Chapter 3

Results and Discussions
3.1 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. When the key signalling pathways are activated, preadipocytes which are fibroblast in nature differentiates into adipocytes. A cocktail containing dexamethasone, insulin and IBMX were used for inducing adipogenesis. A total of 25 different synthetic and natural compounds were used for the study. Initially an MTT assay was carried out to find the toxic concentration of these compounds (Data not shown). Based on the data from toxicity assay, 3 different concentrations for each compound was tested for its effect on adipogenic activity. Compounds were treated alone and also along with the adipogenic cocktail to identify both positive and negative modulators.

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. The representative images of the screening are shown below (Fig 3.1).
Fig 3.1 The modulatory effect of various compounds on adipogenesis in 3T3-L1 adipocyte. Varying concentrations of compound were treated with and without MDI for 2 days. On 8th day of differentiation, cells were stained with Oil Red O, and the lipid accumulation was quantified by measuring the OD of Oil Red O eluted from the cells at 495 nm. Error bars represent mean values ± SD of 3 independent experiments done in triplicate. ( a represents P< 0.05 versus B, b represents P< 0.05 versus MDI, C represents P> 0.05 versus B, d represents P> 0.05 versus MDI. c and d fails to reject null hypothesis that there are no difference between group B and others as well as group MDI and others.
3.2 Functional Characterisation of Positive Modulators Vanillin and 2, 4 Diacetyl Resorcinol

3.2.1 Vanillin is a positive modulator of Adipocyte differentiation

3.2.1.1 Vanillin Induced Adipocyte Differentiation in 3T3-L1 Preadipocyte

Vanillin is a phenolic aldehyde (Fig 3.2.1C) It is mainly used as flavouring agent in foods, beverages, and pharmaceuticals. To evaluate the effect of vanillin on adipocyte differentiation, 3T3-L1 preadipocytes were treated with different concentrations of vanillin for 2 days (Fig 3.2.1A). Cells treated with MDI were used as positive control and DMSO as negative control. After 8 days, morphological changes and increased lipid accumulation were observed in these cells (Fig 3.2.1A). Lipid accumulated cells were stained with Oil Red O and the rate of lipid accumulation was quantified. Vanillin induced lipid accumulation in a concentration dependent manner with a maximum 2.3 fold at 500μM concentration as compared to basal (Fig 3.2.1.B).

![Fig 3.2.1 Effect of vanillin on the differentiation of 3T3-L1 adipocytes.](image)

(A) Phase contrast images showing the morphology and intracellular lipid accumulation of differentiated cells treated with varying concentrations of vanillin (50 μM, 100 μM, 250 μM, and 500 μM), DMSO or MDI (10% FBS, 1 μg/ml insulin, 250 nM dexamethasone, 500 μM IBMX). (B) On 8th day of differentiation, cells were stained with Oil Red O, and the lipid accumulation was quantified by measuring the OD of Oil Red O eluted from the cells at 495 nm. (C) Structure of vanillin. Error bars represents mean values ± SD of three independent experiments done in triplicates.
3.2.1.2 Vanillin Increased Expression of Key Adipogenic Regulator PPARγ and its Target Gene GLUT4

The effect of vanillin on the expression of PPARγ, the key regulator of adipocyte differentiation and its target gene GLUT4 were analysed using western blot. The expression of GLUT4 and PPARγ were analysed on day 2, 4 and 6 after vanillin (500μM) or MDI treatment. Treatment of 3T3-L1 cells with vanillin increased the expression of PPARγ (Fig. 3.2.2A) and its target gene GLUT4 in a day dependent manner (Fig 3.2.2B). Induction with vanillin resulted in a 1.6 fold increase in the expression of PPARγ (Fig. 3.2.2C) and 1.3 fold increase in the expression of GLUT4 (Fig 3.2.2D) on day 6 of differentiation which is almost similar to that observed upon MDI treatment (Fig 3.2.2C, Fig3.2. 2D).

Fig 3.2.2 Effect of vanillin on PPARγ and GLUT4 expression. The cells were treated with DMSO, MDI (10% FBS, 1 μg/ml insulin, 250 nM dexamethasone, 500 μM IBMX) or 500 μM vanillin at two day post confluence (day 0). The cells were harvested 2, 4 and 6 days post treatment, lysed and immunoblotted using PPARγ (A) and GLUT4 (B) antibodies. Actin was used as a loading control. Representative blots from three independent experiments are shown. The graph represents the band intensities of PPARγ (C) and GLUT4 (D) normalized to actin. Error bars denote mean values ± SD.
3.2.1.3 3T3-L1 Preadipocytes Differentiated by Vanillin is Insulin Sensitive

An insulin mediated glucose uptake assay was done to find out whether the cells differentiated by vanillin are insulin sensitive in nature. For this, glucose uptake assay was done (as described in materials and methods) using vanillin (500 μM) differentiated cells, cells differentiated using MDI and DMSO were used as positive and negative controls respectively. In basal condition the cells differentiated using MDI and vanillin showed a 1.9 fold and 1.7 fold increase in glucose uptake respectively as compared to cells differentiated using DMSO (Fig 3.2.3). Under insulin stimulated condition a 3.1 fold and 2.8 fold increase in glucose uptake was observed in cells differentiated using MDI and vanillin respectively. These results suggest that adipocyte differentiated by vanillin retained its insulin sensitivity (Fig 3.2.3).

![Graph showing glucose uptake](image)

Fig. 3.2.3 Vanillin induced differentiated adipocytes are insulin sensitive. Two days post confluent 3T3-L1 preadipocytes were treated with DMSO, MDI (10% FBS, 1 μg/ml insulin, 250 nM dexamethasone, 500 μM IBMX) or vanillin (500 μM). Medium was changed every 2 days. After 8 days of differentiation, glucose uptake was carried out. The insulin used for the stimulation was 100 nM. The fold difference in the glucose uptake under different treatments was determined. The bar represents mean values ± SD from three independent experiments in triplicates.

3.2.1.4. Vanillin Activates the Phosphorylation of Extracellular Signal Regulated Kinase 42/44

We have shown that vanillin increases the expression of PPARγ, a key regulator of adipocyte differentiation, and thus induce adipocyte differentiation. One of the established signalling mechanisms for increasing PPARγ gene expression is mediated by...
ERK. It is possible that vanillin activates ERK and thus increases PPARγ gene expression. To test this, cells were treated with vanillin at different time points (60, 30, 15, and 5 min.) and the phosphorylation status of ERK was analysed. Cells treated with MDI were used as positive control. Vanillin increased the phosphorylation of ERK42/44 in the initial hour of the treatment. Maximum activation of ERK was observed in initial 5 min and there was a gradual decrease in the activation, reaching basal level after an hour of vanillin treatment (Fig 3.2.4B). A similar kinetics in the activation pattern of ERK was observed in MDI treated cells as well. (Fig 3.2.4A). To further confirm the role of ERK42/44 in vanillin induced adipocyte differentiation, cells were preincubated with U0126 (25 μM) for 30 minutes prior to induction of differentiation and after 8 days lipid accumulation were quantified using Oil Red O analysis. A decrease in lipid accumulation was observed in U0126 treated cells when compared to vanillin alone treated cells (Fig 3.2.4C).

![Fig 3.2.4 Effect of vanillin on ERK 42/44 activation. 3T3-L1 preadipocytes were treated with vanillin (500 μM) or MDI (10% FBS, 1 μg/ml insulin, 250 nM dexamethasone, 500 μMIBMX), harvested at different time points and immunoblotted with phospho ERK 42/44 specific antibodies. The phosphorylation status of ERK 42/44 upon MDI (A) and vanillin (B) treatment is shown. (C) Oil Red O analysis of vanillin differentiated and MDI differentiated adipocytes in the presence or absence of U0126 (25μM). The bar represents mean values ± SD from three independent experiments in triplicates.]

3.2.1.5 Vanillin Induces Adipocyte Differentiation Independently of Akt Activation

It has been shown that activation of Akt, one of the key nodal molecules in the insulin signaling pathway, can also increase PPARγ gene expression. Therefore, the effect
of vanillin on Akt activation was investigated. MDI treated cells showed an increase in phosphorylation of Akt, however, no significant change in the phosphorylation status of Akt was seen in vanillin treated and non treated cells (Fig 3.2.5A. Fig 3.2.5B). The role of Akt in vanillin induced adipocyte differentiation was confirmed by differentiating cells in the presence of Akt 1/2 kinase inhibitor (1μM). The Oil Red O analysis show that there was no significant difference in lipid accumulation in vanillin differentiated cells in presence and absence of Akt1/2 kinase inhibitor, AKTi (Fig 3.2.5C). This suggested vanillin induces adipocyte differentiation independently of Akt signaling pathway.

Fig 3.2.5 Effect of vanillin on Akt activation. (A) Immunoblot showing the phosphorylation status of Akt in 3T3-L1 preadipocytes treated with MDI (10% FBS,1 μg/ml insulin, 250 nM dexamethasone, 500 μMIBMX) or vanillin (500 μM). (B) The quantification of Akt phosphorylation from immunoblot data presented in (A). (C) Lipid accumulation in vanillin or MDI differentiated cells in the presence or absence of Akt kinase 1/2 inhibitor AKTi (1 μM). The bar represents mean values ± SD from three independent experiments in triplicates (* represents P<0.05, students T-test).

