SECTION-C

RESULTS & DISCUSSION
4 RESULTS & DISCUSSION

4.1 Development & validation of streptozotocin-induced \(db/+\) mice as an alternate model in antidiabetic drug discovery research

4.1.1 Introduction

Animal models play an important role in drug discovery programs. In evaluating compounds/molecules with potent antidiabetic activity, a suitable animal model is of utmost importance. An appropriate animal model of diabetes mellitus would be of great help in understanding the underlying mechanisms of the disease and in testing novel therapeutic modalities. The ideal model should present physiopathologic and clinical features similar to that of human diabetes mellitus. A number of animal models are currently available for antidiabetic drug screening, including \(db/db\) mice and ob/ob mice. The best studied laboratory animals are the obese and insulin resistant ob/ob (Friedman et al., 1991) and \(db/db\) mice (Rosenbaum et al., 1997), which either lack or suffer from leptin-receptor deficiency.

C57BL/KsJ-\(db/db\) mice are an ideal model of type-2 diabetes mellitus and insulin resistance due to mutation in gene for leptin receptor. The mutation is a unit autosomal recessive with full penetrance, and causes metabolic disturbances in homozygous mice resembling non-insulin-dependent diabetes mellitus in humans. Abnormal deposition of fat at 3-4 week of age is followed by hyperinsulinemia, hyperglycemia, polyurea, and glycosuria (Lubec et al., 1998). These mice are sterile in nature and are produced by crossing between heterozygous \(db/+\) mice which do not become diabetic at any stage but are carrier of diabetic character. Studies in the \(db/+\) mice suggest that the leptin signal is apparently attenuated resulting from reduced number of molecules of the intact long receptor isoform (Lee et al., 1996). The \(db/+\) mice have increased plasma leptin levels relative to +/+ mice (Chung et al., 1998; Ishizuka et al., 1999), suggesting that the receptor is not fully recessive with regard to fat mass and that heterozygosity for the leptin receptor may play a role in susceptibility to environmental conditions favoring obesity.

Although, \(db/db\) mice represent well established animal model for type-2 diabetes mellitus and insulin resistance, breeding and maintenance of these animal is quite expensive and problematic. Further, only a limited percentage of progeny become diabetic; this makes the use of \(db/db\) mouse impractical for the initial screening of a large number of molecules. Cross between two heterozygous \(db/+\) mice leads to approximately 25% \(db/db\) mice and half of the progeny are \(db/+\) type. So, the importance of \(db/+\) mice
till date is only in breeding. In the present study, attempts have been made to establish these db/+ mice as an alternate model for initial screening of synthetic antidiabetic molecules/plant based synthetic analogs. Animals were made diabetic by injecting streptozotocin and establishment of diabetic conditions were monitored by measuring blood glucose level, lipid profile and activity of key regulatory enzymes of carbohydrate metabolism. Further, the effect of standard drug metformin on these diabetic parameters was also evaluated to confirm the validity of the model.

4.1.2 Results

4.1.2.1 Effect on body weight of db/+ mice

Body weight of animals of each group was measured after every five days till day 30 post treatment. As evident from the results shown in fig. 1, initially no statistically significant difference was found in the body weight between the normal (non-diabetic) and STZ-treated groups, but at day 20 a significant increase in body weight was observed in normal group (non-diabetic) in comparison to diabetic group. The body weight of diabetic mice treated with metformin remained stable in the beginning but was found to increase at day 20 of treatment as compared to vehicle-treated diabetic mice and a significant difference was observed post day 30 of treatment.

![Figure 1: Change in body weight of diabetic db/+ mice compared with normal and metformin treated groups. Results are mean ± SD; **p<0.01 & ***p<0.001 (Diabetic group compared with normal group and metformin treated group compared with diabetic group (STZ-induced diabetic db/+ mice).](image)

4.1.2.2 Effect on blood glucose level of db/+ mice

Fig. 2 shows the average ± S.E blood glucose profile of normal group (non-diabetic db/+ mice), untreated diabetic and diabetic-metformin treated groups. It is evident from the data that blood glucose of mice of the normal control group remained within normal limits whereas the diabetic db/+ mice showed hyperglycemia. The % elevation in
blood glucose were 245%, 216.3% and 204%, respectively on day 10, 20 and 30, respectively as compared with non-diabetic normal control. Metformin treated mice were found to have lower blood glucose level by 16.9% \((p<0.05)\) on day 10, 44.6% \((p<0.01)\) on day 20 and 52.6% \((p<0.001)\) on day 30, respectively in comparison to vehicle-treated diabetic groups.

**Figure 2**: Blood glucose profile of diabetic \(db/+\) mice, normal control and metformin treated \(db/+\) mice. Results are mean ± SE, \(N=10\), **\(p<0.01\)**, ***\(p<0.001\)** vs. control.

4.1.2.3 Effect on oral glucose tolerance test in \(db/+\) mice

Glucose tolerance pattern of each animal was tested on day 30 post STZ treatment. Fig. 3 compares the blood glucose profiles at different time intervals post oral glucose load, whereas the animals of the STZ treated group showed abnormal glucose tolerance in comparison to the non-diabetic control group \((p<0.001)\), metformin treated group showed significant improvement in their glucose tolerance pattern \((p<0.001)\) in comparison to untreated diabetic \(db/+\) mice. The overall improvement in glucose tolerance of metformin treated group was calculated to be around 48.1% vs. diabetic control.
Figure 3: Blood glucose levels during OGTT in db/+ mice post 30 days of STZ treatment compared with normal control and metformin treated diabetic animals, ** p<0.01 & *** p<0.001 vs vehicle-treated control.

**4.1.2.4 Effect on biochemical parameters in db/+ mice**

Table 1 depicts the levels of plasma cholesterol, HDL cholesterol (HDL-C) and triglycerides (TG) in normal and experimental groups of db/+ mice after 30 days post STZ treatment. Cholesterol and TG levels were significantly elevated whereas HDL-C level declined in STZ-induced diabetic mice in comparison to normal mice (heterozygote db/+ mice). These parameters were found to be responsive to metformin, as diabetic mice treated with metformin for 30 days resulted in marked decrease in plasma cholesterol and triglycerides levels and increase in HDL-C levels as compared to STZ-induced diabetic mice.

**Table 1: Lipid profile of diabetic, normal control and metformin treated groups of db/+ mice after 30 days of STZ treatment.**

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Plasma lipid profile (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TG (mg/dl)</td>
</tr>
<tr>
<td>Normal control</td>
<td>149 ± 10.44</td>
</tr>
<tr>
<td>Untreated diabetic</td>
<td>174 ± 9.28</td>
</tr>
<tr>
<td>% Change</td>
<td>+17.3</td>
</tr>
<tr>
<td>Metformin treated</td>
<td>153 ± 4.77</td>
</tr>
<tr>
<td>% Change</td>
<td>-12.3</td>
</tr>
</tbody>
</table>

Values are mean ± SD of five animals in each group.

**4.1.2.5 Effect on enzymatic parameters in db/+ mice**

On day 30, animals were sacrificed and levels of key enzymes of carbohydrate metabolism (glucose-6-phosphatase, glycogen phosphorylase, fructose-1, 6-bisphosphatase, phospho-fructokinase, phosphoenolpyruvate carboxykinase and glucokinase) were estimated in liver homogenates of all three groups. As shown in fig. 4 compared to normal control values, the activity level of glucose 6-phosphatase (G 6-Pase), glycogen phosphorylase, fructose-1,6-bisphosphatase (F-1,6-BP) and phosphoenolpyruvate carboxykinase (PEPCK) were found to be increased in the diabetic group. The respective increase in terms of percentage was determined to be around 37.3, 30.2, 44.3 and 31.8%,
in diabetic mice compared to normal mice. Treatment with metformin led to decrease in percentage of these parameters by 20.9, 11.6, 25.2 and 13.8 %, respectively. On the other hand the activity level of enzymes phosphofructokinase (PFK), and glucokinase (GK) was decreased in diabetic mice with a respective percentage of 13.6, and 36.8% and treatment with metformin lead to elevation by 10.5 and 22.6% respectively.

![Bar chart showing activities of G-6-Pase, Glycogen phosphorylase, F-1, 6-BP and GK, PEPCK and PFK key regulatory enzymes of carbohydrate metabolism in diabetic, normal control and metformin treated groups of \( db/+ \) mice. Values are expressed as mean ± SE, n=5.]

**Figure 4:** Activities of G-6-Pase, Glycogen phosphorylase, F-1, 6-BP and GK, PEPCK and PFK key regulatory enzymes of carbohydrate metabolism in diabetic, normal control and metformin treated groups of \( db/+ \) mice. Values are expressed as mean ± SE, n=5.

### 4.1.2.6 Effect of withanolide 4554 K037 in STZ-induced diabetic \( db/+ \) mice

Antihyperglycaemic activity profile of the withanolide 4554 K037 isolated from Withania coagulans in STZ-induced diabetic \( db/+ \) mice is shown in fig. Lowering in the blood glucose was evident from 1 h that continued till 5 h. The peak lowering was obtained at 5 h post 4554 K037 treatment as compared to the vehicle-treated control group. The overall antihyperglycaemic activity was found at different doses to be
mentioned in table 2 & fig. 5 and ED$_{50}$ was calculated to be around 25 mg/kg.body weight.

Table 2: Antihyperglycemic effect of withanolide 4554 K037 in STZ-induced diabetic $db/+\,$mice

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Dose (mg/kg)</th>
<th>% glucose lowering</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>4.79</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>10.4</td>
</tr>
<tr>
<td>4554 K037</td>
<td>15</td>
<td>22.3*</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>30.1**</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>36.5**</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>40.5**</td>
</tr>
</tbody>
</table>

Figure 5: Dose dependent effect of withanolide 4554 K037 in STZ-induced diabetic $db/+\,$mice, values are expressed as mean ± SD; (* p<0.05 & ** p<0.01 as compared to control diabetic $db/+\,$mice).

4.1.2.7 Effect of withanolide 4554 K041 in STZ-induced diabetic $db/+\,$mice

The antihyperglycemic effect of withanolide 4554 K041 isolated from the $W.\,coagulans$ was evaluated in STZ-induced diabetic $db/+\,$mice. Single dose oral administration of withanolide 4554 K041 at different doses like 5, 7.5, 15, 20, 25 and 50 mg/kg body weight, led to significant decrease in blood glucose level by 21.8, 29.0 and
35.3% in diabetic db/+ mice for (doses 20, 25 and 50 mg/kg) respectively as compared to control as shown in table 3 & fig. 6. ED₅₀ was calculated to be around 64.2 mg/kg.

Table 3: Antihyperglycemic effect of withanolide 4554 K041 in STZ-induced diabetic db/+ mice

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Dose (mg/kg)</th>
<th>% glucose lowering</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>2.10</td>
</tr>
<tr>
<td>4554 K041</td>
<td>7.5</td>
<td>7.60</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>21.8*</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>29.0**</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>35.3**</td>
</tr>
</tbody>
</table>

Figure 6: Dose dependent effect of withanolide 4554 K041 in STZ-induced diabetic db/+ mice. Values are expressed as mean ± SD; (* p<0.05 & ** p<0.01 as compared to diabetic db/+ mice).

4.1.3 Discussion

Investigation of newer formulations with antidiabetic properties is the need of time and mouse has become a popular model for different antidiabetic research programmes because it is genetically well defined, has short generation time and environmental factors can be controlled easily in the laboratory because of small size. In db/db mice, a single
gene mutation in leptin receptor gene leads to diabetic condition, and recently it has become an important animal model of type-2 diabetes. The mutation is a unit autosomal recessive with full penetrance, and causes metabolic disturbances only in homozygous condition. The heterozygous counterpart (db/+ ) remains normal and does not become diabetic at any stage of its life span, being a carrier of the mutation. The only importance of these heterozygous mice is in breeding.

