5. AN INVESTIGATION INTO THE MECHANISM UNDERLYING THE MITOGENIC EFFECTS OF JACALIN

5.1 INTRODUCTION

Lectins are capable of recognizing glycan moieties on the cell surface, thereby influencing cell proliferation. However, the involvement of protein-protein interaction on the cellular responses that occurs as a consequence of lectin binding cannot be ruled out [257]. Jacalin, for instance, has been shown to bind to numerous proteins on target cells contributing to downstream signaling [290]. Cav-1 is an integral membrane protein, known to play a crucial part in lipid transport, cell signaling and membrane trafficking [291]. Because the sequence of Jacalin was found to possess a Cav-1-binding site (\(^{120Y-W-L-D-Y-F-S-M-Y}\)\(^{128}\), which resembles the consensus motif \(\Phi XXXX\Phi XXX\Phi\) where \(\Phi\) is Trp, Tyr or Phe) and as Cav-1 was reported to be overexpressed in several cancers, we hypothesized that jacalin may exert its mitogenic effects through Cav-1 [290]. This chapter throws light on the signaling mechanism underlying the mitogenic effects of jacalin on the K562 cells.

Caveolae are specialized flask shaped invaginations that are 50-100 nm in size and are found in the plasma membrane. They are made of cholesterol, sphingolipids and structural proteins referred to as caveolins. Caveolins, that are ubiquitously expressed, occurs in three isoforms, namely Caveolin-1, Caveolin-2 and Caveolin-3 [292]. The highest levels of Cav-1 are found in adipocytes, smooth muscle cells and endothelial cells. Cav-2 is usually colocalized with Cav-1 as Cav-1 is essential for membrane targeting of Cav-2 [293]. Unlike Cav-1 and Cav-2, the expression of Cav-3 is limited to smooth, skeletal and cardiac muscles [294]. In most cases, the expression of Cav-1 was found to correspond quantitatively with the formation of caveolae and the tissues exhibiting high Cav-1 expression are known to have a high density of caveolae and those lacking Cav-1, also lack caveolae [295]. In some studies, recombinant expression of Cav-1 was shown to effectively reconstitute the formation of caveolae in cells that usually lack caveolae [296].
Functionally, the caveolae act as a scaffold for molecules involved in cell growth and survival.

**Implications of Cav-1 in cancer**

Cav-1 is a 22 kDa, scaffolding protein that is associated with the cell surface caveolae. Differential expression of Cav-1 was observed in a diverse tumor cells. While Cav-1 was reported to act as a suppressor of some cancers [297,298]; increased expression has also been associated with cancer progression, metastasis, poor prognosis and drug-resistance in a variety of cancers [299,300]. The role of cav-1 is known to depend on the specific cancer type and stage [301]. The overexpression of Cav-1 has been found to be associated with tumorigenesis and metastasis of several cancers including cancers of the liver, colon, breast and the lungs [302-305]. In hepatocellular carcinoma cells, Cav-1 was shown to prevent autophagy of the cells, thereby favoring tumor growth [306]. Likewise, in a more recent study, Cav-1 was reported to be upregulated in renal carcinoma cells wherein it was observed that besides promoting cell migration and invasion, Cav-1 also increased the resistance of cells to the cancer drug, sunitinib [307].

Cav-1 is known to have multiple binding partners and exhibit differential expression patterns. Many signaling molecules directly interact with Cav-1 through the Cav-scaffolding domain (CSD) (sequence 82-101) [308]. The CSD is responsible for mediating interactions with proteins such as G-protein-coupled receptor, mitogen activated protein kinases (MAPK), endothelial nitic oxide synthase (eNOS), epidermal growth factor receptors (EGFR), protein kinases A and AKT that act as molecular switches within the cells [309,310].
5.2 MATERIALS AND METHODS

5.2.1 Materials

Tween 20 and Ponceau stain were purchased from Himedia. Tris-HCl, Nonidet-P40 (NP-40), SDS, EDTA and NaCl were purchased from SRL. Protease and phosphatase inhibitor cocktail were purchased from Medchem. The following antibodies that were commercially obtained from Santa cruz biotechnology were used for western blot analysis: total-AKT, phospho-AKT (Ser 473), phospho-AKT (Thr 308), total-ERK1/2, phospho-ERK1/2, horseradish peroxidase (HRP) conjugated secondary antibodies anti-mouse and anti-rabbit.

