CHAPTER 5

Piperic acid (PA) & 4-Ethylpiperic acid (EPA) Amides with α-, β- and γ-Amino Acids as Anticancer Agents

5.1 Introduction

Cancer is a group of diseases involving abnormal cell growth with the potential to spread different parts of the body (WHO 2014). It is caused by internal factors which include inherited mutations, hormones and immune conditions and acquired factors such as tobacco, diet, radiation and infectious organisms (Anand et al 2008). It is a genetic disease which reprograms the function of proto-oncogenes and tumor suppressor genes involved in cell growth and division. Various signaling cascades viz MAP kinase and PI3K/AKT/mTOR are highly activated in cancer (Rajagopalan et al 2002). These changes may be inherited or somatic and sternness in these genetic alterations has now paved a greater challenge of drug resistance (Yan et al 2002). Phosphatidylinositol 3-kinase (PI3K)/AKT pathway is constitutively activated in most pancreatic cancers and offers effective targets for therapeutic development (Arlt et al 2003, Liptay et al 2003, Aggarwal et al 2004). Akt is involved in the progression of metastasis through a wide variety of signaling molecules and mechanisms (Liptay et al 2003). Further, transcription factor nuclear factor-kB (NF-kB) which is a direct target of Akt has been correlated with increased metastatic potential in pancreatic cancer, and has been associated with metastasis and invasion in many tumors. (Yebra et al 1995, Tanno et al 2001). In tumor cells the most important biological characteristics are the invasion and metastasis (Liu et al 2011). In metastasis, the cancer cells invade other tissues through a multifarious mechanisms involving numerous signalling pathways that allow lack of involvement of primary cancer cells, motility, degradation of extracellular matrix (ECM), invasion, migration, adhesion to endothelial cells and growth at a new site (Lu et al 2013).

Many chemotherapeutic agents, mostly natural product based, have been used to curb this deadly disease. It has been reported that piperine suppresses tumor growth and metastasis in 4T1 murine breast cancer model (Lai et al 2012). Recently, it has been
shown that proliferation of human osteosarcoma cells were inhibited by piperine via G2/M phase arrest and metastasis by repressing MMP-2/-9 expression (Zhang et al 2015). Yaffe et al has reported that piperine, an alkaloid from black pepper, inhibits growth of human colon cancer cells via G1 arrest and apoptosis triggered by endoplasmic reticulum stress (Yaffe et al 2015). Shimada and Rao et al have reported the anticancer activity and tumor selectivity in piperic acid amides (Rao et al 2012, Shimada et al 2014), which are shown in Figure 5.1.

**Figure 5.1:** Chemical structures of piperine and piperic acid amides

There is another process of programmed cell death called apoptosis, which occurs through various biochemical events that leads to characteristic cellular changes. Apoptosis can be induced through different modes, but the final executing tool remains to be caspases (Rah et al 2015). Poly(ADP-ribose) polymerase-1(PARP-1) has been implicated in two distinct types of cell death, one induced by DNA damage called apoptosis and necrosis. Poly (ADP-ribosyl)ation by PARP-1 is an important mechanism for the maintenance of genomic integrity in response to DNA damage. During the execution phase of apoptosis, PARP-1 is proteolyzed by caspases and the hyperactivated PARP-1 generated promotes apoptosis by preventing DNA repair-induced survival and by known hallmark of apoptosis and has been implicated by DEVD-ase caspases, a family of proteases activated during apoptosis (Leist et al 2001). Further during apoptotic induction, various pro-survival proteins like BCL2, survivin and pro-apoptotic players like BAX, Par-4 etc. are down-modulated and up-regulated respectively (Lin et al 2014, Rah et al 2015). Piperine induces apoptotic cell death in human colon, melanoma, lung
and rectal carcinoma cell lines, (Amours et al 2001, Kim et al 2005, Elmore et al 2007) and inhibits the proliferation of human prostate cancer cells via induction of cell-cycle arrest and autophagy at G0/G1 phase (Fofaria et al 2014). Recently, we have reported that the conjugate of 4-ethylpiperic acid (EPA) with β,β-disubstituted β-amino acid inhibits the invasion of metastasis in pancreatic cancer cells via PI3K/Akt/NF-κB Pathway (Amin et al 2015). β-amino acids are unusual substances in comparison with proteinogenic-L-amino acids in nature. β-amino acids have been found as essential components of bioactive natural products, such as the anticancer agent taxol (paclitaxel) produced by the western yew Taxus brevifolia, anticancer agent bleomycin by streptomyces, and cytotoxic microcystin by cyanobacteria (Kudo et al 2014). Incorporation of β-amino acids in place of α-L amino acids into natural products produces characteristic substances with similar molecular polarity. The chemical structures of piperic acid (PA), 4-ethylpiperic acid (EPA) and their amides which were bioevaluated for the anticancer activity are shown in Figure 5.2.

