

## **Chapter 7**

### **Conclusion and future prospects**

## 7.1 Conclusion

The current study investigated two small molecules, indirubin-3'-monoxime (I3M) and vanillin (VNL), the efficacies and molecular targets in improving insulin sensitivity and reducing inflammation by targeting A<sub>2A</sub>AR signalling activation and combating inflammation by inhibiting TLR4 signalling activation. The present study elucidated I3M as a novel adenosine receptor agonist that markedly prevents lipid-induced inflammation and IR in adipocytes, whereas VNL acts as a novel and potent IRAK4 that significantly mitigate LPS-induced inflammation in macrophages.

Initially, in silico screening and docking approaches were used to find out the potential anti-diabetic compounds which revealed I3M and VNL as two potent molecules among the 142 selected compounds. Indirubin 3' monoxime (I3M), or Vanillin (VNL) treatment notably stimulated A<sub>2A</sub>AR activation and showcased their potential to rescue the lipid induced insulin resistance (Fig 4.1 and 4.2). I3M and VNL incubation markedly prevented FFA-induced impairment of insulin signalling pathway molecule activation and GLUT4 translocation. However, pre-treatment with A<sub>2A</sub>AR antagonist SCH 58261 notably prevented such rescue that conform the involvement of A<sub>2A</sub>AR signalling in I3M and VNL mediated insulin signalling pathway stimulation (Fig 4.3). Interestingly, incubation of I3M and VNL in A<sub>2A</sub>AR silenced cells showed contrasting results, while I3M was unable to improve A<sub>2A</sub>AR signalling, the VNL treatment maintained its effect on A<sub>2A</sub>AR activation (Fig 4.4). This result indicate that VNL could stimulate of A<sub>2A</sub>AR signalling downstream of A<sub>2A</sub>AR receptor due to the post receptor activation. This was also evident from radio-ligand binding experiment as we observed a notable binding affinity between I3M and A<sub>2A</sub>AR, however, VNL failed to show such interaction with A<sub>2A</sub>AR (Fig 4.5). Both I3M and VNL treatment significantly promoted A<sub>2A</sub>AR signalling and strongly reduced adipocyte inflammation which were prevented by SCH 58261 treatment (Fig 4.6 and 4.7). Therefore the initial findings of the present work showcased that indirubin-3' monoxime and vanillin treatments significantly promotes A<sub>2A</sub>AR signalling and attenuates lipid-induced adipocyte inflammation. Also, vanillin prevented lipid-induced insulin resistance in adipocytes through post-receptor activation of A<sub>2A</sub>AR.

The next objective was designed to proof the possible mechanism of I3M's action in the improvement of insulin sensitivity and reduction of inflammation in adipocytes. It was

found that I3M significantly induced A<sub>2A</sub>AR signalling with a notable stimulation of A<sub>2A</sub>AR-dependent cAMP activity with an EC<sub>50</sub> value of 0.12 μM as well as an efficient binding affinity with A<sub>2A</sub>AR (K<sub>i</sub>: 0.52 μM) as confirmed by radioligand binding assay (Fig 5.1). I3M dose-dependently activated A<sub>2A</sub>AR signalling with the stimulation of insulin signalling pathway molecules activation. Application of A<sub>2A</sub>AR inhibitor SCH 58261 significantly attenuated I3M mediated A<sub>2A</sub>AR activation in a dose dependent manner (Fig 5.2). I3M effect on the prevention of FFA-induced adipocyte inflammation and insulin signaling pathway stimulation was strikingly inhibited in A<sub>2A</sub>AR silenced cells (Fig 5.3). I3M dose dependently restored CREB phosphorylation and expression of anti-inflammatory cytokines gene expression in FFA-induced adipocytes and these effects were notably attenuated by SCH 58261 (Fig 5.4). I3M induced anti-inflammatory gene expression was significantly attenuated in A-CREB transfected adipocytes (Fig 5.5) thus leading to the conclusion that I3M is up regulating anti-inflammatory cytokines via the activation of the A<sub>2A</sub>AR-cAMP-CREB pathway. Moreover, FFA induced up-regulation of pro-inflammatory cytokines and gene expression were notably prevented by I3M. However, SCH 58261 pre-treatment significantly prevents I3M effect on the improvement of adipocyte inflammatory status (Fig 5.7).

