2. REVIEW OF LITERATURE

2.1. General

2.1.1. Diabetes: the disease

Diabetes is any disorder characterized by excessive urine excretion. The most common form of diabetes is diabetes mellitus, a metabolic disorder in which there is an inability to oxidize carbohydrate due to disturbances in insulin function. Diabetes mellitus is characterized by elevated glucose in the plasma and episodic ketoacidosis. Additional symptoms of diabetes mellitus include excessive thirst, glucosuria, polyuria, lipemia and hunger. If left untreated the disease can lead to fatal ketoacidosis. Other forms of diabetes include diabetes insipidus and brittle diabetes. Diabetes insipidus is the result of a deficiency of antidiuretic hormone (ADH, also referred to as vasopressin or arginine vasopressin, AVP). The major symptom of diabetes insipidus (excessive output of dilute urine) results from an inability of the kidneys to resorb water. Brittle diabetes is a form that is very difficult to control. It is characterized by unexplained oscillations between hypoglycemia and acidosis.\textsuperscript{150}

Criteria, which clinically establish an individual as suffering from diabetes mellitus, include:

(i) Having a fasting plasma glucose level in excess of 126mg/dL (7mmol/L). Normal levels should be less than 100mg/dL (5.6mmol/L) or:
(ii) Having plasma glucose levels in excess of 200mg/dL (11mmol/L) at two times points during an oral glucose tolerance test, OGTT, one of which must be within 2 hrs of ingestion of glucose.

Glucose tolerance curve for a normal person and non-insulin-dependent diabetes mellitus (NIDDM, Type 2 diabetes) patient is shown in figure RL1.

The earlier a person is diagnosed with diabetes (principally type 2) the better chance the person has of staving off the primary negative consequences which are renal failure, blindness and limb amputations due to circulatory problems. The American Diabetes Association is planning to recommend that physicians consider patients to be pre-diabetic.
if their fasting blood glucose level is above 100mg/dL but less than 125mg/dL and whose glucose levels are at least 140mg/dL but less than 200mg/dL following an oral glucose tolerance test (OGTT).

![Glucose tolerance curve for a normal person and one with non-insulin-dependent diabetes mellitus (NIDDM, Type 2 diabetes). The dotted lines indicate the range of glucose concentration expected in a normal individual.]

**Figure RL1:** Glucose tolerance curve for a normal person and one with non-insulin-dependent diabetes mellitus (NIDDM, Type 2 diabetes). The dotted lines indicate the range of glucose concentration expected in a normal individual.

### 2.1.2. Types of Diabetes Mellitus

Diabetes mellitus is a heterogeneous clinical disorder with numerous causes. Two main classifications of diabetes mellitus exist, **idiopathic** and **secondary**.

Idiopathic diabetes is divided into two main types; insulin dependent and non-insulin-dependent. Insulin-dependent diabetes mellitus, IDDM (more commonly referred to as **type 1 diabetes**) is defined by the development of ketoacidosis in the absence of insulin therapy. Type 1 diabetes most often manifests in childhood (hence, also called juvenile onset diabetes) and is the result of an autoimmune destruction of the β-cells of the pancreas. Non-insulin-dependent diabetes mellitus, NIDDM (more commonly referred to as **type 2 diabetes**) is characterized by persistent hyperglycemia but rarely leads to ketoacidosis. Type 2 diabetes generally manifests after age 40 and therefore has the obsolete name of adult onset-type diabetes. Type 2 diabetes can result from genetics defects that cause both insulin resistance and insulin deficiency. There are two main forms of type 2 diabetes:

(i) Late onset associated with obesity.
(ii) Late onset not associated with obesity.

There is a strong correlation between obesity and the onset of type 2 diabetes with its associated insulin resistance. It should be pointed out that in the United States the proportion of the population under 40 that can be clinically defined as obese now exceeds 25%. Many children are obese and are developing type 2 diabetes at an alarming epidemic rate. The dramatic rise in obesity in the US has lead to an equally alarming increase in the percentage of the population who suffer from the metabolic syndrome. The metabolic syndrome is a clustering of atherosclerotic cardiovascular disease risk factors, one of which involves insulin resistance characteristic in type 2 diabetes. It should be pointed out that obesity alone does not always lead to insulin resistance as some individuals who are obese do not experience insulin resistance and conversely, some individuals who manifest insulin resistance are not obese. These latter observations point to the added role of genetics in the acquisition of insulin resistance.150

Secondary or other specific types of diabetes mellitus are the result of many causes including:

1. Maturity onset type diabetes of the young (MODY) was previously considered to be a third form of type 2 diabetes. However, with the discovery of specific mutations leading to MODY, it is now classified under secondary or other specific types of diabetes. MODY is characterized by onset prior to age 25. All cases to date have shown impaired β-cell function. Patients may also exhibit insulin resistance and late β-cell failure. Evidence indicates that mutations in 10-12 different genes have been correlated with the development of MODY. Mutations in the 8 genes described here are all clearly correlated to MODY:

   **MODY1:** the transcription factor identified as hepatic nuclear factor-4α (HNF-4α; gene symbol = HNF4A). This gene is also called transcription factor-14 (TCF14). Expression of HNF-4α is associated with the growth and normal functioning of the pancreas. Many genes are known to be regulated by HNF-4α including those encoding HNF-1α, PPARα, insulin, glucose-6-phosphatase, GLUT2, the liver pyruvate kinase isoform (L-PK) which is also expressed in the pancreas, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), aldolase B and uncoupling protein 2, UCP2.

   **MODY2:** pancreatic glucokinase

   **MODY3:** the transcription factor HNF-1α (gene symbol = HNF1A). This gene is also called hepatocyte transcription factor-1 (TCF1). HNF-1α is involved in a
regulatory loop with HNF-4α controlling many genes involved in liver function such as the GLUT2 and L-PK genes.

**MODY4:** the homeodomain transcription factor insulin promoter factor-1 (IPF-1). This gene is more commonly called PDX1 which is derived from pancreas duodenum homeobox-1.

**MODY5:** the transcription factor HNF-1β. This gene is also called hepatocyte transcription factor-2 (TCF2). HNF-1β is a critical regulator of a transcriptional network that controls the specification, growth, and differentiation of the embryonic pancreas. In humans, mutations in the HNF-1β gene (symbol = HNF1B) are associated with pancreatic hypoplasia, defective kidney development and genital malformations.

**MODY6:** the bHLH transcription factor NeuroD1. NeuroD1 was first identified as a neural fate-inducing gene. The hamster β2 gene, shown to regulate insulin transcription is identical to NeuroD1 so the gene is often called NeuroD/β2. MODY6 is a rare form of MODY

**MODY7:** the Krüppel-like factor 11 (KLF11) protein is a zinc-finger transcription factor that is involved in activation of the insulin promoter. KLF11 is a TGF-β-inducible transcription factor.

**MODY8:** the carboxyl-ester lipase gene (CEL) which is involved in lipid metabolism. Frameshift deletions in the variable number tandem repeats (VNTR) of the CEL gene are associated with MODY8 which is characterized by pancreatic exocrine and β-cell dysfunction. MODY8 is a rare form of MODY.

2. **Pancreatic disease:** Pancreatectomy leads to the clearest example of secondary diabetes. Cystic fibrosis and pancreatitis can also lead to destruction of the pancreas.

3. **Endocrine disease:** Some tumors can produce counter-regulatory hormones that oppose the action of insulin or inhibit insulin secretion. These counter-regulatory hormones are glucagon, epinephrine, growth hormone and cortisol.

   a) Glucagonomas are pancreatic cancers that secrete glucagon.

   b) Pheochromocytomas secrete epinephrine.

   c) Cushing syndrome results in excess cortisol secretion.

   d) Acromegaly results in excess growth hormone production.

4. **Drug-induced diabetes:** treatment with glucocorticoids and diuretics can interfere with insulin function.

5. **Anti-insulin receptor autoantibodies (Type B insulin resistance).**
6. Mutations in the insulin gene.

7. Mutations in insulin receptor gene which lead to the syndromes listed below. Two clinical features are common in all syndromes that result from mutations in the insulin receptor gene: acanthosis nigricans and hyperandrogenism (the latter being observed only in females).

   a) Donohue syndrome (also referred to as Leprachaunism)
   b) Rabson-Mendenhall syndrome
   c) Type A insulin resistance

8. Gestational diabetes; this syndrome sets in during pregnancy and usually resolves itself following childbirth.

9. Many other genetic syndromes have either diabetes or impaired glucose tolerance associated with them; lipoatrophic diabetes, Wolfram syndrome, Down syndrome, Klinefelter syndrome (XXY males), Turner syndrome, myotonic dystrophy, muscular dystrophy, Huntington disease, Friedreich ataxia, Prader-Willi syndrome, Werner syndrome, Cockayne syndrome, and others such as those indicated above.

2.1.3. Insulin-Dependent Diabetes Mellitus (IDDM) Type 1

2.1.3.1. Etiology of Type 1 Diabetes

Type 1 diabetes has been shown to be the result of an autoimmune reaction to antigens of the islet cells of the pancreas. There is a strong association between IDDM and other endocrine autoimmunities (e.g. Addison disease). Additionally, there is an increased prevalence of autoimmune disease in family members of IDDM patients.

Types of Autoantibodies

1. Islet cell cytoplasmic antibodies: The primary antibodies found in 90% of type 1 diabetics are against islet cell cytoplasmic proteins (termed ICCA, islet cell cytoplasmic antibodies). In non-diabetics ICCA frequency is only 0.5%–4%. The presence of ICCA is a highly accurate predictor of future development of IDDM. ICCA are not specific for the β-cells and recognize antigens in other cell types in the islet. However, the autoimmune attack appears to selectively destroy β-cells. Therefore, the antibodies may play a primary role in the destruction of islet cells. It is an equally likely possibility that the production of anti-islet antibodies occurs as a result of the destruction of β-cells. Whether a direct cause or an effect of islet cell destruction, the titer of the ICCA tends to decline over time.
2. Islet cell surface antibodies: Autoantibodies directed against cell-surface antigens (ICSAs) have also been described in as many as 80% of type 1 diabetics. Similar to ICCAs, the titer of ICSA declines over time. Some patients with type 2 diabetes have been identified that are ICSA positive.

3. Specific antigenic targets of islet cells: Antibodies to glutamic acid decarboxylase (GAD) have been identified in over 80% of patients newly diagnosed with IDDM. Like ICCAs, anti-GAD antibodies decline over time in type 1 diabetics. The presence of anti-GAD antibodies is a strong predictor of the future development of IDDM in high-risk populations. Anti-insulin antibodies (IAAs) have been identified in IDDM patients and in relatives at risk to develop IDDM. These IAA are detectable even before the onset of insulin therapy in type 1 diabetics. IAA are detectable in around 40% of young children with IDDM.

2.1.3.2. Pathophysiology of Type 1 Diabetes

The autoimmune destruction of pancreatic β-cells leads to a deficiency of insulin secretion. It is this loss of insulin secretion that leads to the metabolic derangements associated with IDDM. In addition to the loss of insulin secretion, the function of pancreatic α-cells is also abnormal. There is excessive secretion of glucagon in IDDM patients. Normally, hyperglycemia leads to reduced glucagon secretion. However, in patients with IDDM, glucagon secretion is not suppressed by hyperglycemia. The resultant inappropriately elevated glucagon levels exacerbates the metabolic defects due to insulin deficiency (see below). The most pronounced example of this metabolic disruption is that patients with IDDM rapidly develop diabetic ketoacidosis in the absence of insulin administration. If somatostatin is administered to suppress glucagon secretion, there is a concomitant suppression in the rise of glucose and ketone bodies. Particularly problematic for long term IDDM patients is an impaired ability to secrete glucagon in response to hypoglycemia. This leads to potentially fatal hypoglycemia in response to insulin treatment in these patients.

Although insulin deficiency is the primary defect in IDDM, in patients with poorly controlled IDDM there is also a defect in the ability of target tissues to respond to the administration of insulin. There are multiple biochemical mechanisms that account for this impairment of tissues to respond to insulin. Deficiency in insulin leads to elevated levels of free fatty acids in the plasma as a result of uncontrolled lipolysis in adipose tissue. Free fatty acids suppress glucose metabolism in peripheral tissues such as skeletal
muscle. This impairs the action of insulin in these tissues, i.e. the promotion of glucose utilization. Additionally, insulin deficiency decreases the expression of a number of genes necessary for target tissues to respond normally to insulin such as glucokinase in liver and the GLUT 4 class of glucose transporters in adipose tissue. The major metabolic derangements which result from insulin deficiency in IDDM are impaired glucose, lipid and protein metabolism.  

**Glucose Metabolism:** Uncontrolled IDDM leads to increased hepatic glucose output. First, liver glycogen stores are mobilized then hepatic gluconeogenesis is used to produce glucose. Insulin deficiency also impairs non-hepatic tissue utilization of glucose. In particular in adipose tissue and skeletal muscle, insulin stimulates glucose uptake. This is accomplished by insulin-mediated movement of glucose transporter proteins to the plasma membrane of these tissues. Reduced glucose uptake by peripheral tissues in turn leads to a reduced rate of glucose metabolism. In addition, the level of hepatic glucokinase is regulated by insulin. Therefore, a reduced rate of glucose phosphorylation in hepatocytes leads to increased delivery to the blood. Other enzymes involved in anabolic metabolism of glucose are affected by insulin (primarily through covalent modifications). The combination of increased hepatic glucose production and reduced peripheral tissues metabolism leads to elevated plasma glucose levels. When the capacity of the kidneys to absorb glucose is surpassed, glucosuria ensues. Glucose is an osmotic diuretic and an increase in renal loss of glucose is accompanied by loss of water and electrolytes, termed polyuria. The result of the loss of water (and overall volume) leads to the activation of the thirst mechanism (polydipsia). The negative caloric balance which results from the glucosuria and tissue catabolism leads to an increase in appetite and food intake (polyphagia).

**Lipid Metabolism:** One major role of insulin is to stimulate the storage of food energy following the consumption of a meal. This energy storage is in the form of glycogen in hepatocytes and skeletal muscle. Additionally, insulin stimulates hepatocytes to synthesize triglycerides and storage of triglycerides in adipose tissue. In opposition to increased adipocyte storage of triglycerides is insulin-mediated inhibition of lipolysis. In uncontrolled IDDM there is a rapid mobilization of triglycerides leading to increased levels of plasma free fatty acids. The free fatty acids are taken up by numerous tissues.
(however, not the brain) and metabolized to provide energy. Free fatty acids are also taken up by the liver.

Normally, the levels of malonyl-CoA are high in the presence of insulin. These high levels of malonyl-CoA inhibit carnitine palmitoyltransferase I, the enzyme required for the transport of fatty acyl-CoA's into the mitochondria where they are subject to oxidation for energy production. Thus, in the absence of insulin, malonyl-CoA levels fall and transport of fatty acyl-CoA's into the mitochondria increases. Mitochondrial oxidation of fatty acids generates acetyl-CoA which can be further oxidized in the TCA cycle. However, in hepatocytes the majority of the acetyl-CoA is not oxidized by the TCA cycle but is metabolized into the ketone bodies, acetoacetate and β-hydroxybutyrate. These ketone bodies leave the liver and are used for energy production by the brain, heart and skeletal muscle. In IDDM, the increased availability of free fatty acids and ketone bodies exacerbates the reduced utilization of glucose furthering the ensuing hyperglycemia. Production of ketone bodies, in excess of the organisms ability to utilize them leads to ketoacidosis. In diabetics, this can be easily diagnosed by smelling the breath. A spontaneous breakdown product of acetoacetate is acetone which is volatilized by the lungs producing a distinctive odor.

Normally, plasma triglycerides are acted upon by lipoprotein lipase (LPL), an enzyme on the surface of the endothelial cells lining the vessels. In particular, LPL activity allows fatty acids to be taken from circulating triglycerides for storage in adipocytes. The activity of LPL requires insulin and in its absence a hypertriglyceridemia results.  

**Protein Metabolism:** Insulin regulates the synthesis of many genes, either positively or negatively that then affect overall metabolism. Insulin has a global effect on protein metabolism, increasing the rate of protein synthesis and decreasing the rate of protein degradation. Thus, insulin deficiency will lead to increased catabolism of protein. The increased rate of proteolysis leads to elevated concentrations in plasma amino acids. These amino acids serve as precursors for hepatic and renal gluconeogenesis. In liver, the increased gluconeogenesis further contributes to the hyperglycemia seen in IDDM. IDDM is accompanied by long-term microvascular, neurologic and macrovascular complications. Although the daily management of IDDM is burdensome and the specter of metabolic decomposition ever-present, long-term complications including retinopathy, nephropathy, neuropathy and cardiovascular disease have caused the most morbidity and mortality since the introduction of insulin therapy.
2.1.4. Non-Insulin-Dependent Diabetes Mellitus (NIDDM): Type 2

2.1.4.1. Etiology of Type 2 Diabetes
Type 2 diabetes is characterized by a lack of the need for insulin to prevent ketoacidosis. Type 2 diabetes refers to the common form of idiopathic NIDDM. Type 2 diabetes is not an autoimmune disorder; however, there is a strong genetic correlation to the susceptibility to this form of diabetes. The susceptibility genes that predispose one to NIDDM have not been identified in most patients. This is due in part to the heterogeneity of the genes responsible for the susceptibility to type 2 diabetes. Obesity is a major risk factor that predisposes one to type 2 diabetes. Genetic studies in mice and rats have demonstrated a link between genes responsible for obesity and those that cause diabetes mellitus.

2.1.4.2. Pathophysiology of Type 2 Diabetes
Extensive investigations of the pathophysiology of NIDDM have identified two defects in endocrine functions: insulin resistance and insulin deficiency. Genetic factors contribute importantly to the predisposition to develop NIDDM. Nevertheless, abundant evidence suggests that environmental factors (e.g., nutrition, physical exercise etc.) may also modulate the expression of the diabetic phenotype. Inasmuch NIDDM is a genetic disease; the identity of the cause of the disease is encrypted in the sequence of nucleotides in the patients’ DNA.\textsuperscript{153}

**Insulin resistance:** Insulin is a pluripotent hormone that elicits multiple biological responses. Among its important biological actions insulin accelerates glucose transport in muscle and adipose tissue, regulates the activities of intracellular enzymes, and regulates the transcription of selected genes. Insulin resistance is a pathological condition in which there is a shift in the dose-response curve such that the magnitude of the biological response to insulin is decreased.\textsuperscript{154} The impaired response to insulin either over the entire range of insulin concentration or only at low concentrations of the hormone. Two types of evidence support the conclusion that patients with NIDDM are resistant to the biological actions of insulin. First, patients with NIDDM have diminished response to exogenously administered insulin. The second line of evidence is based on the observations that patients with NIDDM are resistant to the action of endogenously secreted insulin. Hyperinsulinemia in the fasting state is one of the laboratory abnormalities observed relatively early in the natural history of NIDDM. This is a disordered state in which
insulin inadequately stimulates glucose transport in skeletal muscle and fat and inadequately suppresses hepatic glucose production. Glucose tolerance and then diabetes develop as the compensatory hypersecretion of the insulin by β-cells declines. Knowledge of the molecular control of insulin secretion is therefore important for understanding the β-cell dysfunction of T2DM. Zhang et. al. provided compelling evidence that a mitochondrial anion carrier called uncoupling protein 2 is a critical modulator of insulin secretion and that an increase in this protein may cause β-cell dysfunction.¹⁵⁵

**Insulin deficiency:** Pancreatic insulin secretion is subject to regulation by multiple factors. Although the concentration of glucose in the plasma is the most important regulators of insulin secretion, other regulatory influences are important as well (e.g., amino acids, circulating hormones, neurotransmitters, paracrine factors). Insulin deficiency is defined as a pathological condition in which there is an inappropriate decrease in the rate at which the β-cell secretes insulin. Most commonly, normal ranges for the concentration of insulin in plasma are defined as a function of the concentration of glucose in plasma. Nevertheless, because insulin secretion is dynamic process, the levels of insulin in plasma are not constant, but vary from minute to minute throughout the day. Thus, subtle defects in β-cell function may possibly manifest as abnormalities in the rate at which insulin concentrations change as a function of time.¹⁵⁶,¹⁵⁷

As discussed, most patients are hyperinsulinemic early in the natural history of NIDDM.¹⁵⁸-¹⁶³ However, plasma insulin levels usually decline later in the course of the disease, around the time when a patient develops overt NIDDM.

Unlike patients with type 1 diabetes, those with type 2 diabetes have detectable levels of circulating insulin. On the basis of oral glucose tolerance testing (OGTT) the essential elements of type 2 diabetes can be divided into 4 distinct groups; those with normal glucose tolerance, chemical diabetes (called impaired glucose tolerance), diabetes with minimal fasting hyperglycemia (fasting plasma glucose <140 mg/dL), and diabetes mellitus in association with overt fasting hyperglycemia (fasting plasma glucose >140 mg/dL). In patients with the highest levels of plasma insulin (impaired glucose tolerance group) there was also elevated plasma glucose. This indicates that these individuals are resistant to the action of insulin. In the progression from impaired glucose tolerance to diabetes mellitus the level of insulin declines indicating that patients with type 2 diabetes have decreased insulin secretion.
Additional studies have subsequently demonstrated that both insulin resistance and insulin deficiency is common in the average type 2 diabetic patient. Many experts conclude that insulin resistance is the primary cause of type 2 diabetes, however, others contend that insulin deficiency is the primary cause because a moderate degree of insulin resistance is not sufficient to cause type 2 diabetes. As indicated above, most patients with the common form of type 2 diabetes have both defects.

The major clinical complications of type 2 diabetes are the result of persistent hyperglycemia which leads to numerous pathophysiological consequences. As the glucose level rises in the blood the blood becomes more viscous which makes circulation of the blood in the small capillaries difficult. The reduced circulation results in progressive vascular complications leading to diabetic retinopathy (referred to as diabetic blindness), peripheral neuropathy (resulting in numbness in the extremities and tingling in fingers and toes), poor wound healing, and erectile dysfunction. In addition to these major clinical complications, the body reacts by increasing the level of glucose excretion by the kidneys leading to frequent urination which is called polyuria. As the glucose is excreted there is a concomitant loss of water to maintain normal osmolarity of the urine. The water loss leads to excessive thirst called polydypsia.

**Measurement of HbA1c Levels**

The development of hypoglycemia inducing drugs is the major pharmacological focus of type 2 diabetes therapies. Assessment of therapeutic efficacy in the treatment of the hyperglycemia in type 2 diabetes is accomplished by routine measurement of the circulating levels of glycosylated hemoglobin, designated as the level of HbA1c, often designated as just A1C. HbA1 is the major form of adult hemoglobin in the blood and the "c" refers to the glycosylated form of the protein. Since hemoglobin is present in red blood cells and these cells have a limited life span of 120 days in the circulation, measurement of HbA1c levels is a relatively accurate measure of the amount of glucose in the blood and the length of time the level has been elevated. Typical values for HbA1c measurement (using the previous standard Diabetes Control and Complications Trial, DCCT units of %) are shown in the Table below. Beginning in 2011 a new international standard (International Federation of Clinical Chemistry, IFCC units) for the measurement of HbA1c levels will be utilized. This new standard equates the mmole of HbA1c per mole of total measured hemoglobin, Hb (mmol/mol). The method for
calculating the relationship between these two measurement values is to use the following formula:

\[
\text{IFCC-HbA}_{1c} (\text{mmol/mol}) = \left(\text{DCCT-HbA}_{1c} (\%) - 2.15\right) \times 10.929.
\]

To calculate the estimated average glucose (eAG) level in the blood using the DCCT (%) values one would use the following formula:

\[
e\text{AG(mg/dl)} = 28.7 \times \text{A1C} - 46.7 \quad \text{(for glucose level in mM use: } e\text{AG(mM)} = 1.59 \times \text{A1C} - 2.59)
\]

With new IFCC standard the target range of HbA\textsubscript{1c} for healthy levels is 48–59mmol/mol.

### 2.1.5. Diabetes and the Metabolic Syndrome: MetS

Although the metabolic syndrome (also called syndrome X) is not exclusively associated with type 2 diabetes and the associated insulin resistance, the increasing prevalence of obesity and associated development of type 2 diabetes places insulin resistance as a major contributor to the syndrome. The metabolic syndrome is defined as a clustering of atherosclerotic cardiovascular disease risk factors that include visceral adiposity (obesity), insulin resistance, low levels of HDLs and a systemic proinflammatory state. There are key components to the metabolic syndrome which include in addition to insulin resistance (the hallmark feature of the syndrome), hypertension, dyslipidemia, chronic inflammation, impaired fibrinolysis, procoagulation and most telling central obesity.

### 2.1.6. Insulin and Diabetes Mellitus

Diabetes was known as early as the 1st century, A.D. as a disease characterized by Arataeus as "a melting down of the flesh and limbs into urine", although the name diabetes was not yet formally attached to this disease. More understanding of how this disease functioned did not occur until the 19th century when it was found from autopsies that diabetes is accompanied by damage to the pancreas. There was much debate about the specific relationship between the pancreas and diabetes, especially between two scientists from the University of Strasbourg, Oskar Minkowski and Joseph von Mering. They disagreed about the function of pancreatic enzymes in the body, and to solve their argument, they removed the pancreas from a dog and recorded their observations. They found this dog to urinate incessantly, even when taken outside numerous times throughout the day. After testing the sugar concentration in the animal's urine, they
concluded that he had become diabetic. (Research of this sort involving pancreas removal was also conducted on cats, guinea-pigs, rabbits, and rats by a variety of researchers.) Diabetic research continued into the early 1900’s and at the turn of the century Eugene Opie from Johns Hopkins University discovered that diabetes is caused by something contained within cells discovered by Hans Langerhans in 1869, now referred to as islets of Langerhans. Frederick Banting, a graduate of the University of Toronto Medical School, became interested in pancreas and diabetes research in 1920 and approached Professor John Macleod, a well-known researcher in the field of carbohydrate metabolism. Along with Charles Best, a fourth-year medical student at the time, and J.P. Collip, a well-known biochemist, these four scientists discovered a substance that when injected into a diabetic dog, abated the symptoms of diabetes. This discovery was presented in 1922 in a paper that called this substance insulin. In 1923, Banting and Macleod were awarded the Nobel Prize for their discovery of insulin. Banting shared his award with Best, and Macleod shared his award with Collip to acknowledge the invaluable contributions of these men (Bliss, 1982). The primary structure of insulin was determined in 1953 by Frederick Sanger, and despite this being an accomplishment in itself, this was also the first time that the complete amino acid sequence of a protein was determined and proved that proteins have unique covalent structures. The primary amino acid sequence of insulin is shown in figure RL2. The human insulin protein consists of two chains, A and B, which are connected via disulfide bridges.

Figure RL2: The primary amino acid structure of human insulin. As is shown, insulin consists of two peptide chains which are connected by disulfide bonds.
2.1.6.1. **Insulin Secretion**

The major function of insulin is to counter the concerted action of a number of hyperglycemia-generating hormones and to maintain low blood glucose levels. Because there are numerous hyperglycemic hormones, untreated disorders associated with insulin generally lead to severe hyperglycemia and shortened life span.

In addition to its role in regulating glucose metabolism, insulin stimulates lipogenesis, diminishes lipolysis, and increases amino acid transport into cells. Insulin also modulates transcription, altering the cell content of numerous mRNAs. It stimulates growth, DNA synthesis, and cell replication, effects that it holds in common with the insulin-like growth factors (IGFs) and relaxin.

Insulin is synthesized as a preprohormone in the β-cells of the islets of Langerhans. Its signal peptide is removed in the cisternae of the endoplasmic reticulum and it is packaged into secretory vesicles in the Golgi, folded to its native structure, and locked in this conformation by the formation of 2 disulfide bonds. Specific protease activity cleaves the center third of the molecule, which dissociates as C peptide, leaving the amino terminal B peptide disulfide bonded to the carboxy terminal A peptide.

Insulin secretion from β-cells is principally regulated by plasma glucose levels. Increased uptake of glucose by pancreatic β-cells leads to a concomitant increase in metabolism. The increase in metabolism leads to an elevation in the ATP/ADP ratio. This in turn leads to the inhibition of an ATP-sensitive potassium channel (KATP channel). The net result is a depolarization of the cell leading to Ca\(^{2+}\) influx and insulin secretion.

The KATP channel is a complex of 8 polypeptides comprising four copies of the protein encoded by the ABCC8 (ATP-binding cassette, sub-family C, member 8) gene and four copies of the protein encoded by the KCNJ11 (potassium inwardly-rectifying channel, subfamily J, member 11) gene. The ABCC8 encoded protein is also known as the sulfonylurea receptor (SUR). The KCNJ11 encoded protein forms the core of the KATP channel and is called Kir6.2. As might be expected, the role of KATP channels in insulin secretion presents a viable therapeutic target for treating hyperglycemia due to insulin insufficiency as is typical in type 2 diabetes.
Chronic increases in numerous other hormones, such as growth hormone, placental lactogen, estrogens, and progestins, up-regulate insulin secretion, probably by increasing the preproinsulin mRNA and enzymes involved in processing the increased preprohormone.

