APPLICATION OF CELL-BASED SCREENING FOR THE DISCOVERY OF POTENTIAL ANTI TYPE II DIABETES CANDIDATE FROM MALAYSIAN NATURAL PRODUCT USING PPARγ LIGAND AS MODEL

by

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LIST OF ABBREVIATIONS

^{1}H-NMR	Proton NMR
^{13}C-NMR	Carbon NMR
13-HODE	13-hydroxyoctadecadienoic acid
15-dPGJ$_2$	15-deoxy-$\Delta^{12,14}$- prostaglandin J$_2$
9-HODE	9-hydroxyoctadecadienoic acid
AMPK	AMP-activated protein kinase
ATCC	American Type Culture Collection
bp	Base pair
BCS	Bovine calf serum
BuOH	N-butanol
C2C12	Murine myoblast cell
CO_2	Carbon dioxide
COSY	Spectroscopy correlation
CREB	cAMP-response element binding protein
DBD	DNA-binding protein
DEX	Dexamethasone
DM	Diabetes mellitus

DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EtAOC	Ethyl acetate
FBS	Fetal bovine serum
FRIM	Forest Research Institute Malaysia
GC	Gas chromatography
GLUT 4	Glucose transporter 4
HMBC	Heteronuclear Multiple-Bond Correlation
HMQC	Heteronuclear Multiple-Quantum Correlation
hPPARγ	Human peroxisome proliferator activated receptor gamma
Hz	Hertz
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	Half maximal inhibitory concentration
IL-6	Interleukin-6
JNK	Jun -N-terminal kinases
KRPH	Krebs-Ringer-Phosphate-HEPES
LB	Luria-Bertani

LBD	Ligand-binding domain
MHz	Mega Hertz
MEM	Eagle's Minimum essential medium
MeOH	Methanol
mRNA	Messenger RNA
MS	Mass spectrometry
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NBDG	N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deosyglucose)
NIDDM	Non-insulin dependent diabetes
nmol	Nano molar
NMR	Nuclear Magnetic Resonance
OD	Optical density
P300	P300 transactivation coactivator
PBP	PPAR binding protein
PBS	Phoaphate Buffer Saline
PKB	Protein Kinase B
PPAR	Peroxisome proliferator activated receptor

PPARα	Peroxisome proliferator activated receptor alpha
PPARβ	Peroxisome proliferator activated receptor beta
PPARγ	Peroxisome proliferator activated receptor gamma
PPARs	Peroxisome proliferator activated receptors
PPRE	Peroxisome proliferator response element
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RXR	Retinoic X receptor
SRC-1	Steroid receptor coactivator 1
T2D	Type 2 Diabetes mellitus
TE	Tris-EDTA
TLC	Thin layer chromatography
TNFα	Tumor necrosis factor α
TZDs	Thiazolidinrdiones
UV	Ultraviolet
WHO	World Health Organization

PENGGUNAAN PENYARINGAN ASAI BERASASKAN SEL UNTUK PENCARIAN CALON ANTI DIABETIS JENIS II DARI PRODUK SEMULAJADI MALAYSIA DENGAN MENGGUNAKAN LIGAN PPAR- γ SEBAGAI MODEL

ABSTRAK

Pengaktif PPARγ sering digunakan dalam terapi penyakit diabetis jenis 2 dan pembangunan ubat diabetis. Hal ini disebabkan , PPARγ telah terbukti menunjukkan kebolehannya dalam meningkatkan sensitivti insulin di dalam sel. Sejak zaman dulu, tumbuhan ubatan telah banyak digunakan untuk merawat diabetis dan penyelidikan terkini telah membuktikan keberkesanan persediaan herba-herba ini. Dalam kajian ini, potensi 300 ekstrak sumber semulajadi Malaysia ke atas aktiviti anti diabetis telah diuji melalui penyaringan transktivasi PPARγ menggunakan sel hati (HepG2) sebagai model dan aktivitinya dinilai melalui keamatan luminisen yang dihasilkan oleh unsur tindakbalas pengaktif peroksisom (PPRE). Kesan anti-diabetis jenis 2 telah diuiji menggunakan asai pematangan sel lemak memandangkan kebanyakan PPARγ semulajadi hadir di dalam sel lemak. Dalam asai ini, kesan pengaktifan PPARγ ekstrak telah dinilai melalui keupayaannya untuk mematangkan sel pra-lemak tikus (3T3-L1) menjadi sel lemak. Ekstrak daripada pokok aktif yang dikenali sebagai *Rourea mimosoides* merencat kematangan sel lemak, meningkatkan keupayaan pengambilan glukosa dalam sel C2C12 dan meningkatkan ekspresi gen PPARγ sebanyak 6.84 kali ganda pada kepekatan 0.625μg/ml berbanding dengan rosiglitazone (10μM) yang menunjukkan peningkatan ekspresi gen PPARγ sebanyak 6 kali ganda sahaja. Kajian fitokimia telah membawa kepada pengasingan bahan

aktif yang dikenali sebagai Benzene dicarboxylic acid, diisooctyl ester (sebatian **1**) yang berkeupayaan untuk mempertingkatkan transaktiviti PPARγ sebanyak 17.91± 0.65 kali ganda pada kepekatan 1.56nM. Tambahan pula, kesan toksisiti sebatian **1** telah dikaji terhadap sel HepG2, C2C12 dan 3T3-L1 menggunakan asai Alamar biru dan keputusan yang diperolehi menunjukkan yang sebatian **1** adalah tidak toksi kepada sel-sel tersebut. Oleh itu, penemuan kajian ini menunjukkan bahawa PPARγ adalah merupakan molekul sasaran untuk ekstrak *R. mimosoides* dan sebatian aktif **1** yang boleh memberi kefahaman tentang potensi kesan anti-diabetis jenis 2 pokok *R. Mimosoides.*

APPLICATION OF CELL-BASED SCREENING FOR THE DISCOVERY OF POTENTIAL ANTI TYPE II DIABETES CANDIDATE FROM MALAYSIAN NATURAL PRODUCT USING PPARγ LIGAND AS MODEL

