Chapter-1

Introduction
Introduction

1.1. Overview of cancer

Cancer is a complex disease arising from the uncontrolled cell proliferation at a particular part of the body. Cancer cells invade normal tissues and organs and eventually spread throughout the body. The difference between the cancer cells and their normal counterpart is the loss of growth control mechanism. This is due to the accumulated abnormalities in multiple cell regulatory systems and signaling cascades. There are several key signaling pathways and players which are abnormally activated in cancer cells. Tumors may be of two types: benign and malignant. Benign tumor is confined to its original location and does not invade surrounding normal tissue. Malignant tumor can invade surrounding tissue and spread throughout the body with the help of circulatory and lymphatic systems by a process called metastasis. Only malignant tumors are basically referred to as cancer. Cancer can be classified to different types depending upon the type of cell from which it arises. Cancer of a particular organ or tissue may show further difference within the tumor itself (1). Out of the several types of cancer, breast cancer is an important health problem. The growing concern for breast cancer is not only due to its frequent occurrence but also because of the intra and inter tumor heterogeneity. This thesis mainly focuses on the study of breast cancer.

1.2. Breast Cancer

Breast cancer in women is a major public health problem throughout the world. It is the most common cancer among women of both developed and developing countries. Out of every ten new cancers diagnosed worldwide each year, one is female breast cancer (2). An estimated 60,290 new cases of female breast carcinoma in situ are expected to be
diagnosed in 2015, accounting for about 20% of all breast cancers in women in US (3). In India 106,124 cases of breast cancer are expected to be reported in 2015 (4). The etiology of human breast cancer remains unclear. However the risk factors associated with breast cancer can be grouped into three main categories: family history and hereditary factors, hormonal and reproductive factors, and environmental factors including lifestyle (5). Familial relative risk (FRR) for breast cancer is significantly increased for most of the pathological subtype except triple negative (TN) tumors (6). The women who have first degree relatives with a history of breast cancer have greater risk of acquiring the disease. Most of them will develop breast cancer only after the age of 50. However most of the public health programmes aim for early detection of breast cancer amongst women having affected first degree relatives. Hence, these programmes are likely to miss the large majority of women who develop the disease later in their life (7). Reproductive factors like delayed childbirth, nulliparity and early menarche and postmenopausal obesity also increase the risk among some type of breast tumors like hormone receptor-positive breast tumors (8). Thus there are several genetic and heredity factors which make the diagnosis of the breast cancer difficult. Apart from the above factors, it is the heterogeneity of breast cancer which makes it a cause of concern.

1.3. Breast cancer heterogeneity

Breast cancer was considered a single disease until the recent advances in our understanding of the epidemiology, biology, and molecular basis for breast cancer. Breast cancer is now considered as a heterogeneous disease that can be divided into several distinct subtypes. The classification is based on different factors like histological type, tumour grade, lymph node status and the presence of predictive markers such as estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2). It can be classified
into five main subtypes: luminal A, luminal B, HER2, basal and normal. Luminal A tumours are characterised by the presence of estrogen receptor (ER), the presence or absence of progesterone receptor (PR) and the absence of Her2 amplification (9, 10). The molecular characteristics of the different subtypes of the breast cancer and their corresponding cell lines which are commonly used for study are illustrated in the Figure 1.1.

**Figure. 1.1. Breast cancer classification and their representative cell lines.**

Luminal B tumours express Her2 while luminal A lacks its expression. Her2+ tumors are identified by the lack of expression of ER and PR and show the presence of 17q amplification, the chromosomal region that codes for the HER2 tyrosine kinase receptor. Basal-like breast tumours lack the expression of ER, PR and Her2, and epidermal growth factor receptor (EGFR) and cytokeratins 5, 14 and 17 (11). The claudin-low subtype was discovered recently while studying the established human and murine datasets. Initially
they were put under the basal like subtype because of the absence of ERα, PR and HER2. However, claudin low tumors were found to be unique as it showed additional downregulation of claudin-3 and claudin-4, low expression of the proliferation marker Ki67, enrichment for markers associated with the epithelial–mesenchymal transition and expression of features associated with mammary cancer stem cells (CSCs) (12).

