Summary

Cancer is a fatal disease found in heterogeneous population of cells, and is the second leading cause of death worldwide. Even with modern medications, it is still not wholly curable. According to a WHO report published on 12 September 2018, cancer was responsible for about 9.6 million deaths solely in 2018, while it caused one out of six deaths, globally. Among all cancer types, lung cancer stands out with the highest mortality rate (~ 2.09 million) as well as the occurrence (~1.76 million) (Bray et al., 2018).Further, lung cancer is classified into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). The NSCLC accounts for 80-85% of the total incidences of lung cancer with adenocarcinoma as a predominant sub-type, making it a priority disease for which there is a need for accelerated research and development of novel therapeutic.

It has been reported that in majority of the cases the attempt to treat NSCLC type of the cancer with currently available medicines remains unsuccessful due to drug insusceptibility (Musa et al., 2012). However, some synthetic, as well as natural coumarins, when tried at an experimental scale showed good anticancer properties, albeit with attended side-effects at higher doses. Therefore, medicinal chemists synthesized many coumarin derivatives by adding different pharmacophores on the basic coumarin ring, and the preclinical evaluation revealed that some of them have excellent anticancer activity with minimum side effects (Klenkar and Molnar, 2015). Moreover, from literature, it became evident that the substitutions at C-3, C-4, and C-7 are the most crucial ones for enhancing the anticancer property of coumarin (Kaur et al., 2015,Li et al., 2015, Musa et al., 2012). Therefore, it was thought pertinent to synthesize new derivatives of coumarins with modifications at C-3, C-4, and C-7 of coumarin ring followed by testing their efficacy (*in vitro*) as potent alternative to the currently used medicine in the treatment of NSCLC.

A total of eighteen coumarin derivatives (C-4 mono-substituted and C-3, C-7 di-substituted) were synthesized on our demand by a collaborator from the synthetic organic chemistry laboratory of The Department of Chemistry, The Maharaja Sayajirao University of Baroda. The details of the synthesis including the purity of the compounds and their chemical properties are not considered for this thesis, nonetheless, these details are part of a joint publication (Durgapal et al., 2017). **The overall aim of the present study was to find a novel**

synthetic coumarin derivative that can effectively curtail the proliferation and migration of non-small cell lung carcinoma using A549 cell line. This was achieved by conducting five parallel studies listed below, and the results obtained are presented in chapters 1 to 5.

1. Chapter 1: To screen the novel synthetic analogs of coumarin for their potential anticancer property in A549 human lung cancer cell line with concomitant evaluation for side-effects of the derivative showing lowest half-maximal inhibitory concentration in NIH3T3 cell line.
2. Chapter 2: To assess the effect of 4-flourophenylacetamide-acetyl coumarin (4-FPAC - which was found effective in curtailing the proliferation) on the mechanism of cell death in the A549 cell line.
3. Chapter 3: To evaluate the possible anti-metastatic and anti-angiogenic property of 4-FPAC in the A549 cell line.
4. Chapter 4: To study the effect of 4-FPAC on the PI3K/AKT signaling pathway in the A549 cell line.
5. Chapter 5: To evaluate the drug-likeliness, biological reactivity and ligand-target interaction of 4-FPAC*in silico* using DFT, molecular docking and a knowledge-based set of the rule.

As a prelude, these derivatives were screened for their anticancer property using A549, a representative cell line of human lung adenocarcinoma (**Chapter 1**). When A549 cells were incubated with the derivatives for 48h, the 4-fluorophenylacetamide-acetyl coumarin (4-FPAC) derivative of C-4 substitution (with flurophenylacetamide at the acetylated coumarin ring) group showed the lowest half-maximal inhibitory concentration (IC_{50}) of 0.16nM. The possible side-effect of 4-FPAC on neighboring normal cells was assessed using a classical non-cancerous *in vitro* model the NIH3T3 cell line. The half-maximal inhibitory concentration of 4-FPAC for NIH3T3 cells was computed to be 79.58µM post 48h of incubation. This resilience of NIH3T3 cells signifies that a dose of 0.16nM of 4-FPAC (IC_{50} concentration for A549) will effectively curtail the progression of cancerous cells without causing any significant damage to the surrounding non-cancerous cells. Moreover, to understand the duration of the treatment that is ideal to induce maximum cytotoxicity, a time-lapse study was performed, which generated the IC_{50} concentration of 1.19nM, 0.75nM, 0.67nM, 0.16nM and 1.97nM at 6h, 12h, 24h, 48h