### 2.1.6.2. Insulin; Role in Regulation of Metabolism

Insulin, secreted by the β-cells of the pancreas, is directly infused via the portal vein to the liver, where it exerts profound metabolic effects. These effects are the response of the activation of the insulin receptor which belongs to the class of cell surface receptors that exhibit intrinsic tyrosine kinase activity. The insulin receptor is a heterotetramer of 2 extracellular α-subunits disulfide bonded to 2 transmembrane β-subunits. With respect to hepatic glucose homeostasis, the effects of insulin receptor activation are specific phosphorylation events that lead to an increase in the storage of glucose with a concomitant decrease in hepatic glucose release to the circulation (Figure RL3; only those responses at the level of glycogen synthase and glycogen phosphorylase are represented).

**Figure RL3:** Actions of insulin-insulin receptor interactions at the level of insulin receptor substrate-1 (IRS1) and activation of the kinase cascade leading to altered activities of glycogen phosphorylase and glycogen synthase. PI3K = phosphatidylinositol-3-kinase; PIP$_2$ = phosphatidylinositol-4,5-bisphosphate; PIP$_3$ = phosphatidylinositol-3,4,5-bisphosphate; PDK1 = PIP$_3$-dependent protein kinase; Tsc1 and Tsc2 = Tuberous sclerosis tumor suppressors 1 (hamartin) and 2 (tuberin); Rheb = Ras homolog enriched in brain; mTOR = mammalian target of rapamycin. PKB/Akt = protein kinase B/Akt2; GSK3 = glycogen synthase kinase-3; S6K = 70kDa ribosomal protein S6 kinase, also called p70S6K. The insulin-mediated activation of mTOR also leads to changes in protein synthesis (Figure RL5).
In most nonhepatic tissues, insulin increases glucose uptake by increasing the number of plasma membrane glucose transporters: GLUTs. Glucose transporters are in a continuous state of turnover. Increases in the plasma membrane content of GLUTs stem from an increase in the rate of recruitment of the transporters into the plasma membrane, deriving from a special pool of preformed transporters localized in the cytoplasm. GLUT1 is present in most tissues, GLUT2 is found primarily in intestine, pancreatic β-cells, kidney and liver, GLUT3 is found primarily in neurons but also found in the intestine, GLUT4 is found in insulin-responsive tissues such as heart, adipose tissue and skeletal muscle and GLUT5 is expressed in intestine, kidney, testes, skeletal muscle, adipose tissue and brain.

**Figure RL4:** Insulin-insulin receptor actions on glycogen homeostasis showing the role of protein targeting glycogen (PTG) in complexing many of the enzymes and substrates together. PTG is a subunit of PP1. Also diagrammed is the response to insulin at the level of glucose transport into cells via GLUT4 translocation to the plasma membrane. GS/GP kinase = glycogen synthase; glycogen phosphorylase kinase. PP1 = protein phosphatase-1 (Arrows denote either direction of flow or positive effects, T lines represent inhibitory effects).
In liver glucose uptake is dramatically increased because of increased activity of the enzymes glucokinase, phosphofructokinase-1 (PFK-1), and pyruvate kinase (PK), the key regulatory enzymes of glycolysis. The latter effects are induced by insulin-dependent activation of phosphodiesterase, with decreased PKA activity and diminished phosphorylation of pyruvate kinase and phosphofructokinase-2, PFK-2. Dephosphorylation of pyruvate kinase increases its' activity while dephosphorylation of PFK-2 renders it active as a kinase. The kinase activity of PFK-2 converts fructose-6-phosphate into fructose-2,6-bisphosphate (F2,6BP). F2,6BP is a potent allosteric activator of the rate limiting enzyme of glycolysis, PFK-1, and an inhibitor of the gluconeogenic enzyme, fructose-1,6-bisphosphatase. In addition, phosphatases specific for the phosphorylated forms of the glycolytic enzymes increase in activity under the influence of insulin. All these events lead to conversion of the glycolytic enzymes to their active forms and consequently a significant increase in glycolysis. In addition, glucose-6-phosphatase activity is down-regulated. The net effect is an increase in the content of hepatocyte glucose and its phosphorylated derivatives, with diminished blood glucose (Figure RL4).

In addition to the above described events, diminished cAMP and elevated protein phosphatase activity combine to convert glycogen phosphorylase to its inactive form and glycogen synthase to its active form, with the result that not only is glucose funneled to glycolytic products, but glycogen content is increased as well.

All of the post-receptor responses initiated by insulin binding to its receptor are mediated as a consequence of the activation of several signal transduction pathways. These include receptor activation of phosphatidylinositol-3-kinase, PI3K. Activation of PI3K involves a linkage to receptor activation of insulin receptor substrates (of which there are four: IRS1, IRS2, IRS3 and IRS4). Activated PI3K phosphorylates membrane phospholipids, the major product being phosphatidylinositol-3,4,5-trisphosphate, (PIP₃). PIP₃ in turn activates the enzyme protein kinase B, PKB (also called Akt). There are three members of the PKB/Akt family of serine/threonine kinases identified as Akt1, Akt2, and Akt3. It is Akt2 that is important in insulin-mediated glucose homeostasis. Insulin-mediated activation of Akt also results in inhibition of lipolysis and gluconeogenesis and activation of protein synthesis and glycogen synthesis.

Additional enzymes activated by insulin receptor signaling are PIP₃-dependent kinase, (PDK), some isoforms of protein kinase C, PKC (principally PKC-λ) and small ribosomal subunit protein 6 (p70) kinase, (p70S6K). The MAP kinase (MAPK) pathway
is also activated either through insulin receptor phosphorylation of SRC homology 2 containing protein (Shc) which then interacts with growth factor receptor binding protein-2 (GRB2) or via IRS1 activation (Figure RL5).

With respect to insulin responses, activation of PKB and PKC-λ lead to translocation of GLUT4 molecules to the cell surface resulting in increased glucose uptake which is significant in skeletal muscle. Activation of PKB also leads to the phosphorylation and inhibition of glycogen synthase kinase-3 (GSK3), which is a major regulatory kinase of glycogen homeostasis. In addition, PKB phosphorylates and inhibits the activity of a transcription factor (FKHRL1), now called FoxO3a) that has pro-apoptotic activity. This results in reduced apoptosis in response to insulin action.

The role of insulin in the stimulation of protein synthesis occurs at the level of translational initiation and elongation and is exerted primarily via a cascade leading to the activation of mammalian target of rapamycin, mTOR, a protein with homology to a family of proteins first identified in yeast that bind to the immunosuppressant drug, rapamycin. mTOR is a kinase whose catalytic domain shares significant homology with lipid kinases of the PI3K family.
**Figure RL5:** Insulin-mediated cascade leading to enhanced translation (not intended to be a complete description of all of the targets of insulin action that affect translation rates). Also shown is the effect of an increase in the AMP to ATP ratio which activates AMP-activated kinase (AMPK). STK11-LKB1-PJS = serine-threonine kinase 11, Peutz-Jeghers syndrome gene. IRS1 = insulin receptor substrate-1; PI3K = phosphatidylinositol-3-kinase; PIP₂ = phosphatidylinositol-4,5-bisphosphate; PTEN = phosphatase and tensin homolog deleted on chromosome 10; PDK1 = PIP₃-dependent protein kinase; Tsc1 and Tsc2 = Tuberous sclerosis tumor suppressors 1 (hamartin) and 2 (tuberin); Rheb = Ras homolog enriched in brain; mTOR = mammalian target of rapamycin. PKB/Akt = protein kinase B; GSK3 = glycogen synthase kinase-3; 4EBP1 = eIF-4E binding protein; p70S6K = 70kDa ribosomal protein S6 kinase, also called S6K.

Insulin action leads to an increase in the activity of PI3K which in turn phosphorylates membrane phospholipids generating phosphatidylinositol-3,4,5-trisphosphate (PIP₃) from...
phosphatidylinositol-4,5-bisphosphate (PIP$_2$). PIP$_3$ then activates the kinase PDK1 which in turn phosphorylates and activates PKB/Akt. Activated PKB/Akt will phosphorylate TSC2 (tuberin) of the TSC1/TSC2 complex on two residues (S939 and T1462) resulting in altered activity of the complex. The TSC1/TSC2 complex functions as a GTPase-activating protein (GAP) which increases GTP hydrolyzing activity of Rheb. The GAP activity resides in the C-terminal portion of tuberin. The faster the GTPase action of Rheb the faster will be the reduction in Rheb activation of mTOR. When TSC1/TSC2 is phosphorylated by PKB it is less effective at stimulating the GTPase activity of Rheb and therefore Rheb activation of mTOR will remain high as is the case in response to insulin action. AMPK phosphorylates TSC2 at two sites (T1271 and S1387) that are distinct from the sites that are the PKB/Akt targets for phosphorylation. Evidence indicates that the AMPK-mediated phosphorylation of TSC2 promotes the GTPase activity of Rheb resulting in inhibition of mTOR and thus a decrease in protein synthesis. Recent evidence has shown that PKB/Akt actually phosphorylates tuberin at 4 sites (S939, S1130, S1132, T1462) all of which result in inhibition of the Rheb-GAP activity of the TSC1/TSC2 complex (Figure RL5).

The ultimate activation of mTOR leads to phosphorylation and activation of p70S6K which in turn leads to increased phosphorylation of eEF2 kinase. eEF2 kinase normally phosphorylates eEF2 leading to a decrease in its’ role in translation elongation. When phosphorylated by p70S6K, eEF2 kinase is less active at phosphorylating eEF2, thus eEF2 is much more active in response to insulin action. In addition, insulin action leads to a rapid dephosphorylation of eEF-2 via activation of protein phosphatase 2A (PP2A). Taken together, reduced eEF2K-mediated phosphorylation and increased eEF-2 dephosphorylation lead to increased protein synthesis.

Both mTOR and p70S6K have been shown to phosphorylate the regulator of translation initiation, eIF-4E binding protein, 4EBP1. Phosphorylation of 4EBP1 prevents it from binding to eIF-4E. Binding of 4EBP1 to eIF-4E prevents eIF-4E from interaction with the cap structure of mRNAs which is necessary for translational initiation. Thus, the consequences of 4EBP1:eIF-4E interaction is a reduction in translation initiation. As a consequence of the concerted actions of mTOR and p70S6K, insulin results in increased protein synthesis.
PKB activation will also lead to phosphorylation and inhibition of glycogen synthase kinase-3 (GSK3). One of the targets of GSK3, relative to translation, is eIF2B. Phosphorylation of eIF2B prevents it from performing its GTPase activating (GAP) function in association with eIF2 (see the Protein Synthesis page for more details) and as a consequence results in reduced translational initiation. However, when GSK3 is inhibited by PKB phosphorylation the GAP activity of eIF2B remains high and consequently the rate of translational initiation by eIF2 remains high so protein synthesis is favored.

Insulin also has profound effects on the transcription of numerous genes, effects that are primarily mediated by regulated function of sterol-regulated element binding protein, SREBP. These transcriptional effects include (but are not limited to) increases in glucokinase, pyruvate kinase, lipoprotein lipase (LPL), fatty acid synthase (FAS) and acetylCoA carboxylase (ACC) and decreases in glucose 6-phosphatase, fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase (PEPCK).

In contrast, epinephrine diminishes insulin secretion by a cAMP-coupled regulatory path. In addition, epinephrine counters the effect of insulin in liver and peripheral tissue, where it binds to β-adrenergic receptors, induces adenylate cycles activity, increases cAMP, and activates PKA similarly to that of glucagon. The latter events induce glycogenolysis and gluconeogenesis, both of which are hyperglycemic and which thus counter insulin's effect on blood glucose levels. In addition, epinephrine influences glucose homeostasis through interaction with α-adrenergic receptors (Figure RL6).
Figure RL6: Pathways involved in the regulation of glycogen phosphorylase by epinephrine activation of α-adrenergic receptors. See Glycogen Metabolism for details of the epinephrine action. PLC-γ is phospholipase C-γ. The substrate for PLC-γ is phosphatidylinositol-4,5-bisphosphate, (PIP$_2$) and the products are inositol trisphosphate, IP$_3$ and diacylglycerol, DAG. Similar calmodulin-mediated phosphorylations lead to inhibition of glycogen synthase.

2.1.6.3. Insulin Action under the Control of Nutrient Intake and Hormones

Two of the many gastrointestinal hormones have significant effects on insulin secretion and glucose regulation. These hormones are the glucagon-like peptides (principally glucagon-like peptide-1, GLP-1) and glucose-dependent insulinotropic peptide (GIP). Both of these gut hormones constitute the class of molecules referred to as the incretins. Incretins are molecules associated with food intake-stimulation of insulin secretion from the pancreas.

Briefly, GLP-1 is derived from the product of the proglucagon gene (gene symbol = GCG). This gene encodes a preproprotein that is differentially cleaved dependent upon the tissue in which it is synthesized. For example, in pancreatic α-cells prohormone convertase 2 action leads to the release of glucagon. In the gut prohormone convertase 1/3 action leads to release of several peptides including GLP-1. Upon nutrient ingestion GLP-1 is secreted from intestinal enteroendocrine L-cells that are found predominantly in the ileum and colon with some production from these cell types in the duodenum and
jejumum. Bioactive GLP-1 consists of 2 forms; GLP-1(7-37) and GLP-1(7-36)amide, where the latter form constitutes the majority (80%) of the circulating hormone.

The primary physiological responses to GLP-1 are glucose-dependent insulin secretion, inhibition of glucagon secretion and inhibition of gastric acid secretion and gastric emptying. The latter effect will lead to increased satiety with reduced food intake along with a reduced desire to ingest food. The action of GLP-1 at the level of insulin and glucagon secretion results in significant reduction in circulating levels of glucose following nutrient intake. This activity has obvious significance in the context of diabetes, in particular the hyperglycemia associated with poorly controlled type 2 diabetes. The glucose lowering activity of GLP-1 is highly transient as the half-life of this hormone in the circulation is less than 2 minutes. Removal of bioactive GLP-1 is a consequence of N-terminal proteolysis catalyzed by dipeptidylpeptidase IV (DPP IV or DPP4).

2.1.6.4. Wnt Signaling, GLP-1, and Insulin Secretion

Although much of the research that has led to a detailed understanding of the signal transduction pathways initiated by Wnts was carried out in models of early development, evidence has been accumulating demonstrating a significant role for the Wnts in the control of metabolism. In particular Wnt action has been shown to be involved in metabolic control via its actions in both the gut and pancreas. In addition, Wnt signaling has been shown to interact with signaling pathways induced by insulin.

In the gut Wnt has been shown to be involved in regulated expression of the GCG gene. In intestinal enteroendocrine L cells the expression of the GCG gene results in the production of GLP-1. As indicated above, GLP-1 exerts its effects on the gut, the pancreas and in the brain. In the gut its effects lead to a reduced rate of gastric acid secretion and reduced gastric emptying. In the pancreas GLP-1 induced β-cell proliferation and inhibition of β-cell apoptosis. In the brain GLP-1 actins result in increased satiety leading to reduced desire for food intake.

The GCG gene promoter region contains an enhancer that harbors a canonical Wnt response element that binds TCF factors, in particular the TCF7L2 protein. Genome wide screens for polymorphisms associated with type 2 diabetes demonstrated that two single nucleotide polymorphisms (SNPs) in the TCF7L2 gene were the most frequently occurring SNPs associated with this disease. The significance of Wnt in the control of GLP-1 production was demonstrated by the fact that reduction/loss of either β-catenin or
TCF7L2 function completely blocks insulin-stimulated expression of the intestinal GCG gene. In addition, the effects of GLP-1 on the pancreas (i.e. proliferation and anti-apoptosis) are affected via the actions of β-catenin and TCF7L2. In the pancreas insulin inhibits expression from the GCG gene leading to reduced production of glucagon. This action has physiological significance because glucagon is the major counter-regulatory hormone to insulin action. The important role of TCF7L2 in pancreatic function can be demonstrated in experiments that lead to reduction in the levels of TCF7L2. In these types of experiments there is an increased rate of pancreatic β-cell apoptosis, reduced β-cell proliferation, and reduced glucose-dependent insulin secretion.

The demonstration of cross-talk between the Wnt and insulin signaling pathways is important as these observations may eventually lead to novel approaches to the treatment of type 2 diabetes.

2.1.6.5. Mechanism of Insulin Resistance

Insulin resistance (IR) refers to the situation whereby insulin interaction with its receptor fails to elicit downstream signaling events such as those depicted in the Figures above. Metabolically and clinically the most detrimental effects of IR are due to disruption in insulin-mediated control of glucose and lipid homeostasis in the primary insulin-responsive tissues: liver, skeletal muscle, and adipose tissue. IR is a characteristic feature found associated with most cases of type 2 diabetes. In addition, IR is the hallmark feature of the metabolic syndrome (MetS). IR can occur for a number of reasons however, the most prevalent cause is the hyperlipidemic and pro-inflammatory states associated with obesity. How does an abnormal metabolism, as is associated with obesity, lead to the development of IR? The answer to this question can be found in the effects of excess free fatty acids (FFAs) on the insulin receptor-mediated signaling pathways in adipose tissue, liver, and skeletal muscle as well as the pro-inflammatory status induced by the toxic effects of excess FFAs principally in the liver and adipose tissues.

The precise mechanisms that underlie the promotion of a pro-inflammatory state in obese individuals in not completely established. However, both adipose tissue and liver are important mediators of systemic inflammation in obesity.
Figure RL7: Model for how excess free fatty acids (FFAs) lead to insulin resistance and enhanced inflammatory responses in cells such as liver and adipose tissue. Only the major pathways regulated by insulin relative to glucose and lipid homeostasis are shown. Black arrows represent positive actions and red T-lines represent inhibitory actions. JNK = Jun N-terminal kinase. PKC = protein kinase C. IKKβ = inhibitor of nuclear factor kappa B kinase beta. ROS = reactive oxygen species. PI3K = phosphatidylinositol-3 kinase. DAG = diacylglycerol. TAG = triacylglycerols. LCA-CoA = long-chain acyl-CoAs. NFκB = nuclear factor kappa B. Akt is also known as protein kinase B (PKB).

One model proposes that the expansion of adipose tissue that occurs in obesity results in large adipocytes that have metabolic capacities that exceed the local oxygen supply. The resultant hypoxia leads to the activation of cellular stress response pathways causing cell autonomous inflammation and the release of pro-inflammatory cytokines. As a part of the chronic inflammation adipocytes secrete chemokines such as IL-8 and macrophage chemotactic protein-1 (MCP-1) that attract pro-inflammatory macrophages into the adipose tissue. These activated adipose tissue macrophages secrete cytokines that further exacerbate the pro-inflammatory state. In the liver inflammatory processes are also activated due to the excess accumulation of fatty acids and triglycerides which is the consequence of activated stress response pathways. Within the liver, Kupffer cells (resident liver macrophages) become activated by the generation of reactive oxygen species (ROS) and induction of stress responses. These activated Kupffer cells release locally acting cytokines that, like in adipose tissue, exacerbates the pro-inflammatory
environment. Within the vasculature, saturated FFAs can directly activate pro-inflammatory pathways in endothelial cells and myeloid-derived cells resulting in the induction and propagation of a systemic pro-inflammatory state.

Hepatic IR is induced by the excess accumulation of FFAs. Within the hepatocyte, metabolites of the FFA re-esterification process, including long-chain acyl-CoAs and diacylglycerol (DAG), accumulate. Excess FFAs also participate in the relocation of several protein kinase C (PKC) isoforms, from the cytosol to the membrane compartment. These PKC isoforms include PKC-β2, PKC-δ, and PKC-theta (PKC-θ). DAG is a potent activator of these PKC isoforms and the membrane-associated PKCs will phosphorylate the intracellular portion of the insulin receptor on serine residues which results in impairment of insulin receptor interaction with downstream signaling proteins including insulin receptor substrate 1 (IRS1) and IRS2. Loss of IRS1 and IRS2 interaction with the receptor prevents interaction with phosphatidylinositol 3-kinase (PI3K) and its' subsequent activation. In addition to serine phosphorylation of the insulin receptor, various PKCs have been shown to phosphorylate IRS1 and IRS2 further impairing the ability of these insulin receptor substrates to associate with the insulin receptor and downstream effector proteins such as PI3K (Figure RL7).

The FFA-induced down-regulation of insulin signaling pathways results in activation of several kinases involved in stress responses. These kinases include Jun N-terminal kinase (JNK), inhibitor of nuclear factor kappa B kinase beta (IKKβ), and suppressors of cytokine signaling-3 (SOCS-3). Like PKC, JNK activity is also regulated by FFAs and is an important regulator of IR. The target of JNK action is the Ser307 of IRS-1 and this phosphorylation plays an important role in the progression to hepatic IR. Activation of IKKβ (which is required for the activation of nuclear factor kappa B, NFκB) may have the most pronounced effect on inflammatory responses in the liver and adipose tissue. NFκB is the most important transcription factor activating the expression of numerous pro-inflammatory cytokine genes such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-alpha (TNF-α) each of which have been shown to be involved in promoting IR. NFκB-dependent inflammatory mediators produced in hepatocytes act to reduce insulin sensitivity and to promote liver injury.
Analysis of the effects of FFAs on macrophages in cell culture demonstrated that they can activate inflammatory signaling through the toll-like receptors (TLRs), specifically TLR4. The TLRs are a family of cell surface receptors involved in key events triggered via the innate immune system. The TLRs are pattern recognition receptors that recognize structurally conserved molecules from microbial pathogens. TLR4 is responsive to bacterially derived lipopolysaccharide (LPS) which is an endotoxin secreted by gram-negative bacteria. LPS stimulation of TLR4 results in activation of both the JNK and IKKβ signal transduction pathways leading to secretion of pro-inflammatory cytokines such as IL-1β, IL-6, MCP-1, and tumor necrosis factor alpha (TNFα). These cell culture experiments demonstrated that FFA addition to macrophages results in activation of NFκB and that this activation was deficient in macrophages from TLR4 knock-out mice. In the livers of TLR4 knock-out mice there is reduced inflammation even in the presence of hepatic steatosis suggesting that Kupffer cell TLR4 is important in hepatic inflammatory responses to excess FFA loading.

2.1.6.6. **Insulin Action and Endothelial Functions**

The metabolic functions of insulin are primarily reflective of its role in glucose and lipid homeostasis in skeletal muscle, adipose tissue, and liver. However, insulin also exerts important functions in other non-classical insulin target tissues such as the brain, pancreas, and the vascular endothelium. The ability of insulin to exert vasodilator action in the vascular endothelium as a result of increased nitric oxide (NO) production is an important component of the ability of this hormone to enhance glucose uptake by skeletal muscle. The insulin-mediated signaling pathway that triggers production of NO in vascular endothelium involves the same signaling proteins (PI3K, PKD, and Akt/PKB) that are components of metabolic regulatory pathways induced by insulin. Therefore, it is understandable why the same disruptions to insulin signaling that lead to IR (see above) caused by excess FFAs and hyperglycemia result in endothelial dysfunction.

The production of NO in endothelial cells is the result of the activation of endothelial nitric oxide synthase (eNOS). The production and actions of NO and the various NOSs involved are discussed in more detail in the Amino Acid Derivatives page. With respect to insulin action, the activation of endothelial Akt/PKB leads to phosphorylation and activation of eNOS and thus increased NO production. In addition to modulating vascular
tone by activating signaling events in the underlying vascular smooth muscle cells, endothelial cell-derived NO reduces the production of pro-inflammatory cytokines, reduces leukocyte and monocyte recruitment and adhesion to the endothelium, inhibits the proliferation of vascular smooth muscle cells, inhibits apoptosis, and attenuates platelet aggregation. Inactivation of endothelial cell NO production, as occurs due to IR, results in endothelial dysfunction and promotes the development of atherosclerosis. As described above for the liver and adipose tissue, elevated levels of circulating FFAs lead to impaired insulin signaling via the PI3K-PDK-Akt/PKB pathway in vascular endothelial cells.

Insulin exerts its mitogenic, growth promoting, and differentiation effects via a signaling pathway that involves mitogen-activated protein kinase (MAPK) which is distinct from the PI3K-PDK-Akt/PKB pathway that is involved in metabolic regulation by insulin. The MAPK-induced pathway does not play a role in the production of NO by insulin. This MAPK-induced pathway plays a significant role in the development of atherosclerosis in the IR state. When insulin signaling via PI3K-PDK-Akt/PKB is impaired as described above for the IR state, the MAPK signaling pathway in endothelial cells is enhanced. In the endothelium MAPK activation by insulin results in increased expression of endothelin-1 (ET-1), plasminogen activator inhibitor type-1 (PAI-1), and the adhesion molecules intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin. ET-1 is a potent vasoconstrictor and contributes to endothelial cell dysfunction in the presence of IR. The increased expression of numerous cell adhesion molecules accelerates the adherence to the endothelium of pro-inflammatory leukocytes which in turn contributes to the development of atherosclerosis. Therefore, the molecules beneficial to vascular endothelial health that are induced by insulin (e.g. NO) are reduced in the IR state and those that are proatherogenic (e.g. ET-1, PAI-1) are increased.167

2.1.7. Insulin Receptor

The insulin receptor (IR) belongs to the superfamily of transmembrane receptor tyrosine kinases (TKs).168 In contrast to other family members that are monomeric in their structure, IR and its homologue, insulin-like growth factor I receptor (IGF-1R), are intrinsic disulfide-linked dimers of heterodimeric disulfide-linked proteins of the form \((\alpha\beta)_2\). The 135-kDa \(\alpha\) subunit of IR is extracellular, whereas the 95-kDa \(\beta\) subunit
contains an extracellular portion, a single transmembrane sequence, and an intracellular TK domain. The major structural features of the αβ dimer is depicted in figure RL8a. Ligandspecific binding to the α subunits activates the TK, initiating a signal cascade that results in numerous cellular responses. Our understanding of the mechanics of this signal transduction process has been hampered by the unavailability of an atomic structure of the whole IR protein. However, the quaternary structure of the isolated complex of biologically active IR and insulin was recently solved by three-dimensional reconstruction from low dose scanning transmission electron micrographs (STEM) (Figure RL8, b1-b3).

**Figure RL8: Dimeric insulin receptor structure.** a, schematic of IR represented in its (αβ)2 form. Residues at the start of named domains and disulfides (italics) are numbered. TM, transmembrane; CT, C-terminal; juxtamembrane domains not labeled. b, three-dimensional reconstruction from EM. b1, structure at full volume of 480-kDa dimer (side view); b2, structure at 85% of full volume to show domain subdivision (side view); b3, top view of b2; regions 1, 2, and 3 represent epitope locations for monoclonal Fab fragments 18–44, 83–14, and 83–87, respectively (see text and Fig. 3e). b4, ectodomain of EM structure (top view) fitted with atomic domains LC (green), Fn0 (blue), αFn1-αID (red), βFn1-Fn2 (violet); backbone ribbon representation. Second subunit is shown in brown. Arrow indicates orientation of view of Fig. 2a. c, schematic of structural conformation in b2. One monomer is labeled (color) and the second monomer is white. In, insulin; A, activation loop and opposite catalytic site; X, C terminus of α subunit (white) and N terminus of β subunit (black); 1, 2, and 3, disulfides at amino acids 524, 682–685, and between 647 and 872, respectively. Black bar represents 2-fold axis; solid and open arrows point to insulin Phe-B25 and Glu-6 of α subunit.