These db/+ mice can be used as an alternate model for primary screening of antidiabetic molecules as they are susceptible to environmental condition leading to diabetes. Diabetes in these heterozygous mice is induced by intraperitoneal injection of STZ; STZ comprises a glucose molecule with a highly reactive nitrosourea (NSU) side chain that is thought to initiate its cytotoxic action. The glucose moiety directs this agent to the pancreatic β-cells where it binds to a membrane receptor and enters the β-cell via a glucose transporter (GLUT2) and causes alkylation of DNA to generate structural damage. Three important phenomena responsible for β-cell death are process of methylation, free radical generation and nitric oxide (NO) production. Several mechanisms have been postulated to explain STZ induced β-cell damage.

STZ is a potent alkylating agent. The synergistic action of both nitric oxide and reactive oxygen species may also contribute to DNA fragmentation and other deleterious changes caused by STZ. STZ-induced DNA damage activates poly ADP-ribosylation. This process leads to depletion of cellular NAD+, further reduction of the ATP content and subsequent inhibition of insulin synthesis and secretion. The concept of unfavorable consequences of augmented poly ADP-ribosylation as a result of STZ action was confirmed by experiments revealing that the inhibition of this process prevents the toxicity of this diabetogenic agent (Szkudelski, 2001). STZ enters the β-cell via a glucose transporter (GLUT 2) and causes alkylation of β-cell DNA. DNA damage induces activation of poly ADP-ribosylation, a process that activates the nuclear enzyme, Poly (ADP-ribose) synthetase [PARS] that is more important for diabetogenicity of STZ rather than DNA damage (Rerup, 1970). Enhanced ATP dephosphorylation after STZ treatment supplies a substrate for Xanthine oxidase (XOS), resulting in formation of superoxide radicals. STZ liberates toxic amounts of nitric oxide that inhibits aconitase activity and participates in DNA damage (Lenzen et al., 1996).

Injection of streptozotocin led to hyperglycemia in db/+ mice after 48 hr and the animals remainad diabetic throughout the study period. Diabetes mellitus induces a variety of metabolic abnormalities because of insufficient insulin action characterized by glucose
intolerance, abnormalities in lipid profile and more importantly abnormalities in glucose metabolism leading to hyperglycemia (Jung et al., 2004). In the present study, streptozotocin induced \(db/+\) mice were analyzed for all these parameters; they showed persistent hyperglycemia and glucose intolerance. Further, when the lipid parameters were measured, level of triglyceride (TG) and total cholesterol (CHOL) was found to be increased in STZ-treated diabetic \(db/+\) mice in comparison to normal control group. The increase in the plasma lipids of the diabetic subjects are mainly due to the increased mobilization of free fatty acids from peripheral deposits, since insulin inhibits the hormone sensitive lipase (Al-Shamaony et al., 1994). A number of observations indicate that the plasma high density lipoprotein cholesterol (HDL-C) is low in untreated insulin deficient diabetics (Glasgow et al., 1981; Goodman et al., 1982). In our study, a significant decrease was found in plasma HDL-C level in diabetic \(db/+\) mice. Treatment of these diabetic mice with standard drug metformin leads to decrease the elevated level of TG and cholesterol increase the level of HDL-C and validates the model for its sensitivity to the antidiabetic drug.

In general, increased hepatic glucose production, and decreased hepatic glycogen synthesis and glycolysis, are the major symptoms in type-2 diabetes that result in hyperglycemia, and these would seem to be the consequence of the low glucokinase activity and high glucose-6-phosphatase and PEPCK activities in a diabetic state (Guignot and Mithieux, 1999). Hepatic glucokinase is the most sensitive indicator of the glycolytic pathway in diabetes and its increase can increase the utilization of blood glucose for glycogen storage in the liver (Iynedjian et al., 1988). Decreased enzymatic activity of glucokinase and phosphofructokinase has been reported in diabetic animals resulting in depletion of liver and muscle glycogen (Hikino et al., 1989). PEPCK is a rate-limiting enzyme of gluconeogenesis in the liver and plays a key role in the process of glucose homeostasis (Hanson and Reshef, 1997). Diabetic \(db/+\) mice not only showed markedly elevated level of glucose-6-phosphatase, glycogen phosphorylase, fructose-1,6-bisphosphatase and PEPCK activities but also significant lowering in the hepatic glucokinase activity, compared with the normal control (\(db/+\) mice).

Using this model, the antihyperglycemic activity in few natural products was explored and median effective dose (ED\(_{50}\)) of natural compound (K037 & K041) was determined. In conclusion, streptozotocin-induced diabetic \(db/+\) mice show most of the characteristics of diabetes mellitus and can be used as an alternate in-vivo model for
antidiabetic drug discovery research. However, further evaluation of the model in term of molecular mechanism and its resemblance with human diabetes is required.
4.2 Antidiabetic effect of selected plants

4.2.1 Introduction

Plants have served as a natural source of treatments since ancient times and even today they are being used to produce a new generation of therapeutic solutions. In addition, plant based pharmaceuticals are also cost effective. The most commonly used drugs of modern medicine like antimalarials (e.g. Arteether), hypolipidemics (e.g. guggulipid), and hepatoprotective (e.g. Picroliv) and even for the management of diabetes mellitus like metformin from Galega officinalis have originated from natural sources. However, indigenous plants used as remedies against diabetes in the traditional Indian system of medicines (ISM) or in ethno-medicinal practices have not produced any good marketable antidiabetic drugs and this failure may be attributed to the incorrect pharmacognosy of the medicinal plants, incomplete extraction procedure(s), or use of intensive or inadequate animal models (Chandrasekar et al., 1989). Out of an estimated 200,000 and 50,000 higher plants less than 1% have been screened pharmacologically and very few in regard to diabetes mellitus (Grover et al., 2002). Herbal drugs are prescribed widely because of their effectiveness, less side effects and relatively low cost (Venkatesh et al., 2003). Therefore, investigation of such agents from traditional medicinal plants has become more important (Suba et al., 2004). India has a rich history of using various potent herbs and herbal components for treating diabetes. Compound with different structure but with the same therapeutic activity isolated from different plant species act as active moieties for the treatment of various diseases. Some of these active principles originate from edible plants and their inclusion in the diet would undoubtedly be of some value because of their hypoglycemic potential. Several phytomolecules including flavonoids, alkaloids, glycosides, saponins, glycolipids, dietary fibres, polysaccharides, peptidoglycans, carbohydrates, amino acids and others obtained from various plant sources have been reported to act as potent hypoglycemic agent. Conventional antidiabetic agents can affect several pathways of glucose metabolism such as insulin secretion, glucose uptake by target organs as well as nutrient absorption.

In-vivo models are essential the drug discovery process in that all compounds intended for the testing in human patients must first be tested in animals. Recently, a large diversity of animal models has been developed to better understand the pathogenesis of diabetes mellitus and new drugs have been introduced in the market to treat this disease after in-vivo screening. The most popular in-vivo models of diabetes are STZ-induced diabetic rats. It is now well established that STZ selectively destroys the pancreatic β-cells...
and produces hyperglycemia. The hyperglycemia induced by STZ mimics insulin dependent diabetes mellitus (IDDM or Type 1 diabetes) in adult animals.

Therefore, this experimental model has been frequently used for evaluating the antihyperglycaemic/antidiabetic activity of various phytochemicals and synthetic products. In the present study, we evaluated the antihyperglycemic activity of two withanolides (K037 & K041) isolated from the fruits of *Withania coagulans*, 4-hydroxypipecolic acid, isolated from the seeds of *Peganum harmala* and α-amyrin acetate, isolated from the aerial roots of *Ficus bengelensis* in STZ-induced diabetic rats and C57BL/KsJ-db/db mice.

### 4.2.2 Results

#### 4.2.2.1 Antihyperglycaemic activity of K037 in STZ-induced diabetic rats

Table 1 and Fig. 1 present the antihyperglycaemic activity profile of K037 in STZ-induced diabetic rats. Single dose oral administration of K037 at dose of 100 mg/kg body weight for the period of 24 h significantly reduced the hyperglycemia in diabetic rats. An average antihyperglycemic effect was calculated to be around 20.5% (*p*<0.01) whereas standard drug metformin showed 16.5% (*p*<0.05) glucose lowering effect in diabetic rats as compared to vehicle treated control.

**Table 1: Glucose lowering effect of K037 in STZ-induced diabetic rats at 100 mg/kg of dose**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Blood glucose (mg/dl)</th>
<th>% change compared to control</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 60 90 120 180 240 300 1440</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>268.0 ± 8.4 437.4 ± 14.3 539.9 ± 14.0 545.8 ± 9.4 491.4 ± 10.0 444.8 ± 15.8 418.6 ± 9.9 381.1 ± 11.6</td>
<td>-2.2 -7.5 -16.3 -20.4 -25.5 -26.3 -27.4 -24.9</td>
<td>ns ns <em>p</em>&lt;0.05 <em>p</em>&lt;0.05 <em>p</em>&lt;0.01 <em>p</em>&lt;0.01 <em>p</em>&lt;0.01</td>
</tr>
<tr>
<td>K037 (100mg/kg)</td>
<td>262.2 ± 8.6 404.8 ± 13.5 452.0 ± 8.9 434.2 ± 10.3 366.0 ± 10.7 327.8 ± 7.9 296.8 ± 11.2 271.2 ± 8.9</td>
<td></td>
<td>ns ns ns ns ns ns ns ns</td>
</tr>
</tbody>
</table>

Blood glucose values are Mean ± SE of 5 animals per group, Significance: *p*<0.05 and **p*<0.01 ns: not significant.
4.2.2.2 Antihyperglycaemic activity of K041 in STZ-induced diabetic rats

Table 2 and Fig. 2 present the antihyperglycemic activity profile of K041 in STZ-induced diabetic rats. Single dose oral administration of K041 at dose of 100 mg/kg body weight for the period of 24 h significantly declined the hyperglycemia of diabetic rats. An average antihyperglycemic effect were calculated to be around 19.7% (p<0.05) whereas standard drug metformin decreased 16.5% (p<0.05) hyperglycaemia of diabetic rats as compared to vehicle treated control.

Table 2: Antihyperglycaemic effect of K041 in STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Blood glucose (mg/dl) min post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Control</td>
<td>268.0 ± 8.4</td>
</tr>
<tr>
<td>K041 (100mg/kg)</td>
<td>265.2 ± 5.9</td>
</tr>
<tr>
<td>% change compared to control</td>
<td>-1.0</td>
</tr>
<tr>
<td>Statistical significance</td>
<td>ns</td>
</tr>
</tbody>
</table>

Blood glucose values are Mean ± SE of 5 animals per group, Significance: *p<0.05 and **p<0.01 ns: not significant
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4.2.2.3 Antihyperglycemic activity of α-amyrin acetate in STZ-induced diabetic rats

Table 3 & Fig. 3 present the antihyperglycemic activity of α-amyrin acetate in STZ-induced diabetic rats. Single dose oral administration of α-amyrin acetate at dose of 100 mg/kg body weight for the period of 24 h confer the significant decrease in the postprandial hyperglycaemia by 23.6% \((p<0.01)\) in diabetic rats as compared to vehicle treated control. Where as standard drug metformin induces 19.8% \((p<0.05)\) decrease in hyperglycemia of diabetic rats.

Table 3: Glucose lowering effect of α-amyrin acetate in STZ-induced diabetic rats at 100 mg/kg of dose

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Blood glucose (mg/dl) min post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Control</td>
<td>234.3 ± 11.4</td>
</tr>
<tr>
<td>α-AA (100mg/kg)</td>
<td>230.9 ± 5.2</td>
</tr>
</tbody>
</table>

% change compared to control

| Statistical significance | ns | ns | ns | *p<0.05 | **p<0.01 | *p<0.05 | p<0.01 | p<0.01 |

Blood glucose values are Mean ± SE of 5 animals per group, Significance: *p<0.05 and **p<0.01 ns: not significant
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4.2.2.4 Antihyperglycemic activity of 4-HPA in STZ-induced diabetic rats

Table 4 & Fig. 4 present the antihyperglycemic activity of 4-hydroxypipeolic acid (4-HPA) in STZ-induced diabetic rats. Single dose oral administration of 4-HPA at dose of 100 mg/kg body weight caused a significant decrease in hyperglycemia of diabetic rats. The percent antihyperglycemic effect was calculated to be around 27.01% (p<0.01) compared to vehicle treated control. Where as standard drug metformin induces 21.8% (p<0.05) decrease in hyperglycemia of diabetic rats as compared to control.