![Figure 5.2: Chemical structures of piperic acid (PA), 4-ethylpiperic acid (EPA) and their amides](image)
5.2 Experimental Section
The detailed synthesis of piperic acid (PA), 4-ethylpiperic acid (EPA) and their amides shown in Figure 5.2 are described in chapter 3.

5.2.1 Cell Culture and Antibodies
PANC-1, PC-3 and HCT-116 cell lines were purchased from European Collection of Cell Culture (ECACC). Cells were cultured in DMEM/RPMI1640 containing 10 % fetal bovine serum in the presence of 0.1 g/L streptomycin and 70 mg/L penicillin and were incubated at 37 ºC with 5.0 % CO2 and 95 % air. Antibodies were obtained from the following commercial sources: anti-Akt, anti-NF-kB, anti-MMP-2, anti-MMP-9, anti-E-cadherin, anti-mTOR, anti-S6K, anti-TIMP-1, caspase-3, parp, BCL2, XIAP and BAX from Santa Cruz Biotechnology (Santa Cruz, CA), anti-p-Akt and anti p-mTOR from Cell Signaling and anti-beta actin from Sigma Chemical, St. Louis, MO.

5.2.2 Cell viability Assay
In a 96 well tissue culture plate PANC-1, PC-3 and HCT-116 cells were plated at a density of 2.5 x 10^3 cells/well. The cells were treated with different concentrations of indicated compounds, by standard MTT dye uptake method according to the procedure described previously (Koppikar et al. 2010).

5.2.3 Clonogenic Assay
In 6 well tissue culture grade plates PANC-1 cells were plated at a density of 1x10^3 cells / well. After 24 h, culture medium was changed and then new medium was added. Cells were then exposed for 5 days to different concentrations of conjugate 20 along with vehicle DMSO. The clonogenic assay was performed by previously described method (Koppikar et al. 2010).

5.2.4 Scratch Motility (wound healing) Assay
The assay was performed as described previously (Amin et al. 2015). Briefly, PANC-1 cells (5 x 10^5 cells/well) were seeded in a 6 well tissue culture grade plate and allowed to form a confluent monolayer for 24 h then serum starved for another 24 h. The monolayer was then scratched with a sterile pipette tip (200 μL), washed with serum free
medium and photographed (time 0 h). Cells were sequentially treated with medium containing low serum (1.0 %) for 24 h in the presence of different concentrations of conjugate 20 along with vehicle DMSO. Wounded areas were gradually photographed with Nikon D3100 inverted microscope camera (20X magnification).

5.2.5 Matrigel Invasion Assay
The effect of conjugate 20 on cell invasion was determined using BD Biocoat Tumor Invasion Assay System (BD Bioscience, Bedford, MA) according to manufacturer’s instructions. Briefly, PANC-1 cells (1.2 × 10^6) were treated with different concentrations of conjugate 20 or vehicle DMSO. The Matrigel invasion assay was performed according to previously described method (Rah et al 2012).

5.2.6 Cell Scattering Assay
The cell scattering assay was performed as described previously (Amin et al 2015). Briefly, PANC-1 cells were seeded at a density of 1000 cells/well in 6 well plates. After 3-4 days of incubation, distinct, small and cohesive colonies were formed. In the presence of various concentrations of the conjugate 20 in growth medium, cells were then stimulated with VEGF (20 mg/ml). After 24 h, colonies were observed under bright field microscope. Photographs were taken for individual colonies.

