Chapter 4

Study the efficacy of different small molecules on the induction of insulin sensitivity through A_{2A}AR signalling pathway

4.1 Introduction

Natural products based traditional medicine has made a remarkable utilisation in the past 100 years [1]. The frequency of understanding the molecular structure-function relationship of various drug-like candidates increased in the last decade [2].The combination of computational (*in silico*) techniques of discoveringsmall molecules phytochemicals and the testing theirhypothesized bioactivities at molecular level for treating various diseases and disordersboth *in vitro* and *in vivo*animal models has revolutionized pharmacology[3].The various *in silico*toolsapplied are namely (i) druglikeness property based screening from the database satisfying the Lipinski Rule of five, (ii) quantitative structure activity relationship (QSAR) analysis to analyse the relation between physicochemical properties and biological activity, (iii) ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) screening to predict the bioactivity of selected molecules and (iv) molecular docking to study the target protein-ligand interactions, respectively[4].All these methods assist in screening out putative ligands or small molecules showing decent docking with a target protein in its active conformation which could be further subjected to *in vitro* and *in vivo* testing.

Type 2 diabetes (T2D) is one of the leading causes of human deaths in the last few decades with an affected population of around 450million [5]. Sedentary life style along with obesity has become the major reasons for the progression in T2D.Increase in intra abdominal fats and circulatory free fatty acids (FFA) levels trigger the activation of inflammatory signalling pathways causing adipose tissueinflammationthat leads to the development of insulin resistance [6]. Adenosine receptor signalling pathway activation has been reported to improve insulin sensitivity and promoting pancreatic β-cells regeneration thus rescuing the diabetic state [7-10].Various reports suggest the role of adenosine receptors specifically A_{2A}AR and A_{2B}AR in mediating anti-inflammatory effect, although their underlying mechanism remains unexplored [11-13]. In the screening of 142 potential anti-diabetic molecules, we found that two small molecules namely indirubin-3'-monoxime (I3M) and vanillin (VNL) exhibits their binding affinity

with A_{2A}AR agonist conformation. The bioactivity of I3M and VNL was further tested on FFA induced mature 3T3-L1 adipocytes and analysed the downstream effect on the expression and activation status of various inflammatory molecules and cytokines.

4.2 Results:

4.2.1 In-silico studies for screening of potential adenosine 2A receptor (A_{2A}AR) agonists

To find out effective A_{2A}AR agonists of non-adenosine structures, we selected 142 potential anti diabetic compounds of different categories like flavonoid, alkaloids, terpenes and sulfonylurea from the literature (**Table 4.1**) and investigated their binding affinity with the agonist conformation of A_{2A}AR using various in silico approaches.

Sl. No.	Compounds	Sl. No.	Compounds	Sl. No.	Compounds
1.	Acarbose	49.	Glycyrrhizic Acid	97.	Phenformin
2.	Acetovanillone	50.	Gossypol	98.	Phloretin
3.	Aesculin	51.	Gramine	99.	Phlorizin
4.	Allopurinol Sodium	52.	Guanosine	100.	Physcione
5.	Aloin	53.	Hesperetin	101.	Pioglitazone
6.	Ammonium Glycyrrhizinate	54.	Hesperidin	102.	Piperine
7.	Amygdalin	55.	Hordenine	103.	Polydatin
8.	Andrographolide	56.	Honokiol	104.	Puerarin
9.	Apigenin	57.	10-hydroxycamptothecin	105.	Quercetin
10.	Arbutin	58.	5-hydroxytryptophan	106.	Repaglinide
11.	Artesunate	59.	Hyodeoxycholic Acid	107.	Rosiglitazone Hydrochloride
12.	Asiatic Acid	60.	Hypoxanthine	108.	Rotundine
13.	Astragaloside A	61.	Icariin	109.	Rucaparib
14.	Baicalein	62.	Indirubin	110.	Rutaecarpine
15.	Baicalin	63.	Indole-3-butyric acid	111.	Rutin
16.	Berberine Hydrochloride	64.	Inosine	112.	Salicin
17.	Bilobalide	65.	Ipriflavone	113.	Salidroside
18.	Biochanin A	66.	Isoliquiritigenin	114.	Saxagliptin
19.	Bosentan	67.	Kaempferol	115.	Sclareol
20.	Caffeic Acid	68.	Kinetin	116.	Sclareolide
21.	DL-Carnitine Hydrochloride	69.	Lappaconite Hydrobromide	117.	Sesamin
22.	Chlorogenic Acid	70.	Linagliptin	118.	Shikimic Acid
23.	Chrysin	71.	Luteolin	119.	Silibinin
24.	Chrysophanic Acid	72.	Magnolol	120.	Silymarin
25.	Cinchonidine	73.	Manidipine	121.	Sinomenine
26.	Cryptotanshinone	74.	D-Mannitol	122.	Sitagliptin
27.	Cyclocytidine	75.	Matrine	123.	β-Sitosterol
28.	Cytisine	76.	Methyl Hesperidin	124.	Sodium