5.2.2 Preparation of buffers

(i) Radio immunoprecipitation assay (RIPA) buffer [Lysis buffer]

Table 5.1 Preparation of RIPA buffer

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M tris-HCl, pH 7.4</td>
<td>0.5 ml</td>
<td>50 mM</td>
</tr>
<tr>
<td>5 M NaCl</td>
<td>0.5 ml</td>
<td>150 mM</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>0.04 ml</td>
<td>2 mM</td>
</tr>
<tr>
<td>NP-40</td>
<td>0.1 ml</td>
<td>1%</td>
</tr>
<tr>
<td>SDS</td>
<td>0.01 g</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

The final volume was brought to 10 ml with deionized water, the buffer was filtered using a 0.45 µm filter and stored as 1 ml aliquots in -80°C. The protease and phosphatase inhibitor cocktail were added to the buffer, prior to use.
(ii) **Transfer buffer (Towbin buffer)**

To prepare 10X transfer buffer, 14.4 g of glycine and 3.03 g of tris base was dissolved in 80 ml of deionized water and the final volume was made up to 100 ml. The buffer was stored at 4°C.

To prepare 1X transfer buffer (2 L), 200 ml of 10X buffer was mixed with 400 ml of methanol and 1400 ml of deionized water and the buffer was kept at 4°C.

(iii) **Tris buffered saline with 0.1% Tween 20 (TBST)**

To prepare 10X tris buffered saline (TBS), 2.4 g of tris base and 8.8 g of NaCl were dissolved in 80 ml of deionized water. After the pH was brought to 7.6, the final volume was made up to 100 ml. The buffer was syringe filtered and stored at 4°C.

To prepare 1X TBST, 100 µl of tween 20 was added to 10 ml of 10X TBS with continuous stirring and the final volume brought to 100 ml.

(iv) **Blocking buffer (5% BSA in TBST)**

1 g of BSA was added to 15 ml of TBST and the final volume was brought to 20 ml. The buffer was stored at 4°C.

5.2.3 **Reverse transcriptase – PCR analysis of Cav-1 mRNA in jacalin-treated cells**

K562, HeLa and HEK cells were seeded at a density of 1x10⁶ cells per well into a 6 well plate. After 3 h and 6 h of jacalin treatment, the cells were collected by centrifugation. Total RNA was isolated from the jacalin-treated and control cells, as described in chapter 4. 5 µg of total RNA was converted to cDNA. Random primers were used for reverse transcription reaction. The high capacity cDNA reverse transcription kit, obtained from Applied Biosystems, CA was used. cDNA was synthesized as follows: 25°C for 10 mins, 37°C for 120 mins, 85°C for 5 mins. Amplification of Cav-1 mRNA was done using Emerald Amp GT PCR master mix (RR310, Clonetech, Takara).
The amplification conditions were as follows: 95°C for 10 mins, 40 cycles of 95°C for 2 mins, 60°C for 30 secs, 72°C for 30 secs and final extension of 72°C for 10 mins. After amplification, the products were analyzed on a 2% agarose gel and the gel images were acquired using ChemiDoc MP Imager (Bio-Rad).

5.2.4 Quantification of Cav-1 mRNA level in K562, HeLa and HEK cells by real-time quantitative PCR

The mRNA expression of cytokines were quantified by Quantitative real time (qRT-PCR), using SYBR green master mix (Hi-Sybr Master mix, MBT074). The reaction mixture contained 1x Sybrgreen Universal PCR master mix, 500 nM of forward and reverse primers and 1 µl of cDNA. The same universal cycling conditions as mentioned in chapter 4 was used. β-actin was used as the endogenous control to normalize the samples prior to comparison.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Forward primer</strong></td>
</tr>
<tr>
<td>Actin</td>
<td>CTACGTGCCCTGGACTTCGAGC</td>
</tr>
<tr>
<td>Cav-1</td>
<td>GAGCGAGAAGCAAGTGTCGAG</td>
</tr>
</tbody>
</table>

5.2.5 Cell lysis and immunoblotting

K562 cells were seeded at a density of 1X10⁶ cells per well into a 6 well plate and were cultured in the presence of 100 µg/ml jacalin. After 24 h, the cells were pelleted by subjecting to centrifugation at 1500 rpm for 5 mins at RT. After the supernatant was carefully discarded, the cell pellet was washed with PBS.
5.2.5.1 Cell lysis