Atomic structures of subdomains of IR or of highly analogous proteins were fitted into the complex (e.g. Figure RL8, b4), creating the only available atomic structural model of IR. The model reveals structural details of the interaction of insulin with the receptor that lead to the activation of the intracellular TK. Cecil C. Yip et. al in the light of recent
structural data on the unbound receptor, discussed the mechanics of a model of receptor activation arising from insulin binding.\textsuperscript{172}

\textbf{2.1.7.1. The Mechanics of Transmembrane Signaling}

A fundamental question on the mechanism of transmembrane activation of receptors by extracellular ligand binding is how the binding action is transformed into key intracellular biochemical reactions such as receptor autophosphorylation. Inactive monomeric TK receptors, such as the epidermal growth factor receptor and platelet-derived growth factor receptor, are dimerized and activated by ligand binding.\textsuperscript{173} Because IR is intrinsically dimeric without insulin, the distance between the cytoplasmic TKs must be too large for activation without ligand binding. In the three-dimensional reconstruction of the insulin bound IR complex the two TK domains are juxtaposed, and an extended flexible activation loop of one fitted atomic TK structure can just reach the catalytic loop of the other TK.\textsuperscript{170} In a simple mechanical paradigm of activation\textsuperscript{171} the binding of insulin not only overcomes an energy barrier to binding but holds the LCL domains together to permit a lateral shift and approach of the two transmembrane domains to allow \textit{trans} autophosphorylation of the tyrosine residues in the activation loop\textsuperscript{171} (Figure RL9 a and b). The model presupposed an “open” configuration of the free IR. Such a configuration has been confirmed by the three-dimensional reconstruction from STEM micrographs of insulin-bound/free IR dimmers\textsuperscript{174} (Figure RL9 c and d). Moreover, this structure, together with the insulinbound three-dimensional structure, makes it possible to reconcile virtually all structural forms seen previously in electron microscopic examinations of the IR, except for long parallel T-shaped structures occasionally observed.\textsuperscript{175,176} This includes Y, H, V, and X shapes and parallel barlike images, observed by two-dimensional electron microscopy of detergent-solubilized IR, its ectodomain, or vesicle-reconstituted insulin-IR complex.\textsuperscript{175-178} Such configurations can be formed by the projection of the three-dimensional reconstructions at various orientations (Figure RL9e). Moreover, the three-dimensional reconstruction can also explain the images of parallel rods and crosses (Figure RL9e, last column) produced by pairs of antibodies to Fn0, to the $\beta$-ID, and to the CR domain.\textsuperscript{179}
2.1.7.2. Molecular Mechanisms for Insulin-Stimulated Glucose Transport

Whole-body glucose homeostasis is regulated by a balance of glucose supply (nutrients ingested and hepatic glucose output) and glucose uptake by insulin sensitive (muscle, adipose tissue) and insulin-insensitive (brain, splanchnic tissues, blood cells) tissues. Under insulin-stimulated conditions, skeletal muscle plays a predominant role in maintaining glucose homeostasis; it accounts for at least 80% of glucose disposal. Furthermore, under physiological conditions, glucose transport into muscle cells is the rate-limiting step for the regulation of glucose metabolism. Glucose is transported into cells by a process of facilitated diffusion that is mediated by a family of structurally related carrier proteins that are encoded by distinct genes. These glucose transporters (Glut) are expressed in a tissue-dependent manner and constitute a different gene family from the sodium linked glucose transporters. In skeletal muscle and adipose cells, three facilitative diffusion glucose transporters are expressed: Glut1, Glut4, and Glut5. Glut4 is the most abundant and has the highest turnover number (moles of glucose transported/transporter per unit time). Glut5 is a high affinity fructose transporter and...
plays a minimal role in glucose transport into these tissues. Thus, insulin-stimulated glucose transport into muscle and adipose tissue occurs primarily via Glut4, with Glut1 playing a minor role. Insulin stimulation of glucose uptake involves a complex series of cellular events that are illustrated in figure RL10. In the absence of insulin, Glut4 molecules reside primarily in intracellular vesicles. Insulin binding to its receptor elicits a cascade of signalling events those results in the rapid translocation of glucose transporters to the plasma membrane. Signaling events also potentially regulate docking, fusion, and activation of Glut4 at the plasma membrane.

Glut4 transporters are continually recycling from the plasma membrane through endosomes and insulin regulates the kinetics of Glut4 vesicle recycling to enhance exocytosis.\(^{189}\) Insulin also has more chronic effects on Glut4 gene transcription.\(^{190}\) Dysregulation of one or more of these steps could result in whole-body insulin resistance. A hallmark of insulin resistance in rodents and humans is the down-regulation of Glut4 gene expression in adipose tissue but not in skeletal muscle.\(^{191,192}\) Data from studies using rodent models of obesity and diabetes indicate that in skeletal muscle a defect in Glut4 translocation, fusion, or activation, not expression, is responsible, at least in part, for insulin resistance.\(^{191,192}\) Lesions in signaling events emanating from the insulin receptor and (or) defects in Glut4 sorting and trafficking could explain defective Glut4 translocation, fusion, or activation.

Table RL1 gives an account of the tissue distribution and functions of different structurally related carrier proteins for glucose transport.\(^{193}\)

### 2.1.7.3. **Insulin Signaling and Glucose Transport**

Insulin binding to its receptor results in autophosphorylation of the receptor and the subsequent phosphorylation of multiple signaling molecules. Insulin signaling cascades result in diverse and critical effects on cell biology, including the regulation of mitogenesis, gene expression, and metabolic pathways such as glucose transport, antilipolysis, lipogenesis, glycogen synthesis, and amino acid transport (Figure RL11).\(^{192,194,195}\) Although recent studies have led to significant insight into the complexity of the insulin signaling network, little is yet known about how insulin signals metabolic events in the cell.
### Table RL1: Tissue distribution and function of the facilitative glucose transporter family

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Tissue distribution</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>Glut1</td>
<td>Ubiquitous; high expression in cultured cells, brain, endothelial cells, human erythrocytes</td>
<td>Constitutive glucose transport</td>
</tr>
<tr>
<td>Glut2</td>
<td>Kidney and small intestinal epithelial cells, liver, and pancreatic fl cells</td>
<td>Low-affinity transporter; glucose release into blood stream; part of glucose sensor in islets</td>
</tr>
<tr>
<td>Glut3</td>
<td>Neurons and placenta</td>
<td>High-affinity transporter; unabated transport into central nervous system</td>
</tr>
<tr>
<td>Glut4</td>
<td>Skeletal muscle, brown and white adipose tissue and heart</td>
<td>Mediates insulin-regulated glucose transport</td>
</tr>
<tr>
<td>Glut5</td>
<td>Small intestine, kidney, brain, endothelial cells, sperm, adipocytes and muscle</td>
<td>Fructose transporter</td>
</tr>
<tr>
<td>Glut6</td>
<td>Pseudogene</td>
<td></td>
</tr>
<tr>
<td>Glut7</td>
<td>Liver endoplasmic reticulum</td>
<td>Part of the glucose-6-phosphate complex involved in gluconeogenesis</td>
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Intracellular targets of the insulin receptor tyrosine kinase activity include IRS-1, IRS-2, and Shc. Most studies indicate that IRS-1 is a critical upstream signaling molecule in the cascade leading to Glut4 translocation. Transfection of rat adipocytes with epitope-tagged Glut4 and a tyrosine kinase-deficient insulin receptor resulted in no translocation of Glut4 in response to insulin. In cells transfected with IRS-1 antisense ribozyme, there was reduced sensitivity of insulin-stimulated Glut4 translocation with no change in maximal response. In contrast, when IRS-1 was overexpressed in adipocytes transfected with epitope-tagged Glut4, Glut4 translocation was enhanced in the absence of insulin and IRS-1 overexpression restored insulin sensitivity in cells transfected with the IRS-1 antisense ribozyme. The role of IRS-1 in signaling insulin-stimulated glucose transport is further highlighted by studies using mice with targeted disruption of the IRS-1 gene. These mice are growth-retarded with significant reduction of insulin-stimulated glucose transport into isolated adipocytes. However, a
role for other proximal signaling molecules such as IRS-2 and Shc in Glut4 translocation is probable because there is some effect of insulin on glucose transport and translocation of Glut4 in the absence of IRS-1 in adipocytes from these knock-out mice.\textsuperscript{198}

\textbf{Figure RL10:} Sequence of events involved in insulin stimulation of glucose transport in muscle and adipose cells: 1) In the absence of insulin, GLUT4 glucose transporters reside within the cell in a distinct vesicle population. Other proteins that are associated with the GLUT4 vesicle include synaptobrevin (VAMP), gp160/vp165, phosphatidylinositol-4 (PI4) Kinase, Rab4 and secretory component-associated membrane proteins (SCAMPS); 2) when insulin binds to its receptor in the plasma membrane, it initiates a cascade of signals affecting transcription, translocation, and possibly activation of the glucose transporter; 3) In adipocytes Rab4 dissociates from the vesicle and the vesicle translocates to the plasma membrane, where it docks and fuses and glucose transport is activated; 4) GLUT4 vesicles are endocytosed and recycle through endosomes.
**Ras-MAP Kinase Signaling Pathway**

Some of insulin's pleiotropic effects on cell growth and metabolism appear to be mediated by signalling pathways emanating from the low molecular GTP-binding protein, Ras. IRS-1, IRS-2, and Shc, upon phosphorylation by insulin (or other growth factors), act as docking molecules for other proteins via src homology2 (SH2) domains, thereby forming multiple signaling complexes (Figure RL11). Activated IRS-1 and Shc form complexes with Grb2 and SOS proteins, leading to Ras activation, and insulin binding to its receptor has been shown to increase cellular concentrations of GTP-bound, activated Ras.\(^{199,201}\)

Ras activation results in the activation of a serinethreonine phosphorylation cascade that minimally includes the proteins Raf-1, MEK, pp44, and pp42 mitogen activated protein (MAP) kinases and pp90 s6 kinase. The Ras-MAP kinase cascade plays a pivotal role in the regulation of gene expression via phosphorylation of transcription factors. However, the role of the Ras-MAP kinase cascade in the regulation of insulin's metabolic effects such as glucose transport remains controversial.

---

**Figure RL11:** Insulin signal transduction results in mitogenic and metabolic responses. Upon activation, the insulin receptor associates with insulin receptor substrate (IRS)-I and IRS-2, which bind to several proteins containing src homology 2 (SH2) domains, thus activating the ras/MAP kinase cascade and the phosphoinositol-3 (PI3) kinase pathway. These pathways lead to multiple actions including gene transcription, glucose transport, and glucose, protein, and lipid metabolism.\(^{202}\)
Studies using cultured cells to examine the role of Ras-MAP kinase pathways in the stimulation of glucose transport have yielded conflicting results. Vanden Bergh et al. compared the ability of insulin, epidermal growth factor, and thrombin to stimulate Ras-MAP kinase signaling pathways in 3T3-L1 adipocytes and found that Ras activation alone was not sufficient to account for insulin-stimulated glucose transport. Furthermore, enhanced signalling via Ras in Ratl Cells did not alter insulin-stimulated glucose transport. Conversely, when 3T3-L1 adipocytes were transfected with constitutively activated Ras, there was enhanced Glut4 translocation to the plasmamembrane in the absence of insulin, and no further translocation was observed with insulin treatment. A major caveat of this study was the observation that Ras overexpression resulted in a 95% down-regulation of Glut4 expression. The confounding effect of chronic exposure to activated Ras on gene expression was overcome by transiently transfecting rat adipocytes with constitutively activated Ras, which resulted in a significant increase in the translocation of co-transfected epitope-tagged Glut4 to the cell surface in the absence of insulin and no further stimulation with insulin.

Studies using microinjection techniques also provide conflicting data concerning the role of Ras pathways in glucose transport. Manchester et al. reported that microinjection of activated Ras into cardiac myocytes caused a significant increase in glucose transport. However, microinjection of constitutively active Ras or downstream effectors such as activated Ras into 3T3-L1 adipocytes had no effect on the translocation of Glut4 as assessed by the plasma membrane sheet assay. Furthermore, injection of neutralizing antibodies to Ras caused a 50% reduction in insulin-stimulated glucose transport into cardiac myocytes but had no effect in 3T3-L1 adipocytes. The latter finding is supported by the fact that transient transfection of rat adipocytes with dominant negative Ras did not alter translocation of epitopetagged Glut4. Reusch et al. used lovastatin (which inhibits farnesylation of ras and thus inhibits appropriate targeting to the plasma membrane) to neutralize Ras activity and found attenuation of insulin effects on thymidine incorporation and glucose incorporation into glycogen but not glucose transport. However, a major caveat of this study is that lovastatin's effects may not be specific to Ras because it also inhibits the ratelimiting step of cholesterol biosynthesis, geranylgeranylation of proteins, insulin stimulation of PI3- kinase activity, and cell transformation by Raf.
Glucose transport is a pivotal step in whole-body insulin action. Stimulation of glucose transport in insulin-sensitive tissues involves the recruitment of Glut4 from an intracellular vesicle pool to the plasma membrane. This process is regulated by a complex network of signaling pathways. In adipocytes, Glut4 gene expression is reduced in insulin-resistant subjects, including those with obesity and diabetes, whereas in skeletal muscle Glut4 expression is minimally affected. In skeletal muscle, resistance to insulin stimulation seems to result from defects in Glut4 vesicle trafficking. Houseknecht et al. performed studies manipulating Glut4 expression or the expression of specific signaling molecules in insulin-sensitive tissues in transgenic mice which indicated that increasing the number of Glut4 transporters that are functionally inserted in the plasma membrane enhances whole-body insulin action and alters body composition. Thus, they concluded that developing methods to increase the expression of the Glut4 gene or to increase the proportion of cellular Glut4 that are functionally inserted in the plasma membrane are important strategies for developing new therapies to prevent or ameliorate insulin resistance. Such strategies might involve enhancing translocation of the Glut4 vesicle to the plasma membrane, promoting its fusion once at the membrane, increasing the exposure of the Glut4 transporter to the extracellular milieu, or facilitating its activation.

2.1.8. Therapeutic Intervention for Hyperglycemia (Drugs belonging to different chemical classes)

Many, if not all, of the vascular consequences of insulin resistance are due to the persistent hyperglycemia seen in type2 diabetes. For this reason a major goal of therapeutic intervention in type 2 diabetes is to reduce circulating glucose levels. There are many pharmacologic strategies to accomplish these goals. Tables RL 2, 3 and 4 represents the current pharmacological agents, combination therapies and clinical efficacy of the pharmacological therapies used for the treatment of T2D.
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2.1.8.1. Sulfonylureas
Sulfonylureas, derived from sulfonic acid and urea, were initially developed in the 1950s and have remained a cornerstone of therapy for type 2 diabetes. The combination of their proven efficacy in most patients, low incidence of adverse events, and low cost has contributed to their success and continued use. They are frequently classified as either 1st generation or 2nd generation agents. First generation sulfonylureas (acetohexomide, chlorpropamide, tolazamide, and tolbutamide) possess a lower binding affinity for the ATP-sensitive potassium channel, their molecular target (vide infra), and thus require higher doses to achieve efficacy, increasing the potential for adverse events. In addition, the plasma half-life of 1st generation sulfonylureas is extended (e.g. 5-36 h) compared to the 2nd generation agents. Chlorpropamide was once the most commonly used oral agent, but now it is rarely prescribed. Unique complications associated with chlorpropamide are hyponatremia (SIADH) and an alcohol flushing reaction (disulfiram-Antibuse reaction). In addition, tolbutamide, acetohexamide and tolazamide generally require 2 or 3 doses per day and are rarely used.

More recently, 2nd generation sulfonylureas including glyburide (glibenclamide; glipizide and glimepiride (Figure RL12) were introduced, and are now widely used. The 2nd generation sulfonylureas are much more potent compounds (~ 100-fold), possess a more rapid onset of action, and generally have shorter plasma half-lives and longer duration of action compared to the 1st generation agents.

Figure RL12: Structures of Glyburide, Glipizide and Glimepiride
Mechanism of Action
Sulfonylureas are insulin secretagogues, since they control blood glucose levels by directly stimulating first-phase insulin secretion in the pancreatic β-cells. Through the concerted efforts of GLUT2 (the high Km glucose transporter), 217 glucokinase (the glucose sensor), 218 and glucose metabolism, these cells are responsible for sensing and secreting the appropriate amount of insulin in response to a glucose stimulus. 219 Mitochondrial glucose metabolism leads to ATP generation and increases the intracellular ratio of ATP/ADP, which results in the closure of the ATP-sensitive potassium channel (KATP; a 140 kDa membrane protein) on the plasma membrane. Closure of this channel depolarizes the membrane and triggers the opening of voltage-sensitive calcium channels, leading to the rapid influx of calcium. Increased intracellular calcium causes an alteration in the cytoskeleton, and stimulates translocation of insulin-containing secretory granules to the plasma membrane and the exocytotic release of insulin.

The KATP channel is comprised of two subunits, both of which are required for the channel to be functional. One subunit contains the cytoplasmic binding sites for both sulfonylureas and ATP, and is designated as the sulfonylurea receptor type 1 (SUR1). The other subunit is the potassium channel, which acts as the pore-forming subunit. 220 Either an increase in the ATP/ADP ratio or ligand binding (by sulfonylureas, meglitinides) to SUR1 results in the closure of the KATP channel and insulin secretion. Studies comparing sulfonylureas and non-sulfonylurea insulin secretagogues have identified several distinct binding sites on the SUR1 that cause channel closure. Some sites exhibit high affinity for glyburide and other sulfonylureas, while other sites exhibit high affinity for the non-sulfonylurea secretagogues (vide infra).

Efficacy
The over clinical efficacy of sulfonylureas in patients with type 2 diabetes is related to the pre-treatment levels of fasting plasma glucose and HbA1C. The higher the fasting glucose level, the greater the effect will be. In patients with a pre-treatment glucose level of approximately 200 mg/dl (11.1 mmol/l), sulfonylureas typically will reduce glucose by 60-70 mg/dl (3.3-3.9 mmol/l) and HbA1C by 1.5-2% (Table 3). The most responsive patients are those who exhibit mild-to-moderate fasting hyperglycemia (<200-240 mg/dl; <12.2-13.3 mmol/l), along with adequate residual β-cell function (evidenced by elevated fasting C-peptide). When used at maximally effective doses, results from well-controlled clinical trials have not indicated a superiority of one 2nd generation sulfonylurea over
another. Similarly, 2nd generation sulfonylureas exhibit similar clinical efficacy compared to the 1st generation agents. The principal advantage of glimepiride and Glucotrol XL compared to other agents is the once daily dosing regimen. Approximately 10-20% of patients will exhibit a poor initial response to sulfonylureas (primary failures). While these patients are typically those who have severe fasting hyperglycemia (>280 mg/dl; >15.5 mmol/l) and reduced fasting C-peptide levels, these tests are not specific enough to help decide on the usefulness of a sulfonylurea for an individual patient. In addition, treatment with sulfonylureas results in the eventual loss of therapeutic effectiveness (secondary failure) in the range of 3-10% per year.\textsuperscript{216}

**Side Effects**

The major side effect from sulfonylurea treatment is hypoglycemia. This side effect is really just an extension of the therapeutic objective. Mild hypoglycemic events occur in approximately 2-4% of patients and severe hypoglycemic reactions that require hospitalization occur at a frequency of 0.2-0.4 cases per 1000 patient-years of treatment.\textsuperscript{221} In light of this, initiation of treatment with sulfonylureas should be at the lowest recommended dose. An additional undesirable effect of sulfonylurea therapy (as is also the case with insulin therapy) is weight gain. In the UKPDS, sulfonylurea treatment caused a net weight gain of 3 kg, which occurred during the first 3-4 years of treatment and then stabilized.\textsuperscript{222} In contrast, weight gain in response to insulin therapy increased progressively for the duration of the study. As mentioned above, chlorpropamide is associated with hyponatremia (SIADH) and an alcohol flushing reaction (disulfiram-Antibuse reaction). All the agents can cause intrahepatic cholestasis. Rarely maculopapular or urticarial rashes occur.

In renal failure, the dose of the sulfonylurea agent will require adjustment based on glucose monitoring. The half-life of insulin is extended in renal failure and thus there is an increased risk for hypoglycemia. This risk is typically manifest with fasting hypoglycemia.

2.1.8.2. **Meglitinides: Repaglinide and Nateglinide**

The meglitinides are a novel class of non-sulfonylurea insulin secretogogues characterized by a very rapid onset and abbreviated duration of action. Repaglinide (Prandin), a benzoic acid derivative introduced in 1998, was the first member of the
meglitinide class (Figure RL13). Nateglinide (Starlix) is a derivative of the amino acid D-phenylalanine and was introduced to the market in 2001 (Figure RL13). Unlike sulfonylureas, repaglinide and nateglinide stimulate first-phase insulin release in a glucose-sensitive manner, theoretically reducing the risk of hypoglycemic events. The delivery of insulin as an early, transient burst at the initiation of a meal affords several major physiological benefits. These include rapidly suppressing hepatic glucose production and reducing the stimulus for additional insulin that would be required subsequently to dispose of a larger glucose load. Thus, the rapid onset/short duration stimulation of insulin release by meglitinides should enhance control of prandial hyperglycemia, while reducing the risk for post-absorptive hypoglycemia and limiting exposure to hyperinsulinemia.

![Chemical structures of Nateglinide and Repaglinide](image)

**Figure RL13:** Chemical structures of Nateglinide and Repaglinide

**Mechanism of Action**

Similar to sulfonylureas, meglitinides are insulin secretagogues, since they control blood glucose levels by directly stimulating first-phase insulin secretion in the pancreatic β-cells. Receptor-binding studies performed in BTC-3 cells identified a high-affinity repaglinide (KD = 3.6 nmol/l) site having lower affinity for glyburide (14.4 nmol/l), and one high-affinity glyburide (25 nmol/l) site having lower affinity for repaglinide (550 nmol/l). Repaglinide is approximately 5 times more potent than glyburide in stimulating insulin secretion. Unlike glyburide (and other sulfonylureas), repaglinide does not stimulate insulin secretion in vitro in the absence of glucose. Rather, it enhances glucose-stimulated insulin secretion especially at 180 mg/dl (10 mmol/l) glucose. The mechanism of action of nateglinide also involves the binding to and closure of the KATP channel resulting in membrane depolarization, an influx of calcium, and insulin exocytosis. The kinetics of
interaction of nateglinide with the KATP channel are distinct compared to both rapaglinide and sulfonylureas, and accounts for its rapid insulinotropic effects. The onset of action of nateglinide is similar to that of glyburide but three-fold more rapid than that of rapaglinide. When nateglinide is removed from the KATP channel, its effect is reversed twice as quickly as glyburide and five times more quickly than rapaglinide. Thus, nateglinide initiates a more rapid release of insulin that is shorter in duration compared to rapaglinide, despite having an in vivo pharmacokinetic profile that is similar.

**Efficacy**

The efficacy of rapaglinide, when used as a monotherapy, is similar to sulfonylureas. Repaglinide treatment of patients with type 2 diabetes reduced fasting plasma glucose by approximately 60 mg/dl and HbA1C by 1.7%. In a double-blind placebo-controlled study, rapaglinide had similar effects on lowering HbA1C (0.5-2%) and fasting plasma glucose (65-75 mg/dl; 3.6-4.2 mmol/l) compared to glyburide. Repaglinide is also efficacious when used in combination with either metformin or troglitazone (a thiazolidinedione withdrawn from the market). In patients treated with rapaglinide and metformin, HbA1C was decreased from 8.3% to 6.9% and fasting plasma glucose by 40 mg/dl (2.2 mmol/l). Although lowered, the changes observed in subjects treated with either repaglinide or metformin monotherapy were not significant for HbA1C (0.4 and 0.3% decrease, respectively), or fasting plasma glucose (9 mg/dl (0.5 mmol/l) increase and 5.4 mg/dl (0.3 mmol/l) decrease, respectively). Significant increases in body weight occurred in the both repaglinide and combined therapy groups (2.4 Â± 0.5 and 3.0 Â± 0.5 kg, respectively). The combination therapy of rapaglinide and troglitazone showed a significant reduction in mean HbA1C values (1.7%) that was greater than with either type of monotherapy. Repaglinide monotherapy resulted in a reduction of HbA1C values that was significantly greater than troglitazone (0.8% vs. 0.4%). In addition, combination therapy was more effective in reducing fasting plasma glucose (80 mg/dl) than either repaglinide (43 mg/dl) or troglitazone (46 mg/dl) monotherapies. Repaglinide is also efficacious when used in combination with other available thiazolidinediones, rosiglitazone (Avandia) and pioglitazone (Actos).

The efficacy of nateglinide when used as a monotherapy is similar to sulfonylureas and repaglinide. However, several therapeutically attractive features distinguish nateglinide from repaglinide and sulfonylureas. Nateglinide produces a more rapid post-prandial
increase in insulin secretion, and its duration of response is shorter than that of glyburide. Thus, the risk of post-absorptive hypoglycemia should be lower than with either sulfonylureas or rapaglinide, but this has not been demonstrated in studies to date. The efficacy of nateglinide treatment has been evaluated alone and in combination with metformin in patients with type 2 diabetes. In this randomized double-blind study, patients with an HbA1C level between 6.8 and 11.0% during a 4-week placebo run-in received 24 weeks treatment with 120 mg nateglinide before meals (n = 179), 500 mg metformin three times a day (n = 178), combination therapy (n = 172), or placebo (n = 172). At the study conclusion, HbA1C and fasting plasma glucose were significantly reduced from baseline with nateglinide [0.5% and 12.6 mg/dl (0.7 mmol/l), respectively] and metformin [0.8% and 28.8 mg/dl (1.6 mmol/l), respectively], but was increased with placebo [0.5% and 7.2 mg/dl (0.4 mmol/l), respectively]. Combination therapy was additive [HbA1C, 1.4% and glucose, 43.2 mg/dl (2.4 mmol/l)]. Although only preliminary data are available, nateglinide also appears effective when used in combination with thiazolidinediones.

In a direct comparison of Repaglinide and Nateglinide, the known pharmacodynamic differences in the drugs are evident on the clinical outcomes. The longer acting Repaglinide has significant effects on fasting glucose levels while Nateglinide does not. These clinical findings are important and can be incorporated into clinical decision making. For example, if the main issue for the patient is postprandial hyperglycemia, and fasting glucoses are near normal, an agent that has limited effect on the fasting glucose would be beneficial.

**Side Effects**
In 1-year trials, the most common adverse events reported in repaglinide recipients (n = 1,228) were hypoglycemia (16%), upper respiratory tract infection (10%), rhinitis (7%), bronchitis (6%) and headache (9%). The overall incidence of hypoglycemia was similar to that recorded in patients receiving glibenclamide, glipizide or gliclazide (18%; n = 597); however, the incidence of serious hypoglycemia appears to be slightly higher in sulphonylurea recipients. Weight gain does occur in patients treated with repaglinide, but the magnitude is significantly less compared to treatment with glyburide. In patients switched from sulfonylureas to repaglinide, no weight gain was observed; in drug-negative patients, repaglinide-treatment increased body weight by approximately 3% (6lb).
The clinical trials of nateglinide carried out to date have found the drug to be safe and well tolerated. Dosage regimens ranging from 60 to 240 mg have been evaluated. The most common adverse effects are nausea, diarrhea, dizziness, and lightheadedness. The incidence of mild hypoglycemia is lower with nateglinide than for rapaglinide and no reports of severe hypoglycemia, consistent with the mechanism of action of nateglinide. In the clinical studies carried out to date, there have been no reports of any increase body weight gain.

**Table RL3: Fixed Combination Therapies Treat Type 2 Diabetes**

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<th>Drug 2</th>
<th>Brand</th>
<th>Manufacturer</th>
<th>Generic Available</th>
<th>Available Doses (mg Drug1/ mg Drug 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyburide</td>
<td>Metformin</td>
<td>Glucovance</td>
<td>Bristol-Myers Squibb</td>
<td>yes</td>
<td>1.25/250; 2.5/500; 5/500</td>
</tr>
<tr>
<td>Glipizide</td>
<td>Metformin</td>
<td>Metaglip</td>
<td>Bristol-Myers Squibb</td>
<td>yes</td>
<td>2.5/250; 2.5/500; 5/500</td>
</tr>
<tr>
<td>Glimepiride</td>
<td>Pioglitazone</td>
<td>Duetact</td>
<td>Takeda</td>
<td>no</td>
<td>2/30; 4/30</td>
</tr>
<tr>
<td>Glimepiride</td>
<td>Rosiglitazone</td>
<td>Avandaryl</td>
<td>Glaxo SmithKline</td>
<td>no</td>
<td>(restricted use in US) 1/4; 2/4; 4/4</td>
</tr>
<tr>
<td>Sitagliptin</td>
<td>Metformin</td>
<td>Janumet</td>
<td>Merck</td>
<td>no</td>
<td>50/500; 50/1000</td>
</tr>
<tr>
<td>Saxagliptin</td>
<td>Metformin</td>
<td>Kombiglyze</td>
<td>Bristol-Myers Squibb</td>
<td>no</td>
<td>5 mg /500; 5/1000; 2.5/1000</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>Metformin</td>
<td>ACTOS</td>
<td>Takeda</td>
<td>no</td>
<td>15/500; 15/850</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>Metformin</td>
<td>PrandiMet</td>
<td>Novo Nordisk</td>
<td>no</td>
<td>1/500; 2/500</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>Metformin</td>
<td>Avandamet</td>
<td>Glaxo SmithKline</td>
<td>no</td>
<td>(restricted use in US) 1/500; 2/500; 4/500; 2/1000; 4/1000</td>
</tr>
</tbody>
</table>
Table RL4: Clinical Efficacy of Pharmacological Therapies to Treat Type 2 Diabetes When Used as Monotherapy or as Add On Treatment

<table>
<thead>
<tr>
<th>Agent Class</th>
<th>↓ Fasting Plasma Glucose (mg/dl)</th>
<th>↓ HbA1C (%)</th>
<th>Insulin</th>
<th>Lipids</th>
<th>Body Weight</th>
<th>Major Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfonylureas</td>
<td>60-70</td>
<td>3.3-3.9</td>
<td>0.8-2.0</td>
<td>Increase</td>
<td>No effect</td>
<td>Increase Hypoglycemia</td>
</tr>
<tr>
<td>Meglitinides</td>
<td>65-75</td>
<td>3.6-4.2</td>
<td>0.5-2.0</td>
<td>Increase</td>
<td>No effect</td>
<td>Increase Hypoglycemia</td>
</tr>
<tr>
<td>Biguanide (Metformin)</td>
<td>50-70</td>
<td>2.8-3.9</td>
<td>1.5-2.0</td>
<td>Decrease</td>
<td>↓TG, ↓LDL, ↑HDL</td>
<td>Decrease GI disturbances; LA1</td>
</tr>
<tr>
<td>Thiazolidinediones</td>
<td>60-80</td>
<td>3.3-4.3</td>
<td>1.4-2.6</td>
<td>Decrease</td>
<td>↓TG, -LDL, ↑HDL</td>
<td>Increase Fluid retention; decreased Hb, Congestive Heart Failure, fractures</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Glucosidase inhibitors</td>
<td>25-30</td>
<td>1.9-2.2</td>
<td>0.7-1.0</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect GI disturbances</td>
</tr>
<tr>
<td>DDP-4 inhibitor</td>
<td>12-28</td>
<td>0.6-1.5</td>
<td>0.5-0.8</td>
<td>Increase</td>
<td>No effect</td>
<td>No effect Risk of Pancreatitis (not proven)</td>
</tr>
<tr>
<td>Bile Acid Sequestrant</td>
<td>15</td>
<td>0.83</td>
<td>0.5</td>
<td>No effect</td>
<td>-TG, ↓ LDL, -HDL</td>
<td>No effect Esophageal obstruction, bowel obstruction, fecal impaction, dysphagia, pancreatitis, nausea, constipation</td>
</tr>
<tr>
<td>Dopamine Agonist</td>
<td>0-18</td>
<td>0-1.0</td>
<td>0.1-0.6</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect Nasal Stuffiness, Nausea, headache, constrictive pericarditis, neuroleptic malignant syndrome, hypotension</td>
</tr>
<tr>
<td>GLP-1 agonist</td>
<td>9-28</td>
<td>0.5-1.5</td>
<td>0.7-9</td>
<td>Increase</td>
<td>No effect</td>
<td>Decrease Nausea, constipation, Risk of pancreatitis (not proven)</td>
</tr>
<tr>
<td>Amylin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Decrease</td>
<td>Nausea</td>
</tr>
</tbody>
</table>

↓: Decrease; ↑: Increase; ↓HbA1C (%)
Note: caution must be taken in comparing the results presented above. Head to head comparisons of these agents are limited. The groups studied were often different, with significantly different baseline HgA1c levels. There is a strong correlation between baseline HbA1c and the magnitude of effectiveness of these agents (i.e. ability to decrease HbA1c). Significantly greater reductions in both fasting plasma glucose and HbA1c were observed in groups with higher baseline HbA1c. For those patients whose HbA1c was <8.0%, the reduction from therapy was only 0.1-0.2%
2.1.8.3. Biguanides

Metformin (dimethylbiguanide; Glucophage®) is a synthetic analog of the natural product guanidine, whose history as a treatment for diabetes can be traced to medieval times.\textsuperscript{240} Metformin has surpassed the sulfonylureas as the most prescribed oral agent for type 2 diabetes in the US. In the major European markets, metformin is the second most prescribed agent after glyburide.\textsuperscript{241} The widespread acceptance of metformin evolved after the realization that lactic acidosis was not a major problem in individuals with normal renal function. Phenformin, a structurally similar analog of metformin, was previously withdrawn from the market in many countries due its propensity to induce lactic acidosis. Metformin is recommended as a first-line therapy in newly diagnosed individuals, and can be used in combination with an insulin secretagogue (sulfonylurea or meglitinide), thiazolidinedione, α-glucosidase inhibitor, exenatide, DPP-4 inhibitor or insulin.\textsuperscript{221,242,243} When used as a monotherapy, metformin decreases HbA1c by 1.5-2.0%, increases insulin sensitivity, does not promote weight gain, and has an acceptable side effect profile.