					Danshensu
29.	Dapagliflozin	77.	Miglitol	125.	Sophocarpine
30.	Dihydroartemisinin	78.	MK-8245	126.	Sorbitol
31.	Dihydromyricetin	79.	Morin Hydrate	127.	Synephrine
32.	Dioscin	80.	Myricetin	128.	TAK-875
33.	Diosmetin	81.	Myricitrin	129.	Tangeretin
34.	Diosmin	82.	Nalidixic Acid	130.	Tanshinone I
35.	Emodin	83.	Naringenin	131.	Tanshinone II A
36.	Enoxolone	84.	Naringin	132.	Taxifolin
37.	Epigallocatechingallate	85.	Nateglinide	133.	Tetrandrine
38.	Ferulic Acid	86.	Neohesperidin	134.	Triptolide
39.	Fisetin	87.	Neohesperidin Dihydrochalcone	135.	Troxerutin
40.	Formononetin	88.	2-Nitroimidazole	136.	Ursolic Acid
41.	Gastrodin	89.	Nobiletin	137.	D-Usnic Acid
42.	Gliclazide	90.	Oleanolic Acid	138.	Vanillin
43.	Glimepiride	91.	Oridonin	139.	Vanillylacetone
44.	Glipizide	92.	Orotic Acid	140.	Violdagliptin
45.	Gliquidone	93.	Osthole	141.	Xanthone
46.	Glyburide	94.	Oxymatrine	142.	Yohimbine Hydrochloride
47.	Glycyrrhizic Acid	95.	Paeonol		
48.	Gossypol	96.	Parthenolide		

Table 4.1: List of compounds (142 in number) used in present investigation

The comparative analysis has suggested that vanillin, indirubin and cytisine are forming stable complexes after an effective docking into the binding cavity of A_{2A}ARvia the formation of hydrogen bonding at Phe168 or Glu169 residues of A_{2A}AR that are responsible for active site binding interaction.Visualization of the receptor-ligand complexes in Ligplot (Fig. 4.1) showed the presence of hydrogen bond and the hydrophobic interactions. The selected compounds, having optimal binding energy, hydrogen bonding and non-bonded interaction with the active site residues, have a potential binding affinity towards the A_{2A}AR.

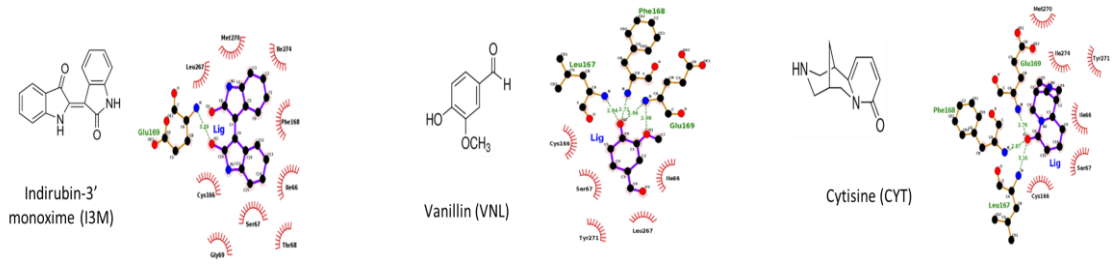
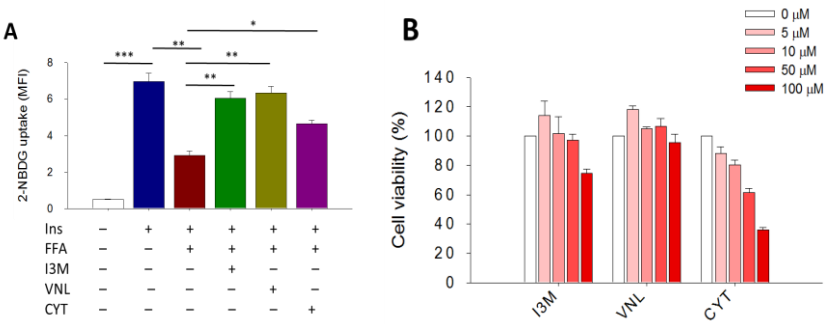


Fig 4.1 Images representing chemical structure of indirubin- 3'-monoxime (I3M), vanillin (VNL), and cytisine (CYT), with their interactions with A_{2A}AR using ligplot.

4.2.2 Vanillin (VNL)and indirubin-3'-monoxime (I3M)prevents lipid induced insulin resistance through the activation of A_{2A}AR:

Activation of insulin signalling pathway leads to cellular glucose uptake and thus maintaining glucose homeostasis. Impairment of this signalling cascade by palmitate, a saturated free fatty acid, is known to be associated with the reduction of glucose transport implementing a state of insulin resistance [15-17].In order to observe whether impairment of insulin signaling by palmitate could be prevented by vanillin, cytisine and indurubin, we incubated these compounds with 3T3-L1 adipocytes followed by the treatment with palmitate in absence or presence of insulin.Insulin stimulated 2-NBDG uptake by 3T3-L1 adipocytes was significantly inhibited by palmitate, however, such inhibition was significantly waived by vanillin, cytisine, and indirubin treatment. Interestingly, vanillin and indirubin effect is more pronounced in comparison to cytosine (Fig. 4.2A). To test the cytotoxicity of these compounds, we performed MTT assay on 3T3-L1 adipocytes. The result showed that vanillin and indirubin does not have any toxic effect ontheir tested concentrations, which is more prevalent in cytisine treatment (Fig. 4.2B). Based on these results, we selected vanillin and indirubin for further study. VNLand I3M incubation dose dependently stimulates glucose uptake (Fig. 4.2C). Interestingly, the beneficial effect of VNL and I3M on insulin stimulated glucose uptake was prevented when cells were pre-treated with caffeine or SCH-58261, AR and A_{2A}AR receptor antagonists, respectively. Moreover, vanillin effect was not inhibited by adenosine deaminase voiding the possibility of extracellular adenosine accumulation in response to vanillin which may activate A_{2A}AR (Fig. 4.2D).



