DEVELOPMENT OF THERMOSTABILIZED PCR METHOD FOR THE SIMULTANEOUS DETECTION OF SNPS OF CYP2B6 AND OPRM1 GENES

by

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µl	-Micro liters
1X	-1 times
ADRs	- Adverse Drug Reactions
AIDS	-Acquired Immuno-Deficiency Syndrome
bp	-Base pairs
CCR-5	-C-C chemokine receptor type 5
CYP	-Cytochrome P450
CYP2B6	-Cytochrome P450 2B6 subtype
CYP2C19	-Cytochrome P450 2C19 subtype
CYP2C9	-Cytochrome P450 2C9 subtype
CYP2D6	-Cytochrome P450 2D6 subtype
CYP3A4	-Cytochrome P450 3A4 subtype
ddNTPs	-dideoxynucleotide triphosohates
diln	-Dilution
DNA	-Deoxyribonucleic Acid
EDTA	-Ethylene di-amide tetra acetic acid
EtOH	-Ethanol
FDA	-Food and drug administration
FRET-PCR	-Flourscent Resonance Energy Transfer-Polymerase Chain Reaction
GPCRs	-G-protein coupled receptors
GRKs	-GPCR kinases
HAART	-Highly Active Antiretroviral Therapy
HIV	-Human immunodeficiency virus
KRAS	-V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MAC	-Malaysian AIDS Council
mBar	-Millibars
Mg	-Milligram
MMT	-Methadone Maintenance Therapy

NaOAc	- Sodium acetate
NAT2	-N-acetyltransferase 2
nm	-Nanometers
NNRTI	- Non-nucleoside reverse transcriptase inhibitor
OD	-Optical density
OPRM1	-Mu-opioid receptor gene
PCR	-Polymerase Chain Reaction
PCR-RFLP	-Polymerase chain reaction-restriction fragment length polymorphism
PMol	-Picomoles
Rpm	-Revolutions per minutes
RT-PCR	-Real-time polymerase chain reaction
SNP	-Single nucleotide polymorphism
SSCP	-Single strand conformation polymorphism
TBE	-Tris Borate EDTA
Tm	-melting temperature
UTR	-Untranslated Regions
UV	-Ultra Violet

ABSTRAK

PEMBANGUNAN TINDAK BALAS BERANTAI POLIMERASE BEKU KERING UNTUK PENGENALPASTIAN BERTERUSAN POLIMORFISME SATU NUKLEOTIDA BAGI GEN CYP2B6 DAN GEN OPRM1

Penggunaan heroin secara haram dan HIV adalah dua ancaman utama yang dihadapi oleh Malaysia dan juga negara lain. Majlis AIDS Malaysia melaporkan jumlah keseluruhan kes HIV di Malaysia pada Jun 2009 adalah sebanyak 86,127 kes dan jumlah ini telah meningkat sebanyak 3 kali ganda jika dibandingkan dari tahun 1986. Kadar peningkatan kes HIV dijangka akan menyebabkan 24 juta rakyat Malaysia dijangkiti dengan HIV/AIDS pada tahun 2020 di mana jumlah keseluruhan populasi pada ketika itu dijangka sebanyak 40 juta. Punca utama jangkitan HIV di Malaysia dan juga Asia Timur Selatan adalah disebabkan oleh perkongsian jarum suntikan tercemar di kalangan penagih dadah. Statistik menunjukkan 71% kes HIV di Malaysia adalah kerana perkongsian jarum oleh penagih dadah. Metadon dan antiretroviral digunakan secara besar-besaran untuk membasmi masalah ini. Kedua-dua dadah ini dipengaruhi oleh CYP2B6 manakala reseptor opiat μ turut mempengaruhi metadon. Kedua-duanya adalah polymorfisme dari segi genetik. Objektif kami adalah untuk menghasilkan “multiplexed allele- specific PCR” untuk deteksi/pengesanan polymorfisme ini dan mengoptimumkan dengan terapi metadon dan antiretroviral di samping menghasilkan kaedah PCR beku kering supaya memudahkan penggunaan rutin dalam makmal diagnostik. Bahagian gen reseptor opiat μ (OPRM1) dan CYP2B6 digandakan dahulu sebelum digunakan sebagai

