Chapter 4

The 4-FPAC targets the PI3K/AKT pathway to exert its anti-proliferative, anti-metastasis, and anti-angiogenic activity in the A549 cell line.

Phosphoinositide 3-kinase (PI3K) signaling pathway is the complex and well-regulated network that is responsible for the normal function of the cellular process, including growth, proliferation, metabolism, and survival (Cantley, 2002). Any mutation in the regulators of the pathway can lead to several diseases, including cancer. Many studies on the role of PI3K in cancer was carried out and reported that in most of the cancer, including non-small cell lung cancer (NSCLC), PI3K was overactivated (Engelman et al., 2008). The hyperactivation of the pathway leads to various hallmarks of cancer, namely uncontrolled tumor cell proliferation, inhibition of apoptosis, sustained angiogenesis, increased invasion, enhanced migration, and adhesion-independent tumor growth and metastasis (Slomovitz and Coleman, 2012, Wu and Hu, 2012). This increased expression is either due to direct mutation or increased amplification of *AKT1* or loss of *PTEN*. Its expression can also get elevated due to the overactivation of any upstream regulators. In most of the cancer, including that of the lung, there was a reported mutation in the key mediator of the pathway - *PI3CA* (Pérez- Ramírez et al., 2015). *PI3CA* is the catalytic subunit of PI3K known as p100α and has a cluster of hot spots of mutation. The mutated *PI3CA* send constitutive signaling even in the absence of growth factor. In transgenic mice, lung-specific induction of p110α, a kinase mutant domain H1047R, developed the lung adenocarcinoma (Fumarola et al., 2014). In NSCLC, the PI3K pathway was heavily implicated in both tumorigenesis and the progression of the disease. There are numbers of study which suggest that PI3K signaling pathway is central to NSCLC growth and survival. The NSCLC cell lines in which the PI3K signaling pathway was inhibited showed an increased sensitivity to apoptotic responses (Brognard et al., 2001, Cheng et al., 2014). Several PI3K pathway inhibitors were evaluated for their anticancer property and are in the clinical and in a pre-clinical trial. These PI3K inhibitors inhibit not only tumor cell proliferation but also the angiogenesis and metastasis (Massacesi et al., 2016). Therefore, PI3K signaling can be a good target for novel anticancer therapeutics.

PI3Ks belongs to the lipid kinases family that phosphorylates the 3-hydroxyl group on phosphoinositides, generating secondary messengers, which in turn regulates many pathways

responsible for normal functioning as well as diseases (Cantley, 2002; Vivanco and Sawyers, 2002). Three classes of PI3Ks have been reported, however, the majority of evidence suggested the importance of Class I_A PI3Ks in human cancer. Class I_A PI3K is activated in the presence of the growth factor, as growth factor binds to the receptor tyrosine kinase (RTK) it relieves the catalytic subunit, which localizes the PI3K to the plasma membrane where its receptor phosphatidylinositol 4,5-bisphosphate (PIP2) residue is present. PI3K phosphorylates PIP2 on the 3'OH position and converts it to PIP3 (PI (3,4,5) P3). This signaling is antagonized by the tumor suppressor gene called PTEN (phosphatase and tensin homologue deleted on chromosome 10), which dephosphorylates the PIP2 and PIP3. Once PIP3 gets activated, it activates PDK1, which further phosphorylates and activates the AKT (Engelman et al., 2006).

AKT is the downstream mediator and activator of the PI3K signaling pathway, which plays a critical role in cell survival and termination of apoptosis, increased activation of this cascade due to any factor leads to the rapid proliferation of cells and malignancy. AKT activation contributes to the neoplastic phenotype by negatively affecting the apoptotic pathway by disabling the pro-apoptotic regulators of Bcl-2 family members -BAD and BAX. The activated AKT phosphorylates BAD, a pro-apoptotic regulator that dissociates from the Bcl-2 complex and hence losses its pro-apoptotic activity (Datta et al., 1997). Similarly, activated AKT phosphorylates the BAX and takes away its pro-apoptotic function by inhibiting the mitochondrial-mediated release of cytochrome c and then apoptosis (Xin and Deng, 2005). In response to the growth factor, AKT inhibits the p53 activity through phosphorylating and activating its inhibitor, MDM2. Activated AKT also promotes cell cycle progression by enhancing the cyclins D1 and D3 mRNA translation, which leads to increased cyclin-dependent kinase and E2F (Testa and Bellacosa, 2001). AKT also stabilizes the cell cycle inhibitors p21 and p27 and inhibits their transport into the nucleus and thereby inhibiting cell cycle progression. In short, it shows anti-apoptotic function by inhibition of cytochrome *c* release, stimulation of glucose uptake and utilization, inactivation via phosphorylation of BAD, BAX, and pro-caspase 9, activation of NF-κB and overexpression of Bcl-2 (Plas and Thompson, 2002).