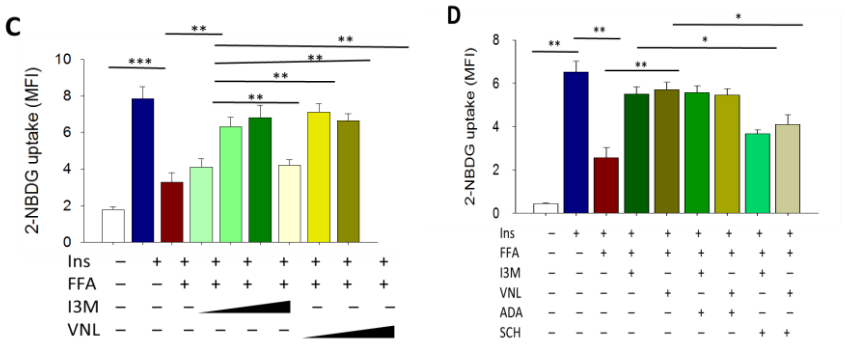


Figure 4.2. (A) Analysis of 2-NBDG uptake by 3T3-L1 adipocytes in response to Insulin (Ins, 100 nM) or Ins + FFA (palmitate, 0.75 mM) in presence or absence of indirubin-3'-monoxime (I3M, 10 µM), or vanillin (VNL, 100 nM) or cytisine (CYT, 10 mM). (B) Determination of viable cells (%) in response to indicated concentrations of I3M, VNL, and CYT treated 3T3-L1 adipocytes. (C) Dose dependent effect of I3M (2, 5, 10 µM) or VNL (50, 100, 200 nM) on 2-NBDG uptake by 3T3-L1 adipocytes. (D) Effect of adenosine deaminase (ADA, 0.00138 U/ml), or SCH 58261 (SCH, 300 nM) on the I3M (10 mM) mediated attenuation of lipid-induced impairment of 2-NBDG uptake in 3T3-L1 adipocytes. All experiments were performed in triplicate. Each value is the mean ± SEM of three independent experiments. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

Activation of insulin signalling cascade initiates when insulin binds to its receptors on target cellsand phosphorylates insulin receptor (InR) and insulin receptor substrate-1 (IRS-1) on its tyrosine residues. Phosphorylation of IRS-1 recruits and activates different signalling molecules that ultimately lead to the activation of Protein Kinase B/Akt which facilitates glucose transporter-4 (Glut4) migration from cytosol to plasma membrane inflicting cellular glucose uptake [15-16]. It could be seen from Fig. 4.3A that palmitate markedly inhibits insulin stimulated IRS-1 and Akt phosphorylation. Although vanillin did not alter the IRS-1 phosphorylation, however, intriguingly notable stimulation of Akt activation was observed suggesting that vanillin possibly stimulates post-receptor events. SCH-58261 treatment abrogates vanillin action suggesting the involvement of A_{2A}AR. These results indicate two important aspects; (i) vanillin action is independent of InR and IRS-1, upstream mediators of insulin signalling cascade, and (ii) it may act on the downstream of insulin signalling through the activation of A_{2A}AR. To examine further about vanillin stimulation of insulin sensitivity and participation of A_{2A}AR, we investigated Glut-4 migration, an important marker for insulin action, in L6 myotubes. Insulin effected Glut-4 migration from cytosol to membrane was prevented by palmitate which was markedly waived by vanillin incubation. However, vanillin effect was compromised in cells when treated with A_{2A}AR antagonist SCH-58261 (Fig.

4.3B). Our results reinforce that vanillin prevents lipid induced insulin resistance via the mediation of $A_{2A}AR$ activation.