Table 4: Glucose lowering effect of 4-HPA in STZ-induced diabetic rats at 100 mg/kg of dose

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Blood glucose (mg/dl) min post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Control</td>
<td>234.3 ± 10.2</td>
</tr>
<tr>
<td>4-HPA (100mg/kg)</td>
<td>234.6 ± 9.8</td>
</tr>
<tr>
<td>% change compared to control</td>
<td>0.1</td>
</tr>
<tr>
<td>Statistical significance</td>
<td>ns</td>
</tr>
</tbody>
</table>

Blood glucose values are Mean ± SE of 5 animals per group, Significance: *p<0.05 and **p<0.01 ns: not significant

Figure 3: Blood glucose profile of vehicle and α-amyrin acetate or metformin treated STZ-induced diabetic rats at different time intervals
RESULTS & DISCUSSION: Antidiabetic effect of selected plants

4.2.2.5 Antihyperglycemic activity of withanolides (K037 & K041) in C57BL/KsJ-db/db mice

Effect on hyperglycemia

Fig. 5 depicts an overall antihyperglycaemic effect of the withanolides (K037 & K041) on the postprandial blood glucose level in db/db mice. As evident from figure, multiple oral gavage of withanolides K041 and K037 at dose of 100 mg/kg body weight for the period of 10 days caused a gradual reduction in hyperglycemia of db/db mice by 28.4% and 22.0% on day 5 and 53.9% and 45.3% on day 10 respectively, whereas standard drug rosiglitazone lowered the blood glucose level by 27.4% on day 5 and 52.2% on day 10 respectively as compared to control.

Figure 5: Effect of withanolides on hyperglycemia in db/db mice. Values are expressed as mean ± SD, N=5. *p<0.05, ** p<0.01 & *** p<0.001 as compared to control.
Effect on oral glucose tolerance test

Fig. 6 presents antihyperglycemic effect of withanolides (K037 & K041) on glucose tolerance in db/db mice post 10 days consecutive treatment. Overnight fasted db/db mice were subjected to an oral glucose tolerance test and found that the baseline fasting blood glucose level significantly lower in treated group compared to control. It was also observed that withanolides prevent rise in the postprandial blood glucose level of the treated mice post glucose load of 3.0 g/kg body weight. An average antihyperglycemic effect was calculated to be around 42.7% ($p<0.01$) and 48.4% ($p<0.001$) respectively, while standard drug rosiglitazone improved glucose tolerance by 46.2% ($p<0.001$) as compared to control.

![Figure 6: Effect of K037 & K041 on glucose tolerance in db/db mice](image)

Effect on lipid profile in db/db mice

Fig. 7 presents the effect of withanolides (K037 & K041) on plasma lipid profiles in db/db mice. Multiple oral gavage of withanolides for the period of 10 days significantly lowered the plasma triglyceride by 26.4% ($p<0.01$) and 20.2% ($p<0.05$) and total cholesterol by 21.0% ($p<0.01$) and 15.2% respectively and enhanced the cardio protective HDL-cholesterol by 22.9% ($p<0.01$) and 12.5% respectively as compared to control. Whereas rosiglitazone decreased the plasma triglyceride by 29.0% and total cholesterol by 12.1% respectively and did not show any change in HDL-cholesterol level but it increased the ratio of HDL-cholesterol to total cholesterol by 15.7% ($p<0.05$) as compared to control. Withanolides treatment also increased the HDL-cholesterol to total cholesterol ratio by 55.2% ($p<0.01$) and 44.7% ($p<0.01$) respectively as compared to control.
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**Figure 7:** Effect of withanolides (K037 & K041) on lipid profile in db/db mice. Values are expressed as mean ± SD; (* p<0.05 and ** p<0.01 vs. control db/db mice).

**Effect on plasma insulin level**

Fig. 8 presents the effect of withanolides (K037 & K041) on the plasma insulin level in db/db mice. Oral gavage of withanolides for the period of 10 days caused significant decrease in plasma insulin level by 31.3% and 22.9% respectively. Whereas rosiglitazone lowered 32.7% hyperinsulinemia of db/db mice as compared to control.

**Figure 8:** Effect of withanolides (K037 & K041) on plasma insulin level in db/db mice, Values are expressed as mean ± SD; (* p<0.05 & ** p<0.01 vs. control db/db mice)

**4.2.2.6 Antihyperglycemic activity of 4-hydroxypipenicolic acid (4-HPA) in db/db mouse**

**Effects of 4-HPA on hyperglycemia in C57BL/KsJ-db/db mice**
Fig. 9 depicts antihyperglycemic effect of 4-HPA on postprandial blood glucose level in hyperglycemic \(db/db\) mice. Multiple oral gavage 4-HPA at 100 mg/kg for the period of 10 days significantly decreased postprandial blood glucose level by 35.2% and 56.8% on day 5 and day 10 respectively. Whereas standard drug rosiglitazone reduces postprandial blood glucose level by 38.7% and 51.4% respectively as compared to control.

\[
\begin{array}{c|c|c|c}
\text{Day 0} & \text{Day 5} & \text{Day 10} \\
\end{array}
\]

\[
\begin{array}{c|c|c|c}
\text{Control} & 4-HPA & Rosi \\
\end{array}
\]

**Figure 9:** Effect of 4-hydroxy piperolic acid on hyperglycemia in C57BL/6J-db/db mice, values are expressed as mean ± SD; (\(** p<0.01 \) & \(*** p<0.001 \) vs. control \(db/db\) mice).

**Effect on oral glucose tolerance test in \(db/db\) mice**

Oral glucose tolerance test performed in overnight fasted \(db/db\) mice post 10 day treatment showed that the 4-HPA helps in normalizing the oral glucose tolerance curve of \(db/db\) mice is shown in fig.10. It is presumed that 4-HPA prevents rise in the postprandial blood glucose level significantly after a post glucose load of 3 gm/kg. The percent antihyperglycemic effect of 4-HPA was calculated to be around 33.2% (\(p<0.01\)), while rosiglitazone confer 34.2% (\(p<0.01\)) antihyperglycemic effect as compared to control.

\[
\begin{array}{c|c|c|c}
\text{Control} & 4-HPA (100mg/kg) & Rosi (100mg/kg) \\
\end{array}
\]

**Figure 10:** Effect of 4-hydroxy piperolic acid on oral glucose tolerance in \(db/db\) mice
**Effect on plasma insulin in db/db mice**

Fig. 11 presents the effect of 4-HPA on plasma insulin level of db/db mice. It was found that the treatment of 4-HPA for the period of 10 days confer significant ($P<0.05$) reduction in plasma insulin concentration by 41.6%. Whereas rosiglitazone reduces 22.9% hyperinsulinemia of db/db mice as compared to control.

![Figure 11](image)

*Figure 11:* Effect of 4-HPA on plasma insulin level in db/db mice, values are expressed as mean ± SD; (** p<0.01 & *** p<0.001 vs. control db/db mice).

**Effect on plasma lipid profile in db/db mice**

Fig. 12 depicts the effect of 4-HPA on plasma lipid profiles in db/db mice. 4-HPA significantly lowered the plasma triglycerides level by 28.4% and total cholesterol level by 21.7% respectively. It also increased the cardio protective HDL-cholesterol level by 36.3% and HDL-cholesterol to total cholesterol ratio by 73.6% as compared to control. Whereas rosiglitazone treatment declined the plasma triglyceride and total cholesterol level by 14.0% and 12.4% respectively. It also enhanced the cardio protective HDL-cholesterol by 14.5% and HDL-C to T-CHOL ratio by 31.7% respectively as compared to control.

![Graphs](image)
4.2.2.7 Antihyperglycemic effect of α-amyrin acetate in C57BL/Js-L-db/db mice

Effect on hyperglycemia in db/db mice

Fig. 13 presents the antihyperglycemic effect of α-amyrin acetate in db/db mice. Multiple oral administration of the α-AA at dose of 100 mg/kg body weight for the period of 10 days conferred significant lowering in postprandial blood glucose level by 31.4% and 50.1% respectively on day 5 and 10 of post treatment, whereas standard drug rosiglitazone induces 23.9% and 44.8% reduction in postprandial blood glucose level of db/db mice respectively as compared to control.

![Bar chart showing blood glucose levels over days 0, 5, and 10 for control, α-AA, and rosiglitazone treatments.]

**Figure 13:** Effect of α-AA on hyperglycemia in C57BL/Js-L-db/db mice, values are expressed as mean ± SD; (** p<0.01 & *** p<0.001 vs. control db/db mice).
Fig. 14 presents the effect of α-amyrin acetate on glucose tolerance in \( db/db \) mice post 10 days treatment. Overnight fasted \( db/db \) mice were subjected to an oral glucose tolerance test. It was found that α-AA improved the impaired fasting blood glucose level and recovered the glucose tolerance of \( db/db \) mice. It is presumed that α-AA prevents rise in the postprandial blood glucose level significantly after a post glucose load of 3 g/kg. The average antihyperglycemic effect of α-AA was calculated to be around 37.7% (p<0.01), whereas rosiglitazone improved the glucose tolerance by 30.3% vs. control.

![Graph of Blood glucose levels](image)

**Figure 14:** Effect of α-amyrin acetate on glucose tolerance in C57BL/KsJ-\( db/db \) mice.

**Effect on plasma insulin level in \( db/db \) mice**

Fig. 15 presents the effect of α-amyrin acetate on plasma insulin level in \( db/db \) mice after 10 days consecutive treatment. The supplementation of α-AA caused significant reduction in plasma insulin level by 36.2% (p<0.01) of \( db/db \) mice, whereas rosiglitazone induced 28.2% reduction in plasma insulin level of \( db/db \) mice as compared to control.

![Graph of Plasma insulin levels](image)

**Figure 15:** Effect of α-amyrin acetate on plasma insulin level in \( db/db \) mice, values are expressed as mean ± SD; (**p<0.01 & ***p<0.001 vs. control \( db/db \) mice).
**Effect on plasma lipid profile in db/db mice**

Fig. 16 presents effect of α-AA on plasma lipid profile of db/db mice post 10 days consecutive treatment. Treatment of α-AA significantly lowered the plasma triglycerides and cholesterol by 19.6% and 26.1% respectively. Besides, it also increased the cardio protective HDL-cholesterol level by 16.0% and HDL-cholesterol to total cholesterol ratio by 29.2% as compared to control, whereas rosiglitazone lowered the plasma triglycerides and cholesterol by 25.4% and 17.4% respectively and also increased the level of cardio protective HDL-cholesterol and HDL-C/T-CHOL ratio by 7.16% and 18.7 respectively as compared to control.

![Graphs showing lipid profile](image)

**Figure 16:** Effect of α-AA on plasma lipid profiles in db/db mice, values are expressed as mean ± SD; (* p<0.05 & ** p<0.01 vs. control db/db mice).
4.2.3 Discussion

Diabetes is a disorder of carbohydrate, fat and protein metabolism attributed to diminished production of insulin or mounting resistance to its action. Herbal treatments for diabetes have been used in patients with insulin-dependent and non-insulin-dependent diabetes, diabetic retinopathy, diabetic peripheral neuropathy etc. Scientific validation of several Indian plant species has proved the efficacy of botanicals in reducing the sugar level. From the reports of their potential effectiveness against diabetes, it is assumed that the botanicals have a major role to play in the management of diabetes and therefore, merit further exploration for development of drugs and nutraceuticals (Mukherjee, 2001, 2002). However, many herbal remedies used today have not undergone careful scientific assessment and some have the potential to cause serious toxic effects and major drug-to-drug interaction. Continuing research is necessary to elucidate the pharmacological activities of herbal remedies now being used to treat diabetes mellitus. Our aim was to evaluate the antihyperglycemic effect of phytochemicals isolated from different plants in STZ-induced diabetic rats and db/db mice.

