DISCOVERY OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA AGONIST

BY

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ABBREVIATIONS

ADT	AutoDockTools
AF-1	Activation Function 1
AF-2	Activation Function 2
BMI	Body Mass Index
CADD	Computer Aided Drug Design
CAP	Cbl Associated Protein
Cbl	Casitas b-lineage lymphoma
CIP4/2	Cdc42-Interacting Protein 4/2
CBP	CREB Binding Protein
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DPF	Dock Parameter File
DPP	Diabetes Prevention Program
EC_{50}	Enzymatic Constant
FBE	Free Binding Energy
FBS	Fetal Bovine Serum
FFA	Free Fatty Acid
GAP	GTPase-Activation Protein
GDM	Gestational Diabetes Mellitus
GLUT 4	Glucose Transporter 4
GM	Genetic Modified
GPF	Grid Parameter File
H-Bond	Hydrogen Bond
IDDM	Insulin Dependent Diabetes
IFD	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
IR	Insulin Receptor
LB	Luria-Bertani
LBD	Ligand Binding Domain

MAP4K4	Mitogen-Activated Protein Kinase 4
MCP-1	Monocyte Chemoattractant Protein-1
NCI	National Cancer Institute
NIDDM	Non-insulin Dependent DM
NTC	Non-Transfected Cell
PBF	Phosphate Buffered Saline
PDB	Protein Data Bank
PDK1	PI3K-Dependent Serine/Threonine Kinases
PI3K	Phosphatidylinositol 3 Kinase
PIP3	Phosphatidylinositol 3,4,5 triphosphate
PPARγ	Peroxisome Proliferator Activated-Receptor Gamma
PBP	PPAR Binding Protein
PGC-1	PPARγ co-Activator 1
PRIP	PPAR Interacting Protein
PTP1B	Protein-Tyrosine-Phosphate-1B
QSAR	Quantitative Structure Activity Relationship
RLU	Relative Light Unit
RMSD	Root Mean Square Deviation
SBDD	Structure-Based Drug Design
SERM	Selective Estrogen Receptor Modulator
SHIP2	SH domain Containing Inositol 5-Phosphate
SPPARM	Selective PPAR Modulator
SRC-1	Steroid Receptor co-Activator
T1DM	Type-1-Diabetes Mellitus
T2DM	Type-2 Diabetes Mellitus
TNFα	Tumour Necrosis Factor-α
TZD	Thaizolidinediones
WHO	World Health Organization

PENEMUAN AGONIS UNTUK PENGAKTIFAN PEROKSISOM PROLIFERATE GAMMA

ABSTRAK

Pengawalaturan Pembezaan Reseptor aktivasi Pembiakan Peroksisom Gamma (PPARγ) adalah superfamili reseptor nuklear faktor transkripsi yang diaktifkan oleh ligan. Dalam. Sistem biologi, PPARγmemainkan peranan yang penting dalam mengawal metabolism glukosa and lipid. Disebabkan oleh itu, PPARγ telah menarik perhatian yang besar sebagai sasaran yang berpotensi untuk mereka bentuk ubat yang baru untuk merayat penyakit diabetes jenis-2. PPARγ agonis, rosiglitazone, adalah ubat barisan pertama bagi miningkatkan sensitiviti insulin diabetes jenis-2. Walau bagaimanapun, kajian baru-baru ini menunjukan bahawa rosigkitazone menyebabkan kesam sampingan yang tidak diingini seperti pertambahan berat badan, edema dan pembendungan air. Oleh itu, adalah perlu untuk mengenalpasti agonis PPARγ yang kurang komplikasi.Dalam kajian ini, saringan virtual menggunakan pendekatan dok molekul telah dilakukan terhadap 5000 sebatian, 3000 sebatian adalah dari pengkalan data Nature-Based Drug Discovery (NADI) dan 2000 sebatian telah diambil dari pengakalan data National Cancer Institute (NCI) Diversity set 1. 100 calon sebatian yang teratas mengikut tenaga pengikatan terendah seterusnya dianalisis berdasarkan nod pengikatan. Sebanyak empat sebatian telah dipilih dari pengkalan data NCI and dua tumbuhan (Temu kunci and Ketumbar) telah dipilih dari sumber asli Malaysia untuk menjalankan ujian in-vitro terhadap PPARγ. Dari hasil ujian, sebatian yang paling aktif dari pangkalan data NCI adalah NCI37245, PPARγ yang diaktifnya dengan kandungan EC_{50} 43.6 nM. Manakala bagi tumbuhan – tumbuhan yang terpilih, ekstrak mentah metanol Temu kunci adalah lebih aktif

berbanding dengan ketumbar. Ekstrak mentah Temu kunci mengaktifkan PPARγ pada kira-kira 1.5 kali gnda lebih tinggi berbanding dengan ketumbar (Prubahan kali ganda = 1.6) dengan kandungan EC50, 0.13μg/mL. Oleh itu, secara kesimpulannya, pendokan berasaskan teknik saringan virtual telah berjaya dibangunkan dan boleh digunakan untuk memilih ligan PPARγ yang berpotensi daripada molekul-molekul kecil.

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DISCOVERY OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA AGONIST

ABSTRACT

Peroxisome Proliferator- Activator Receptor gamma (PPARγ) is a nuclear receptor superfamily of ligand activated transcription factor. In biological system, PPARγ plays a crucial role in regulating glucose and lipid metabolism. For that reason, PPARγ has drawn enormous attention as a potential target for designing novel drug to treat type-2- diabetes. PPARγ agonist, Rosiglitazone, is the first line drug for improving insulin sensitivity of type 2 diabetes. However, recent studies indicated that Rosiglitazone are causing undesired side effects like weight gain, oedema and fluid retention. Thus, it is necessary to search for PPARγ agonist for a less complication alternative. In this study, virtual screening using molecular docking approach was done against 5000 compounds; 3000 compounds were from Nature-Based Drug Discovery (NADI®) Database and another 2000 compounds were taken from National Cancer Institute (NCI) Diversity Set 1 library. The top 100 hits with lowest free energy of binding were further analyzed based on their favorable binding mode. A total of four compounds were selected from NCI library and two plants (Temu kunci and Ketumbar) were selected from Malaysia natural resources to test against PPARγ *in vitro*. From the assay result, the most active compounds from NCI database were NCI37245, which activated PPARγ at EC_{50} of 43.6nM. While for the selected plants, the methanol crude extracts of Temu kunci was more active compared with Ketumbar. The crude extracts of Temu kunci activate PPARγ at about 1.5 fold higher compared with Ketumbar (1.6 fold) with the EC_{50} of 50.0ng/mL. Thus, it can be concluded that, docking based virtual

screening technique has been successfully developed and can be used to select potential PPARγ ligand from a pool of small molecules.