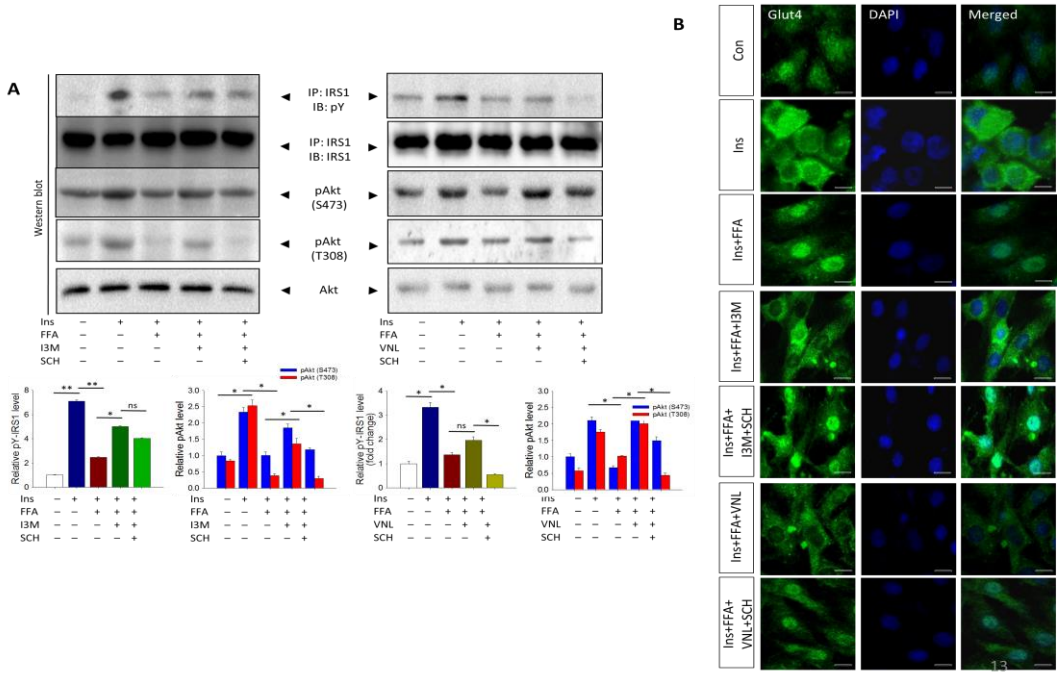


Figure 4.3. (A) Western blot (upper) and its quantification (lower) showing pY-IRS1 and pAkt (S473 and T308) abundance in 3T3-L1 adipocytes in response to insulin (Ins, 100 nM) or Ins + FFA (palmitate, 0.75 mM) in absence or presence of I3M (10 μM) and VNL (100nM) without or with SCH 58261 (SCH, 300 nM). IRS-1 and Akt were used as loading controls. (B) Representative immunofluorescence images (upper) and its quantification (lower) showing Glut4 abundance and localization in indicated incubations of L6 cells. DAPI used for nuclear counterstaining. Scale bar, 20 mm. All experiments were performed in triplicate. Each value is the mean ± SEM of three independent experiments, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns = nonsignificant.

4.2.3 Vanillin stimulates $A_{2A}AR$ signalling and promotes anti-inflammatory state of adipocytes:

It is well known that stimulation of the $A_{2A}AR$, a G-protein α-s coupled receptor, caused activation of MAPK for promoting cellular proliferation and stimulation of adenylyl cyclase dependent cAMP production which confers resolution of inflammation [18-23]. Interestingly, we observed noticeable stimulation of ERK1/2 phosphorylation without any significant change in p38 activation in response to VNL and I3M treatment. VNL and I3M stimulated increased ERK1/2 phosphorylation was strikingly annihilated by $A_{2A}AR$ inhibitor SCH-58261 (Fig. 4.4A). Activation of $A_{2A}AR$ caused increased production of secondmessenger cAMP which by activating protein kinase A (PKA) stimulates cAMP responsive element binding protein (CREB) phosphorylation and thus

regulates downstream genes expression [18]. As illustrated in Fig. 4.4 B, increased abundance of phosphorylated CREB in 3T3-L1 adipocytes in response to vanillin was notably relinquished by SCH-58261. These results indicate the agonistic behaviour of vanillin.

