4 Discussion

The IGF signalling system cascade is regulated by 2 cognate ligands (IGF-I and IGF-II), cell surface receptors (IGF-IR, IGF-IIR, IR and hybrid receptors) and its 6 IGF binding proteins (IGFBPs) (Pollak, Schernhammer and Hankinson, 2004; Bao et al., 2010). Evidences from research has demonstrated that the signal transmitted by IGF-IR is essential for normal cell growth, cell proliferation as well as for the regulation of overall development and growth of organism (Stuard, Titone and Robertson, 2020). Altered signalling of IGF-IR plays a significant role in cancer cell survival, proliferation and progression, and cancer therapeutic resistance too, thus has provoked great interest as a promising target for cancer treatment (Bao et al., 2010; Osher and Macaulay, 2019).

Role of IGF-IR in many tumorigenesis has been identified that is correlated with overexpression of IGF1R and amplification of IGF-IR. One of the potent factors involved in neuroblastoma metastatic progression is IGF-IR (van Golen et al., 2006; Cao and Yee, 2021). Data derived from experimental studies suggest the feasibility of targeting IGF-IR for target specific therapy in NB. The cyclolignan picropodophyllin was selected for study as inhibitor of IGF-1R, on the basis of literature analysis that suggests PPP is a specific inhibitor of IGF-IR and it does not affect the highly homologous insulin receptor. The NB cell lines are sensitive to the IGF-1R inhibitor (Menu et al., 2006a; Thiery et al., 2009; Yin et al., 2010).

Aberrant IGF signaling due to overexpression of IGFs and IGF-IR has been observed in NB. However, in NB, consistent with the other tumors of neuronal origin, activating mutations of ALK are also observed. The occurrence rate of ALK mutations in the NB patients at the time of diagnosis is about 10% with even higher incidence at relapse. IGF-IR and ALK, both are surface receptor and controls carcinogenesis. Strategy to develop novel therapeutic compound involves

targeting these overexpressed surface markers. If simultaneous inhibition of IGF-IR and ALK is achieved by use of a single therapeutic agent, it could aid in development of better treatment strategies for NB. George et. al, 2019 demonstrated through in vitro and in vivo studies that combined inhibition of IGF-IR and ALK, using their respective inhibitors, proved to be an effective method for treatment of lymphoma cancer (George et al., 2019).

PPP is a phytochemical that is known to inhibit IGF-IR, a receptor tyrosine kinase by binding to the ATP binding domain and reducing its phosphorylation. We had hypothesized that PPP could be able to inhibit ALK too, as it is a receptor tyrosine kinase and PPP is ATP binding competitor. In order to analyze binding affinity of PPP to ALK, current study performed molecular docking which showed that the docking score/binding affinity of PPP with ALK is more (-8.8 kcal/mol) as compared to that of the IGF-IR (-7.5 kcal/mol). These strongly suggests that PPP can be a potent inhibitor of ALK as well. In support of molecular docking, our gene and protein expression study reported downregulation of ALK significantly. This justifies the derived binding affinity with ALK. PPP could emerge as an alternative approach for NB treatment capable of inhibiting both IGF-IR and ALK.

After 24 hours of PPP exposure, derived IC50 value on SH-SY5Y neuroblastoma cells was 0.501µM. During experiment of cytotoxicity, it is noticed that SH-SY5Y cells were highly sensitive to PPP that leads to cell death. Cell death is induced due to loss of mitochondrial function. Therefore, it is mandatory to select a dose that only induces apoptosis by PPP. Because other modes of cell death like necrosis or mitotic catastrophe were also noticed in response to PPP treatment. Hence dose selection for current study was one of the main target. On exposure of PPP, low IGF-IR expression seemed to confer an increased tendency for responding to PPP at 16nM dose with apoptosis, whereas weak apoptosis response was observed when treated at 501nM dose of PPP. Thus, 16nM PPP dose was selected for proposed study. The result is in support with other

study that also demonstrated that low concentration of PPP induces high level of apoptosis (Bao et al., 2010; Yin et al., 2010; Waraky et. al., 2014; Simpson et al., 2017).

