Synopsis

Adipocyte plays a major role in regulating energy homeostasis both by acting as a fat reservoir and an endocrine organ. The formation of adipocytes from precursor cells, termed as adipogenesis is a coordinated process orchestrated by various signaling pathways and the transcriptional machinery. Adipogenesis is mainly accompanied by morphological changes, difference in gene expression and insulin sensitivity. A transcriptional cascade is initiated by different signals emanating from intracellular and extra cellular environment resulting in the expression of various target genes involved in the lipid and glucose metabolism. Peroxisome proliferator-activated receptor gamma (PPARγ) is the key transcription factor that regulates this process. Signalling pathways mediated by insulin receptor (IR/Akt) and extra cellular signal regulated kinase play a major role in this process. Adenosine monophosphate-activated protein kinase (AMPK) and β–catenin signaling are known to negatively regulate adipocyte differentiation.

The proper functioning of adipocytes is very crucial for maintaining normal physiological condition. Any dysfunction to adipocyte formation leads to serious metabolic complications like obesity, insulin resistance, type 2 diabetes and cardiovascular diseases. Expanded adipose tissue (obese state) secretes proinflammatory adipocytokines such as Interleukin 6 (IL-6), Monocyte chemoattractant protein-1 (MCP-1), Tumor necrosis factor-alpha (TNF–alpha), resistin and visfatin which ultimately leads to whole body insulin resistance and type 2 diabetes. The chronic inflammation of adipocytes leads to the activation of hormone sensitive lipase resulting in the release of free fatty acids. Studies have shown that excess free fatty acids can induce insulin resistance, a hallmark of type 2 diabetes. In addition to this, obese state also leads to hyperleptinemia which is considered as an important factor for developing type 2 diabetes.

The present study focuses on the identification and functional characterization of small molecule modulators of adipogenesis. Compounds that regulates adipogenesis and adipokine expression will not only serves as a tool to understand the hitherto unknown mechanisms of adipogenesis but also will help us to identify lead compounds that may combat obesity and related disorders like insulin resistance and type 2 diabetes.
Screening for modulators of adipogenesis

A set of synthetic and natural compounds were used for preliminary screening of the modulators of adipocyte differentiation. 3T3-L1 cell line, an ideal model system for understanding adipocyte development was used for this study. This fibroblast preadipocytes has the potential to differentiate into adipocytes, when the key signaling pathways are activated. Oil Red O analysis was used for screening modulators of adipogenesis. A few compounds have shown modulatory effect on adipogenesis. Vanillin and 2, 4 diacetyl resorcinol were identified as positive modulators whereas ellagic acid and arjunolic acid were found to be negatively regulating adipogenesis.

Vanillin induces adipocyte differentiation in 3T3-L1 preadipocytes.