5.2.7 Immunoblotting
PANC-1 cells were exposed to the indicated concentrations of conjugate 5 and 20 along with DMSO. Cells were then lysed with lysis buffer and equal quantity of protein from each sample was subjected to western blot analysis as described previously (Sinha et al 2013).

5.2.8 Fluorescent Gelatin Degradation Assay
Cross-linked-fluorophore (FITC)-conjugated gelatin matrix-coated coverslips were prepared as described (Amin et al 2015). Gelatin-coated coverslips were quenched with RPMI containing 10% fetal bovine serum at 37°C for 60 min prior to plating cells. To measure the formation of invadopodia and degradation of FITC-gelatin matrix, PANC-1 cells were cultured on FITC gelatin-coated coverslips. After 16 h, cells were treated with
indicated concentrations of conjugate 20 for 24 h followed by DAPI mounting media and then observed under FLoid Cell Imaging Station for determining gelatin degradation. In Image-J software images were further processed for determining the threshold area of degradation.

5.2.9 Preparation of Rat Tail Collagen

Rat tail collagen was prepared according to the procedure described previously (Katz et al 2011). Briefly, tails were removed from adult rats (100-150g, b.w.) already sacrificed for other experimental purposes in the animal house of Indian institute of integrative medicine, canal road, Jammu. Tails were then cleaned by washing with 70% ethanol. The skin was peeled away. Each tendon was separately dissected and fibers were rinsed briefly in 70% ethanol, weighted, and incubated in 0.2% acetic acid. The flask was stirred for three to five days at 4°C. Insoluble debris was separated by centrifugation. The collagen rich material was resuspended in 0.25 M acetic acid at 4°C and dialyzed against 1:1000 acetic acid at 4°C for 3 days. The collagen solution was then sterilized by centrifugation.

5.2.10 3D Invasion Assay

3D invasion assay was performed as described previously (Evensen et al 2013). Briefly, PANC-1 cells were combined with neutralized collagen, placed into the center of each well of a 96-well plate, and allowed to solidify, forming a cell-collagen hemisphere with a distinct boundary. Following solidification, the cell collagen hemispheres were embedded within a cover-layer of neutralized collagen and allowed to solidify. Then to each well, media was added and the cells were allowed to invade into the surrounding matrix in presence or absence of indicated concentrations of conjugate 20.

5.2.11 Apoptosis by DAPI Staining

Following the treatment with 5, Panc-1 cells (1x10⁵ cells/well) were harvested by trypsin digestion and washed twice with chilled PBS. Cells were fixed with 4% paraformaldehyde for 10 min followed by incubation with DAPI (Sigma), containing mounting medium for 15 min at room temperature in the dark and apoptosis was detected by fluorescence microscopy (100X magnification).
5.2.12 Caspase Activity Assay
The intracellular caspase activity was measured through a commercial Caspase-Glo (3/7) assay kit (Promega). Briefly, cells (10 × 103 per well) in a 96 well plate were treated with different doses of compound 5 and incubated for 24 h. The plate was brought to room temperature and 100 μL of Caspase-Glo (3/7) reagent was added into the wells. After 30 min of further incubation, the reading was taken with the help of a luminometer microplate reader (TECAN, Infinite M200 PRO, and Switzerland). The values were analyzed and expressed as relative luminescence unit (RLU).

5.2.13 Cell Cycle Analysis
Cell cycle analysis was carried out with Premo-Fucci cell cycle sensor reagent (Invitrogen), briefly cells were seeded in a chamber slide containing complete medium and incubated overnight. 24 h after treatment Premo Fucci cell cycle sensor was added to the cells and again incubated for 16 h or more and then cell cycle progression in populations of cells was visualized using fluorescence microscopy. Cells change from red in the G1 to yellow in the G1/S interphase and green in S, G2 and M phases, as fusions of emGFP and TagRFP coupled to two cell cycle-regulated proteins are expressed and degraded.

5.2.14 Statistical Analysis
All assays were carried out in triplicate. Data were expressed as means with standard deviations. Student’s t-test was applied to compare the mean of each group with that of the control group. ‘p’ values < 0.05 were considered as statistically significant.