CHAPTER 1

INTRODUCTION

Diabetes mellitus (DM) is a complicated metabolic disorder characterized by chronic hyperglycemia or high blood glucose level with fasting blood sugar reading of 126 mg/dl or higher. Usually diabetes is caused by the defection of insulin secretion, insulin resistance of targeted cells or both (American Diabetes Association, 2012). Insulin is a hormone which plays important roles in glucose and lipid metabolism. Poor control of excessive glucose in the blood will damage the circulation system and organs. This eventually brings to the long term complication of diabetes such as heart disease, stroke, high blood pressure, retinophathy, nephropathy, amputation (Fajans and Nutting, 1993), Neuropathy (Maser et al., 2003, Martin et al., 2006) and as well as bond fracture (Janghorbani et al., 2006, Lipscombe et al., 2007).

DM is a well known disease recognized as a health threat worldwide. According to WHO, 346 million people in the world have diabetes and in 2011, diabetes was the cause of more than 3.5 million deaths in developing country which is almost 80% of total diabetes death. Compared with high income countries like United States (US), the mortality rate are much lower than the middle income countries. In the same year of 2011, about 180000 people died in US (Scully, 2012).

The case of DM is increasing at an alarming rate and has just reached epidemic proportion. According to international Diabetes Federation, the prevalence of diabetes was estimated to rise from 171 million in 2000 to 366 million in 2030 due to population growth, urbanization, increasing prevalence of obesity, physical inactivity and aging of the world population (Wild et al., 2004). However, what makes it more alarming is that,

most of the people are not aware that they are actually having diabetes. A survey conducted by Malaysian Diabetes Association, there were approximately 1.2 million people suffer from diabetes in 2007 and it was estimated that more than half are believed to have it without realizing they do (Hasan et al., 2011).

1.2 Classification of Diabetes

There are a few types of DM but the major ones are Type 1 and Type 2 DM. Other types of diabetes include gestation diabetes, prediabetes (American Diabetes Association, 2012), Latent Autoimmune Diabetes in Adults (LADA) (Pozzilli and Di Mario, 2001) and type 3 DM (Monte de la and Wands, 2008).

1.2.1 Prediabetes

Prediabetes is a new term for impaired fasting glucose (IFD) or impaired glucose tolerance (IGT). It is characterized by high glucose levels in blood plasma but are yet to be diagnosed as diabetes (American Diabetes Association, 2012). Recent data show that at least 57 million adults in the U.S. had pre-diabetes in 2007. Usually there are no symptoms observed on those who have pre-diabetes but, if nothing is done to bring down their glucose levels, they are at great risk of developing diabetes within about 10 years. Experts are recommending that everyone who has any of the risk factors for type 2 diabetes to be tested for prediabetes (Bray, 2008).

Basically, prediabetes can be diagnosed based on fasting glucose level or glucose tolerance level. People with prediabetes usually having impaired fasting glucose (IFG) levels of 100 mg/dl (5.6 mmol/l) to 125 mg/dl (6.9 mmol/l), or impaired glucose

tolerance (IGT) of 140 mg/dl (7.8 mmol/l) to 199 mg/dl (11.0 mmol/l) (American Diabetes Association, 2012). The physiological condition of prediabets can be reversed in several manners such as physical exercise, weight loss and healthy diet. According diabetes prevention program (DPP), reduction of fat and calories intake together with physical activites can prevent or delay the development of diabetes from prediabetes (Knowler et al., 2002).

1.2.2 Gestational Diabetes Mellitus

Gestational Diabetes Mellitus (GDM) is a hyperglycemia condition that happens in pregnant women, usually in late second trimester of pregnancy. Studies indicated that gestational diabetes is due to the hormones from the placenta which affect the function of insulin in maintaining euglycaemic control and finally, causing elevated blood glucose levels (Bottalico, 2007). According to American Diabetes Association, about 7% of pregnancies women are complicated with GDM resulting in more than 200000 cases annually (American Diabetes Association, 2012). Historical studies on type 2 diabetes women showed that women with gestational diabetes history ussualy have as high as 20% risk of developing overt diabetes (Hayes, 2012) and their offsprings are at risk of being obese which might later develop type 2 diabetes (Clausen et al., 2008). Healthy lifestyle and metformin therapy are recommended by DPP to reduce the percentage of developing diabetes of historical GDM women (Ratner et al., 2008).

1.1.3 Type 1 Diabetes (T1DM)

Type 1 DM is also known as insulin dependent diabetes (IDDM) or juvenile onset diabetes (American Diabetes Association, 2012). Type 1 DM results from the cellular mediated autoimmune destruction of pancreatic β-cells in the islets of Langerhans that lead to absolute insulin deficiency (American Diabetes Association, 2012). According to American Diabetes Association, only 5-10% of all diabetes patients in the world encountered as Type 1 DM (American Diabetes Association, 2012). Type 1 DM is closely related with genetic makeup of pancreatic beta cells and usually occuring in genetically susceptible individuals. Genetical profile shown that, 90% of Type 1 DM patients are reported to have mutation in genes that encode for Human Leukocyte Antigen, HLA (Koshland, 1958). HLA is a protein that plays an important role in human body defense system. It presents molecules to the body T-cells to differentiate self cells and non-cells. Mutation of HLA genes in T1DM patient had let to autoimmune inflammatory attack that destroys the pancreatic beta cells. Thus, insulin injection will be the mainstay treatment for Type 1 DM patients (Casey, 2012).

1.1.4 Type 2 Diabetes

Type 2 DM, previously called as non-insulin dependent DM (NIDDM), is the most common diabetes and is characterized by insulin resistance and defection in insulin secretion (Butler et al., 2003, American Diabetes Association, 2012). According to World Health Organization (WHO), approximately 90% of all diabetic patients in the world is Type 2 DM. This phenomenon is usually due to the changes of lifestyle in the developing country, where urbanization and economic development have brought to

negative impact of lifestyle such as diet changes, less physical activity, and so on. This incidence, have also caused to the rise of upper body obesity in the developing country (Yoon et al., 2006). Obesity is well known as a main factor that contribute to insulin insensitivity of targeted cells (Fradkin, 2012). Obesity was defined as an excess of body adiposity (Caballero, 2007). Body mass index (BMI) was used as a standard tools to measure body fat based on height and weight, for obese people the BMI is more than 30 (BMI, 2012). Despite lifestyle and diet changes, psychological conditions (stressed and insomnia) (James et al., 2005) and genetic makeup of human (O'Rahilly and Farooqi, 2006) are also associated with obesity as shown in Figure 1.1.