AKT is not only reported in tumor growth but also in the process of metastasis and angiogenesis. PI3K/AKT signaling pathways serve to regulate TGF-β induced EMT in the A549 cell line (Chen et al., 2012). Chen et al. reported that Luteolin, a natural flavonoid, inhibits the TGF-β induced EMT and invasion of NSCLC cells by inactivating in the PI3K/AKT/NF-κB-Snail pathway (Chen et al., 2013). In the presence of active AKT, the E-cadherin promoter is less active, and this repression appears to be the consequence of the upregulation of the transcription repressor

Snail. Indeed, Snail induces EMT by repressing E-cadherin transcription (Cano et al., 2000). It also modulates angiogenesis by Hypoxia-induced, HIF-1α mediated release of VEGF. However, activation of the PI3K/AKT pathway in tumor cells can also increase VEGF secretion, both by HIF-1α dependent and independent mechanisms. The PI3K/AKT pathway also modulates the expression of other angiogenic factors such as IL-8, nitric oxide, and angiopoietins (Karar and Maity, 2011).

NF-κB transcription factor also plays a crucial role in the PI3K induces cellular functions, including apoptosis, cell cycle control, adhesion, invasion, and metastasis (Sonenshein, 1997). It is a downstream mediator to AKT. Activated AKT releases the NF-κB from its inhibitor IκB to perform its function. NF-κB is found to be constantly active in lung cancer cells and mediate EMT, invasion, angiogenesis, metastasis, proliferation, and also preventing apoptosis (Lin et al., 2010, Yun et al., 2013). NF-κB inactivation leads to reduced expression of EMT-associated transcription factors such as Twist1, Slug, and Zeb2, which lead to the failure of the NSCLC invasion (Kumar et al., 2013). It also suppresses the cellular stress-mediated apoptosis through the removal of reactive oxygen species by increasing the expression of MnSOD (Ju et al., 2007). Thus NF-κB inhibits apoptosis via both the mitochondrial and death receptor-mediated extrinsic pathways. It also suppresses apoptosis by antagonizing p53, possibly through competition for transcriptional co-activators (Kaltschmidt et al., 2000). Finally, NF-κB downregulates the expression of PTEN to activate AKT to promote cell survival and proliferation (Kim and Lee, 2005). Inhibiting NF-κB with different approaches inhibited lung cancer cell's survival and proliferation. NF-κB in inflammatory cells activates the secretion of a variety of angiogenesis factors such as VEGF, TNF-α, IL-8, IL-6, and matrix metalloproteinases (MMPs) (Grivennikov et al., 2010). TGF-β activates PI3K/AKT-mediated NF-κB activation, contributing to the migration of human lung cancer cells. Blocking NF-κB activity downregulates of MMP-2 and MMP-9 expressions, resulting in suppression of lung cancer invasion (Huang et al., 2009) Additionally since TNF-α is involved in inflammation-associated lung carcinogenesis and blocking NF-κB promotes TNF-α induced apoptosis in lung cancer cells, NF-κB blockage may convert TNF-α from a tumor promoter to a tumor suppressor (Lin et al., 2008)

Loss of PTEN and mutation in AKT is also reported in many NSCLC patients of different stages (Marsit et al., 2005). Interestingly, about 44% of early tumor revealed a complete loss of PTEN, however, the patient with PTEN showed improved survival than the patient lacking it. Therefore, the presence of PTEN can be potentially correlated with the improved survival rate in chemotherapy (O'Byrne et al., 2011). The AKT inhibitor, Perifosine, and MK2206 are in phase

II trial and they showed satisfying responses in NSCLC patients (Henderson et al., 2006; Lara et al., 2015). Many inhibitors for the pathways, especially for PI3K, AKT, PTEN, and mTOR, are currently in various phases of the pre-clinical and early clinical trials of NSCLC and showing satisfying results. Therefore, the PI3K/AKT signaling pathway is an attractive target for anticancer therapy for NSCLC.