template bagi PCR spesifik alel kedua. Tiga “single nucleotide polymorphism” (SNPs) dari CYP2B6 dan tiga SNPs dari OPRM1 disasarkan. Satu set daripada kaedah PCR pertama (bahagian gen masing-masing yang merangkumi SNPs sasaran) dan tiga set daripada PCR kedua (masing-masing menggandakan dua SNPs sasaran) akhirnya dihasilkan. SNPs daripada OPRM1 dan CYP2B6 berjaya digandakan dengan menggunakan kaedah yang telah dibangunkan. Hasil keputusan disahkan dengan menggunakan jujukan DNA secara langsung. Kawalan positif untuk kesemua mutasi dihasilkan dengan menggunakan kaedah “site-directed mutagenesis”. Kawalan positif ini divalidasi dengan menggunakan jujukan DNA secara langsung. Kawalan positif diuji terhadap kaedah PCR yang dibangunkan menunjukkan hasil yang tepat. Kaedah “nested allele-specific” PCR berjaya dihasilkan untuk deteksi SNP daripada OPRM1 dan CYP2B6. Kaedah PCR yang dihasilkan seterusnya dibeku kering dengan menggunakan enzim stabilizer yang bersesuaian. Kaedah PCR beku kering didapati sensitif mengesan sehingga 5 ng DNA yang terdapat dalam sampel. Ujian kestabilan pantas menunjukkan PCR beku kering adalah stabil sehingga setahun pada suhu 4oC. Kaedah ini akan digunakan secara rutin dalam makamal diagnostic untuk mengoptimumkan terapi metadon dan antiretroviral bagi engurangkan risiko penularan HIV.

ABSTRACT

DEVELOPMENT OF THERMOSTABILIZED PCR METHOD FOR THE SIMULTANEOUS DETECTION OF SNPS OF CYP2B6 AND OPRM1 GENES

Illicit heroin use and HIV are double threats faced by Malaysia and some parts of the world. Malaysian AIDS Council reported the total number of HIV cases in Malaysia to be 86,127 by June2009, was merely 3 in the year 1986. With the rate of increase in HIV it is estimated that about 24 million Malaysians will be infected or affected by HIV/AIDS by the year 2020 where the total population is estimated to be 40 million. In Malaysia and parts of South East Asia, the prime vehicle for HIV transmission is the sharing of contaminated injection needles by drug users. Statistics shows that 71% of the HIV cases in Malaysia are due to sharing of needles/syringe by the Injecting drug users. To combat the threats, methadone and antiretrovirals are widely used. These drugs are influenced by CYP2B6 and for methadone, μ Opiate-receptor is also involved. Both are genetically polymorphic. Our objective was to develop a multiplexed allele-specific PCR for the simultaneous detection of these polymorphisms to optimize therapies with methadone and antiretrovirals and to freeze dry the PCR method so as to make it feasible for routine diagnostic laboratory use. Regions from μ Opiate-receptor gene (OPRM1) and CYP2B6 were first amplified and then used as templates for parallel allele-specific second PCR. Three single nucleotide polymorphisms (SNPs) from CYP2B6, and three SNPs from OPRM1were targeted. One set of first PCR method (regions in the respective gene covering the targeted SNPs) and three sets of

second PCR (each amplifying two targeted SNPs) were finally devised. The targeted SNP's from OPRM1 and CYP2B6 were successfully amplified using the developed methods. The results were confirmed by direct DNA sequencing. Positive controls for all these mutations were devised using site-directed mutagenesis approach. These positive controls were validated by direct DNA sequencing. These controls tested against the developed PCR method showed accurate results. A nested allele-specific PCR method was successfully developed for the simultaneous detection of SNP's from OPRM1 and CYP2B6. The PCR method hence developed was freeze dried by using suitable enzyme stabilizer. The freeze dried PCR method was found to be sensitive enough to detect up to 5 ng of DNA present in the sample. Accelerated stability study showed the freeze dried PCR stable up to 1 year at 4°C. This method will be used in routine diagnostic laboratories to optimize therapies with methadone and antiretrovirals to reduce risks for HIV transmission.