Each subtype has different prognosis and treatment response (13). Because ER is a therapeutic target, the luminal A and luminal B subtypes are susceptible to hormonal therapy. Similarly the HER2 group are possible candidates for trasuzumab (antibody based) therapy. In the current absence of expression of a recognised therapeutic target, basal tumours are difficult to treat, more biologically aggressive and often have a poor prognosis. Because the basal phenotype is characterised by the lack of expression of ERα, PR and HER2, it is sometimes referred to as triple-negative. Even though the basal and triple-negative phenotypes appear to be similar, they are not identical (14).

Majority of breast tumours display intra-tumour heterogeneity at the time of diagnosis. Earlier the source of intra-tumour heterogeneity was explained by the existence of clonal selection and later by the stem cell hypothesis. The clonal selection hypothesis states that in a particular microenvironment, only best fit clones will be selected during tumour progression. On the other hand, the cancer stem cell concept proposes that the tumor stem cells are responsible for the maintenance and progression of tumours as they possess the unique ability to both self-renew and differentiate into the bulk of tumour non-stem cells (15). Experimental evidence supports the existence of both cancer stem-like cell differentiation and clonal selection of particular cell subpopulations contribute to the breast cancer heterogeneity (15, 16).

Our understanding of the biology of breast cancer has made tremendous advances using different breast cancer cell lines. The concept of studying one marker in one cell line has
now been replaced. The use of multiple cell lines or cell line panels prove to be more effective as experimental models for studying specific subgroups of breast cancer (17). Thus different breast cancer cell lines were included in this study for better understanding of breast cancer as a disease.

Breast cancer shows modulation of different cell signalling pathways and networks. In this thesis, we have tried to address the modulation of the cell signalling pathways in breast cancer which eventually leads to cell death. The modulation of the cell signalling pathway is achieved by two entirely different anticancer agents. One is a traditional painkiller which is present in the market for a long time. The second agent is a natural product whose role is well established in cancer.

1.4. Cancer and cell signalling

There are multiple cellular signalling networks and cascades which regulate a normal cell growth, function and behaviour. The development of cancer at any particular site or organ is due to the malfunctioning or abnormality of these signalling pathways. The alterations in signalling pathways which normally control cell growth, motility and survival allow the cancer cells to evade cell death. By overcoming the checkpoints of cell proliferation, cancer cells grow at an abnormal rate and eventually invade surrounding normal tissues and organs. Hence cancer originated at a particular site may eventually spread throughout the body. Thus understanding the complexity of the cellular signalling networks will help us in having better understanding of the tumor cell behaviour and implementing this knowledge for cancer therapy (18).

We are giving a brief overview of some of the cellular pathways that have been shown to affect breast cancer in this thesis. Even though there are several other signalling cascades
and networks which have certain role in breast cancer development and progression, their description have not been provided here, as they are not relevant to this thesis.

1.5. Akt pathway overview

The Akt/ PKB (Protein Kinase B) which include Akt 1, Akt 2, Akt 3 is the key signaling molecule involved in regulating cell survival, proliferation, metabolism and angiogenesis (19) as illustrated in Fig. 1.2. The Akt is the most commonly activated signaling element in several human cancers like lung, prostate, skin, colon, endometrium, breast and cervix (20). The PI3K/Akt pathway can be activated by Receptor tyrosine kinases (RTK) (21), activated Ras (22), activation of G protein coupled receptors (GPCR) (23) or inactivation of the tumour suppressor, PTEN (24). The PI3K phosphorylates the phosphatidylinositol lipids at the 3’ position of the inositol ring forming the product phosphatidylinositol-3,4,5- triphosphate (PIP3) which in turn facilitates phosphorylation and activation of Akt (25). Once active, Akt can control important cellular signalling processes by phosphorylating substrates involved in apoptosis, cell cycle progression, protein synthesis and glucose uptake (26, 27). Akt signaling leads to protection from apoptosis and may induce uncontrolled cell cycle progression culminating in tumor cell survival (28). Akt can regulate autophagy through the mTOR pathway as discussed earlier. Besides, Akt can also inhibit autophagy by interacting directly with the core autophagy machinery. Akt-mediated phosphorylation of Beclin 1 enhanced its interactions with 14-3-3 and vimentin intermediate filament proteins, and vimentin depletion increased autophagy and inhibited Akt-driven transformation (29).
1.6. Akt in breast cancer