3.2.1.6 Vanillin Binds to Olfactory Receptor 56

In this study it was identified that vanillin activates ERK 42/44 and enhances adipocyte differentiation. However the upstream molecule that activates ERK 42/44 in this process is unknown, therefore we performed an affinity chromatography with vanillin coated beads to identify vanillin binding proteins. To capture and identify vanillin
targeted molecules, vanillin was immobilized on to epoxy activated beads. Non specific absorption was removed by exhaustive washing with cell lysis buffer. Untreated beads were used as control. 3T3-L1 pre-adipocyte cell lysate was allowed to bind to this vanillin activated beads, VAB. Non specifically bound proteins were removed by multiple washing with lysis buffer followed by stringent washing with lysis buffer containing 0.35 M to 1M NaCl. Beads was again equilibrated with lysis buffer and then eluted with 1mM vanillin. All the fractions were analysed on SDS PAGE and detected by silver staining. A prominent 33 kDa protein along with a few faint bands were observed in vanillin eluted fraction from VAB and were not observed in the control beads. These bands were excised and digested with trypsin and subjected to MALDI MS. The raw data was processed using MASCOT software. The analysis resulted in the identification of olfactory receptor 56 (OFR-56) with a significant score. The mass of this receptor was in agreement with the mass of the protein excised from the gel. This suggested a possibility that vanillin binds to OFR-56 and may mediate the downstream signalling for inducing adipogenesis.
3.2.1.7 Vanillin Binds to the Predicted Cavity of the Olfactory Receptor 56 Homolog Model

To know whether vanillin binds OFR-56, a homology modeling and molecular docking approach was used. Since there were no structural details available for OFR56, a homology model was generated for this protein using bovine rhodopsin as the template. The template was identified using fold recognition method. The target and the template
sequence were then aligned using the CLUSTALW program. The multiple sequence alignment was manually corrected based on the details from TM prediction, multiple sequence alignment and binding site prediction. This modified sequence alignment between the target and template sequences were used for the molecular modelling. The known ligand binding residues of mouse ordorant receptor for ethyl vanillin (mOR-EV) and eugenol (mOR-EG) were included in the comparative modelling. The loop regions were predicted using the advanced loop prediction tool in the modeller program. The energy minimized model has been used for the further validation and structural analysis. According to the PROCHECK result, the stereochmical quality of the model was comparable with crystal structure. Ramachandran plot has shown that 0.4% residues are in the disallowed region and these residues belong to loop regions. Cavities present in the receptor have been identified using the CASTp server. The cavity with a volume of 827.7 Å³ was identified as the largest cavity. Conserved residues, Val-77, Phe-220, Phe-174 and other residues, Met-175, Phe-180, Leu-219, Ile-227 surrounds this cavity (Fig 3.2.7A and B). The known ligand of mOR-EG, eugenol, binds to this cavity with a lower binding free energy of -5.39 kcal mol⁻¹ and an estimated inhibition constant, Kᵢ of 101.60 uM. Therefore this cavity was assumed as the ligand binding pocket of OFR-56. Vanillin also binds to the same cavity when docked to this receptor with a mean binding energy of -4.78 kcalmol⁻¹ and an estimated inhibition constant, Kᵢ of 298.85 uM. Pentanal and toluene were docked to this model as negative controls and as expected these compounds did not bind to the predicted cavity. Docking analysis has shown that Gln-128 of OFR forms hydrogen bonds with the hydroxyl and methoxy group present in the vanillin. A hydrophobic interaction between aromatic ring of vanillin and Phe-220 of OFR was also observed. (Fig 3.2.7C).
3.2.1.8 Identification of Differentially Expressed Proteins upon Vanillin Treatment in 3T3-L1 cells

To identify the downstream effectors of vanillin mediated adipogenesis a detailed proteomic study was carried out using iTRAQ in vitro labelling and mass spectrometry. Cell lysate of untreated and vanillin treated (500μM for 48 hrs) 3T3-L1 preadipocytes were subjected to tryptic digestion and the peptides from two sets were in vitro labelled with iTRAQ reagents. Two different isobaric tags consisting of charged reporter ions of mass 114, 116 were used. The labelled samples were subjected to strong cation exchange chromatography to fractionate the sample into various fractions and these fractions were identified using LTQ-Orbitrap Fourier transform mass spectrometer (LTQ-Orbitrap Velos, Thermo Fisher) The data obtained from different fractions was merged and processed.
together using the commercially available SEQUEST software in Proteome Discoverer platform (Fig 3.2.8). The data was searched against mouse RefSeq protein database. The analysis identified 1000 different proteins of which 126 protein were differentially modulated. The up regulated proteins includes, proteins associated with cytoskeletal rearrangement, basic transcriptional and translational machinery, protein folding, sorting and transport, enzymes involved in metabolism, extra cellular proteins and several others. Some proteins have also been down regulated in the process (Table 3.2.1). The data obtained is in agreement with the known adipogenic proteome.

Fig 3.2.8 Steps involved in iTRAQ proteomic analysis. 3T3L1 preadipocytes were treated with and without vanillin for 48 hours and the cells were lysed and equal amounts were digested with trypsin followed by labeling with iTRAQ reagents. The peptides were seperated using strong cation exchange chromatography and the fractions were subjected to mass spectrometry. The raw data obtained were analysed using sequest in the proteome discoverer platform and the identification and quantification of proteins were performed.
<table>
<thead>
<tr>
<th><strong>Upregulated proteins</strong></th>
<th><strong>Myeloid-associated differentiation marker</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione S-transferase</td>
<td>Pyridoxal kinase</td>
</tr>
<tr>
<td>Epoxy dehydrase</td>
<td>Complement component 1 Q subcomponent binding protein</td>
</tr>
<tr>
<td>6-phosphogluconate dehydrogenase</td>
<td>72 kDa type IV collagenase</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td><strong>Metallothionein-1</strong></td>
</tr>
<tr>
<td>2-oxoglutarate dehydrogenase</td>
<td>Small nuclear ribonucleoprotein F</td>
</tr>
<tr>
<td>Acetyl-CoA hydrolase</td>
<td>Heterogeneous nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>Amine oxidase</td>
<td>60S ribosomal protein L35a</td>
</tr>
<tr>
<td>S-methyl-5'-thioadenosine phosphorylase</td>
<td>PREDICTED: 40S ribosomal protein S13-like</td>
</tr>
<tr>
<td>Isoleucyl-CoA dehydrogenase</td>
<td>Protein arginine N-methyltransferase 1</td>
</tr>
<tr>
<td>S-adenosylmethionine synthase</td>
<td>40S ribosomal protein</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>Histone H2A type</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>Cold shock domain-containing protein</td>
</tr>
<tr>
<td>Maleimide dehydrogenase</td>
<td>Splicing factor I6a subunit 3</td>
</tr>
<tr>
<td>Estradiol 17-beta dehydrogenase</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>Dihydrolipoyl dehydratase</td>
<td>60S ribosomal protein L18s</td>
</tr>
<tr>
<td>Transmembrane cAMP phosphodiesterase</td>
<td>Elongation factor 1-beta</td>
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<tr>
<td>Voltage-dependent anion-selective channel protein 3 isoform 2</td>
<td>DNA replication licensing factor MCM3</td>
</tr>
<tr>
<td>ATP synthase subunit b, mitochondrial precursor</td>
<td>Transformer-2 protein homolog beta</td>
</tr>
<tr>
<td>Calcinin</td>
<td>CCR4-NOT transcription complex subunit 1 isoform 2</td>
</tr>
<tr>
<td>Lysosome membrane protein 2</td>
<td>P04 and SFRS1-interacting protein</td>
</tr>
<tr>
<td>ATP-binding cassette sub-family F member 1</td>
<td></td>
</tr>
<tr>
<td>Monocarboxylate transporter 1</td>
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</table>

Table 3.2.1 List of upregulated and down regulated proteins upon vanillin treatment in 3T3-L1 cells.
Adipocyte differentiation is mediated by the programmed regulation of gene expression coordinated by various metabolic and hormonal signaling pathways and transcriptional regulatory machinery. This is generally accompanied by changes in morphology, alterations in gene expression and sensitivity to hormones (Farmer, 2006). It has been shown that adipocyte differentiation can occur spontaneously if preadipocytes are maintained in serum medium for several weeks (Green and Meuth, 1974). Many molecules such as insulin, glucocorticoids and phosphodiesterase inhibitors are known to accelerate this process (Rubin et al., 1978). In addition to these, studies have identified several other compounds activating adipogenesis independently or in combination with insulin. In this study, we report the adipogenic effect of vanillin, a dietary component, commonly seen in confectionaries, pharmaceuticals and beverages. Vanillin induced adipogenesis in 3T3-L1 cells. The differentiated adipocytes with vanillin were characterized by morphological differences and enhanced lipid accumulation similar to

Table 3.2.1. List of proteins upregulated and down regulated during vanillin induced adipocyte differentiation.