ABSTRACT

Peroxisome proliferator –activated receptor (PPAR)-γ activators are commonly used in type 2 diabetes therapy or even drug research as they improved the sensitivity of insulin receptors in cell. Since ancient, medicinal plants have been used to treat diabetes mellitus and recent research proved the efficacy of these preparations and some of which are remarkably effective . In this study, anti-type 2 diabetes properties of the methanol extract of a total of 300 Malaysian natural products were subjected to PPARγ transactivation activity assay using liver hepatocellular carcinoma (HepG2) cell as model and the activity was assessed through the intensity of the luminescence produced by peroxisome proliferator response element (PPRE) . The anti type 2 diabetes mellitus properties was also tested using adipocyte differentiation assay as most of the naturally accuring PPARγ is present in adipose cell. In the assay, the PPARγ activation properties of the extracts were assessed through its ability to differentiate the mouse pre-adipocyte (3T3-L1) cell into adipose cell. Extract from active plant identified as *Rourea mimosoides* was shown to inhibit adipocyte differentiation, increased the ability to enhance glucose uptake in C2C12 myoblast cells and increased the PPARγ gene expression by 6.84 fold at the concentration of 0.625μg/ml as compared to rosiglitazone (10μM) which showed 6 fold increase of the PPARγ gene expression. Phytochemical investigation has led to the isolation of the active compound identified as benzenedicarboxylic acid, diisooctyl ester

(compound **1**) which was found to increase the PPARγ transactivation by 17.91± 0.65 fold at the concentration of 1.56nM. Furthermore, the toxicity of compound **1** was investigated on HepG2, C2C12 myoblast and 3T3-L1 cells through Alamar blue assay and the results suggested that the compound was not toxic to the cells. Thus, the findings indicated that PPARγ is a molecule target of *Rourea mimosoides* extract and active compound **1** that may provide a better understanding of the potential of anti-diabetes type 2 properties of *R. mimosoides*.

CHAPTER 1

INTRODUCTION

1.1 Diabetes Mellitus

Metabolism is a process where ingested food is broken-down into glucose and utilized for energy generation and cell growth purposes. Glucose is then transported to the body parts via blood stream. However, glucose is unable to enter cells or tissues in the absence of insulin, a type of hormone which facilitates the diffusion of glucose from blood stream into cell or tissue. Deficiency of insulin action will cause glucose to remain in the blood and over time, the level of blood glucose will increase causing diabetes mellitus (DM) or diabetes (Kuzuya et al., 2002); a metabolic disease resulting from chronic hyperglycemia. A Greek physician used this term to describe a patient having high blood glucose, passing too much water or polyuria, and feeling thirsty all the time or polydipsia. DM may be caused by several factors depending on its type, but in general DM may be caused by a sedentary life style (for example: low exercise level, cigarette smoking, high glucose intake, alcohol consumption, obesity and etc.) (Ripsin et al., 2009), genetic factors (for example: defect of β cell function, mutation of insulin receptor and hereditary) and environmental toxin (Roder et al., 2000).

In 1980, Expert Committee on Diabetes Mellitus of WHO was classified diabetes mellitus into Type 1 Diabetes, Type 2 Diabetes, Malnutrition-Related Diabetes Mellitus (MRDM), and Gestational Diabetes Mellitus (GDM) according to the insulin deficiency degreed by WHO in 1985. While in year 1999, it was reported by WHO, diabetes mellitus is tended to be classified according to the causes of the disease rather than by the insulin defiency level. The terms Insulin Dependent

Diabetes Mellitus (IDDM) and Non-insulin Dependent Diabetes Mellitus (NIDDM) are used to describe the stages of diabetes and the various degree of insulin deficiency (World Health Organization). According to WHO diabetes mellitus type 1 or IDDM is refer to the diabetes which caused by the failure of insulin secretion in betal cell and eventually the patient diagnosed with diabetes mellitus type will needed insulin injection to control their blood glucose level. While diabetes mellitus which caused by the low sensitivity of insulin or insulin resistance is classified as diabetes mellitus type 2 (Kuzuya et al., 2002). Like other clinical classes' diseases, diabetes mellitus can be divided into non-diabetic, pre-diabetes, diabetic with no insulin therapy required and diabetic with insulin therapy required. At the last stage, insulin therapy is required to reduce the blood glucose level for survival purposes (ADA, 1997 & WHO, 1999). Clinically, history of disease, glycemic level, plasma insulin and C-peptide assay in urine are required in the evaluation of the insulin deficiency degree, and tend to be used to detect the stages of diabetes (Chikazawa et al., 1985).

Symptom is a departure from normal functioning which is noticeable by patient and thus symptoms may indicate diseases or abnormalities being present. Diabetes mellitus can be suspected with the presence of some particular symptoms such as: being constantly tired, unexplained weight loss, polyuria, polyphagia, polydipsia, poor wound healing ability, and unclear vision (Cooke & Plotnick, 2008; Robert, 2011). However not all symptoms will appear in a diabetes patient. The most common ones are polyuria, where a patient suffers from excessive urination (Cooke & Plotnick, 2008). This may be caused by the excretion of excessive glucose in the bloodstream into urine, and lead to dehydration (where large volumes of water are also needed to transport glucose out from the body) (Robert, 2011). Other than

polyuria, polydipsia is one of the most common symptoms appearing in diabetes mellitus patient. Diabetes mellitus patient may have increased thirsty (Porth, 1990; Cooke & Plotnick, 2008; Robert, 2011) all the time, and they may increase the volume of water consumed to dilute the concentrated glucose in their blood (Robert, 2011). This action was stimulated by human homeostasis which encourages the body to take in more water to dilute the glucose concentrated blood and bring the blood glucose level back to normal level. Usually polydipsia or excessive thirst, is the initial symptom for DM (Porth, 1990). Figure 1.1 shows illustration about the diabetes' symptoms.