Chapter 1

General Introduction

1.1 Introduction

Spread of HIV/AIDS has been threatening the fundaments of Malaysian society along with other parts of Asia. Malaysian AIDS Council reports total number of HIV cases in Malaysia to be 86,127 by June2009, which was merely 3 in the year 1986 (MAC, 2008). With the rate of increase in HIV it is estimated that about 24 millions Malaysians will be infected or affected by HIV/AIDS by the year 2020 where the total population is estimated to be 40 million (MAC, 2008). Demography may change because of the preponderance of the disease among specific groups such as the young males, the gender ratio as of 2008 is 4 men to every women. In Malaysia and parts of South East Asia, the prime vehicle for HIV transmission is the sharing of contaminated injection needles by drug users. Statistics shows that 71% of the HIV cases in Malaysia are due to sharing of needles/syringe by the Injecting drug users (MAC, 2008). Thus, the dual threat of illicit drug use and HIV is increasing in Malaysia. To encounter the problem Malaysian government introduced Methadone Maintenance Therapy (MMT) in the year 2006, with the objective of removing the need for the sharing of contaminated needles by injecting drug users as well as to reduce overall harms associated with illicit drug use (Nasir et al, 2010).

MMT is the most effective management of opiate dependence. It have been used world wide to reduces illicit opiate use along with the objectives to improves personal and

social functioning and also reduces drug related crimes. Most importantly it controls HIV spread through the sharing of injection equipment among drug users (Nasir Mohamad, 2010). Methadone is generally given as a racemic mixture. Although, the activity of Methadone is attributed to the R-enantiomer, the S-enantiomer may be important in cardiotoxicity and undergoes stereo-selective metabolism with CYP2B6(Totah *et al.*, 2008). Patient on MMT might also be receiving drugs from Highly Active Antiretroviral Therapy (HAART) for AIDS. This can lead to potential Drug-drug interaction as antiretroviral agents used in HAART like Efavirenz, Nevarapine are metabolized by CYP2B6 (Haas *et al.*, 2009).

1.2 Pharmacogenetics

Various factors like age, sex, ethnicity, body weight, dietary habits, concomitant drug use and exposure to chemicals are responsible for varied drug response (Brazell *et al.*, 2002). Genetic variation is recognized as one of the factors influencing the drug response this research area is defined as Pharmacogenetics (Wolf *et al.*, 2000) . Though defined in 2000, there were cases where researchers have realized the potential influence of enzyme variation in some adverse drug events (Meyer, 2000).

Koo and Lee, define Pharmacogenetics as the study of heredity and response to drugs. The aim of Pharmacogenetics is based on genetic tests provide aid for giving right medicine with right dose which attempts to achieve maximum efficacy and minimum toxicity (Koo and Lee, 2006). ADR is an important consequence of drug therapy, 6.7% patients in US develop Adverse Drug Reactions (ADRs) out of which 0.32% develop

fatal reactions (Lazarou *et al.*, 1998). Similarly, 11.5%, 13.0% and 16.0% of hospital admission are due to for ADRs in Norway, France and UK respectively (WHO, 2002). It may be appropriate to adjust drug doses in accordance to the results of genetic tests. This facility of tailoring medicines will be effective to prevent ADRs and also be helpful in reducing the direct costs of therapy and indirect cost of hospitalization. The information from Pharmacogenetic tests can also be used in drug development and design targeted for specific population (Brazell *et al.*, 2002). It can also be used for preventive purposes, by identifying the gene/ genetic variation causing certain ADRs that can be then prevented using Pharmacogenetics. Prescription of drugs with narrow therapeutic index and metabolic polymorphism are the most important benefits with Pharmacogenetics (Wolf *et al.*, 2000). During drug development knowledge of Pharmacogenetic variations helps to avoid unnecessary costs of adverse drug events due to genetic traits (Wolf *et al.*, 2000).

Pharmacogenetics has progressed rapidly in the past years, after the term was coined in 1959 by Vogel. After the publication of the guidelines by FDA for submission of Pharmacogenomics data, pharmacogenomics have formally entered the field of healthcare (FDA, 2005). About 10% of the FDA approved drug labels contain pharmacogenetic information. The genetic biomarkers are intended to play a role in identify the Responders and Non-Responders, to avoid toxicity and to adjust the dose to ensure efficacy and safety. The genetic biomarkers are classified according to their specific use like clinical response and differentiation; risk identification; dose selection guidance; susceptibility, resistance and differential disease diagnosis; and polymorphic

drug targets (FDA). There are certain biomarkers classified as Valid Biomarkers by the FDA and are required in the labels of relevant drugs. CCR-5, CYP2C19 variants, CYP2C9 variants, CYP2D6 variants, NAT2 variants are some of the selected biomarkers and are indicated to be included in drug labels. A typical label example as required by the FDA is,