Statistical studies on obesity and diabetes prevalence in Asia showed that the proportions of people with type 2 diabetes and obesity have increased throughout Asia. Asia alone accounts for 60% of world's diabetes population (Chan et al., 2009) and the prevalence of overweight and obesity is increasing parallel with economic development and rapid urbanization (Hu, 2011).

In general, once the cells become resistance to insulin, the function of insulin in controlling glucose and lipid metabolism has been cut off and the sugar will remain in the blood stream. In order to overcome this problem, pancreatic β-cells will tend to produce more insulin to take down the blood sugar level. Prolonging this situation will cause hyperinsulinemia where excess level of insulin is found in the circulation system. Ultimately, it will affect the β-cell mass to become lesser and lesser and eventually lead to insulin dependency and type 2 DM (Butler et al., 2003, Holness and Sugden, 2012).

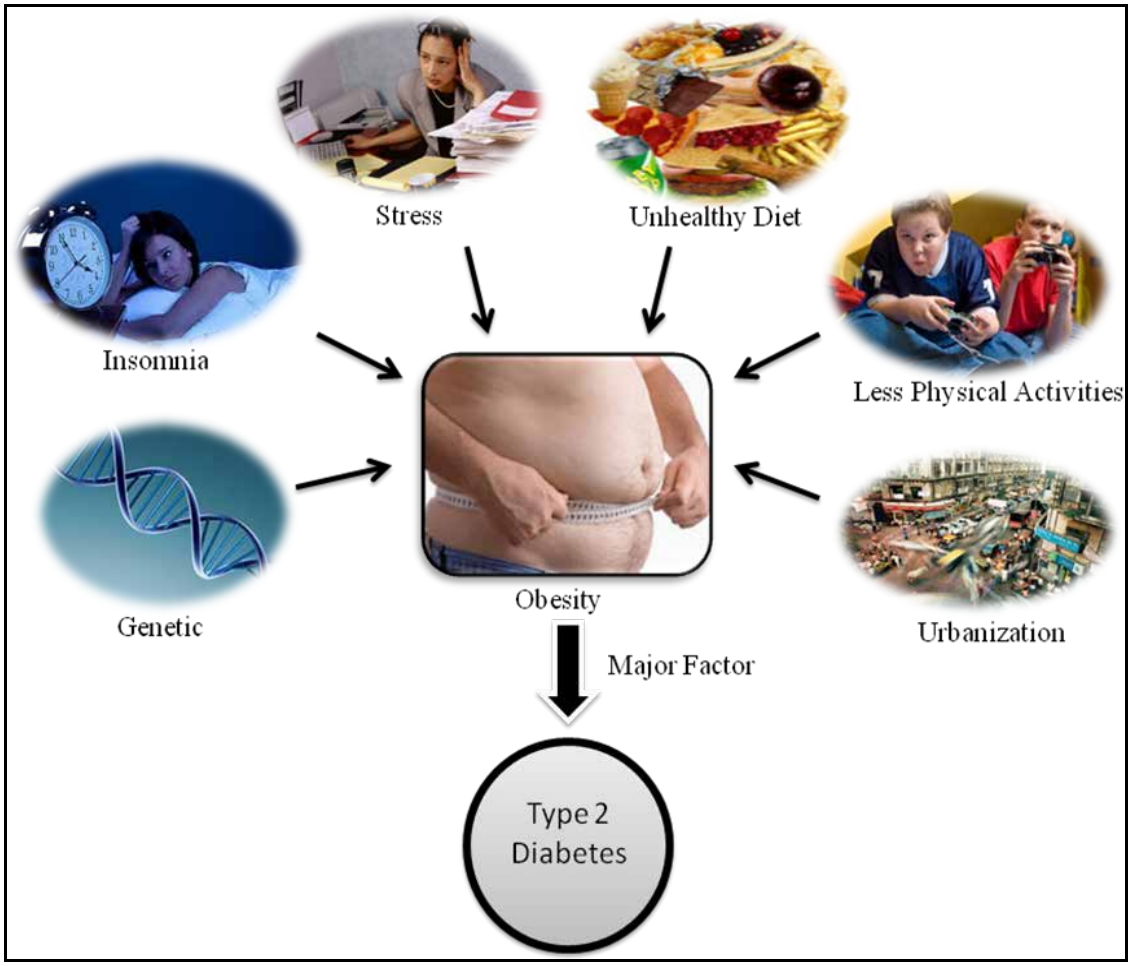

Figure 1.1 Factors contribute to obesity and Type 2 Diabetes Mellitus. All pictures were downloaded from Google image (www.google.com).

1.2 The Mechanism of Insulin and Glucagon Action in Glucose Homeostasis

Insulin is an antagonist hormone that helps to maintain glucose homeostasis together with glucagon. Inside the cell, glucose was utilized as an energy source and stored in the form of glycogen in the muscle and liver cells or as triglyceride in the adipose tissues (Albright and Stern, 1998). Glucose is required for cellular respiration and also as a building block of organic compounds such as lipid and glycogen as for the energy source. Therefore maintaining glucose level at a normal rank of 90mg/mL in the body is a crucial step for cell metabolism process.

In a normal condition, when glucose level is high, β-cells of pancreas will be detected and insulin will be released into the blood. Insulin then acts like a key to unlock the door on the membrane cell to allow glucose to go inside the cell to bring down blood sugar level (Aronoff et al., 2004); on the other hand, if the blood glucose is lower than the normal rank, pancreatic α-cells are stimulated to release glucagon into the bloodstream and these glucagon will regulate gluconeogenesis where the stored glycogen will be converted into glucose inside the liver and released into bloodstream to bring back glucose level in the body (Orci et al., 1975, Gerich et al., 1979).

Insulin and glucagon are working in the opposition role but they are interconnected to each other to regulate glucose homeostasis. In other words, if one of them fails or unable to perform their role in glucose homeostasis then the body will become unbalance. In the case of T2DM, excessive glucose is found in the bloodstream and the body cells are unable to take up the glucose due to insulin resistance of the cells. Therefore is it worth for us to look inside the molecular mechanism of insulin action on glucose homeostasis to understand the main reason for the cells to become insulin

resistance. This will hopefully allow us to search for the possible solution to overcome the problem.