The whole cell lysates were prepared using the RIPA buffer. The protease and phosphatase inhibitor cocktail were added to the lysis buffer prior to use. Briefly, the cell pellet was redissolved in ice-cold RIPA buffer (0.5 ml/10⁶ cells) and mixed well using an insulin syringe. The cell suspension was incubated for 30 mins on ice. The tubes were vortexed every 10 mins to completely lyse the cells. The tubes were subjected to centrifugation at 12,000 rpm for 10 mins at 4°C. The supernatant was aspirated to a fresh pre-cooled micro centrifuge tube kept on ice and the pellet was discarded. The concentration of the cell lysates were estimated by Lowry’s method and were stored as aliquots, at -80°C until use.

5.2.5.2 Western blotting

The lysed samples that were subjected to separation by SDS-PAGE (10%) were transferred to a nitrocellulose membrane by wet transfer method (Hoeffer, TE22). The conditions used for transfer of proteins onto the membrane were 100 mA for 100 mins. The blot was then stained using ponceau stain to check if the transfer was successful. After repeated washing to remove traces of the ponceau stain, the non-specific regions on the membranes were blocked by incubating the blot in 5% BSA (in TBST) for 1 h at RT. The membranes were washed 3 times for 5 mins each in TBST, prior to overnight incubation at 4°C in the specific primary antibody (1:1000 dilution). The membranes were again washed 3 times for 5 mins each in TBST and probed with the HRP conjugated mouse anti-rabbit or rabbit anti-mouse secondary antibody (1:3500 dilution) for 1 h at RT, with gentle shaking. After washing, blots were developed using enhanced chemiluminescence reagent (Cyanagen, Srl). The blot was incubated for 5 mins in a mixture containing Luminol and peroxide in equal volumes, prior to image acquisition using ChemiDoc MP Imager (Bio-Rad).
5.3 RESULTS AND DISCUSSION

5.3.1 Differential effects of jacalin on the proliferation of K562, HeLa and HEK cells

Jacalin was found to exert differential effects on proliferation of K562, HeLa and HEK cells (figure 5.1). While jacalin was found to stimulate the proliferation of K562 cells, it inhibited the proliferation of HeLa cells. In contrast, the rate of proliferation of normal HEK cells remained unchanged at jacalin concentrations of up to 125 μg/ml; while at higher concentrations, jacalin stimulated the proliferation of HEK cells. Such differential effects may be attributed to the biochemical differences between these cells [311].

Figure 5.1 Differential effects of jacalin on the proliferation of K562, HeLa and HEK cells. The respective cells were cultured with varying concentrations of jacalin. After 24 h, viability percentage was determined by MTT assay. Untreated cells grown in 1% FBS were regarded as 100% cell growth and the viability of the treated cells were calculated accordingly. Error bars indicate the standard deviations.
5.3.2 Differential expression of Cav-1 mRNA in jacalin-treated K562, HeLa and HEK cells

The role of Cav-1 in tumorigenesis was found to be controversial. As the expression of Cav-1 is known to be high in several tumors, it was deemed significant to analyze if the expression of Cav-1 correlates with the differential effects of jacalin on cells of different lineage. While some lymphocytes and neuroblastoma cells do not express Cav-1, they are abundantly expressed in the fibroblasts and the normal mammary epithelium [312,313]. Likewise, the basal level expression of Cav-1 was shown to be high in some cancers while it was observed to be less in other cancers. As shown in [figure 5.2a (i)], the basal level expression of Cav-1 mRNA was found to be low in K562 cells. This is in accordance with previous reports [314]. Likewise, while Cav-1 mRNA was shown to be moderately expressed in HeLa cells, the expression was high in control HEK293 cells.