IGF1R expression was observed to be downregulated when SH-SY5Y cells were exposed to PPP. Result of western blot also demonstrated that PPP reduces IGF-IR-phosphorylation. The result of gene and protein expression, both were statistically significant ($p \leq 0.0001$). Our result about inhibition of IGF-IR is in accordance with the studies of Vasilcanu et al., 2004, 2006; Yin et al., 2010. Researchers have performed *in vivo* tumour regression studies on NB tumour on exposure of IGF-IR inhibitor. Their results shows effective inhibition of IGF-IR autophosphorylation. PPP specifically blocks phosphorylation of the residue Tyr1136 in the activation loop of IGF1R kinase (Menu et al., 2006). Present result is supported by cellular localization of IGF-IR. Image obtained by ICC reflects low level of expression of IGF-1R. Intensity of fluorescence depends on localization of IGF-IR in NB cell lines. Intensity of fluorescence is proportionate to cellular level of *IGF1R* (Fig. 3.10).

Inhibition of IR function leads to dysregulation of glucose metabolism and change in physiology. IR is one of the vital protein to maintain homeostasis of glucose metabolism. Dual inhibition of IGF1R and IR causes side effects due to disruption of IR function. IGF-IR and IR share 100% similarity in their ATP binding site (Vasilcanu et al., 2004; Aleem et al., 2011; Cao and Yee, 2021). Glucose metabolism might not be altered as IR activity is not affected on exposure of PPP as it is a selective inhibitor for IGF-IR. On PPP exposure for 24 hours to NB cell lines, low basal level of IR expression was observed which was statistically not significant whereas IGF-IR expression was found statistically significant. Nonsignificant reduction of IR activity was observed because IR and IGF-IR receptors share 85% sequence homology (Sabbatini et al., 2009). Treatment of PPP to NB cells, reduced the activity of ALK. Reduction in ALK expression was confirmed at both the mRNA and protein level in comparison with untreated cells. PPP is ATP binding competitor hence it can be predicted that PPP can bind to ALK proteins' ATP pocket and

inhibit autophosphoraylation of ALK. Sabbatini et al. (2009) observed a reduction in ALK activity when cells were exposed to IGF-IR inhibitor.

The effect of PPP was measured for downstream cascade of kinases involved in IGF-IR mediated signal transduction. Result demonstrated that PPP treatment reduced IGF-1R-dependent phosphorylation of PI3K and AKT in NB cells. *PI3K* and *AKT* transcript level of expression was observed low which was statistically significant ($p \leq 0.0001$). It is known that the PI3K/AKT pathway has regulatory role in cell proliferation via phosphorylation, so it is conceivable that the observed low level of transcript expression may result into low level of protein after post translation modification that attenuates PI3K/AKT phosphorylation. Attenuation of PI3K/AKT phosphorylation may be mediated by PPP induced inhibition of proliferation of NB cells. Observation of cell proliferation rate after treatment of PPP reveals that it was slowed down in comparison to untreated cells. Obtained result on low expression of PI3K and AKT is supported with the research data of researchers where they show PPP treatment induced inhibition of PI3K/AKT pathway (Neudauer and McCarthy, 2003; Vasilcanu et al., 2004; Duan et al., 2009; Yin et al., 2010; Wu et al., 2015).

CDK is a master regulator of cell cycle that phosphorylates and activates enzymes regulating the mitosis-specific microtubule reorganization (Meunier and Vernos, 2012a; Waraky et al., 2014; Patterson et al., 2021). Aberrant activity of CDK is common in cancer metastasis (Lim and Kaldis, 2013; Kohlmeyer et al., 2020). In comparison to untreated cells, expression of *CDK* was decreased. This result was in resemblance with Waraky et al. (2014), who observed similar results on A549 and MCF7 cell line after treatment. There was increased fold change in CDK expression upto 8 hours of treatment. Downregulation of CDK and pCDK expression was observed after 24 hours of PPP exposure (Waraky., 2014; Zhou et al., 2016). On suppression of CDK activity, CDK/Cyclin B1 complex formation is inhibited hence it induces cell cycle arrest in G_2/M phase. A remarkable observation made by researchers to explain the apoptotic phenomenon was that cell

cycle gets arrested in G2/M phase on exposure of IGF-IR inhibitor (Waraky et al., 2014; Carrasco-Garcia et al., 2018).

Persistence CDK activation at the transition from interphase to mitosis induces important changes in microtubule dynamics (Fourest-Lieuvin et al., 2006; Shawky et al., 2021). Tubulin expression was measured in the study with postulation that on inhibition of CDK 1, organization of microtubule may alter that induces apoptosis via stabilizing G_2/M phase arrest. With reference to CDK down expression, low mRNA expression of TUBULIN was observed in PPP treated SH-SY5Y cells in comparison of untreated NB cell line. Result is in accordance with Waraky et al., (2014). Their results suggested that PPP increased soluble tubulin and decreased spindle-associated tubulin within minutes, indicating that it interfered with microtubule dynamics. Treatment with PPP leads to collapse of the mitotic spindle that promotes programmed cell death (Tanenbaum and Medema, 2010; Medema and Lindqvist, 2011; Meunier and Vernos, 2012b; Baas et al., 2021; Sun et al., 2022a).