Hence, in this chapter, based on the above literature, we evaluated the effect of 4-FPAC on the PI3K/AKT signaling pathway in the A549 human lung cancer cell line.

MATERIAL AND METHODS

Dose and Duration

The A549 cell line was treated with 4-FPAC as per the method mentioned in the previous chapters. The selected concentration of 0.16nM was prepared in DMF (0.5%) and used to treat the cells for 48h.

Western blot analysis

$1x10^6$ cells/well were seeded in a six-well plate and allowed to adhere overnight and after that treated with 0.16nM of 4-FPAC. Post 48h incubation, the media was removed, and cells were washed with cold PBS and homogenized in lysis buffer, centrifuged, and the supernatant was taken. The quantification of protein was done using Bradford reagent. 40µg of protein was used for the SDS-PAGE electrophoresis. The separated sample was transferred onto the PVDF membrane at 100mA for 20min. After that, blocking was done with TBS containing 0.1% Triton X-100 and 5% skimmed milk for 1h. Then, the membrane was incubated with primary antibodies which were, monoclonal anti-GRB7 IgG mouse 0.5µg/ml (DSHB Iowa), anti-PI3K IgM mouse 0.5µg/ml (DSHB Iowa), anti-AKT IgG rabbit 0.1µg/ml (Sigma Aldrich USA), anti-pAKT IgG rabbit 0.1µg/ml (Sigma Aldrich USA), and anti-β-actin IgG mouse 0.1µg/ml (Santa Cruz Biotechnology, USA) at 4°C for 16h. Followed by three washes with wash buffer (50mM Tris HCl of pH 7.6, 150mM NaCl and 0.1% Tween 20), and each wash last for 15min. Then incubated with corresponding biotinylated secondary antibodies (0.5µg/ml) for 45min at room temperature, followed by three washes. After that, the membrane was incubated with ALP conjugated streptavidin (0.5µg/ml) for 45min, washed thrice, as mentioned earlier. Bands were developed upon the addition of the BCIP-NBT substrate.

Quantitative real-time PCR

Total RNA was isolated from control, and 0.16nM treated A549 cell line using TRIzol reagent, and purity of RNA was checked by the ratio of A_{260}nm by A_{280}nm. 1µg of DNAase free RNA was reverse transcribed into cDNA using cDNA Synthesis Kit (Applied Biosystems, USA). Real-time RT-PCR (LightCycler 96 Roche Diagnostics, Switzerland) was performed using primers for genes, namely *NF-κB and AKT* (Primer sequence is provided in Appendix 1). *18sr*RNA was used as an endogenous control for normalization of data. Gel electrophoresis and melt curve analysis were used for confirmation of specific product formation. Fold change was calculated using the Livak method ($2^{-\Delta\Delta Cq}$) (Livak and Schmittgen, 2001).

STATISTICAL ANALYSIS

All values are reported as Mean ± Standerd Deviation of Mean (SD). Experiments were performed in triplicates. The GraphPad Prism 5 software (GraphPad Software Inc., USA). was used for Statistical analysis. The difference between groups was analyzed using Student's t-test. The level of significance was kept at 95%.

RESULT

4-FPAC reduces the expression of AKT and NF-κB at transcript level in A549 cell line

PI3K is a key regulator of cell division (Kumar and Carrera, 2007), cell survival (Hsieh et al., 2011), apoptosis (Parcellier et al., 2008), EMT (Xu et al., 2015), invasion, and angiogenesis (Jiang et al., 2000). AKT is the positive regulator and the downstream mediator of the PI3K signaling pathway. The result of the qRT-PCR analysis did not show any significant change in the expression of *AKT* in the 4-FPAC treated cells compared to that of control (Figure 4.1A, Table 4.1). However, a significant decrease in the *NF-κB* was observed when compared to control (Figure 4.1A, Table 4.1). NF-κB is also a downstream mediator of the PI3K signaling pathway, which gets activated once its inhibitor IκB relieved from its catalytic site due to the activation of AKT.