![Figure RL14: Structure of Metformin](image)

**Mechanism of Action**

An elevated rate of basal hepatic glucose output is the primary determinant of elevated fasting blood glucose levels in patients with type 2 diabetes.\textsuperscript{244} The primary effect of metformin (Figure RL14) is the suppression of basal hepatic glucose production, thereby reducing fasting plasma glucose.\textsuperscript{241,245,246} Despite the large number of studies both in vitro and in humans that have established this mode of action, the molecular target of metformin action still awaits identification. Metformin does not stimulate insulin
secretion; in contrast, metformin reduces fasting plasma insulin and improves whole-body insulin-stimulated glucose metabolism (insulin sensitivity). \(^{241,246}\) While it is possible that the beneficial effect of metformin on insulin sensitivity is mediated directly, a more likely explanation is that it is secondary to a reduction in hyperglycemia, triglycerides, and free fatty acids.

Recent in vitro and in vivo evidence has shown that metformin activates the AMP-activated protein kinase (AMPK), \(^{247}\) a major cellular regulator of lipid and glucose metabolism. \(^{248}\) As a result, acetyl-CoA carboxylase activity was reduced, fatty acid oxidation induced (due to decreased malonyl-CoA), and the expression of lipogenic enzymes along with SREBP-1, a key lipogenic transcription factor, suppressed. \(^{247}\) The use of a novel AMPK inhibitor indicated that AMPK activation was required for the inhibitory effect of metformin on glucose production in hepatocytes. In isolated rat skeletal muscles, metformin stimulated glucose uptake coincident with AMPK activation. These results are intriguing in that they implicate the activation of AMPK as a unified explanation for the beneficial effects of metformin.

**Efficacy**

A large number of well-controlled clinical studies have established that metformin monotherapy consistently reduces fasting plasma glucose by 60-70 mg/dl (3.3-3.9 mmol/l) and HbA1c by 1.5-2.0%. \(^{219,221,246}\) Thus, the efficacy of metformin is in the same range as that observed for monotherapy treatment with sulfonylureas. Similar to the sulfonylurea treatment, the overall magnitude of response to metformin is directly related to the starting fasting plasma glucose concentration. Metformin also reduces fasting plasma insulin, triglycerides, and free fatty acids. \(^{246}\) Unlike sulfonylurea treatment, metformin monotherapy is not associated with weight gain and even promotes a modest weight loss. When used in combination with other oral agents or insulin, weight gain is not observed. Metformin is the only oral agent that when used as a monotherapy has been reported to reduce the risk of developing macrovascular complications. \(^{249}\)

Historically, when patients with type 2 diabetes in the US had mean HbA1c of approximately 10% and fasting plasma glucose approximately 200-240 mg/dl, in patients of this sort, monotherapy with either metformin or a sulfonylurea generally decreased plasma glucose to <140 mg/dl (<7.8 mmol/l) in about 25-30% of patients. In contrast,
combined metformin and sulfonylurea therapy increased the percentage of patients who achieve this level of control to approximately 60-70%.246 When added to a sulfonylurea, the effects of both agents are additive, consistent with their different mechanisms of action. Interestingly, in patients that no longer responded to sulfonylurea treatment (secondary failures) and were removed from treatment, addition of metformin had minimal effects.250 Thus, in these patients; sulfonylurea treatment was still eliciting an effect, emphasizing the need to continue treatment with both agents.

While investigating the additive effect of metformin and sulfonylurea therapy, it was found there was no change in glucose levels when the sulfonylurea was changed to metformin. However, when the metformin was added, there was a dramatic decrease in plasma glucose. In fact, this pattern is seen in virtually all studies comparing two oral agents. This concept is illustrated in figure RL15. When a patient is on drug A and they are changed to drug B, no improvement in glucose control will be seen. However, if drug B is added to drug A, there is an improvement. This concept can often be extended by the addition of drug C, drug D, etc.

![Figure RL15](image_url)

**Figure RL15:** Efficacy when oral agents are used as add-on therapy. When a patient is on drug A and they are changed to drug B, C, or D, often no improvement in glucose control will be seen. However, if drug B is added to drug A, there is an improvement. This concept can often be extended by the addition of drug C, drug D.
Pioglitazone (Actos) and rosiglitazone (Avandia) are members of the thiazolidinedione class of insulin sensitizing compounds originally discovered and characterized for their glucose- and lipid-lowering activity. These compounds decrease insulin resistance and enhance the biological response to endogenously produced insulin, as well as insulin administered by injection. Until September 23, 2010, each drug was approved for use in the US as monotherapy, which results in a significant reduction in fasting plasma glucose by 60-80 mg/dl and in HbA1c by 1.4-2.6%. In addition, pioglitazone is approved for use in combination with insulin, metformin, or a sulfonylurea, and rosiglitazone is approved for use in combination with metformin or a sulfonylurea. Troglitazone (Rezulin), another member of this chemical class, was withdrawn from US, European, and Japanese markets in 2000 due to idiosyncratic hepatic reaction leading to hepatic failure and death in some patients. Although there are some data from animal studies suggesting that hepatic toxicity might be characteristic of the thiazolidinedione class, current clinical evidence indicates that pioglitazone and rosiglitazone treatment do not result in liver toxicity. As of September 2010, as a result of cardiovascular concerns (see below) Rosiglitazone was removed from the European market and use in the United States was restricted by the FDA. Use of rosiglitazone was restricted to patients who could not use any other medication. In addition, the patient must be informed about the potential cardiovascular risks.

![Chemical structures of Rosiglitazone and Pioglitazone](image)

**Figure RL16:** Chemical structures of Rosiglitazone and Pioglitazone
Mechanism of Action

The primary effects of pioglitazone and rosiglitazone (Figure RL16) are the reduction of insulin resistance and improvement of insulin sensitivity, resulting in a reduction of fasting plasma glucose, insulin, and free fatty acids. Unlike other existing anti-diabetic medications that possess a very rapid onset of activity, pioglitazone and rosiglitazone exhibit a characteristic delay from 4-12 weeks in the onset of their therapeutic benefits. This is likely related to their mode of action, which involves the regulation of gene expression. Pioglitazone and rosiglitazone are selective agonists for the peroxisome proliferator-activated receptor γ (PPARγ), a member of the superfamily of nuclear hormone receptors that function as ligand-activated transcription factors. The PPAR family, which also includes PPARα and PPARδ, functions as receptors for fatty acids and their metabolites (e.g. eicosanoids) and, consequently, plays a critical physiological role in the regulation of glucose, fatty acid, and cholesterol metabolism. PPARα is the receptor for the fibrate class of lipid-lowering drugs, and PPARδ is involved in the regulation of high-density lipoprotein metabolism. The structure-activity relationship between PPARγ agonists and their glucose lowering activity in vivo has been established. In the absence of ligand, PPARs bind as heterodimers with the 9-cis retinoic acid receptor (RXR) and a multi-component co-repressor complex to a specific response element (PPRE) within the promoter region of their target genes. Once PPAR is activated by ligand, the co-repressor complex dissociates allowing the PPAR-RXR heterodimer to associate with a multi-component co-activator complex resulting in an increased rate of gene transcription. The target genes of PPARγ include those involved in the regulation of lipid and carbohydrate metabolism. It does not appear that rosiglitazone and pioglitazone improve insulin sensitivity and glucose disposal by direct effects on either liver or muscle. PPARγ is expressed chiefly in adipose tissue, and its expression in liver and skeletal muscle is low. Thus, it is more likely that the primary effects of these drugs are on adipose tissue, followed by secondary benefits on other target tissues of insulin. The ability of rosiglitazone and pioglitazone to decrease circulating free fatty acids could lead to an improvement in insulin action in the periphery. More recently, PPARγ agonists have been reported to increase the expression and circulating level of adiponectin (Acrp30), an adipocyte-derived protein with insulin sensitizing activity, in diabetic rodents and in patients with type 2 diabetes. Recognition of the importance of PPARγ in the overall regulation of carbohydrate and lipid metabolism along with growing realization that the adipocyte is an
endocrine organ suggests that investigations in this area will intensify, and perhaps uncover additional mechanisms by which rosiglitazone and pioglitazone improve insulin sensitivity and glucose disposal.

**Efficacy**

**Rosiglitazone**

The clinical efficacy of rosiglitazone and pioglitazone therapy has been extensively reviewed. Two 26-week, double blind, placebo-controlled clinical studies have established that rosiglitazone monotherapy reduces fasting plasma glucose and HbA1c in patients with type 2 diabetes. Treatment with rosiglitazone at 4 mg/day reduced fasting plasma glucose by approximately 30-45 mg/dl and HbA1c by 0.8-1.0%, compared with placebo. Treatment with rosiglitazone at 8 mg/day reduced fasting plasma glucose by approximately 45-65 mg/dl and HbA1c by 1.1-1.5%, compared with placebo. In patients with type 2 diabetes inadequately controlled with metformin, rosiglitazone produced a significant reduction in HbA1c compared to metformin treatment alone. In another study in which rosiglitazone was directly compared to a maximum stable dose of glyburide (15 mg/day), rosiglitazone reduced fasting plasma glucose by 25 mg/dl at 4 mg/day, and 40 mg/dl at 8 mg/day. The reduction in HbA1c was 0.7% for glyburide, 0.3% for rosiglitazone at 4 mg/day, and 0.5% for rosiglitazone at 8 mg/day.

**Pioglitazone**

Double blind, placebo-controlled studies with pioglitazone as monotherapy, have established that this agent reduces fasting plasma glucose HbA1c in patients with type 2 diabetes. Patients treated with 15, 30, or 45 mg (once daily) pioglitazone had significant mean decreases in HbA1c (range -1.00 to -1.60% difference from placebo) and fasting plasma glucose (-39.1 to -65.3 mg/dl difference from placebo). The decreases in fasting plasma glucose were observed as early as the second week of therapy; maximal decreases occurred after 10-14 weeks and were maintained until the end of therapy (week 26). There was no evidence of drug-induced hepatotoxicity, or elevated alanine aminotransferase activity.

The efficacy and tolerability of pioglitazone in combination with metformin has been assessed in patients with type 2 diabetes mellitus. Patients receiving pioglitazone (30 mg) + metformin had statistically significant mean decreases in HbA1c (-0.83%) and
fasting plasma glucose levels (-37.7 mg/dl) compared with placebo + metformin. Decreases in fasting plasma glucose levels occurred as early as the fourth week of therapy, the first time point at which fasting plasma glucose was measured. The pioglitazone + metformin group had significant mean percentage changes in levels of triglycerides (-18.2%) and high-density lipoprotein cholesterol (+8.7%) compared with placebo + metformin. Mean percentage increases were noted in low-density lipoprotein cholesterol levels (7.7%, pioglitazone + metformin; 11.9%, placebo + metformin) and total cholesterol (4.1%, pioglitazone + metformin; 1.1%, placebo + metformin), with no significant differences between groups. In the extension study, patients treated with open-label pioglitazone + metformin for 72 weeks had mean changes from baseline of -1.36% in HbA1c and -63.0 mg/dl in fasting plasma glucose. In this study, there was no evidence of drug-induced hepatotoxicity.

The efficacy and tolerability of pioglitazone in combination with a sulfonylurea has been also assessed in patients with type 2 diabetes mellitus. Patients receiving pioglitazone (30 mg) + metformin had statistically significant mean decreases in HbA1c (-0.83%) and fasting plasma glucose levels (-37.7 mg/dl) compared with placebo + metformin. Decreases in fasting plasma glucose levels occurred as early as the fourth week of therapy, the first time point at which fasting plasma glucose was measured. The pioglitazone + metformin group had significant mean percentage changes in levels of triglycerides (-18.2%) and high-density lipoprotein cholesterol (+8.7%) compared with placebo + metformin. Mean percentage increases were noted in low-density lipoprotein cholesterol levels (7.7%, pioglitazone + metformin; 11.9%, placebo + metformin) and total cholesterol (4.1%, pioglitazone + metformin; 1.1%, placebo + metformin), with no significant differences between groups. In the extension study, patients treated with open-label pioglitazone + metformin for 72 weeks had mean changes from baseline of -1.36% in HbA1c and -63.0 mg/dl in fasting plasma glucose. In this study, there was no evidence of drug-induced hepatotoxicity.

The efficacy and tolerability of pioglitazone in combination with a sulfonylurea has been also assessed in patients with type 2 diabetes mellitus. Twenty-three diabetic patients treated with a stable dose of sulfonylurea were randomly assigned to receive either placebo (n = 11) or pioglitazone (45 mg/day) (n = 12) for 16 weeks. Before and after 16 weeks of treatment, all subjects received a 75-g oral glucose tolerance test (OGTT) and peripheral insulin sensitivity was measured with a two-step euglycemic insulin clamp. After 16 weeks pioglitazone treatment significantly decreased fasting
plasma glucose, mean plasma glucose during OGTT, and HbA1c without changing fasting or glucose-stimulated insulin/C-peptide concentrations. Fasting plasma free fatty acid (FFA) and mean plasma FFA during OGTT also decreased significantly after pioglitazone treatment. Pioglitazone treatment significantly decreased endogenous glucose production, whereas insulin-stimulated total and non-oxidative glucose disposal was significantly increased indicative of an improvement in hepatic and peripheral (muscle) tissue sensitivity to insulin. Subsequent work has indicated that pioglitazone at doses of 30 and 45 mg/day (but not at doses of 7.5 or 15 mg/day) improves β-cell function along with whole-body insulin sensitivity.263

Side Effects
The major side effects of this class of drugs are edema, weight gain, decreased hematocrit and hemoglobin, and elevated (but reversible) alanine aminotransferase activity. Unlike troglitazone, idiosyncratic hepatic reaction does not appear to be a problem with rosiglitazone or pioglitazone. The edema ranges from bothersome trace to anasarca. The mechanism of the edema production is not known. Clinically, diuretics have minimal effect on reducing the edema, though spironolactone may have more benefit than other diuretics.296 While there are no published studies on this subject, it does appear that the edema is dose dependent. Weight gain may be a modest 2-4 pounds to >20 pounds.297 Due to their mechanism of action, the risk of hypoglycemia with rosiglitazone or pioglitazone monotherapy is low. Mild to moderate hypoglycemia has been reported during combination therapy with sulfonylureas or insulin.298,299

Cardiovascular Disease
A general listing of the cardiovascular effects of thiazolidinediones is shown in Table RL5. In a meta-analysis by Nissen and Wolski, 161 published literature and clinical trial registries were searched for cardiovascular end points such as MI and death from cardiovascular causes. Data were combined by means of a fixed-effect model. Forty-two trials were selected. In the rosiglitazone group, as compared with the control group, the odds ratio (OR) for MI was 1.43 (95% CI, 1.03-1.98; P=0.03), and the OR for death from cardiovascular causes was 1.64 (95% CI, 0.98-2.74; P=0.06). The authors concluded that rosiglitazone was associated with a significant increase in the risk of MI and with an increased risk of death from cardiovascular causes that had borderline significance. There were significant limitations to the study including lack of access to original data, which
would have enabled time-to-event analysis, no confirmation of events, many studies had only zero or 1 report, and these trials were not designed to capture or adjudicate events. With correction of some of these limitations, another Meta analysis failed to show any statistical increased risk. Since that time there have been multiple meta analyses looking at variations of the same data with variable results.

**Table RL5: Effects of Thiazolidinediones (pioglitazone/rosiglitazone) on Cardiovascular Risk Factors**

| Lipid profile | ↑ LDL cholesterol particle size |
|               | ↑ Lipoprotein a |
|               | ↑ HDL cholesterol |
|               | ↓/-- LDL/HDL ratio |
|               | ↓/↑ Triglycerides |
| Coagulation and fibrinolysis | ↓ Fibrinogen |
|                         | ↓ Platelet aggregation |
| Hemodynamic and vascular parameters | ↑ Intravascular volume |
|                                     | ↓ Blood pressure |
|                                     | ↓ Intima-media thickness |
|                                     | ↑ Endothelial function |
|                                     | ↓ Urine albumin excretion |
| Inflammatory Markers | ↓ C-reactive protein |
|                       | ↓ Adiponectin |
|                       | ↓ Tumor necrosis factor-α |

A meta analysis of cardiovascular events using patient-level data from randomized trials comparing pioglitazone with a range of alternative regimens determined that death, MI, or stroke occurred in 375 of 8,554 patients (4.4%) receiving pioglitazone and 450 of 7,896 patients (5.7%) receiving control therapy (HR, 0.82; 95% CI, 0.72-0.94; P = .005). The authors concluded that treatment with pioglitazone was associated with a significantly lower risk of death, MI, or stroke compared with any of the alternative regimens. Serious heart failure was increased in patients on pioglitazone, although without an associated increase in mortality (HR, 1.41; 95% CI, 1.14-1.76; P =0.002).
There have been two prospective, randomized studies specifically looking at cardiovascular outcome and thiazolidinediones. The study looking at pioglitazone (PROactive study) failed to show a significant benefit of treatment on the primary composite end points (hazard ratio [HR], 0.90; 95% confidence interval [CI], 0.80-1.02; P = 0.095). However, pioglitazone reduced risk for the main secondary end points, including death from any cause, nonfatal MI, and stroke (HR, 0.84; 95% CI, 0.72-0.98; P = 0.027). Addition of rosiglitazone to glucose-lowering therapy (RECORD Study) was confirmed to increase the risk of heart failure, but the data remained inconclusive about any possible effect on myocardial infarction. Rosiglitazone did not increase the risk of overall cardiovascular morbidity or mortality compared with standard glucose-lowering drugs.

**Fractures**

In 2006, an increased risk of fractures was discovered in subjects receiving rosigliatzone participating in the ADOPT Study (A Diabetes Outcome and Progression Trial). Since then there have been multiple reports on the association of thizolidenediones and fractures with a Meta-analysis of 10 randomized controlled trials (13,715 participants) and from 2 observational studies (31679 participants) showed a significant increased risk of fractures in women (OR 2.23, 95%cl 1.65-3.01; p<.001), but not in men. There has been no explanation for the sex difference, but one may speculate that the age of the men in these studies is generally younger than one expects to see significant risk of fractures. Several studies have shown that bone mineral density declines with use of these medications.

**2.1.8.5. α-Glucosidase Inhibitors**

Acarbose (Precose, Glucobay) and miglitol (Glycet) are members of the α-glucosidase inhibitor class of oral anti-hyperglycemic compounds that function by blocking the enzymatic degradation of complex carbohydrates in the small intestine. These compounds lower post-prandial glucose and improve glycemic control without increasing the risk for weight gain or hypoglycemia. Each drug is approved for use in the US as monotherapy, which results in a significant reduction in fasting plasma glucose by 25-30 mg/dl, post-prandial glucose by 40-50 mg/dl, and HbA1c by 0.7-1.0%. In addition,
Acarbose is approved for use in combination with insulin, metformin, or a sulfonylurea, and miglitol is approved for use in combination with a sulfonylurea. The effects of these compounds on glycemic control are additive when used in combination, presumably since their mechanism of action is different. Neither drug is approved in the US for use in combination with a meglitinide or thiazolidinedione. α-glucosidase inhibitors are suitable approaches for patients that have mild to moderate hyperglycemia, or those patients prone to hypoglycemia or at risk for lactic acidosis.

![Chemical structure of Acarbose](image)

**Figure RL17: Chemical structure of Acarbose**

**Mechanism of Action**

α-Glucosidase inhibitors are competitive, reversible inhibitors of pancreatic α-amylase and membrane-bound intestinal α-glucosidase hydrolase enzymes. Acarbose, the first α-glucosidase inhibitor discovered, is a nitrogen-containing pseudotetrasaccharide (Figure RL17), while miglitol is a synthetic analog of 1-deoxynojirimycin. The mechanism of action of these inhibitors is similar but not identical. They bind competitively to the oligosaccharide binding site of the α-glucosidase enzymes, thereby preventing enzymatic hydrolysis. Acarbose binding affinity for the α-glucosidase enzymes is: glycoamylase >
sucrase > maltase > dextranase.\textsuperscript{308,310} Acarbose has little affinity for isomaltase and no affinity for the β-glucosidase enzymes, such as lactase.\textsuperscript{308} Miglitol is a more potent inhibitor of sucrase and maltase that acarbose, has no effect on α-amylase, but does inhibit intestinal isomaltose.\textsuperscript{308}

**Efficacy**

Clinical trials conducted to date have established that the antihyperglycemic effectiveness of acarbose and miglitol is less than 50% than that of either sulfonylureas or metformin. When used as monotherapy, acarbose primarily affects post-prandial glucose levels, which is reduced by 40-50 mg/dl after meal.\textsuperscript{308,309,311-313} In most studies, α-glucosidase inhibitors have no significant effects on either fasting insulin or whole body insulin sensitivity in patients with type 2 diabetes. However, there is some evidence that acarbose and voglibose, a structural analog of miglitol in clinical development in Japan, reduces post-prandial hyperinsulinemia in glucose intolerant individuals.\textsuperscript{314,315} Some but not all studies have reported small decreases in fasting or post-prandial triglycerides.\textsuperscript{308} Since the mechanism of action of α-glucosidase inhibitors is different from other oral agents, their effects on glycemic control are additive when used in combination. As summarized by Lebovitz\textsuperscript{171} addition of acarbose to sulfonylurea therapy decreases HbA1c by 0.85%; addition of acarbose to metformin therapy decreases HbA1c by 0.73%; and addition of acarbose to insulin therapy decreases HbA1c by 0.54%. As for monotherapy, the predominant improvement is on post-prandial hyperglycemia. Treatment with α-glucosidase inhibitors appears to have a lower rate of secondary failures characteristic of sulfonylurea and metformin therapy.

**Side Effects**

The major side effects of the α-glucosidase inhibitors are related to gastrointestinal disturbances. These occur in approximately 25-30% of diabetic patients, and include flatulence, diarrhea, bloating, and abdominal discomfort. These side effects can often be minimized by careful dose titration, and sometimes diminish with time. Acarbose is contraindicated in patients with inflammatory bowel disease, cirrhosis, or elevated plasma creatinine (>$177$ μmol/l). This class of drugs is associated with dose-dependent hepatotoxicity, and serum transaminase levels require monitoring for patients receiving high doses (>200 mg three times daily). Transaminase elevations, which are often asymptomatic, are reversible upon cessation of treatment. Hypoglycemia does not occur in patients on α-glucosidase inhibitor monotherapy. If hypoglycemia occurs while a
patient is taking an α-glucosidase inhibitor simultaneously with a sulfonylurea, insulin or a meglitinide, the recommended action is oral administration of pure glucose, dextrose or milk.

2.1.8.6. Dipeptidyl Peptidase IV (DPP-IV) Inhibitors

As described above, DPP-IV inhibitors are compounds that increase the concentration of endogenous incretins, including GLP-1, by limiting the proteolytic cleavage by DPP-IV. As shown in Figure , above, GLP-1 is an insulinotropic hormone secreted by L-cells of the small intestine that stimulates insulin secretion in a glucose-specific manner, inhibits gastric emptying, suppresses glucagon secretion, and has central anorexic activity. Although it possesses multiple effective clinical activities, administration of GLP-1 is not an ideal approach since it cannot be administered orally. Furthermore, endogenous (and exogenously administered) GLP-1 has undesirable pharmacokinetics; after it is secreted, it is rapidly cleaved and inactivated [plasma half-life < 1 min] by the enzyme dipeptidyl peptidase IV (DPP-IV). Thus, inhibition of DPP-IV has been suggested as a feasible alternative to elevate circulating GLP-1 levels, and circumvent the limitations of GLP-1 administration.

The initial, orally active DPP-IV inhibitor, NVP-DPP728, was identified and characterized in vitro and in vivo. Inhibition of DPP-IV by NVP-DPP728 resulted in a significantly amplified early phase of the insulin response to an oral glucose load in obese fa/fa rats, and restoration of glucose excursions to normal. In contrast, DPP-IV inhibition produced only minor effects in lean FA/FA rats. Inactivation of GLP-1 (7-36) amide was completely prevented by DPP-IV inhibition suggesting that the effects of this compound on oral glucose tolerance were mediated by increased circulating concentrations of GLP-1 (7-36) amide. In DPP-IV(+) [but not in DPP-IV(-)] transgenic rats fed either standard chow or a high-fat diet, NVP-DPP728 significantly suppressed glucose excursions after glucose challenge by inhibiting the plasma DPP-IV activity, associated with the stimulation of early insulin secretion. NVP-DPP728 also improved the glucose tolerance after an oral glucose challenge by potentiating the early insulin response by inhibition of plasma DPP-IV activity in aged DPP-IV(+) Wistar and F344 rats. In contrast, NVP-DPP728 did not affect the glucose tolerance after an oral glucose challenge in aged DPP-IV(-)F344 rats.

Taken together, these results indicate that treatment with NVP-DPP728 ameliorates
glucose tolerance in vivo by the direct inhibition of plasma DPP-IV activity, and presumably the subsequent increase in endogenous GLP-1 action.

The clinical activity of this compound as a monotherapy was reported in patients at an early stage of type 2 diabetes.\textsuperscript{329} Compared with placebo, NVP-DPP728 at 100 mg (tid; n = 31) significantly reduced fasting glucose by 18 mg/dl (1.0 mmol/l), prandial glucose excursions by 21.6 mg/dl (1.2 mmol/l), and mean 24-h glucose levels by 18 mg/dl (1.0 mmol/l). Similar reductions were seen in the 150-mg (bid; n = 32) treatment group. Mean 24-h insulin was significantly reduced by 26 pmol/L in both groups. In the combined active treatment groups, HbA1c was significantly reduced from 7.4 Â± 0.7% to 6.9 Â± 0.7%. Laboratory safety and tolerability were good in all groups. These results provided clinical proof of concept that inhibition of DPP-IV is a feasible approach for the treatment of type 2 diabetes in the early stage of the disease.

**Sitagliptin**

Sitagliptin (Januvia), a selective DPP-IV inhibitor (Figure RL18), received regulatory approval in the US\textsuperscript{330} in 2006.