Vanillin (VNL) has already been reported for its anti-oxidant, anti-inflammatory, anti-carcinogenic, and anti-diabetic efficacies in both in-vitro and in-vivo systems. To date, the underlying mode of VNL action and its molecular target remain largely unexplained. The current study elucidated the anti-inflammatory effect of VNL's action on THP1 macrophages and revealed it as a potent IRAK4 inhibitor to combat LPS-induced TLR4 signaling and inflammation. VNL treatment significantly downregulated LPS induced NF-κB activation and its transactivation potential in cells with agonist-stimulated TLR signalling. VNL also reduced LPS induced nuclear localization of NF-κB and prevented the binding of NF-κB to the IL-6 pro-inflammatory cytokine gene promoter (Fig. 6.1 & 6.2). However, VNL failed to down regulate IRF3 transactivation potential as indicated by the promoter-reporter luciferase assay. Moreover, VNL could successfully suppress the pro-inflammatory marker CD80 and promoted anti-inflammatory marker CD206 (Fig. 6.3). Furthermore, VNL's anti-inflammatory behaviour was further supported prior the results of inflammatory activation molecules and the different pro- and anti-inflammatory cytokines expression profile. The results

showcased that VNL incubation significantly down regulated LPS-induced MCP-1, TNF- $\alpha$ , IL-1 $\beta$ , iNOS, and IL-6 gene expressions and the protein expressions of TNF- $\alpha$  and IFN- $\gamma$ . Vanillin notably reduced the activation of NF- $\kappa$ B, IRAK4, IRAK1, TAK1, IKK $\alpha$ / $\beta$ , c-Jun, c-Fos, JNK, and p38 MAPK. Interestingly, it was observed that VNL was unable to alter the LPS-induced Type I interferons (IFN- $\alpha$ , and IFN- $\beta$ ) gene expression activated by IRF3. This indicates that VNL might be acting by inhibiting TIRAP/MyD88-dependent signaling without posing any significant effect via TRAM/TRIF-mediated pathway (Fig. 6.4 & 6.5). To validate the molecular target of VNL, THP1 macrophages were transfected with constitutively activated forms of different TLR4 signaling molecules and it was evident that VNL could impair NF- $\kappa$ B activation, inflammatory cytokine IL-1 $\beta$  gene expression, and  $\kappa$ B luciferase activity in constitutively activated IRAK4 and IRAK1 macrophages. We also studied VNL'S effect on the TLR4 signaling molecules and observed that VNL incubation had a noticeable inhibition of IRAK4-MyD88, IRAK4-IRAK1, and IRAK1-TRAF6 association. Also, VNL reduced the NF- $\kappa$ B activation and IL-1 $\beta$  gene expression in PANC1 and MIAPaCa2 cell lines that constitutively expressed IRAK4 (Fig. 6.7, 6.8 & 6.9). These observations envision IRAK4/1 to be a molecular target of vanillin's effect. Surface plasmon resonance (SPR) analysis revealed direct bonding action of vanillin with IRAK4 as suggested by moderate binding [KD: 2.63E-04 M; Chi<sup>2</sup> (RU<sup>2</sup>) = 0.243] and in-vitro IRAK4 kinase assay showcased significant attenuation of IRAK4 kinase activity attenuation (IC<sub>50</sub>: 0.0213  $\mu$ M), respectively (Fig. 6.10). In-silico study of vanillin-IRAK4 interaction helped in identifying the probable site such as Asp329, Tyr262, and Val263 residues of the IRAK4 that could be involved its interaction with the 3OMe and 4OH side groups of vanillin (Fig. 6.11). Mice pre-treated with vanillin and then induced with LPS displayed reduced serum pro-inflammatory cytokine levels (TNF- $\alpha$  and IL-6), down regulated levels of iNOS, IL-1 $\beta$ , MCP1, and IL-6 gene expression in isolated macrophages, prevention of macrophage pro-inflammatory M1 polarization state and the reduced levels of IRAK4 and NF- $\kappa$ B activation in the peritoneal macrophages (Fig. 6.12).