4-FPAC reduces the expression of PI3K and AKT at the protein level in the A549 cell line

As growth factor binds to Receptor tyrosine kinase, it phosphorylates and activates the tyrosine residue associated with the SH2 domain of the GRB7, which in turn, through mediators, activate the PI3K signaling pathway and contributes toward cell cycle turn over and cell migration. Western blot images revealed appreciable reduction in the expressions of AKT, pAKT, and PI3K in the treated cells (Figure 4.1B, Table 4.2). It is phosphorylated AKT, which in turn activates

its downstream regulators. Moreover, no significant change in the protein level expression of GRB7, an upstream regulator of PI3K, was noticed in the treatment group (Figure 4.1B, Table 4.2), which vividly indicates that the derivative is affecting the pathway downstream of GRB7 at PI3K level.

DISCUSSION

After the screening of an array of coumarin derivatives, 4-FPAC was selected, which exerts its cytotoxicity toward non-small cell lung cancer cell line - A549 at a very minimal dose (0.16nM at 48h). Interestingly at this concentration and duration of exposure, the 4-FPAC did not induce cytotoxicity to the non-cancer fibroblast cell line of mouse origin namely NIH3T3. In the previous chapters, it was observed that 4-FPAC initiates apoptosis by elevating the ROS beyond its threshold concentration and upregulate p53 via downregulating the MDM2. It also affects the key regulator of the extrinsic and intrinsic pathways to activate the apoptosis, which involves initiator as well as effector caspases. The 4-FPAC at the selected concentration also influences the decisive steps of metastasis and angiogenesis to exerts its effect. It decreases the EMT by elevating the E-cadherin and downregulating the vimentin via p53 mediated snail pathway, invasion by TGF-β mediated downregulation of MMP-9 and MMP-2. It also affects angiogenesis by decreasing IL-8. Based on the literature, most of the affected mediators of 4-FPAC treated cell line are also the downstream targets of the PI3K signaling pathway (Datta et al., 1997, Plas and Thompson, 2002, Xin and Deng, 2005, Lin et al., 2008, Huang et al., 2009). Therefore, here, in this chapter, the role of the PI3K signaling pathway was investigated to understand the entire mechanism by which 4-FPAC check the progression of A549 human lung cancer cell line.

The PI3K signaling pathway is one of the primary mediators of cell survival, EMT, metastasis as well as angiogenesis and hence, a potential target for anticancer therapy (Lin et al., 2004). Recently it has been reported that one of the coumarin derivatives successfully hampered the PI3K/AKT pathway in K562 cells (Ma and Liu, 2017). Moreover, it is stated that upon activation, PI3K phosphorylates AKT, a downstream target and positive regulator of the pathway, which subsequently phosphorylates and inactivates both BAX and BAD two pro-apoptotic proteins as well as the pro-caspase 9 of the intrinsic pathway of apoptosis (Simonyan et al., 2016). In addition, phosphorylated AKT is known to enhance the MDM2 expression and hence, counteracts the expression of p53 (Abraham and O'Neill, 2014). In the present study, a significant reduction of AKT and MDM2 was observed in the A549 cells treated with 0.16nM of 4-FPAC. Moreover, it was also noted that BAD, BAX at mRNA levels, and p53 at both mRNA

and protein levels remained significantly high in the treated cells. Therefore, it is possible that exposure to 4-FPAC might have reduced the expression of AKT either directly or by reducing the PI3K expression, as both were found to be downregulated in the treatment group, which resulted in the reduction of *MDM2* level, leading to the activation of p53, *BAD* and *BAX* hence, could have induced apoptosis in the 4-FPAC treated A549 cells.