<table>
<thead>
<tr>
<th>Up-regulated proteins</th>
<th>Down-regulated proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nascent polyepitope-associated complex subunit alpha isoform b</td>
<td>Fibulin 2 isoform a</td>
</tr>
<tr>
<td>Coreceptor subunit</td>
<td>Fibronectin precursor</td>
</tr>
<tr>
<td>T-complex protein 1 subunit gamma</td>
<td>Protein delta homolog 1 isoform 2 precursor</td>
</tr>
<tr>
<td>Alpha-soluble NSF attachment protein</td>
<td>Protein delta homolog 1 isoform 2 precursor</td>
</tr>
<tr>
<td>Peptidyl-prolyl cis-trans isomerase FKBP3</td>
<td></td>
</tr>
<tr>
<td>EH domain-containing protein 1</td>
<td></td>
</tr>
<tr>
<td>ER:CaM protein retaining receptor 1</td>
<td></td>
</tr>
<tr>
<td>Lysosomal protease protein isoform b</td>
<td></td>
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<tr>
<td>Exorin</td>
<td></td>
</tr>
<tr>
<td>Tubulin beta-2C chain</td>
<td></td>
</tr>
<tr>
<td>Collin 1</td>
<td></td>
</tr>
<tr>
<td>Protein S100-A4</td>
<td></td>
</tr>
<tr>
<td>Transmembrane emp24 domain-containing protein</td>
<td></td>
</tr>
<tr>
<td>Voltage-dependent anion-selective channel protein 3 isoform 2</td>
<td></td>
</tr>
<tr>
<td>ATP synthase subunit b, mitochondrial precursor</td>
<td></td>
</tr>
<tr>
<td>Calcinin</td>
<td></td>
</tr>
<tr>
<td>Lysosome membrane protein 2</td>
<td></td>
</tr>
<tr>
<td>A TP-binding cassette sub-family F member 1</td>
<td></td>
</tr>
<tr>
<td>Neutral carbohydrate transporter 1</td>
<td></td>
</tr>
<tr>
<td>Myeloid-associated differentiation marker</td>
<td></td>
</tr>
<tr>
<td>Pyruvical kinase</td>
<td></td>
</tr>
<tr>
<td>Septin 7</td>
<td></td>
</tr>
<tr>
<td>WD repeat-containing protein 1</td>
<td></td>
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</table>

3.2.1.9. Discussion

Adipocyte differentiation is mediated by the programmed regulation of gene expression coordinated by various metabolic and hormonal signaling pathways and transcriptional regulatory machinery. This is generally accompanied by changes in morphology, alterations in gene expression and sensitivity to hormones (Farmer, 2006). It has been shown that adipocyte differentiation can occur spontaneously if preadipocytes are maintained in serum medium for several weeks (Green and Meuth, 1974). Many molecules such as insulin, glucocorticoids and phosphodiesterase inhibitors are known to accelerate this process (Rubin et al., 1978). In addition to these, studies have identified several other compounds activating adipogenesis independently or in combination with insulin. In this study, we report the adipogenic effect of vanillin, a dietary component, commonly seen in confectionaries, pharmaceuticals and beverages. Vanillin induced adipogenesis in 3T3-L1 cells. The differentiated adipocytes with vanillin were characterized by morphological differences and enhanced lipid accumulation similar to
what was generally been observed in adipocytes differentiated by normal MDI. Compounds like reversine in combination with MDI and magnolol along with insulin were known to enhance the normal rate of adipocyte differentiation but did not show any adipogenic effect independently (Choi et al., 2009; Kim et al., 2007). However, vanillin induced adipocyte differentiation in 3T3-L1 cells without the involvement of any other adipogenic molecules. In a previous study it was shown that harmine a small molecule (alkaloid belonging to beta-carboline family of compounds) enhanced the rate of adipogenesis. Both vanillin and harmine mediate its effect on adipogenesis by increasing the expression of PPARγ, the master regulator of adipogenesis and its target gene GLUT4 (Waki et al., 2007). The adipocytes differentiated by these molecules were insulin sensitive in nature.

Next, we analyzed the mechanism by which vanillin differentiates 3T3-L1 preadipocytes to adipocytes. There are mainly two kinase systems namely, PI3K/Akt and Ras ERK 1/2, involved in adipocyte differentiation. In this study, no appreciable differences in Akt phosphorylation (Ser 473) were observed. It is possible that a small change at the activation level of Akt which could not be observed at the level of western blot is sufficient for mediating its effect (Hoehn et al., 2008). To rule out this possibility the cells were differentiated in the presence of a known Akt kinase 1/2 inhibitor along with vanillin. Such treatment did not show any inhibition on vanillin mediated adipogenesis. This suggested that vanillin activates a different kinase which could possibly be ERK42/44. Studies have shown that ERK 42/44 gets activated in the initial phase of adipogenesis (Prusty et al., 2002). Similar results were obtained in our studies where vanillin activated ERK 1/2 in the initial hour of adipocyte differentiation leading to increased expression of PPARγ and lipid accumulation. U0126, a MEK/ERK inhibitor markedly reduced this effect suggesting a direct role for ERK42/44 in vanillin mediated adipogenesis. Compound like H89, protein kinase A inhibitor, enhanced differentiation by activating both Akt and ERK 42/44 (Kato et al., 2007). Treatment of preadipocytes with reversine a synthetic molecule plus MDI increased adipocyte differentiation by down regulating Akt but without affecting ERK 42/44 signaling (Kim et al., 2007). However vanillin neither activated nor downregulated Akt.

Further experiments were carried out to understand the upstream and downstream players of vanillin mediated ERK 42/44 signaling. An affinity chromatography was performed with vanillin activated beads using 3T3-L1 preadipocyte cell lysate, followed by mass spectrometry to identify vanillin binding protein. A protein of 33 kDa, olfactory
receptor 56, was identified as a vanillin binding protein. This receptor protein belongs to rhodopsin class A rhodopsin-like family of G protein-coupled receptors. Wang et al have used a similar affinity chromatography approach to identify resveratrol targeted proteins (Wang et al., 2004). Bioinformatics docking analysis was an ideal strategy to confirm this binding of vanillin with OFR 56, but a three dimensional structure for this protein was not available. Therefore a homology model of this protein was made using comparative modeling method which was commonly been employed to predict the structure of membrane proteins. The basic principle of this modeling approach is that, the evolutionary related sequence will have a similar three dimensional structure. Therefore, the model of the template protein (protein of interest) can be built from the three dimensional model of the target (evolutionary related) protein, provided they share a significant sequence or fold similarity (Sali A. and Blundell 1993). In this case, bovine rhodopsin was identified as a template and homology model was made and validated using various approaches. Several ordorant receptors are identified so far and ethyl vanillin, eugenol have shown to bind with different ordorant receptors, mouse ordorant receptor for ethyl vanillin, mOR-EV and mouse ordorant receptor for eugenol mOR-EG. These proteins belong to a family of G-protein coupled receptors and their ligand binding sites are well characterized (Baud et al., 2010). Information about the ligand residues from these studies were incorporated for predicting the ligand binding sites of OFR 56. Homology model of OFR 56 was used for docking purpose. The docking was performed and found that vanillin binds to the predicted ligand binding cavity. Binding analysis with eugenol showed that it binds to the cavity but with a lower binding energy. However pentanal and toluene did not bind to the cavity but bound at a different site. These observations suggest that vanillin binding to OFR 56 is highly specific in nature though this binding need to be validated in wet lab.

To further characterize the downstream events, a detailed proteomic analysis was performed with vanillin treated and untreated 3T3-L1 preadipocytes using in vitro iTRAQ labeling and mass spectrometry. Thousand different proteins were identified; however the quantification results show that 126 different proteins were differentially expressed. The differentially expressed proteins include several molecules that are important in adipocytes differentiation in general. It is important to note that initial phases of adipocyte differentiation involves clonal expansion and is therefore metabolically very active. Also known to undergo extensive cytoskeleton remodeling during this phase of adipogenesis. Various proteins identified include those associated with transcription and translation,
metabolism, cytoskeletal dynamics, and extra cellular proteins. A number of proteomics study has been carried out by different groups to understand the players of adipogenesis. Study was carried out at different levels, either to analyze the global expression of proteins during adipogenesis (Welsh et al., 2004) or to understand the expression of proteins under particular treatments. The expression profile of proteins upon rosiglitazone treatment in 3T3-L1 adipocytes were carried out and it was observed that a number of proteins critical for insulin signaling and GLUT4 translocation were up regulated during this condition (Ahmed et al., 2009). Most of the proteins identified upon vanillin treatment were in general was shown to be important for adipogenesis.