CYP2C9 Variants	CYP2C9 Variants PM and EM genotypes and drug exposure; "Patients who are known or suspected to be P450 2C9 poor metabolizers based on a previous history should be administered celecoxib with caution as they may have abnormally high plasma levels due to reduced metabolic clearance."	Celecoxib

The information on CYP2C9 variant as appeared on Celecoxib labels (FDA, 2010).

1.3 CYP Family

Cytochrome P450 are of heme containing enzymes responsible for phase-I metabolism of drugs and other exogenous and endogenous compounds. They get this name due to their characteristics optical property of absorbing light at 450 nm. This distinctive property is due to the presence of reduced heme, which when bound to drugs can bind with cabon monoxide to form complexes possessing this typical characteristic (Omura, 1999). Factors effecting variation on CYP450 activity include 1. Genetic polymorphisms, 2. Environmental factors, 3. Physiological status and 4. Disease state. The variations on CYP enzymes are responsible for variable response to drugs leading to adverse reaction or lack of efficacy (Amal Al Omari, 2007, Omura, 1999).

CYP enzymes are classified into families and subfamilies according to the homology they have with each other. This standard nomenclature system was introduced in 1996. Forty percent homology is assigned as a family represented by a number, 55% homology is assigned a subfamily represented with a letter and the individual enzymes are again designated an individual number. For most of the enzymes the "wild-type" is designated *1, and other alleles with different numbers with "*" (Nelson *et al.*, 1996). There are more than 57 active human CYP450 genes and 58 pesudogenes. The majority are polymorphic(Rodriguez-Antona and Ingelman-Sundberg, 2006).

Despite the presence of the large number of CYPs, only CYP1, CYP2, and CYP3 families have major roles in drug metabolism. Other CYPs are involved in the

metabolism of endogenous compounds. In general only about 10 CYP enzymes are involved in drug metabolism.

Table 1.1: Major enzymes contributing drug metabolism

Enzyme	**Estimated contribution to drug metabolism (% of drug metabolized by the enzyme)**
CYP1A1/2	3
CYP2A6	3
CYP2B6	3
CYP2C8/9	12
CYP2C19	8
CYP2D6	19
CYP2E1	1
CYP3A4/5	51

Adapted from (Amal Al Omari, 2007).

Out of all the CYPs, CYP2 family is the largest family involved in the metabolism of drugs in mammals. The family is subdivided into CYP2A, CYP2B, CYP2C, CYP2D and CYP2E. CYP2B6 is a member of the CYP2B subfamily.

1.3.1 CYP2B6

CYP2B6 is found in small quantities of 3-5% in liver cells; its presence is very low in extrahepatic tissues like the brain, lungs, intestine and the kidneys (Wang and Tompkins, 2008). Though, earlier thought to be not clinically important, recent studies showed that CYP2B6 is involved in the metabolism of many drugs; ranging from therapeutically important drugs to recreational drugs. Moreover, it is also involved in the metabolism of endogenous chemicals and pesticides and it is also involved in the activation of procarcinogens (Ekins and Wrighton, 1999). Some of the common compounds involving CYP2B6 in their metabolism include, chemotherapeutics like cyclophospamide, ifosfamide, tamoxifen; anti retrovirals like, efavirenz, nevarapaine; antidepressants like, bupropion; anti epileptic like mephobarbital, valporic acid; anesthetics like, propofol, ketamine; opioids like, methadone, pethidine; psychotropic like, diazepam, midazolam; steroids like, estrogen and testosterone and procarcinogens like; alfatoxins and nitrosamines are activated by CYP2B6 (Hesse *et al.*, 2000) (Chang *et al.*, 1998) (Chung *et al.*) (Xie *et al.*, 2003).