NF-κB, a downstream signaling molecule of the PI3K/AKT pathway as well as an essential transcription factor of several mediators, was found to be downregulated in 4-FPAC treated A549 cell line. When AKT gets activated due to phosphorylation, it activates the NF-κB to perform vital functions, including inhibition of ROS induced apoptosis, p53 mediated p21 dependent arrest of cell cycle progression, a decrease of the cyclin D and D3, and TNF-α mediated extrinsic pathway of apoptosis. Hence, PI3K/AKT/NF-κB inhibits the extrinsic as well as an intrinsic pathway of apoptosis by downregulating BAD, BAX, pro-caspase 9, and TNF-α. Since 4-FPAC treatment reduced the PI3K/AKT mediated activation of *NF-κB* as observed at the transcript level study, it might be the reason behind 4-FPAC induced arrest of apoptosis and cell cycle in the A549 cell line.

Additionally, the PI3K/AKT pathway is known to upregulate the potent inducers of EMT, namely Snail, Twist, MMPs, and TGF-β (Hong et al., 2009; Zuo et al., 2011). The ablation of the AKT pathway is also reported to inhibit the nuclear accumulation of β-catenin and, thereby, EMT (Fang et al., 2007). Phosphorylated AKT also minimizes the E-cadherin expression via upregulating the Snail and Zeb1 directly or with the upregulation of NF-κB to strengthen the EMT process. NF-κB also acts as a positive regulator of AKT as it downregulates the negative regulator of the pathway – PTEN (Vasudevan et al., 2004). The pathway also supports the metastasis in cancer by increasing the cellular invasion through MMPs and angiogenesis through upregulating IL-8 (Li et al., 2013). PI3K/AKT signaling pathway is also a downstream target pathway of VEGFR. When VEGF binds to VEGFR, it activates the PI3K/AKT signaling pathway to stimulate the growth of endothelial cells involves in angiogenesis (Cunningham et al., 1995, Autiero et al., 2003). In the previous chapter (Chapter 3), it was observed that there is an increase in *VEGF-α* and *kdr* (VEGFR). However, a decrease in angiogenesis noticed in the HET-CAM assay might be because of the downregulated PI3K signaling pathway, which became insufficient to generate the response of VEGF into angiogenesis.

Overall, in this study, we found that 4-FPAC downregulates the PI3K/AKT/NF-κB pathway, thereby increasing the apoptosis and concomitantly decreasing the EMT, invasion, and

angiogenesis in the 4-FPAC treated A549 cells. Therefore, on the basis of various experiments conducted so far, it could be construed that 4-FPAC induced downregulation of PI3K/AKT/NF-κB pathway is responsible for the observed arrest of EMT, invasion and angiogenesis in the NSCLC *in vitro* model selected for the current study.

SUMMARY

PI3K/AKT pathway is vital for the NSCLC cell proliferation, metastasis, and angiogenesis. Inhibiting the pathway using pharmacological agents against the key nodes of the pathway is the current need of research. Many novel therapeutics were designed and are in various stages of research as an inhibitor of the pathway with an aim to become a potent treatment for NSCLC in the long run. Many coumarin derivatives were also reported to have potential as an inhibitor of the pathway. In the previous chapters (Chapter 2 and 3), it was investigated that 4-FPAC is downregulating the key molecule of apoptosis as well as metastasis to exerts its anticancer property. Those key molecules are also the downstream mediator and the target molecules of the PI3K signaling pathway. Therefore, in this chapter, we evaluated the potential of 4-FPAC as an inhibitor of the PI3K signaling pathway. The transcript level study showed the downregulation of *AKT*, as well as *NF-κB* in treated group, the western blot analysis of protein expression, also revealed the decrease of PI3K, AKT, pAKT level in the treatment group, however, no significant change was observed in the level of upstream mediator GRB7 which signify that 4-FPAC affect the PI3K signaling downstream to GRB7. These observations confirmed that 4-FPAC exerts its anticancer effect by downregulating the PI3K/AKT/NF-κB pathway. Further support for this finding was provided by the previous chapters investigation of key molecules involved in apoptosis, EMT, invasion, and angiogenesis. The entire work and results are graphically summarized in Figure 4.2.