1.2.1 Insulin mediate Glucose Transporter 4 translocation

Insulin mediate cellular glucose uptake through the translocation of Glucose Transporter 4 (GLUT 4) from intracellular to plasma membrane. GLUT 4 is a protein, primarily found in adipose tissue and muscle, which facilitated glucose uptake through passive transport by forming an aqueous pore across the plasma membrane (Bell et al., 1993, Bryant et al., 2002a). Translocation of GLUT4 involves two important pathway stimulated by insulin which is PI3 kinase /Akt pathway and CAP/Cbl pathway as shown in Figure 1.2 (Bryant et al., 2002b, Jun et al., 2008).

Insulin will first bind to the Insulin Receptor (IR) on the membrane surface and then activates the tyrosine domain (Yu and Czech, 1984). The activated insulin receptor then trans-phosphorylates and subsequently recruits insulin receptor substrate 1-4 protein, Shc and Cbl (Holland et al., 2008). These events led to the activation of downstream signaling pathway which involves glucose transport, glycogen and protein synthesis as well as mitogenesis (Kohn et al., 1996, White, 1998, Alessi and Cohen, 1998). One of the important pathways involves in glucose uptake and glycogen synthesis is PI3 kinase/Akt pathway (Bryant et al., 2002b).

1.2.1.1 PI3 kinase/Akt pathway

PI3 kinase /Akt pathway plays a pivotal role in GLUT4 translocation. Validation of the important function of PI3K is derived from the pharmacological inhibition of

PI3K which blocked the GLUT 4 translocation to the membrane cell and glucose uptake (Shang et al., 2008). Phosphorylation of IRS stimulated the activation of Phosphatidylinositol-3 kinase (PI3K) by binding to the regulator p85 subunit and activates its catalytic p110 subunit. Activation of PI3K lead to the formation of Phosphatidylinositol 3,4,5 triphosphate (PIP3) and subsequently recruit the PI3K-dependent serine/threonine kinases (PDK1) and activate the Akt. There are evidence supports that activated Akt is requiring for insulin-dependent translocation GLUT4 (Hill et al., 1999, Wang et al., 1999, Kohn et al., 1996)

The activated Akt will to lead phosphorylation of 160kDa Akt substrate (AS160). AS160 contains a GTPase-activation protein (GAP) domain for Rabs protein which plays critical roles in vesicle formation, movement, and fusion. Phosphorylation of AS160 causes the dissociation of AS160 from the GLUT4 storage vesicle, preventing AS160 from converting the Rab protein to its inactive GDP form. The activated Rab–GTP complex then promotes docking and/or fusion of the GLUT4 storage vesicle with the plasma membrane and facilitated glucose transportation (Hardie, 2007, Sano et al., 2003).

1.2.1.2 Cbl/CAP pathway

Cbl/CAP pathway is another important pathway which works in parallel with PI3k/Akt pathway in GLUT4 translocation (Saltiel and Pessin, 2003). Insulin stimulates tyrosine phosphorylation of c-Cbl leading to the translocation of Cbl-CAP complex from intracellular site to lipid rafts plasma membrane. In the lipid rafts, CAP binds with flotillin, a protein within lipid rafts, while Cbl recruits Crkll (adaptor protein) along with

C3G, which lead to activation of TC10. Finally, the activated TC10 with it effectors (CIP4) will then mediate GLUT4 translocation (Chiang et al., 2001, Symons and Rusk, 2003). The pivotal role of CAP-Cbl in GLUT4 translocation has been studied by expression of CAP mutant, which showed it completely inhibit insulin stimulate uptake and GLUT4 translocation (Chunqiu Hou and Pessin, 2003).

Therefore, it can be concluded that PI3K/Akt and Cbl/CAP pathways are important pathways in GLUT4 translocation. Selective inhibition of these pathways could contribute to the development of insulin resistance (Shang et al., 2008, Chunqiu Hou and Pessin, 2003). This also in turn suggests that, PI3K/Akt and Cbl/CAP pathway might be an important pathway for pharmacological study in search for a novel anti-diabetes drug candidate (Zhang and Moller, 2005). In term of safety issue, Cbl/CAP pathway will be a better choice for drug target. This is because, constitutively activation of PI3K might lead to tumourigenesis as PI3K is also involved in various cellular processes such as cell survival and growth factor transduction pathway (Jaiswal et al., 2009). Apart from that, down regulation of PI3K regulator subunit could bring to protein-protein interactions which may interrupt the metabolism process in the body (Okkenhaug and Vanhaesebroeck, 2001, Koyasu, 2003, Mellor et al., 2012). Thus, agent that can stimulate Cbl/CAP pathway in GLUT4 translocation may represent new therapeutic approaches for insulin resistance.