Thousands of different odorant receptors have been identified so far. Though the receptors have specificity for odorants, most of its signaling is mediated through cAMP (Pace et al., 1975; Sklar et al., 1986; Lowe et al., 1989; Breer et al., 1990). It is shown that in olfactory sensory neuron, odorants mediates its effect by activating ERK 42/44 followed by CREB activation leading to transcription of many target genes. It was also shown that cAMP level increases upon odorant activation in these neurons (Watt and storm 2001). Adipogenic factors have shown to elevate cAMP levels in 3T3-L1 preadipocytes leading to enhanced CREB phosphorylation and transcriptional activity. Moreover ERK 42/44 is known to phosphorylate in the initial hour of adipocytes differentiation and it is known to elicit its effect via CREB phosphorylation (Reusch et al., 2000 ). Interestingly, the present study found vanillin treatment activates ERK1/2 in the initial hour of adipogenesis. Further analysis has shown that vanillin binds to an olfactory receptor 56. Together these data suggest a novel role for odorant receptor signaling in adipogenesis. The proteomic data has shown that among the proteins up regulated there are proteins that have known to be elevated upon cAMP and CREB activity. Metallothionein-1 level was increased in 3T3-L1 cells upon the induction with bromo-cAMP. Metallothionein is an early marker of adipocyte differentiation. The proteomic study show that Metallothionein is up regulated during vanillin induced adipocytes differentiation (Trayhurn et al., 2000). The expression of Protein S100-A4, a calcium binding protein is involved in cellular differentiation process was increased during vanillin induced adipogenesis. Expression of this protein is elevated upon cAMP activity in neurons (Kiryushko et al 2006). A study by Chen et al has shown that ERK 1/2 activation, upregulated protein S100A16 expression in breast cancer cell lines (Chen et al., 2007). Though no studies are available to understand its effect on adipogenesis, a detailed study of protein S100A16, a close homologue of Protein S100-A4 with similar
function belongs to S100 super family of protein, was reported. This studies show that S100A16 is a pro adipogenic protein (Leu et al., 2011). Taken together these data suggest a possibility that vanillin may be eliciting its effect via OFR 56/ERK 42/44/CREB pathway leading to induction of various proadipogenic genes (Fig 3.2.8).

![Figure 3.2.9 Proposed model of vanillin signaling. Vanillin by binding to olfactory receptor 56 activates ERK 42/44, which in turn activates CREB leading to the expression of adipocyte specific genes. Vanillin may also elicits its effect via cAMP signaling.](image)

### 3.2.1.10 Conclusion

Vanillin induces adipogenesis in 3T3-L1 cells by increasing the phosphorylation of ERK1/2. The proadipogenic effect of vanillin may be via binding to olfactory receptor 56 and there by inducing ERK1/2 phosphorylation which ultimately leads to the expression of adipogenic genes. This study therefore reports a novel role for an odorant receptor in adipogenesis.
3.2.2. 2, 4 Diacetyl Resorcinol (DAR) Enhances Adipogenesis in 3T3L-L1 cells

3.2.2.1. 2, 4 DAR Enhances Adipocyte Differentiation in 3T3-L1 Cells

To evaluate the effect of 2, 4 DAR on adipogenesis, untreated and MDI treated 3T3-L1 preadipocytes were incubated with 2, 4 DAR for 2 days in the initial screening. 50 μM of 2, 4 DAR was used for initial assay. Cells treated with MDI were used as a positive control and 0.2 % DMSO as negative control. On day 8 it was observed that 2, 4 DAR enhanced adipocyte differentiation in cells treated with and without MDI, but the rate of differentiation was significantly high in cells treated with MDI. Cells treated with 2, 4 DAR and MDI has shown 1.9 fold increase in adipogenesis compared to cells treated with MDI alone. When cells were treated with 2, 4 DAR alone the fold increase in adipogenesis was only 1.3 fold compared with vehicle treated cells (Fig 3.3.1A). Since 2, 4 DAR has shown an enhanced effect on adipogenesis when treated along with MDI, a concentration dependent effect of 2, 4 DAR in presence of MDI was analyzed. A dose dependent increase in adipogenesis was observed with a maximum increase of 2.1 fold at 100 μM (Fig 3.3.1B and C).
Fig 3.3.1 Effects of 2, 4 DAR on adipocyte differentiation of 3T3-L1 cells. Two-day post confluent 3T3-L1 preadipocytes were treated with 2, 4 DAR in the presence and absence of MDI. Medium was changed every 2 days for 8 days. Cell treated with 0.2% DMSO was used as negative control and MDI as positive control. On day 8 the rate of adipocyte differentiation was quantified using Oil Red O analysis. Intracellular lipid accumulation in 2, 4 DAR treated cells (A). A dose dependent increase in lipid accumulation in 2, 4 DAR and MDI treated cells (B). Oil Red O staining in 2, 4 DAR and MDI treated 3T3-L1 cells (C). The bars represent mean values ± SD.

3.2.2.2. 2, 4 DAR Enhances Expression of Adipocyte Marker Gene GLUT4

The expression of adipocyte marker gene GLUT4 was analyzed to confirm the effect of 2, 4 DAR on adipocyte differentiation. MDI induced and non-induced 3T3-L1 preadipocytes were treated with 2, 4 DAR for 2 days. GLUT4 expression was analyzed on day 8. Western blot analysis shows that 2, 4 DAR increases the expression of GLUT4. The expression of GLUT4 further increased in the case where the cells were treated with both DAR and MDI (Fig 3.3.2)
Fig 3.3.2 Effect of 2, 4 DAR on the expression of adipocyte marker gene GLUT4. Two day post confluent 3T3-L1 preadipocytes were treated with 0.2% DMSO, MDI, 2, 4 DAR (100μM) in the presence and absence of MDI for 48 hrs. The cells were harvested on day 4 after the treatment and immunoblotted using GLUT4 antibodies. The expression of GLUT4 on 2, 4 DAR treatments are shown (A). Student T-test has been performed and the P<0.05.

3.2.2.3 2, 4 DAR Binds to PPARγ Ligand Binding Domain

Ligand mediated PPARγ activation is known to play a major role in the induction of adipogenesis. In this study it is shown that when added along with MDI, 2,4 DAR increased adipocyte differentiation. It is possible that 2,4 DAR exerts its adipogenic effect by binding to ligand binding domain (LBD) of PPARγ and thus leading to its activation. To analyze this possibility, a docking study was performed with LBD of PPARγ and 2, 4 DAR. In the docking study it was observed that the 2, 4 DAR binds to the ligand binding domain of PPARγ with minimum free energy. In the detailed binding analysis, formation of hydrogen bonds were observed between acetyl and hydroxyl groups of 2, 4 DAR and the Tyr 473, His 323, Tyr327 and F282 residues of PPARγ LBD domain. These residues
are the known interacting sites for the synthetic ligand of PPARγ, rosiglitazone (Fig3.3.3).

Fig 3.3.3: Docking analysis showing the interaction between PPARγ LBD residues and 2, 4 DAR. Residues in LBD interacting with 2, 4 DAR is shown in yellow colour, 2, 4 DAR is shown in blue colour and the red colour dotted lines indicate the hydrogen.

3.2.2.4. 2, 4 DAR Increases GLUT4 Expression Possibly by Acting as a PPARγ Ligand

To further confirm the role of 2, DAR as a PPARγ ligand, the expression of PPARγ target gene, GLUT4 was analyzed in MDI induced cells. 3T3-L1 preadipocytes were pretreated with MDI for 2 days and then treated with 100μM 2, 4 DAR for another 2 days. 2, 4 DAR untreated but MDI pretreated 3T3-L1 cells were used as control. Both PPARγ and GLUT4 expression were analyzed. An increase in GLUT4 expression was observed in 2, 4 DAR treated cells with no change in the expression levels of PPARγ. (Fig3.3.4). This observation further suggests 2, 4 DAR as a possible agonist of PPARγ.
Fig 3.3.4 Effect of 2, 4 DAR on the expression of GLUT4 and PPARγ. Two days post confluent 3T3-L1 preadipocytes were treated with MDI for 2 days and then with and without 2, 4 DAR (100μM) for another 2 days. The cells were harvested on day 4 after the treatment and immunoblotted using GLUT4 and PPARγ antibodies. The expression of GLUT4 and PPARγ after 2, 4 DAR treatments are shown.

3.2.2.5 2, 4 DAR Increases Basal and Insulin Stimulated Glucose Uptake in 3T3-L1 Adipocytes

PPARγ ligands are known to increase glucose uptake in 3T3-L1 adipocytes. To determine whether 2, 4 DAR has a direct effect on insulin sensitivity and glucose metabolism, glucose uptake assay was performed. 3T3-L1 adipocytes were treated with 100 μM 2, 4 DAR for 2 days and then glucose uptake assay was performed under insulin stimulated and unstimulated condition. Untreated 3T3-L1 adipocytes were used as negative control. 2, 4 DAR increases basal and insulin stimulated glucose uptake. Under basal and insulin stimulated conditions 2, 4 DAR has shown 1.3 fold and 1.7 fold increase in glucose uptake respectively (Fig 3.3.5).
Fig 3.3.5: Effect of 2, 4 DAR on glucose uptake in 3T3-L1 cells. Two days post confluent 3T3-L1 preadipocytes were treated with, MDI (10% FBS, 1 μg/ml insulin, 250 nM dexamethasone, 500 μM IBMX) for 48 hours. Medium was changed every 2 days interval. After 6 days cells were treated with and without 2, 4 DAR (100 μM) as indicated for 48 hours and glucose uptake was carried under insulin stimulated (30 minutes) and unstimulated conditions. 0.2 % DMSO was used as negative control. The insulin used for the stimulation was 100 nM. The fold level differences in the glucose uptake under different treatments were determined. The bar represents mean values ± SD. (*= P <0.05, ** = P< 0.005).