Vanillin is the primary component of vanilla beans used as flavoring agents in foods, beverages and pharmaceuticals. In order to understand the effect of vanillin in adipocyte differentiation, 3T3-L1 cells were treated with various concentrations of Vanillin and measured the rate of adipogenesis. A dose dependent effect of vanillin on adipogenesis was detected with a maximum increase of 2.3 fold at a concentration of 500μM. In order to confirm this inducing effect of vanillin on adipocyte differentiation, expression levels of key adipogenesis marker proteins such as PPARγ and glucose transporter 4 (GLUT4) were analysed. It was found that PPARγ and GLUT4 expression were increased during vanillin mediated adipogenesis in a day dependent manner. Induction with vanillin resulted in a 1.6 fold increase in the expression of PPARγ and a 1.3 fold increase in the expression of GLUT4. Insulin sensitivity is a characteristic feature of fully differntiated adipocytes. To analyse insulin sensitivity of vanillin differentiated cells, glucose uptake assay was performed. Under insulin stimulated condition a 3.1 fold and 2.8 fold increases in glucose uptake over basal vehicle treatment was observed in cells differentiated using MDI and vanillin respectively. Adiponectin expression in known to promote insulin sensitivity. The effect of vanillin on adiponectin expression was analysed using real time polymerase chain reaction and a 2.7 fold increase in expression was observed. In order to understand the molecular mechanism involved in vanillin mediated differentiation, the phosphorylation status of key molecules in the signaling pathways were analyzed. ERK 42/22 was activated in the initial hour of adipogenesis while Akt failed to show any activation. ERK inhibitor,U0126, markedly reduced vanillin mediated adipogenesis whereas Akt inhibitor did not show any effect in this process. These results suggest that vanillin induces adipogenesis in 3T3-L1 cells in an ERK 42/22 dependent manner. Further studies were done to identify the upstream molecules involved
in vanillin signaling. Vanillin has been shown to elicit specific responses in different cell types through its interaction with distinct membrane receptors. Therefore we hypothesized that vanillin mediates its effects by binding to some specific cell surface receptor. Affinity chromatography was performed with vanillin coated beads using 3T3-L1 cell lysate to identify the putative receptor protein. The vanillin bound proteins were eluted from the beads and subjected to mass spectrometry analysis to identify protein/s bound to the column. A protein called olfactory receptor 56 was identified as a putative receptor for vanillin. To know whether vanillin binds to this receptor, a bioinformatics approach was resorted to and a homology model of this receptor was made using rhodopsin receptor as a template. Docking analysis with the model identified that, vanillin binds to the predicted ligand binding pocket of olfactory receptor 56. In addition to this, a proteomic analysis was used to identify the global protein expression changes in adipocytes upon vanillin treatment. We have identified several proteins that are regulated by vanillin and further characterization of these proteins are in progress. In conclusion, this part of the study identified vanillin as a positive regulator of adipogenesis and also identified a novel role for olfactory receptors in adipocyte differentiation.

2, 4 diacetyl resorcinol increases adipogenesis in 3T3-L1 cells.

2, 4 diacetyl resorcinol was one of the small synthetic compound synthesized by our chemistry division and no studies have been carried out to understand its biological activity. To understand its adipogenic activity, this molecule was included in our preliminary screening and it was found to increase the rate of adipogenesis. 2, 4 diacetyl resorcinol when added along with adipocytes differentiation medium has shown a maximum of 1.6 fold increase in triglyceride accumulation at 100μM. This modulatory effect of 2, 4 DAR on adipocyte differentiation was evidenced by an increase in GLUT4, a target gene of PPARγ. This observation led to speculate whether 2, 4 DAR is a possible agonist of PPARγ. To analyse this possibility, a docking study of 2, 4 DAR with the ligand binding domain (LBD) of PPARγ was performed. The analysis has shown that 2, 4 DAR binds to the LBD of PPARγ and the interacting residues were very similar to the known ligands of PPARγ. 2, 4 DAR enhances insulin sensitivity as evidenced by an increase in insulin induced glucose uptake in 2, 4 DAR treated adipocytes. The expression of anti-inflammatory and insulin sensitizing protein adiponectin was increased by 2 fold, upon 2, 4 DAR treatment on mature adipocytes. Adiponectin expression was known to be increased upon PPAR γ activation. These data together suggest that 2, 4 DAR exerts its
effects possibly by acting as a PPARγ agonist.