5.3 Results and Discussion
All the amides were bioevaluated by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay against a panel of three cancer cell lines i.e. human prostate cancer (PC-3), pancreatic cancer (Panc-1) and colorectal carcinoma (HCT-116). Among all the tested amides, 5 and 20, a novel amides of piperic acid (PA) and 4-ethylpiperic acid (EPA) with β,β-disubstituted-β-amino acids, β3,3-Pip(Bzl)-OH 2-(4-amino-1-benzylpiperidin-4-yl) acetic acid and β3,3-Pip-OH 2-(4-aminopiperidin-4-yl)acetic acid) exhibited promising cytotoxicity against pancreatic cancer (Panc1) cell line with IC₅₀ of 7.0 and 4.0µM, respectively. The conjugate 20 was chosen for further
study. Conjugate 20 was found to inhibit the invasion and metastasis on pancreatic cancer cells by abrogating PI3K/Akt/NF-kB pathway. The conjugate 20 was synthesized by coupling of Valeryl-β\(^3\)-Pip(NH)-NH-NH-Ph with EPA using EDCI.HCl and NMM in dry DCM as described in the experimental section. In order to make out the inhibition of invasion and metastasis we perform several experiments to demonstrate that the conjugate 20 is the potent inhibitor for pancreatic cancer cells.

**Table 5.1:** Cytotoxic effects of piperic acid and 4-ethylpiperic acid and conjugates on a panel of three different cancer cell lines viz: PC-3, PANC-1 and HCT-116 through cell viability (MTT) assay. Data were compared with untreated control and IC\(_{50}\) values were expressed as mean ± s.d. of three independent experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PC-3 IC(_{50}) (μM) ± s.d.</th>
<th>PANC-1 IC(_{50}) (μM) ± s.d.</th>
<th>HCT-116 IC(_{50}) (μM) ± s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2(PA)</td>
<td>84.3± 0.5</td>
<td>93.4± 0.2</td>
<td>91.1± 0.5</td>
</tr>
<tr>
<td>7(EPA)</td>
<td>79.2± 0.2</td>
<td>81.1± 0.2</td>
<td>87.7± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>88.4± 0.2</td>
<td>92.3± 0.5</td>
<td>76.4± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>73.1± 0.3</td>
<td>89.4± 0.2</td>
<td>78.9± 0.3</td>
</tr>
<tr>
<td><strong>5</strong></td>
<td><strong>36.8± 0.2</strong></td>
<td><strong>07± 0.5</strong></td>
<td><strong>68.8± 0.5</strong></td>
</tr>
<tr>
<td>6</td>
<td>81.2± 0.2</td>
<td>49.6± 0.2</td>
<td>87.8± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>43.5 ± 0.4</td>
<td>6.7 ± 0.1</td>
<td>47.1 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>80 ± 0.2</td>
<td>48.7± 0.2</td>
<td>82.8± 0.2</td>
</tr>
<tr>
<td>11</td>
<td>41.9± 0.3</td>
<td>&gt;100 ± 0.6</td>
<td>48.3± 0.5</td>
</tr>
<tr>
<td>12</td>
<td>16 ± 0.3</td>
<td>42.2± 0.5</td>
<td>49.8± 0.4</td>
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<tr>
<td>14</td>
<td>&gt;100 ± 0.4</td>
<td>&gt;100 ± 0.4</td>
<td>&gt;100 ± 0.3</td>
</tr>
<tr>
<td>15</td>
<td>77.6± 0.2</td>
<td>98.1± 0.4</td>
<td>92.5± 0.2</td>
</tr>
<tr>
<td>17</td>
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<td>49.1± 0.2</td>
<td>39.5± 0.5</td>
</tr>
<tr>
<td>18</td>
<td>45.2± 0.3</td>
<td>10 ± 0.2</td>
<td>47.6± 0.2</td>
</tr>
<tr>
<td><strong>20</strong></td>
<td><strong>15 ± 0.1</strong></td>
<td><strong>4 ± 0.5</strong></td>
<td>&gt;100 ± 0.1</td>
</tr>
</tbody>
</table>