and 72h respectively. The result revealed that 4-FPAC exerted maximum inhibitory effect at 48-hour post-treatment. Therefore, all the further analyses were performed after 48 hours of treatment with 0.16nM of 4-FPAC.

When the cell loses its control over death and starts proliferating beyond a limit, it forms a tumor- a heterogeneous mass of cells under different phases of the cell cycle, referred as primary cancer. In most of the cancer types, the primary tumor is curable and can be dissected out by surgical intervention, however, in case of lung, the primary tumor is as life-threatening as the metastasized one (Lowe et al., 2004). Incidentally, cell death is a highly organized critical activity that is essential for the maintenance of the homeostatic balance and the failure of which leads to cancer. It has been observed that, the cell death (apoptosis) is under the regulation of a myriad of pro-apoptotic factors such as BAD, BAX, BID, along with anti-apoptotic mediators like BCL-2 (Danial and Korsmeyer, 2004). Any flaw in the expression or function of these molecules can lead to a misbalanced equilibrium between cell death and survival (Wong, 2011). A tumor suppressor gene TP53 is an oxidative stress-regulated transcription factor, which acts as the 'guardian gene' by playing a pivotal role in the maintenance of genomic integrity. It also protects the cell against ROS induced DNA damages (Hussain et al., 2004). When a chemotherapeutic agent increases the ROS beyond the threshold limit, it activates p53, which in turn acts on its downstream mediators thereby inducing either DNA repair or apoptosis. Cell cycle is arrested to boost the former process but if the repair machinery fails to make such amends, then the cell is pushed towards apoptosis.

In the preliminary screening (first objective) it was noticed that 4-FPAC at a concentration of 0.16nM induced appreciable cytotoxicity and therefore, meticulous efforts were made to unravel the mechanistic details of 4-FPAC induced cell death in the A549 cell line (**Chapter 2**). It was found that 4-FPAC exerts its anticancer activity via apoptosis rather than necrosis. The fluorescent staining and NAC (N-Acetylcysteine) -an inhibitor of ROS confirmed the involvement of Reactive Oxygen Species (ROS) in evoking cell death. The results of the biochemical estimations of antioxidant enzymes such as catalase and glutathione peroxidase (GPx) were in conformity with the above finding. Supplementary immunocytochemical studies revealed that, the ROS load altered the mitochondrial as well as the lysosomal membrane potential to release the apoptotic mediators such as cathepsin B, and cytochrome c to activate the intrinsic pathway of apoptosis. ROS also activated TNF-α, as evident from its protein

expression, and led the cell towards the extrinsic pathway of apoptosis. The transcript level study of *BID, BAX, BAD, AIF, PARP1, β-catenin, cathepsin B, calpain 2, caspase 3,7, 8, 9 cytochrome c, TRADD, FADD,* and *BCL-2* confirmed the activation of ROS dependent extrinsic as well intrinsic pathways of apoptosis which culminates with activation of caspase 3. 4-FPAC also exerts a cytostatic effect and arrests the cell cycle at the G0/G1 phase via DNA damage-induced p53 dependent and p21 mediated downregulation of PCNA, *CDK2/cyclin E* and *CDK4/cyclin D*. It maintains the p53 level by downregulating *MDM2*. The increased levels of p53 (confirmed at transcript as well as protein level) also activates BAX and contributes towards apoptosis. Therefore, based on the results of Chapter 2, it can be concluded that 4-FPAC exerts its anti-proliferative effects by permeabilizing mitochondrial and lysosomal membranes, activating extrinsic as well as an intrinsic pathway of apoptosis via ROS mediated p53 dependent pathway and causes cell cycle arrest by p53 dependent p21 mediated downregulation of CDK2/CDK4-cyclins.