The PI3K/Akt pathway is abnormally activated in breast cancer with mutations occurring in most of the breast cancer. The majority of mutations are in PIK3CA, encoding the catalytic p110α subunit, and are nonrandomly localized in three “hot spots” resulting in single amino acid substitutions: E545K and E542K in the helical domain (exon 9) and H1047R in the kinase domain (exon 20). These mutations increase the enzymatic function, enhance downstream signaling elements including Akt and promote oncogenic transformation (30). The frequency of the PI3K mutations in breast cancer have been summarized in Fig. 1.3.
Several groups have shown that breast cancer cells can evade cell death by using several different resistance mechanisms, Akt activation being one of them (31). Akt may be considered the first and foremost molecular support that breast cancer cells uses to escape cell death on exposure to toxic stimuli. There is a constitutive activation of Akt in breast cancer cells suggesting that Akt can be targeted for directly decreasing tumor cell survival. Moreover, inhibiting the activation of Akt may eliminate the molecular crutch that tumor cells rely on to escape cell death. Targeting the Akt directly influences the decision to undergo apoptosis and increases the therapeutic effectiveness of standard agents (32). There are multiple signalling pathways responsible for regulating the PI3K/Akt pathway but we will be discussing the players relevant to our study in details.

1.7. Reactive oxygen species

Amongst the several players responsible for regulating Akt signaling, reactive oxygen species (ROS) seems to be an important upstream mediating molecules for the Akt/ASK1/p38 signaling pathway in apoptosis induction (33). ROS are the by-products of cellular

Figure 1.3. PI3K mutations in breast cancer.
metabolism formed upon incomplete reduction of oxygen and includes the superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and the hydroxyl radical (HO•). Under normal physiological conditions, ROS are involved in maintaining cell homeostasis (34) in regulating cell signaling (35) and protection against pathogens and bacteria (36). However excessive production of ROS can lead to occurrence of various kinds of disease processes like inflammation, ageing, fibrosis, neurological disorders and carcinogenesis (37, 38). Several studies have indicated that the presence of ROS leads to induction of apoptosis in different kinds of cancer cells (39-43). ROS levels in cancer cells can act as double-edged swords by sending survival signals at lower concentration and triggering cell death at higher concentrations as illustrated in Fig. 1.4 (44, 45). ROS levels are also known to regulate the alternate cell death pathway called autophagy in the cells (46).

![Figure 1.4. ROS, a double edged sword.](image-url)
The anticancer agents which are generally used to target the pathways mentioned above, eventually aim to cause cell death in cancer cells. Thus it is essential to study the mechanism of cell death pathways in the chemotherapy. We have tried to study the effect of two anticancer agents in inducing cell death by modulating certain cell signaling networks in this study.

1.8. Cell death pathways in cancer chemotherapy

Cell death and cell renewal are balanced throughout the life of a multicellular organism. The development of animal starts with the rapid cell proliferation of the embryonic cells which then further differentiate to various specialized types of cells that make up adult organs and tissues. This complex process of development not only involves cell proliferation and differentiation but also cell death. Although cells can die due to numerous unpredictable traumatic events like exposure to toxic chemicals but most of the cells of multi cellular organism die undergoing certain programmed cell death mechanisms. Programmed cell death in adults is responsible for regulating cell proliferation and maintaining constant cell numbers in tissues undergoing cell turnover. Apart from this programmed cell death also provides defense mechanism by which the damaged and potentially dangerous cells can be eliminated for the good of the whole organism (47). In contrast to the accidental cell death which occurs by necrosis, programmed cell death are of four types: apoptosis, autophagy, necroptosis and pyroptosis. These pathways may together decide the fate of cancer cells (48).

1.8.1. Apoptosis

Apoptosis is an active process involving a sequence of cellular changes. During apoptosis chromosomal DNA is fragmented due to cleavage in the nucleosome. The chromosome
condenses and the nucleus breaks down into small pieces. The cell then shrinks and breaks up into membrane enclosed fragments called apoptotic bodies. A vast array of signalling cascades and key signalling players regulate the process of apoptosis. The morphologic characteristics of apoptosis include cell membrane blebbing, cell shrinkage, chromatin condensation, and nucleosomal fragmentation. Cells which undergo apoptosis are normally recognized by macrophages, or neighboring cells that consume the cells fractionated carcasses. Apoptosis has been considered a major mechanism of chemotherapy-induced cell death, and thus pathways that regulate apoptosis are very important for many preclinical drug discovery investigations (47).