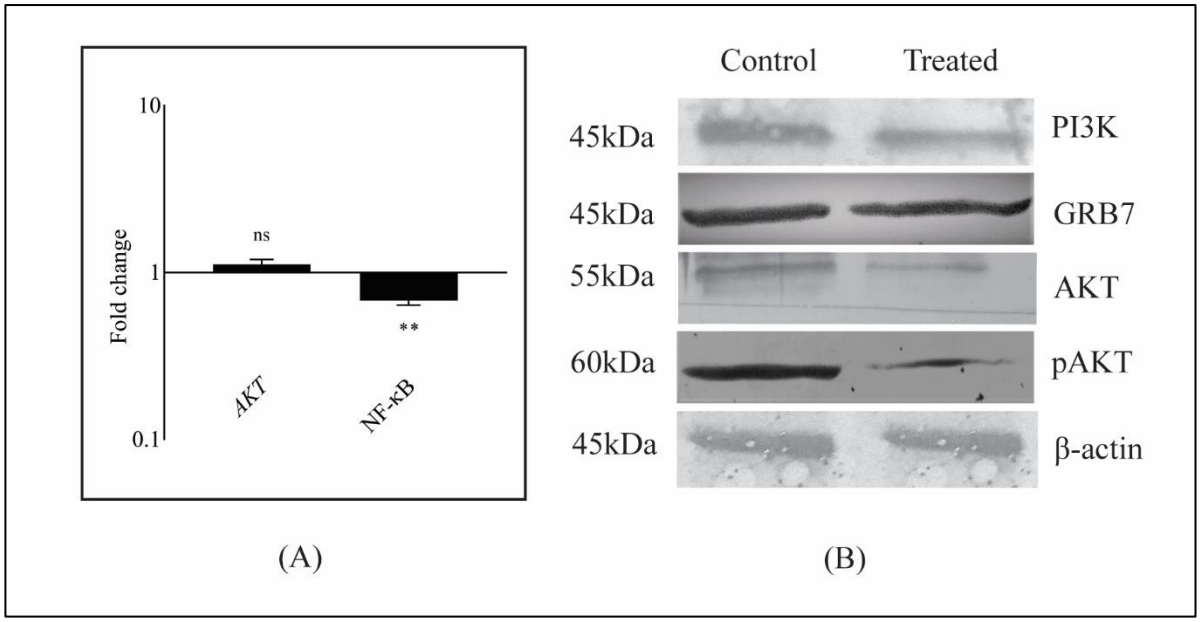

Figure 4.1. 4-FPAC affects the PI3K/AKT signaling pathway. (A) Quantification of AKT and NF-κB at the transcription level by qRT-PCR, graph represents the fold change of *AKT* and *NF-κB*. The expression level of control mRNA was assigned as 1.0. The experiment was performed in triplicate. Data are represented as Mean ± Standerd Error of Mean (SEM), * *p≤0.01, ns= not significant. (B) Western blot analysis of PI3K, AKT, pAKT, and GRB7 in control (0nM) and treated cell line (0.16nM). β-actin was taken as an internal control.

Gene	Fold change (Mean±SEM)
AKT	1.12 ± 0.082^{ns}
NF-κB	$0.68\pm0.04^{**}$

Table 4.1. qRT-PCR analysis of *AKT* and *NF-κB* in 4-FPAC treated A549 cell line. The fold change value of the control group was 1.0. The level of significance is denoted as, **p≤0.01, ns=not significant.

Protein	Relative Density (Fold change)
PI3K	$0.79\pm0.03^{*}$
AKT	$0.86\pm0.02^{*}$
pAKT	$0.63\pm0.02^{**}$
GRB7	1.03 ± 0.05^{ns}

Table 4.2. Quantification of western blots using plugin Fiji (Image J ver 2.0, USA), represented as Relative Density (fold change) of PI3K, AKT, pAKT, and GRB7. The comparison for statistical significance was made for 4-FPAC treated group (0.16nM) with the control group (0nM). Data is represented as Mean ± Standerd Error of Mean (SEM), and the level of significance is denoted as, **p≤0.01, **p≤0.01 *p≤0.05, ns=not significant**.**

