corresponds to the ventricular repolarization. Hence, the QT interval of an ECG, which is measured in milliseconds (ms), demonstrates the duration of the ventricular depolarization and repolarization, plus the time associated with transmission across the cardiac muscles (Figure 2.1) (Finlayson *et al.*, 2004; Amin *et al.*, 2010; Qu *et al.*, 2014).

The generation of cardiac action potential is contributed by the selective permeability of ion channels distributed on the cardiomyocytes membrane, as shown in Figure 2.2. The inward depolarizing currents, primarily through Na^+ and Ca^{2+} channels, contribute to normal depolarization, whereas outward repolarizing currents, predominantly through K^+ channels, result in repolarization (van Noord *et al.*, 2010). The human ventricular action potential consists of five sequential phases (phases 0 to 4). Initially, the cell is polarized near to the equilibrium potential for potassium ions

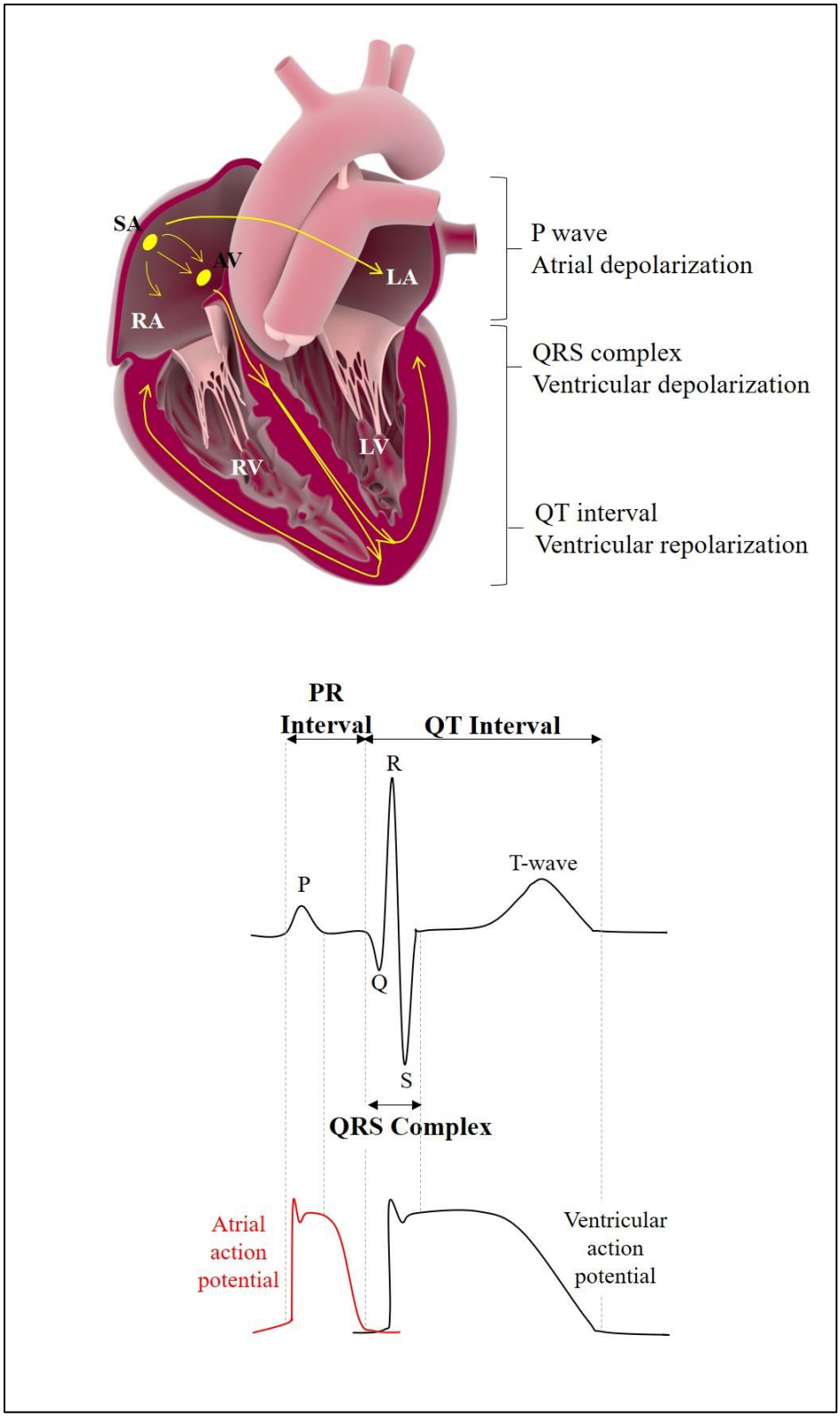


Figure 2.1 The electrical activity of the heart presented on the surface electrocardiogram (ECG). The 'P' wave reflects the combined electrical activity of action potential depolarization in the atria. The QRS complex represents the action potential depolarization in the ventricles, whereas the T wave corresponds to the ventricular repolarization. (Adapted from Amin *et al.*, 2010 and Qu *et al.*, 2014)