Constantly being tired might also be one of the more common symptoms shown in diabetes patients. Due to the deficiency of insulin in the body, glucose fails to diffuse into cells and thus, the cell tend to uses up lipid and fats as fuel sources. In this case, more energy is required to breakdown the components and thus the body might feel tired easily (Robert, 2011). Another common symptom is weak wound healing; this happens due to the white blood functioning abnormally in the prevention of bacterial infection and dead cell digestion. High glucose levels in the blood may affect the function of the white blood cells, thus the wound site may be infected by bacteria or viruses frequently as the wound site needs a longer time to heal (Robert, 2011).

A prolonged, untreated diabetes mellitus may lead to death or mortality (Stamler et al., 1993 & Ko et al., 2000). According to the statistic by World Life Expectancy, the mortality rate for diabetes was categorized into four levels which are high mortality, middle high, middle, and low mortality rate. Mauritius, Guyana, Mexico and Jordan were categorized as high diabetes mortality rate countries as a

result of their mortality rate which was more than 61, out of 100, 000 patients . The mortality rate of diabetes mellitus in Mauritius was the highest where out of every 100,000 patients, 176 will die from diabetes. In Malaysia, the mortality rate of diabetes mellitus is still at the low level, where out of every 100,000 patients only 19 will die from diabetes mellitus (Wild et al., 2004). However, researchers should not ignore the seriousness of diabetes mellitus in Malaysia, as diabetes is a life threatening disease and in these few years, the diabetic incidence is shifting to the young generation.

According to the study of Detournay et al., (1999) and Charpentier et al., (2003) diabetes mellitus might be the risk factor which leads to cardiovascular disease, stroke, and peripheral vascular disease. Besides, diabetes mellitus may also damage human body and small blood vessel where it will lead to two diseases which are diabetic nephropathy, which affects the blood vessel in the kidney and the function of the kidney and diabetic retinopathy, which affects the blood vessel in the eyes and retina and thus may cause the blurring of vision (Boussageon et al., 2011). The summary of the complication caused by diabetes mellitus is also shown in Figure 1.2 Therefore, a lot of effort had been done by the researchers and also medical practitioners, through the use of special diets in diabetes mellitus patients, such as control of glucose intake and levels in the body (Danne et al., 2005 & Kemp et al., 2005), oral medication, and insulin injection (Rys et al., 2011) to treat or prevent diabetes mellitus from affecting the world's population especially in the younger generation.

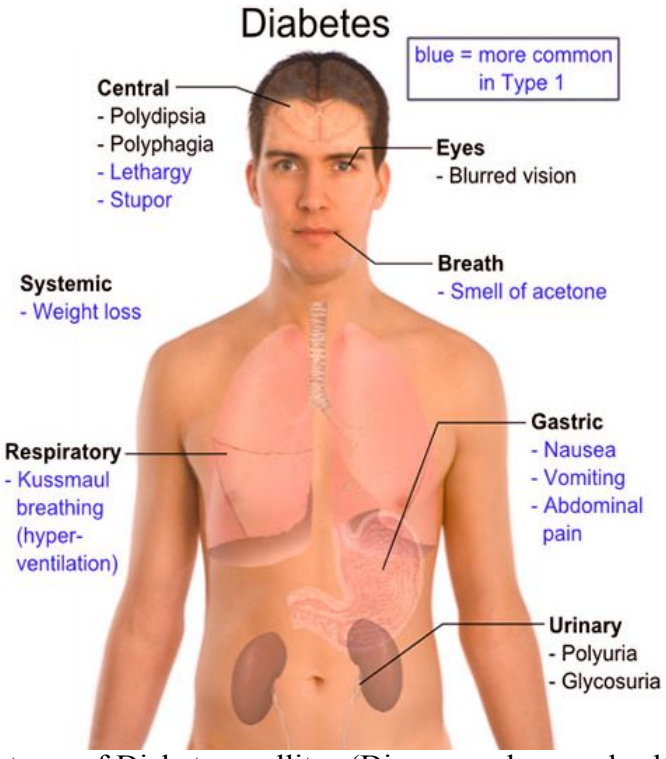

Figure 1.1: Symptoms of Diabetes mellitus (Disease and cures, health tips. Retrieved on 7th October 2015)[ebloghealth.com]

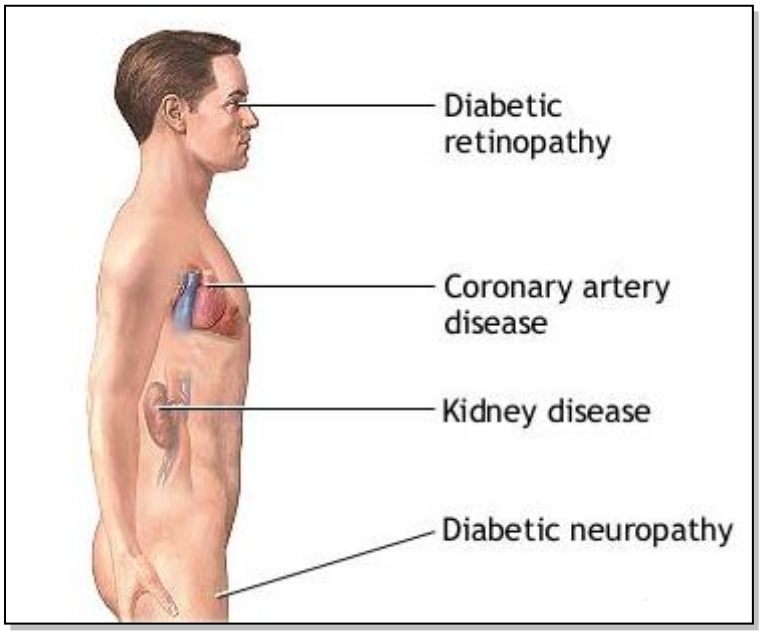

Figure 1.2: Vascular complication caused by Diabetes mellitus (Disease and cures, health tips. Retrieved on 7th October 2015)[tabletsmanual.com]