IC\(_{50}\) values are indicated as the mean ± s.d. of three independent experiments performed (s.d. = standard deviation).
As pancreatic cancer invasion and metastasis remains the most critical determinant of respectability and hence survival, so we aimed to study the effect of conjugate 20 on the invasive potential of PANC-1 cells in vitro. Wound healing assay was performed to conclude whether conjugate 20 could inhibit motility of Panc-1 cells. After 48 h of incubation with 2.0 and 4.0 μM of conjugate 20, motility of PANC-1 cells inhibited significantly (p < 0.05), similar to Staurosporine (Figure 5.3). Whereas, the cells treated with vehicle DMSO had totally migrated through the wounded area to close the wound.

![Figure 5.3: Wound healing assay was performed by treating PANC-1 cells with different concentrations of conjugate 20 and sequentially to assess the degree of wound healing. Scratched areas were photographed (20x magnification) at zero hour and at 24 h.](image)

Effect of conjugate 20 on cell scattering, the main trait of mesenchymal property of a cancer cell, PANC-1 cells were treated with indicated concentrations of 20, after stimulation with VEGF. Scattered colonies were achieved by the distinctive change in morphology characterized by acquisition of a migratory, fibroblast-like phenotype and cell-cell dissociation in vehicle treated cells and that ultimately decelerated by treatment with different concentrations of conjugate 20 (Figure 5.4).
Figure 5.4: In presence of VEGF alone or in combination with indicated concentrations of conjugate 20, PANC-1 cells were incubated for 24 h. Cells were then observed under an inverted microscope and individual colonies were photographed from the random fields.

To determine the ability of conjugate 20 to inhibit the formation of colonies in Panc-1 cells, The Panc-1 cells were treated to different concentrations of conjugate 20 and performed a colony formation assay (Figure 5.5). As the images show, the formation of colonies was inhibited progressively with increasing concentrations of conjugate 20. This suggests that conjugate 20 inhibits the clonogenicity of Panc-1 cells in a significant manner (P < 0.05).

The critical event in tumor invasion and metastasis is the ability of tumor cells to invade through the extracellular matrix, allowing tumor cells to move beyond the limits of primary tumor environment (Kessenbrock et al 2010) Boyden chamber invasion assay was carried out to study the effect of conjugate 20 on cell invasion. As shown in (Figure 5.6), treatment with 20 (2.0 and 4.0 μM) inhibited cell invasion (P<0.05).

The effect of conjugate 20 on cell invasion was observed using 3D invasion assay. As shown in (Figure 5.7), DMSO treated cells showed increased number of invasive cells protruding beyond the original cell collagen boundary, whereas indicated treatment of conjugate 20 suppressed the cell invasion in a dose dependent manner. The above results
Figure 5.5: Colony formation assay was carried out against PANC-1 cells (1 x 10^3 cells/well) and treated with various concentrations of conjugate 20 along with DMSO for five days. The number of crystal violet stained colonies was counted randomly, quantified and images were captured under inverted microscope at 20x magnification.

Figure 5.6: Cells were treated with various concentrations of 20 for 24 h and analyzed for the invasive ability through matrigel invasion assay. The invaded cells from five random fields in each treatment group were counted and photographed under an inverted microscope (20x magnifications). Data from three independent experiments were subjected to statistical analysis. (n = 3, *p<0.05).
The above results collectively specify that EPA conjugate of $\beta^{3,3}$-Pip-OH, 20 alter the invasion and metastatic potential of PANC-1 cells.

Metastasis and invasion in cancer are the two processes that MMPs are thought to mediate. Type IV collagen acts as a substrate for MMP-2 and MMP-9 and reports have connected overexpression of these enzymes in invasion and metastasis (Bramhall *et al* 1997). Cells were cultured on a cross-linked fluorophore (FITC)-conjugated-gelatin matrix-coated coverslips for 24 h in order to examine the ability of pancreatic cancer cells to degrade the matrix. Figure 5.8 clearly shows that higher concentrations of conjugate 20 inhibited the matrix gelatin degradation by aggressive Panc-1 cells (indicated by arrows). Image-J software highlights the degraded area, which support the spots of gelatin matrix degradation.