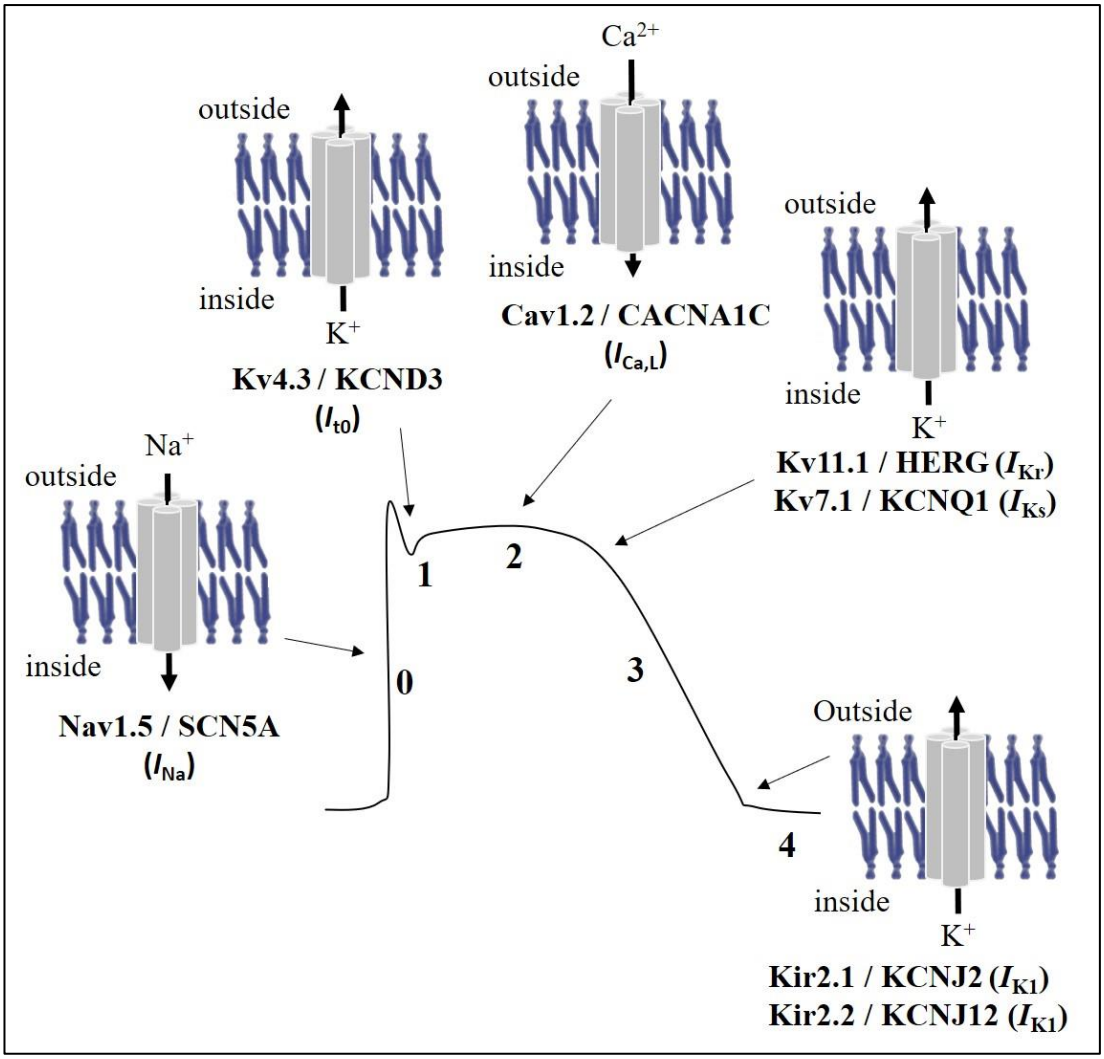


Figure 2.2 The ventricular cardiac action potential and cardiac ion channels. The upstroke (phase 0) is triggered by the large inward Na^+ current (I_{Na}). The rest of the action potential (phase 1 to 4) is controlled by the interplay of depolarizing Ca^{2+} channels (mainly Cav1.2) and repolarizing K^+ channels that regulate the transient outward current (I_{t0}), the rapid and slow delayed rectifiers currents (I_{Kr} and I_{Ks}) and the inward rectifier current (I_{K1}). (Adapted from Pollard *et al.*, 2010 and George, 2013)