![Figure RL18: Structure of Sitagliptin](image)

**Mechanism of Action**

As described above, DPP-IV inhibitors are compounds that increase the concentration of endogenous incretins, including GLP-1, by limiting the proteolytic cleavage by DPP-IV. The clinical effect is to stimulate insulin secretion in a glucose-specific manner and suppress glucagon secretion.

**Efficacy**

In sitagliptin monotherapy studies HgA1c was reduced from baseline by .28% to 0.76% at 12 to 24 weeks.\textsuperscript{331-337} As an add on agent, there were HgA1c reductions of 0.45% to
1.0% at 24 to 30 weeks.\textsuperscript{334-341} In the studies discussed above, there were no significant effects on weight reported.

\textit{Side Effects}

As monotherapy, the incidence of hypoglycemic was reported between 0.5% to 2.2%, with no severe episodes.\textsuperscript{335-337} When used in combination with a sulfonylurea, hypoglycemia occurred in 12.2% of patients, but no severe events were reported.\textsuperscript{338} Gastrointestinal events rarely occur. Headaches (incidence of 1.8% to 5.7%), nasopharyngitis (2.9% to 9.1%), and upper respiratory tract infection (0% to 8.8%) have been reported.

\textbf{Saxagliptine}

Saxagliptin (Ongyza), a selective DPP-IV inhibitor (\textit{Figure RL19}), received regulatory approval in the US in 2009.

\textbf{Mechanism of Action}

As described above, DPP-IV inhibitors are compounds that increase the concentration of endogenous incretins, including GLP-1, by limiting the proteolytic cleavage by DPP-IV. The clinical effect is to stimulate insulin secretion in a glucose-specific manner and suppress glucagon secretion.

\textbf{Efficacy}

When added to a sulfonylurea, Saxagliptin at doses of 2.5 mg and 5 mg, decreased HgA1c by 0.54% to 0.64%. The same doses decreased HgA1c by 0.66% to 0.94% when
added to a thiazolidinedione (0.30% decrease seen with a thiazolidinedione plus placebo).  

**Side Effects**  
At doses of 2.5 and 5 mg, saxagliptin was associated with a hypoglycemia incidence of 2.7% to 4.1% when combined with a thiazolidinedione and 13.3% to 14.6% when combined with a sulfonyl urea.

2.1.8.7. **Emerging Approaches**  
The development pipeline for new oral therapeutic agents for type 2 diabetes is encouraging and continues to expand. These intensive research and development efforts are in response to the increasing prevalence of the disease and related co-morbidities, realization by care givers that successful glycemic control will likely require combination therapy, a growing understanding of the pathophysiology of the disease, and the identification and validation of new pharmacological targets. These targets include receptors and enzymes that: enhance glucose-stimulated insulin secretion, suppress hepatic glucose production, increase skeletal muscle glucose transport and utilization, increase insulin sensitivity and intracellular insulin signaling, and reduce circulating and intracellular lipids. Due to their promise for future clinical success and because they exhibit mechanisms of action distinct from current therapies, two such emerging approaches are highlighted here.

**Selective Cannabinoid-1 Receptor (CB-1) Antagonism**  
The appetite-stimulating activity of Cannabis sativa has long been known through traditional recreational and medicinal use. The active molecule, delta-9-tetrahydrocannabinol (THC), was initially purified in 1964, but the discoveries of cannabinoid receptors and their endogenous ligands did not occur until more recently, within the last 15 years. The endocannabinoid system is composed of two distinct G-protein-coupled cannabinoid receptors (CB1 and CB2), two major endogenous ligands (anandamide and 2-arachidonyl glycerol), and the enzymes responsible for ligand synthesis (N-acylphosphatidylethanolamine-specific phospholipase D and diacylglycerol lipase) and degradation (fatty acid amide hydrolase and monoacylglycerol lipase). CB1 receptors are widely distributed on central and peripheral neurons, and also present in
adipose tissue, liver, muscle, pancreas, heart, vasculature, and the gastrointestinal tract.\textsuperscript{347,348} The distribution of CB2 receptors is more limited, where they are mainly associated with the immune system and hematopoiesis.\textsuperscript{347,348} Ligand stimulation of the receptors results in decreased adenylate cyclase activity and cAMP production, along with a reduction in intracellular calcium.

The endocannabinoid system was identified as playing a significant role in the regulation of food intake and energy metabolism. There is some evidence that the endocannabinoid system is constitutively active in certain regions of the brain that are known to control energy balance, suggesting that tonic activation favors energy intake, storage, and weight gain.\textsuperscript{349} In rodents, activation of CB1 (but not CB2) receptors by endogenous ligands or THC stimulates feeding,\textsuperscript{350-352} while CB1-deficient mice are protected against diet-induced obesity.\textsuperscript{353,354} In human obesity, it has been reported that fatty acid amide hydrolase, the enzyme that degrades anandamide, is reduced,\textsuperscript{355} and that a missense mutation is associated with increased body mass index.\textsuperscript{356} Taken together, these data have provided the scientific rationale for the idea that pharmacological antagonists of CB1 might offer a promising approach to treat obesity.

Rimonabant is a selective CB1 receptor antagonist for obesity and smoking cessation.\textsuperscript{357-360} Animal studies have convincingly demonstrated that this compound exerts a beneficial effect on reducing hunger, caloric intake, and body weight, and for increasing satiety.\textsuperscript{360} In humans, rimonabant was equally effective as judged by initial clinical studies,\textsuperscript{361-364} but due to concern over its depressive side effects, the drug was not approved for use in the United States and after initial approval in Europe was removed from the market. Nevertheless, this mechanism of action may be utilized in the future.

Despite the magnitude of the disease, the choice of oral antihyperglycemic drugs for type 2 diabetes was limited to sulfonylureas for over 40 years. The last 11 years have witnessed the introduction of five new classes of oral antihyperglycemic therapies. Each possesses a distinct mechanism of action, which enables their use independently and, in some cases, as combination therapy. This is important since most patients with type 2 diabetes will require combination therapy to reach an acceptable level of glycemic control.\textsuperscript{365} However, despite these advances; there is still plenty of room and necessity for improvement.
The successful long-term management (and hopefully prevention) of type 2 diabetes and its related co-morbidities undoubtedly requires an aggressive, comprehensive approach. This includes intervention at the pre-diabetes stage (e.g. obesity, impaired glucose tolerance) including both changes in lifestyle (i.e. dietary modification and exercise), along with pharmacological intervention that might delay or even prevent the development of the disease and/or its complications. However, once the disease is established and beyond the control of lifestyle modifications, treatment must be initiated and carefully monitored using existing drugs that address the current understanding of the pathophysiology: impaired insulin secretion, increased hepatic glucose production, and peripheral tissue insulin resistance. It is anticipated that emerging agents will also have a beneficial impact on these processes, but with greater efficacy and safety due to a higher degree of selectivity for their molecular targets. In addition, new information regarding the biochemistry, cell biology, and pathophysiology of the disease process is rapidly providing additional exciting opportunities for target identification, validation and subsequent drug development.

The biological effects of emerging agents will not be limited to lowering blood glucose. Already, there are some new agents in clinical development that have been designed to afford a combination of benefits, including the reduction of both lipids and glucose. Other new therapies will likely address the emerging belief that there is a significant inflammatory component of the disease. Perhaps, this might be why tight glycemic control alone has experienced limited success at reducing the risk of macrovascular complications of diabetes. Targeting the risk factors for heart disease clearly requires serious attention and concomitant therapy. The complexity of type 2 diabetes and associated co-morbidities will continue to present a formidable challenge for successful pharmacological treatment. However, based on the growing sophistication of 21st Century research approaches along with the realization of the consequences of failure, there is ample support for the optimistic view point that the current selection of orally active treatment options will continue to expand.

2.1.9. Peroxisome Proliferator Activated Receptors (PPARs)
The PPARs were first cloned as the nuclear receptors that mediate the effects of synthetic compounds called peroxisome proliferators on gene transcription. It soon became clear that eicosanoids and fatty acids can also regulate gene transcription through PPARs. At the molecular level, PPARs act in a similar manner to other nuclear hormone receptors. First, they bind a specific element in the promoter region of target genes. PPAR and some other nuclear hormone receptors bind the promoter only as a heterodimer with the receptor for 9-cis retinoic acid, RXR (retinoid X receptor). Second, they activate transcription in response to binding of the hormone (ligand) (Figure RL20a). For the PPAR:RXR heterodimer, binding of the ligand of either receptor can activate the complex, but binding of both ligands simultaneously is more potent. \(^\text{376}\)

Three PPAR isotypes have been identified: \(\alpha\), \(\beta\) (also called \(\delta\) and NUC1) and \(\gamma\). PPAR\(\alpha\) is expressed most in brown adipose tissue and liver, then kidney, heart and skeletal muscle. PPAR\(\gamma\) is mainly expressed in adipose tissue, and to a lesser extent in colon, the immune system and the retina. PPAR\(\beta\) is found in many tissues but the highest expression is in the gut, kidney and heart. \(^\text{376}\)

**Figure RL20a:** PPAR at the basic level. Basic mechanism of action of nuclear hormone receptors. Nuclear hormone receptors bind to a specific sequence in the promoter of target
genes (called hormone response elements), and activate transcription upon binding of ligand. Several nuclear hormone receptors, including the retinoic acid receptor, the vitamin D receptor and PPAR, can bind to DNA only as a heterodimer with the retinoid X receptor, RXR, as shown.

PPARs are ligand-dependent transcription factors: activation of target gene transcription depends on the binding of the ligand to the receptor. Some ligands are shared by the three isotypes, such as polyunsaturated fatty acids and probably oxidized fatty acids. Several compounds bind with high affinity to PPARα, including long-chain unsaturated fatty acids such as linoleic acid, branched, conjugated and oxidized fatty acids such as phytanic acid and conjugated linolenic acid, and eicosanoids such as 8S-HETE and leukotriene (LT) B4\textsuperscript{376-379} (Figure RL20b). This last compound is particularly interesting, as a membrane receptor for LTB4 has also been cloned. The functional relationship between these two types of receptor is not clear. The prostaglandin 15-deoxy-D\textsubscript{12,14}-prostaglandin J2 is the most potent natural ligand of PPARγ, but the extent to which its in vivo effects are mediated through PPARγ is not known.\textsuperscript{380}

2.1.9.1. PPAR function at the cellular level

Much of the function of PPARs can be extrapolated from the identity of their target genes, which so far all belong to pathways of lipid transport and metabolism. PPARα has
mostly been studied in the context of liver parenchymal cells, where it is highly expressed. The target genes of PPARα are a relatively homogenous group of genes that participate in aspects of lipid catabolism such as fatty acid uptake through membranes, fatty acid binding in cells, fatty acid oxidation (in microsomes, peroxisomes and mitochondria) and lipoprotein assembly and transport (Figure RL21). Whereas PPARα operates in the catabolism of fatty acids in the liver, PPARγ influences the storage of fatty acids in the adipose tissue (Figure RL21). With the C/EBP transcription factors, PPARγ is part of the adipocyte differentiation program that induces the maturation of pre-adipocytes into fat cells. Most of the PPARγ target genes in adipose tissue are directly implicated in lipogenic pathways, including lipoprotein lipase (LPL), adipocyte fatty acid binding protein (A-FABP or aP2), acyl-CoA synthase and fatty acid transport protein (FATP).

2.1.9.2. PPARs in whole body physiology
The role of these transcription factors in whole body human physiology and metabolism can best be illustrated by comparing two opposite nutritional states: early absorptive period or fed state and late post-absorptive period or fasting state. In the fed state, which in humans is up to 4 h after a large meal, carbohydrates and fat enter the circulation in the form of glucose and chylomicrons, respectively (Figure RL22a). Most glucose is taken up by the liver and, if glycogen stores are already filled, used for lipogenesis. The amount of the transcription factor sterol response element binding protein 1 (SREBP1, also called ADD1) rises in the fed state, which promotes the glycolytic conversion of glucose into acetyl-CoA and subsequently the synthesis of fatty acids from acetyl-CoA8,9. Fatty acids are converted to triglycerides and packaged into very low density lipoproteins (VLDL).
Figure RL21: Action of PPARα and PPARγ at the cellular level. PPARα stimulates oxidation of fatty acids in various organelles, such as mitochondria, peroxisomes and microsomes. It also stimulates uptake of fatty acids and synthesis of lipoproteins. PPARγ stimulates lipolysis of circulating triglycerides and the subsequent uptake of fatty acids into the adipose cell. It also stimulates binding and activation of fatty acids in the cytosol, events that are required for synthesis of triglycerides. FA: fatty acid; HDL: high density lipoprotein.

In adipose tissue, the amounts of SREBP and PPARγ are elevated, probably because of regulation by insulin. PPARγ is a direct target gene of SREBP, which emphasizes the cooperative and additive functions between these two types of receptor. In addition, SREBP1 may be involved in producing an endogenous ligand (probably fatty acid) for PPARγ. The overall effect is stimulation of the uptake of glucose and fatty acids, and their subsequent conversion to triglycerides.

Triglyceride storage causes increased production of the hormone leptin in the adipose tissue. Leptin is the protein product of the ob gene, whose deletion leads to severe obesity in mice. Its expression is increased by long-term overfeeding as part of a feedback mechanism to limit further food intake and weight gain. Consistent with the role of PPARγ in promoting lipogenesis, production of leptin in adipose tissue is under negative control by PPARγ. Paradoxically, expression of both PPARγ and leptin is reduced by fasting and increased by feeding. In the latter case, PPARγ may attenuate the increase in leptin expression to limit wasteful lipolysis and fatty acid oxidation, processes which are
stimulated by leptin. If the above scenario is correct, decreased PPARγ expression may lead to increased leptin levels and, as a result, to lower food intake and weight gain. Studies with PPARγ+/- mice indicate that this is the case. A different situation exists in the late post-absorptive or fasting state (Figure RL22b). In the liver, fatty acids are oxidized to acetyl-CoA and subsequently to ketone bodies, such as acetoacetate and β-hydroxybutyrate. Both processes are strongly stimulated by PPARα, expression of which is elevated upon fasting. Fatty acids are ligands for PPARs, so it is possible that the large amounts of fatty acids liberated from the adipose tissue can stimulate their own metabolism by activating PPARα. Experiments with PPARα null mice show that PPARα is important in the hepatic response to fasting. When fasting, these mice suffer from a defect in fatty acid oxidation and ketogenesis, resulting in elevated plasma free fatty acids, hypoketonaemia, hypothermia and hypoglycaemia. The hypoglycaemia emphasizes the important interplay between fatty acid and glucose metabolism in energy homeostasis.

In adipose tissue, expression of SREBP and PPARγ is low under fasting conditions. Under a strong adrenergic stimulus, triglycerides are hydrolysed to fatty acids and glycerol, but some of the released fatty acids are re-esterified to triglycerides, in a reaction that requires synthesis of glycerol from gluconeogenic precursors. The rate-limiting step for glyceroneogenesis is catalysed by phosphoenolpyruvate carboxykinase, whose transcription is positively controlled by PPARγ. Thus, even under highly catabolic conditions such as fasting, lipogenesis continues and is dependent upon PPARγ.

### 2.1.9.3. Therapeutic potential of PPAR ligands

In developed societies, metabolic disorders such as hyperlipidaemia, atherosclerosis, diabetes and obesity rarely occur in isolation, but are usually part of a complex phenotype of metabolic abnormalities called syndrome X. Synthetic agonists for both PPARα (fibrates) and PPARγ (thiazolidinediones; TZDs) are useful in the treatment of the diseases that are part of this syndrome.
**Figure R1.22:** Overview of the roles of PPARα and PPARγ during feeding and fasting. (a) PPARγ is mainly active in the fed state when it stimulates accumulation of fat in the adipose tissue. (b) PPARα is mainly active in the fasting state when it stimulates oxidation of fatty acids in the liver. The particular metabolic steps that are regulated by PPARα and PPARγ are shown in orange and red, respectively. The roles of the transcription factors C/EBP, c-myc, HNF4 and SREBP, which are transcriptional regulators of metabolism, have been illustrated. The expression of the indicated proteins is increased (O) or decreased (P). AA, amino acids; CHYL, chylomicrons; FA, fatty acids; TG, triglycerides.

Synthetic PPARγ ligands are used for their potent antidiabetic effects. In the United States, three TZDs, troglitazone (Rezulin), rosiglitazone (Avandia) and pioglitazone (Actos), are approved for use in type II diabetic patients. They bind PPARγ with
moderate (troglitazone) to high (rosiglitazone) affinity, so it is believed that their hypoglycaemic effect is exerted by activating PPARγ. However, a direct connection between PPARγ and glucose homeostasis has not been easy to establish because skeletal muscle, which accounts for the TZD-mediated increase in glucose disposal, expresses only trace amounts of PPARγ. To explain this paradox, a mechanism has been proposed by which TZDs divert fatty acids away from skeletal muscle by increasing their uptake in adipose tissue, and so reduce the deleterious effects of fatty acids on muscle insulin action. In this model, the hypoglycaemic effect of TZDs is secondary to their hypolipidaemic effect. However, mice that lack adipose tissue can still benefit from the action of TZDs, indicating that adipose tissue is dispensable for mediating the hypoglycaemic effects of TZDs. It is also possible that the effect of TZDs on glucose homeostasis might be via an alternative mechanism not involving PPARγ, as is the case for the inhibitory effect of troglitazone on cholesterol synthesis.

New ligands specific for PPARγ will be useful in refining these observations and working out the mechanisms of glucose homeostasis. Fibrates are potent hypolipidaemic drugs. In the past few years they have been used increasingly to treat cardiovascular disease. Fibrates, which include gemfibrozil, bezafibrate and fenofibrate, bind PPARα with high affinity and it is believed that most of their effects on disease progression are mediated by PPARα. Fibrates lower plasma triglyceride levels markedly and increase high density lipoprotein (HDL) levels. The former effect occurs by stimulating hepatic fatty acid oxidation and reducing apoCIII expression, whereas the latter effect is due to induction of apolipoprotein-AI and apolipoprotein-AII expression, both mediated by PPARα.

Fibrates may also have a hypoglycaemic and thus anti-diabetic effect, as a consequence of their hypolipidaemic action. Accordingly, it is interesting to compare PPARα agonists and PPARγ agonists. Though they act through different receptor isotypes, both groups of compound have potent hypolipidaemic properties which may be at the basis of their hypoglycaemic effect. Future studies will have to establish whether fibrates or other PPARα agonists may be applicable in the treatment of type II diabetes. The heterodimer between PPAR and RXR can also be activated by ligand binding to RXR. Synthetic ligands specific for RXR have been tested as an alternative treatment for diabetes.
2.1.10. Functional and Structural insight of PPARγ

PPARγ binds to DNA as a heterodimer with the retinoid X receptors (RXRs), receptors for the vitamin A metabolite 9-cis-retinoic acid (9cRA). The three RXR subtypes (α, β, γ) bind to DNA and activate transcription in response to 9cRA as homodimers. However, the RXRs also serve as common heterodimeric partners for ~15 of the class 1 nuclear receptors, including the retinoic acid, thyroid hormone, and vitamin D receptors. Thus, RXRs are essential components of multiple endocrine signaling pathways. Some of the RXR heterodimers, including those formed between the PPARs and RXRs, are “permissive” in that they can be activated by 9cRA. RXRs also form permissive heterodimers with LXR and FXR, nuclear receptors for oxysterols and bile acids, respectively. The dualmodulation of these permissive heterodimers by either cognate ligand provides an additional level of regulation of these hormone signaling pathways.

The effects of hormones on PPARγ, RXR, and other nuclear receptors are mediated through the ligand-binding domain (LBD), a conserved region of ~250 amino acids in the C-terminal half of the receptor. In addition to its role in hormone binding, the LBD also contains dimerization and transactivation functions, including the transcriptional activation function 2 (AF-2). Upon hormone binding, the LBD undergoes a conformational change, most notably in the AF-2 domain. These conformational changes result in the displacement of corepressor proteins, such as NCoR and SMRT, that inhibit transcription and the recruitment of coactivator proteins, such as p160 and DRIP/TRAP, family members that are involved in transcriptional activation.

The structure of the PPARγ/RXRα heterodimer complex contains six components: the two receptor LBDs, their two respective ligands, and two SRC-1 peptides (Figure RL23). The complex is butterfly shaped, with both LBDs adopting the conserved “helical sandwich” fold for other ligand-bound nuclear receptors (front view in Figure RL23). PPARγ contains 13α helices and 4 short β strands, and the gross structure resembles the PPARγ homodimer. RXRα is composed of 11 a helices and 2 short b strands, but the ligand-bound structure shows large conformational changes in H3, H10 and the AF-2 helix compared with the apo-RXR structure.
The PPARγ and RXRα ligands occupy their respective ligand-binding cavities, which lie in the lower half of the LBDs. Both PPARγ and RXRα are in the active configuration, such that the AF-2 helix is folded against the main body of their respective LBDs. The AF-2 helix, together with H3 and H4, form a hydrophobic groove into which the coactivator peptide binds. Two SRC-1 peptides are present in each heterodimer complex where residues 630–639 of the peptide are ordered in the RXRα complex and residues 628–643 are ordered in the PPARγ complex. The overall structure of the coactivator-binding site and the docking modes of the SRC-1 peptide are similar to those observed in the other nuclear receptor coactivator structures (Figure RL23). In each receptor, the coactivator-binding site is a groove formed by H3, H39, and H4 at the top and by the AF-2 helix at the bottom. The floor and the sides of this groove are composed of hydrophobic residues. The SRC-1 peptide contains a two-turn amphipathic α helix, which is positioned over the hydrophobic groove between the charge clamp, formed by the lysine and the glutamate residues, as seen in the PPARγ homodimer structure with SRC-1.

**Figure RL23:** Overall Structure of the PPARγ/RXRα Heterodimer Complex

The front and top views of the PPARγ/RXRα heterodimer complex are presented in solid rendering (a helices in cylinders, and b strands in arrows). RXR is colored in yellow, PPARγ in blue, and the two SRC-1 peptides in purple. The two compounds (9cRA and GI262570) are shown in space-filling representation with carbon, oxygen, and nitrogen atoms depicted in green, red, and blue, respectively.
**Binding of Rosiglitazone and GI262570 to PPARγ**

The PPARγ ligand-binding site is much larger (~1440Å) and more convoluted than is the corresponding pocket of RXR. Rosiglitazone occupies only 25% of the available pocket. Interestingly, the conformation of rosiglitazone bound to the PPARγ/RXR heterodimer shows significant differences compared with the previously determined rosiglitazone-PPARγ homodimer structure. In the heterodimer, rosiglitazone binds into the large PPARγ pocket in a U-shaped conformation, with the TZD headgroup oriented toward the AF-2 helix (Figure RL24). Hydrogen bonds are made between the TZD headgroup and H449, Y473, H323, and S289. However, the hydrogen bond that was seen between the TZD and Q286 in the homodimer structure is absent. The pyridyl tail is directed into a lipophilic pocket adjacent to the β sheet. In the heterodimer, the rosiglitazone side chain adopts a different gauche conformation than it does in the homodimer. In the heterodimer structure, the N-methyl group is directed into a lipophilic pocket between H6 and H7, where it contacts C285, M364, and L353. The pyridyl nitrogen makes a hydrogen bond within the ligand pocket that was not observed in the homodimer. These differences suggest that heterodimerization has a direct effect on the bound conformation of rosiglitazone.

GI262570 is a tyrosine-based molecule that binds to the PPARγ/RXRα heterodimer with a $K_i$ 51 nM. This binding affinity is ~50-fold higher than that of rosiglitazone. The X-ray structure of GI262570 in the PPARγ/RXRα heterodimer shows that GI262570 also binds to PPARγ in a U-shaped conformation, occupying ~40% of the pocket (Figure RL24b). The carboxyl group of GI262570 makes hydrogen bonds with the same four residues (S289, H323, H449, and Y473) that interacted with the TZD headgroup of rosiglitazone. GI262570 contains a 5-methyl-2-phenyloxazole tail that adopts a gauche conformation, with its methyl group directed into the H6/H7 pocket (Figure RL24c). The oxazole nitrogen makes a hydrogen bond with the bound water molecule. As a result, the phenyloxazole tail of GI262570 is inserted ~2.3 Å deeper than is the tail of rosiglitazone into the hydrophobic ligand-binding pocket, while maintaining critical interactions with the H6/H7 pocket and the bound water molecule (Figure RL24). At the other end of GI262570, the benzophenone group attached to the tyrosine nitrogen reaches ~7 Å into a large lipophilic pocket formed by H3, H7, and H10. These hydrophobic interactions, provided by the benzophenone group of GI262570, are not available to rosiglitazone or
other TZDs. Thus, the additional hydrophobic interactions of GI262570 with both ends of the ligand-binding pocket reveal the structural basis for its increased PPARγ binding affinity compared with rosiglitazone.

There are several marked differences between the RXRα and PPARγ subunits of the heterodimer with respect to ligand binding. Unlike the enclosed 9cRA-binding pocket in RXR, the PPARγ pocket has a solvent-accessible channel between H3 and the β strands. Rosiglitazone and GI262570 can access the PPARγ pocket via this open channel without the protein undergoing a significant conformational change. Furthermore, rosiglitazone and GI262570 interact directly with the PPARg AF-2 helix, whereas the RXR AF-2 helix is held in place by 9cRA exclusively through hydrophobic interactions. Thus, ligand binding and activation of PPARγ occur through a mechanism that is distinct from that for RXR. However, the intramolecular interactions between the AF-2 helix and the body of the PPARγ LBD are similar to those seen in the 9cRA/RXR complex. Hydrophobic residues V446, V450, L453, and I456 from H10, together with residues F282, Q286, V290, and V293 from H3 and V322 from H5, form a large hydrophobic pocket for binding of the AF-2 helix. The hydrophobic residues of the AF-2 helix (L468, L469, I472, and Y473) face directly toward this hydrophobic pocket and make extensive interactions with these surrounding residues. The carbonyls of residues 471, 472, and 474 from the PPARg AF-2 helix are also capped by K319, a conserved positively charged residue from the end of H4. The importance of these capping interactions is underscored by the conservation of these interactions across many nuclear receptors and by the fact that a mutation in the corresponding conserved charged residue in RAR (K264A) abrogates the activity of the receptor. These observations demonstrate that although PPARγ ligands directly with the AF-2 helix, and RXRα ligands do not, these two receptors employ a conserved set of intramolecular interactions to stabilize the active AF-2 conformation.
Figure RL24: Recognition of Rosiglitazone and GI262570 by PPARγ
Electron density maps for the rosiglitazone (a) and GI262570 (b) molecules within the PPARγ pocket. The maps were calculated in the absence of the compounds with 2Fo-Fc coefficients and contoured at 1.0 σ. (c) Interactions between GI262570 and PPARγ. Residues involved in hydrogen bond interactions and hydrophobic interactions are indicated in blue and white, respectively. (d) Comparisons of the docking modes of GI262570 (green carbons) and rosiglitazone (purple carbons) in the PPARγ ligand-binding pocket (gray surface). The pockets that are not filled by rosiglitazone but are occupied by the benzophenyl group (“A”) and the benzyl tail (“B”) of GI262570 are indicated, as is the Y473 residue from the AF-2 helix.

2.1.11. Structural insight of PPARα

The overall structure of hPPARα LBD encompasses residues 199–468. The domain consists of 12 helices arranged in an antiparallel helix sandwich. In addition, a 3-stranded antiparallel β sheet is situated in the core of hPPARα LBD. Residues 449–457, preceding the C-terminal helix (helix 12 or AF2 helix) segment is tightly packed onto the core of the domain in PPARα. The ligand binding site in hPPARα is located in the central core of the
LBD, flanked by helices 3, 5, 7, 11, and 12. It is situated in a large T-shaped cavity. The central cavity spans the domain between the AF2 helix and the 3-stranded antiparallel β sheet. At the level of the β sheet, the cavity splits upward and downward along an axis roughly parallel to helix 3. These extensions are referred to as the upper and lower distal cavities (Figure RL25). An apparent entrance to the ligand binding site is found between helix 3 and the 3-stranded antiparallel β sheet. The region of residue 254 to 264 constituting a loop is highly flexible and partly covers the entrance to the ligand binding site. The entrance is further restricted by Tyr334, which forms a hydrogen bond with Glu282. Due to these steric hindrances, a great deal of flexibility is probably required in order for large ligands such as AZ 242 to enter the ligand binding site.

![Figure RL 25](image-url)

**Figure RL 25**: A stereo view of a superposition of hPPARγ LBD (red) and hPPARδ LBD (yellow) on hPPARα LBD (blue). Only the Cα trace is shown. Ligands from the three respective structures are shown in green. The central cavity is marked with a “C”, and the upper distal and lower distal cavities are marked with an “U” and “L”, respectively.
2.1.12. Comparison of structures of LBDs and binding modes to ligands among the three PPAR subtypes

The structure of hPPARα LBD is very similar to both hPPARγ LBD and hPPARδ LBD (Figure 25400 and 2675). The domain consists of 12 helices arranged in an antiparallel helix sandwich. In addition, a 3-stranded antiparallel β sheet is situated in the core of hPPARα LBD. The largest deviation between the three different crystal structures is found in a region comprising residues 231–265, referred to as the omega loop. This region displays the highest B factors in hPPARα LBD and differs among the various reported crystal structures of hPPARγ LBD.396,401 Residues 449–457, preceding the C-terminal helix (helix 12 or AF2 helix), constitute a second region exhibiting a large degree of conformational variation between the three subtypes. This segment is more tightly packed onto the core of the domain in PPARα compared with the other two PPAR subtypes.