CDK induces mitosis and cell proliferation whereas p53, a tumour suppressor gene, limits the cell cycle. Suppression of p53 activity and metastasis is associated with tumorigenesis. IGF-1R inhibition at the transcriptional level is one of the critical mechanism by which p53 exerts its tumour-suppressor function (van Leeuwen et al., 2011; Worrall et al., 2017). In comparison to non-treated NB cells, expression of p53 transcript was estimated 5.625-fold upregulated on PPP treatment. Down regulation of CDK and upregulation of p53 transcript expression is in support of expression pattern of mitogenic transcription factor that we have selected to prove the hypothesis. Downregulation of p53 and aberrant expression of *MYC* induces uncontrolled cell proliferation. MYC, transcription factor, amplification is a hallmark characteristic of NB. MYC binds to *IGF 1R* gene and regulates its expression. Amplification of MYC induces metastasis and stabilize cell survival that makes cell resistance to apoptosis (overexpression of BCL2) while maintaining EMT. MYC is an important target for MAPK signaling pathway for cell proliferation. MYC

amplification and MAPK phosphorylation is an acknowledged phenomenon of cancerous cells (Navas et al., 2006; Gilan et al., 2020; Mancarella, Morrione and Scotlandi, 2021). Result of present research shows suppression of *MYC* and *IGF1R* gene on PPP exposure at 16nM in comparison to untreated cells. Down regulation of both the genes were statistically significant in proposed study. The knockdown of IGF-IR decreased the expression of MYC. Knockdown of IGF-IR activates the MAPK signalling pathway, which may lead to an increase in the expression of the pro-apoptotic gene *p21*, and a decrease in the pro-proliferative gene, MYC (Li et al., 2014; Xu et al., 2014; He et al., 2020). PPP increased the expression of p21 and reduced the expression of MYC in K562 cell lines (Paul et al., 2019; He et al., 2020).

Expression of IGF-1 enables the activation of the STAT3 and NF-κB signalling pathway, which enhances the invasive ability of tumor cells (Ma et al., 2020; Wang et al., 2020; Ianza et al., 2021). Moreover, NF-κB and MYC activity is associated with tumorigenesis (Yuan et al., 2016). NF-κB is a heterodimer composed of p50 and p65 (RelA) subunits, p65 translocates into nucleus from the cytoplasm and maintains characteristics of cancer cell (Ghosh and Karin, 2002; Lee et al., 2007; Jana et al., 2017). In current study, PPP exposure at 16nM for 24 hours to SH-SY5Y cells, induced down regulation of *NF-κB* transcript expression. This data is supported by immunocytochemical localization of NFkB. Cellular level expression in nucleus was observed low in comparison to control cells of NB. The data was statistically significant ($p \leq 0.0001$). Scientist suggested that PI3K-dependent p65 NF-κB downregulation results in increased transcription of MDM2 and degradation of p21 (Meng et al., 2012; Bauer et al., 2015; Jana et al., 2017). On degradation of p21, there is termination of CDK/p21 complex formation. Hence cancer cells transit into death pathway (Nennig and Schank, 2017; Lehman et al., 2018; Loria et al., 2018). This event is in support of current result, where expression of *CDK*, *PI3K* and *NF-κB* was observed low with characteristic of apoptotic cell death as visualized in AO/EtBr staining.

CDK/p21 stable complex is a hallmark for cell invasive state too. Invasiveness of the cell, accompanying with mesenchymal transition of cancer cells, marks them negative for anchoring. To analyze this phenomenon, gene expression of EMT marker was included in current study. E-cadherin was selected as epithelial marker whereas SNAIL, TWIST and NANOG as mesenchymal markers. EMT is a mechanism in which E-cadherin expression is lost during cancer progression. Additional signaling pathways, such as RTK, Rho GTPases, PI3K and Hippo affects cadherin cell-cell adhesion and also contribute to tumor progression and metastasis (Kaszak et al., 2020). Our results are in support of this phenomenon. Calculated qPCR fold change expression of E-cadherin transcript was upregulated. The fold expression of E-cadherin was 19.58, upregulated after PPP treatment of 24 hours.