There are two known signalling pathways mediating apoptosis: the intrinsic and extrinsic pathways as illustrated in Fig. 1.5. The intrinsic pathway is initiated inside the mitochondria while the extrinsic pathway is mediated by cell surface death receptors. Caspases (cysteine aspartic acid specific proteases) are the central regulatory proteins in both the types of apoptotic pathways. These proteins are synthesized as inactive pro-caspases which are cleaved into active caspases in a sequential manner leading to the activation of ‘executioner’ caspases that are common to both signalling pathways. These executioner caspases then cleave a variety of proteins essential for cell survival such as cytoskeletal proteins and DNA repair proteins resulting in cell death (49).

1.8.1.1. Intrinsic pathway

The intrinsic or mitochondrial pathway is so called because it is initiated inside the cell, particularly, within the mitochondria. Cellular stress signals like DNA damage, hypoxia, a defective cell cycle and loss of cell survival factors leads to activation of the intrinsic pathway. The pathway of apoptosis is tightly regulated by a balance of pro-apoptotic and anti-apoptotic proteins. The anti-apoptotic members belong to the Bcl-2 (B cell lymphoma
2) and include Bcl-2 related gene A1 (Bcl-A1), Bcl-2, Bcl-2-related gene, long isoform (Bcl-XL), Bcl-w, and myeloid cell leukemia 1 (MCL-1). These proteins mainly maintain the integrity of the outer mitochondrial membrane (OMM). The intrinsic pathway, when activated by cellular stress signals, leads to upregulation of pro-apoptotic BH-3 only proteins such as BAD (Bcl-2 antagonist of the cell death), BID (BH3 interacting domain death agonist), BIM (Bcl-2 interacting mediator of the cell death), BMF (Bcl-2 modifying factor), PUMA (p53 upregulated modulator of apoptosis), and Noxa. These proteins in turn bind to the anti-apoptotic members of the family and inhibit their actions (50). One subgroup of BH-3 only proteins includes direct activators (BID and BIM) which are able to bind and inhibit anti-apoptotic proteins but also can activate the effector proteins, BAK and BAX (51). The other group called sensitizers includes BAD, Hrk (Harakiri), Noxa and PUMA (52). These proteins bind the hydrophobic groove of anti-apoptotic Bcl-2 proteins, therefore preventing any future interactions between anti-apoptotic and pro-apoptotic proteins (53). Once the pathway is activated, BAK and BAX homo-oligomerize and form pores in the OMM, leading to outer membrane permeabilization. This results in release of pro-apoptotic proteins like cytochrome-c and Smac/DIABLO (second mitochondria-derived activator of caspases/ direct inhibitors of apoptosis proteins-binding protein with low ph) into the cytosol. Cytochrome c forms a complex with APAF-1 and pro-caspase 9, whereas Smac/ DIABLO binds to inhibitors of apoptosis proteins (IAPs). These steps lead to activation of caspase 9, and subsequent activation of effector caspases 3 and the pro-apoptotic phenotype (54).

1.8.1.2. Extrinsic pathway

The extrinsic pathway is activated by members of two protein families, the tumor necrosis factor (TNF) family and the receptors for these ligands (TNFR) (55). Most TNF family
members bind receptors that activate signals involved in pro-inflammatory responses and do not signal cell death. The TNF ligands that can induce apoptosis are TNF-α, FasL (also known as CD95L), and TNF receptor apoptosis-inducing ligand (TRAIL; also known as Apo2L) (56, 57). After extracellular ligand binding, the cytoplasmic end of the TNFR recruits initiating caspases. TRAIL binding to its death inducing receptors acts in a manner similar to FasL, while TNF-mediated signaling is more complex (58). The ligand-bound Fas or TRAIL death receptors (DR4 and DR5) recruit the adapter protein Fas-associating death domain-containing protein (FADD) (59). Bound FADD recruits initiator caspase-8 and caspase-10, and this assembly of proteins (receptor, FADD, and caspases) is termed the death-inducing signaling complex (DISC) (60). The proximity of the initiator caspases-8/10 to each other within the DISC serves to facilitate their autocatalytic activation allowing them to activate ‘effector’ caspases 3, 6 and/ or 7 and converging into the intrinsic pathway of apoptosis (61, 62).
**1.8.2. Autophagy**