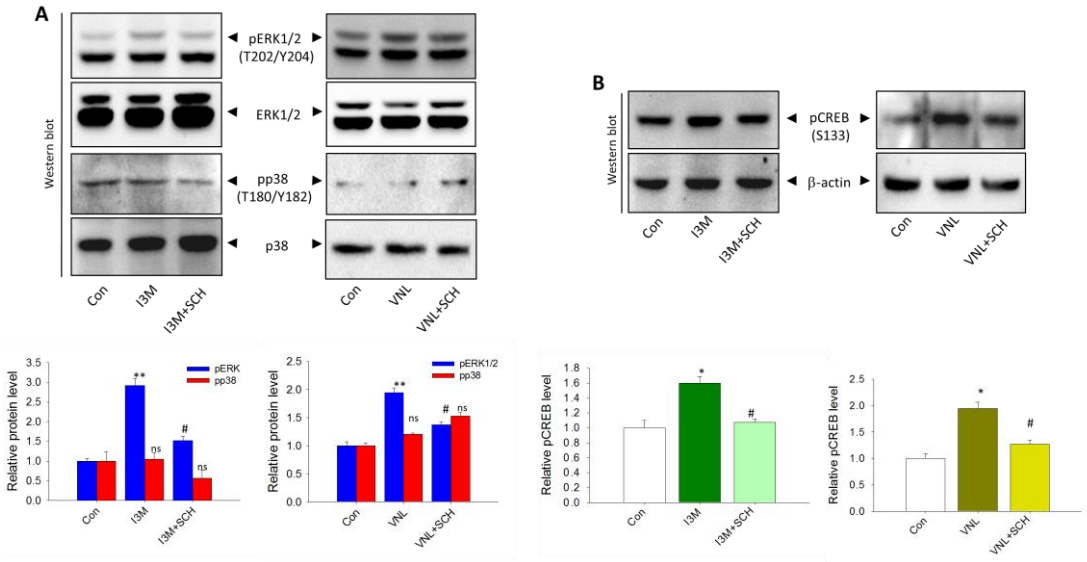
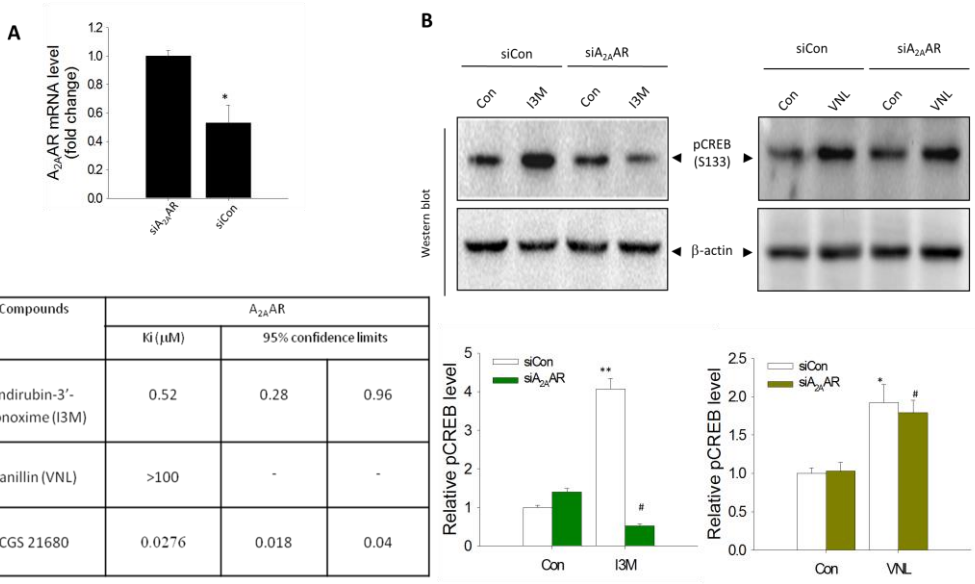


Figure 4.4. (A) Western blots (upper) and its quantification (lower) showing pERK1/2 (T202/Y204) and pp38 (T180/Y182) abundance in 3T3-L1 adipocytes incubated without or with I3M (10 μM) in absence or presence of SCH 58261 (SCH, 300 nM). ERK1/2 and p38 were used as loading controls. Each value is the mean ± SEM of three independent experiments, ** P < 0.01, * P < 0.05 vs Con, # P < 0.05 vs I3M, VNL (B) Western blots (upper) and its quantification (lower) showing pCREB (S133) abundance in 3T3-L1 adipocytes incubated without or with I3M (10 μM) in absence or presence of SCH 58261 (SCH, 300 nM). β-actin was used as a loading control. Each value is the mean ± SEM of three independent experiments, * P < 0.05 vs Con, # P < 0.05 vs I3M, VNL

To secure direct evidence of $A_{2A}AR$ involvement in I3M or VNL effect, we examined the efficacy of I3M and VNL on CREB activation in $A_{2A}AR$ silenced 3T3-L1 cells (Fig 4.5 A). I3M induced CREB phosphorylation was considerably attenuated in $A_{2A}AR$ knockdown cells but intriguingly, we noticed significant enhancement of CREB phosphorylation in VNL treated $A_{2A}AR$ silenced cells. This result indicates the participation of $A_{2A}AR$ in I3M mediated effect, whereas, VNL's action does not necessitate the requirement of $A_{2A}AR$ signalling (Fig. 4.5 B). To confirm further, a radio ligand binding assay was performed which displayed a potent high affinity binding of I3M with $A_{2A}AR$ (Ki: 0.52 μM) and was selective over $A_{2B}AR$, but, it was observed that VNL did not directly bind with $A_{2A}AR$ (Fig 4.5 C)



Compounds	$A_{2A}AR$		
	Ki (μM)	95% confidence limits	
Indirubin-3'-monoxime (I3M)	0.52	0.28	0.96
Vanillin (VNL)	>100	-	-
CGS 21680	0.0276	0.018	0.04

Figure 4.5: (A) Abundance of $A_{2A}AR$ gene expression in Control siRNA or $A_{2A}AR$ siRNA transfected 3T3-L1 cells by RT-qPCR analysis, **(B)** Western blots (upper) and its quantification (lower) showing pCREB (S133) abundance in control siRNA (siCon) and $A_{2A}AR$ siRNA ($siA_{2A}AR$) transfected 3T3-L1 adipocytes incubated without or with I3M (10 μM), VNL(100nM). β-actin was used as a loading control. Each value is the mean ± SEM of three independent experiments, ** $P < 0.01$ vs control siRNA transfected cells control cells, ## $P < 0.01$ vs control siRNA transfected cells treated with I3M,VNL, **(C)** Competition of putative agonist (I3M) or agonist (CGS 21680) for [3H]NECA binding at $A_{2A}AR$ and the effect of I3M or CGS 21680 on adenylyl cyclase activity/cAMP level via $A_{2B}AR$