Streptozotocin (STZ)-induced diabetic rats are widely used as experimental animal models for diabetes. STZ exerts diabetogenic action when it is administered parenterally: intravenously, intraperitoneally or subcutaneously. The dose of this agent required for inducing diabetes depends on the animal species, route of administration and nutritional status. According to the administered dose of this agent, syndromes similar to either type 1, type 2 diabetes mellitus or glucose intolerance can be induced (Lenzen et al., 1996; Mythili et al., 2004). STZ is an antibiotic obtained from Streptomyces achromogenes and cytotoxic action of this diabetogenic agent is mediated by reactive oxygen species (Lei et al., 2005). STZ enters the pancreatic β-cell via a glucose transporter (GLUT-2) and causes alkylation of deoxyribonucleic acid (DNA). Furthermore, STZ induces activation of poly adenosine diphosphate ribosylation and nitric oxide release. As a result of STZ action, pancreatic β-cells are destroyed by necrosis (Mythili et al., 2004). In adult rats, 60 mg/kg is the most common dose of STZ to induce insulin dependent diabetes (Patel et al., 2006), but higher doses are also used. A model of type 2 diabetes can be induced in rats by either i.v. (tail vein) or i.p. treatment with STZ in the first days of life. At 8-10 weeks of age and thereafter, rats neonataly treated with STZ manifest mild basal hyperglycemia, an impaired response to the glucose tolerance test, and a loss of pancreatic β-cell sensitivity to glucose (Pascoe and Storlien, 1990).
C57BL/KsJ-db/db mice have been commonly and extensively used for the investigation of type 2 diabetes/diabetic dyslipidaemia and screening of variety of agents such as insulin mimetic and insulin sensitizers (Himms-Hagen and Danforth, 1996; Ramarao and Kaul, 1999; Reed and Scribner, 1999; Zhang et al., 1999; Nuss and Wagman, 2000; Knouff and Auwerx, 2004; Kumar et al., 2005; Reifel-Miller et al., 2005).

An aqueous extract of fruits of *W. coagulans* has been shown to exert hepatoprotective (Budhiraja et al., 1986), anti-inflammatory (Rajurkar et al., 2001) and antidiabetic activity (Hemalatha et al., 2004) and also hypolipidemic activity in triton and high fat diet (HFD) induced hyperlipidemic rats (Hemalatha et al., 2006). In the present study, we reported for the first time the antihyperglycemic effect of withanolides (K037 & K041) from fruits of *W. coagulans* in STZ-induced diabetic rats and db/db mice. It was found that the compound K037 & K041 significantly lowered the hyperglycemia and improved the glucose tolerance of STZ-induced diabetic rats and db/db mice. Theses also improved the hyperlipidemia and hyperinsulinemia in db/db mice.

Various active components isolated from the *Ficus bengelensis*, including the dimethoxy derivative of leucocyanidin 3-O-beta-d-galactosyl cellobioside, dimethoxy derivative of perlargonidin 3-O-alpha-L rhamnoside, glycoside of leucopelargonidin, leucodelphinidin (Fig.) isolated from the bark of this plant have been reported for their hypoglycemic activity (Deshmukh et al., 1960; Kumar and Augusti, 1994). It has also been reported that these derivatives stimulate insulin secretion from beta cells of islets of langerhans (Achrekar et al., 1991; Cherian et al., 1992; Augusti et al., 1994) and inhibit insulin degradation. In the present study we isolated α-AA for the first time from the aerial roots of *F. bengelensis* and evaluated it for antidiabetic effects in diabetic rats and db/db mice, the treatment of α-AA significantly reduced the blood glucose level and improved the diabetic condition, whereas it also improved the diabetic condition in db/db mice by decreasing the hyperglycemia, hyperlipidemia and hyperinsulinemia and improved the glucose tolerance.
In Moroccan traditional medicine, seeds of *P. harmala* were used as powder decoction, maceration or infusion for fever, diarrhoea, abortion and subcutaneous tumors. It is still widely used as a remedy for rheumatic pain, painful joint and intestinal pain. *P. harmala* L. is rich in alkaloids (β-carboline harmala) that have a wide spectrum of pharmacological actions in various areas. In the present study, we report for the first time the hypoglycemic effect of *P. harmala* in diabetic rats; we also isolated the phytoconstituent 4-hydroxypipeolic acid from seeds of this plant and evaluated their antidiabetic effect in STZ-induced diabetic rats and *db/db* mice, data have been communicated.

The overall studies suggest that the lowering in hyperglycemia following treatment with withanolides (K037 & K041), α-AA and 4-HPA could be due to the action of the potential bioactive component that suppresses the generation of free radicals induced by STZ in diabetic rats or it could regenerate the damaged β-cells (a result of STZ administration) or increase the sensitivity towards insulin in *db/db* mice. Interestingly, all the four antihyperglycemic agents were found to lower postprandial hyperglycemia as well as improve the glucose tolerance of one of the most popular model of type 2 diabetes Thus the glucose lowering effect of these phytoconstituents in diabetic rats and *db/db* mice proves that these might be useful in both type 1 and type 2 diabetes.
4.3 Antidiabetic effect of plant based synthetic isoflavone derivatives

4.3.1 Introduction

Diabetes mellitus is one of the most common endocrine disorder and a major global health problem affecting 5% of world population (Zimmet et al., 2001). The disease is characterized by chronic hyperglycemia due to a relative or absolute lack of insulin, or the action of insulin on its target tissue or both (Kumar and Clark, 2002). Both forms (type 1 and type 2) of diabetes are associated with major long-term complications, including cardiomyopathy, angiopathy, neuropathy, retinopathy, nephropathy, diabetic foot and digestive insufficiencies (Ahmed et al., 2004).

It is well known that the prevalence of type 2 diabetes mellitus is rising globally but its impact is most marked in developing countries like India and China. Some of the important risk factors associated with diabetes are mostly similar in all countries but their expression and intensities vary widely between races, regions and countries. Asian Indians have a racial predisposition and other unique risk factors to develop diabetes to a greater extent. In India there is increasing urbanization and industrialization which has led to physical inactivity, sedentary lifestyle, psychosocial stress and obesity leading to progressive increase in prevalence of diabetes mellitus (Gupta and Phatak, 2003). At present, therapy for type 2 diabetes relies mainly on several approaches intended to reduce the hyperglycemia itself: sulphonylureas which increase insulin secretion from pancreatic beta cells, Metformin which acts to reduce hepatic glucose production, peroxisome proliferator activated receptor-γ agonists which enhance insulin action and α-glucosidase inhibitors interfere with gut glucose absorption. These therapies have limited efficacy, limited tolerability and mechanism-based toxicity. Therefore, the development of new antidiabetic agents with no or minimum side effects is an unmet need.

In the present chapter we investigated the antihyperglycemic effect of isoflavone derivatives, RS-853 and DL-857 and their resolved enantiomer (S-853 & R-853) and (D-857 & L-857) in STZ-induced diabetic rats and C57BL/KsJ-db/db mice.

4.3.2 Results

4.3.2.1 Antihyperglycemic effect of RS-853 and their resolved enantiomer S-853 and R-853 in STZ-induced diabetic rats

Table 1, 2 & 3 and fig. 1, 2 & 3 presents the antihyperglycemic activity profile of isoflavone derivative RS-853 and their resolved enantiomer S-853 & R-853 in STZ-induced diabetic rats. Oral administration of compounds at a single dose each of 100 mg/kg body weight, conferred significant inhibition in the postprandial blood glucose...
level in diabetic rats post sucrose load of 2.5 g/kg body weight as compared to vehicle treated control group. An average antihyperglycemic effect was calculated to be around 26.1%, 27.8% and 20.8% respectively, whereas standard drug metformin showed 19.0% antihyperglycemic effect as compared to vehicle treated control group.

Table 1: Glucose lowering effect of RS-853 in STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Blood glucose (mg/dl)</th>
<th>% change compared to control</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>Control</td>
<td>281.5</td>
<td>524.3</td>
<td>501.7</td>
</tr>
<tr>
<td>± 13.4</td>
<td>± 10.0</td>
<td>± 18.0</td>
<td>± 18.4</td>
</tr>
<tr>
<td>RS-853 (100mg/kg)</td>
<td>279.6</td>
<td>470.8</td>
<td>401.8</td>
</tr>
<tr>
<td>± 12.5</td>
<td>± 14.0</td>
<td>± 11.6</td>
<td>± 15.0</td>
</tr>
<tr>
<td>% change compared to control</td>
<td>-0.7</td>
<td>-10.2</td>
<td>-19.9</td>
</tr>
<tr>
<td>Statistical significance</td>
<td>ns</td>
<td>ns</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

Blood glucose values are Mean ± SE of 5 animals per group, Significance: *p<0.05 and **p<0.01 ns: not significant

Figure 1: Blood glucose profile of vehicle and RS-853 or metformin treated STZ-induced diabetic rats at different time intervals
Table 2: Glucose lowering effect of S-853 in STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Blood glucose (mg/dl)</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
<th>180</th>
<th>240</th>
<th>300</th>
<th>1440</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>281.5±13.4</td>
<td>524.3±10.0</td>
<td>501.7±18.0</td>
<td>498.7±18.4</td>
<td>470.9±10.2</td>
<td>458.6±14.7</td>
<td>442.3±13.0</td>
<td>415.2±14.1</td>
</tr>
<tr>
<td>S-853 (100mg/kg)</td>
<td></td>
<td>280.5±13.5</td>
<td>450.2±11.2</td>
<td>390.0±15.0</td>
<td>378.0±16.1</td>
<td>340.0±14.5</td>
<td>301.0±14.0</td>
<td>270.0±13.6</td>
<td>240.0±12.4</td>
</tr>
</tbody>
</table>

% change compared to control:

-0.3 -14.1 -22.3 -24.2 -27.8 -34.4 -39.0 -42.2

Statistical significance:

ns ns p<0.05 p<0.01 p<0.01 p<0.01 p<0.01 p<0.01

Blood glucose values are Mean ± SE of 5 animals per group, Significance: *p<0.05 and **p<0.01 ns: not significant

Figure 2: Blood glucose profile of vehicle and S-853 or metformin treated STZ-induced diabetic rats at different time intervals
Table 3: Glucose lowering effect of R-853 in STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Blood glucose (mg/dl)</th>
<th>min post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Control</td>
<td>281.5</td>
<td>524.3</td>
</tr>
<tr>
<td>± 13.4</td>
<td>± 10.0</td>
<td>± 18.0</td>
</tr>
<tr>
<td>R-853 (100mg/kg)</td>
<td>285.7</td>
<td>464.9</td>
</tr>
<tr>
<td>± 14.5</td>
<td>± 13.2</td>
<td>± 16.0</td>
</tr>
</tbody>
</table>

% change compared to control

| Statistical significance | ns | ns | ns | p<0.05 | p<0.05 | p<0.01 | p<0.01 | p<0.01 |

Blood glucose values are Mean ± SE of 5 animals per group. Significance: *p<0.05 and **p<0.01 ns: not significant

Figure 3: Blood glucose profile of vehicle and R-853 or metformin treated STZ-induced diabetic rats at different time intervals
RESULTS & DISCUSSION: Antidiabetic Effect of Synthetic Compounds

4.3.2.2 Antihyperglycemic effect of DL-857 and their resolved enantiomers D-857 and L-857 in STZ-induced diabetic rats

Table 4, 5 & 6 and fig.4, 5 & 6 presents the antihyperglycemic activity profile of compounds DL-857 and their resolved D-857 & L-857 enantiomers in STZ-induced diabetic rats. Oral administration of all three compounds at 100 mg/kg body weight conferred significant inhibition in the postprandial blood glucose level in diabetic rats post sucrose load of 2.5 g/kg body weight. An average antihyperglycemic effect was calculated to be around 24.4% (p<0.01), 27.3% (p<0.01) and 19.7% respectively, whereas metformin illustrate 15.7% antihyperglycemic effect in diabetic rats as compared to control.