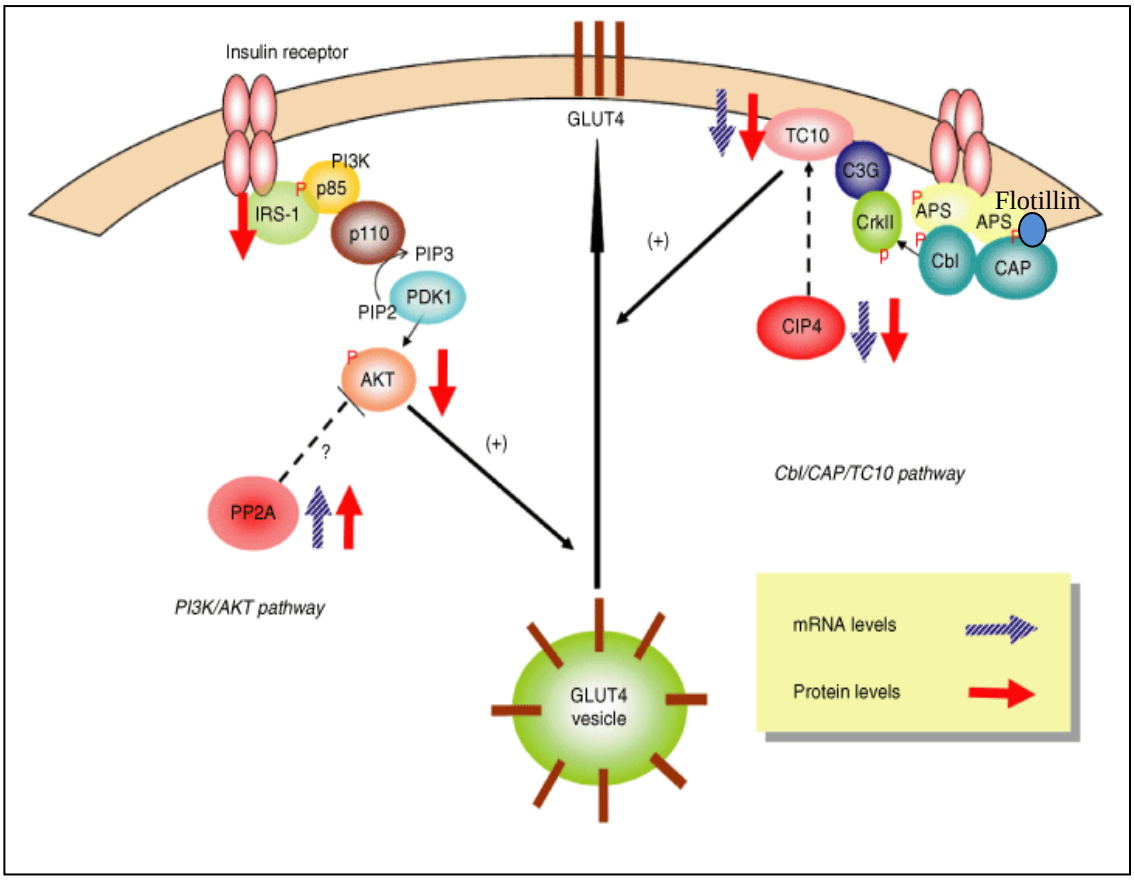

Figure 1.2 Model of PI3K/AKT and Cbl/CAP/TC10 pathway in GLUT4 translocation (Jun et al., 2008).

1.3 Insulin Resistance

Looking back, insulin resistance is actually the major cause of T2DM (Kahn et al., 2006). Insulin resistance is a metabolic syndrome closely link to various type of disease such as T2DM, polycystic ovary syndrome (Dunaif, 1997), obesity and hypertension (Bloomgarden, 2002, Saad et al., 2004). However, here focus is set only on the relationship between insulin resistance and T2DM and how this relationship can bring to discovery of anti-diabetes drug (Kahn et al., 2006).

Insulin resistance is a physiological condition where the body cells especially muscle and fat cells become resistance to insulin. In other word, the insulin become less effective to bring down glucose level in blood stream which eventually will lead to hyperglycemia, a major consequence of insulin insensitivity (Lee et al., 2006, Jellinger, 2009).

In order to study the mechanism behind the insulin resistance, genetic modification of genes that encode protein which directly or indirectly involved in insulin signaling pathway will provide crucial information of the genes that related to insulin insensitivity (Saad et al., 2004). Table 1.1 showed the phenotypic effect of genetic modified mouse towards insulin resistance.

Genetically inhibited gene expression that encode IR, IRS1 & 2, P85α and β, Akt2, Glut4 and PPARγ have shown to cause insulin resistance and diabetes in GM mice, while genes that encode protein-tyrosine-phosphate-1B (PTP1B), SH domain containing inositol 5-phosphate (SHIP2), TNFα, TNFα receptor and Adiponectin have show to improved the insulin sensitivity which mean that agents that stimulated the expression of PTP1B, SHIP2, TNFα, TNFα receptor and Adiponectin gene would be

Table 1.1 Show the phenotypic effect of genetic modified mouse towards insulin resistance.

Target Gene	Genetic Modification	Physiological Respond	References
IR	-/-	Severe diabetes	(Joshi et al., 1996, Accili et al., 1996)
IRS1	-/-	Insulin Resistance	(Araki et al., 1994, Tamemoto et al., 1994)
IRS2	-/-	Insulin Resistance with diabetes	(Kubota et al., 2000)
IRS3	-/-	No effect on glucose homeostasis	(Liu et al., 1999)
IRS4	-/-	Mild effect on glucose homeostasis	(Fantin et al., 2000)
P85α and β	-/-	Lethal	(Fruman et al., 2000, Ueki et al., 2002)
P85α and β	+/-	hypoglycemia	(Fruman et al., 2000, Mauvais-Jarvis et al., 2002)
Akt1	-/-	No diabetes	(Lawlor et al., 2002)
Akt2	-/-	Insulin resistance with diabetes	(Cho et al., 2001, Garofalo et al., 2003, Peng et al., 2003)
Glut4	-/-	Diabetes	(Rossetti et al., 1997)
Glut4	+/-	Diabetes	(Stenbit et al., 1997)
PTP1B	-/-	enhance insulin sensitivity	(Klaman et al., 2000)
SHIP2	-/-	Improve insulin sensitivity	(Clement et al., 2001)
TNFα	-/-	Improve insulin sensitivity	(Uysal et al., 1997)
TNFα receptor	-/-	Improve insulin sensitivity	(Uysal et al., 1998)
Adiponectin	-/-	Improve insulin sensitivity	(Maeda et al., 2002)
PPARγ	-/-	Severe diabetes	(Kubota et al., 1999)
PPARγ	+/-	Improve insulin sensitivity	(Kubota et al., 1999)

the main factor to contribute to insulin resistance. All in all, genetically modified on selected genes of mice have provided a very clear picture on the genes which are involved in the molecular mechanism leading to insulin resistance.

Apart from that, there are lots of scientific proves that insulin resistance is closely related with obesity especially in upper body obesity. Obesity is a medical condition in which excess fat accumulated in the body and these fat will store inside a specify tissues known as adipose tissue (Kopelman, 2000).

Adipose tissues are making up of adipocytes or fat cells, it is mainly distribute in the hips and thighs for women and upper body for males (Arner, 1997). Previously, adipocyte is merely known as an energy or fat storage and has been ignored by most scientists for centuries. But after a few decade of intense investigation, it was found out that adipocyte is not merely fat storage but served as an endocrine organ, a major factory to secrete variety factors that potentially mediate insulin resistance (Mohamed-Ali et al., 1998).

As shown in Figure 1.3, In lean state, small adipocytes store incoming fatty acids as triglyceride, which can be used to generate energy when caloric need. However excessive intake of caloric increases triglyceride input and leads to inflammation of adipocyte. This eventually affects the physiological activities of adipocyte by over secreting monocyte chemoattractant protein-1 (MCP-1) which takes on more macrophage within adipose tissue. Activation of macrophage by cytokine secrete large amount of tumour necrosis factor-α (TNFα) (Xu et al., 2003, Sartipy and Loskutoff, 2003, Weisberg et al., 2003).