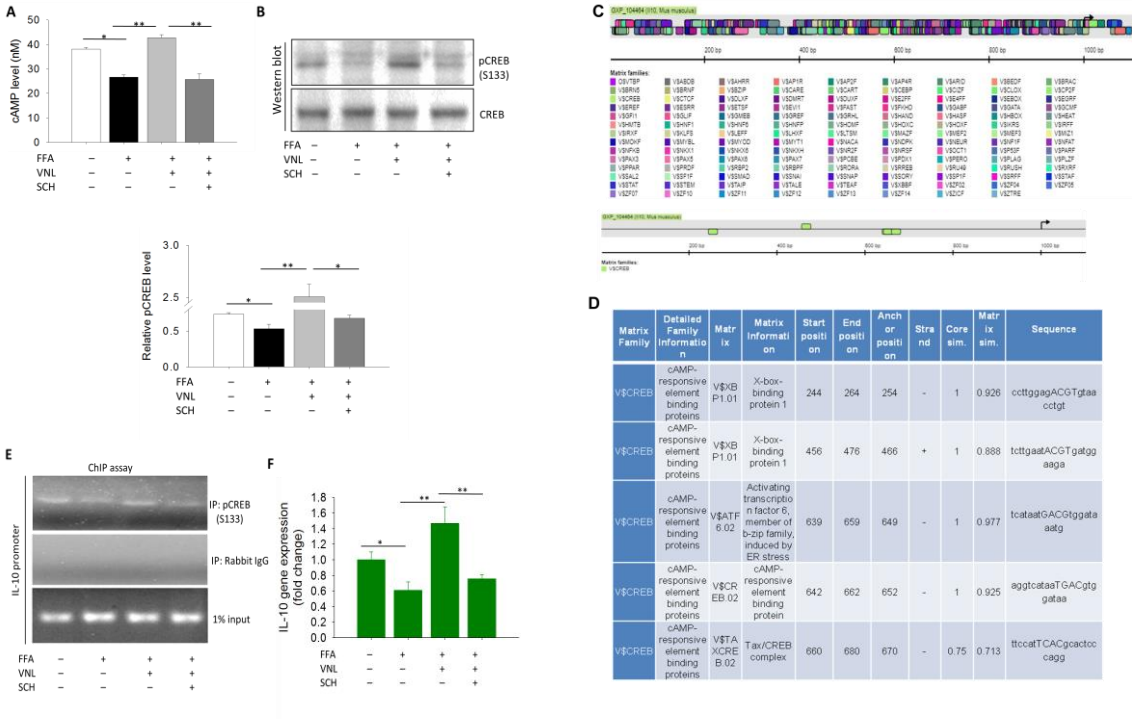
We next explored the efficacy of vanillin and involvement of $A_{2A}AR$ therein in the *in-vitro* model of lipid induced insulin resistant 3T3-L1 adipocytes. Palmitate significantly lowered the intracellular cAMP level in 3T3-L1 adipocytes which was waived by vanillin; however, treatment with SCH-58261 conspicuously opposed vanillin action (Fig. 4.6A).

To investigatewhether stimulation of $A_{2A}AR$ by vanillin could lead to the activation of CREB, we observed that vanillin evoked increased phosphorylation of CREB in adipocytes which could be suppressed by $A_{2A}AR$ antagonist SCH-58261 (Fig. 4.6B) suggesting a potential role of CREB activation in mediating vanillin effected $A_{2A}AR$ stimulation.

Recent studies have indicated the involvement of activated CREB in the induction of IL-10 anti-inflammatory cytokine gene expression [24]. We then searched for putative CREB-binding sites on the IL-10 promoter and found four probable binding sites as detected by MatInspector Professional program with considering sequence homology of classical CREB-response elements (Fig.4.6C,D). Considering that increased occupation

of activated CREB in IL-10 promoter may be involved in vanillin mediated induction of IL-10 gene expression, we performed ChIP assay and RT-qPCR analysis.

Vanillin incubation distinctly averts palmitate effect causing increased binding of phosphorylated CREB to the IL-10 promoter (Fig. 4.6E) which resulted in the enhanced level of IL-10 gene expression (Fig. 4.6F). However, vanillin effect on activated CREB binding on IL-10 promoter and IL-10 gene expression were considerably attenuated by SCH-58261 (Fig. 4.6E,F). Collectively, these results suggest that vanillin strongly promotes anti-inflammatory state in adipocytes through the activation of A_{2A}AR-cAMP-CREB pathway.