Table 4: Glucose lowering effect of DL-857 in STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Blood glucose (mg/dl) min post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Control</td>
<td>210.0 ± 12.6</td>
</tr>
<tr>
<td>DL-857 (100mg/kg)</td>
<td>199.8 ± 10.5</td>
</tr>
<tr>
<td>% change compared to control</td>
<td>-4.9</td>
</tr>
<tr>
<td>Statistical significance</td>
<td>ns</td>
</tr>
</tbody>
</table>

Blood glucose values are Mean ± SE of 5 animals per group, Significance: *p<0.05 and **p<0.01 ns: not significant

Figure 4: Blood glucose profile of vehicle and DL-857 or metformin treated STZ-induced diabetic rats at different time intervals
Table 5: Glucose lowering effect of D-857 in STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Blood glucose (mg/dl)</th>
<th>min post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Control</td>
<td>210.0 ± 12.6</td>
<td>423.0 ± 9.7</td>
</tr>
<tr>
<td>D-857 (100mg/kg)</td>
<td>207.5 ± 11.1</td>
<td>329.6 ± 5.7</td>
</tr>
</tbody>
</table>

% change compared to control
-1.2 -22.1 -18.5 -20.0 -22.3 -29.1 -42.3 -41.4

Statistical significance
ns p<0.05 p<0.05 p<0.05 p<0.01 p<0.01 p<0.01

Blood glucose values are Mean ± SE of 5 animals per group, Significance: *p<0.05 and **p<0.01 ns: not significant

Figure 5: Blood glucose profile of vehicle and D-857 or metformin treated STZ-induced diabetic rats at different time intervals
**RESULTS & DISCUSSION:** Antidiabetic Effect of Synthetic Compounds

Table 6: Glucose lowering effect of L-857 in STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Blood glucose (mg/dl)</th>
<th>min post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Control</td>
<td>210.0 ± 12.6</td>
<td>423.0 ± 9.7</td>
</tr>
<tr>
<td>L-857 (100mg/kg)</td>
<td>209.8 ± 13.2</td>
<td>378.8 ± 9.0</td>
</tr>
<tr>
<td>% change compared to control</td>
<td>-0.1</td>
<td>-10.4</td>
</tr>
</tbody>
</table>

Statistical significance: ns, *p<0.05*, **p<0.01**

Blood glucose values are Mean ± SE of 5 animals per group. Significance: *p<0.05* and **p<0.01** ns: not significant

![Figure 6: Blood glucose profile of vehicle and L-857 or metformin treated STZ-induced diabetic rats at different time intervals](image)

4.3.2.3 Antihyperglycemic effect of RS-853 and their resolved enantiomers S-853 & R-857 in C57BL/KsJ-db/db mice

*Effect on hyperglycemia*

Fig. 7 depicts an overall effect of the isoflavone derivative RS-853 and their enantiomers S-853 and R-853 in C57BL/KsJ-db/db mice, when compared to the vehicle
treated control group before and during 10 days treatment. As evident from the fig., the S-enantiomer caused significant reduction in the hyperglycemia by 55.4% (p<0.001) compared to less active R-enantiomer which decreased the hyperglycemia by 34.1% (P<0.05), whereas RS-853 racemic mixture declined the blood glucose level by 38.3% (p<0.01) when compared to vehicle treated control db/db mice, whereas rosiglitazone induces 42.1% reduction in hyperglycemia of db/db mice as compared to control.

![Graph showing the effect of RS-853 racemic mixture and their enantiomers S-853 and R-853 on hyperglycemia in db/db mice, values are expressed as mean ± SD; (**p<0.05 and ***p<0.001 as compared to control).](image)

**Figure 7:** Comparative effect of RS-853 racemic mixture and their enantiomers S-853 and R-853 on hyperglycemia in db/db mice, values are expressed as mean ± SD; (**p<0.05 and ***p<0.001 as compared to control).

**Effect on oral glucose tolerance test**

Fig. 8 presents antihyperglycemic effect of RS-853 and enantiomer S-853 & R-853 on glucose tolerance in db/db mice post 10 days consecutive treatment. Overnight fasted db/db mice were subjected to an oral glucose tolerance test. The fasting baseline blood glucose values at 0 min were found lower in all the treated groups compared to vehicle treated control group at the corresponding time. The entire compound significantly inhibited the rise in postprandial blood glucose level of db/db mice post glucose load of 3.0 g/kg body weight. The treatment of S-853 enantiomer showed promising antihyperglycemic effect by 43.9% (p<0.01) whereas R-853 showed 27.0% (p<0.05) antihyperglycemic effect in db/db mice and RS-853 racemic mixture showed 30.3% (p<0.01) antihyperglycemic effect, whereas rosiglitazone treatment improved glucose tolerance of db/db mice by 35.9% (p<0.01) as compared to control.
Figure 8: Comparative effect of RS-853 and their enantiomers S-853 and R-853 on oral glucose tolerance in db/db mice post 10 days treatment.

Effect on lipid profile in db/db mice

Fig. 9 presents the effect of RS-853 racemic mixture and their resolved S-853 and R-853 enantiomer on plasma lipid profiles in db/db mice. Multiple oral administrations of all three compounds at dose of 100 mg/kg body weight for the period of 10 days significantly lowered the plasma triglycerides by 20.1%, 27.4% and 14.1% respectively. Plasma total cholesterol level was found to decrease by 22.3%, 30.7% and 15.9% respectively and enhanced the level of cardio protective HDL-cholesterol by 20.9%, 33.2% and 8.30% respectively, whereas rosiglitazone lowered the TG and T-CHOL by 24.5% and 24.8% respectively and also enhanced the HDL-cholesterol level by 6.98% as compared to control. Test compound and rosiglitazone treatment also increased the HDL-cholesterol to total cholesterol ratio by 26.0%, 57.8%, 5.0% and 15.7% respectively as compared to control.
**RESULTS & DISCUSSION:** Antidiabetic Effect of Synthetic Compounds

**HDL**

<table>
<thead>
<tr>
<th>HDL Means + st.dev.</th>
<th>70</th>
<th>60</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>Control</td>
<td>RS-853</td>
<td>S-853</td>
<td>R-853</td>
</tr>
</tbody>
</table>

**HDL-C/CHOL ratio**

<table>
<thead>
<tr>
<th>HDL-C/CHOL (mg/dl)</th>
<th>0.4</th>
<th>0.3</th>
<th>0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>RS-853</td>
<td>S-853</td>
<td>R-853</td>
</tr>
</tbody>
</table>

**Figure 9:** Comparative effect of RS-853 and their enantiomer S-853 and R-853 on plasma lipid profiles in *db/db* mice, values are expressed as mean ± SD; (* p<0.05, ** p<0.01 & *** p<0.001 vs. vehicle treated control).

**Effect on plasma insulin level**

Fig. 10 presents the effect of RS-853 and their resolved enantiomer S-853 and R-853 on the plasma insulin level in *db/db* mice after 10 days consecutive treatment. Repeated oral gavages of compounds at dose of 100 mg/kg body weight for 10 consecutive days caused significant decrease in plasma insulin level by 30.6%, 53.4% and 18.0% respectively compared to vehicle treated control group, whereas rosiglitazone treatment reduced the hyperinsulinemia of *db/db* mice by 45.3% as compared to control.

**Effect on body weight**

As shown in the fig. 11, body weight in vehicle treated control group was increased from 40.6 ± 2.9 g on day 0 to 42.6 ± 2.7 g on day 10 during the experiment, whereas mice treated with S-853 enantiomer showed significant decrease in body weight from 39.9 ± 3.0 g on Day 0 to 30.6 ± 3.9g on Day 10 (*P* < 0.05), whereas RS-853 and R-853 treatment do not showed significant change in body weight gain of *db/db* mice. While

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rosiglitazone treatment significantly increased the body weight from 40.6 ± 2.9 g on day 0 to 50.4 ± 2.7 g on day 10 ($P<0.01$) as compared to control.

**Figure 11:** Comparative effect of RS-853 and their enantiomer S-853 and R-853 in db/db mice. Values are expressed as mean ± SD; (*$p<0.05$ & **$p<0.01$ as compared to control).

4.3.2.4 Antihyperglycemic effect of isoflavone derivative DL-857 and their enantiomer D-857 and L-857 in db/db mice

**Effect on hyperglycemia**

Fig. 12 presents the effect of DL-857 and their resolved enantiomer D-857 and L-857 at a dose of 100 mg/kg body weight on the hyperglycemia of db/db mice after 10 days consecutive treatment. As evident from the figure that the D-857 enantiomer caused significant reduction in the hyperglycemia by 48.0% and L-857 which decreased the hyperglycemia by 20.8%, whereas DL-857 declined the hyperglycemia by 34.4% as compared to control, whereas rosiglitazone treatment reduced the hyperglycemia by 35.1% of db/db mice as compared to control.

**Figure 12:** Comparative effect of DL-857 and their enantiomer D-857 and L-857 on hyperglycemia in db/db mice. (*$p<0.05$ & **$p<0.01$ as compared to control).
Effect on oral glucose tolerance test

Fig. 13 depicts the effect of DL-857 and their resolved enantiomer D-857 & L-857 on glucose tolerance in db/db mice. The treatment of D-857 enantiomer showed significant antihyperglycemic effect (46.4%, p<0.01) and L-857 enantiomer showed 29.3% antihyperglycemic effect. DL-857 showed 35.5% (p<0.01) antihyperglycemic effect compared to vehicle treated db/db mice, while rosiglitazone treatment illustrate 43.3% antihyperglycemic effect as compared to vehicle-treated mice.

Figure 13: Comparative effect of DL-857 and their D-857 and L-857 enantiomer on oral glucose tolerance in db/db mice. Values are expressed as mean ± SD, N=5.

Effect on lipid profile in db/db mice

Fig. 14 presents the effect of DL-857 and their resolved enantiomer D-857 and L-857 on plasma lipid profiles in db/db mice. Repeated oral gavage of DL-857, D-857 and L-857 for the period of 10 days significantly lowered the plasma triglycerides level by 14.2%, 21.0% and 14.1% and total cholesterol by 15.3%, 27.0% and 11.2% respectively and enhanced the cardio protective HDL-cholesterol level by 11.8%, 23.1% and 3.59% as compared to vehicle treated control, whereas rosiglitazone treatment illustrates 23.6% and 32.6% lowering in TG and CHOL level and enhanced the level of HDL-cholesterol by 6.90% respectively. It was also observed that the treatment of test compound and rosiglitazone increased the HDL-cholesterol to total cholesterol ratio by 36.4%, 72.7%, 18.2% and 63.6% as compared to control.
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**Figure 14:** Comparative effect of DL-857 and their resolved enantiomer D-857 and L-857 on plasma lipid profiles in db/db mice.

**Effect on plasma insulin level**

Fig. 15 presents the effect of DL-857 and their enantiomer D-857 and L-857 on the plasma insulin level in db/db mice post 10 days treatment. Multiple oral gavages of test compound significantly decreased the plasma insulin level by 22.3%, 39.2% and 9.10% respectively as compared to control, whereas rosiglitazone reduced 40.5% hyperinsulinemia of db/db mice as compared to control.

**Figure 15:** Effect of DL-857 and their enantiomer D-857 and L-857 on plasma insulin level in db/db mice. Values are expressed as mean ± SD; (**p<0.01 & *** p<0.001 as compared to control).
Effect on body weight

As shown in the fig. 16, none of the groups show any significance change in body weight of \textit{db/db} mice but rosiglitazone significantly increased the body weight of \textit{db/db} mice (from 39.8 ± 3.6 g on day 0 to 46.5 ± 4.3 g on day 10 ($P<0.05$) as compared to control.  

**Figure 16:** Effect of DL-857 and their enantiomer D-857 and L-857 on body weight in \textit{db/db} mice post 10 days treatment.