1.1.1 Diabetes Mellitus Type 1

Diabetes Mellitus type 1(T1D) or Insulin-Dependent Diabetes Mellitus (or commonly called juvenile diabetes) is a catabolic disorder where the autoimmune destruction of β-cell in the pancreas has happened and leads to insulin production deficiency (Kuzuya et al., 2002). Insulin deficiency in a patient of T1D actually is affected by the dysfunction of β-cell or Islet of Langerhans (caused by infection of virus or environmental toxin) and the infiltration of lympohotic in the pancreas (Khardori et al., 2011). According to the study of Yoon et al., (1989) and Dotta et al., (2007), viruses are one of the factors which caused the dysfunction of the β-cell and lead to T1D (Figure 1.3). The *Encephalomyocarditis* (EMC) virus, *Coxsackie* B4 virus, *Mengo* virus 2T, Rubella, Mumps, Epstein-Barr virus, Varicella zoster virus are those detected viruses that infect the Islet of Langerhans cell (Ginsberg-Fellner et al., 1984; Chikazawa et al., 1985; Surcel et al., 1987; Hyoty et al., 1988; Yoon et al., 1989; Jali & Shankar, 1990). Infection of these viruses may cause the pancreatic β-cell to be auto-immune. The body's immune system may produce anti- insulin antibodies to attack the insulin produced by pancreas (Khardori et al., 2011). Thus the deficiency of insulin production may lead to ketosis, hyperglycemia and abnormal lipid and protein metabolis.

The credibility of the virus infection leading to diabetes type 1 still was an issue among scientists. King et al., (1983) proved that Coxsackie B4 viruses had been detected from the children who were diagnosed with diabetes type 1, this finding was then further supported by the study of Banatvala et al., (1985). However this finding had been overthrown through the study of Yoon et al., (1986), in their study, they found that not all monkeys had high glucose levels or developed

abnormalities in insulin secretion when injected with Coxsackie B4 virus. Thus, Yoon and the research team concluded that Coxsackie B4 viruses may cause glucose intolerance in certain condition. Even though some papers discredited the idea of viral infections leading to auto-immune β-cells and diabetes type 1, there are still some studies which stated that viral infections are associated with autoimmunity in diabetes. Rayfield et al., (1986) and Suenaga and Yoon, (1988) reported that rubella viruses and retroviruses are factors which caused the autoimmunity in non-obese diabetic mouse. The effect from the autoimmunity in two types of mice causes the increase in blood glucose level. Moreover, the rubella virus caused hypoinsulinemia and mononuclear cell filtrate of the islet in Syrian hamster (Rayfield et al., 1986). However, the mechanism of rubella viruses in diabetes induction still remains unclear.

1.1.2 Diabetes Mellitus Type 2

Non-insulin dependent diabetes mellitus (NIDDM) or type 2 Diabetes Mellitus(T2D) represents a major global public issue since 2007**.** The disease is a more heterogeneous disease, in which both insulin secretion and insulin sensitivity are impaired and usually diagnosed in adults above the age of 40. Currently this disease is affecting more than 100 million people in the world (Wild et al., 2004). Through the expression of the diabetic patient number, the incidence was projected to increase up to 300 million people in year 2030. According to the WHO report year 2010, the diabetes type 2 incidence in Malaysia was predicted to be raised from 3.48% to 6.38% in 2030. To date diabetes type 2 remains a serious metabolic disorder, despite the numerous preventative strategies and medications. Diabetes type 2 might lead to chronic damages to body systems if the disease is not properly managed in the long term.

Diabetes type 2 is caused by the complex interactions of many factors. The two major factors that cause diabetes type 2 are insufficient insulin secretion in the pancreas and impaired glucose uptake in the muscles, liver and adipose cell, which is caused by the resistance of the action of insulin (Cheng & George Fantus , 2005). Insulin plays an essential role in glucose homeostasis, in order to regulate the cascade of fatty acid and glucose uptake through a series of protein mechanisms (Koopmans et al., 1999). During the glucose homeostasis, adipose and muscle cells are stimulated by insulin to uptake the blood glucose and further breakdown into pyruvate or glycogen, and stored in the muscles and liver cells (Bergam, 1997). However, in the case of diabetes type 2, adipose and muscle cells are not sensitive to the action of insulin, thus the cells are unable to uptake the glucose. Hence, it causes

a high level of glucose being detected in the blood, which is called hyperglycemia (Krssak & Roden, 2004).

Moreover, excessive free amino acids or fat, may cause an impairment of glucose uptake. Diabetes type 2 patients are highly associated with obesity, which increases the risk of insulin resistance and blood glucose elevation. A high level of fatty acids in the bloodstream will stimulate the secretion of cytokines in the adipose cell and subsequently increase the insulin resistance. In addition, free fatty acids cause the damages of beta cell in pancreas. Thus, the pancreatic insulin production and secretion will be affected, which leads to the impairment of glucose uptake, causing high glucose levels in the bloodstream (Cheng & George Fantus, 2005). As reported in Centre of Diseases Control (CDC) national fact sheet in year 2011, diabetes type 2 was diagnosed also in youngsters instead of adult; this is due to the increase in the levels of obesity among children and youngster in recent year. According to the report, most of the diabetes type 2 young patient possesses family histories of type 2 diabetes as well as insulin resistance.

1.2 PPARs family

Peroxisome proliferator-activated receptors (PPARs) are one of the transcriptional factors in the nuclear receptors superfamily, which regulate gene expression through ligand activation (Desvergne & Wahli , 1999; Ogata et al., 2002). PPARs can be activated either by natural ligand, such as fatty acids, steroid hormones, thyroid hormones and vitamin D, or synthetic compound such as thiazolidinediones (TZD) class drug, troglitazones and rosiglitazones (Ogata et al., 2002; Katherine et al., 2009). In addition to this, PPARs form a heterodimer with the Retinoid X Receptor

(RxR) prior to binding with the Peroxisome Proliferator Response Element (PPRE) to regulate gene expression which not only controls lipid metabolism, glucose homeostasis and inflammation, but also embryo implantation (Schoonjans et al., 1996; Fajas et al., 1997; Desvergne & Wahli, 1999; Ogata et al., 2002). Besides, PPARs has also been implicated in many biological-related diseases treatments and drug development such as hypolipidaemia agent, anti-inflammatory agent, anti-cancer agent and oral anti-diabetes agent.