3.2.2.6 2, 4 DAR Increases Adiponectin Expression in 3T3-L1 Adipocytes

Adiponectin is adipokine increases insulin sensitivity and its expression is known to enhance during PPARγ activation. To determine whether 2, 4 DAR enhances adiponectin expression, 3T3-L1 adipocytes were treated with 2, 4 DAR for 48 hrs and the mRNA expression was analyzed using real time PCR. A 1.9 fold increase in adiponectin mRNA expression was observed in 3T3-L1 adipocytes treated with 2, 4 DAR compared to untreated 3T3-L1 adipocytes (Fig 3.3.6).
Fig 3.3.6 Effect of 2, 4 DAR on adiponectin expression. 3T3-L1 adipocytes were treated with and without 2, 4 DAR (100 μM) for 12 hours. Cells treated with 0.2% DMSO were used as control. After treatment, total RNA was isolated and subjected to quantitative analysis of adiponectin by real-time PCR. Relative expression was analysed using 2$^{-ΔΔCT}$. The relative fold difference in adiponectin expression upon 2, 4 DAR is shown. Error bars represent mean values ± SD of three independent experiments done in triplicates.

3.2.2.7 Discussion

Present study demonstrates the effect of a novel synthetic molecule 2, 4 DAR on 3T3-L1 adipocyte differentiation. 2, 4 DAR enhanced adipocytes differentiation in 3T3-L1 cells. Differentiated adipocytes were characterized by morphological differences and enhanced lipid accumulation (Farmer 2006). 3T3-L1 preadipocytes treated with 2, 4 DAR has shown elevated lipid accumulation when compared to vehicle treated 3T3-L1 preadipocytes and this lipid accumulation was further increased when cells were treated with 2, 4 DAR along with MDI. Hence a dose dependent treatment of 2, 4 DAR along with MDI was performed in 3T3-L1 preadipocytes. A dose dependent increase in adipogenesis was observed when treated with 2, 4 DAR. These observations were further confirmed by analyzing the expression of adipocyte marker gene GLUT4. The expression of GLUT4 was increased upon 2, 4 DAR treatment and a significant difference was observed when treated along with MDI. GLUT4 is known to be a target gene of PPARγ. PPARγ expression increases upon the induction of adipogenic medium. When PPARγ gets activated by various signals, it induces cells to exit from the cell cycle, and in cooperation with C/EBP α, stimulates the expression of many metabolic genes such as GLUT4 (Tontonoz et al., 1994). Since there was a further increase in GLUT4 expression in 3T3-L1 preadipocytes upon combined treatment of 2, 4 DAR with MDI lead to speculate that 2, 4 DAR may be exerting its effect through PPARγ.
It was already shown that various small molecules such as Phloretin, a chalcone class of flavonoids, present in strawberries, magnolol a compound from *Magnolia officinalis* and emodin (3-methyl-1,6,8-trihydroxyanthraquinone) from *Rheum palmatum* L can enhance adipogenesis by activating PPARγ (Choi et al., 2009; Hassan et al., 2007 and Yang et al., 2007). Synthetic PPARγ agonists such as thiazolidinediones (TZDs), have shown to be effective as insulin-sensitizing agents, reducing insulin resistance and lowering plasma glucose levels in patients with type-2 diabetes. To determine whether 2, 4 DAR elicits its effects via direct binding and activation of PPARγ, a molecular docking analysis was performed with the LBD of PPARγ and 2, 4 DAR. Analysis showed that 2, 4 DAR binds to the LBD of PPARγ with minimum free energy. Hydrogen bonds are formed between the hydroxyl and acetyl group of the 2, 4 DAR and the side chains residues Tyr327, Phe282, H323 and Tyr473 of LBD. These residues are reported to be the ligand binding sites of potent PPARγ agonists TZD and rosiglitazone. Binding of ligands to these residues increases the transcriptional activity of PPARγ by inducing conformational changes directly in the coregulator binding site or indirectly in the distal part of the ligand binding domain (Sheu et al., 2004). In our docking studies it was shown that acetyl group and hydroxyl group of 2, 4 DAR hydrogen bond with Tyr 473 of AF-2 region. In general such an interaction between ligand and LBD of PPARγ is very important and plays a critical role in coactivator binding to AF-2 domain, the ligand dependent activation domain of PPARγ (Itoh et al., 2008). The very same fact that such an important interaction was observed between 2, 4DAR and Tyr 473 of AF-2 clearly indicating the possibility of 2, 4 DAR act as an agonist of PPARγ. If it were to true, then one would expect increased expression of GLUT4, a PPARγ target gene. To confirm this possibility, a western blot analysis was performed to check whether cells treated with 2, 4 DAR for a definite time increases GLUT 4 expression. The analyses showed that GLUT4 expression is increased with no detectable change for the expression level of PPARγ (2.4). This indirectly demonstrates that 2, 4 DAR activates GLUT4 by binding to PPARγ. Magnolol showed a similar effect like 2, 4 DAR and showed increased expression of target genes such as GLUT4 keeping PPARγ expression constant.

The ligands of PPARγ are known to increase glucose uptake in 3T3-L1 adipocyte (Ribon et al., 1998). To determine the effect of 2, 4 DAR on glucose uptake, differentiated 3T3-L1 adipocytes were treated with 2, 4 DAR for 48 hours and glucose uptake assay was done under insulin stimulated and unstimulated conditions. An increase in glucose
uptake assay was observed in 2, 4 DAR treated cells under both basal and insulin stimulated conditions (Fig 2.5). Various studies have shown that the rate of glucose uptake in insulin sensitive tissues is correlated with the amount of glucose transporters present in the cells (Gnudi et al., 1995; Wood and Trayhurn 2003). In this study an increase in GLUT4 expression was observed in 2, 4 DAR treated 3T3-L1 adipocytes. The increase in glucose uptake may be associated with the increase in GLUT4 expression by 2, 4 DAR. PPARγ ligands are known to increase number of small adipocytes and insulin sensitivity (Staels and Fruchart 2005). The ability of 2, 4 DAR to increase number of adipocytes and insulin sensitivity suggests a role for 2, 4 DAR as PPARγ ligand.

Adiponectin is an adipocyte specific protein and is the abundant circulating anti-inflammatory adipokine in the body. Decreased level of adiponectin is associated with insulin resistance and type 2 diabetes (Kadowaki et al., 2006). PPARγ activation enhances the expression of adiponectin (Iwaki et al. 2003). The effect of 2, 4 DAR on adiponectin expression was analysed using real time PCR analysis. The analysis showed that 2, 4 DAR increased the mRNA level of adiponectin. This increase in adiponectin expression may be via PPARγ activation by 2, 4 DAR.

3.2.2.8 Conclusion

Our experiments prove that 2, 4 DAR is a positive modulator of adipogenesis. Further it has shown that 2, 4 DAR increases adipogenesis in a PPARγ dependent manner. Though further experiments need to be carried out to evaluate the physiological relevance of this compound, this study reports a novel molecule with adipogenic activity.
3.3. Functional Characterisation of Negative Modulators of Adipogenesis.

3.3.1 Ellagic Acid Inhibits Adipocyte Differentiation in 3T3-L1 Cells

3.3.1.1. Ellagic Acid Reduces Adipogenesis in 3T3-L1 Cells

The effect of ellagic acid (EA) on adipocyte differentiation was determined. To examine the effect, 2 day post confluent 3T3-L1 cells were treated MDI or MDIEA (MDI along with ellagic acid) for 8 days and Oil Red O analysis was performed. Oil Red O analysis showed that EA reduces adipocyte differentiation in 3T3-L1 cells (Fig 3.4.1A). A dose dependent decrease in adipocyte differentiation was observed with a maximum of 37 \% inhibition was observed at a concentration of 10μM (Fig 3.4.1B). At this concentration, EA did not show any toxicity to the cells (Data not shown).

![Fig 3.4.1 Effect of ellagic acid on adipocyte differentiation of 3T3-L1 cells. Two day post confluent cells were treated with MDI, MDI and different concentrations of ellagic acid (100 nM, 1 μM, 10 μM), or DMSO. The medium was changed every 2 days. On 8th day of differentiation, cells were stained with Oil Red O, and the lipid accumulation was quantified by measuring the OD of Oil Red O eluted from the cells at 495 nm. Oil Red O stained images of MDI differentiated or MDI differentiated cells in presence of ellagic acid (10 μM) (A. Oil Red O quantification results of cells differentiated in presence of ellagic acid (B). Structure of ellagic acid (C). Error bars represents mean values ± SD of three independent experiments done in triplicates.](image-url)
3.3.1.2 Ellagic Acid Downregulates the Expression of Key Adipogenic Transcription Factors PPARγ, CEBPα, and SREBP-1c

In the present study, it is shown that EA downregulates adipogenesis and various transcription factors could play a role in this EA mediated anti-adipogenic effect. PPARγ, CEBPα, and SREBP-1 are known to play a key role in adipogenesis and it was interesting to see whether EA had any effect on the expression levels of these proteins. The effect of EA on the mRNA expression of these adipogenic transcription factors was determined. For this, 3T3-L1 preadipocytes were treated with MDI in presence and absence of EA (10 μM) was used. At day 4, expression of adipogenic transcription factors were determined by real time PCR analysis and the results show that the expression of transcription factors were reduced when compared to control cells. The mRNA expression level of PPARγ, CEBP alpha and SREBP-1c was reduced by 36 %, 45 % and 42 % respectively (Fig 3.4.2).