In summary, the current study involving in-silico and in-vitro investigation led us to identify small molecules with bioactive attributes. Comparative analysis of vanillin and indirubin-3'-monoxime demonstrated their efficacies in curbing insulin resistance and

inflammation. Vanillin prevents lipid-induced IR by targeting A<sub>2A</sub>AR pathway post-receptor pathway in lipid-induced insulin resistant adipocytes. I3M exhibit insulin sensitive and anti-inflammatory action in FFA-induced adipocytes by specifically activating A<sub>2A</sub>AR-cAMP-CREB pathway. I3M act as a potent and selective novel A<sub>2A</sub>AR agonist with a therapeutic potential in the prevention and/or treatment of type 2 diabetes. Also, it is evidently proved by our study that vanillin specifically interacted with the IRAK4 inhibiting myddosome assembly and IRAK4 kinase activity; therefore, vanillin has immense value as a novel and potent IRAK4 inhibitor.

## 7.2 Future Prospects

### 7.2.1 Analysing the insulin sensitive and anti-inflammatory efficacy of vanillin and indirubin-3'-monoxime *in vivo*

In-vitro study with 3T3-L1 adipocytes, we have shown that indirubin-3'-monoxime have the capacity to improve insulin sensitivity and impart anti-inflammatory effect to prevent free fatty acid induced insulin resistance. However, these findings can be supported more strongly with the results from the in-vivo mice models and therefore further study in this direction with strengthen our in-vitro findings.

Although, our investigation revealed Indirubin-3'-monoxime posed as a potent A<sub>2A</sub>AR agonist in attenuating lipid-induced adipocyte insulin resistance, however the specificity of I3M's binding on the active conformation of A<sub>2A</sub>AR remains unclear and need to be explored further. The structure-function relationship of A<sub>2A</sub>AR and I3M can render in visualizing the specific and targeted binding conformation between A<sub>2A</sub>AR and I3M.

Our study depicted a multifaceted attribute of indirubin-3'-monoxime. I3M could significantly up regulated glucose uptake through increased phosphorylation of insulin signalling pathway molecules and glucose uptake as well as it was able to improve the inflammatory status by reducing the expression levels of pro-inflammatory cytokines in FFA induced mature adipocytes. However, the presence of a specific cellular linking between these two potential bioactivities showcased by I3M still remains unexplored and thus requires a meaningful attention in this view.

Vanillin incubation significantly reduced TLR4 and TLR2/1 activation dependent phosphorylation of IRAK4. It would be meaningful to examine the different TLR subtypes dependent activation of IRAK including IRAK4 and effect of vanillin therein.

Although SPR analysis revealed a moderate binding affinity between IRAK4 and vanillin with a KD of 2.63E-04 M and  $\chi^2$  (RU<sup>2</sup>) =0.243 indicating interaction specificity. However, it will be fascinating to understand the physical interaction between vanillin and the active site mutants of IRAK4 (Tyr262, Val63, and Asp329 residues).

Vanillin holds a fair bioavailability percentage with a probable enterohepatic circulation (EHC) after administration [1]. Countable amount of work is already performed in finding out the ways to improve the bioavailability of vanillin as well as to maintain the structural stability both in vitro and in vivo. However, future research in this direction to improve the bioavailability of vanillin is essential for the efficient inhibition of the IRAK4 activation in order to combat inflammation and associated inflammatory diseases.

Adipokines are known to be associated with both insulin sensitivity and insulin resistance. While, adipokine such as adiponectin is involved in improving insulin sensitivity, the obesity-induced adipokines and cytokines are associated with insulin resistance and T2DM. Thus, it would be interesting examine the effect of vanillin and indirubin-3'-monoxime on the expression profile of different adipokines.

We have found that vanillin and its analogues are potent inhibitor of IRAK4 myddosome assembly and kinase activity. Since IRAKs play a vital role in the regulation of TLR mediated inflammation, therefore, it will be interesting to acknowledge this finding in screening of various natural products against IRAKs in addressing various inflammatory diseases.

## Bibliography

- [1] Beaudry, F., Ross, A., Lema, P. P., & Vachon, P. Pharmacokinetics of vanillin and its effects on mechanical hypersensitivity in a rat model of neuropathic pain. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, 24(4): 525-530, 2010.