The LBD is situated in a large T-shaped cavity similar in size to that of hPPARγ LBD396 and hPPARδ LBD,402 with a volume of about 1300Å. An apparent entrance to the ligand binding site is found in hPPARα between helix 3 and the 3-stranded antiparallel β sheet in a region similar to the proposed entrance in hPPARγ LBD and hPPARδ LBD. The equivalent residues to Tyr334 in hPPARα LBD are Glu341 in hPPARγ LBD, which appears to be flexible,396 and Asn307 in hPPARδ LBD. Most known PPAR agonists share features such as a hydrophilic head group, a central hydrophobic part, and a flexible linker to the tail. The binding mode of these compounds are highly similar, with the head group interacting with the AF2 helix, the central ring systems forming hydrophobic interactions (Figure RL27),400 and the tail extending toward the lower or upper distal cavity. PPAR agonism is caused by a conserved hydrogen bonding pattern involving the AF2 helix. This hydrogen bonding network is also present in hPPARδ LBD402 and is therefore conserved in the entire PPAR family. The overall similarity in the binding mode for hPPARα LBD and hPPARγ LBD also extends to the coordination of water molecules.
**Figure RL. 26:** The x-ray crystal structures of PPARα (pink worm), PPARγ (yellow worm), and PPARδ (cyan worm). Each PPAR is complexed to a high-affinity ligand (not shown). PPARα and PPARγ are complexed to LXXLL peptides (purple worms). For each PPAR, the solvent-accessible ligand binding pocket is displayed as an off-white surface.

**Figure RL27:** $\sigma$A-weighted Fo-Fc electron density omit map calculated around AZ 242. Residues forming hydrophobic contacts with AZ 242 are represented in green, and the residue forming the hydrogen bonds with the propionic head group are represented in blue.
2.1.13. Insight of structure, activity and side effects of PPAR agonists (Synthetic ligands)

Many synthetic PPAR ligands have been reported in the literature since the discovery of PPARs. Data from a number of crystallographic studies exploring these compounds reveal a fairly clear view of the agonist binding mode in the LBD. Nearly all the compounds conformed to a three-module structure, with a binder group involved in a series of hydrogen bonds in front of the AF-2, a linker mainly arranged around central helix 3 and an effector end occupying the large cavity of the binding site (Figure RL 28).

2.1.13.1. PPARα Agonists

♦ Fibrates

PPARα is the predominant target for fibrates, a class of drugs well-established in the therapy of dyslipidemia and hypertriglyceridemia. The hypolipidemic fibrate drugs are currently used in clinical practice for treatment of dyslipidemia (in particular, low HDL cholesterol and elevated triglyceride levels) and for reducing cardiovascular risk. In 1962, Thorp and Waring showed that clofibrate (1) (Figure I11), the ethyl ester of α-parachlorophenoxyisobutyric acid, among a series of α-aryloxyisobutyric acid derivatives, was most effective at decreasing the concentration of cholesterol and other lipids in rat plasma and liver. In 1965, 1 became the first PPARα agonist (EC$_{50}$ = 55 μM) to be used in clinical therapy in the treatment of dyslipidemia. However, it was withdrawn from the market because of severe adverse effects, as it was shown to increase mortality among treated patients. In an attempt to improve the pharmacological profile of clofibrate, some analogues were synthesized and biologically evaluated. These compounds were referred to as “second generation fibrates” and include fenofibrate (2), bezafibrate (3) (Figure I11), ciprofibrate (17), and the dimethylphenoxypentanoic acid derivative gemfibrozil (18) (Figure RL 29). These compounds increased the synthesis of lipoprotein lipase, thereby increasing clearance of triglycerides; however, they showed only modest selectivity over the other PPAR subtypes. Furthermore, the structures of these compounds were relatively small compared to the wide pocket they occupied, fostering the development of a newer generation of fibrate analogues. Attemps to identify more potent compounds led to the synthesis of a series of ureidofibrates that were
active at lower doses in rodent models of hyperlipidemia. The first reported examples of selective PPARα agonists were ureido-based fibric acids 19 (GW9578) and 20 (GW7647) with their respective EC50 values for human PPARα being 6 and 50 nM, respectively. α,α-Dimethylcarboxylic acids 19 and 20 showed 20-fold and 200-fold selectivity, respectively, compared to PPARγ and PPARβ/δ and possessed potent lipid-lowering activities in a cholesterol/cholic acid fed rat assay compared to fenofibrate. Researchers at Lilly also reported their phenoxyphenylpropanoic acid 21 (LY518674) to be a potent PPARα agonist with 200-fold binding selectivity compared to PPARγ and PPARβ/δ receptors. In order to develop PPARα agonists more potent and selective than the fibrate class, Sierra et al. modified their selective PPARβ/δ agonist (GW501516) (structure not disclosed) to incorporate the 2-aryl-2-methylpropionic acid group of fibrates, which led to compound 22 (GW590735), shown to be a potent and selective PPARα agonist with EC50 = 4 nM on PPARα and at least 500-fold selectivity versus others subtypes. This compound offered the potential for significantly improving therapeutic benefits compared to the fibrates in dyslipidemia and hypertriglyceridemia. Meyer et al. reported another PPARα agonist, 23 (K111), which differed from the classical fibrate class by the long chain alkyl moiety, α-dichlorododecanoic acid.
**Figure RL29:** Fibrate class of PPARα agonists (synthetic ligands)

Compound 23 was an insulin sensitizer with PPARα selectivity compared to PPARγ and PPARβ/δ. This compound led to significantly decreased body weight and improved hyperinsulinemia, insulin sensitivity, hypertriglyceridemia, and HDL cholesterol levels, without adipogenesis or significant effects upon fasting glucose, 24 h urine glucose excretion, systolic and diastolic blood pressure, plasma fibrinogen, total cholesterol, and chemistry and hematology profiles. These benefits were similar to the health-improving effects of caloric restriction, providing preliminary evidence that 23 has excellent potential as a calorie-restricting mimetic agent. The results also showed that it is a potent antidiabetic and hypolipidemic drug in nonhuman primates. More recently, 24 (CP-900691), with a phenylpiperidine carbamate moiety, has been reported to be a highly selective PPARα agonist in T2DM monkeys, with favorable effects upon dyslipidemia as well as upon glycemic control, body weight, and inflammation. Indeed, treatment of diabetic monkeys with 24 has been shown to increase HDL cholesterol and apoA1, reduce plasma triglycerides and apoB, improve the lipoprotein index, decrease body weight and CRP, and increase adiponectin. Li et al. reported hybrid compounds obtained by a combination of the classical fibrate “headgroup”, a linker with the appropriate length, and a chalcone known for over a century to have advantageous biological activities, including antioxidant, anti-inflammatory, anticancer, and antinfective properties. Compound 25 (DE027) was described as being the most prominent, with EC50 = 60 nM. This compound was identified using a virtual screening approach based on the crystal structure of PPARα. Compound 25 has provided a promising novel family of chalcones with a potential hypolipidemic effect.
Non-Fibrates
Using a cell-based transactivation assay, 26 (WY14643), a non-fibrate selective PPARα agonist with a thioether moiety and a pyrimidine linker part, was identified as being a micromolar activator of murine PPARα (Figure RL30). The Kyorin group reported a highly potent human PPARα-selective agonist, 27 (KCL). This compound is a non-fibrate phenylpropanoic acid derivative that is a potent human PPARα agonist in vitro, possessing selectivity for PPARα compared to the other subtypes.412 Human PPARα selectivity of 14 is mainly due to a specific interaction between the hydrophobic tail part of 27 (the 4-trifluoromethyl group) and the key amino acid Ile272 located on the helix 3 region of human PPARα LBD. Compound 27 (Figure RL30) activates human and rat PPARα with EC50 of 0.06 and 5.2 μM, respectively. Thus, the transactivation activity of KCL for PPARα is approximately 100-fold less potent in rats than in humans. NNC 61-4655,413 a phenylpropargyl derivative, is a nonselective but PPARα-prefering potent PPAR agonist with excellent pharmacokinetic properties; it has been shown to have more efficacious in vivo effects in male db/db mice than PPARγ selective agonists such as rosiglitazone and pioglitazone. Kuwabara et al. reported a highly potent PPARα agonist, NS-220,414 a dioxanecarboxylic acid derivative that presents PPARα agonist activity 1000 times more potent than fibrates, suggesting that the dioxanecarboxylic acid moiety is responsible for its high potency and selectivity for the PPARα subtype. This compound led to amelioration of metabolic disorders in type 2 diabetic mice. A series of 2,3-dihydrobenzofuran-2-carboxylic acids have been described that led to development of a novel class of PPARα agonists,415 represented by compound 28 (Figure RL30), which displays the best selectivity among PPARα selective agonists reported to date. Indeed, in animal models, it displays very high potency (EC50 < 10 nM) and subtype selectivity (>1000-fold), as well as highly potent and effective hypolipidemic activity. It is believed that the inherent selectivity of this class of compounds is primarily due to conformational constraints rendered by the structurally unique 2,3-dihydrobenzofuran ring. In male Syrian hamsters, 24 h drug exposure to 28 and fenofibrate administered in a series was measured following the final dose of each compound. It was found that 28 required only about 1/500 of the exposure to fenofibrate to achieve comparable lipid-lowering efficacy, suggesting that the in vivo efficacy of these two compounds is closely correlated with their potency as PPARα agonists in vitro. Researchers at Lilly also reported a tetrazole ring binder, LY-171883, a leukotriene D4 antagonist that induces peroxisome
proliferation in rodent liver.\textsuperscript{416} Like many peroxisome-proliferating agents, it causes transient lipid accumulation along with other changes in hepatic lipid metabolism. Study of the effect of \textit{LY-171883} on lipid metabolism revealed that it increased triglycerides in liver, associated with a 3-fold increase in fatty acid synthetase. Some sulfamide-derived analogues of OEA with potent selective binding affinity for PPAR\(\alpha\) have been reported and have led to an N-octadecyl-N\(^{\prime}\)-propylsulfamide (29). This compound has been shown to induce satiety, reduce food intake in rats, reduce body weight, and act as a lipid-lowering agent.\textsuperscript{417} On the basis of the structure of another famous natural compound, resveratrol, which showed a beneficial effect at lowering the risk of cardiovascular diseases in animal models, pterostilbene (30) and its phosphate derivative (31)\textsuperscript{418} (Figure RL30) have been reported, with the trans conformation being more active than cis analogues. Compound 31 is the most active compound in this series, with decreased cholesterol levels in animal models and higher potency than ciprofibrate. Moreover, reports of interest in the research of developing PPAR\(\alpha\) antagonists can also be found in the literature.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Glitazone class of PPAR\(\gamma\) agonists (synthetic ligands)}
\end{figure}

\subsection*{2.1.13.2. \textit{PPAR}\(\gamma\) Agonists}

\begin{itemize}
\item Glitazones
\end{itemize}
Following the discovery of PPARγ, the first class of synthetic ligands to specifically bind it consisted of thiazolidine-2,4-diones (TZDs) or glitazones. TZDs, introduced on the market in the past 10 years, constitute the first class of insulin sensitizers. Their antidiabetic action occurs through improvement in hyperglycemia and hyperinsulinemia and lowering of insulin resistance. Since the pioneering discovery by scientists at the Takeda Pharmaceuticals Company of ciglitazone (32), which effectively reduces insulin resistance by potentiating insulin action in genetically diabetic and/or obese animals, several new TZDs have been developed. Troglitazone (33) was derived from 32 by replacing the lipophilic tail (methylcyclohexyl methyl ether moiety) with a vitamin E residue. Pioglitazone (4) and rosiglitazone (5) were developed by modifications based on the metabolites of 32. These compounds possess a pyridyl tail group. Some modifications were made with physical cyclization of the linker; indeed, rigidification of the ethoxy in a chromane structure led to englitazone (34). This had the effect, however, of increasing the complexity of the compound because of the introduction of a chiral center, probably dooming an otherwise interesting possibility. It is noteworthy, however, that 34, which lack a large effector module, were shown to have less potency than 4 and 5. Three of these TZDs have been marketed: 33, withdrawn from clinical use in 1997 because of its severe hepatotoxicity; 4 and 5, currently used as third-intention drugs in treatment of type 2 diabetes. The hepatotoxicity of 33 did not seem to apply to the other glitazones. SAR studies of this family have shown the effectiveness of the methylene moiety as a linker between benzene and the TZD ring. Moreover, the 4-oxylbenzyl group appears to be essential for enabling the compound to exert favorable hypoglycemic and hypolipidemic activities. However, replacement of the thiazolidine-2,4-dione ring by an oxazolidine-2,4-dione ring or a 1-oxa-2,4-diazolidine-3,5-dione ring led to a decrease in activity, while other heterocycles, such as rhodamine, hydantoin, thiohydantoin, and 2-thioxo-5-thiazolidinedione, resulted in complete loss of activity. Nevertheless, because of their ability to induce gene expression in adipocytes and to enhance adipocyte differentiation, TZDs induced weight gain in often already obese patients as a mechanism-related class effect. In addition, other undesirable side effects such as fluid retention, hemodilution, potent cardiac hypertrophy, liver toxicity, and an increased risk of cardiovascular-related death reported in preclinical and clinical studies emphasize the need for safer insulin sensitizers to treat metabolic syndrome and associated complications. Thus, there has been considerable interest in designing novel PPARγ-modulating drugs that retain efficacious insulin-sensitizing properties while
minimizing potential adverse side effects, leading to conventional chemistry-led pharmacomodulation studies to improve the glitazones. Indeed, in order to synthesize novel TZDs with better safety and efficacy, a second generation of glitazones has been developed, focusing most intensively on the tail part. The two glitazones that are still marketed share a common trait: their effector module is fairly small compared to the large pocket in which it is bound. In fact, the binder part is common to the entire class and the linker has also been well conserved, having been modified only in the most recent glitazones.

Indeed, it was the tail group that bestowed upon each of the glitazones their originality in chemical terms but also their specific biological activity. This module is the most probable candidate for explaining differences in affinity and potency between very similar glitazones, thus entitling it to the status of effector module of the compound. While maintaining a TZD moiety as the binder group, several attempts were made to modify the linker module as well as the effector. The effector module of 28, the N-methyl-2-pyridyl moiety, was first modified with different heterocycles. Replacement by a 4-oxazolyl moiety led to 35, which exhibited potent hypoglycemic and hypolipidemic activities and displayed the same biological profile as 32. Compound 36 (DRF-2189), an indole-based tail group, was also reported to be analogous to 5. Pharmacological studies showed equal potency for 36 and 5. The plane shape of indole appears to be important for its activity. As a potential backup for 5, 37 (BRL-48482) was developed in the same family, differing from its predecessors by its slightly larger effector module, a bicyclic benzoxazole. More drastic modifications in the effector module were also carried out: for example, its replacement by a dithiolane, leading to compound 38. An in silico docking study of this compound was carried out to elucidate its satisfactory activity; it showed a supplementary hydrogen bond with Ser-432 at its effector amide carboxyl, reinforcing hydrophobic contacts formed by the dithiolane cycle. When compared with biological data available for the compounds, docking results led to the hypothesis that the position of the effector in the pocket, either above the plane of the linker (in the upper arm of the Y-shaped pocket) or below it (in the lower arm), was a key to the affinity and probably functional activity of the ligands. Compound 38 was developed as a derivative of potent antioxidant vitamin α-lipoic acid and has been shown to be a potent PPARγ agonist, with improved insulin sensitivity and reduced triglyceride levels in Zucker rats. These new compounds may prove to be efficacious in the treatment not only of type 2 diabetes but also of atherosclerosis and prevention of vascular restenosis and inflammatory skin
diseases. Rivoglitazone presents a benzimidazoly tail.\textsuperscript{428} It was reported to be approximately 3 times more active than \textit{5} in a cell-based PPAR\(\gamma\) transfection assay but had little effect on PPAR\(\alpha\) and PPAR\(\delta\) activity in luciferase reporter assays. In vivo, it was found to be more potent than \textit{5}, which can be explained not only by enhanced in vitro activity but also by a longer half-life. Daiichi Sankyo reported that a phase 3 clinical trial was discontinued in May 2009, but the drug was being reused in phase 2 to treat xerophthalmia. \textit{39 (BM13.1258)} and \textit{40 (BM15.2054)} possess a benzothiophene ring as a linker. They are potent PPAR\(\gamma\) activators in transient assays in vitro. Considerable evidence indicates that the therapeutic effects of TZD are mediated via activation of PPAR\(\gamma\), resulting in insulin sensitization and improved glucose metabolism. The results of that study did not challenge this hypothesis but suggested that \textit{39} and \textit{40} may, in addition, affect muscle glucose metabolism via other biochemical pathways.\textsuperscript{429} That conclusion was supported by the absence of a correlation between chronic oral effects of \textit{39} and \textit{40} on glycogenic and glycolytic fluxes and by acute insulin-dependent catabolic stimulation of glucose metabolism in vitro. Maintenance of the 4-oxazolyl moiety as the effector module and modification of the 4-ethoxybenzyl linker with a phenone moiety led to darglitazone, which showed better oral potency than \textit{4} or \textit{5}. However, that compound caused dramatic effects upon white and brown adipose tissue and unexpected morbidity/mortality in rats and monkeys.\textsuperscript{430} Efatutazone, with a similar scaffold as that of Rivoglitazone where the methoxy group of present in the lipophilic tail of Rivoglitazone has been replaced by a 3,5-dimethyl 4-amino phenoxy group; very potently activates PPAR\(\gamma\)-mediated transcription, more than 40-fold that of \textit{5}, and demonstrated high selectivity for PPAR\(\gamma\) within the PPAR family. To date, several reports have shown preclinical antitumor activity for PPAR\(\gamma\) agonists in certain models, including carcinogenesis and xenograft models. However, pilot clinical trials in cancer patients using PPAR\(\gamma\) agonists have been inconclusive. The antitumor activity of efatutazone has been investigated and was demonstrated in vitro and in vivo.\textsuperscript{431}

A series of antidiabetic unsaturated TZDs have also been reported in order to develop agonists without a stereogenic center at C-5 of TZD and have led to compounds \textit{41} and \textit{42 (Figure RL32)}.\textsuperscript{432} Surprisingly, these compounds show little or no in vitro activity toward PPAR\(\gamma\) but more effective euglycemic and in vivo triglyceride-lowering activities than \textit{33}. These observations raise the possibility that some TZDs mediate their antidiabetic activity via mechanisms other than PPAR\(\gamma\), although activation of PPAR by
metabolites of these drugs cannot be ruled out. Furthermore, these unsaturated compounds have a better pharmacological profile than their saturated analogues. More recently, a new series of glitazones with a different scaffold and promising levels of glucose uptake activity have been described. The highest activity was shown for compound 43, which possesses a 1,3-oxazolidin-2,4-dione headgroup connected to the linker part, with a double bond and an anisidine tail part. This compound displays satisfactory antihyperglycemic activity in the presence of insulin.

![Figure RL32: Glitazone class of PPARγ agonists (synthetic ligands)](image)

However, the TZD ligands contain a stereogenic center at C-5 of their headgroup, and one drawback of this class of compounds is the rapid racemization of the resident chiral center under physiological conditions, leading to development of the TZDs as racemates. By use of a PPARγ binding assay, it has been shown that only the (S)-enantiomers of the TZDs bind to the receptor with high affinity. In fact, the antidiabetic activity of 5 in humans is only due to the (S)-(−)-enantiomer. This suggests that only 50% of the drug substance in the currently approved TZDs binds to the target receptor while 50% of the drug substance is inactive but can be converted to the active enantiomer if not first metabolized.

♦ Non-Glitzazones

To overcome this problem, several groups have identified acyclic head groups that are less prone to racemization. The acidic functionality of the TZD ring is considered essential for its binding to PPARγ, so some PPARγ agonists have been developed as bioisosteres of the TZD ring, conserving acidic properties by replacement of this ring with acyclic structures such as carboxylated hydroxyureas, α-heteroatom- or α-carbon-substituted carboxylic acids, and 1,3-dicarbonyl compounds. The first acyclic non-
carboxylic acid derivative was compound 44, a carboxylated hydroxyurea able to normalize the blood glucose level in an in vivo study (Figure RL33). The α-heteroatom-substituted-β-phenylpropionic acid series was based on replacement of the TZD moiety of insulin sensitizer 37 by α-heteroatom-substituted-β-phenylpropionic acids and maintenance of the 4-oxazolyl effector module. These compounds, typified by α-ethoxy acid 45 (SB-213068), have been shown to be potent PPARγ agonists. In contrast to the TZDs, α-benzyloxy acids do not undergo racemization in vivo. Within this series, the (S)-enantiomers were shown to have higher binding affinity for PPARγ and were more potent than their (R) counterparts in adipocyte differentiation assays. Compound 45 is one of the most potent antihyperglycemic agents yet reported. It was assumed that the role of acidic TZD was played by carboxylic acid in these compounds and that an appropriate substituent at the α-position could alter the chemical environment around the carboxylic acid in such a way that the entire group would mimic the TZD ring. Some benzofuran non-TZD derivatives have also been described, based on incorporation of oxygen into a benzofurane ring, as described above for englitazone but without creating a stereocenter. These modifications led to compounds 46 and 47 which possess α-alkoxy and α-thioether carboxylic acid groups, respectively. For both compounds, the effect of stereochemistry at the α-position of the carboxylic acid group was examined, and it was shown that the activity resided entirely within the (S)-enantiomers. These two compounds were shown to stimulate glucose uptake at low nanomolar concentrations in adipocyte cell lines. Some acetic acid derivatives bearing a benzoxazinone core have been synthesized and have led to compounds 48 and 49. Substitution on the amide of the benzoxazinone ring by lipophilic aliphatic chains enhanced receptor activation while generally maintaining bioavailability and resistance to oxidative metabolism. It should be noted that these compounds have an (R) absolute configuration, and show potent efficacy in vivo in a db/db mouse model of type 2 diabetes. An aminomethyl cinnamate (AMC) series of PPARγ agonists is represented by compound 50 (Figure RL33), which possesses a large linear effector module, a biphenyl-4-oxazolyl moiety. The α-heteroatom of the linker was moved to the ortho position of the aromatic ring, eliminating the epimerizable stereocenter and producing highly selective PPARγ compounds. With this large linear effector moiety, further investigation led to the discovery of tetrahydroisoquinolines (THQs) represented by compound 51, a non-TZD.
This compound exhibits potent PPARγ affinity for the isolated receptor and in cellular assays while showing good selectivity against PPARα. Compound 51 has an excellent in vivo profile in db/db and ZDF models of type 2 diabetes and also possesses desirable pharmacokinetic properties. Lin et al. recently reported new fibrate derivatives 441 52 and 53 with potent selective PPARγ agonist activities. These compounds were synthesized based on structural modification of a previously described indole fibrate derivative dual PPARα/γ agonist in which indole was replaced by a tetrahydroquinoline core. X-ray cocrystal analyses revealed that selective PPARγ agonist activity is conferred by the tail part building block 4-phenylbenzophenone. Cell-based reporter assays of two enantiomeric fibrate-like derivatives showed that (R)-enantiomer 54 is a full agonist of PPARγ whereas the (S)-enantiomer is a less potent partial agonist. 442 Analysis of the
Crystal structures of the PPARγ ligand binding domain, complexed with the (R)- and the (S)-enantiomers, respectively, showed that the differing degree of stabilization of helix 12 induced by the ligand determined its behavior as a full or partial agonist. Other structurally diverse PPARγ agonists have been described in the literature. Henke et al. reported selective PPARγ agonists in a series of indole-5-carboxylic acids represented by 55 (GW0207). This compound had in vitro potency toward PPARγ similar to or better than that of the three currently marketed thiazolidinedione antidiabetic agents, along with an excellent pharmacokinetic profile in rats. Sauerberg et al. studied the concept of dimeric ligands in the design of new PPAR agonists. A dimeric ligand with a common group 56 or a full dimeric ligand 57 (Figure RL33) gave PPARγ agonists with retained or increased potency compared to 5 (Figure I12). The dimeric agonists altered the PPAR subtype profile compared to their monomeric counterparts, suggesting that the dimeric design concept can be used to finetune the subtype selectivity of PPAR agonists.

![Chemical structures](image)

**Figure RL34:** L-Tyrosine analogs (PPARγ synthetic ligands)

**L-Tyrosine Analogs**

An alternative to α-heteroatom-substituted β-phenylpropanoic acids was found in the development of L-tyrosine analogues (Figure RL34). Farglitazar (58) was the first example of the binding mode of the second large class of PPARγ agonists, the glitazars. Compared with 5, 58 was primarily characterized by its large binder module, an N-(2-benzoylphenyl)-L-tyrosine rather than a TZD. It nevertheless shared the same binding mode, forming hydrogen bonds with both His-323 and Ser-289, and Tyr-473 with its acid moiety. TZD and carboxylic acid moieties of 28 and 56, respectively, were
imperative for AF-2 helix interaction. Although these compounds have an asymmetric center, the (S)-enantiomers have been shown to possess greater binding affinity and functional activity at PPARγ than the corresponding (R)-enantiomers and are conveniently synthesized from naturally occurring L-tyrosine. Unlike the TZDs, these tyrosine-based compounds do not undergo racemization in vivo. The X-ray crystal structure of 58 bound to PPARγ showed that the benzophenone group was inserted into a lipophilic pocket while the tyrosine nitrogen and the benzophenone carbonyl formed an intramolecular hydrogen bond. This intramolecular hydrogen bond reduces the basicity and polarity of the tyrosine amino group. Compound 58 leads to potent reduction in glucose activity, reduction of triglycerides, and a rise in HDL cholesterol in diabetic patients. The positive lipid effects of 58 may be due to residual PPARα activity in the compound. However, it failed to pass phase 3 of clinical trials because of the emergence of adverse effects such as peripheral edema. Other tyrosine-based PPARγ agonists have been developed, exemplified by 59 (GW1929) and 60 (GW7845). This series also contains some of the most potent PPARγ agonists reported to date, with a number of analogues having subnanomolar activity toward human PPARγ. In addition, these compounds showed >1000-fold selectivity for PPARγ over the PPARα and PPARβ/δ subtypes in cell-based transactivation assays. Compound 59 (Figure RL34) demonstrated antihyperglycemic activity equipotent to that of troglitazone at >100-fold lower plasma concentrations in ZDF rats, which paralleled their differences in PPARγ binding and activation. Still other L-tyrosine analogues were developed, with alternative N-substituents that added a small lipophilic substituent while mimicking some of the effects of the intramolecular hydrogen bond present in the benzophenone analogue, leading to pyrrole derivative 61 (Figure RL34). This compound incorporates a pyrrole as a low molecular weight N-substituent, and its low basicity may be the key to its potent PPARγ activity. Unfortunately, as for the TZD class and despite excellent potency, several compounds from the L-tyrosine analogue class present unwanted therapeutic profiles marked by fluid retention, weight gain, and potential cardiac hypertrophy.

Figure RL35: 1,3-Dicarbonyl analogs (PPARγ synthetic ligands)
1,3-Dicarbonyl Analogs
Replacement of the TZD ring by a noncyclic 1,3-dicarboxyl moiety led to compounds 62 and 63\(^{449}\) based on replacement of the 2-phenyloxazole of 60 by a 2-(3H)-benzothiazolone (Figure RL35). These compounds show interesting insulin-sensitizing and hypoglycemic activities, with levels of glucose and triglyceride correction comparable to those of 5 in ob/ob mouse studies.

![Figure RL36: Other PPARγ synthetic ligands](image)

Other Classes of ligands
Several compounds have been described as PPARγ agonists without belonging to the classical three-module structure ligands previously described. Thus, some N-sulfonyl-2-indolecarboxamide compounds derived from indole-derived PPARγ binding agents were reported\(^ {450}\) and led to identification of 64 (Figure RL36). Several of these compounds were found to stimulate osteoblast differentiation in a cell-based assay, thus suggesting potential applications in treatment of osteoporosis. Some indenone derivatives, possessing a simple structure containing neither TZD nor the carboxylic acid moiety, were synthesized and led to the discovery of compound 65\(^ {451}\) which displays the most active agonistic activity in this series and a new binding mode in the X-ray cocystal structure. In contrast to 5 (Figure I12), the indenone moiety of 65 occupies the base part of the PPARγ LBD pocket and the phenylpropyl group stretches deeply into the upper part of the cavity. The indenones seem to be a novel and interesting chemical class that might be further optimized for treatment of type 2 diabetes. Derivatives of the nonsteroidal anti-inflammatory drug (NSAID) sulindac sulphide were recently described as potential selective agonists of PPARγ, without the COX inhibitory activity of the parent NSAID; they have led to compound 66\(^ {452}\) equipotent to 5. Compound 66 (Figure RL36) induces expression of a liver fatty acid binding protein (L-FABP) and an adipocyte fatty acid
binding protein (aP2), two established PPARγ target genes. Taken together, these compounds represent potential leads in the development of novel PPARγ agonists.