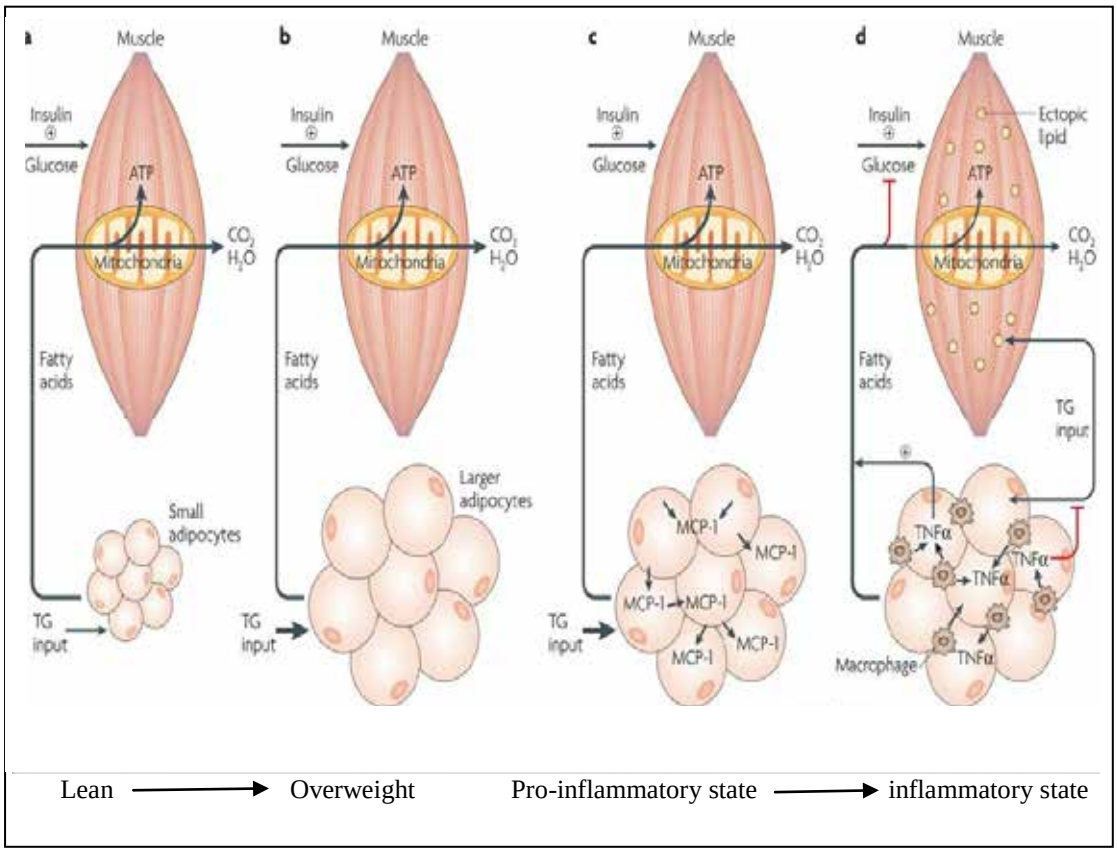

Figure 1.3 Physiological condition of adipocyte in lean state, pro-inflammatory state and inflammatory state (Guilherme et al., 2008).

Tumor Necrosis Factor α , TNFα is one of the important factors that contribute to insulin resistance that inactivates several molecules which take part in insulin signaling pathway such as down regulation of IR, defect of the IR tyrosine kinase activity and decreased activity of IRS-1 and PI3 kinase. (Hotamisligil and Spiegelman, 1994, Hotamisligil et al., 1995). Study also showed that TNFα secretion led to increased in lipolysis and decreased in triglyceride synthesis (Guilherme et al., 2008, Ruan and Lodish, 2003). At the same time, it also increased level of circulating FFA and accumulation of activated lipids in skeletal muscle, and liver (Lagathu et al., 2006). The activated lipid and fatty acid derivatives are known to affect normal metabolic process or causing lipotoxicity in the body (Unger, 1995, Unger, 2002). Lastly, prolonged caloric overload will also cause adipocyte dysfunction and disrupting functions such as insulin-stimulated glucose transport, thus triggering insulin resistance (Guilherme et al., 2008)

But the question is what will be the right target on controlling the inflammatory process in adipose tissue that brings to adipocyte dysfunction? Many studies have emphasized on the effect of TNFα on insulin resistance, adipogenesis and triglyceride storage. However, highlighted here showed that down regulation of peroxisome proliferator activated-receptor gamma (PPARγ) is a key element in controlling adipocyte inflammation. This has been proven by adding extra TNFα into adipocytes that result in rapid decrease of PPARγ mRNA level, inhibit post-transcription process of PPARγ protein by up-regulating mitogen-activated protein kinase 4 (MAP4K4) and degrading PPARγ protein. It is clear that, down regulation of PPARγ by TNFα have decreased triglyceride storage and increase FFA circulation which in turn lead to insulin insensitivity and T2D (Guilherme et al., 2008).

It is interesting to note that by activating PPARγ by its agonist, such as thiazolidinediones, have shown a very clear effect on decreasing TNFα and antagonize the inhibition of insulin signaling by TNFα as well as reversing the overall physiological condition of adipocyte that due to TNFα (Sandra C. Souza et al., 1998, Dıaz-Delfin et al., 2007). Besides that, the activated PPARγ also stimulates c-Cbl-associated protein (Lagathu et al.) expression in adipose tissue and increased insulin-stimulated c-Cbl phosphorylation pathway in GLUT4 translocation which facilitate glucose uptake as discussed previously (Section 1.2.1.2) and at the same time increase insulin sensitivity (Ribon et al., 1998, Balasubramanyam and Mohan, 2000). Therefore, it will be valuable to study on PPARγ as a potential target on designing drug for T2D as it appears to be the principle target in the pathogenesis of T2D.

1.4 Peroxisome Proliferator Activated Receptor Gamma

Peroxisomal Proliferator Activated Receptor gamma (PPARγ) is one of the three types of PPAR isoforms, PPAR alpha, PPAR delta and PPAR gamma, and it belongs to nuclear receptor superfamily of ligand activated transcription factor. PPARγ plays an important role in regulation of adipocyte gene expression and differentiation, insulin sensitivity, glucose homeostasis and lipid metabolism. (Berger and Moller, 2002).

There are two isoforms of PPARγ that have been found in mice (Tontonoz et al., 1994a), γ1 and γ2. Both γ1 and γ2 are expressed from the same gene but the levels of expression are varying among the tissues. PPARγ1 is expressed in adipose, liver, skeletal and cardiac muscle, and macrophages, while PPARγ2 is expressed predominantly in adipose tissue (Tontonoz et al., 1994b, Brun et al., 1996). The two

isotypes of PPARγ are form due to the post-translational modification process which involved different RNA splicing of the same gene and used of different promoter (Zhu et al., 1995). The PPARγ2 has an additional 28 amino acids in N-terminal compared to PPARγ1.