Once a genetically altered cell grows beyond 23mm in size, its inner mass becomes hypoxic, and induces the secretion of angiogenic growth factors thereby attracting the endothelial cells towards the tumor mass and eventually starting the process of neovascularization. The process of tumor-associated sprouting of new vessels from the existing ones is often referred as 'tumor angiogenesis' (Folkman, 1971). These new blood vessels not only provide nourishment to the tumor cells but also act as their entry point into the circulation. When these primary tumor cells get transformed, in response to any genetic or epigenetic stimulation, they start invading the surrounding tissue and colonize in the distant organ of the body to attain the characteristics of metastatic tumors. Now they are called a secondary tumor, and the process is known as metastasis, a principal reason for cancer-related deaths (Hanahan and Weinberg, 2000). Metastasis is a multi-step process in which primary tumor cells first undergo epithelial to mesenchymal transformation. It initiates with the loss of cell-cell adhesion (i.e. decrease of E-cadherin, β-catenin with simultaneous increase of N-cadherin and vimentin) and activation of transcriptional inhibitors of Snail1, Snail2, ZEB2 and Twist. This eventually shifts the epithelial characteristics, and changes the gene expression pattern to acquire the features of a mesenchymal, migratory phenotype (Thiery, 2003, Thiery et al., 2009). Once the tumor cell gets transformed and attains the characteristics of a mesenchymal cell, it starts secreting the proteases (MMPs) to degrade the basement membrane for the active translocation of neoplastic cells across ECM (Mareel and Leroy, 2003). These metastasized cells start invading the nearby

as well as the distant organs through a circulatory or lymphatic system in search of a new niche.

Chemokines are also known to help these secondary tumor cells in migration, survival, and establishment in the new environment. The transforming growth factor-β (TGF- β), which plays a central role in tumor progression, upregulates the transcriptional repressors of E-cadherin (Thiery, 2002). During metastasis in A549 cell line, IL-8 is one of the closely associated molecules to EMT and angiogenesis (Koch et al., 1992). IL-10 which has an immunosuppressive property, when expressed in tumor cell, it suppresses the production of pro-inflammatory cytokines such as TNF-α, IL-6,and IL-1β, thereby supporting metastasis and angiogenesis (Changkija and Konwar, 2012). Most of the non-small cell lung cancer cells also secrete TNF-α and IL-1β, which promote EMT, invasion, and metastasis (Shang et al., 2017) as they act on cancer cells by establishing crosstalk with autocrine factors like MMPs, VEGF-α, IL-8, IL-6, and TNF-α. These mediators further facilitate invasion and angiogenesis (Voronov et al., 2003).

The **third chapter** of this study therefore, focussed on uderstanding the anti-metastatic and anti-angiogenic properties of 4-FPAC on A549 cell line. The results revealed that 4-FPAC minimizes the EMT process by downregulating the expression of N-cadherin and upregulating the E-cadherins via reduction in the transcripts of *Snail1, Snail2, and ZEB1*. This downregulation of Snail might be a result of increased p53, which controls the expression of the former, by degrading it. In addition, 4-FPAC reduces migrating, clonogenic and independently anchoring properties of A549 cells, to eventually increase their aggregation, thus eliciting the anti-metastatic action. The derivative (4-FPAC) also minimizes the invasion and ECM remodeling by increasing the expression of *TIMP2* and *TIMP4*, which reduced the expressions of *MMP2* (at transcription level) and *MMP*9 (at both protein and transcription level expression). Moreover, 4-FPAC reduces angiogenesis as observed in the HET-CAM assay by downregulating IL-8 without affecting VEGF-α. Elevated expression of IL-10, TGF-β, and concomitant decrease in the expression of IL-1β and IL-6, reinforced the anti-metastatic effect of 4-FPAC in A549 cell line. Therefore, it is prudent to believe that 4-FPAC ameliorates metastasis by p53 dependent downregulation of Snail, while it curbs invasion via downregulation of MMP-9 and MMP-2. On the other hand, it also hinders angiogenesis by reducing the levels of IL-8.