![Graph showing relative fold difference in expression of PPARγ, C/EBPα, and SREBP-1c with EA treatment](image)

*Fig 3.4.2 Effect of EA on the expression of key adipogenic factors PPARγ, C/EBPα and SREBP-1c. 3T3-L1 preadipocytes (day 0) were treated with MDI or MDIEA (10μM) for 48 hours. Medium was changed every 2 days. On day 4 of differentiation, total RNA was isolated and subjected to quantitative analysis of PPARγ, C/EBPα and SREBP-1c by real-time PCR. Relative expression was analysed using \(2^{-ΔΔCT}\). The relative fold difference of PPARγ, C/EBPα and SREBP-1c expression upon EA treatment is shown. Error bars represents mean values ± SD of three independent experiments done in triplicates.*
3.3.1.3 Ellagic Acid Reduces the Expression of Adipogenic Marker Gene GLUT4

To confirm the effect of EA on adipocyte differentiation, the expression of adipogenic marker gene, GLUT4, was analyzed. 3T3-L1 preadipocytes were treated with MDI in the presence and absence of 10 μM EA. At day 6, GLUT4 expression was analyzed using western blot. The expression of GLUT4 was reduced in MDIEA treated cells when compared to MDI treated cells (Fig 3.4.3).

![Western blot image showing reduced GLUT4 expression in MDIEA treated cells]

Fig 3.4.3 Effect of EA on the expression of GLUT4. 3T3-L1 pre-adipocytes (day 0) were treated with MDI or MDI along with EA (10 μM) for 48 hours. Medium was changed every 2 days. Cells were harvested on day 4 of differentiated and immunoblotted using GLUT4 antibodies. The expression of GLUT4 in MDI and MDIEA treated cells are shown.

3.3.1.4 Ellagic Acid Reduces ERK 1/2 Activation in 3T3-L1 Preadipocytes

To analyse the mechanism by which EA inhibits adipocyte differentiation, the status of ERK phosphorylation upon MDIEA treatment was analysed. 3T3-L1 preadipocytes were treated using MDI, MDIEA or 0.2% DMSO for 15 minutes. MDIEA reduced phosphorylation of ERK 42/44 in 3T3-L1 preadipocytes when compared to MDI in the initial 15 minutes of induction (Fig3. 4.4).
Fig 3.4.4 Effect of EA on ERK 42/44 activation. 3T3-L1 preadipocytes were treated with MDI, MDIEA (10 μM), or DMSO for 30 minutes. The cells were harvested and immunoblotted with phospho ERK 42/44 antibodies. The phosphorylation status of ERK 42/44 is shown.

3.3.1.5 Ellagic Acid Activates AMPK in 3T3-L1 Cells

AMPK activation is known to reduce adipocyte differentiation in 3T3-L1 cells. To analyse whether AMPK has any role in the reduction of adipocyte differentiation by EA, AMPK phosphorylation status was analysed in 3T3-L1 cells treated using MDIEA and MDI. DMSO was used as negative control and berberine as positive control. EA enhanced AMPK phosphorylation similar to berberine (Fig 3.4.5).

Fig 3.4.5 Effect of EA on AMPK activation. 3T3-L1 preadipocytes were treated with MDI, MDIEA (10 μM), or DMSO for 1 hour. Berberine was used as positive control. The cells were harvested and immunoblotted with phospho pAMPK antibodies. The phosphorylation status of AMPK is shown.
3.3.1.6 Ellagic Reduces the Expression of Inflammatory Adipokines in Fat Cells and Non Fat Cells

In obese condition, the dysfunction of adipose tissue leads to altered circulation of inflammatory adipokines. This may contribute to the development of insulin resistance and type 2 diabetes. Since EA has shown an inhibitory effect on adipocyte differentiation, it was highly probable that it could also modulate the expression of adipokines. Therefore, the effect of EA on inflammatory adipokine was analysed. Raw macrophages 264.7 were treated with LPS and differentiated 3T3-L1 adipocytes treated with spent media of LPS stimulated raw macrophages to induce inflammation. These cells were then incubated with and without EA for 12 hours and mRNA expression of TNFα, IL-6 and MCP-1 were analysed. The expression of these adipokines was reduced upon EA treatment in both 3T3-L1 adipocytes and raw macrophages (Fig 3.4.6).

**Fig 3.4.6** The effect of EA on inflammatory adipokine expression in 3T3-L1 adipocytes and raw macrophages. 3T3-L1 adipocytes were treated with and without EA for (10 μM) for 12 hours. Cells treated with 0.2% DMSO were used as control. After treatment, total RNA was isolated and subjected to quantitative analysis of IL-6, TNFα and MCP-1 by real-time PCR. Relative expression was analysed using 2^(-ΔΔCT). The relative fold difference in IL-6, TNFα and MCP-1 expression in EA treated and untreated inflammation induce raw macrophages (A) and adipocytes (B) are shown. Error bars represents mean values ± SD of three independent experiments done in triplicates.
3.3.1.7 Ellagic Acid Modulates the Expression of Adipokines Involved in Insulin Resistance

Resistin and leptin are the two adipokines secreted from fat cells that contributes to insulin resistance and type 2 diabetes. The effects of EA on the expression of these adipokines were also analysed using real time PCR. 3T3-L1 cells were treated with and without EA for 18 hours and the mRNA expression of resistin and leptin were analysed. The analysis showed a 70% and 85% reduction in the expression levels of leptin and resistin respectively (Fig 3.4.7).

![Figure 3.4.7](image)

Fig 3.4.7 Effect of EA on leptin and resistin expression. 3T3-L1 adipocytes were treated with and without EA for (10 μM) for 12 hours. Cells treated with 0.2% DMSO were used as control. After treatment, total RNA was isolated and subjected to quantitative analysis of leptin and resistin by real-time PCR. Relative expression was analysed using $2^{-\Delta\Delta CT}$. The relative fold difference of leptin and resistin expression in EA treated and untreated cells. Error bars represents mean values ± SD of three independent experiments done in triplicates.

3.3.1.8 Ellagic Acid Reduces the Expression of Hormone Sensitive Lipase in 3T3-L1 Adipocytes

Obesity is associated with elevated level of free fatty acids. These free fatty acids interfere with insulin signaling pathway and contributes to insulin resistance and
associated complications. Hormone sensitive lipase is the key enzyme involved in free fatty acid synthesis. To understand whether EA has any effect on HSL expression, 3T3-L1 adipocytes were treated with or without EA for 18 hrs and the mRNA levels were detected by real time PCR. The results show that EA reduce HSL expression by 73 % in 3T3-L1 adipocytes (3.4.8).

![Graph showing effect of EA on hormone sensitive lipase expression in 3T3-L1 cells.](image)

**Fig 3.4.8** Effect of EA on hormone sensitive lipase expression in 3T3-L1 cells. 3T3-L1 adipocytes were treated with and without EA for (10 μM) for 12 fours. Cells treated with 0.2% DMSO were used as control. After treatment, total RNA was isolated and subjected to quantitative analysis of hormone sensitive lipase by real-time PCR. Relative expression was analysed using $2^{-\Delta\Delta\text{CT}}$. The relative fold difference of HSL expression in EA treated and untreated cells. Error bars represents mean values ± SD of three independent experiments done in triplicates.

### 3.3.1.9 Discussion

Ellagic acid (EA) is commonly present in blue berries, raspberries and pomegranate fruits. It is well known for its anticancer and antioxidant activities (Umesalma and Sundhandiran G 2011; Murugan et al., 2009). Though many plants extract containing ellagic acid has shown antiobesity effects, (Plant extract that have 10.6% EA has shown 54% decrease in body weight gain in mice when fed with 800mg/Kg) there are no direct evidences to show the antiadipogenic effect of EA (Koh et al., 2011; Ratnam et al., 2009; Lei F et al., 2007). Therefore in this study, the effect of EA on adipocyte differentiation was determined. EA reduced adipocytes differentiation in 3T3-L1 cells in a dose dependent manner with a maximum inhibition at 10μM. Many phytochemicals such as Guggulsterone, isorhamentin, esculetin, and genistein with
antioxidant and anti-inflammatory properties exhibited anti-adipogenic activity (Lee et al., 2008; Yang et al., 2008; Yang et al., 2006; Hwang et al., 2005).