Apart from metabolizing many drugs, CYP2B6 generally also shares substrates with CYP3A4. In parallel with the increasing role of CYP2B6 in the metabolism of

xenobiotics, there is an increase in the chances of interactions mediated by CYP2B6. Bupropion is considered to be a probe drug for the study of CYP2B6 activity. Several, studies however reveal that other CYP enzymes are also involved in the metabolism of bupropion. Production of a specific metabolite and differences in affinity are the characteristic feature of CYP2B6 metabolism of this drug compared with other CYPs like CYP3A4, CYP2C9 and CYP2C19. Recent studies show that CYP2B6 selectively catalyzes the hydroxylation of S-bupropion (Hesse *et al.*, 2004). Generally, brupopion is given as a racemic mixture of R and S for therapeutic purposes. This characteristic of catalyzing the hydroxylation of S-bupropion by CYP2B6 is utilized for using brupopion as a probe drug by identifying the specific metabolite S, S-hydroxybupropion (Joy *et al.*). This stereo-selective property of CYP2B6 is also seen with methadone (Totah *et al.*, 2008).

Methadone is a synthetic opioid used in Methadone Maintenance Therapy for opiate users. It has been used for the treatment of opiate dependence for more than 30 years. Methadone binds to the opiate receptor, to prevent opiate withdrawals and to enable drug users to live a normal life.(Nasir et al, 2010).

Methadone is given as a racemic mixture. Only the R-methadone binds to the mu-opiate receptors to exert its opiate effects. S-methadone doesn't exert similar effect but inhibits the cardiac potassium channel. S-methadone is responsible for the cardiac toxicity of methadone that even sometimes leads to sudden death.

1.3.1.1 SNPs in CYP2B6

Unlike well established CYP enzymes like CYP2D6 or CYP2C19, studies on the polymorphisms of CYP2B6 have only recently been studied. In CYP2B6 SNPs are present in both the coding and non-coding regions. SNPs in the coding region i.e. in nine codons represent nonsynonymous and silent mutations.

There are many SNPs in the non coding region as well. SNPs in the regulators or introns also influence the gene expression. Since, CYP2B6 is highly inducible this facts leads it to be a potential area for studying the SNPs in these regions.

Table 1.2: SNPs at both coding and non coding region of CYP2B6 and their effects

Allele	Nucleotide changes	Enzyme activity	References
CYP2B6*1B	-2320T>C	N/A	Lamba et al., 2003
CYP2B6*1C	-2320T>C; 14593C>G; 15582C>T	N/A	Lamba et al., 2003

CYP2B6*1E	-1578C>T; -757C>T	N/A	Lamba et al., 2003
CYP2B6*1F	-1224A>G	N/A	Lamba et al., 2003
CYP2B6*1G	-750T>C	N/A	Lamba et al., 2003
CYP2B6*1H	-2320T>C; -750T>C	N/A	Hesse et al., 2004
CYP2B6*1J	-2320T>C; -1778A>G; -1186C>G; -750T>C	N/A	Hesse et al., 2004
CYP2B6*1K	-1972C>T; -1578C>T; -750T>C	N/A	Hesse et al., 2004
CYP2B6*2B	64C>T; 12740G>C	N/A	Lamba et al., 2003
CYP2B6*4B	-2320T>C; -1778A>G; -1186C>G; -750T>C; 18053A>G	N/A	Hesse et al., 2004
CYP2B6*4C	18053A>G; 12917A>T	N/A	Jacob et al., 2004

CYP2B6*5B	-2320T>C; -750T>C; 25505C>T	N/A	Hesse et al., 2004
CYP2B6*6B	-1456T>C; -750T>C; 15631G>T;18053A>G	N/A	Hesse et al., 2004
CYP2B6*7B	-1456 T>C; -750T>C; 15631G>T;18053A>G; 25505C>T	N/A	Hesse et al., 2004
CYP2B6*8	13072A>G	Decreased expression of the enzyme	Lamba et al., 2003 Lang et al., 2004
CYP2B6*9	-1456T>C; 15631G>T; 21563C>T	N/A	Lamba et al., 2003
CYP2B6*10	62A>T; 64C>T; 12740G>C	N/A	Lang et al., 2004
CYP2B6*11A	136A>G	Decreased activity of enzyme	Lang et al., 2004

1.3.1.2 Clinical implications of CYP2B6 polymorphism

As stated, CYP2B6 is involved in metabolism of many drugs including cyclosporine, efavirenz, nevarapine and many other important drugs. Altered expression, enzyme inhibition or polymorphisms of CYP2B6 have mainly been studied with cyclosporine and efavirenz (Xie *et al.*, 2003)(Ramachandran *et al.*, 2009). Both these drugs have narrow therapeutic indices and show large interindividual variation in plasma drug concentration (Penzak *et al.*, 2007).