4.3.3 Discussion

Diabetes mellitus is a complex metabolic syndrome in which the carbohydrate and lipid metabolism is altered. It is a very fast growing global health problem and every third person is prone towards this syndrome. In the later stages, diabetic patients suffer from various serious complications including diabetic neuropathy, nephropathy and retinopathy. Type 2 diabetes mellitus constitutes more than 90 percent of the total diabetic population. Conventional antidiabetic drugs that are available in the market including insulin, thiazolidinediones, sulfonylureas, metformin suffer from one or the other side effects related to body weight gain and induction of hypoglycemia. Hence, it becomes essential to search for novel antidiabetic lead molecules in order to develop new antidiabetic drugs having lesser side effects as compared to the conventional antidiabetic drugs.

Natural products from both plants and animal sources have been of great use in treating various diseases and disorders from the ancient times. With the advancement of modern techniques and with better understanding of the etiology of the diseases, these natural products have regained their importance. Most of the present day promising drug in use have either been isolated from the natural sources as active principles or have been synthetically modified to improve the efficacy. Flavonoids are naturally occurring plant
polyphenols found in abundance in diets rich in fruit, vegetables and plant derived beverages such as tea. Epidemiological studies have shown that the consumption of vegetable, fruits and tea is associated with a decreased risk of cancer and cardio-vascular diseases. Flavonoids produce biological effects through their free radical scavenging antioxidant activities and metal ion chelating abilities. These data have attracted attention towards the use of flavonoids as possible chemoprotective or chemotherapeutic agents. These properties led us to utilize isoflavones for the synthesis of hybrid molecules as antidiabetic and antidyslipidemic agents.

Among the synthetic analogues based on the leads from plants, many compounds have shown promising antihyperglycemic effect in STZ-induced diabetic rats and db/db mice. Most of these compounds belong to the subclasses of flavonoids, namely, chalcones, isoflavones and isoflavones. The studies include in this chapter demonstrate the antidiabetic potential of the plant based synthetic isoflavones derivatives RS-853 and DL-857 and their resolved enantiomer (S-853/R-853) and (D-857/L-857) in standardized experimental animal models of diabetes.

*In-vivo* antihyperglycemic activities were performed in the STZ-induced diabetic rats and db/db mice. The STZ-induced diabetic rats used in this study have been widely used as experimental animal models of diabetes. The test compounds (RS-853/S-853 & R-853) and (DL-857/D-857 & L-857) were primarily screened for antihyperglycemic activity in STZ-induced diabetic rats. The oral administration of test compounds conferred significant reduction in glucose level of hyperglycemic rats. The comparative study indicates that the S-853 enantiomer had greater antidiabetic potential than RS-853 and R-853 compound, whereas compounds of 857 series also showed similar profile i.e. D-857 enantiomer was more active than DL-857 and L-857. Lowering in hyperglycemia following treatment with isoflavone derivatives could be due to the suppression of the generation of free radicals or it could regenerate the β-cells destroyed as a result of STZ-administration. Sensitization of the insulin receptor to insulin or stimulation of the β-cell of islets to restore plasma insulin level may also be the possible mechanisms of action of isoflavones derivatives. These compounds were further evaluated in the most popular model of type-2 diabetes i.e. db/db mice. Db/db mice exhibit an initial phase of hyperglycemia, hyperinsulinemia, hyperphagia and obesity. Interestingly, all these test compounds were found to lower the postprandial hyperglycemia as well as improve the glucose tolerance in db/db mice. The level of plasma lipids are usually raised in diabetic conditions and the results demonstrated these antihyperglycemic lead molecules produce a
significant decrease in plasma triglycerides and total cholesterol as well as significantly increase the level of cardio protective plasma HDL-cholesterol, which is desirable. As plasma insulin decreased in db/db mice as a result of treatment with these compounds; it is reasonable to infer that the effect of the treatment on hyperglycemia is not through an increase in insulin concentration. Thus there, might be the possibility that the compound improved the sensitivity to insulin or it could be presumed that their antihyperglycemic activity may be attributed to extra pancreatic effect which is needed to be explored.
4.4 Mechanism(s) of action of novel lead antidiabetic molecules from natural as well as synthetic origin

4.4.1 Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by dysregulation in carbohydrate, protein and lipid metabolism caused by the complete or relative insufficiency of insulin secretion and/or insulin action (O'Brien and Granner, 1996), leading to hyperglycemia. Insulin resistance underlies the pathogenesis of type 2 diabetes (DeFronzo et al., 1992). Gluconeogenesis is the main cause of the elevated hepatic glucose output in type-2 diabetes contributing 50-60% of the released glucose in hyperglycemia (Hundal et al., 2000). The rate of gluconeogenesis is regulated by the activity of the key gluconeogenic enzymes viz. Phosphoenolpyruvate Carboxykinase (PEPCK), Fructose-1, 6-bisphosphatase (F-1,6-BPase) and Glucose-6-Phosphatase (G-6-Pase) and elevated activities of these enzymes result in synthesis of new glucose molecules from non-carbohydrate sources like lactate, amino acids and glycerol. Alternatively, decrease in the activities of enzymes of the glycolysis pathway viz. Glucokinase, Pyruvate kinase and Phosphofructokinase lead to insufficient glucose uptake and utilization in the insulin dependent tissues, thus contributing to hyperglycemia. In addition, increased activity of the key enzyme of glycogenolysis (Glycogen phosphorylase) leads to break down of glycogen thereby releasing more glucose in the circulation.

The regulation of glucose metabolism in the liver: In the hepatocytes, insulin stimulates the utilization and storage of glucose as lipid and glycogen while repressing glucose synthesis and release. This is accomplished through a coordinated regulation of enzyme synthesis and activity. Insulin stimulates the expression of genes encoding glycolytic enzymes (glucokinase, phosphofructokinase and pyruvate kinase) and fatty-acid synthetic enzymes (acetyl-CoA carboxylase, fatty-acid synthase), while inhibiting the expression of those encoding gluconeogenic enzymes (glucose-6-phosphatase, fructose-1, 6-bisphosphatase, phosphoenolpyruvate carboxykinase). These effects are mediated by a series of transcription factors and co-factors, including sterol regulatory element binding protein (SREBP)-1, hepatic nuclear factor (HNF)-4, the forkhead protein family (Fox) and PPAR-γ co-activator-1 (PGC-1). The hormone also regulates the activities of some enzymes, such as glycogen synthase and citrate lyase, through changes in phosphorylation state. Results of the studies made in previous chapter indicate that the identified antidiabetic lead molecules ameliorated hyperglycemia, hyperinsulinemia and improved
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glucose tolerance in diabetic rats and db/db mice as well as improved various parameters of lipid metabolism including TG, CHOL and HDL-cholesterol. Therefore, the present study was designed to explore the precise biochemical mechanism(s) that could be responsible for improving hyperglycemia in the experimental animal models either by ameliorating insulin resistance or by targeting regulatory enzymes of glucose metabolism, thus decreasing hepatic glucose output or increasing peripheral glucose utilization.

In this chapter we tried to explore the mechanism of action of identified natural (K037, K041, 4-HPA and α-AA) and synthetic (RS-853 and DL-857 and their resolved enantiomers S-853/R-853 & D-857/L-857) antidiabetic lead molecules in db/db mice

4.4.2 Results

4.4.2.1 Effect of withanolides (K037 & K041) on regulatory enzymes of carbohydrate metabolism in db/db mice

Withanolide (K037 & K041) treatment at a dose of 100 mg/kg for the period of 10 days significantly increased the enzyme activity of glucokinase and phosphofructokinase by 27.7, 16.2 and 23.5, 27.5% respectively as compared to control (fig. 1). Withanolide (K037 & K041) also significantly increased the activity of pyruvate kinase by 19.8% and 26.2% as compared to control (fig. 2). Repeated oral administration of withanolides significantly decreased the enzyme activity of glycogen phosphorylase (fig. 2). Withanolide (K037 & K041) treatment also significantly decreased the enzyme activity of PEPCK, F-1,6 BPase and G-6-Pase by 17.3, 33.2, 26.9% and 31.9, 46.8 & 43.5% respectively as compared to control (fig. 3).

Figure 1: Hepatic glucokinase and phosphofructokinase activities in db/db mice treated with withanolides (K037 & K041). Values are expressed as mean ± SD; (*p<0.05 & **p<0.01 as compared to vehicle treated mice).
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Figure 2: Effect of withanolides (K037 & K041) on activity of pyruvate kinase and glycogen phosphorylase in db/db mice. Values are expressed as mean ± SD; (*p<0.05 & **p<0.01 as compared to control).

Figure 3: Effect of withanolides (K037 & K041) on gluconeogenic enzyme activities in db/db mice. Values are expressed as mean ± SD; (*p<0.05 & **p<0.01 as compared to control).

### 4.4.2.2 Effect of 4-HPA and α-AA on regulatory enzymes of carbohydrate metabolism in db/db mice

Figure 4, 5 & 6 presents the effect of 4-HPA and α-AA at a dose of 100 mg/kg body weight in db/db mice. Repeated oral administration of 4-HPA & α-AA for the period of 10 days significantly increased the enzyme activity of glucokinase, phosphofructokinase and pyruvate kinase by 18.7, 19.6, 15.8% and 25.0, 16.6, 6.89% respectively as compared to control. 4-HPA and α-AA treatment also significantly reduced the activity of glycogen phosphorylase enzymes by 46.8 and 27.8% as compared to control. In addition, 4-HPA & α-AA significantly reduced the activity of G-6-Pase, F-1,6-BPase and PEPCK by 43.9, 31.6, 23.6% and 56.9, 45.0, 28.2% respectively as compared to control.
Figure 4: Effect of 4-HPA and α-AA on glucokinase and phosphofructokinase activities in db/db mice. Values are expressed as mean ± SD; (*p<0.05 & **p<0.01 as compared to control).

Figure 5: Effect of 4-HPA and α-AA on pyruvate kinase and glycogen phosphorylase enzyme activity in db/db mice. Values are expressed as mean ± SD; (*p<0.05, **p<0.01 & ***p<0.001 as compared to vehicle-treated mice).

Figure 6: Effect of 4-HPA and α-AA on gluconeogenic enzymes in db/db mice. Values are expressed as mean ± SD; (**p<0.01 & ***p<0.001 as compared to control).
4.4.2.3 Effect of isoflavone derivative RS-853 and their resolved enantiomers S-853 & R-853 on regulatory enzyme of glucose metabolism in db/db mice

The treatment of isoflavone derivative RS-853 and their enantiomers S-853 & R-853 at a dose of 100 mg/kg body weight for the period of 10 days results in the increase in activity of GK (by 13.4, 21.6, 8.16%), PFKCK (by 15.2, 20.5, 15.6%) and PK (by 14.2, 20.1, 12.3 respectively as compared to control (Fig. 7, 8). Administration of isoflavone derivative RS-853 and its resolved enantiomers S-853 and R-853 also significantly reduced the glycogen phosphorylase activity by 25.2%, 31.2% and 17.7% respectively (Fig. 8). In addition, isoflavone derivative RS-853 and their enantiomers S-853 & R-853 treatment significantly reduced the enzyme activity of glucose-6-phosphatase (by 35.5, 53.9, and 41.4%), fructose-1,6-bisphosphatase (by 28.6, 36.8 and 24.4%) and phosphoenolpyruvate carboxykinase (by 24.3, 28.8 and 17.4%) respectively as compared to control (Fig. 9).

Figure 7: Effect of RS-853 and its enantiomers S-853 & R-853 on GK and PFK enzymes in db/db mice. Values are expressed as mean ± SD; (*p<0.05 & **p<0.01 vs. control).

Figure 8: Effect of isoflavones derivatives RS-853 and its resolved enantiomers S-853 & R-853 on pyruvate kinase and glycogen phosphorylase activity in db/db mice. Values are expressed as mean ± SD; (*p<0.05 & **p<0.01 as compared to control).
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4.4.2.4 Effect of isoflavones derivative DL-857 and its resolved enantiomers D-857 & L-857 on regulatory enzyme of glucose metabolism in db/db mice

Figure 10, 11 & 12 presents the effect of isoflavone derivative DL-857 and its resolved enantiomers D-857 & L-857 on enzyme activity of carbohydrate metabolism in db/db mice. The isoflavone derivative DL-857 and their enantiomers D-857 & L-857 at a dose of 100 mg/kg body weight for the period of 10 days significantly increased the enzyme activity of GK (by 15%, 25.3% and 14.9%), PFK (by 26.2%, 42.2% and 20.5%) and PK (by 18.2%, 26.1% and 9.20%) respectively (Fig. 10, 11). as well as significantly reduced the glycogen phosphorylase enzyme activity by 33.9%, 41.2% and 14.6% respectively as compared to control (fig. 11).