To verify if the mRNA expression of Cav-1 corresponds to the differential effects of jacalin on the cell growth, the expression of Cav-1 mRNA in jacalin-treated K562, HeLa and HEK cells was analyzed. As shown in figure 5.2a (i) & (ii), the mRNA expression of Cav-1 was found to be upregulated in 3 h and 6 h jacalin-treated K562 cells. A two fold and twelve fold increase in mRNA levels of Cav-1 was observed in 3 h and 6 h jacalin-treated K562 cells, respectively. Further, the relative mRNA levels of Cav-1 was found to be slightly downregulated in 3 h and 6 h jacalin-treated HeLa cells [figure 5.2b (i) & (ii)]. In case of HEK cells, no significant change in expression of Cav-1 mRNA was observed upon 3 h and 6 h jacalin treated cells [figure 5.2c (i) & (ii)].
Figure 5.2a Differential levels of Cav-1 mRNA in Jacalin-treated K562 cells

(i) mRNA levels of Cav-1 in jacalin-treated K562 cells assessed by agarose gel electrophoresis. (ii) Quantification of Cav-1 mRNA level in jacalin-treated K562 cells. 100 μg/ml jacalin was added to the K562 cells. After 3 and 6 h, the mRNA expression of Cav-1 was analyzed with respect to untreated control. Bar graph denotes the specific fold change. β-actin serves as the housekeeping gene. *p < 0.05.
Figure 5.2b Differential levels of Cav-1 mRNA in jacalin-treated HeLa cells. (i) mRNA levels of Cav-1 in jacalin-treated HeLa cells assessed by agarose gel electrophoresis. (ii) Quantification of Cav-1 mRNA level in jacalin-treated HeLa cells. 100 µg/ml jacalin was added to the HeLa cells. After 3 and 6 h, the mRNA expression of Cav-1 was analyzed with respect to untreated control. Bar graph denotes the specific fold change. β-actin serves as the housekeeping gene. *p < 0.05.
Figure 5.2c Differential levels of Cav-1 mRNA in jacalin-treated HEK cells

(i) mRNA levels of Cav-1 in jacalin-treated HEK cells assessed by agarose gel electrophoresis. (ii) Quantification of Cav-1 mRNA level in jacalin-treated HEK cells. 100 μg/ml jacalin was added to the HEK cells. After 3 and 6 h, the mRNA expression of Cav-1 was analyzed with respect to untreated control. Bar graph denotes the specific fold change. β-actin serves as the housekeeping gene. *p < 0.05.
5.3.3 Differential expression of Cav-1 in jacalin-treated K562 cells

To substantiate the increased mRNA levels of Cav-1 in 3 h and 6 h jacalin-treated K562 cells, the protein level of Cav-1 in 24 h jacalin-treated K562 cells were detected by western blot analysis. As presumed, the level of Cav-1 was found to be upregulated in the 24 h jacalin-treated K562 cells (figure 5.3).

![Western blot analysis of Cav-1 expression in jacalin-treated K562 cells.](image)

**Figure 5.3 Western blot analysis of Cav-1 expression in jacalin-treated K562 cells.** Lysates of untreated (Unt) and 24 h jacalin-treated (Jac T) K562 cells were subjected to western blot analysis using anti-Cav-1 polyclonal antibody. β-actin served as control for equal protein loading.
5.3.4 Increased phosphorylation of ERK1/2 in jacalin-treated K562 cells

Aberrant phosphorylation of ERK1/2 is common in most cancers. Cav-1 is known to regulate a number of signaling pathways such as EGFR, PDGFR and focal adhesion kinase (FAK) that lead to ERK1/2 activation. Also, there are reports to show that Cav-1 promotes cancer cell growth through ERK1/2 activation; for instance, Cav-1 was found to promote metastasis and survival of melanoma cells through Src-dependent activation of ERK1/2 [315]. Thereupon, the phosphorylation status of ERK1/2 in jacalin-treated K562 cells was investigated. As shown in figure 5.4, a slight increase in phosphorylation of ERK1/2 was observed in jacalin-treated K562 cells.

Figure 5.4 Western blot analysis of p-ERK1/2 and total-ERK1/2 in jacalin-treated K652 cells. Lysates of untreated (Unt) and jacalin-treated (Jac T) K562 cells were subjected to separation by SDS-PAGE analysis, transferred to nitrocellulose membrane and immunoblotted for p-ERK and total ERK.
5.3.5 Increased phosphorylation of AKT at Thr 308 in jacalin-treated K562 cells

AKT is a protein kinase that is known to play a prominent role in diverse tumorigenic activities. Therefore, activation of AKT alone can promote cancer progression. The activation of AKT involves membrane translocation followed by phosphorylation at Thr 308 and Ser 473 \[316\]. Once activated, AKT regulates the functions of its substrates which are implicated in cell-cycle progression.