Adipocyte differentiation is regulated by the sequential expression of different adipogenic transcription factor such as PPARγ, CEBPα and SREBP1-c. Down regulation of these genes have shown to inhibit the development of preadipocytes to mature fat cells (Guo et al., 2000). The effect of EA on the expression of these adipogenic factors was determined. EA down regulates the expression of PPARγ, CEBα and SREBP1-c. Several compounds have shown to inhibit adipogenesis by down regulating these transcription factors. Leutolin, Chitosan oligosaccharides, Isorhamnetin inhibits adipocyte differentiation by down regulating PPARγ and CEBPα (Cho et al., 2008; Park et al., 2009; Lee et al., 2009). To further confirm the inhibition of adipocytes differentiation by EA, protein expression of adipogenic marker gene GLUT4 was analysed. The expression of GLUT4 was decreased during EA induced adipocytes differentiation. This reduction in expression may be due to the inhibition of the expression of its regulator PPARγ.

To elucidate the mechanism by which EA inhibits adipocytes differentiation, the phosphorylation status of key signaling molecules were analyzed. EA inhibited MDI induced phosphorylation of ERK 42/44 in the initial hour of adipogenesis. ERK 42/44 is considered crucial in the initial period of adipocyte differentiation. ERK 42/44 phosphorylates signaling molecules that are involved in the induction of PPARγ and CEBPα thereby inhibiting it (Prusty et al., 2002). Fucoidan, a polysaccharide and pigment epithelium-derived factor (PEDF) reduces adipocyte differentiation by inhibiting ERK in the initial period (Kim et al., 2010). These findings suggest that ERK 42/44 play a major role in initiating adipogenesis and any compound or biofactor capable of inactivating ERK 42/44 in the initial period of differentiation down regulates the conversion of preadipocyte to adipocyte. In this study, it was shown that MDI reduced AMPK activation whereas EA increased the phosphorylation of AMPK. Phytochemicals such as Cinamaldehyde, genistein, EGCG, capsaicin, berberine, dioxinodehydroeckol, and various plant extracts like Artemisia sacrorum Ledeb, Momordica charantia inhibits adipogenesis via the AMPK signaling pathway in 3T3-L1 adipocytes. (Huang et al., 2011; Yuan and Piao 2011; Choi et al., 2006; Nerurkar et al., 2010). AMPK phosphorylates
acetyl CoA carboxylase and inhibits fatty acid synthesis and triglyceride accumulation in 3T3-L1 cells. There are several phytochemicals shown to inhibit adipogenesis either by inhibition of ERK or activation of AMPK. Treatment with U0126 an MEK/ERK inhibitor with MDI has restored the AMPK activity in 3T3-L1 cells (data not shown) and another study has shown an inhibitory cross talk between ERK and AMPK during the proliferation of cardiac fibroblast (Du et al., 2008) which strongly suggest that EA may be activating AMPK via ERK inhibition.

Since EA has shown an inhibitory effect on adipogenesis, it is highly possible that it could also modulate adipokine expression. The expression of inflammatory adipokines such as TNFα, IL-6 and MCP-1 in fat cell and non fat cells were analyzed upon EA treatment. The analysis shows that EA treatment reduced the expression of the chemokines and cytokines in both adipocytes and macrophages. These molecules are involved in the inflammation associated with insulin resistance and obesity. Elevated levels of TNFα in obese patients are shown to induce insulin resistance and type 2 diabetes. Expanded adipose release TNFα which enhances the recruitment of M1 macrophages to adipose tissue (Carey Lumeng and Alan Saltiel 2001). These macrophages also secretes TNFα and IL-6 and increases the degree of inflammatory status leading to insulin resistance. IL-6 expression in adipose tissue is positively correlated with metabolic syndrome including obesity and type 2 diabetes. MCP-1 has markedly increased in obese state and targeted deletion of MCP-1 in adipose tissue has shown reduced macrophage infiltration and increased insulin sensitivity (Gnacińska et al 2009). The inhibitory effect of EA on the expression of these inflammatory molecules will potentially enable to alleviate the inflammatory status in obesity. The expression of other adipokines that are elevated in obese condition and associated with type 2 diabetes and insulin resistance are leptin and resistin. The analysis show that EA reduced mRNA levels of leptin and resistin. Hyperleptinemia is associated with insulin resistance and elevated resistin leads to beta cell dysfunction resulting in diabetes (Maffei et al., 1995). The level of free fatty acid in circulation is very high in obese condition which contributes to insulin resistance. Free fatty acids interfere with insulin signaling pathway and reduces insulin sensitivity. Hormone sensitive lipase is the key enzyme involved in the synthesis of free fatty acids from triglycerides. The effect of EA on HSL expression in 3T3-L1 adipocytes was analyzed. The analysis demonstrates that EA reduces HSL expression in 3T3-L1 adipocytes. This reduction in HSL expression may lead to reduced
synthesis of free fatty acids from triglycerides.

**3.3.1.10 Conclusion**

Ellagic acid reduces adipogenesis and expression of major adipokines that are known to play a role in insulin resistance, obesity and type 2 diabetes. EA exerts its effect by inhibiting ERK signaling and activating AMPK phosphorylation. It is also observed that EA treated adipocytes show an enhanced glucose uptake (data not shown). EA being an antiadipogenic and anti-inflammatory molecule, could be a lead molecule in the treatment of obesity and associated complications.
3.3.2 Arjunolic Acid Downregulates Adipocyte Differentiation in 3T3-L1 Cells

3.3.2.1 Arjunolic Acid Reduces Adipocyte Differentiation in 3T3-L1 Cells

To evaluate the effect of arjunolic acid (AA) on adipocyte differentiation, 3T3-L1 preadipocytes were treated with different concentrations of arjunolic acid along with MDI (MDIA). Cells treated with MDI were used as positive control and DMSO as negative control. After 8 days, lipid accumulations were determined in these cells using Oil Red O analysis. Arjunolic acid reduced lipid accumulation in a concentration dependent manner and a maximum inhibition of 30% was obtained at 50 μM without any cytotoxic effects. (Fig 3.5.1A and B).

Fig 3.5.1 Effect of Arjunolic acid (AA) on adipocyte differentiation of 3T3-L1 cells. Two day post confluent cells were treated with MDI, MDI and different concentrations of arjunolic acid (1μM, 10μM or 50 μM), or DMSO. The medium was changed every 2 days. On 8th day of differentiation, cells were stained with Oil Red O, and the lipid accumulation was quantified by measuring the OD of Oil Red O eluted from the cells at 495 nm. Oil Red O stained images of MDI differentiated or MDI differentiated cells in presence of AA (50 μM) (A). Oil Red O quantification results of cells differentiated in presence of AA (B). Structure of arjunolic acid. Error bars represents mean values ± SD of three independent experiments done in triplicates.
3.3.2.2 Arjunolic Acid Reduced the Expression of PPARγ and its Target Gene GLUT4

To further confirm the role of AA on adipocyte differentiation, the expression level of PPARγ, the key regulator of adipocyte differentiation, and its target gene GLUT4 were analysed. 3T3-L1 cells were treated with MDI or MDIA for 48 hrs and then switched to normal media. On day 6 the expression level of GLUT4 and PPARγ were analysed using western blot. Treatment of 3T3-L1 cells with AA decreased the expression of GLUT4 and PPARγ (Fig. 3.5.2).

![Figure 3.5.2](image)

**Fig 3.5.2** Effect of AA on the expression of GLUT4 and PPARγ. Two day post confluent 3T3-L1 preadipocytes were treated with MDI or MDI with 50 μM arjunolic acid for 2 days. Medium was changed 2 days. The cells were harvested on day 4 after the treatment and immunoblotted using GLUT4 and PPARγ antibodies. The expression of GLUT4 and PPARγ upon MDI and MDIA treatments is shown.

3.3.2.3 Effect of AA on Adipocytes Differentiation was Independent of Akt

Akt is known to play a role in adipocytes differentiation and to investigate its role in AA mediated inhibition of adipocyte differentiation, 3T3-L1 cells treated with MDI or MDIA for 60 minutes and the phosphorylation status of Akt was analysed. No change in Akt phosphorylation was observed in MDIA when compared to MDI treated cells suggesting, the inhibitory effect of AA on adipocyte differentiation is independent of Akt (Fig 3.5.3).
Fig 3.5.3 Effect of AA on Akt phosphorylation. 3T3-L1 preadipocytes were treated with MDI, MDIA (50 μM), or DMSO for 1 hour. The cells were harvested and immunoblotted with phospho Akt antibodies. The phosphorylation status of Akt upon MDI or MDIA treatment in 3T3-L1 cells.

3.3.2.4 Prolonged Activation of ERK Extracellular Signal Regulated Kinase 42/44 by Arjunolic Acid.

To understand the mechanism by which AA mediates its effect, the phosphorylation status of key signaling molecules involved in adipocyte differentiation were analysed. To analyse the phosphorylation status of ERK upon AA treatment, 3T3-L1 preadipocytes were treated with MDI or MDIA for 60 mins and the phosphorylation status was analysed using western blot. The activation of ERK sustained even after 60 mins by MDIA treatment, while in MDI treated cells the activation of ERK dropped after 30 mins. (Fig 3.5.4).