(E_K) due to the high K^+ conductance at rest (phase 4). A rapid depolarization of the membrane (phase 0) is triggered by the large inward Na^+ current. Repolarization is much slower and occurs in three phases. A brief partial repolarization, which lasts only a few milliseconds, follows rapidly (phase 1); this is caused by inactivation of fast Na^+ channels and a transient outward current (I_{t0}), comprising of two components, one a Cl^- current, and the other by a K^+ current. A much slower rate of repolarization, known as the plateau phase (phase 2), of the action potential then follows; this is a dynamic equilibrium between inward movement of Ca^{2+} through L-type calcium channels and outward movement of K^+. The plateau phase delays the cardiac action potential duration because the K^+ channels that repolarize the membrane either activate slowly, inactivate rapidly, or conduct less current at positive transmembrane potentials. A long action potential allows sufficient time for influx of extracellular Ca^{2+} for optimum excitation-contraction coupling and also renders the cardiomyocytes comparatively refractory to premature excitation. Subsequently, the cell membrane slowly repolarizes when K^+ efflux exceeds Ca^{2+} influx. This repolarization (phase 3) increases the conductance of some K^+ channels while reduces the Ca^{2+} current, resulting in a net outward positive current. The augmenting K^+ current rapidly induces complete repolarization and returns the membrane potential of the cardiac muscle cells to its original resting level (Finlayson *et al.*, 2004; Sanguinetti and Tristani-Firouzi, 2006; Zünkler, 2006; Pollard *et al.*, 2010; George, 2013).

There are three major K^+ currents involving in the repolarization of ventricular myocytes action potential, namely the transient outward K^+ current (I_{t0}), the delayed rectifier K^+ current (I_K) and the inward rectifier K^+ currents (I_{K1}). I_{t0} contributes to the phase 1 repolarization of the action potential, whereas I_{K1} maintains the resting membrane potential and leads to the late repolarization phase (Zünkler, 2006; Hancox

et al., 2008; Jeevaratnam *et al.*, 2017). I_K is the major outward current that is responsible in mediating cardiac action potential repolarization, and thus, is crucial in determining the duration of the action potential. Noble and Tsien (1969) first provided evidence for two kinetically distinct components of the I_K in cardiac myocytes, which they named I_{x1} and I_{x2} (Noble and Tsien, 1969). In 1990, Sanguinetti and Jurkiewicz demonstrated that the two components differed in pharmacological sensitivity, in which certain class III antiarrhythmic drugs selectively blocked the rapid component, which they named I_{Kr}, whereas the slow component, I_{Ks} was highly sensitive to activation by β-adrenergic stimulation (Sanguinetti and Jurkiewicz, 1990). Subsequently, the presence of both components was proven in human ventricular myocytes (Li *et al.*, 1996; Grant, 2009; Jeevaratnam *et al.*, 2017).

2.3 Long QT syndrome (LQTS)

Long QT syndrome (LQTS) is a disorder portrayed by delayed ventricular myocyte repolarization, which is illustrated by abnormal prolongation of the QT interval on the body surface ECG (Figure 2.3) (van Noord *et al.*, 2010; Barsheshet *et al.*, 2014; Gintant *et al.*, 2016). The QT interval is influenced by heart rate and hence the QT interval is normally corrected for heart rate (QTc). Apart from that, the normal and lengthened QTc values rely on gender and age. The American Heart Association, the American College of Cardiology Foundation and the Heart Rhythm Society (AHA/ACCF/HRS) Recommendations for the Standardization and Interpretation of the Electrocardiogram recommended that the QTc greater than 450 ms and 460 ms in adult males and adult females, respectively, be regarded as a prolonged QT interval, whereas QTc shorter than 390 ms be considered a short QT interval (Rautaharju *et al.*, 2009; Postema and Wilde, 2014). Patients with QTc greater than 500 ms are