**Ellagic acid decreases adipocyte differentiation by activating AMPK.**

Ellagic acid (EA) was the second negative modulator of adipogenesis identified in our screening. It is a natural antioxidant found in different berries and plant foods. It is well known for its anticancer, antiatherogenic and anti-thrombotic properties. In this study EA has shown an anti-adipogenic effect in 3T3-L1 cells. This decrease in adipogenesis was evidenced by a 37% reduction in lipid accumulation at 10μM and a significant down regulation of key adipogenic regulators PPARγ, CCA-AT/enhancer binding protein alpha (CEBP alpha), Sterol regulatory element-binding protein 1 (SREBP-1) and GLUT4. Unlike AA (Arjunolic acid) 3T3-L1 cells with EA resulted in the ERK 42/22 inhibition in the initial hour of adipogenesis followed by AMPK activation. Activation of ERK 42/22 during the induction of differentiation enhances mitotic clonal expansion leading to adipogenesis. AMPK activation is known to inhibit adipocyte differentiation and lipolysis in mature adipocytes. These results suggest that EA mediates its antiadipogenic effect by inhibiting ERK 42/22 and activating AMPK. Since AMPK is known to reduce lipolysis in adipocytes, the expression of hormone sensitive lipase, the key enzyme involved in lipolysis was analysed. EA significantly reduced hormone sensitive lipase expression. The secretion of different inflammatory cytokines are known to increase from both fat cell and non fat cell during adipose hypertrophy leading to insulin resistance. EA reduced the expression of inflammatory adipokines such as MCP-1, IL-6, and TNF alpha, in both Raw macrophages and adipocytes. The expression of other adipokines related to insulin resistance, leptin, adiponectin and resistin were analysed. Leptin, resistin and adiponectin expression was also modulated by ellagic acid. Together this data suggest that EA has the ability to not only reduce adipogenesis but also to inhibit the obesity related inflammatory status that contributes to insulin resistance.

**Arjunolic acid downregulates adipocyte differentiation in 3T3-L1 cells.**

Arjunolic acid (AA) a triterpenoid is known for its anti-inflammatory and anticancer activity but there are no reports on its effect on adipogenesis. In the preliminary screening, AA was found to reduce the adipocyte differentiation. A maximum of 30% reduction in lipid accumulation was observed at 50μM. In order to confirm this observation, the expression levels of known adipocyte markers such
as PPARγ and GLUT4 were analysed. A significant reduction in the expression levels of these marker proteins were noticed using western blots. Further studies were carried out to understand how AA mediates its antiadipogenic effect. A prolonged activation of ERK 42/22 was observed with no significant difference in Akt activation. ERK 42/22 is known to have a biphasic effect on adipogenesis with an increase in ERK 42/22 phosphorylation during the initial hour of adipocyte differentiation and a subsequent gradual reduction in its expression. However, a sustained ERK 42/22 activation reduces adipogenesis. Studies in different cell types have shown that prolonged activation of ERK 42/44 leads to the inhibition of Glycogen synthase kinase-3β (GSK-3β) activity. To know whether sustained activation of ERK 42/44 leads to GSK-3β inhibition, the phosphorylation status of GSK-3β (Ser 9) was analysed. It was observed that AA significantly increased GSK-3β phosphorylation resulting in the reduction of its activity. It is known that GSK-3β phosphorylates β-catenin and targets it for degradation by the ubiquitin-proteasomal system. Inhibition of GSK-3 activity leads to accumulation of β-catenin in the cytoplasm and its subsequent translocation to the nucleus and activation of target genes that inhibits adipogenesis. Since GSK-3 inhibition was observed, the expression level of β-catenin was analysed. AA treatment has stabilized β-catenin expression. Together these data suggest that AA inhibits GSK-3β and this inhibition facilitates both ERK 42/22 activation and β-catenin stabilization leading to reduction of adipocyte differentiation. Expansion of adipocytes is accompanied with an increase in secretion of inflammatory molecules. It is highly probable that AA being an anti-adipogenic and anti-inflammatory molecule, it could reduce the expression of inflammatory adipokines, therefore a real time polymerase chain reaction was performed to analyse adipokine expression in AA treated 3T3-L1 adipocytes. TNF-alpha, IL-6, MCP-1 expression were decreased upon AA treatment in 3T3-L1 adipocytes. This part of the study thus report the identification of AA as the negative modulator of adipogenesis and the characterisation of molecular mechanism suggest the involvement of GSK-3-β-catenin pathway. Additionally, real time PCR data suggest that AA reduce the expression levels of anti-inflammatory adipokines.

In conclusion a collection of small molecules was screened for modulators of adipocyte differentiation. Vanillin and 2,4-D were found to increase adipogenesis whereas AA and EA exerts an inhibitory effect on adipocyte differentiation. Further studies to elucidate the signaling pathways regulated by these molecules suggested that each of these molecule used distinct molecular mechanism to mediate its modulatory effect on adipogenesis.