Autophagy is a self degradative process which is evolutionarily conserved from yeast to mammals (63). Autophagy is activated in response to nutrient starvation, differentiation, and developmental triggers. It is an adaptive process to cope up with the metabolic stresses and results in degradation of intracellular proteins and organelles (64, 65). During autophagy, portions of the cytoplasm are encapsulated in a double-membrane structure referred to as an autophagosome. Autophagosomes then fuse with lysosomes where the contents are delivered, resulting in their degradation by lysosomal hydrolases. Autophagy occurs at the basal levels in most of the tissues to maintain the normal turnover of the
cytoplasmic contents. While autophagy can promote cell adaptation and survival during stresses such as starvation, it can also cause cell death when excess. (63).

There are three defined types of autophagy: macro-autophagy, micro-autophagy, and chaperone-mediated autophagy. Macro-autophagy delivers cytoplasmic cargo to the lysosome through a double membrane-bound vesicle called autophagosome, which then fuses with the lysosome to form an autolysosome. In micro-autophagy, cytosolic components are directly taken up by the lysosome itself through invagination of the lysosomal membrane. Both macro- and micro-autophagy are able to engulf large structures through both selective and non-selective mechanisms (66). In chaperone-mediated autophagy (CMA), targeted proteins are translocated across the lysosomal membrane in a complex with chaperone proteins (such as Hsc-70) that are recognized by the lysosomal membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A), resulting in their unfolding and degradation (67).

There are five key stages in which autophagy is mediated at the molecular level: (a) phagophore formation or nucleation (b) Atg5–Atg12 conjugation, interaction with Atg16L and multi-merization at the phagophore (c) LC3 processing and insertion into the extending phagophore membrane (d) capture of random or selective targets for degradation and (e) fusion of the autophagosome with the lysosome, followed by proteolytic degradation by lysosomal proteases of engulfed molecules (66). The schematic representation of autophagy is illustrated in Fig. 1.6.

1.8.2.1. Phagophore formation

In mammalian cells, phagophore membranes appear to initiate primarily from the ER [11,12] in dynamic equilibrium with other cytosolic membrane structures, such as the trans-Golgi and late endosomes (68, 69). Phagophore can even derive membrane from the
nuclear envelope under restricted conditions (70). Autophagosomal membranes lack the transmembrane proteins, so one cannot rule out de novo membrane formation from cytosolic lipids in mammalian cells. Atg1 kinase in a complex with Atg13 and Atg17 is required for phagophore formation in yeast, by regulating the recruitment of the transmembrane protein Atg9. This acts by promoting lipid recruitment to the expanding phagophore (71). The energy sensing TOR kinase regulates this step by phosphorylating Atg13, preventing it from interacting with Atg1 (72) and rendering initiation of autophagy sensitive to growth factor and nutrient availability. Ulk-1, a mammalian homologue of Atg1 is critical for autophagy in maturing reticulocytes (73). But whether Ulk-1, or Ulk-2 (a second Atg1 homologue), functions analogously in promoting autophagy in mammalian systems remains elusive. Class III PI3-kinases, notably Vps34 (vesicular protein sorting 34) and its binding partner Atg6/Beclin-1, play an important role in phagophore formation and autophagy in mammalian systems. Vps34 is involved in various membrane-sorting processes in the cell but is selectively involved in autophagy when complexed to Beclin-1 and other regulatory proteins (74). Vps34 is unique amongst PI3-kinases in only using phosphatidylinositol (PI) as substrate to generate phosphatidyl inositol triphosphate (PI3P), which is essential for phagophore elongation and recruitment of other Atg proteins to the phagophore (75).

1.8.2.2. Atg5-Atg12 conjugation

There are two important ubiquitin-like systems in autophagy (69, 76) acting at the Atg5–Atg12 conjugation step and at the LC3 processing step. In the conjugation step, Atg7 acts like an E1 ubiquitin activating enzyme and activates Atg12 in an ATP-dependent manner. It does so by binding to its carboxy terminal glycine residue. Atg12 is then transferred to Atg10, an E2-like ubiquitin carrier protein that facilitates covalent linkage of Atg12 to
lysine 130 of Atg5. Conjugated Atg5–Atg12 complexes then pairs with Atg16L dimers to form a multimeric Atg5–Atg12–Atg16L complex that associates with the extending phagophore. The association of Atg5–Atg12–Atg16L complexes is thought to induce curvature into the growing phagophore through asymmetric recruitment of processed LC3B-II. Atg5–Atg12 conjugation is not dependent on activation of autophagy and once the autophagosome is formed, Atg5–Atg12–Atg16L dissociates from the membrane, making conjugated Atg5–Atg12 a relatively poor marker of autophagy (77).