Fig 3.5.4 Effect of AA on ERK 1/2 phosphorylation. 3T3-L1 preadipocytes were treated with MDI, MDIA (50 μM), or DMSO for 60 minutes. The cells were harvested and immunoblotted with pERK 42/44 antibodies. The phosphorylation status of ERK 42/44 in MDI or MDIA treated 3T3L-1 cells.
3.3.2.5 Arjunolic Acid Phosphorylates GSK3 and Inhibits its Activity

Phosphorylation of ERK 42/44 is known to phosphorylate GSK3-β in other cell types, and in this study a sustained activation of ERK 42/44 was observed upon AA treatment. To analyze whether prolonged activation of ERK 42/44 lead to GSK3-β phosphorylation upon AA treatment, cells were treated with MDI and MDIA for 60 mins and the phosphorylation status was analysed using western blot. MDI treated cells has shown an increase in the phosphorylation status of GSK3-β (Ser9) when compared to MDIA (Fig 3.5.5).

![Western Blot Image](image)

*Fig 3.5.5 Effect of AA on GSK3-β phosphorylation. 3T3-L1 preadipocytes were treated with MDI, MDIA (50 μM), or DMSO for 60 minutes. The cells were harvested and immunoblotted with phospho GSK3-β antibodies (Ser9). The phosphorylation status of GSK3-β upon MDI or MDIA treatment in 3T3-L1 cells.*

3.3.2.6 β-catenin Expression was Stabilised Upon Arjunolic Acid Treatment

Since AA has shown to inhibit GSK activity by enhancing the phosphorylation at Ser9, the expression of its effector molecule β-catenin was analysed. 3T3-L1 preadipocytes were treated with MDI, MDIA and vehicle for 48 hours and the expression was analyzed using western blot. MDI treated cells has shown a reduction in β-catenin expression when compared to untreated cells as seen in Fig 3.5.6, AA stabilized the levels of β-catenin
3.3.2.7 ERK 42/44 Inhibits GSK3 Activation

To analyze whether GSK3-β inactivation is mediated by ERK 42/44 phosphorylation, the phosphorylation status of GSK3-β upon AA treatment was analyzed in U0126 (an ERK inhibitor) pretreated 3T3-L1 cells. In U0126 pretreated 3T3-L1 cells the phosphorylation of GSK3-β was reduced compared to untreated cells. This results show that the Ser9 phosphorylation of GSK3-β is depended on ERK 42/44 phosphorylation (3.5.7).

3.3.2.8 Arjunolic Acid Reduced Inflammatory Adipokine Expression

Since AA is well known for its anti inflammatory activity in different cell types including macrophages, its effect on inflammatory adipokine expression was analysed
using real time PCR analysis. 3T3-L1 adipocytes were treated with and without arjunolic acid for 12 hours and the expression of IL-6, TNFα, and MCP were analysed. The analysis show that AA reduced IL-6, TNFα and MCP-1 by 80%, 40% and 42% respectively (Fig 3.5.8).

![Bar chart showing the effect of AA on inflammatory adipokine expression in 3T3-L1 adipocytes.](image)

**Fig 3.5.8** The effect of AA on inflammatory adipokine expression in 3T3-L1 adipocytes. Adipocytes were treated with and without AA for (50 μM) for 12 hours. Cells treated with 0.2% DMSO were used as control. After treatment, total RNA was isolated and subjected to quantitative analysis of adiponectin by real-time PCR. Relative expression was analysed using 2^ΔΔCT. The relative fold difference in IL-6, TNFα and MCP-1 expression in AA treated and untreated cells are shown. Error bars represents mean values ± SD of three independent experiments done in triplicates.

### 3.3.2.9 Discussion

Arjunolic acid (AA) is a triterpenoid having various biological functions. AA ameliorates cytotoxicity in hepatocytes, it is well known for its anti-inflammatory, anticancer and antiasthmatic activity but there are no reports on its effect on adipogenesis (Manna et al 2007; Hemalatha et al. 2010). In the preliminary screening, AA was found to reduce adipocyte differentiation. A maximum of 30 % reduction in lipid accumulation was observed at 50 μM. Triterpenoids like oleanolic acid and oleanolic triterpenoid 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole (CDDO-Im) is known to inhibit adipogenesis in 3T3-L1 cells. Oleanolic acid is known to inhibit adipocyte differentiation at a non toxic concentration of 25 μM (Sung et al., 2010; Hughes et al., 2008). Arjunolic acid has a similar structure like oleanolic acid that might corresponds to this antiadipogenic effect. In order to confirm the antiadipogenic effect, the expression levels
of the master regulator of adipogenesis PPARγ and its target gene GLUT 4 were analyzed and a reduction in the expression levels of these marker proteins were noticed using western blots. Oleanolic acid has also shown to inhibit adipocyte differentiation by reducing PPARγ expression.

Further studies were carried out to understand how AA mediates its antiadipogenic effect. The effect of AA on ERK and insulin signaling pathways was demonstrated. A prolonged activation of ERK 42/44 was observed with no detectable difference in Akt activation. ERK 1/2 is known to have a biphasic effect on adipogenesis with an increase in ERK 42/44 phosphorylation during the initial hour of adipocyte differentiation and a subsequent gradual reduction in its expression. However, it was also shown that a sustained ERK 42/44 activation can also reduce adipogenesis (Prusty et al., 2002). For example, Kim et al has shown that a sustained expression of Pre-adipocyte factor 1, Pref1, was sufficient to maintain the status of preadipocytes. Deletion of this gene from preadipocyte was sufficient for its differentiation. The antiadipogenic effect of this gene was due to its role in the prolonged activation of ERK (Kim et al., 2007). Tanabe et al used mechanical stretching to inhibit adipocyte differentiation and has shown that in this case also a prolonged ERK activation was sufficient for this inhibition. (Tanabe and Nakayama 2004). Oncostatin M mediates its antiadipogenic effect by activating ERK 42/44 (Miyaoka et al., 2006).

A prolonged activation of ERK 42/44 leads to inhibition of GSK-3β activity and there by up regulating β-catenin level (Ding et al 2005). Therefore, we analysed the phosphorylation status of GSK-3 β upon AA treatment. Interestingly it was found that AA induced phosphorylation of GSK-3 β leading to its inhibition. GSK-3 β/ β-catenin signaling is known to play a role in adipogenesis. It is known that GSK-3 β phosphorylates β-catenin and targets it for degradation by the ubiquitin-proteasomal system. Inhibition of GSK3-β 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. Hence the β-catenin levels were also analysed. The study has shown that AA stabilized the levels of β-catenin. Further to confirm the role of ERK in GSK3 inhibition, MEK/ERK inhibitor, U0126, pretreated 3T3-L1 cells were treated with AA and the phosphorylation status of GSK3-β was analysed. Analysis showed that U0126 reduced phosphorylation of GSK3-β. These results show that ERK/GSK3-β/β-catenin
play a role in AA mediated inhibition of adipocyte differentiation. Compounds like curcumin and isorhamnetin has shown to inhibit adipocyte differentiation by stabilizing β-catenin (Ahn et al., 2010; Lee et al., 2010).

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 PCR was performed to analyse adipokine expression in AA treated 3T3-L1 adipocytes. TNFα, IL-6, MCP-1 expression were decreased upon AA treatment in 3T3-L1 adipocytes. This decreased expression of anti-inflammatory cytokines could potentially ameliorate chronic inflammation in obese conditions which otherwise could lead to insulin resistance. A molecule like arjunolic acid which is shown to exhibit both anti adipogenic and anti inflammatory effect need to be studied in detail to understand the efficacy of this compound in treating obesity induced insulin resistance and type 2 diabetes. Obesity is associated with chronic inflammation which ultimately leads to the development of insulin resistance and type 2 diabetes. TNFα, IL-6, MCP-1 contributes to obesity induced insulin resistance. Molecules that could reduce inflammatory status will ameliorate obesity associated complications.

3.3.2.10 Conclusion

Arjunolic acid has shown to down regulate inflammatory adipokine expression and reduce adipocyte differentiation. ERK/GSK-3 β/ β-catenin pathway is involved in AA mediated reduction of adipogenesis. This study enabled us to identify AA as an antiadipogenic molecule which has the potential to be a lead molecule for treating obesity related complications.
Key findings from this study.

- Vanillin was identified as a novel positive modulator of adipocyte differentiation. Vanillin induces adipogenesis by activating extracellular signal regulated kinase 42/44 in the initial hour of adipogenesis. Olfactory receptor 56 was identified as a vanillin binding protein. Further proteomic studies have identified a set of proteins that have been upregulated during vanillin mediated adipocyte differentiation which includes early marker of adipocyte differentiation, metallothionein and a calcium binding protein, PS100-A4.

- A synthetic molecule 2, 4 DAR induced adipocyte differentiation in 3T3L1 cells. 2, 4 DAR binds to LBD of PPARγ. The compound modulated adiponectin expression and exhibited insulin sensitivity.

- Ellagic acid was identified as a negative modulator of adipocyte differentiation. Ellagic acid inhibited ERK 44/42 phosphorylation and enhanced AMPK activation. It also modulates the expression of major adipokines involved in insulin resistance.

- Arjunolic acid was identified as an antiadipogenic agent. Arjunolic acid exerts its effect via ERK 44/42/GSK3-β/β-catenin signaling pathway. The expression of inflammatory adipokines was reduced by arjunolic acid.