While the clinical benefits of PPARγ agonists in treating metabolic disorders have been clearly demonstrated, the current generation of glitazone drugs has been associated with undesirable side effects. Thus, there has been significant interest in the design of novel PPARγ-modulating drugs. A moderate reduction in PPARγ activity has been shown to prevent insulin resistance and obesity induced by a high-fat (HF) diet. Therefore, appropriate functional modulating of PPARγ activity could be a logical approach to protection against obesity and related diseases such as type 2 diabetes. For this reason, efforts are being made to identify partial agonists. Genetic and pharmacologic studies suggested that moderate activation of PPARγ can lead to better therapeutic outcome than activation of the receptor with a high-affinity, potent full agonist. Partial agonists are defined as weak activators of PPARγ that elicit the same activation pattern and show linked dose–response curves but with lower maximal activity compared to full agonists. They might induce alternative receptor conformation and thus recruit different coactivators, resulting in distinct transcriptional effects compared to classical glitazones.

PPARγ antagonists appear to have effects similar to those of PPARγ agonists in rodents, that is, generation of small adipocytes and attenuated secretion of adipokines that interfere with insulin signaling. Consequently, not only PPARγ agonists but also PPARγ antagonists could be of potential clinical use, as such compounds would increase insulin sensitivity while preventing adipogenesis and obesity; thus, in contrast to TZD, they would not induce weight gain. A moderate reduction in PPARγ activation with a PPARγ antagonist was shown to decrease triglyceride (TG) content in white adipose tissue, skeletal muscle, and liver. These inhibitors were shown to ameliorate high-fat-diet-induced obesity and insulin resistance.

### 2.1.13.3. Dual PPAR Agonists

The development of a single compound displaying multitarget capacities would provide enhancement of efficacy and/or improvement in safety compared to the present one-drug–one-target methods. Potent agonists with mixed receptor selectivity toward PPARα and PPARγ, also called dual PPARα/γ agonists, were thought to have significant potential...
as novel antidiabetic agents. In recent years, dual PPARδ/γ, PPARα/δ, and pan-PPAR α/δ/γ agonists have also emerged.

2.1.13.3.1. Dual PPARα/γ Agonists

Given the importance of controlling both glucose and lipid levels in type 2 diabetes, the concept of identifying ligands that bind to and activate both PPARα and PPARγ represents a logical continuation in the field of PPAR research. PPARγ agonists are associated with improved insulin sensitivity and glucose tolerance but at the cost of increased weight gain and other side effects, including liver dysfunction and bone loss. The fibrates have shown specific efficacy in reducing angiographic progression of coronary heart disease in type 2 diabetes mellitus, and this effect is most likely related to correction of atherogenic dyslipidemia. In addition to their beneficial effects upon lipid metabolism, there were reports in the literature that fibrates reduced body weight gain in rodents without affecting food intake, leading to the hope that activation of PPARα would mitigate the weight gain induced by PPARγ activation seen in humans. The hypothesis that PPARα/γ dual agonism could provide additive and possibly synergistic pharmacology has resulted in an intensive effort by the pharmaceutical industry to develop and evaluate these agents. Dual activation of PPARα and PPARγ could, in theory, also limit the occurrence of side effects associated with TZD therapy. Thus, combined PPARα/γ activation has emerged as an interesting concept and spawned the development of various coagonists. A number of PPARα/γ dual agonists have been reported in this class of compounds and have shown robust insulin-sensitizing and hypolipidemic activities in clinical trials.

Figure RL37: TZD analogs (dual PPARα/γ synthetic ligands)
TZD Derivatives

The Kyorin Pharmaceutical Company modified the linker module of classical glitazone in an SAR study centered around a previous hit. They achieved an interesting result with 6 (KRP-297) (Figure I14), which showed similar affinity for PPARγ and PPARα. In vivo, 6 was reported to improve abnormal lipid metabolism in liver and to elicit hypoglycemic, hypoinsulinemic, and hypolipidemic effects in obese rats. However, further development of 6 was terminated in 2003 because of findings of a rare malignant tumor in mice.

Another modification consisted of including nitrogen in a lactone cycle, leading to 67 (DRF-2519). This benzoazininone analogue of TZD more satisfactorily improved hyperglycemia, hyperinsulinemia, abnormal lipid metabolism, and hypertension than PPARα or PPARγ selective ligands. Indeed, in vivo studies showed that 119 possessed better efficacy than both 5 (Figure I12) and 6 (Figure I14). 67 (Figure RL37) might be beneficial for a whole range of complications associated with type 2 diabetes. A series of 5-arylthiazolidine-2,4-diones were identified as belonging to a class of dual PPARα/γ agonists. Changing the point of attachment of the thiazolidine-2,4-dione ring on the phenyl ring from a para- to a meta-orientation with respect to the three-carbon methylene tether, as was the case for compound 68 (Figure RL37), transformed PPARγ selective agonists into dual PPARα/γ agonists. This compound has shown better efficacy than 5 in the db/db mouse model of type 2 diabetes and presents satisfactory pharmacokinetic parameters. Bioisosteric replacement of the corresponding TZD ring with an oxazolidine-2,4-dione and structural modification of the aryloxy substituent with a cyclohexylphenyl tail group led to compound 69. This compound was found to be equal in both binding affinity and functional potency to the corresponding TZD analogue 68. Compound 70 (JTT-501), an isoxazolidine-3,5-dione, is less potent than its parent 35 (Figure RL31), a TZD with a phenyl oxazolidine tail group. However, the side effects profile is improved. It was found that 70 is a PPARγ agonist with some PPARα activity. This compound has been reported to activate PPARα at concentrations approximately 10-fold higher than those required for activation of PPARγ. Its activity is probably mediated through a malonic amide metabolite by hydrolysis of the binder heterocyclic ring. Compound 70 (Figure RL37) improves hyperglycemia, hyperinsulinemia, and hypertriglyceridemia and enhances insulin-stimulated glucose oxidation in adipose tissues in noninsulin-dependent
diabetes mellitus models. In particular, the triglyceride-lowering activity of 70 is a unique characteristic compared to thiazolidinedione counterparts.456

**α-Alkoxy/aryloxy-β-phenylpropionic Acid Derivatives**

The first alkoxypropionic acid to display dual PPARα/γ agonist activities was described in 2001 and called tesaglitazar (7) (Figure I14), developed by AstraZeneca. The crystal structures of PPARα and PPARγ in complex with 7 revealed a conserved hydrogen bonding network involving a Tyr in the AF2 helix that had to be formed in order to stabilize LBD in the active conformation throughout the entire PPAR family. Compound 7 monotherapy was reported to improve markers of glycemic control and atherogenic dyslipidemia at doses of 0.5 and 1 mg daily in subjects with manifestations of insulin resistance or type 2 diabetes.462 A phase 3 program was conducted to examine the effects of 7 when given to patients with type 2 diabetes. However, in 2006, its development was discontinued in phase 3 of clinical trials after the emergence of several adverse effects, elevated serum creatinine and associated decreases in the glomerular filtration rate.463

Scientists at Dr. Reddy’s Research Foundation also came out with a series of α-ethoxy-β-phenylpropionic acid derivatives exemplified by ragaglitazar (8) (Figure I14), which contains a phenoxazine group as the lipophilic tail portion of the molecule.464 Compound 8, by virtue of its dual PPARα- and PPARγ-activating property, acted on both the liver and adipose tissue and thereby shows greater improvement not only of hyperglycemia and hyperinsulinemia but also of abnormal lipid metabolism than marketed PPARα- or PPARγ-selective agonists. Despite demonstrating advantages in preclinical models, 8 was suspended from clinical studies because of development of bladder tumors in rodents.465 To further understand the SAR generated in this series of 124 derivatives, additional tricyclic analogues were designed and synthesized and led to carbazole 9 (Figure I14),466 which has a better dual PPARα/PPARγ activity profile than 8.
Naveglitazar (71) (Figure RL38) was a γ-dominant PPAR α/γ dual agonist somewhat like farglitazar. This compound showed significant reduction in mean fasting serum glucose levels and triglyceride levels. It was selected for clinical evaluation in the treatment of type 2 diabetes. Cardiac effects in rats treated with 71 were similar to those reported for other PPARγ agonists and α/γ dual agonists. Furthermore, treatment of rats with 71 was associated with an increased incidence of sarcomas in males and urothelial tumors in females, thus leading to discontinuation of this compound in clinical studies.\(^{467}\)

Modification of the linker of 71, by replacing the oxygen directly bound to the central aryl ring with an alkyne moiety, provided compound 72 (LSN862). The alkyne linker did not affect the PPARα/γ selectivity profile and was still flexible enough to be adapted to both receptors. This compound was reported to be a high-affinity PPARγ partial agonist with relatively weaker but still significant PPARα agonist activity.\(^{468}\) Compound 73 (E3030), a calcium salt of α-isopropyloxy-β-phenylpropionic acid, was reported to be a potent dual activator of PPARα and PPARγ in animal models. Experimental results suggested that this compound had potential for use in treatment of various types of metabolic dysfunction in type 2 diabetes, including dyslipidemia, hyperglycemia, hyperinsulinemia, and impaired glucose disposal.\(^{469}\) The Dong-A Pharmaceutical Company characterized the pharmacological profile of 74 (PAR-5359), reported to be a well-balanced coagonist of PPARα and PPARγ and which demonstrated excellent antihyperglycemic and hypolipidemic activities. However, the pharmaceutical group decided not to enter into clinical development of 74 because of failure to guarantee a sufficient safety margin based on long-term toxicological studies.\(^{470}\) Compound 12

Figure RL38: α-alkoxy β-phenylpropionic acid derivatives (dual PPARα/γ synthetic ligands)
(LY510929) (Figure 114), an α- aryloxy-α-methylhydrocinnamic acid, possessed potent dual PPARα/γ activity. In vivo studies showed that this compound improved insulin sensitivity and potently reversed diabetic hyperglycemia while significantly improving overall lipid homeostasis. Clinical trials were stopped because of severe side effects. Treatment with suprapharmacologic doses of 12 caused dose-dependent increases in heart weight in male and female rats, characterized by increased left ventricular lumen volume and wall thickness. Aleglitazar (75) (Figure RL38), a dual PPARα/γ agonist developed by Hoffman-LaRoche, is currently in phase 3 of clinical trials. A phase 2 trial showed that therapy with this agent reduced hyperglycemia and favorably modified levels of HDL-C and triglycerides, with an acceptable safety profile. Compound 75 is currently being studied in large-scale clinical trials to assess whether it reduces the risk of major cardiovascular end points (death, myocardial infarction, or stroke) among patients with diabetes and coronary artery disease. If ongoing studies confirm the theoretical benefits and safety of dual PPARα/γ agonism, 75 may become the first therapy demonstrated to reduce macrovascular complications in patients with diabetes.472

♦ Fibrate Derivatives

A series of fibrates endowed with dual agonism of PPARα and -γ have also been reported. Some fibrate analogues, clofibrac acid and fenofibrac acid, the active metabolites of clofibrate and fenofibrate, were shown to be dual PPARα/γ activators with 10-fold selectivity for PPARα.456 Thus successful studies were carried out to derive potent dual PPARα/γ agonists from PPARα selective fibrates.

Figure RL39: Fibrate derivatives (dual PPARα/γ synthetic ligands)
In an indole-based series of PPAR agonists, Mahindroo et al. reported a new fibrate derivative, 76 (Figure RL39), a dual PPARα/γ agonist. In this compound, the fibrate acid group is attached to the 5-position of indole and the N-terminal is attached via a threecarbon linker to the tail part hydrophobic naphthophenone moiety. In research on new PPARα selective agonists, some ureidofibrates have been described. One of them, 77 (GW2331), binds to both PPARα and PPARγ with high affinity in the nanomolar range. Starting from a selective PPARα agonist, pyrazole 78 (Figure RL39) has been generated with equipotent PPARα/γ dual activities. 473 Compound 78 demonstrates in vivo activity toward Zucker fatty rats and has also been tested in a rat 7-day toxicological study. No safety issues have been raised that would preclude further development. The 2-aryloxy-2-methylpropionic acid derivative 79 (Figure RL39) was identified as a PPARα/γ dual agonist with relative PPARα selectivity and has demonstrated potent efficacy in lowering both glucose and lipids in animal models without causing body weight gain. The PPARα activity of 79 appears to have played a significant role in lowering glucose levels in db/db mice. 474 A series of novel antidiabetics based on a (2R)-chromane-2-carboxylic acid, considered to be restricted fibrate analogues, have been developed and have led to compound 80. 475 It has shown antihyperglycemic activity comparable to rosiglitazone, 5 (Figure I12) in db/db mouse type 2 diabetes.

In addition, the lipid-lowering activities of 80 were demonstrated in a Syrian hamster and a dog model. Unlike the currently marketed glitazone class, 80 is a single enantiomer invulnerable to racemization. It was recently demonstrated that 80 induces growth arrest and apoptosis in both imatinib-sensitive and -resistant chronic myeloid leukemia (CML) cell lines. These data suggest that PPARα/γ dual ligands could represent novel therapy for CML even when the cells are refractory to imatinib. 476

♦ L-Tyrosine Derivatives

Compound 16 (GW409544) (Figure I14) contains a vinylogous amide substituent and possesses three fewer carbon atoms than the benzophenone found in 58 (Figure RL34). Aside from this difference, the chemical structures of the compounds are identical. In contrast with 58, 16 is a potent activator of both PPARα and PPARγ, with a <10-fold difference between its PPARα and PPARγ activities. Because of the larger steric size of Tyr-314 in PPARα compared with His-323 in PPARγ, 16 occupies a position in which it
binds deeper into the PPARα ligand binding pocket than 58 in the PPARγ pocket (Figure RL40). Thus, the potent dual PPARα/γ agonist activity of 16, in which three carbon atoms are removed from farglitazar, is the result of re-engineering of the ligand to accommodate the larger size of the Tyr-314 residue in PPARα (Figure RL40). 16 is also a potent PPARγ ligand. The different type interactions of 16 with PPARγ protein is shown in figure RL41. Replacement of phenyl in the benzophenone of 58 with a furan ring led to 81 (C333H) (Figure RL42), reported to be a more potent agonist of PPARα than 2 (Figure I11); indeed, it was found to have a PPARγ activation effect similar to that of 5. It not only controls glucose and lipid metabolism but also promotes preadipocyte differentiation and improves insulin resistance. Further studies should be carried out to develop 138 as a novel therapy for metabolic disease. A series of compounds containing the piperazine ring, represented by 82 (P633H), have been designed on the basis of previously described compounds 58 (farglitazar) and 16 (GW409544). Preliminary studies in mice showed that 82 (Figure RL42) is well-tolerated in mice, with favorable pharmacokinetics and excellent oral bioavailability. Thus, 82 displays satisfactory druglike characteristics. Although 82 was reported to be a high-potency PPARα/γ dual agonist with good functional activity in vitro, it produces opposing antidiabetic effects, promoting hepatic gluconeogenesis while increasing blood glucose levels in diabetic mice.
Figure RL40: X-ray crystal structure of the PPARα LBD. (A) The PPARα protein is displayed as a red worm with the AF2 helix in red and K292 in dark blue. The LxxLL peptide is displayed as a purple worm. The agonist ligand GW409544 is displayed in space-filling representation colored by atom type. The solventaccessible ligand binding pocket is shown as a white dot surface. Helices 2, 2’, 3, 9, and 10 are indicated. The amino acids Phe-273, Lys-292, Tyr-314, Glu-462, and Tyr-464 are displayed in light blue. (B) A 2Fo -Fc omit map showing the electron density (1σ) of the ligand and the surrounding residues. (C) Key hydrogen bonding (yellow line) and hydrophobic (white broken line) interactions between GW409544 and the PPARα protein and a bound water molecule.
**Figure RL41**: X-ray crystal structure of the PPARγ/RXRα LBDs. (A) The PPARγ protein is displayed as a yellow worm and the RXRα protein as a dark blue worm with the LxxLL peptides in purple. The agonist ligands GW409544 and 9-cis-retinoic acid are displayed in space-filling representations colored by atom type. (B) A 2F₀ - Fc omit map showing the electron density (1σ) of the ligand and the surrounding residues. The amino acids Phe-282, Tyr-323, and Tyr-473 are indicated. (C) Key hydrogen bonding (yellow line) and hydrophobic (white broken line) interactions between GW409544 and the PPARγ protein and two bound water molecules.
**O-Arylmandelic Acid Derivatives**

The only mandelic acid PPARγ agonist **83** (Figure RL43) was discovered to be a PPAR ligand with micromolar PPARγ affinity but nanomolar affinity for PPARα. The novel structural features of **83**, coupled with its advantageous in vitro biological profile, prompted Merck Research Laboratories to embark on further investigation and led to trifluoromethylbenzisoxazole mandelic derivative (**S-84**). This compound is an effective antidiabetic dual PPARα/γ agonist with reduced PPARγ mechanism-based side effects. SAR developments, X-ray crystallography studies, and various in vivo evaluations of a new series of S-aryloxyphenylacetic acids culminated in the discovery of benzoxazolone **85** and benzisoxazolone **86** (Figure RL43). These compounds are characterized by their balanced binding affinity for both PPARα and PPARγ receptors, along with “super”-agonist activity in a PPARα-GAL4 transactivation assay and weak or partial agonist activity toward PPARγ. Most notably, they demonstrate excellent antihyperglycemic efficacy in the db/db mouse and hypolipidemic activity in hamster and dog models, without provoking typical PPARγ-mediated toxicity in a rat tolerability model.

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**Figure RL42:** Tyrosine analogs (dual PPARα/γ synthetic ligands)

**Figure RL43:** O-aryl mandelic acid analogs (dual PPARα/γ synthetic ligands)
2-BABA derivatives (dual PPARα/γ synthetic ligands)

2-BABAs

2-BABA, or the 5-substituted 2-benzoylaminobenzoic acids family, has been described, via identification of 87 (BVT.142), as a new class of PPARα/γ modulators (Figure RL44). Variations at the 5-position with a 2-thienylmethoxy and 2-(3-thienyl)ethoxy led to more potent compounds 88 (BVT.762) and 89 (BVT.763), respectively. X-ray crystallographic data showed that these compounds utilize a novel binding epitope not involving classical agonist characteristic interactions of thiazolidinediones. Compound 88 (Figure RL44) occupies a region in proximity to helix 3, and its carboxylic acid group interacts with the backbone nitrogen of Ser-342. 2-BABA derivatives act by binding at the entrance of the ligand pocket and activate the receptor without direct interaction with helix 12.

Other Synthetic PPARα/γ Agonist Ligands

Another study of pure pharmacochemistry was rewarded by the discovery of muraglitazar (13) (Figure I14), with a large binder module similar to that of 58 (Figure RL34). Contrary to the preceding compounds, 13 does not bear a chiral center in its binder module, therefore considerably simplifying its synthesis. In early clinical studies, 13 demonstrated a significant glucose-lowering reduction in triglycerides and an increase in HDL-C in patients with type 2 diabetes. The drug completed phase 3 clinical trials. However, in 2006, its further development was discontinued. Meta-analysis of phase 2 and phase 3 clinical trials revealed that it was associated with a greater incidence of myocardial infarction, stroke, and transient ischemic attacks. SARs of the oxybenzylglycine series were developed by examining the effects of a conformational restriction at the carbamate N-acid moiety, leading to the triazole acid analogue 90 (Figure RL45). This compound exhibited very potent dual PPARα and PPARγ agonist
activity in vitro and also showed excellent antidiabetic and antidyslipidemic activity in a diabetic db/db mouse model. Imiglitazar (91) was a novel oxyiminoalkanoic acid derivative with potent PPARα/γ activities. In various rodent models of insulin resistance and type 2 diabetes mellitus, 91 showed favorable pharmacokinetic properties, with good absorption and duration, and exhibited marked glucose- and lipid-lowering activities without causing significant body weight gain. Unfortunately, 91 was placed on clinical hold because of findings of abnormalities in liver enzymes in a small number of patients during the course of phase 3 studies. Cevoglitzar (92), developed by Novartis, is a very potent pharmacological agent that improves insulin sensitivity, glucose tolerance, and plasma lipid and inflammatory markers in animal models predictive of type 2 diabetes and metabolic syndrome in humans. More remarkably, 92 shows a desirable antiobesity effect in both genetically obese and diabetic ob/ob mice and obese insulin-resistant cynomolgus monkeys.

\[ \text{Imiglitazar (90)} \]

\[ \text{Imiglitazar (91)} \]

\[ \text{Cevoglitzar (92)} \]

\[ \text{CG301269 (95)} \]

\[ Z = \text{double bond (93)} \]
\[ Z = \text{single bond (94)} \]

**Figure RL45:** Other dual PPARα/γ agonists (synthetic ligands)

These preclinical results demonstrated that 92 (Figure RL45) holds promise in the treatment of diabetes and obesity-related disorders because of its unique beneficial effect on energy balance, in addition to improving glycemic and metabolic control. A series of benzopyran derivatives were synthesized, and their insulinsensitizing activities were evaluated in 3T3-L1 cells. Compounds 93 and 94 (Figure RL45) exhibit more potent insulin-sensitizing activity than 5 (Figure I12). When comparing these compounds, the single-bond and double-bond derivative did not show much difference in activity.
Compound 94 shows PPARα/γ dual agonist activity and exerts potent and efficacious hypoglycemic, hypolipidemic, and insulin-sensitizing effects in ob/ob mice. It not only lowers lipids in peripheral circulation but also reduces triglyceride deposits in skeletal muscle. Various substitutions on the phenyl ring of the 2-(5-methyl-2-aryloxazol-4-yl)ethanol block were designed but led to compounds less potent than 94. Recently, a novel PPARα/γ dual agonist, 95 (CG301269), has been reported to enhance fatty acid oxidation in vitro and ameliorate insulin resistance and hyperlipidemia in vivo. In db/db mice, 95 (Figure RL45) reduced inflammatory responses and fatty liver without body weight gain. These data suggest that 95 might be a potential lead compound against obesity and related metabolic disorders.

![Figure RL46: Dual PPARγ/δ agonists (synthetic ligands)](image)

2.1.3.2. Dual PPARγ/δ Agonists

In recent years, an interest in PPARγ/δ coagonists has developed. Both PPARδ and PPARγ improve insulin sensitivity and glucose tolerance. Furthermore, activation of PPARδ is beneficial in terms of plasma lipoprotein levels and reduced adiposity. Combined activation is believed to correct metabolic disorders associated with T2DM with greater efficacy than single PPAR activation. The beneficial effects on lipid homeostasis and the ability to stimulate reverse cholesterol transport are expected to significantly impede progression of atherosclerosis, which could contribute to lowering of the mortality rates of type 2 diabetic patients. Furthermore, the propensity of PPARδ activation for improving insulin sensitivity and increasing fatty acid oxidation suggests that a dual PPARγ/δ agonist could attenuate the undesired weight gain occurring with
selective PPARγ agonists. However, reports of PPARγ/δ dual agonists are limited. Berger et al. first reported a series of phenylacetic acid derivatives such as 96 (L-165461), 97 (L-783483), and 98 (L-796449), which showed potent activity toward PPARγ and PPARδ, and were able to normalize glucose and triglyceride levels (Figure RL46). Liu et al. developed general solid-phase synthesis of lipophilic carboxylic acids that led to identification of a potent PPARγ/δ dual agonist fibrate derivative, 99. This compound showed good plasma exposure in rats and demonstrated antihyperglycemic and antihyperlipidemic efficacy in diabetic fatty Zucker rats. However, no data regarding other key end points such as effects on body weight or inflammation markers were reported. Currently, some pharmaceutical organisations are involved in research for developing PPARγ/δ dual agents.

![Figure RL47](image)

**Figure RL47:** Dual PPARα/δ agonists (synthetic ligands)

### 2.1.3.3. Dual PPARα/δ Agonists

The observation that both PPARα and PPARδ agonists increased HDL-C levels in human and animal models prompted development of new molecules targeting both PPAR isotypes, with the hope of potentiating individual effects on HDL-C induction. Only a few dual PPARα/δ agonists have been reported and are still at the discovery stage, such as the fibrate analogue 100 (GW2433) (Figure RL47). Some optically active α-ethylphenylpropionic acid derivatives have also been reported, represented by 101, based on structural modifications of the potent PPARα selective agonist 27 (Figure RL30). This new PPARα/δ dual agonist shows potent human nanomolar effects on PPARα and PPARδ compared to PPARγ. In this compound, the three-atom unit linker -CH₂-NH-CO-of 27 was modified to a -CO-NH-CH₂- linker that appeared to increase both PPARα and PPARδ transactivation activities. Furthermore, the activity of this compound resides almost exclusively in the (S)-enantiomer. Dual PPARα/δ agonists of other chemical scaffolds have also been reported.
2.1.13.4. Pan-Agonists

Along with the dual agonists, PPAR pan-agonists that would combine the agonistic activities of PPARγ, PPARα, and PPARβ/δ in a single ligand are also being explored and may prove to be the ultimate combination of PPAR activities for treatment of type 2 diabetes and its further complications. PPAR pan-agonists would regroup then beneficial effects of the three PPARs by normalizing insulin resistance, plasma lipids, and adiposity. They are believed to be more effective than dual PPAR agonists. Some PPAR panagonistic compounds with potential antihyperglycemic, lipidmodulating, and insulin-sensitizing activities have been recently reported and have elicited much interest due to their pharmacology. The reported PPAR pan-agonists effectively lowered plasma glucose levels, improved insulin sensitivity, and exerted positive effects on lipid and cholesterol homeostasis by reducing serum triglycerides and elevating HDL cholesterol, with lower weight gain and less fluid retention than for classical glitazones. Bezafibrate was the first pan-PPAR agonist used in therapy. First developed as a PPARα selective agonist, this fibrate analogue was shown, in further pharmacological evaluations, to activate all three subtypes with the same effectiveness.

Several promising agents have been developed, a few to mention; Sodelglitazar (102) (Figure RL48), an oral PPAR pan-agonist destined for treatment of type II diabetes, was discontinued in phase 2 of clinical trials in October 2009 because of adverse effects. Some phenylpropionic acids have also been reported, represented by compound 103, a potent pan-PPAR agonist identified from SAR evaluations of the known dual PPARα/γ
activator 71 (Figure RL38). It displayed equal potency and efficacy on all three receptors. This compound was synthesized by maintaining the acidic part of a dual PPAR activator unchanged and modifying only the carbazole lipophilic part with a diphenylpropene moiety. GlaxoSmithKline has reported a series of \(\alpha\)-isopropoxyphenylpropanoic acids containing oxadiazole tails initially developed as PPAR dual agonists; however, some of these compounds have shown weak agonistic activity toward PPAR\(\delta\) as, for example, compound 104 (Figure RL48). The in vitro binding and transactivation studies of 105 (BPR1H036), a compound belonging to a new chemical class with indole as a core skeleton, revealed that this compound was a potent PPAR pan-agonist. In vitro biochemical studies of glucose uptake, adipocyte differentiation, and insulin-sensitizing activity have shown that 166 is an excellent candidate for further studies as an antidiabetic agent. The compound has demonstrated highly efficacious glucose-lowering activity in in vivo studies in KKAy mice and an excellent pharmacokinetic profile. The same authors further explored new hydrophobic building blocks such as the 6-benzoil-1-propynaphthalen- 2-yl tail part of the indole-based PPAR agonists. Several other efforts are in progress for developing successful pan agonists.

New pharmacological compounds having the ability to simultaneously target more than one receptor, with the exception of the PPAR subtypes, have also been described. Dual functional agents as PPAR\(\gamma\) agonists and 11\(\beta\)-HSD1 inhibitors, dual functional agents as PPAR agonists and cannabinoids, dual functional agent as a PPAR agonist and COX inhibitor etc are some interesting areas where research for developing successful agents is going on.

2.1.14. Partial Agonists to keep away/reduce adverse effects

Especially in the case of PPAR\(\gamma\), companies are developing partial agonists with the goal of retaining the beneficial effects of this class of agents, while diminishing their adverse effects. Companies that are developing such agents include Metabolex, Roche, Evolva and Astellas.
The lead candidate, metaglidasen (Figure RL49), of Metabolex has a different chemical structure and mechanism of action than the insulin sensitizers currently in the market. Unlike pioglitazone and rosiglitazone, metaglidasen is a non-TZD. Whereas drugs from the TZD class are full agonists of the PPARγ and selectively modifies gene expression needed for insulin sensitization without activating the genes responsible for weight gain and edema. On June 10, 2006, Metabolex announced that it had initiated a Phase II/III clinical trial of metaglidasen in patients with T2D. In a previous Phase II clinical trial, metaglidasen was well tolerated and lowered blood glucose and triglycerides. Unlike PPAR full agonists, metaglidasen did not cause weight gain or edema.147

At the Keystone Symposia Scientific Conference in Hayward, CA in January 2006, Metabolex announced the results of a preclinical study of metaglidasen, which indicated that it is a partial agonist of PPARγ. As with other partial agonists of nuclear receptors, it recruits coactivators as an agonist rosiglitazone. This may explain different manner than the full PPARγ the observed different pattern of gene expression seen with metaglidasen as opposed to rosiglitazone. Specifically, metaglidasen appears to be less active in activating pathways that result in adipocyte differentiation and enlargement. In animal models, metaglidasen improved glycemic control in an equivalent manner to rosiglitazone, but with less weight gain and less cardiac hypertrophy. Preclinical studies also indicate that metaglidasen treatment preserves the function of insulin-producing pancreatic beta cells. Because beta-cell function is often lost over time in type 2 diabetics, resulting in the need for insulin treatment, this is a potential advantage of metaglidasen if this finding is confirmed in human clinical trials.