Engelman (2008) from his study has opined that in most of the cancers, including NSCLC, the PI3K/AKT signaling pathway remains overactivated (Engelman et al., 2008). The hyperactivation of this pathway can facilitate 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). Therefore, it was thought relevant to study the effect of 4-FPAC on the PI3K/AKT signaling pathway (**Chapter 4**). In brief, when A549 cells were treated with 0.16nM of 4-FPAC for 48h, it downregulated the PI3K signaling pathway, which in turn, suppressed its downstream mediators and their functions. AKT-a downstream mediator and a positive regulator of the pathway when activated, induces the NF-κB, which in turn initiates the process of invasion and vascularization via MMPs and IL-8. It also suppresses the extrinsic pathway of apoptosis by NF-κB mediated downregulation of TNF-α. Activated AKT also downregulates the expression of p53,which halts the process of cell cycle progression through p21 dependent pathway. The activated AKT suppresses the p53 mediated degradation of Snail, thus inducing the EMT process. Similarly, it also inhibits BAX mediated intrinsic pathway of apoptosis through p53 dependent pathway. In nutshell, 4-FPAC, by downregulating the PI3K/AKT/NF-κB pathway curbed the whole process of proliferation, EMT, invasion, and angiogenesis, while activating the process of apoptosis.

Lastly, an *in silico* based study was performed to predict the interactions between the targeted protein data bank (PDB) and the derivative ,to evaluate the chemical, as well as biological reactivity of the compound, and to check the druggability along with ADME property of the 4-FPAC (**Chapter 5**). The chemical reactivity and kinetic stability of 4-FPAC were analyzed using Density Function Theory (DFT), it includes the HOMO-LUMO (frontier molecular orbitals) and MESP map generation using Jaguar - an *ab initio* quantum chemical program, by applying Becke's three-parameter exchange potential and the Lee-Yang-Parr correlation functional (B3LYP) (Gill et al., 1992). The frontier molecular orbitals, HOMO (Highest Occupied Molecular Orbital) and LUMO (Lowest Unoccupied Molecular Orbital) were distributed overdifferent non-overlapping molecules of the 4-FPAC. The HOMO orbitals were located over the 4-fluorobenzene ring, and LUMO orbitals were located on the 3-acetyl coumarin ring, distributed around the amine, oxygen, carbon, and fluorine groups, indicating the participation of these moieties during protein-ligand interactions. The calculated HOMO, LUMO, and HOMO-LUMO energy gaps for 4-FPACare -0.2430eV, -0.1052eV, and -0.1378eV, respectively. The negative eigenvalues of orbital energies and gap energies

characterized 4-FPAC as a highly reactive, easily polarizable, and chemically soft molecule. Based on the Koopman's theorem for closed-shell molecules (Pearson, 1986), the global reactivity descriptors were also analyzed by using HOMO and LUMO energies of the compound which again signifies that, the 4-FPAC is a highly reactive soft molecule that has both the electron donor and acceptor properties. The MESP map of 4-FPAC was also generated, which mapped the region having the most negative potentials (over the oxygen and fluorine atom) and the region having the most positive potentials (over the hydrogen atom and the methyl group) of the derivative. This highly reactive nature of the compound might be responsible for its anticancer activity.