The antiretroviral drug efavirenz is a non-nucleoside reverse transcriptase inhibitor (NNRTI) which is one of the drugs given in combination for HIV infected individuals. Efavirenz is shown to have high plasma concentration variation. It has been reported that higher plasma concentrations of efavirenz causes symptoms like difficulty in sleeping, dizziness, depression responsible for discontinuation of the drug and treatment failures. Studies using human liver microsomes and recombinant CYPs by Ward et al. showed that CYP2B6 was involved in the metabolism of efavirenz; 8-hydroxy-efavirenz and 8,14-dihyrroxy-efavirenz were identified as primary metabolites. Further pharmacogenetic studies showed that CYP2B6*6, specifically SNP 516 G>T was associated with elevated plasma concentrations.(Ramachandran *et al.*, 2009). Subsequently, population genotyping was used as a tool for efavirenz dose optimizing. CYP2B6*6 is common in west African and Papua New Guinean populations, countries with serious problems with HIV (Mehlotra *et al.*, 2007) (Penzak *et al.*, 2007).

Identification of “slow metabolizers’ was helpful for dose optimization and to decrease adverse events. A similar study in Japan used to decreases efavirenz dose by 33% to 66% but the plasma concentration was maintained leading to better therapeutic outcomes (Tsuchiya *et al.*, 2004).

1.4 Opioid receptors

Opioids have been used by human since ancient times and they were cultivated in Mesopotamia as early as 3400 BC. It has been in use for thousands of years for pain reliving. The first scientific work with opioids dated back to 1973, when a graduate student used radioactive morphine to locate the site of action for morphine (Trescot *et al.*, 2008). It was observed that morphine was specifically attached to a specific area termed “morphine receptors”. Though this phenomenon was observed in 1973, opioid receptor research was only started in the fifties when clinical interaction studies were carried out. By the sixties there were already proposals for the structure and mechanism of opioid receptors (Chahl, 1996).

The first key on the biological receptors was depicted by Lee on using snake toxin and its effect on nicotine acetylcholine receptors. Subsequently studies showed the presence of opioid receptors, the presence of endogenous opioids and by 1976 three different opioid receptors were proposed that were further subdivided (Wang *et al.*, 1994).

Morphine is agonist to mu-receptors. The mu receptor has subtypes that include mu1 and mu2. Mu1 mediates analgesia and euphoria whereas mu2 is associated with respiratory depression and sedation. On the other hand, Kappa receptor has Ketocyclazocine as its agonist. Kappa receptor mediates spinal analgesia, sedation, dyspenea and dependence. For Delta receptor its agonist is delta-alanine-delta-leucine-enkephalin. They are responsible for psychometric and dysphoric effect. Sigma receptors are no more considered as opioid receptors and are classified under targets for Phencyclidine and analogues (Trescot *et al.*, 2008).

Table 1.3: Different opioids and their effect at different receptors

	Mu (μ)	Delta (δ)	Kappa (κ)
	Mu 1- analgesia Mu 2- sedation, vomiting, respiratory depression, pruritus, euphoria, anorexia, urinary retention, physical dependence	Analgesia, spinal analgesia	Analgesia, sedation, dyspnea, psychomimetic effects, miosis, respiratory depression, euphoria, dysphoria
Endogenous Peptides			
Enkephalins	Agonist	Agonist	
B-endorphin	Agonist	Agonist	
Dynorphin A	Agonist		Agonist
Agonists			
Morphine	Agonist		Weak agonist
Codeine	Weak agonist	Weak agonist	
Fentanyl	Agonist		
Meperidine	Agonist	Agonist	
Methadone	Agonist		
Antagonists			
Naloxone	Antagonist	Weak antagonist	Antagonist
Naltrexone	Antagonist	Weak antagonist	Antagonist