Structurally, PPARγ is made up of four domain structures. That are the ligand-binding domain (LBD) at the COOH- terminal region, which is made up of around 250 amino acids, a conserved DNA-binding domain encoding, two zinc fingers and the NH2-terminal ligand independent transcriptional activation function (AF-1) domain, which regulates PPAR activity and a docking domain for cofactor (Weatherman et al., 1999, Wilson et al., 2001, Nettles, 2008, Farce et al., 2009).

Ligand binding domain acts as a hormone binding and also contains dimerization motif and transcriptional activation function (AF2) domain associated with helix 12 (H12) (Sheu et al., 2005, Freedman, 1999). Structural studies indicated that precise position of AF2 helix is important for co-activator recruitment and selective genes regulation (Gampe et al., 2000, Motani et al., 2009b).

There are 12 α-helices and 4 short β-sheet folded into sandwich like hydrophobic cavity of LBD (Uppenberg et al., 1998). A special feature of LBD of PPARγ is that, it has a big Y- shape cavity within the protein with a total volume of 1300 to 1400 Å (Zoete et al., 2007). The cavity is made up of flexible entrance between H3 and β-strands, which extend from the surface of the protein to create solvent-accessible channel and two branching arms with approximately 12.0 Å in length. Flexibility of the entrance allows the ligand to enter the binding site of the protein without significantly changing the conformation of the LBD (Xu et al., 1999, Lu et al., 2006). While the two

arms act as a binding site for the ligand. Arm I is made up of polar residues like His^{323}, Tyr^{327}, Lys^{367}, His^{449} and Tyr^{473} to create a polar surface while the second arm is delimited by Phe^{226}, Pro^{227}, Ile^{296} and Met^{329}. Both entrance and Arm II are more hydrophobic, thus usually occupied by hydrophobic tail of the ligand (Uppenberg et al., 1998).

Typically, the binding pockets of PPARγ are more hydrophobic in nature compared to the surface and this phenomenon enhances the effect of ionic interaction. Ionic interaction is usually important for initial interaction as the drug enters the binding site and it is the strongest intermolecular bonds which takes place between two functional groups having opposite charge.

PPARγ can be activated by specific nutrients, non-nutrient endogenous ligands, and drug such as thiazolidinediones and glitazone. In the inactivated state, PPARγ forms heterodimer with RXR. The PPARγ-RXR complex associated with a group of co-repressor to prevent deacetylation of Histone to inhibit the gene transcription process. Once the ligand bound with PPARγ, the LBD undergoes a conformational change in such a way that the AF-2 helix, together with H3 and H4 form a hydrophobic groove (Gampe et al., 2000). This results in the dissociation of co-repressor such as SMRT (silencing mediator for retinoid and thyroid hormone receptor) from the PPARγ-RXR heterodimer and the co-activator molecule is associated to fully activate the PPARγ (Lavinsky et al., 1998, Zhu et al., 2000, Glass and Rosenfeld, 2000). The activated PPARγ-RXRα complex binds to DNA motif element, PPAR respond elements (PPRE), in promoter region of the targeted gene for acyl-CoA oxidase to modulate the gene

transcription process as showed in Figure 1.4 (Glass and Rosenfeld, 2000, Balasubramanyam and Madras, 2000).

Currently there are number of co-activators identified and characterized such as CREB binding protein (CBP), P300, steroid receptor co-activator (SRC-1), PPAR binding protein (PBP), PPARγ co-activator-1 (PGC-1) and PPAR interacting protein (PRIP). The recruitment of co-activator is totally depending on the structure of PPARγ which in turn is determined by the ligand type (Balasubramanyam and Madras, 2000, Gelman et al., 2007).

As discuss previously, the unique characteristic of PPARγ LBD with big hydrophobic cavity and hydrophilic Arm II has created an environment which can recruit a wide variety of natural and synthetic ligand to give different respond. Ligand for PPARγ can be classified into three major classes: PPARγ against, PPARγ antagonist, and selective PPARγ modulator. But the most famous studied were PPARγ agonists and selective PPARγ modulators.