There are 3 distinct PPAR subtypes namely, PPAR-Alpha (PPARα), PPAR – beta (PPARβ) and PPAR-gamma (PPARγ). Each subtype is encoded by separate genes with specific tissue distribution pattern (Dreyer et al., 1992; Kliewer et al., 1994; Pineda et al., 2002). PPARα is highly expressed in those tissues rich with mitochondria such as the liver, heart and muscle tissue. While PPAR-β is found in most body tissues but rarely found in adipose tissue. PPARγ is mainly found in adipose tissue. Each subtype has its own specific functions, for example PPARα regulate the gene expression in lipoprotein metabolism and PPARγ is mainly involved in glucose homeostasis (Sugii et al., 2009). Based on the research report, PPARγ is also the molecule target for TZD which is an anti type 2 diabetic drug (Chawla et al., 2001; Chinetti et al., 2001; Park et al., 2001; Boitier et al., 2003).

1.3 Peroxisome Proliferator-activated Receptor (PPAR)γ

PPARγ is one of the members belonging to nuclear hormone receptor (NHR) superfamily, which functions as ligand-activated transcription factors. The first discovery of PPARγ was in 1992 by Dreyer and the research team through the homology cloning in the Xenopus frog's liver. PPARγ was discovered as a potent therapy target for various metabolic syndromes such as obesity, diabetes etc (Zaman et al., 2000; Marx et al., 2002). There are two isoforms of PPARγ, including PPARγ-1 and PPARγ-2, which are distributed at different part of body tissues. PPARγ-2 contains an additional 30 amino acids at the N-terminal and is mostly found in adipose tissues which play an essential role in adipocyte differentiation, lipid and glucose homeostasis. PPARγ-1 is found in most of the body tissue (liver, skeletal muscle, breast, colon and the immune system) (Greene et al., 1995; Vidal-Puig et al., 1996; Spiegleman, 1997; Desvergne and Wahli, 1999; Fajas et al., 1999; Maeda et al., 2001).

Like other PPARs members, PPARγ functions as transcriptional factors through the formation of the heterodimer complex with RXRα. This complex is further bound to specific DNA response elements which are called Peroxisome proliferator response element (PPRE) at domain E/F to regulate the upstream action (Kliewer et al., 1992; Mangelsdorf and Evans, 1995). According to the study of Nakshatri and Chambon (1994), PPAR-RXR binds directly at the sequences of –AGGTCA- situated with the DR1 elements. As reported, the binding of PPARγ/RXRα heterodimer will cause the interactions between PPARγ and upstream extended core hexamer of DR1 to occur (Jpenberg et al., 1997). Hence, recognition of DR1 in PPRE is important for the docking purposes of PPARγ/RXRα .

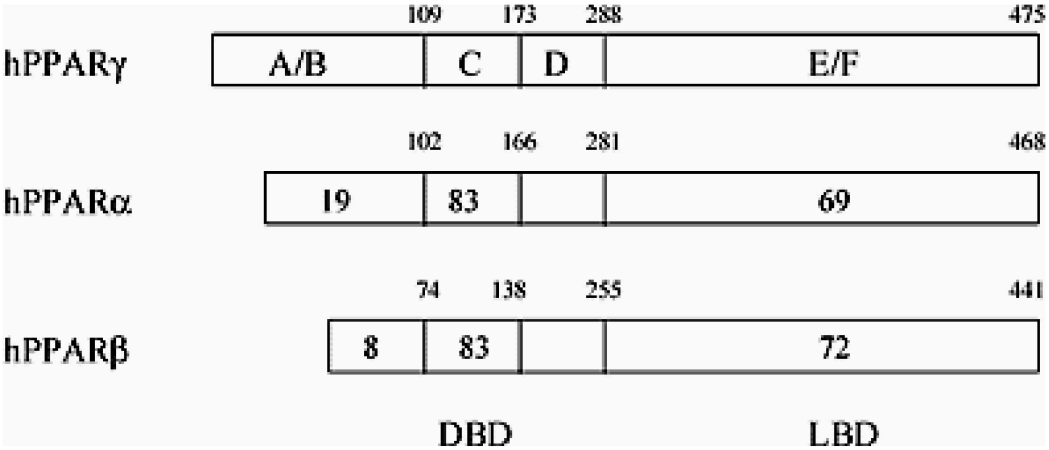

Figure 1.3: Structure domains of human PPARs (Techibana et al., 2008)

PPAR is built up with four distinct function domains which included A/B, C, D, E/F (Figure 1.3). N-terminal A/B domain with ligand independent activation function 1(AF-1) is responsible for phosphorylation, DNA binding domain (DBD), hinge region for cofactors docking, and C-terminal ligand-binding domain (LBD) with activation function 2 (AF-2) to promote the recruitment of cofactors required for gene transcription respectively. The number inside each domain corresponds to the percentage of amino acid sequence identity of human PPARγ and PPARβ relative to PPARα (Ranjan, 2002; Techibana et al., 2008)

1.4 Mode of action of PPARγ

The mechanism of actions of the PPARs members had been studied in the past twenty years. However, the mode of action of PPARγ had been widely studied compared to the other PPARs isoforms (PPARα and PPARβ/δ). Recently, scientists reported that PPARγ not only plays an important role in cellular homeostasis and is highly related to metabolic diseases; it also acts as the target of anti-diabetic drug in T2D therapy (Issesmann and Green, 1990). The mechanism of actions of PPARγ had been reported as being similar to PPARα and PPARβ/δ. Principally, four distinct functional domains which included domain A/B, C, D and E/F had been identified in PPARγ protein (Desvergne and Wahli, 1999). As reported, N-terminal domain or A/B domians which consists of ligand independent activation function 1(AF-1) is responsible for phosphorylation at DNA binding domain (DBD) (Figure 1.4). To promote the binding of PPARγ to PPRE, two zinc fingers in C domain will recognize the specific DNA response region and hold the PPARγ and PPRE in the promoter region of targeted genes (Kliewer et al., 1992; Werman et al., 1997). As discussed in previous topic, PPARγ function through the ligand –activation; thus, to trigger the upstream regulation of PPARγ prior binding to PPRE, E/F domain or ligand binding domain (LBD) exhibits a strong ligand dependent activation function (AF-2) which only allow a specific ligand to bind with the receptor to trigger the activation of the PPARγ binding process (Ranjan, 2002; Techibana et al., 2008).