**Figure 5.7:** PANC-1 cells were combined with neutralized collagen, placed into the center of each well of a 96-well plate, and allowed to solidify, forming a cell-collagen hemisphere with a distinct boundary as described in materials and methods. Fresh media were then added to each well and the cells were allowed to invade into the surrounding matrix in presence or absence of indicated concentrations of conjugate 20.

**Figure 5.8:** The anti-metastatic effect of conjugate 20 was assessed by culturing PANC-1 cells on FITC conjugated gelatin matrix. Image-J software was used to process and analyze the threshold areas of degradation. Degradation zone was indicated by arrows. Statistical analysis was performed on data obtained from three independent experiments. ($n = 3$, *p*<0.05).
Further, we ought to investigate the effect of conjugate 20 on MMP-2 and MMP-9 expression in PANC-1 cells. The immunoblot experiments demonstrate that treatment of PANC-1 cells with conjugate 20 leads to down regulation of both MMP-2 and MMP-9 expression. As TIMP-1 has an ability to inhibit matrix metalloproteinases and seen to suppress metastasis, (Jimenez et al 2000) so we further studied the effect of conjugate 20 on TIMP-1 expressions. The western blot results demonstrated that the treatment of conjugate 20 resulted in upregulation of TIMP-1 expression in a dose dependent manner (Figure 5.9A). The expression of E-cadherin or its cell surface localization is often lost in advanced tumors and has been linked to a higher incidence of metastasis and tumor recurrence (Birchmeier et al 1994, Berx et al 2009, ), both loss of E-cadherin and the overexpression of MMPs are common features of an invasive phenotype, so we further studied the effect of conjugate 20 on the expression of E-cadherin. The western blot experiments demonstrated that the treatment of conjugate 20 results in upregulation of E-cadherin in PANC-1 cells (Figure 5.9A).

Figure 5.9: Conjugate 20 inhibits MMP-2 and MMP-9 expression through the Inhibition of PI3K/Akt/NF-KB Pathway in PANC-1 cells (A,B,C) and were treated with indicated concentrations of conjugate 20 and checked for the expressions of indicated proteins by western blotting.

Akt is regarded as one of the strong promoters of tumorigenecity in pancreatic cancer (Blum et al 2014). Also in majority of pancreatic tumors, NF-kB is constitutively
activated through a phosphatidylinositol 3-kinase (PI3K)-dependent activation of IKK (Wang et al 1999, Arlt et al 2003, Liptay et al 2003). Also MMP expression is regulated primarily through nuclear factor-κB (NF-κB) at the level of transcription through PI3K/Akt pathway, their cell surface localization and activators or inhibitors regulate at the post transcriptional and protein level (Westermarck et al 1999, Chen et al 2011). So, we thought to study the effect of conjugate 20 on phosphorylation of Akt, expression of PI3K and NF-κB. The immunoblot experiments showed that conjugate 20 treatment resulted in decrease in phosphorylation of Akt (S473) and also down regulation of PI3K P85α, P110α and NF-κB in a dose dependent manner (Figure 5.9B). As Akt is known to regulate NF-KB via mTOR, (Dan et al 2008) we studied the effect of conjugate 20 on mTOR and S6K. The immunoblot results demonstrate that conjugate 20 treatments resulted in decrease in phosphorylation of mTOR along with downregulation of S6K. There is no effect on total mTOR expression following conjugate 20 treatment (Figure 5.9C). The above results demonstrate that EPA conjugate of β3,3-Pip-OH inhibits PANC-1 cell invasion and metastasis by inhibition of matrix metalloproteinases via suppression of PI3K/Akt/NF-κB signalling pathway.