**Figure 9:** Effect of isoflavone derivative RS-853 and its resolved enantiomers S-853 & R-853 on gluconeogenic enzyme in db/db mice. Values are expressed as mean ± SD; (*p<0.05, **p<0.01 & ***p<0.001 as compared to control).

**Figure 10:** Effect of isoflavone derivatives DL-857 and its enantiomers D-857 & L-857 on gluconeogenic enzymes in db/db mice. Values are mean ± SD; (*p<0.05, **p<0.01 & ***p<0.001 as compared to control).
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Figure 11: Effect of isoflavone derivatives DL-857 and its enantiomers D-857 & L-857 on pyruvate kinase and glycogen phosphorylase enzymes activities db/db mice. Values are mean ± SD; (*p<0.05, **p<0.01 & ***p<0.001 as compared to control).

The activities of gluconeogenic enzyme were markedly elevated in control db/db mice group. Administration of isoflavone derivative DL-857 and its enantiomers D-857 & R-857 significantly reduced the enzyme activities of glucose-6-phosphatase (by 40.9%, 50.4% and 35.5%), fructose-1, 6-bisphosphatase (by 26.6%, 36.2% and 13.3%) and phosphoenolpyruvate carboxykinase (by 18.3%, 25.1% and 12.3%) respectively as compared to control (fig.12).

Figure 12: Effect of isoflavone derivatives DL-857 and its resolved enantiomers D-857 and L-857 on gluconeogenic enzymes activities in db/db mice. Values are mean ± SD; (*p<0.05, **p<0.01 & ***p<0.001 as compared to control).
4.4.3 Discussion

In diabetes mellitus, deficiency of or insensitivity to insulin causes derangement in carbohydrate metabolism. In the present study, the activities of the enzymes of glycolytic, glycogenolytic and gluconeogenic pathways were significantly altered in diabetic condition. The activity of regulatory enzymes of carbohydrate metabolism was measured, to find out whether these antihyperglycemic leads implicated as antihyperglycemic agent modulate key enzymes involved in glucose metabolism during its course of action. The severity of diabetic state as indicated by the degree of hyperglycemia bears a significant correlation with the alteration in hepatic glycolytic, glycogenolytic and gluconeogenic enzyme activity. The enzyme activity abnormality in the tissue of diabetic db/db mice has been well documented (Herberg and Coleman, 1977). Results of the present studies indicate lower levels of the hepatic glycolytic enzymes while elevated levels of hepatic gluconeogenic enzymes in the diabetic animals. These results are consistent with earlier published results (Grover et al., 2000; Raju et al., 2001; Rathi et al., 2002). Both these actions are directly responsible for decreased uptake and utilization of glucose in the liver.

In this study, we showed that the supplementation of antidiabetic lead molecules exerts an antidiabetic effect in type 2 diabetic mice, at least in part, by enhancing the hepatic glucose and lipid metabolism. The traditional focus of antidiabetic agents was mainly to reduce fasting plasma glucose but convincing evidence now exists showing the importance of controlling postprandial hyperglycemia (Avignon et al., 1997). The db/db mice at 12 weeks of age exhibited most of the human characteristics of type 2 diabetes including hyperglycemia in the fasting and fed states, hyperinsulinemia and insulin resistance, as already reported (Shafrir, 1992).

The increased hepatic glucose production is crucial to the maintenance of fasting and postprandial hyperglycemia (DeFronzo et al., 1992) and was observed in spite of high serum insulin levels in the db/db mice (Coleman and Hummel, 1967), indicating relative insensitivity of the liver to insulin. In this study we observed the hepatic G-6-Pase, F-1, 6-Pase and PEPCK activity were higher in the db/db mice. However, the supplementation of identified antidiabetic leads significantly lowered the G-6-Pase activity compared to the control db/db mice. The G-6-Pase is a key enzyme controlling hepatic gluconeogenesis and glucose output in liver (Nordlie et al., 1993; Hanson and Patel, 1994) and is normally suppressed by the action of insulin (Pilkis and Granner, 1992). The hepatic gluconeogenic enzyme activity in diabetic animals was found much higher as compared to the non
diabetic animals. These antidiabetic agents helped in the significant restoration of the elevated enzyme activity of glucose-6-phosphatase in the diabetic animals post treatment. Glucose-6-phosphatase enzyme plays an important role in glucose homeostasis in hepatic tissue (Berg et al., 2001). Activity of other two enzymes of gluconeogenesis i.e. fructose-1, 6-bisphosphatase and phosphoenolpyruvate carboxykinase was also found to be suppressed after treatment with these antihyperglycemic agents. Thus these antihyperglycemic agents may have acted by decreasing hepatic glucose production by gluconeogenic pathway. The hallmark of diabetes mellitus is an inability to control blood glucose level (Nathan, 1994). Maintenance of normoglycemia by therapeutic interventions, does not fully prevent the onset of micro-vascular complications and delays progression of complication in diabetes (Bolli, 1999). The results of the present study are indicative of the potential of the compounds (K037, K041, 4-HPA and α-amyrin acetate and synthetic isoflavone derivatives RS-853 and DL-857 and its resolved enantiomers) in reversing the alterations in the activity of enzymes playing key roles in synthesis and degradation of glucose that are largely responsible for maintaining hypoglycaemic condition.
4.5 PPARs and LXRs agonist activity of selected antidiabetics

4.5.1 Introduction

Type 2 diabetes accounts for >90% of the cases of diabetes and is caused by defective insulin secretion and insulin resistance. Type 2 diabetes is often of polygenic origin, but the molecular defects are still not fully known. However, the genetic background of maturity-onset type 2 diabetes of the young is known. A class of nuclear receptors has recently become the focus for its important role in cholesterol, lipid, and carbohydrate metabolism, namely the peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR). The main target tissues for PPAR and LXR action are liver and adipose tissue, where their functions have been studied in detail. Recent studies with these two nuclear receptors have suggested their role in lipid and carbohydrate metabolism in muscle as well. Important target tissues of insulin action are also liver, muscle, and adipose tissue, where insulin stimulates the uptake of excess glucose from blood and inhibits hepatic glucose production. A salient feature of type 2 diabetes is insulin resistance in these tissues, which leads to hyperglycemia and hyperlipidemia, both pathological conditions.

NRs are typically organized in main structural and functional domains. The amino-terminal A/B domain contains a ligand-independent transactivation function. It has been shown that its phosphorylation state contributes to the modulation of NRs activity (Hu et al., 1996; Shalev et al., 1996; Zhang et al., 1996; Adams et al., 1997; Camp and Tafuri, 1997; Juge-Aubry et al., 1999). The central DNA-binding domain (C domain) is highly conserved, with its two zinc finger-like structures and its α-helical DNA-binding motif. The ligand-binding domain (E/F domain; LBD) contains the ligand-dependent transactivation function. The interaction of NRs with their ligands, because of the conformational changes that are induced, allows the recruitment of co-activators, such as steroid receptor coactivator-1 (Onate et al., 1995; Wahl, 1997) CREB-binding protein (Dowell et al., 1997), and the release of co-repressors, such as nuclear receptor co-repressor and silencing mediator of retinoid and thyroid hormone receptor (DiRenzo et al., 1997; Zamir et al., 1997; Dowell et al., 1999). Their ligand-dependent activity makes nuclear receptors good pharmacological targets.
Fig. 1: Schematic representation of the functional domains of NRs. NRs is composed of four distinct functional regions. The A/B domain located at N-terminal with AF-1 is responsible for phosphorylation, the domain C is implicated in DNA binding, domain D is the docking region for cofactors and domain E/F is the ligand specific domain, containing AF-2, which promotes the recruitment of cofactors required for the gene transcription.

Table 1: Nuclear receptors involved in glucose and lipid metabolisms

<table>
<thead>
<tr>
<th>Nuclear receptor subtypes</th>
<th>Distribution</th>
<th>Physiological involvement</th>
</tr>
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<tbody>
<tr>
<td>PPAR-α</td>
<td>Liver, kidney, heart, skeletal muscle, adipose tissue</td>
<td>Lipid catabolism and oxidation, gluconeogenesis</td>
</tr>
<tr>
<td>PPAR-β</td>
<td>Ubiquitous</td>
<td>Adipocyte differentiation</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Adipose tissue, muscle, large intestine, haematopoietic cells, liver</td>
<td>Glucose and fatty acid uptake, gluconeogenesis, lipogenesis, glycogenesis, adipocyte differentiation, macrophage maturation, modulation of inflammation</td>
</tr>
<tr>
<td>LXR-α</td>
<td>Liver, intestine, adipose tissue, spleen and macrophages</td>
<td>Role in lipid and cholesterol metabolism, atherosclerosis</td>
</tr>
<tr>
<td>LXR-β</td>
<td>Ubiquitous</td>
<td></td>
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PPAR: peroxisome proliferator-activated receptors, LXR: Liver X Receptor

i) Peroxisome proliferator-activated receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) are ligand-inducible transcription factors that belong to the nuclear hormone receptor superfamily. PPARs play essential roles in the regulation of cellular differentiation, development, and metabolism (carbohydrate, lipid, and protein) of higher organisms. Transcriptional regulation by PPARs is achieved through PPAR-RXR heterodimerisation. Dimerization is essential for the activity of PPARs, as it is for most of the other members of the nuclear hormone receptor superfamily. They heterodimerize with the 9-cis retinoid X receptor (RXR),
forming a complex that is able to bind to the PPAR response elements located in the promoter of PPAR target genes. PPARs regulate the expression of target genes involved in a variety of important physiological pathways such as lipid metabolism, insulin sensitivity, cell differentiation, inflammation (Willson et al., 2000; Moraes et al., 2006).

Figure 2: Activation of the PPAR receptors leads to an accumulation in the nucleus, where they heterodimerize with RXR. The PPAR: RXR heterodimer binds to DNA sequences called PPAR response elements (PPRE), leading to the transcription of the responsive gene. Activation of PPAR-α can cause peroxisome proliferation, and increased lipid metabolism; PPAR-γ causes cellular lipid accumulation in susceptible cells and the down regulation of monocyte activation by cytokines; while the function of PPAR-β is not known.

ii) Liver X Receptor

Liver X receptors are ligand-inducible transcription factors that belong to the nuclear hormone receptor superfamily. LXRs appear to serve as key sensors of intracellular sterol levels by regulating the expression of genes that control cholesterol absorption, storage, transport, and elimination. LXRs are also involved in fatty acid metabolism by their ability to increase the expression of sterol regulatory element-binding protein 1c (SREBP-1c). These findings define LXRs as potential therapeutic targets for the treatment of lipid disorders. One particular therapeutic target, known as transcription factor liver X receptor (LXR), regulates Reverse cholesterol transport (RCT) by functioning as a sensor of cholesterol levels in tissues (Repa and Mangelsdorf, 2002; Tontonoz and Mangelsdorf, 2003; Kino and Chrousos, 2004). At present, two LXR isoforms have been characterized.
LXR-α is primarily expressed in the liver, intestine, adipose tissue, spleen and macrophages, whilst LXR-β is ubiquitously expressed (Repa and Mangelsdorf, 2002).

The ligand-activation of the LXR transcription factors results in the formation of obligate heterodimers with the retinoid X receptor. The complexes subsequently bind to the consensus response element sequence DR4 in the promoter regions of several target genes, including cholesterol 7α-hydroxylase, cholesterol ester-transfer protein, ATP-binding cassette (ABC) transporter proteins (ABCA1, ABCG5 and ABCG8), apolipoprotein E (apoE), lipoprotein lipase and SREBP-1c (Mohan and Heyman, 2003; Kino and Chrousos, 2004).