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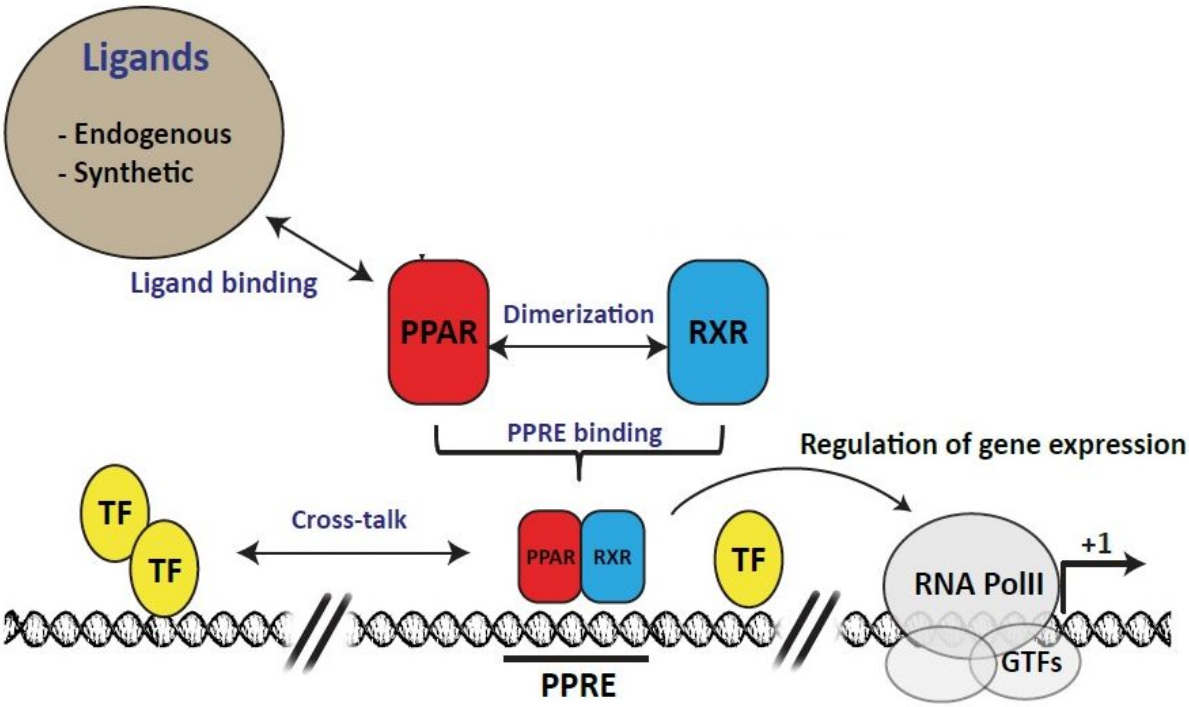


Figure 1.4 : Schemic view of PPAR action. After a ligand binds to PPAR, PPAR dissociates from corepressor, recruits coactivator, heterodimerizes with 9-cis-retinoic acid receptor (RA), and then binds to a DR1 or DR2 response element, which either activates or represses PPAR target genes. PPAR activity is also controlled by both protein degradation and phosphorylation (Chen et al., 2003)

PPRE: PPAR- responsive element; RXR : retinoid X receptor

1.5 Cofactors and Ligand of PPARγ

The function of PPARγ is regulated by common co-factors. Co-factors for PPARγ can be separated into co-activators or co-repressors of the nuclear receptor family (Powell et al., 2007). Examples of co-activators are steroid receptor co-activators 1(SRC-1), CREB binding protein(CBP), P160 steroid receptor co-activator family, and TRAP220/Med 1(McInerney et al., 1998).To activate the transcription process of PPARγ, a co-activator binding site is created when there is the conformational change in ligand binding d555omain (LBD), triggered by the binding of ligand to the receptor. Leucine amino aci (LXXLL) motifs in each co-activator's protein will cause the co-activators to bind or attach with the co-activator binding groove in LBD which triggers the docking process further. Leucine amino acid (LXXLL) motifs are present in most of the co-activators where the L denotes leucine and X denotes any amino acid (Heery et al., 1997; Torchia et al., 1997 and McInerney et al., 1998).

Binding of different PPARγ to the nuclear receptor will trigger different conformational changes in the LBD, thus it will induce different downstream biological effects. With this specificity of conformational changes; recruitment of nuclear receptors are promoted and stimulate the transcription of their targeted genes (McKenna & O'Malley, 2002)

Most of the PPARγ ligands are soluble in fat or hydrophobic ligand. Thus to associate the binding between PPARγ and ligand; intracellular lipid binding proteins (iLBP) (member of the family of fatty acid-binding proteins, FABPs) play an important role to act as a transportation medium to allow ligand to travel in cytosol before the ligand diffused into the nucleus to bind with PPARγ to form a heterodimer

with RxR-α (Adida and Spener 2002). Upon the formation of PPARγ - RxRα complex; transcriptional co-activators will be promoted (Torchia et al., 1997). As mentioned, the conformational changes in LBD will recruit the transcriptional co-activators; these co-activators will now bind to PPRE and trigger the expression of the targeted genes. Activation of PPARγ is mainly regulated by ligand binding; either natural ligand or a synthetic ligand.

1.6 PPARγ in Adipogenesis

PPARγ is found abundant in adipocytes cells and it plays a very important role in adipogenesis and lipid metabolism in mature adipocytes (Tontonoz et al., 1994a). In general, mature adipocyte plays a role in energy homeostasis, storing and energy production (Spiegelman and Flier, 1996).