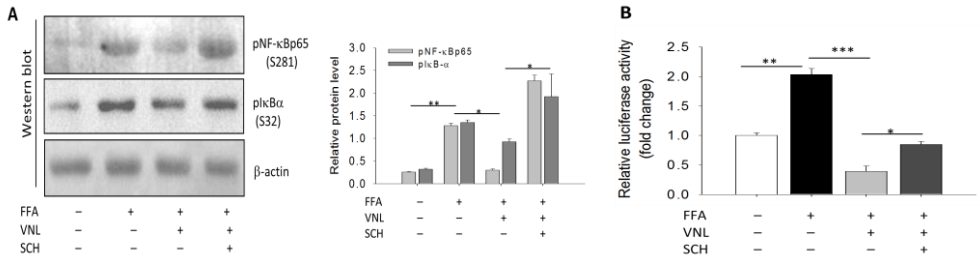


Matrix Family	Detailed Family Information	Matrix	Matrix Information	Start position	End position	Anchor position	Strand	Core sim.	Matrix sim.	Sequence
V$CREB	cAMP-responsive element binding proteins	V$XBP1.01	X-box-binding protein 1	244	264	254	-	1	0.926	ccttggagACGTgtaacctgt
V$CREB	cAMP-responsive element binding proteins	V$XBP1.01	X-box-binding protein 1	456	476	466	+	1	0.888	tcttgaatACGTgatggaaga
V$CREB	cAMP-responsive element binding proteins	V$ATF6.02	Activating transcription factor 6, member of b-zip family, induced by ER stress	639	659	649	-	1	0.977	tcataatGACGtggataaatg
V$CREB	cAMP-responsive element binding proteins	V$CREB.02	cAMP-responsive element binding protein	642	662	652	-	1	0.925	aggtcataaTGACgtggataa
V$CREB	cAMP-responsive element binding proteins	V$TAXCREB.02	Tax/CREB complex	660	680	670	-	0.75	0.713	ttccatTCACgcactccagg

Figure 4.6. A) 3T3-L1 adipocytes were incubated with FFA for 4h followed by the incubation without or with VNL in absence or presence of SCH-58261 (SCH). On termination of incubations, cAMP level was estimated. (B) Western blot (and its quantification) showing abundance of pCREB (S133) level in 3T3-L1 adipocytes incubated without or with FFA in absence or presence of VNL or VNL+SCH-58261. CREB was used as loading controls. (C) IL-10 promoter analysis by MatInspector database revealing putative binding sites for different transcription factors (top) and CREB (bottom). The mouse IL-10 promoter region GXP_104464 spanning -680/-244 fragment contains five putative CREB binding sites which are responsible for IL-10 transactivation (D) Table showing detailed description of individual CREB binding sites with start and end position on mouse IL-10 promoter region along with the putative binding sequences. (E) ChIP assay showing pCREB (S133) binding to IL-10 promoter in 3T3-L1 adipocytes in response to FFA incubation without or with VNL or VNL+SCH-58261. Rabbit IgG used as negative control and 1% input as loading control. (F)Real time quantitative PCR analysis showing fold change of IL-10 mRNA expression in 3T3-L1 adipocytes incubated without or with FFA in absence or presence of VNL or VNL+SCH-58261. Gapdh served as an internal control for normalization. Each value is the mean ± SEM of three independent experiments, **p < 0.01 vs Con, *p < 0.05.

4.2.4 Activation of $A_{2A}AR$ by vanillin attenuates lipid induced adipocyte inflammation:

Several investigators have shown that FFA-mediated activation of nuclear factor-κB (NF-κB) stimulates increased production of pro-inflammatory cytokines from inflamed adipose tissue that play a role in the impairment of the insulin signaling pathway. As expected, incubation of palmitate in 3T3-L1 adipocytes enhanced NF-kBp65 and IkBa phosphorylation which was substantially impeded by vanillin. However, vanillin effect was compromised when cells were treated with SCH-58261 (Fig. 4.7A). To have a better insight of vanillin effect and the contribution of $A_{2A}AR$ in attenuating palmitate stimulation of NF-κB transactivation potential, we investigated κB luciferase reporter activity in 3T3-L1 adipocytes. Palmitate enhanced luciferase reporter activity was significantly reduced in vanillin incubated cells; however, vanillin effect was reversed when cells were pre treated with SCH-58261 indicating that vanillin down regulates NF-κB transactivation by activating $A_{2A}AR$ (Fig. 4.7B). We then examined the expression of NF-kB regulated downstream genes and found that vanillin incubation remarkably prevents palmitate induced upregulation of MCP-1, IL-6, IL-1β and TNF-α cytokines gene expression which was inhibited by SCH-58261 (Fig. 4.7C). All these results suggest that vanillin effect maybe via $A_{2A}AR$ activation alleviate lipid induced adipocyte inflammation.