The molecular interactions between various target proteins and 4-FPAC were made with the help of the Schrödinger based molecular docking protocol named Glide. The targeted proteins were p53(5GLY), BAX(2K7W), caspase 8 (4ZBW), caspase 3 (4QTX), SNAIL (4QLI), VEGFR(5EW3), PTEN(5BZX) and HIF-1α (5L9B). These are the major proteins, which were involved in the 4-FPAC induced anti-proliferative, anti-metastatic, and anti-angiogenic effects in the A549 cell line. When 4-FPAC was docked with p53, it generated a docking score of -5.89kcal/mol by interacting with the active site amino acids (six hydrogen bonds and two pi-pi bonds). BAX which is a pro-apoptotic protein also interacts with two hydrogen bonds with Trp17 and Gly166, and three pi-pi interactions with Phe165 in presence of 4-FPAC. These interactions generated a docking score of -11.7kcal/mol, which is very high and signifies the strong interaction between the derivative and BAX.

Similarly, caspase 8 interacts with two stable H-bonds at the active site amino acid residues and generated a docking score of -5.563kcal/mol. However, caspase 3 generated a docking score of -3.462kcal/mol by establishing an interaction with three hydrogen and two pi-pi bonds. SNAIL, a downstream mediator of p53, when interacts with 4-FPAC, it generated a docking score of -2.89kcal/mol by interacting with Lys122 and Arg129 and this interaction was not as strong as with other PDBs. Therefore, it can be deduced that it is the strong activation of p53 with 4-FPAC which is responsible for the Snail mediated downregulation of epithelial to mesenchymal transformation. VEGFR is also docked with the compound and got a docking score of -9.66kcal/mol (interacted with two hydrogen bonds and one pi-cations). These interactions might have placed the ligand at the active pocket or near the site at which VEGF binds due to which it was not able to further bind and initiate the process of angiogenesis in 4-FPAC treated cells. HIF-1α also generates a docking score of -8.893kcal/mol

with three hydrogen bonds and three pi-cations. PTEN is a negative regulator of the PI3K signaling pathway whose activation leads to the downregulation of AKT activity. Since a significant reduction in AKT activity was observed in 4-FPAC treated cells, it was presumed to function for activating PTEN. To validate this notion, PTEN was docked with 4-FPAC, which generates a docking score of -5.557kcal/mol by interacting with active site amino acid Lys128, Gly129, and Thr167 by three H-bonds. These interactions might be activating PTEN, which further downregulates AKT to exert its anticancer activity.

The studies so far revealed that 4-FPAC has the potential to emerge as a good anticancer drug against NSCLC and therefore an *in silico* based study was done to predict the drug-likeliness and ADME property of the compound. Incidentally, *in silico* studies at the early stage of drug development, lower the cost of discovery and reduce the chance for the failure of the drug at a later stage during clinical trials. In addition, this computational analysis is widely accepted as a reliable predictive tool to know the competence of a derivative to enter into an advanced stage of the drug development process and the likeliness to become a drug of choice for the future treatment regimen. The drug-likeliness properties were checked with the criteria for Lipinski's Rule of Five (Lipinski et al., 1997). The result revealed that 4-FPAC has a molecular weight of 355.3, hydrogen bond donor - 1, hydrogen bond acceptor - 7.5, and QPlogPo/w octanol-water partition coefficient to be 2.329. These criteria satisfy Lipinski's Rule of Five and are found to be under the acceptable limits. The pharmacokinetic property of 4-FPAC was analyzed using ADME (Absorption, Distribution, Metabolism, and Excretion) which includes, Blood-Brain Barrier (QPlogBB) penetration and distribution. All the parameters were found to be under the acceptable limit, with 1.27 (QPlogBB), 319.5 (QPPCaco),and 85.41% oral absorption property. The result revealed that 4-FPAC did not violate any of the Lipinski's Rule of Five and hence, can be considered as a drug-like candidate with lesser toxicity and good bioavailability.

Altogether, a carefully planned *in vitro* study on A549 cell line, confirmed that 4-FPAC at a dose of 0.16nM has the potential to reverse all the deviant processes which are infamous for the development of cancer (in this case NSCLC) namely uncontrolled proliferation, metastasis and angiogenesis (Figure I). The supplementary, computational analysis (*in silico*) confirmed that 4-FPAC is a good candidate for anticancer drug development with lesser side-effects, good druggability, ADME, and lower toxicity, therefore, it can be taken further for the advanced drug development process for non-small cell lung cancer.