Adipogenesis is influenced by external signals such as combining high concentration of cyclic AMP, dexamethasone ans insulin. Furthermore , transcription factors such as CCAAT/enhancer-binding protein α (C/EBPα) (Tang et al, 2004), peroxisome proliferator-activated receptor ((PPAR) γ and differentiation factor 1 (ADD-1) are act to control the adipogenesis which involved in triglyceride metabolism during the final stage of terminal differentiation. Besides, hormone such as insulin also play a part in adipocyte differentiation along with adipogenesis factor which including fatty acid, prostaglandins and 15-deoxy-$\Delta^{12,14}$- PGJ_2 (15d-PGJ_2).

Adipocytes usually secrete a number of proteins which perform a very specific function. Adipokines, serine protease adipsin, leptin, adiponectin, interleukin-6(IL-6), tumor necrosis factor-α (TNF-α), resistin, and visfatin are proteins which are secreted by adipocytes (Cook et al., 1987; Hwang et al., 1997; Berg et al., 2002; Fain et al., 2004; Banerjee et al., 2004 and Storka et al., 2008). Moreover their biological effect

in metabolism was well documented. These adipocytes-secretes molecules are high contributors to obesity-associated metabolic disorder development, as well as cardiovascular diseases, cancers and insulin resistance (Chaldakov et al., 2003).

1.7 Types of PPARγ ligands

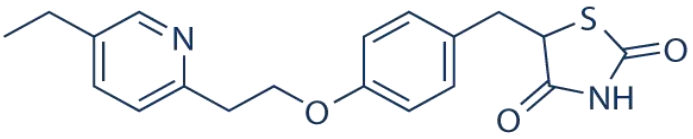
1.7.1 Natural endogenous PPARγ ligands

Natural endogenous PPARγ ligands are made mostly of fatty acid. In 1995, fatty acid and eicosanoids were reported and implicated as being PPARγ ligands by Forman et al. 1995. In their research, they managed to demonstrate that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ2) bind with PPARγ in adipose cell with a moderate binding affinity which is 2.5μM. Besides this, some long-chain unsaturated fatty acids were also reported as PPARγ activators (Desvergene & Wahli, 1999). Another group of fatty acids which included 15-lipoxygenase-derived metabolites of linoleic acid, 13-hydroxyoctadecadienoic (13-HODE), 9-HODE, as well as their metabolites 9-oxooctadecadienoic acid (9-oxoODE) and 13 –oxoODE have also been identified to serve as ligands of PPARγ (Nagy et al., 1998). However, from their research, these substances were shown to be weak PPARγ agonists with a low binding affinity (Nagy et al., 1998). Thus, to maintain a high binding affinity of these natural PPARγ agonists, fatty acid was used at high concentration (30μM and above) to ensure it is able to stimulate the downstream physiological effect.

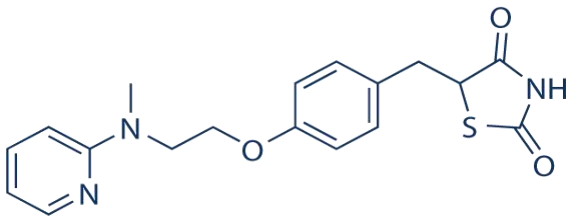
1.7.2 Synthetic PPARγ ligands

In the past 15 years, few synthetic PPARγ ligand have been identified and one of the most common synthetic ligand would be in the TZDs class. The three members of the TZDs class include rosiglitazone, pioglitazone and troglitazone (Figure 1.5). A number of researches have been carried out to assess the activities of TZDs on the hypoglycemia effect; Fortunately, TZDs showed their ability to improve the insulin sensitivity in cell as well as to enhance the uptake of glucose in the body and hence to reducing the blood glucose level. Thus, in 2001,rosiglitazone and troglitazone were approved to be used in T2D treatment (Kaplan et al., 2001 and Moller, 2001).

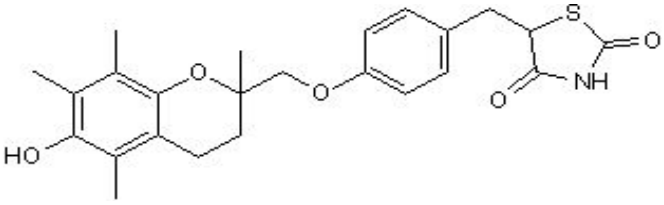
Discovery of TZDs and its downstream biological effect on PPARγ, opened a new path for T2D drug developments and design. A series of bioactive compounds which include GI262570, GW1929, GW7845, L796449, L764406 and SB219994 have been synthesized and developed (Murakami et al., 1998; Berger et al., 1999 and Elbrecht et al., 1999). Trans-activation activity of each compound has been tested, and they showed high potential in activating PPARγ at extremely low concentrations. These compounds were reported to possess similar effects as TZDs; they reduce the blood glucose levels and triglycerides levels effectively (Desvergne and Wahli 1999 and Corton et al., 2000).



(a) Pioglitazone



(b) Rosiglitazone



(c) Troglitazone

Figure 1.5 Molecular structure of TZDs class - (a) Pioglitazone, (b) Rosiglitazone, (c) Troglitazone are oral anti-diabetic type 2 agent.

1.8 Thiazolidinediones (TZDs) and its side effects

Rosiglitazone and troglitazone have been approved to be used in T2D therapy. TZDs class drug are highly recommended to be used in T2D treatment because of its high binding affinity toward PPARγ. The binding of these drugs trigger a few body metabolisms such as carbohydrates and lipid metabolism, as well as improve insulin sensitivity through the reduction of the circulating free fatty acids. However TZDs, especially rosiglitazone and pioglitazone, give several side effects to patient. The major sides effects caused by rosiglitazone are weight gain, anemia, pulmonary edema and congestive heart failure (Herz et al., 2003).