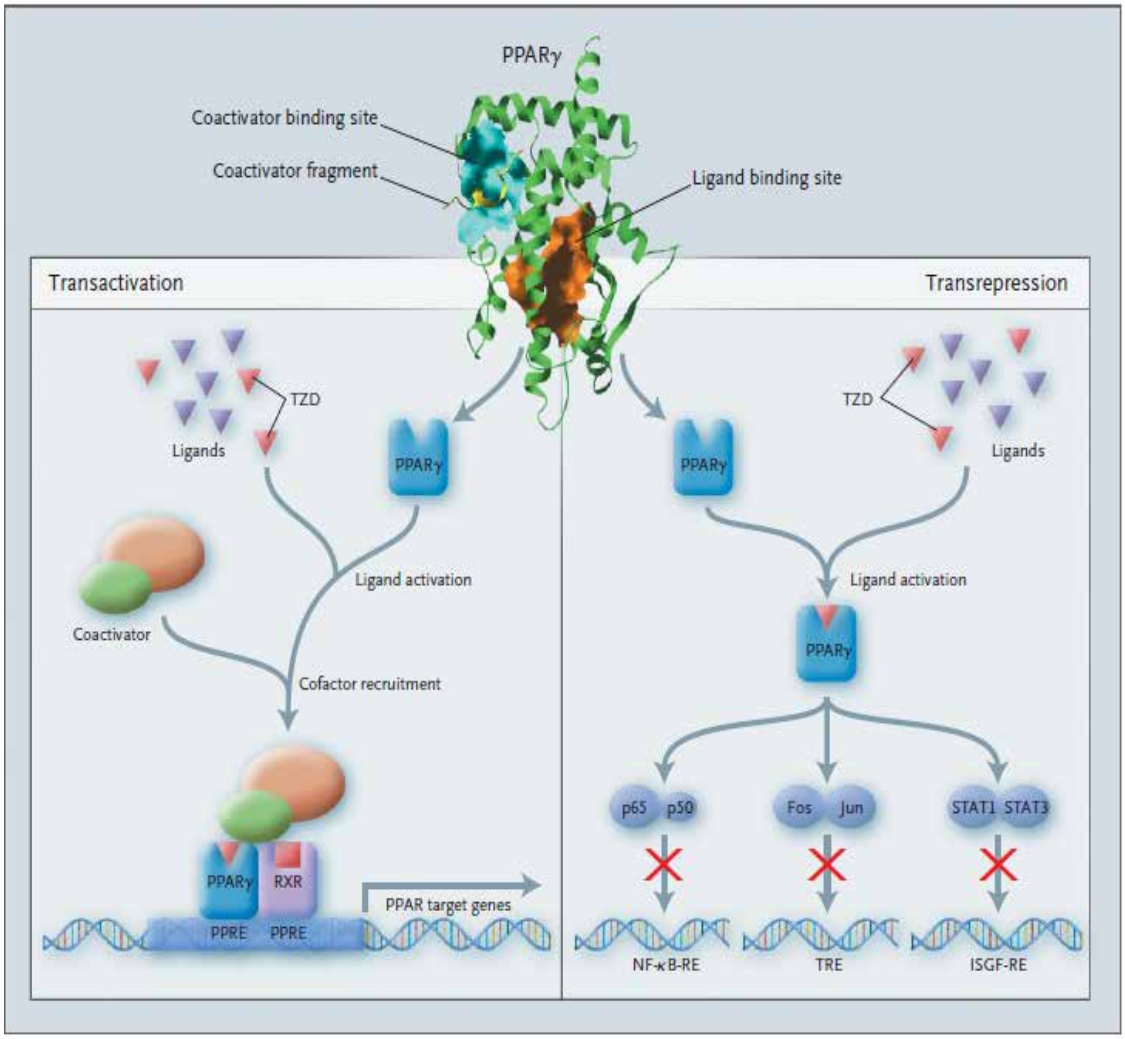

Figure 1.4 Mechanism of PPARγ activation and transcriptional regulation of target genes (Yki-Jarvinen, 2004).

1.4.1 PPARγ Agonists

PPARγ agonist is a receptor ligand which fully activates PPARγ receptor to induce full response of its activity. PPARγ has been shown to be the molecular target for thaizolidinediones (TZD), a new class of antidiabetic drug that includes troglitazone (Rezulin), rosiglitazon (Avandia), and pioglitazone (Actos) (Shearer and Billin, 2007). Pharmaceutically, TZD was used as insulin sensitizer in T2D patients. Administration of TZD class drugs such as Rosiglitazon has showed in consistently lower fasting and postprandial glucose concentrations as well as free fatty acids and insulin concentration in several manners (Nolan et al., 1994, Miyazaki et al., 2001, Bajaj et al., 2004).

The activated PPARγ stimulates the expression of different set of genes particularly involved in lipid and glucose metabolism. According to the "fatty acid steal" hypothesis, PPARγ agonists promote fatty acid uptake and storage in adipose tissues by enhancing adiponectin secretion. This however increases the adipose tissues mass and protects muscle, liver and possibly pancreatic beta cells from exposing to harmful metabolic effect of high fatty acid concentration (Martin et al., 1998, Yki-Jarvinen, 2004). Apart from that, activation of PPARγ by its agonist also stimulates adipogenesis and block the action of TNFα (Peraldi et al., 1997), which inhibits insulin signaling pathway as well as TNFα induced glycerol and non-esterified fatty acid release (Souza et al., 1998). Hence, activation of PPARγ decreases various signaling molecules such as free fatty acids, leptin, and TNFα, which are able to counteract the hypoglycemic action of insulin.

PPARγ agonist molecule usually possesses an acidic head involved in the hydrogen bond network, a central aromatic moiety and a hetero-aromatic hydrophobic

tail. This topology is preserved in a large number of synthetic PPARγ ligands, which belong mainly to five chemical classes: thiazolindinediones, tyrosined-based, indole-based, propionic-acid and phenylacetic acid derivatives (Giaginis et al., 2009).

Troglitazone is the first approved TZD drug on the market but was withdraw from the marketplace because of the idiosyncratic liver toxicity (Shearer and Billin, 2007). There are some other PPARγ agonist's marketed drugs such as rosiglitazon and pioglitazon. Both rosiglitazon and pioglitazon have been published by the DrugBank with the accession number DB00412 and DB01132 respectively.

Nevertheless, the marketed TZD classes of PPARγ agonist have been proven to have some side effect such as weigth gain, mascular and pulmonary oedema and fluid accumulation (Nesto et al., 2003). Oedema is the most undesired side effect for PPARγ agonist and is shown in 10-15% of patients treated with TZD. The accumulation of fluid and oedema will increase the incident of heart failure (Scheen, 2004). Is it believed that, PPAPγ is selectively expressed in the medullar collecting duct and induces the expression of the epithelial Na+ channel of γ subunit, which may play an important role in PPARγ mediated sodium reabsorption and increase fluid retention (Guan et al., 2005, Zhang et al., 2005, Nicholas et al., 2001). Thus, identification of PPARγ-specific target gene is necessary for designing a new TZD class PPARγ agonist, which is free from fluid retention and oedema.

The second major problem of TZD treatment is the increased the body weight of treated patients. PPARγ is known to be involved in adipogenesis; stimulation of PPARγ by TZD will lead to the formation of new adipose tissue that promotes the adipocyte

differentiation and lipid accumulation and lastly contributes to increase body weight (Shearer and Billin, 2007).

1.4.2 Selective PPARγ Modulator

Selective PPAR modulator (SPPARM) is selective combinations of agonist and antagonist compound, which induced spesific changes of receptor conformation and regulate a specific subset of target genes by recruiting particular coregulator. The concept of SPPARM was based on the discovery of selective estrogen receptor modulator (SERM) drug. Compared with estradiol, a natural ligand for estrogen receptor, SERM (tamoxifen) antagonist the activity in endometrium which can lead to endometrial hyperplasia and increase the susceptibility to cancer (Shang et al., 2000).

The functional profile of SERM had brought to the new stage of drug discovery involving nuclear receptor by providing a better understanding on the importance of selective recruitment of coregulator by nuclear receptor in genes regulation as well as emerging the idea of designing selective PPARγ modulaotr. The objective for designing the SPPARM is to improve the therapeutic index by modulating the desired biological activity while reducing the undesired side effect (Gelman et al., 2007, Shearer and Billin, 2007).

SPPARM can be characterized by its function as full agonist in particular tissue with sufficient expression of coactivator and partial agonist or antagonist in the selective tissues with adequate concentration of coactivator. Therefore the activities of SPPARM are varied according to the cell type and its context (Olefsky and Saltiel, 2000, Miles et al., 2000).