GRAPHICAL SUMMARY

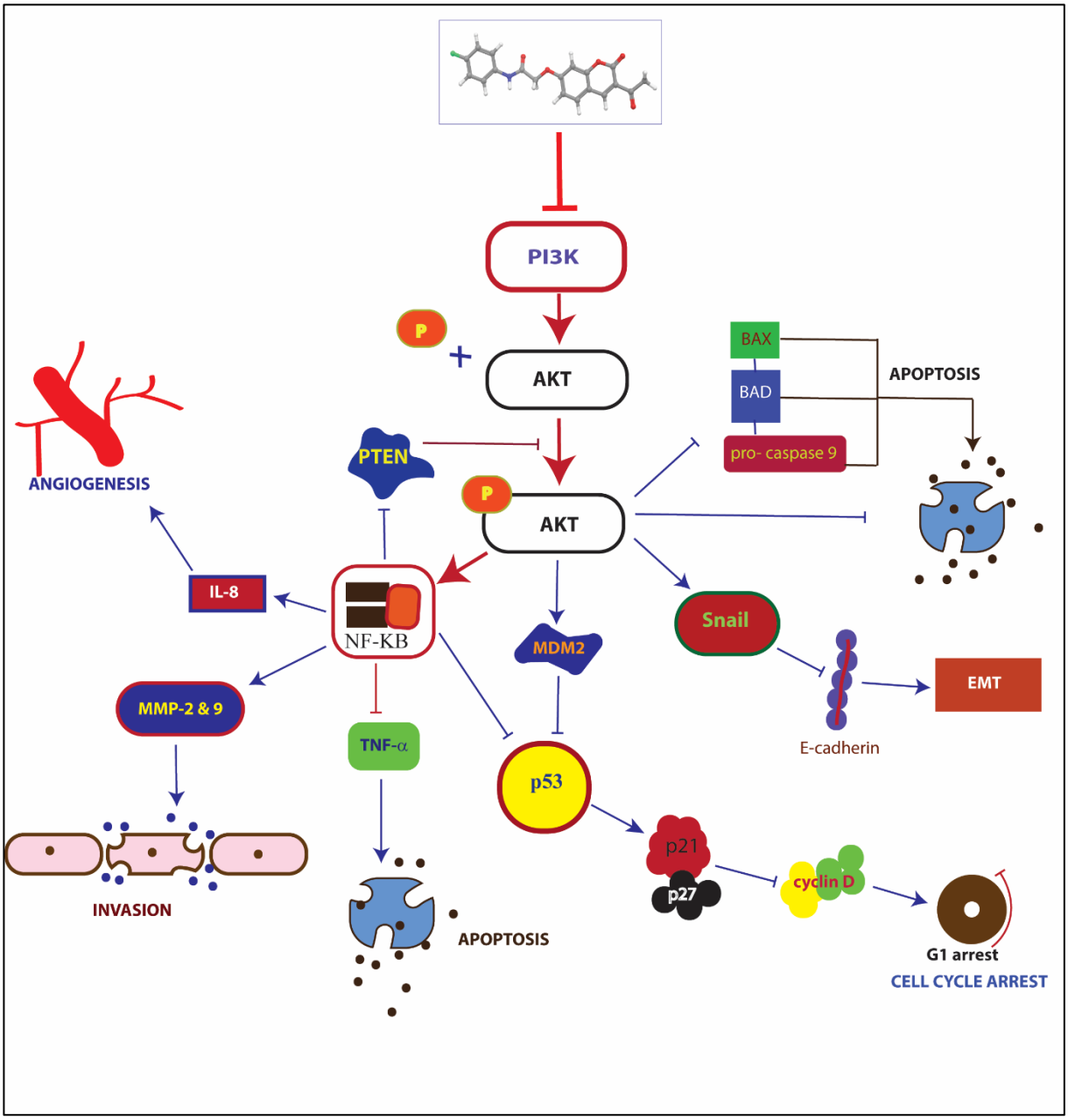

Figure 4.2. 4 - FPAC induced anti-cancerous activity via PI3K/AKT pathway.