PPARγ are found not only in liver cells and muscle cells; it is also found most abundance in adipose cells. Thus activation of PPARγ by TZDs drugs might not only lower the plasma glucose level; it will also stimulate the differentiation of adipocyte cells and hence lead to weight gain (Herman et al., 1997). Besides, over-doses of TZDs will also promote the terminal differentiation of malignant breast epithelial cells which will cause cancer. On top of that, toxic effects caused by high doses of TZDs on animals have also been reported (Herman et al., 1997).

Although the occurrences of side-effects are quite common, TZDs is the only synthetic PPARγ agonists approved in clinical anti-diabetes treatment. They actually play the role of insulin sensitizer, which are mainly designed to improve insulin sensitivity in body cells, in order to reduce the glucose level in the bloodstream as well as hepatic glucose level. rosiglitazone (Avandia) and pioglitazone (Actos) (Table 1.1) were approved respectively by the US Food and Drug Administration(FDA). Earlier, a thiazolidinedione class drug, troglitazone which was introduced in 1997, was eventually banned due to its chronic hepatoxicity risk (Schoonjans and Auwerx, 2000; Mudaliar and Henry, 2001).

Table 1.1 Other synthetic oral hypoglycemia drugs (Prabhu et al., 2005).

Drugs	Administration time	Possible side effects
Glibenclamide	Taken with meal or 15 to 30 minutes before meal	Hypoglycemia, Obesity
Gliclazide	Taken with meal	Hypoglycemia
Metformin	Take during or immediately after a meal to minimize gastrointestinal side effects	GI disturbances
Acarbose	Swallow whole with liquid before meal or chew with the first few mouthfuls of food	GI disturbances
Repaglinide	Taken with meal	Hypoglycemia
Pioglitazone	Taken with meal	Hypoglycemia

1.9 Anti Diabetic Potential Plants

In the past 20 years, a number of drugs have been developed and used in diabetes therapy. One of the most popular drugs was TZDs; however the side effects caused by the drugs have raised community's concern. Hence, herbs or plants were preferred to be used to treat diabetes instead of modern medicine. Traditionally, plants have always been used as sources of drugs (Alarcon-Aguilara et al., 1998); various medical plants or herbs were documented for their anti diabetic therapeutic properties, which are highly effective in lowering blood glucose level and are low cost as well as less side effects (Rohini and Vikrant , 2011).

A phenolic compound isolated from Korean red pepper has been reported to possess anti-diabetic properties. The mechanism of the phenolic compound from Korean red pepper towards enhancing the glucose uptake is well documented. It was shown that the phenolic compound found in this plant exhibited anti-diabetic activities through the activation of AMPK and PPARγ signaling pathway (Yang, et al., 2012). In previous research, phenolic compounds such as chlorogenic acid and berberine were reported to stimulate the glucose uptake in mammalian cells through the enhancing of GLUT 4 and PPARγ gene expression (Prabhakar & Doble, 2009).

1.9.1 Potential PPARγ Ligand from Plants

Natural active compounds which serve as PPARγ natural ligand are also found in the *Punica granatum* flower extract. From the research result, beneficial effects of *Punica granatum* flower extract and its fractionated components as anti-diabetics was evidenced through their biological activities (Schubert et al., 1999). In the research, these active components were used to treat macrophage cell line and they showed the ability to enhance PPARγ mRNA and protein expressions in

macrophage cell line. One of the compounds labeled as Compound X induced the PPARγ gene expression at a concentration of 50μg/ml. Moreover, the extracts also show the activities in enhancing glucose uptake through the expression of GLUT-4 protein (Tom Huang, et al., 2005).

PPAR γ transactivation properties of monascin (yellow pigment) a secondary metabolite derived from Monacus fermented product have also been studied (Lee et al., 2011). Monascin was reported as PPARγ agonist as it is able to induce the expression of the PPARγ gene. Monascin was also acclaimed to increase the sensitivity of C2C12 myotubes toward insulin through protein kinases B (PKB) pathway and inhibiting the Jun-N-terminal kinase (JNK) activation. Besides, glucose uptake ability of monascin treated C2C12 myotubes was stimulated and also induced the expression of GLUT-4 protein in TNF-α- induced insulin resistance cell (Lee et al., 2011).

One of the common natural sweeteners, stevioside which is isolated from *Stevia rebaudiana,* has also been used in diabetes treatment for a period of time. Stevioside has been shown to induce the secretion of insulin in the pancreas in order to lower blood glucose level (White et al., 1994 and Jeppesen et al., 2002). Besides, rebaudioside, a synthetic diterpene glycoside from *Stevia rebaudiana,* was also shown to possess the same properties as stevioside in type 2 diabetes therapy (Jeppesen et al., 2003). In addition, a number of anti-diabetic potential phytochemical constituents which included alkaloids, glycosides, galactomannan, polysaccharides, peptidoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ion had been isolated from plants, fruits and herbs (Figure 1.7) (Grover et al., 2002). Some anti-diabetic plants and the solvent used to extract the constituents are tabulated in Table 1.2.

Table 1.2 Plants with anti-diabetic potential and the extraction solvent used

(Grover et al., 2002)

Plants	Common Name	Extraction solvent	Active part
Allium cepa	Onion	Petroleum ether Chloroform	Flesh
Allium sativum	Garlic	Ethanol Petroleum ether Ethyl ether	Flesh
Artemisia pallens	Davana	Methanol	Aerial
Caesalpinia bonducella	Fever nut	Aqueous alcoholic	Seed
Citrullus colocynthis	Bitter apple	Aqueous extract	Fruit
Hibiscus rosa-sinesis	Shoe-flower	Ethanol	Whole plant
Mangifera indica	Mango	Aqueous	Seed and fruit
Momordica charantia	Bitter Gourd	Aqueous Acetone Ethanol	Fruit pulp, seed and leaves
Ocimum sanctum	Holy basil	Ethanol	Leaves
Punica granatum	Pomegranate	Aqueous-Ethanolic	Flower
Salacia oblonga	Ponkornati	Aqueous Petroleum ether Ethyl acetate	Root bark