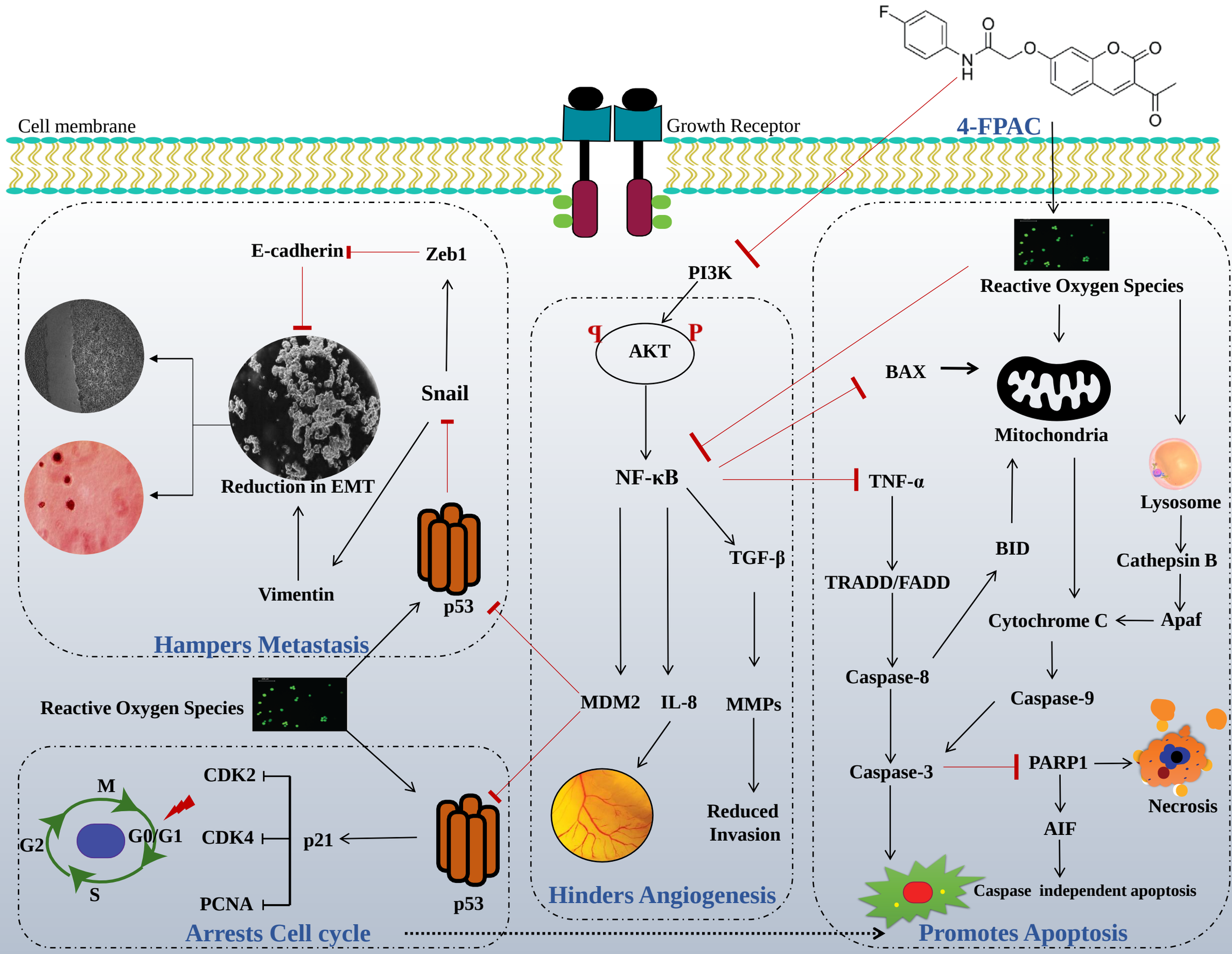

Figure I: A pictorial overview of the current work