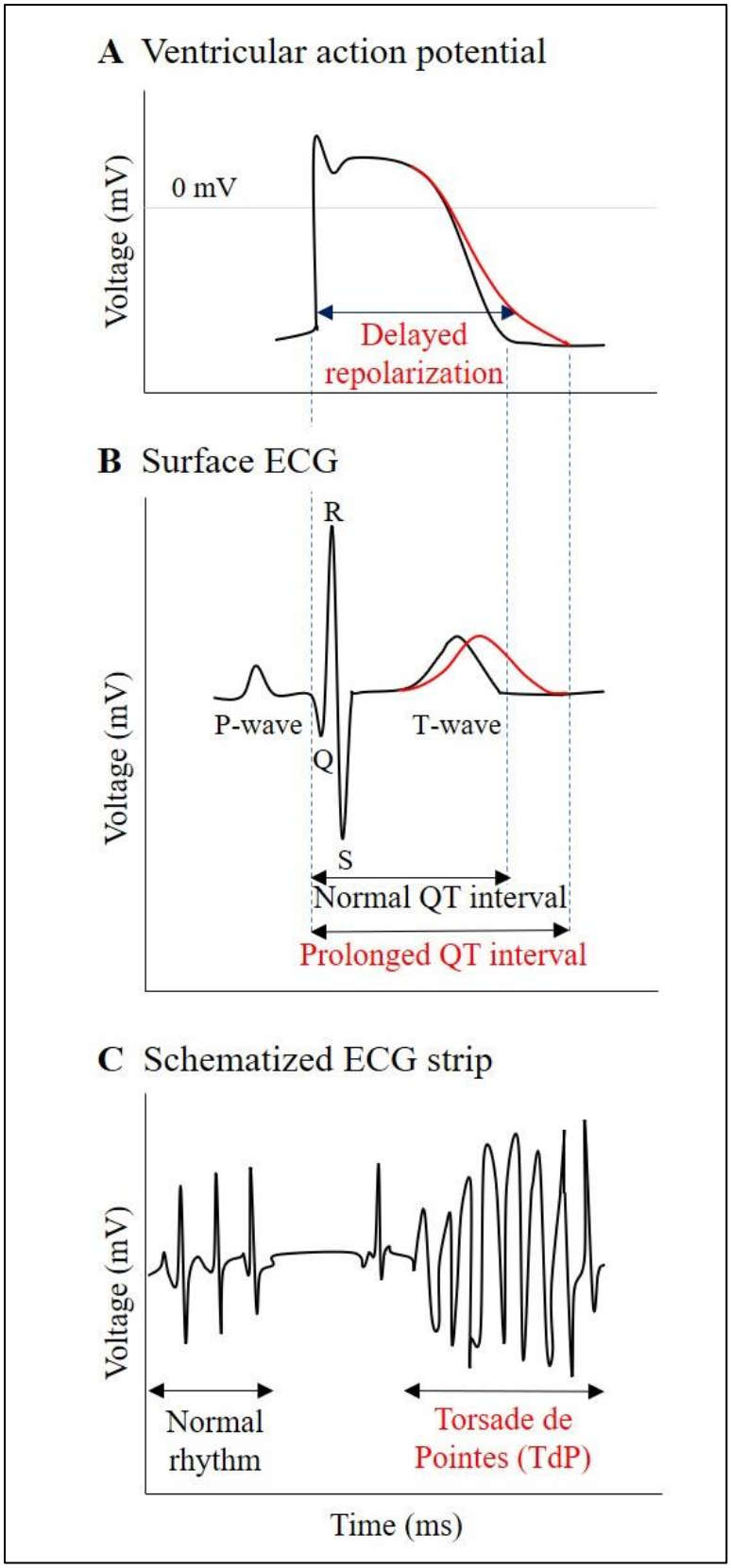


Figure 2.3 Relationship between (A) delayed repolarization, (B) prolonged QT interval and (C) Torsade de Pointes (TdP) cardiac-arrhythmia (Adapted from Gintant *et al.*, 2016).

predisposed to a ventricular tachyarrhythmia, specifically Torsade de Pointes (TdP), characterized by continuously twisting of the QRS complexes around the isoelectric line of the ECG, which either spontaneously terminates or degenerates into ventricular fibrillation, leading to syncope and sudden death (Postema and Wilde, 2014).

2.3.1 Congenital LQTS

Congenital LQTS is an inherited form of LQTS affecting young otherwise healthy individuals and is related to recurrent spontaneous syncope, which often results in cardiac arrest and sudden cardiac death due to ventricular tachyarrhythmia and fibrillation. The estimated prevalence of LQTS is about 1 in 2,500 live births (Finlayson *et al.*, 2004; Crotti *et al.*, 2008; van Noord *et al.*, 2010; Barsheshet *et al.*, 2014).

The first incidence of congenital LQTS may have been depicted by Meissner in Germany in 1856 (Meissner, 1856). He described a deaf girl who collapsed and died while being reprimanded at school. The girl had two brothers who also died suddenly after a severe fright or intense fury. This report preceded the development of ECG and thus the QT intervals of this family were not interpreted (Meissner, 1856; Vincent, 2002; Finlayson *et al.*, 2004). It was more than a century later when Jervell and Lange-Nielsen (1957) presented the electrocardiographic basis for LQTS. In a family with six siblings, four were deaf and suffered repeated syncope during exercise or without apparent cause. Three of them died suddenly, at the ages of 4, 5 and 9 years respectively. Electrocardiographic studies in three of the cases demonstrated a distinct QT interval prolongation. No apparent abnormality of the heart was observed (Jervell and Lange-Nielsen, 1957; Vincent, 2002; Finlayson *et al.*, 2004). Thereafter, Romano and Ward reported families with a cardiac abnormality almost identical to that described by Jervell and Lange-Nielsen but with normal hearing, suggesting a similar