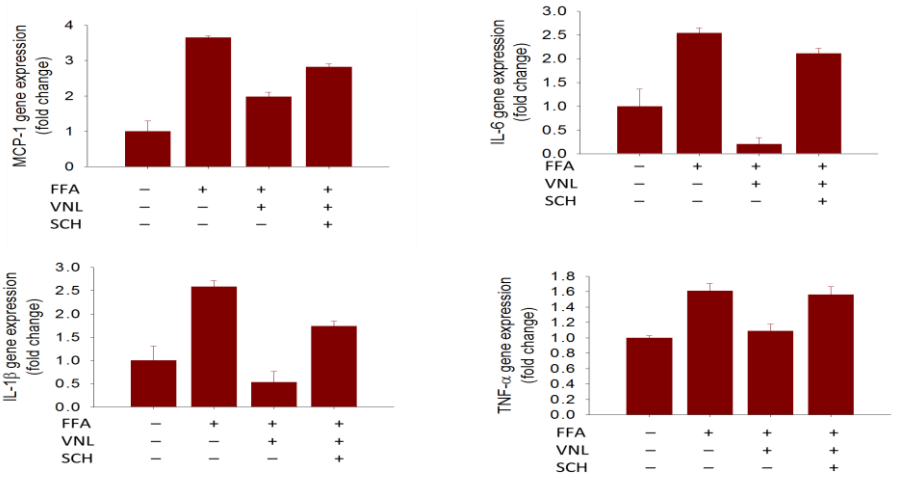


Figure 4.7: (A) Western blot and its quantification showing pNF-kB (S281) and pIkB-(S32) abundance in 3T3-L1 adipocytes incubated without or with FFA in absence or presence of VNL or VNL+SCH-58261. β-actin serves as loading control (B) 3T3-L1 adipocytes transfected with kB luciferase plasmid were incubated without or with FFA in absence or presence of VNL orVNL+SCH-58261. On termination of incubations, cells were lysed and luciferase activity wasmeasured by multimode reader. (C) Real time quantitative PCR analysis showing fold change ofMCP-1, IL-1β, IL-6 and TNF-α mRNA expression in 3T3-L1 adipocytes incubated without orwith FFA in absence or presence of VNL or VNL+SCH-58261. GAPDH served as an internalcontrol for normalization. Each value is the mean ± SEM of three independent experiments, ***p< 0.001; **p < 0.01; *p < 0.05.

4.3 Discussion:

Natural products and plant derived products have been explored for their various bioactive attributes from time immemorial with many reported to have active anti-microbial, anti-cancer, anti-inflammatory effects [25-28].Adenosine signalling pathway pose an effective role in circumventing insulin resistance [29-31] and thus in the quest of finding smallmolecules that can act as activeadenosine receptor A_{2A}AR agonists the current study was designed.The current study explored the potential bioactive aspects of two natural products namely vanillin (VNL) and indiubin-3'-monoxime (I3M), respectively. In our attempt to find potential A_{2A}AR agonists, we selected 142 different molecules based on two different aspects; (i) it should not have structural similarity with adenosine and therefore be devoid of a ribose moiety and (ii) should have an anti-diabetic effect published in the literature. Screening of these compounds with the active conformation of A_{2A}ARthrough *in-silico* approaches yielded six molecules with lower toxic values. Out of the six molecules, only indirubin-3'-monoxime (I3M), vanillin (VNL) and cytisine (CYT) could develop hydrogen bonding with the active site

residues Phe168 and Glutamate169 as depicted in the Ligplot analysisof the docked compounds (Fig 4.1).

CYT was excluded from further analysis as it imparted cytotoxic affect thus narrowing down to I3M and VNL. Investigating the efficacy of I3M and VNLfor preventing insulin resistance, we utilized a well established *in-vitro* model of insulin resistance by incubating 3T3-L1 adipocytes with a saturated free fatty acid palmitate. Palmitate incubation significantly impairs insulin signaling represented by attenuated insulin stimulated glucose uptake (Fig 4.2), compromised insulin signaling pathway molecules activation and reduced Glut4 migration from cytosol to the plasma membrane (Fig 4.3). The potential activation of A_{2A}AR signalling pathway is marked by the up regulated phosphorylation status of MAPK molecule ERK1/2 and adenylyl cyclase dependent cAMP production which confers resolution of inflammation [32].I3M and VNL mediated significant up regulation of ERK1/2 and CREB phosphorylation which was notably reduced by SCH 58261 (Fig 4.4) Thus, supporting the probable A_{2A}AR agonistic effect of VNL and I3M. Although some significant observations bent the direction of the research as it was observed that VNL treatment did not attenuatedCREB phosphorylation in A_{2A}AR silenced 3T3-L1 cells and also showed a very high Ki value (>100μM) thus indicating that VNL might not be directly binding to A_{2A}AR. On the contrary, I3M proved to act directly by binding A_{2A}AR with a potent high affinity binding(Ki: 0.52 μM) with A_{2A}AR (Fig 4.5).

Interestingly VNL imparted anti-inflammatory effect via the activation of A_{2A}AR-cAMP-CREB pathway as supported by the waived cAMP level with an up regulated CREB phosphorylation in FFA treated 3T3-L1 cells. VNL treatment alsoresulted in an enhanced IL-10 gene expression via the phosphorylated CREB binding to the IL-10 promoter thus supporting theresearch finding which state that CREB activation is mandatory to mediate the induction of IL-10 anti-inflammatory gene expression [33]and all these effects were compromised by A_{2A}AR antagonist SCH-58261 (Fig 4.6). It is established that FFA induces pro-inflammatory status via activation of nuclear factor-κB (NF-κB)[34] and VNL significantly down regulated the FFA mediated up regulation of MCP-1, IL-6, IL-1β and TNF-α cytokines gene expression via the attenuation of enhanced NF-kBp65 and IkBα phosphorylation in 3T3-L1 adipocytes (Fig 4.7). Although VNL did not establish a direct binding with adenosine 2A receptor, however,

vanillin incubation notably stimulates A_{2A}AR signalling, which could be due to the post-receptor activation upon VNL incubation.Therefore, future study in this direction to examine the molecular target(s) of VNL for the stimulation of A_{2A}AR signalling will be helpful to delineate its molecular mechanism of action. In conclusion, the present study provides evidence that I3M and VNL has potential to inhibit inflammation and improve insulin responsiveness in adipocytes.

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