In vitro studies by researchers from Astellas and Roche also indicate that their PPARγ partial agonists (FK614 and PA-082, respectively) activate pathways that ameliorate insulin resistance without stimulating fat accumulation in adipocytes.500,501

![Chemical structure of Metaglidasen](image)

**Figure RL49:** Chemical structure of Metaglidasen
Intriguingly, in vitro and animal studies indicate that two angiotensin II receptor blockers (ARBs) approved for the treatment of hypertension, telmisartan (Abbott/Boehringer Ingelheim’s Micardis) and irbesartan (Sanofi-Aventis/BMS Avapro) are also partial agonists of PPARγ. This is not a class effect of all ARBs (and is independent of angiotensin II blocking activity), and telmisartan in particular appears to function as a PPARγ partial agonist at pharmacologically relevant concentrations. Both of these agents have established safety profiles. Because the metabolic syndrome includes hypertension as well as insulin resistance, these findings with telmisartan and irbesartan suggest the potential of developing safe and effective drugs that treat both aspects of the syndrome.

Studies with partial agonists of PPARγ suggest that a focus on partial PPAR agonists may be a way of developing agents that have the desired efficacy of PPAR agonists without at least some of their potential adverse effects. However, development of PPAR agonists remains a challenging endeavor.

**Benzimidazole, Indole, Acridone- heterocycles of great pharmacological importance**

It is well documented in the literature that benzimidazole, indole and acridone scaffolds are important structural core in medicinal chemistry because their derivatives exhibit illustrious biological and pharmacological activities. The synthesis of benzimidazole derivatives has gained importance during recent years because of their broad spectrum of activities as antiviral, antitumor, antioxidant, anticoagulant, antihypertensive and antiparasitic agents.

Indole nucleus is another heterocyclic pharmacophoric component of immense medicinal significance. Suitably substituted or fused indole ring containing compounds demonstrate antiproliferative activity in many types of human cancer cells, agonism to cannabinoid receptors, antitumor activity, free radical scavenging activity, anti-inflammatory activity.

Similarly acridone nucleus is also indispensable in drug discovery research. The compounds containing acridone structural unit may act potentially as antiviral agents, antitumor agents, NS3 helicase inhibitor (that inhibits hepatitis C virus replication), antimalarial agents.
The antihypoglycemic activity of the α-alkylthio and α-alkoxy carboxylic acid based compounds has been previously investigated thoroughly and reported. The heterocyclyl linked benzylidine based molecules are also reported to be active and show euglycemic activity. The benzylidene 1,3-diesters are also reported to show antihypoglycemic activity.
2.2. Computational

2.2.1. Molecular Modeling Techniques
In recent years, as computational modeling of binding and biological activity has matured into a well accepted field of drug discovery, there has been a concomitant increase in the application of computational modeling to Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties.

2.2.1.1. Computational Methodology
The currently available molecular modeling techniques can be broadly classified into four major heads: a) Quantum mechanical methods, b) Correlation methods, c) Docking methods and d) Mapping methods.

Analysis based on all these techniques start with the identification of the bioactive conformation of the molecule concerned. The bioactive conformation may be obtained from X-ray crystal structure analysis. If such information is not available, global minimum conformation is considered as the bioactive conformation. Global minimum structure of any given drug molecule can be obtained by carrying out a series of molecular modeling exercises. The general procedure for these exercises involves estimation of energy using either quantum chemical or molecular mechanical methods (details given below), followed by performing energy minimization. Thus a possible conformation of a drug molecule with reasonably reliable chemical representation of the structure can be obtained. This conformation may be local minimum on the potential energy surface of the drug molecule. To obtain the global minimum we have to perform conformational search by manual or automated methods. Energy estimation using quantum chemical methods, molecular mechanics methods, energy minimization, conformational analysis are presented below.

Energy Minimization
Molecular structures generated in the computer have to be optimized to find the individual energy minimum state. This is normally carried out with molecular mechanics or quantum mechanical methods. In the course of minimization procedure, the molecular
structure is relaxed. The internal strain in the structures due to small deviation in the bond lengths and angles are rectified. Before starting the geometry optimization, van der Waals contacts should be removed. Several advantages like speed, sufficient accuracy and the broad applicability on the small molecules as well as on large systems have established the force field geometry optimization as the most important standard method. The common energy minimization procedures used by molecular mechanics include steepest descent minimizer, conjugate gradient method and Newton-Raphson method.\textsuperscript{519} Hence the choice of minimizer depends upon the size of the system and the current state of the optimization. The convergence criterion in non-gradient methods like the steepest descent is the increments in the energy or the coordinates while for the gradient systems atomic gradients are used like root mean square gradient of the forces on each atom of the molecule.

**Conformational Analysis**

Each molecule containing freely rotatable bonds exists at each moment in many different conformations. The transformation from one conformer to the other is related to the change in torsion angles. The changes in molecular conformations can be regarded as movements on a multi-dimensional surface that describes the relationship between the potential energy and the geometry of a molecule. Each point on the potential energy surface represents the potential energy of a single conformation. Stable conformations of a molecule correspond to local minima on this surface. The relative population of a conformation depends on its statistical weight which is influenced by potential energy and entropy. Therefore global minimum having the lowest potential energy is not necessarily the structure with the highest statistical weight. The biological activity of a drug molecule is supposed to depend on a single unique conformation on the potential energy surface which is called as the bioactive conformation that can bind to the active site of the molecule. It is widely accepted that the bioactive conformation is not necessarily the lowest-energy conformation. Thus identification of various conformations is an important task in medicinal chemistry. Various automated methods of conformational analysis are known which include, the systematic search procedures, Monte Carlo methods, random search methods, genetic algorithms, expert systems, molecular dynamics etc.\textsuperscript{520} The systematic search is the most natural of all the different conformational analysis methods. It is performed by varying systematically each of the torsion angles of a molecule to generate all possible conformations. The step size for torsion angle change is normally 30°.
The first step in the data reduction is a van der Waals screening or "bump check". The screening procedure excludes all the conformations where a van der Waals volume overlap of atoms, which are not directly bound to each other, is detected. The validity of the conformation is detected by the sum of the van der Waals radii of non-bonded atoms. The next step is the calculation of the potential energy of the conformers. It is in general calculated by neglecting the electrostatic interactions. After the calculation of the conformational energies, another possibility to reduce the number of conformations is the use of energy window. It is based on the fact that the conformations containing much more energy than those close to the minimum are neglected. This method is of advantage to relatively rigid molecules. Monte Carlo methods or random search procedures follow a different route and are of statistical nature. Molecular dynamics is another method of carrying out systematic conformational search for a flexible molecule.

2.2.1.1.1. Quantum Mechanical Methods

Quantum mechanics is a fundamental physical theory which extends and corrects Newtonian mechanics, especially at the atomic and subatomic levels. It gives a set of laws that describe the behavior of small particles such as electrons and nuclei of atoms and molecules. It is the underlying framework of many fields of physics and chemistry, including condensed matter physics, quantum chemistry, and particle physics. The term quantum (Latin, “how much”) refers to the discrete units that the theory assigns to certain physical quantities, such as the energy of an atom at rest. Quantum chemistry is a branch of theoretical chemistry. It is the application of quantum mechanics to problems in chemistry. The description of the electronic behavior of atoms and molecules pertains to their reactivity is an application of quantum chemistry. Quantum chemistry lies on the border between chemistry and physics, and significant contributions have been made by scientists from both branches of science. Organic chemists use quantum chemistry to estimate the relative stabilities of molecules, to calculate properties of reaction intermediates, to investigate the mechanisms of chemical reactions, to predict the aromaticity of compounds and to analyze the NMR spectra. It is also applied by analytical chemists for using the spectroscopic methods extensively and by inorganic chemists to study the properties of determines the natural atomic orbitals, natural hybrid orbitals, natural bond orbitals, natural localized molecular orbitals and uses them to perform natural population analysis (NPA) and other tasks pertaining to localized analysis of wave function properties. This program can be used extensively to study various kinds of
second order interactions present in the molecules hence is a tool to understand the different electronic interactions in the molecules under investigation. 521,522

2.2.1.1.2. Quantitative Structure Activity Relationship (QSAR)

It is almost 40 years since QSAR is in practice in the fields of agrochemistry, pharmaceutical chemistry, toxicology and other facets of chemistry. It constitutes an important ligand based technique as a drug design approach. This method employs the construction of QSAR models to design new molecules. More than a century ago indications came from various groups that physiological action of a substance is dependent on its chemical composition. QSARs attempt to relate physical and chemical properties of molecules to their biological activities. This can be achieved by using easily calculable descriptors (for example, molecular weight, number of rotatable bonds, Log P). Further developments over the years and contributions of Hammett and Taft laid the mechanistic basis for the development of QSAR paradigm by Hansch and Fujita. Initially 2D QSAR or the Hansch approach took different kinds of descriptors, which did not involve any 3D properties. It was known that the structural changes that affect biological properties are electronic, steric and hydrophobic in nature. These properties were described by Hammett as the electronic constants, Verloop Sterimol parameters and hydrophobic constants. These type of descriptors are simple to calculate and allow for a relatively fast analysis but often fail to take into account the 3D nature of chemical structures (which obviously play a part in ligand-receptor binding, and hence activity).

The development in the field of molecular modeling and X-ray crystallography spawned 3D QSAR methodologies that were based on molecular shape approaches developed independently by Simon et. al. and Hopfinger, distance geometry method by 'Crippen'. 3D QSAR uses probe-based sampling within a molecular lattice to determine three-dimensional properties of molecules (particularly steric and electrostatic features) and can then correlate these 3D descriptors with biological activity. These are routinely used in CADD. Then evolved the most successful module CoMFA (Comparative Molecular Field Analysis) developed by Crammer in 1988523 Similar approach was adopted in developing modules like CoMSIA (Comparative Molecular Similarity Index Analysis), SOMFA (Self Organizing Molecular Field Analysis) and COMMA (Comparative Molecular Moment Analysis). The broad history of the QSAR is described in table 1.4. Utilization and predictivity of CoMFA itself has improved sufficiently in accordance to the objectives to be achieved by it.525 Despite the formal differences between the various methodologies, any QSAR method must include some identifiers of chemical structures,
reliably measured biological activities and molecular descriptors. In 3D QSAR, structural descriptors are of immense importance in every QSAR model. Superimposition of the molecules is necessary to construct good models. The main problems encountered are related to improper superposition of molecules, greater flexibility of the molecules, uncertainties about the bioactive conformation and about different binding modes of ligands. The major assumptions in 3D QSAR include, the effect is produced by modeled compound and not it's metabolites, the proposed conformation is the bioactive one, the binding site is the same for all modeled compounds, the biological activity is largely explained by enthalpic processes, entropic terms are similar for all the compounds, the system is considered to be at equilibrium, and kinetics aspects are usually not considered, pharmacokinetics parameters like solvent effects, diffusion, transport are not included. While considering the template, conformation from the crystal can be extracted or conformation at the binding site can be taken. Otherwise conformational search can be performed using anyone of the options like grid search, systematic search, random search or distance, geometry (NMR-NOE). Alignment of Molecules is carried out using RMS atoms alignment, moments alignment or field alignment. The relationship between the biological activity and the structural parameters is obtained by linear or multiple linear regression analysis.

**CoMFA (Comparative Molecular Field Analysis)**

The CoMFA methodology is a 3D QSAR technique which allows one to design and predict activities of molecules. The database of molecules with known properties is suitably aligned in 3D space according to various methodologies. After consistently aligning the molecules within a molecular lattice, a probe atom (typically carbon) samples the steric and electrostatic interactions between the probe atom and the rest of the molecule. Charges are then calculated for each molecule at a level of theory deemed appropriate. These values are stored in a large spreadsheet within SYBYL, from where they are accessed during the partial least squares (PLS) routines. One then attempts to correlate these field energy terms with a property of interest by the use of PLS with cross-validation, giving a measure of the predictive power of the model. Electrostatic maps are generated indicating red contours around regions where high electron density (negative charge) is expected to increase activity, and blue contours represent areas where low electron density (partial positive charge) is expected to increase activity. Steric maps indicate areas where steric bulk is predicted to increase (green) or decrease (yellow)
activity.$^{523,525}$

**CoMSIA (Comparative Molecular Similarity Index Analysis)**

A technique similar to CoMFA called CoMSIA is also in use. Due to the problems associated with Lennard-Jones potential used in CoMFA methods, Klebe *et al.* have developed a similarity indices-based method named as CoMSIA. It uses Gaussian-type functions to model the probe interactions which allows for much smoother sampling of the fields around the molecules, as well as incorporating new field information such as hydrophobic and hydrogen bonding fields instead of the traditional CoMFA potentials.

The clear advantage of CoMSIA lies in the functions used to describe the molecules as well as the resulting contour maps. It also avoids the cutoff values used in CoMFA to prevent the potential functions from assuming unacceptably large values.$^{524}$

**Apex-3D**

Apex-3D is used to build 3D QSAR models which can be used for activity classification and prediction. The importance of Apex-3D methodology lies in the automated identification of pharmacophores. These biophores can be used for constructing 3D QSAR models when good quantitative data is available, and for database searching. Combination of a 3D pharmacophore with a quantitative regression equation is unique to the Apex-3D approach.$^{526,527}$

**4D QSAR**

4D QSAR analysis developed by Vedani *et al.* incorporates the conformational, alignment, and pharmacophore degrees of freedom in the development of 3D QSAR models. It is used to create and screen against 3D-pharmacophore QSAR models and can be used in receptor-independent or receptor-dependent modes. 4D QSAR can be used as a CoMFA pre-processor to provide conformations and alignments in combination with CoMFA to combine the field descriptors of CoMFA with the grid cell occupancy descriptors (GCOD) of 4D QSAR to build a "best" model, or in addition to CoMFA because it treats multiple alignments, conformations and embedded pharmacophores which are CoMFA limitations.$^{528}$

**5D QSAR**

4D QSAR concept has been extended by an additional degree of freedom, the fifth
dimension, allowing for a multiple representation of the topology of the quasi-atomistic receptor surrogate. While this entity may be generated using up to six different induced-fit protocols, it has been demonstrated that the simulated evolution converges to a single model and that 5D QSAR, due to the fact that model selection may vary throughout the entire simulation, yields less biased results than 4D QSAR where only a single induced-fit model can be evaluated at a time (software Quasar).\textsuperscript{529}

**6D QSAR**

The program Quasar generates a family of quasi-atomistic receptor surrogates that are optimized by means of a genetic algorithm. The hypothetical receptor site is characterized, the surface of which mimics the 3D shape of the binding site. It maps properties of interest, such as hydrophobicity, electrostatic potential, and hydrogen-bonding propensity. The fourth dimension (4D QSAR) takes in account an ensemble of conformations, orientations, and protonation states, of each ligand molecule thereby reducing the bias in identifying the bioactive conformation and orientation. Quasar evaluates the manifestation and magnitude of the induced fit of ligands to the target protein, up to six different induced-fit protocols (5D QSAR). The most recent extension of the Quasar concept to six dimensions (6D QSAR) allows for the simultaneous consideration of different solvation models.\textsuperscript{530} This can be achieved explicitly by mapping parts of the surface area with solvent properties (position and size are optimized by the genetic algorithm) or implicitly. In Quasar, the binding energy is calculated as:

\[
E_{\text{binding}} = E_{\text{ligand-receptor}} - E_{\text{desolvation, ligand}} - T\Delta S - E_{\text{internal strain}} - E_{\text{induced fit}}
\]

**2.2.1.3. Molecular Docking**

There are several possible conformations in which the ligand may bind to active site called as binding modes. Molecular docking involves a computational process of searching for a conformation of the ligand that is able to fit both geometrically and energetically into the binding site of a protein. Docking calculations are required to predict the binding mode of new hypothetical compounds. Docking procedure consists of three interrelated components, i.e. identification of binding site, a search algorithm to effectively sample the search space (the set of possible ligand positions and conformations on the protein surface) and scoring function. In most docking algorithms, the binding site be predefined, so that the search space is limited to a comparatively small
region of protein. The search algorithm effectively samples the search space of the ligand-protein complex. The scoring function used by the docking algorithm gives a ranking to the set of final solutions generated by the search. Different energy calculations are needed to identify the best candidate. The stable structures of a small molecule correspond to minima on the multidimensional energy surface. Different forces that are involved in binding are considered - electrostatic origin, electrodynamic origin, steric forces and solvent related forces. The free energy of a particular conformation is equal to the solvated free energy at the minimum with the small entropy correction. All energy calculations are based on the assumption that the small molecule adopts a binding mode of lowest free energy within the binding site. The free energy of binding is the change in free energy that occurs on binding.

\[ \Delta G_{\text{binding}} = G_{\text{complex}} - (G_{\text{protein}} + G_{\text{ligand}}) \]

Where \( G_{\text{complex}} \) is the energy of the complexed protein and ligand, \( G_{\text{protein}} \) is the free energy of noninteracting separated protein and \( G_{\text{ligand}} \) is the free energy of noninteracting separated ligand. The common search algorithms used for the conformational search, that provide a balance between the computational expense and the conformational search include, molecular dynamics, Monte Carlo methods, genetic algorithms, fragment-based methods, point complementary methods, distance geometry methods, tabu searches, systematic searches. 531

Scoring functions are used to estimate the binding affinity of a molecule or an individual molecular fragment in a given position inside the receptor pocket. Three main classes of scoring functions are known which includes force field based methods, empirical scoring functions and knowledge based scoring functions.

The force field scoring function uses nonbonded energies of existing, well established molecular mechanics force fields for estimating binding affinity. The AMBER and CHARMM nonbonded terms are used as scoring functions in several docking programs. The van der Waals term of force fields is mainly responsible for penalising docking solutions with respect to overlap between receptor and ligand atoms. It is often omitted when only the binding of experimentally determined complex structure is analysed. A new contribution to the list of force field based scoring methods is OWFEG (One Window Free Energy Grid) by Charifson and Pearlman.
In case of empirical scoring functions the binding free energy of the noncovalent receptor-ligand complex can be factorised into some of localised, chemically intuitive interactions. Such decompositions are useful to gain insight into binding phenomenon even without analysing 3D structures of receptor-ligand complexes. Empirical scoring functions usually contain individual terms for hydrogen bonds, ionic interactions, hydrophobic interactions and binding entropy, as in the case of SCORE employed in DOCK4 and Bohm scoring functions used in FlexX. Empirical scoring functions regard only explicit interactions. Less frequent interactions are usually neglected.

Knowledge based scoring functions overcome this drawback and try to capture the knowledge about protein-ligand binding that is implicitly stored in the protein data bank by means of statistical analysis of structural data, for example PMF and DrugScore functions, Wallqvist scoring function, the Verkhivker scoring function.

Various molecular docking softwares are available of which widely used ones are FlexX, Flexidock, DOCK, AUTODOCK, etc.

**FlexX**
FlexX is fragment-based method. It handles the flexibility of the ligand by decomposing the ligand into fragments and performs the incremental construction procedure directly inside the protein active site. It allows conformational flexibility of the ligand while keeping the protein rigid.

**MCDOCK**
MCDOCK is a non conventional Monte Carlo simulation technique in which Monte Carlo simulation method is used to search the global minimum. The scoring function used is based upon molecular mechanics as used in CHARMM and AMBER programs. It allows full flexibility of ligands by explicitly sampling all the rotatable bonds of the ligand and the ligand conformational energy is assessed by the atomic interactions of the ligands. A number of techniques have been integrated to optimize its docking efficiency.

**FlexE**
It addresses the problem of protein structure variations during docking calculations. It can
dock flexible ligands into an ensemble of protein structures which represent the flexibility, point mutations or alternate models of a protein. It is based on the united protein description generated from the superimposed structures of the ensemble. For varying parts of the protein discrete alternative conformations are explicitly taken into account which can be combinatorially joined to create new valid protein structures. FlexE is based on FlexX.\textsuperscript{541}

DREAM++

DREAM++ (Docking and Reaction programs using Efficient search Methods) is a set of programs to design combinatorial libraries that uses techniques of a hybrid algorithm between backtrack and incremental construction algorithms and inheritance of conformations through reactions. The correlation between the binding affinity and the number of conformations can be examined to find the best way to screen virtually generated molecules.\textsuperscript{542}

2.2.1.4 Pharmacophore Mapping

The pharmacophore is an important and unifying concept in rational drug design which embodies the notion that molecules are active at a particular receptor because they possess a number of key features that interact favorably with this receptor and which possess geometric complementarity to it. It is the spatial arrangement of key chemical features that are recognized by a receptor and are thus responsible for ligand-receptor binding. Pharmacophore models are typically used when some active compounds have been identified but the three dimensional (3D) structure of the target protein or receptor in unknown. It is possible to derive pharmacophores in several ways, by analogy to a natural substrate, by inference from a series of dissimilar biologically active molecules (active analog approach) or by direct analysis of the structure of known ligand, and target protein. The active compounds are superimposed to determine their common features to provide a pharmacophore map that explains ligand-receptor binding. Given a set of active molecules, the mapping of a pharmacophore involves two steps: (i) analyzing the molecules to identify pharmacophore features, that is to identify atoms that interact with a receptor and (ii) align the active conformations of the molecules to find the best overlay of the corresponding features. The main difficulty in pharmacophore mapping is in handling conformational flexibility. The major differences between the programs lie in the algorithms used for the alignment and in the way in which conformational flexibility
Besides ligand-based approach, pharmacophores can also be generated manually. Common structural features are identified from a set of experimentally known active compounds. Conformational analysis is carried out to generate different conformations of the molecules and interfeature distances are inferred to develop the final models. The receptor mapping technique is also currently in practice to develop pharmacophores. The pharmacophore models can be derived using the receptor active site. The important residues required for binding the pharmacophores are identified, which are employed for generating the pharmacophores. The structure of protein can be used to generate interaction sites or grids to characterize favorable positions for ligands. Four types of interactions sites, which are hydrogen-bond donors, hydrogen-bond acceptors, aliphatic and aromatic groups, are characterized. Aliphatic and aromatic side chains are important as they also pack closely to form hydrophobic core or proteins. Molecular dynamics simulation for generating diverse protein conformations have been proposed to introduce protein flexibility in the pharmacophore development.

After a pharmacophore map has been derived there are two ways to identify molecules which share its features and thus elicit the desired response. (a) First is the de novo drug design which seeks to link the disjoint parts of the pharmacophore together with fragments in order to generate hypothetical structures that are chemically novel. (b) The second is the 3D database searching, where large databases comprising 3D structures are searched for those that match to a pharmacophoric pattern. One advantage of this method is that it allows the ready identification of existing molecules which are either easily available or have a known synthesis. The two distinct approaches being used for 3D database searches include shape based methods in which a protein structure is used to formulate the database query, to search for compounds whose structure complements the receptor's steric characteristics and pharmacophore based methods which search for compounds whose structure satisfies a certain pharmacophoric pattern. Generally pharmacophores are taken from the literature and are used as 3D search queries or are taken from protein crystal structure.

To propose the 3D requirements for a molecule to exhibit a particular bioactivity, bioactive conformation and a superposition rule for every active compound needs to be
supplied. For this pharmacophore mapping, generation and optimization of the molecules, the location of ligand points and site points (projections from ligand atoms to atoms in the macromolecule) is carried out. Typical ligand and site points are hydrogen bond donors, hydrogen bond acceptors, and hydrophobic regions such as centers of mass of aromatic rings. A pharmacophore map identifies both the bioactive conformation of each active molecule and how to superimpose, compare in 3D, the various active compounds. The map identifies what types of points match in what conformations of the compounds. A complication for pharmacophore mapping is that different ligands might approach a polar site point from different directions, the result is that in a pharmacophore map, the positions of the ligand atoms may not overlap even though their projections to the macromolecule do, in the case of hydrogen bonding interactions. Hence to match these features in the ligands superimposition of only the ligand based points is done. For the pharmacophore mapping of potent small molecules, it is preferred that the proposed bioactive conformation be a low energy one. It is assumed that the interacting atoms of the biomolecule can move slightly and with little energy cost to make optimal interaction with a ligand. Thus a larger tolerance is accepted between overlapping points if the resulting pharmacophore map includes significantly lower energy ligand structures. Experience suggests that it is computationally expensive to search conformational space of a molecule. Especially if a molecule is flexible, even 100 structures may not be enough. Using molecular graphics to compare many conformations of several molecules can take much human time. Moreover these comparisons can be biased by the order in which one studies them. The pharmacophore mapping softwares therefore sought an objective and fast method that would compare all low energy conformations of all molecules simultaneously. Of the various methods employed to superimpose two defined 3D structures when the knowledge of the corresponding atoms is unknown, the most attractive solution is a method given by Brint and Willett based on clique detection technique. DISCO softwares became popular for performing pharmacophore mapping.

**Distance Comparison method (DISCO)**
The various steps involved in DISCO are conformational analysis, calculation of the location of the ligand and site points, to find potential pharmacophore maps and graphics analysis of the results. In the process of conformational search, 3D structures can be generated using any building program like CONCORD, from crystal structures or from conformational searching and energy minimization with any molecular or quantum
mechanical technique. Comparisons of all the duplicate conformations are excluded while comparing all the conformations. If each corresponding interatomic distance between these atoms in the two conformations is less than a threshold (0.4Å), then the higher energy conformation is rejected. DISCO calculates the location of site points to be considered for the pharmacophore. These points can be the location of ligand atoms, or other atom-based points, like centers of rings or a halogen atom are points of potential hydrophobic groups. The other point is the location of the hydrogen bond acceptors or donors. The default locations of site hydrogen bond donor and acceptor points are based on literature compilations of observed intermolecular crystallographic contacts in proteins and between the small molecules. Hydrogen bond donors and acceptors such as OH and NH₂ groups can rotate to change the locations of the hydrogen atom.

During the process of performing pharmacophore mapping in DISCO, its set up iterates the tolerance used to set inter-point distances the same. The starting and the final values and an iteration increment are given. Alternatively, the user may input the tolerance for each type of inter-point distance. The user may direct DISCO algorithm to consider all the potential points and to stop when a pharmacophore map with a certain total number of points is found. Alternatively they may specify the types of fewer features an inhibitor maps to, the poorer its fit to them and lower its predicted affinity.⁵⁴⁶,⁵⁴⁷

2.2.1.2 Virtual screening

Virtual screening also called as \textit{in silico} screening is a new branch of medicinal chemistry that represents a fast and cost effective tool computationally screening compound databases in search for novel drug leads. Various changes have occurred in the ways of drug discovery, the major ones taking place in the field of high throughput synthesis and screening techniques. The basic goal of virtual screening is the reduction of the huge chemical space of synthesized molecules and to screen them against a specific target protein. Thus the field of virtual screening has become an important component of drug discovery programs. Substantial efforts in this area have been put in by large pharmaceutical companies. However, the area of virtual screening is highly diverse and is evolving rapidly. Various reports describing the development of new methods are coming up. Many chemists and biologists perceive this field as an ‘algorithmic jungle’ that lacks well defined standards.⁵³³

\textit{Types of virtual libraries}
Two types of virtual libraries can be generated. One is produced by computational design. The basic idea behind this is of course not to synthesize all compounds (as in combinatorial library design, for example) but to select only preferred molecules. Other libraries focus on compounds that already exist from commercial sources or company inventories. It is possible to extract millions of compounds from these sources and create virtual library. These libraries are useful in the absence of knowledge about the specific drug targets for virtual screening. More focused libraries are important sometimes to save resources as it may be more prudent not to run the entire HTS file against the target protein instead of focused library with higher chances of containing hits may be scrutinized.\textsuperscript{544}

**Applications**

Virtual screening can be applied to target-based subset selection from the data bases. Statistical approaches like binary QSAR or recursive partitioning can be applied to process HTS results and develop predictive models for biological activity. The developed models are employed to select candidate molecules from databases. Similarly, hits from HTS are used in fingerprint searches or compound classification analysis to identify set of similar molecules. Based on these results, a few compounds are selected for additional testing. Many assumptions are made in virtual screening and a positive outcome can not be guaranteed every time. However, the overall process is extremely cost effective and fast. Virtual screening has the ability to produce leads that otherwise may not have been identified. In silico mining of compounds no longer requires an expensive infrastructure. Clustering of low-cost computers make it possible to process, analyze, and search a large amount of data that is otherwise difficult to handle. With the merging of bioinformatics and cheminformatics, virtual screening is expected to become increasingly better established. Improvements in current techniques and the development of new methods will continue at a fast pace, keeping the areas stimulating and interesting.\textsuperscript{533,544}