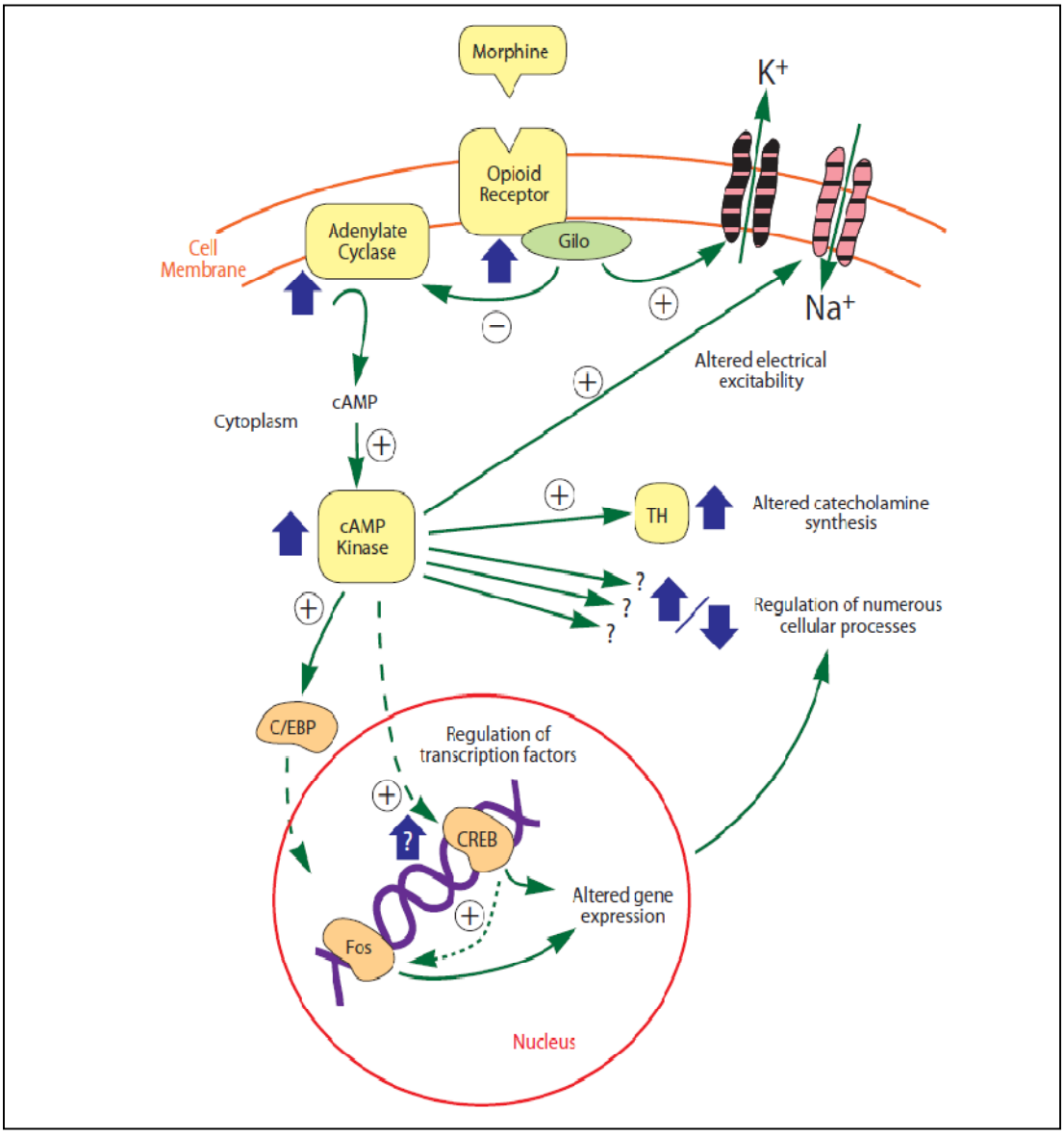
Adapted from (Trescot *et al.*, 2008).

1.4.1 Mu-opioid receptors

Mu opioid receptors are so named because they have morphine as agonists. They are primarily found in the brain stem and medial thalamus. They are responsible for supraspinal analgesia, euphoria, sedation, respiratory depression, decrease gastrointestinal motility and physical dependence. There are two subtypes of mu-receptors mu1 and mu2. Mu1 receptors are related to analgesia, euphoria and serenity. Mu2 receptors are related to respiratory depression, pruritus, prolactin release, dependence, anorexia and sedation.