Interestingly, immunoblot analysis revealed the protein levels of p-AKT (Thr 308) alone to be significantly increased in 24 h jacalin-treated K562 cells (figure 5.5). However, there was no profound change in phosphorylation of AKT at Ser 473. While phosphorylation at Thr 308 is known to be essential for AKT activation, Ser 473 phosphorylation has little effects on activity of AKT \[317\]. Thus, aberrant activation of AKT in jacalin-treated K562 cells can be considered to be responsible for driving the K562 cell proliferation.

![Figure 5.5](image)

**Figure 5.5** Western blot analysis of p-AKT (Thr 308), p-AKT (Ser 473) and total AKT in jacalin-treated K652 cells. Lysates of untreated (Unt) and jacalin-treated (Jac T) K562 cells were subjected to separation by SDS-PAGE analysis, transferred to nitrocellulose membrane and immunoblotted for p-AKT (Thr 308), p-AKT (Ser 473) and total AKT.
5.4 CONCLUSION

In this chapter, the possible mechanism behind the mitogenic facet of jacalin has been investigated. Initially, jacalin was shown to exhibit differential effects on the growth of K562, HeLa and HEK cells. Interestingly, mRNA expression of Cav-1 correlated with the contrastive effects of jacalin on growth of cells of different lineages. Likewise, the protein expression of Cav-1 was found to be upregulated in jacalin-treated K562 cells.

Further, to elucidate the mechanism through which Cav-1 may promote K562 cell proliferation, the activation of other key proteins involved in signal transduction cascade was analyzed. Cav-1 is a known modulator of mitogenic signaling. In several studies, increased expression of Cav-1 has been associated with increase in P13K/AKT signaling. Further, Cav-1 has been shown to increase the activities of signaling proteins such as PDK1, AKT, and ERK1/2 by interacting with and inhibiting their negative regulators. For instance, deletion of cav-1 in prostate cancer cells significantly reduced phosphorylated AKT and cell viability [318]. In another study, si-RNA mediated knockout of Cav-1 resulted in AKT dephosphorylation in human pancreatic cells [319]. Similarly, in HCT116 cells, overexpression of Cav-1 inhibited the negative regulators of AKT, resulting in increased amount of phosphorylated AKT [320].

As for the potential downstream target of Cav-1, it has been observed that Cav-1 activates AKT by interacting with and inhibiting PP2A in prostate cancer cells [318]. PP2A is a serine/threonine phosphatase that is ubiquitously expressed and plays a major role in regulation of cell growth. It acts as a tumor suppressor by controlling the activities of signaling phosphoproteins such as AKT, c-Myc, p-53, ERK1/2 and β-catenin by readily dephosphorylating them. Notably, PP2A is known to preferentially dephosphorylate AKT at Thr 308 [321-323]. Besides, PP2A is also known to be a negative regulator of ERK1/2 phosphorylation. Interestingly, the catalytic subunits of PP2A possess a consensus Cav-1-binding site (ΦXXΦXXXXΦ where Φ is Trp or Tyr or Phe) [324]. Cav-1 is known to bind to PP2A through the scaffold binding domain.
and hinder the normal catalytic functions of the enzyme. Thus, PP2A can be presumed as the target of Cav-1 and it can be postulated that the Cav-1 overexpressed by jacalin-treated K562 cells binds to and disturbs the catalytic activity of PP2A, resulting in incessant phosphorylation of AKT and ERK1/2. AKT and ERK1/2 phosphorylation further activates other downstream targets culminating in incessant cell growth and survival. A schematic representation of mechanism underlying the anti-proliferative effects of jacalin on A431 cells [290] and a proposed mechanism underlying the mitogenic effects of jacalin on the K562 cells are shown in figures 5.6 (i) and (ii), respectively.
Figure 5.6  Proposed mechanisms underlying the effects of jacalin on (i) A431 cells: Upon jacalin treatment, there is activation of PP2A that in turn dephosphorylates MEK and AKT. This prevents activation of downstream signaling proteins, thereby resulting in inhibition of cell proliferation. (ii) K562 cells: Upon jacalin treatment, there is an increase in expression of Cav-1 that binds to the PP2A through the scaffold binding domain, thereby hindering the normal catalytic functions of the enzyme. As PP2A is the negative regulator of MEK and AKT phosphorylation, Cav-1-PP2A binding leads to incessant phosphorylation of ERK1/2 and AKT, resulting in increased proliferation of K562 cells.