Transcription factors such as Snail, Slug, Twist and ZEB1, markers of EMT, participate in the malignant transformation of tumors cells from healthy cells. These transcription factors downregulate expression of the epithelial markers such as E-cadherin and keratin (Nieto et al., 2016; Škovierová et al., 2018; Guenther et al., 2019). Our result for epithelial marker, E-cadherin, was upregulated whereas expression of SNAIL, TWIST and NANOG were observed to be downregulated on exposure of IGF-IR inhibitor. Results of Wang et al., (2019) results suggest that the knockdown of IGF1R inhibits EMT of NB cells via reduction in the phosphorylation of AKT, and STAT3. NANOG is stemness related gene which enhances capacities for self-renewal cloning, growing, metastasizing, homing, and re-proliferating during cancer as well as trigger cancer relapse after regression (Wang et al., 2019). NANOG maintains metastasis through STAT3 signaling axis (Zhou et al., 2014; Ching et al., 2017; Tsui, Chan and Ng, 2020). On exposure of PPP, low level of transcript expression of *NANOG* was measured that suggests that on treatment with PPP, NB cells losses its self-renewing properties. Present result suggests that on exposure of PPP, NB cells loss its metastasis properties via gaining anchoring properties on expression of E-cadherin that lead anoikis in NB cells.

Research on PPP inhibition on other cancer type suggested that cell death induced by PPP is through mitochondrial activated pathway and is caspase independent. PPP treatment to HepG2 cells, resulted in decreased expression of antiappoptotic protein (Bcl2) and also phosphorylated Akt; increased level of pro apoptotic protein (Bax), the ratio of Bax/Bcl2 is elevated in dose dependent manner and led to apoptosis via mitochondria triggered caspase dependent pathway. Result obtained in this study is in accordance with the study of Lee et al., (2021). Result of present work shows suppression of transcript expression of BCL2 and amplification of expression of BAX. *BCL 2* and *BAX* both are mitochondrial specific genes. High level of BCL 2 and low level of BAX is a characteristic of tumor cell which aids in developing resistance against apoptosis. Down regulation of BCL2 induces mitochondrial dependent cell death. Wang et al. (2019) also observed IGF-IR-mediated mitochondrial dependent apoptosis in colon cancer on knockdown of IGF-1R (Wang et al., 2019).

It is known that miRNAs are associated with the malignancy by regulating signaling pathways involved in immune checkpoints, apoptosis, cell proliferation, cell cycle and invasion. Some miRNA overexpression suppresses the expression of oncogenic mRNA whereas downregulation of some enhances the function of tumor suppressor genes. miR-223 is is widely reported to be suppressed in several cancer condition (Aziz et al., 2022). Since, one miRNA may regulate more than one mRNA, Jia et al (2011) predicted target genes along with IGF-IR that are LMO2, STMN1, Mef2C, FBXW7 and NF1A. Author has identified IGF-IR to be a prominent target of miR-223 at both mRNA and protein level and suggested that 3' UTR of *IGF1R* can be a strong interactive match for miR-223. They also noticed that miR-223 suppresses the IGF-IR pathway and downstream signaling molecules (Jia et al., 2011) Consistent with these results, present study initially identified miR-223 and let-7 affinity to bind with *IGF1R* on the basis of binding scoring generated by miRDB. Additionally, increased expression of miR-223 is also observed with inhibition of *IGF1R*. Previous study also demonstrated that miR-223 induces apoptosis and G_2/M

arrest by downregulating IGF1R and CDK2 in LLC cells (Nian et al., 2013). Cell death reported in present study is also in accordance with other studies, where they observed apoptosis with the downregulation of IGF1R.

Likewise, expression of Let-7a miRNA expression is suppressed in many cancers. It exerts tumor suppressor activity by affecting several genes involved in various signaling pathway, AKT being one of those (Iwasaki et al., 2015; Tang et al., 2016). In Papillary thyroid carcinoma, let-7a inhibits cell growth by directly targeting PI3K/AKT pathway (Zhou et al., 2017). Correspondingly, our study also observed reduced expression level of PI3K and AKT with increased expression of let-7a. Besides, it is observed that let-7a-3p induces the apoptosis by inhibiting *BCL2* gene expression in glioblastoma cells (Dong et al., 2017). Present study reported upregulation of miRNA let-7a-3p, which might lead to apoptosis by downregulating *BCL2* gene expression.