Since, the mu opioid receptor is a member of G-protein coupled receptors (GPCRs) its regulation involves different processes like desensitization, internalization, resensitization and down regulation. GPCR kinases (GRKs) and β arrestins play major role in these processes. When GPCRs are stimulated by agonists, GRKs initiate receptor phosphorylation. This leads to recruitment of β arrestins which uncouples the receptor and G protein thus preventing further signaling. This is termed as desensitization. β arrestins also facilitate the internalization of inactivated receptors with possible promotion of receptor recycling to plasma membrane or downregulation by receptor degradation. This process of signaling holds true for morphine and is different for different opioids. Agonists like morphine, etorphine, methadone and fentanyl can activate the receptors with similar efficacy but they differ in their ability to promote desensitization and internalization. Morphine and heroin do not promote receptor

endocytosis but other agonists like methadone, etorphine and fentanyl promotes the endocytosis (Wang *et al.*, 1994).



Adapted from: (Chahl, 1996)

Fig 1.1: Opioids action via receptors

1.4.1.1 SNPs in mu-receptor gene (OPRM1)

Mu-opioid receptor gene is located on chromosome 6 at position q25.2 and is composed of 4 exons (Wang *et al.*, 1994). There have been studies on SNPs of μ-opioid receptor and its association with opiate addiction and alcohol dependence. SNPs at OPRM1 are present at 5' UTR region, exons, introns, and 3' UTR regions. Below is the list of the SNPs at OPRM1 genetic loci with their corresponding amino acid changes.

Table 1.4: SNPs at OPRM1 gene

Position	Location in the gene	Amino Acid change
-1045 A/G	5'UTR	-
-995 C/A	5'UTR	-
-692 G/C	5'UTR	-
-554 G/A	5'UTR	-
-488 G/T	5'UTR	-
-245 A/C	5'UTR	-
-236 A/G	5'UTR	-
-172 G/T	5'UTR	-
-133 C/T	5'UTR	-
-111C/T	5'UTR	-
-54 G/T	5'UTR	-
-38 C/A	5'UTR	-

17 C/T	Exon 1	Ala/Val
24 G/A	Exon 1	Synonymous
118 A/G	Exon 1	Asn/Asp
440 C/G	Exon 2	Ser/Cys
454 A/G	Exon 2	Asn/Asp
IVS2+31 G/A	Intron 2	-
IVS2+106 T/C	Intron 2	-
IVS2+397 T/A	Intron 2	-
IVS2+438 G/A	Intron 2	-
IVS2+480 T/C	Intron 2	-
IVS2+534 C/T	Intron 2	-
IVS2+691 C/G	Intron 2	-
779 G/A	Exon 3	Arg/His
794 G/A	Exon 3	Arg/His
802 T/C	Exon 3	Ser/Pro
820 G/A	Exon 3	Asp/Asn
942 G/A	Exon 3	Synonymous
IVS3+37 A/C	Intron 3	-
401 G/C	3'UTR	-

Table modified from (Xin and Wang, 2002).

1.4.1.2 Clinical implication of OPRM1 polymorphism

As listed above there are many SNPs at OPRM1 and many studies have been carried out on these SNPs. However, most of the studies focus on the most prevalent SNP which is a nucleotide substitution at position 118 (A118G) (Bond *et al.*, 1998) (Kim *et al.*, 2009) (Coller JK, 2009). This SNP predicts an amino acid change at a putative N-glycosylation site (Bond *et al.*, 1998). This variant has also been shown to be of physiogenetically (genetically based difference in physiology) and pharmacogenetically important. The frequency of this variant varies widely across populations, occurring at less than 2% in some populations (Bond *et al.*, 1998) and up to 50% in other populations (Gelernter *et al.*, 1999).

Bond *et al* (1998) also studied the binding of β-endorphin on the A118G variant and its prototype. The cellular activities of μ-opioid receptors were also studied, and the outcome showed that there is a similarity in the activity of the prototype and the variant receptor. on the other hand, β-endorphin showed a 3-fold greater potency in the activation of K+ current in A118G variant compared to the prototype. Persons who carry this variant gene may show altered function of the μ-opioid receptor.

Other studies have suggested that this polymorphism (A118G) may also result in altered pharmacogenetic response; with individuals carrying the 118G allele having different physiological and analgesic response to morphine (Lotsch *et al*., 2002). Also, heterozygous and homozygous mutant genotype of this polymorphism have been