**Figure 3:** Model describing the LXR regulatory pathways governing lipid metabolism. In liver, intestine, and macrophages, LXRs regulate the expression of genes involved in bile acid synthesis, fatty acid synthesis, and cholesterol efflux and transport. LXRs sense intracellular cholesterol levels to help maintain cholesterol homeostasis by increasing bile acid excretion, reducing cholesterol absorption, and increasing cholesterol transport back to the liver for catabolism and storage. (C, cholesterol; CE, cholesterol ester; BA, bile acid; FA, fatty acid; TG, triglyceride; HDL, high density lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein.

In this chapter we study, whether the antidiabetic and antidyslipidemic properties of plant based synthetic isoflavone derivatives and chemical entities isolated from *Ficus bengelensis, Peganum harmala* and *Withania coagulans* could be mediated through PPAR and LXR activation, similar to the results obtained with many plant-derived products. We also confirmed the anti-adipogenic and glucose uptake properties of these compounds in
3T3 L1 cell line. Since PPAR and LXR are the therapeutic targets for cholesterolemia, hyperglyceridemia and insulin resistance.

4.5.2 Results

4.5.2.1 PPAR-α luciferase activity of synthetic and natural compounds

In the present study, we evaluated the PPAR-alpha luciferase activity of synthetic and natural compounds in COS-7 cells. The COS-7 cells treated with compounds, S-0516, S-0517, S-1405, K041 and S-1084 at 0.1μM and 1μM concentration, after 48 hour of treatment cells were lysed and the luciferase activity was measured. The compounds S-0516, S-0517 and S-1084 showed significant PPAR-α luciferase activity by 1.49, 1.80 and 1.64 fold at 1μM concentration as compared to vehicle (DMSO) treated control, whereas the treatment of positive control GW-7647 at 1μM concentration confer 1.93 fold PPAR-α luciferase activity (fig. 4).

![Figure 4: The effect of synthetic isoflavone derivatives and natural products on PPAR-α luciferase activity in COS-7 cells. Values are expressed as mean ± SD, n=3. p<0.05 (*), p<0.01 (**) and p<0.001 (***) as compared to vehicle treated control.](image)

4.5.2.2 PPAR-γ luciferase activity in COS-7 cells

COS-7 (1x10^6 cells/well) cells were transfected with the reporter plasmid (PPRE-luc), pCMX-RXR expression plasmid, expression plasmid (pCMX-PPAR-γ plasmid), and the internal control plasmid (pRL-TK-luc plasmid). The transfected cells were treated with
phytoconstituents (K041, K037, α-AA and 4-HPA) and isoflavone derivatives (RS-853/S-853 & R-853 and DL-857/D-857 & L-857) at 5 μM concentration. Rosiglitazone was used as positive control and DMSO as a vehicle. Among the tested compounds none of the showed PPAR-γ luciferase activity over vehicle treated control (fig. 5 & 6).

**Figure 5:** The effect of isolated phytoconstituents (4-HPA, α-AA, K037 & K041) on PPAR-γ luciferase activity in COS-7 cells. Values are expressed as mean ± SD.

![Graph](image)

**Figure 6:** The effect of isoflavone derivatives (RS-853/S-853 & R-853 and DL-857/D-857 & L-857) on PPAR-γ luciferase activity in COS-7 cells.

4.5.2.3 LXR-α luciferase activity

After transfection cells were treated with positive control (GW-3965) at 1μM concentration and test compounds (0516, 0517, 1084 and K041) at 0.1 μM and 1.0 μM concentration and then cells were lysed after 48 hr treatment and measured the reporter
luciferase activity. All the treated compounds increased LXR-α reporter luciferase activity significantly over vehicle treated control (fig.7).

**Figure 7**: The effect of synthetic isoflavone derivatives and phytochemicals on LXR activation. Luciferase activity was regulated by the PPRE-containing reporter plasmid and was cotransfected into COS-7 cells using the LXR-α expression plasmids. Cells were incubated with 1µM of GW-3965 as a positive control and test compounds (0516, 0517, 1084 and K041) at 0.1µM and 1.0 µM concentration, or DMSO as a vehicle. Data are expressed as the means ± SD derived from three experiments (p<0.05 (*) and p<0.01 (**) as compared to vehicle treated control).

4.5.2.4 Effects of phytoconstituents on the differentiation of 3T3-L1 preadipocytes

3T3-L1 preadipocytes were differentiated to adipocytes using insulin, dexamethasone, and IBMX. The differentiation protocol required 8 days to obtain >80% adipocyte differentiation in culture. First the effect of phytoconstituents on 3T3-L1 cell viability was assessed under adipocyte differentiation conditions. Fig. 8A shows that treatment of 3T3-L1 preadipocytes with 1–10 mM K041, K037, 4-HPA and α-amyrin acetate was used for the entire period of differentiation and compared with
control cells differentiated without test compound. At the end of 8 days incubation, lipid-bound Oil Red O was extracted, and optical density of samples was determined at 490 nm. Fig. 3B showed that K041 significantly inhibited the adipogenesis of 3T3-L1 preadipocytes by 8.90%, 18.7% (P < 0.05), and 33.4% (P < 0.01) at 1, 5, and 10 mM, respectively, compared to control cells (Fig.5B). Other compounds α-amyrin acetate and 4-hydroxypipelic acid showed moderate anti-adipogenic activity (fig. 8B).

![Graph-image](image-url)

**Fig. 8A**: Effect of incubation with elevated levels of different phytochemicals, on the MTT assay for 3T3-L1 cells. There were no differences in the results of the MTT assay for the wells containing cells treated with any of the various concentrations of phytochemicals K041, K037, α-AA and 4-HPA.
Figure 8B: Phytoconstituents of different plant inhibits adipogenesis of 3T3-L1 cells; Adipogenesis under various treatment conditions was quantitated by extracting lipid-bound Oil Red O and optical density of samples were determined at 490 nm as described in materials and methods. Data are expressed as percent inhibition in OD by different phytochemicals treatment over control.
4.5.2.5 Effect on glucose uptake in L6 cells

These phytochemicals (K041, K037, 4-HPA & α-AA) also screened for glucose uptake in L6 cells, out of them only withanolide K041 significantly increased (by 89.9%, p<0.01) glucose uptake in L6 cells. 4-hydroxypipecolic acid also had moderate glucose uptake potential (by 66.7%, p<0.05) in L6 cells over vehicle (DMSO) treated control as shown in fig 9.

![Glucose Uptake (L6 cells)](image)

**Figure 9:** Effect of phytoconstituents (K041, K037, 4-HPA & α-AA) on 2-deoxyglucose uptake. L6 myotubes were incubated for 24 hr with 5 μg/ml concentration of different test samples or 400 μM of metformin at 37 °C. 1μCi/well of ³H-2-deoxyglucose (10 μM) was added for 10 min and uptake was measured as described in materials and methods. Values are mean ± SE of three independent experiments. *p<0.05, **p<0.001 vs. vehicle treated control.

4.5.3 Discussion

Nuclear receptors (NR) are a superfamily of ligand-activated transcription factors that regulate development, reproduction, metabolism of lipids, drugs and energy. The importance of this family of proteins in metabolic diseases is exemplified by NR agonists and antagonists used in the clinic or under drugs development for the treatment of diabetes mellitus, dyslipidemia, hypercholesterolemia, or other metabolic abnormalities.

The aim of our study was to evaluate the PPARs and LXRs mediated antidiabetic antidyslipidemic and antiadipogenic action of pytochemicals and synthetic isoflavone derivatives. Our results indicate that the synthetic compounds S-0516, S-0517, 1084 and one of the natural product K041 significantly activates PPAR-α and LXR-α nuclear receptors. In separate study, we also observed the PPAR-γ activity of some chemical entities (α-amyrin acetate, 4-hydroxypipecolic acid and withanolide K041&K037) and
synthetic isoflavone derivatives (RS-853/S-853 & R-853 and DL-857/D-857 & L-857) in COS-7 cells, it was found none of the compounds activate PPAR-γ nuclear receptor over vehicle treated control. In addition, phytochemicals isolated from different plants also moderately inhibits differentiation of 3T3-L1 preadipocytes into adipocytes. Since 3T3-L1 differentiation to adipocyte is accomplished by treatment with a mixture of insulin, dexamethasone, and IBMX, whether most potent compound K041 specifically blocks any one or all of them need to be determined. K041 also have glucose uptake potential in 3T3 L1 pradipocytes.

The antidiabetic and antidyslipidemic action of these compounds might be through the following pathway discussed here. LXR is a type II nuclear receptor that consists of two isoforms, LXR-α and LXR-β. The former is important for the regulation of cholesterol metabolism, and is expressed in the liver, spleen, adipose tissue, lung, and pituitary gland, whereas the latter is expressed ubiquitously. Activated LXR-α stimulates transcription target genes which are involved in lipid and cholesterol metabolism like cholesterol 7α-hydroxylase (CYP7A1), cholesterol ester–transfer protein (CETP), ATP-binding cassette proteins (ABC), apolipoprotein-E (ApoE), lipoprotein lipase (LPL), and sterol response element-binding protein 1c (SREBP-1c). Through transcriptional regulation of these and other target molecules, LXR-α decreases circulating LDL and tissue cholesterol by 1) facilitating cholesterol excretion in the gall bladder and catabolism through bile acid formation in the liver, 2) reducing cholesterol absorption in the intestine, and 3) promoting cholesterol efflux from peripheral tissues such as resident macrophages (Fig. 1) (Repa and Mangelsdorf, 2000; Tontonoz and Mangelsdorf, 2003). LXR-α agonists also increase circulating levels of HDL cholesterol by stimulating the expression of ABCA1, apoE, and phospholipids transfer protein (PLTP) (Jiang et al., 2003). Administration of LXR-α agonists in addition to conventional cholesterol-lowering compounds could produce added benefit in the treatment/ prevention of atherosclerosis.

The apparent beneficial effects of LXR-α agonists on lipid metabolism, including a decrease of LDL and an increase of HDL, however, are associated with increased lipogenesis and production of triglycerides in the liver with resultant hypertriglyceridemia, an independent risk factor for atherosclerosis (Fig 1) (Schultz et al., 2000; Tontonoz and Mangelsdorf, 2003). This activity of LXR-α is mediated by its induction of SREBP-1c, fatty acid synthase (FAS), sterol coenzyme A desaturase I (SCD-1), and acyl coenzyme A carboxylase (ACC) via direct activation of their promotors (Tontonoz and Mangelsdorf, 2003). SREBP-1c, a helix-loop-helix type transcription factor, plays a central role in LXR-
α-mediated lipogenesis by stimulating the transcription rates of several genes whose products are associated with fatty acid metabolism (Schultz et al., 2000).

Recently, Beyer et al. attempted to diminish the pro-lipogenesis and hypertriglyceridemia effect of LXR-α agonists by co-administering peroxisome proliferator activator receptor-α (PPAR-α) agonists, known anti-lipogenic compounds (Beyer et al., 2004; Bocher et al., 2002). Like the LXRs, PPAR-α belongs to the nuclear receptor superfamily and is distributed in tissues that have high lipid-metabolizing activity, such as the liver, brown fat, kidney, heart and skeletal muscles. Fatty acids, eicosanoids, and the fibrates (e.g., fenofibrate, clofibrate, and WY14643), a well-known class of hypolipidemic drugs, are PPAR-α ligands (Chiang, 2002). As with the LXRs, PPAR-α stimulates the transcriptional activity of its responsive genes by binding to its consensus sequence, DR1, located in their promoter regions. PPAR-α activates β-oxidation in the liver, generating energy by catabolizing fatty acids, by increasing the transcription rates of the genes of several enzymes involved in this process. Furthermore, PPAR-α increases the production of the apolipoproteins apoAV and apoCIII, which results in decreased levels of triglycerides in the circulation (Bocher et al., 2002). Thus, activation of PPAR-α reduces the fatty acid content of the liver and the circulating levels of triglycerides—exactly the reverse of the effects of LXR-α activation (Fig. 1). Some of the PPAR-α agonists also slightly increase HDL cholesterol in humans (Staels et al., 1998), thus working in the same direction with the LXR-α agonists.