Further it was interesting to notice that piperic acid amide 5 has a different mechanism of action to block proliferation and induce apoptosis in cancer cells. The mechanistic examination of compound 5 induced anti-proliferative and apoptosis inducing activity revealed that compound 5 inhibits phosphorylation/activation of p38-MAPK and ERK1/2. The compound 5 having IC50 value of 7.0 μM was tested in Panc-1 cell line for antiproliferative activity. In order to verify the induction of apoptosis by compound 5, western blot experiment was performed with poly (ADP ribose) polymerase-1 (PARP-1) and procaspase-3 in Panc-1 cells. PARP-1 is responsible for the synthesis of poly (ADP-ribose), its cleavage prevents DNA repair-induced survival and blocks energy depletion-induced necrosis and hence promotes apoptosis (Amours et al 2001). The levels of procaspase-3 are often elevated in the cancer cells, and have an important role in the development of cancer (Peterson et al 2009). Here we observed that compound 5 induced PARP-1 and procaspase-3 cleavage in a concentration dependent manner (Figure 5.10A). The apoptotic induction by PARP-1 and procaspase-3 cleavage was further confirmed by caspase 3/7 glow assay and DAPI staining, which also showed a dose dependent increase
in relative caspase 3/7 activity percentage and nucleus distortion respectively Figure 5.10B, C and D. Further, we examined the expression of some important cancer cell survival related pro-pro-proliferative markers viz XIAP and BCL2 and it was found that the expression of these markers was reduced drastically by 5 in a concentration dependent manner. Further, we found consistent increase in BAX, a pro-apoptotic protein, expression due to 5 exposure. Moreover, cell cycle analysis was carried using Premo-Fucci cell cycle sensor reagent and observed that 5 provoked cell death is associated with G2 arrest as shown in Figure 5.11A and B.

Thus these results demonstrate that 5 is a potent cytotoxic and apoptosis inducing agent in Panc-1 cells, which deterred the down-stream pro-survival and anti-apoptotic effects of p38-MAPK/ERK1/2 pathway and induced apoptosis Figure 5.12A and B.

**Figure 5.10:** Conjugate 5 is a potent apoptosis inducer. (A) Cells after indicated treatments of 5 were analysed, through western blotting, for caspase-3 and parp cleavage, the expression of BAX, BCL2 and XIAP was also checked. Beta-actin was used as a loading control. (B) Graph is a presentation of relative caspase 3/7 activity in 5 treated Panc-1 cells. (C and D) Figure shows and represents the bright distorted apoptotic nuclei obtained after DAPI staining. Data given are representatives of three independent experiments performed, *p < 0.05.
Figure 5.11: Conjugate 5 arrests Panc-1 cells in G2 phase of cell cycle. (A) To the given cells after treatment with the indicated doses of 5 Premo-Fucci cell cycle sensor reagent was added and visualized under fluorescent microscope. The cells turned red and green represents the cells in G1 and G2 phase respectively. (B) Representation of percentage of cells in G2 phase after 5 treatments. Data given are representatives of three independent experiments performed, *p < 0.05, **p < 0.01

Figure 5.12: Conjugate 5 inhibits p38-MAPK/ERK pathway. (A) Shows the activation /Phosphorylation analysis of p38-MAPK and ERK1/2 by Immunoblotting. Beta-actin was taken as a loading control. (B) Represents the dentiometric analysis of western blots. Data given are representatives of three independent experiments performed *p < 0.05.
5.4 Conclusions
In conclusion, piperic acid and 4-ethylpiperic acid amides with non-protein amino acids were tested for anticancer activity against PC-3, Panc-1, HCT-116 cancer cell lines. The conjugate of $\beta^{3,3}$-Pip-OH, 20 significantly decreases invasion and metastasis in PANC-1 cells by downregulating MMP-2 and MMP-9 via suppression of PI3K/Akt/NF-kB signaling pathway. The conjugate 5 displayed a promising antiproliferative activity, which was cancer specific, particularly in pancreatic cancer cells. The western-blot experiment with PARP-1 and procaspase-3, backed with DAPI staining and caspase glow assay, indicated that 5 induces apoptotic cell death in Panc-1 cells. Further, 5 has a potential to halt cells in G2 phase of growth cycle. The mechanistic study of 5 suggested that 5 deterred the down-stream pro-survival and anti-apoptotic effects of p38-MAPK/ERK1/2 pathway and induced apoptosis. Therefore EPA conjugate with $\beta^{3,3}$-Pip-OH can be used as an effective anti-metastatic agent against advanced pancreatic cancer. The presence of N-substituted piperidine ring at $\beta$-position in the $\beta$-amino acid is responsible for cytotoxicity and apoptosis.