Similarly, miR-9 is important for cell proliferation, migration and invasion. CDK being a potent target of miR-9, it controls *CDK* mRNA half-life and regulates cell cycle progression. Upregulation of miR-9 downregulates the expression of CDK. It is also reported that MYC induced miR-9, which promotes mesenchymal characteristics by downregulating E-cadherin. Additionally, it also contributes to upregulates genes involved in vascular endothelial growth factor (VEGF) (Ma. et al., 2010). miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. Nature cell biology, 12(3), 247-256.). Current study observed downregulation of miR-9 while treating PPP. Simultaneously, expression of E-cadherin is elevated and EMT marker genes are downregulated with reduced level of miR-9.Regulatory role of miR-9 in migration, invasion and angiogenesis via upregulating PI3K/AKT pathway is observed by. Yao et al. (2020). miR-9 promotes cell proliferation and invasion, which is inhibited by its downregulation in current study. In current study, on exposure of 16nM dose of PPP for 24 hours to SH-SY5Y cell line, miR-223, let-7 was upregulated whereas expression of miR-9 was downregulated.

Molecular level analysis of transcript and protein expression suggests that PPP has potency to provoke cell death in NB cell line. To confirm this mechanism, present study was supported by cell response study to prove inhibition of cell proliferation and loss of survival pathway. It is known that IGFs are potent chemotactic agents and that they can stimulate cancer cell migration (Tarnowski et al., 2015). PPP specifically blocks phosphorylation of the residue Tyr1136 in the activation loop of IGF1R kinase. PPP can cause the complete regression of various types of human solid malignancies through reduction of cell migration (Menu et al., 2006; Langer et al., 2014; Tarnowski et al., 2017). On exposure of PPP to SH-SY5Y cells, gap formed in scratch assay was not occupied in comparison to untreated NB cells. The gap was found more in treated cells that suggests that PPP inhibits cell proliferation that may suppress the migration/ invasive properties of NB cells. IGF signaling regulates survival and proliferation, alteration of which results into immortal cancer pathogenesis. On IGF-IR inhibition, cells lose their neoplasmic feature and are guided for death pathways. This also is in support of low expression of *CDK* and *TUBULIN* suggest that cells are arrested in G_2/M phase. Due to arrest in mitosis phase, rate of cell proliferation is inhibited, resulting into prominent gap in scratch assay.

Result of *BAX* and *BCL2* gene expression, provides the proof for apoptotic cell death. To analyse cell death phenomenon on exposure of PPP, FACS and AO/EtBr staining were performed. Cells were treated by PPP for 24 hours and used for FACS. Cells death % was observed higher in treated cells than compared to untreated cells. 49.17% cells underwent for apoptosis whereas in control the percent for apoptotic cell death was observed 12.48%. Positive control cells, treated by H_2O_2, showed 46.85% programmed cell death. Substanicial evidence suggests that PPP inhibition induces apoptosis *in vitro* as well as *in vivo* (Vishwamitra et al., 2011; Tarnowski et al., 2017; Sun et al., 2022). PPP treated LP-1 cells showed 6 times higher apoptosis than non-treated LP-1 cells. Additionally, elevated fraction of cells observed in G_2/M phase from 10% to 78% in PPP treated LP-1 cells. (Strömberg et al., 2006). Girnita et al., 2004 observed, a typical apoptotic cell death in

PPP treated P6 and FM 55 cells and no cell death was observed in IGF1R negative cell line when treated by PPP. Similarly, results from the current study on the SH-SY5Y cell death analysis revealed increased apoptosis in PPP treated cells compare to untreated control cells. Observed apoptotic percent cell death was four times higher than the non-treated SH-SY5Y cells which clearly indicates that PPP induces programmed cell death on inhibition of IGF-I receptor.

By staining with AO/EtBr, morphological changes of cell can be studied on the basis of staining pattern. Acceptance of stain by cells depends on that physiological state of cell. Green fluorescent emitted by cells when they are alive and stained by AO whereas necrotic cells give red fluorescent as they get stained by EtBr. Cells undergoing programmed cell death emit yellow to orange fluorescent. Cells with more yellow fluorescent is a characteristic of early apoptotic state whereas as orange with late apoptotic state (Fig. 3.15). Cells with low level of BCL2 and high level of BAX expression, losses the mitochondrial membrane potential, leading to apoptotic cell death.

Taken together, we suggest that cyclolignan PPP interfering with the IGF-1 along with ALK and induces cell death in NB cells via modulation of kinases, cell proliferation regulator transcription factor, EMT markers & miRNA and offering a novel and selective therapeutic strategy for Neuroblastoma patients.