1.8.2.3. LC3 processing

The second ubiquitin-like system involved in auto-phagosome formation is the processing of microtubule-associated protein light chain 3 (LC3B), which is encoded by the mammalian homologue of Atg8. LC3B is a full-length cytosolic protein expressed in most of the cells. Upon induction of autophagy, it is proteolytically cleaved by Atg4, a cysteine protease, to generate LC3B-I. The carboxyterminal glycine exposed by Atg4-dependent cleavage is then activated in an ATP-dependent manner by the E1-like Atg7. Activated LC3B-I is then transferred to Atg3, a E2-like carrier protein. After that phosphatidylethanolamine (PE) is conjugated to the carboxyl glycine to generate processed LC3B-II. Recruitment and integration of LC3B-II into the growing phagophore is dependent on Atg5–Atg12. LC3B-II is found on both the internal and external surfaces of the autophagosome, where it plays a role in both hemifusion of membranes and in selecting cargo for degradation. The synthesis and processing of LC3 is increased during autophagy, making it a key readout of levels of autophagy in cells (77).
1.8.3. Necroptosis

Necrosis was initially recognised as non-programmed cell death pathways. But recent research shows that its execution can be controlled by specific signal-transduction pathways and catabolic mechanism (78-80). This alternative form of necrotic programmed cell death is termed as necroptosis. It is induced by tumor necrosis factor (TNF) receptor signaling that involves activation of the receptor-interacting protein (RIP) family. Upon
inhibition of apoptotic pathway by the caspase inhibitor, activation of RIP1 and RIP3 kinase leads to mitochondrial instability and cell death (81). Phosphorylated RIP1 and RIP3 generate a molecular complex called the necrosome, which initiates necroptosis. ROS production under necroptosis has been shown to facilitate TNF-α-induced cell death by sustaining c-Jun N-terminal kinase activation (82). Necroptosis can also be executed via stimulation by apoptosis-inducible ligands such as TNFα, FasL, or TRAIL. Thus inhibition of caspases by z-FAD (caspase inhibitor) in many experimental models leads to the facilitation of the necroptotic pathway by the TNFR activation. PARP-1 and Akt were recently shown to be directly activated by RIP1 leading to necroptosis by reducing the ATP levels and activating the JNK pathway. PARP-1 overactivation consumes large amount of NAD+ which results in massive ATP depletion. PARP-1, thus acts as molecular switch between apoptosis and necroptosis by regulating the ATP levels in the cell (83). Notably, cytotoxic agents are shown to induce necrotic cell death in apoptosis-defective cancer cells (84), probably because necroptosis is principally induced when a cell cannot die via apoptotic pathways (48, 85). The schematic diagram of necroptosis is illustrated in Fig. 1.7.
1.8.4. Pyroptosis

Pyroptosis is a recently indentified form of programmed cell death which is stimulated by microbial infections, non-infectious stimuli like myocardial infarction and cancer. In contrast to apoptosis, pyroptosis is uniquely mediated by caspase-1 activity. It is triggered by the formation of a cytosolic complex termed the ‘inflammasome’, which results in highly inflammatory outcomes. Pyroptotic cells represent morphological characteristics, some of which are shared with apoptosis and necrosis. The function of activated caspase-1 is to cleave proteolytically the proforms of the proinflammatory cytokines, IL-1β and IL-
18, to their active forms (86). Pyroptosis has been intensively studied in the context of bacteria-infected macrophages (87). It can also be triggered in human cancer cells infected with recombinant herpes simplex virus 2 (HSV-2) (88). Pyroptotic cancer cells induced by microbial infection have been recently shown to facilitate phagocytosis by macrophages, through their phosphatidylserine exposure and ATP release (89).

We have studied the effect of an anticancer agent and its derivative in inducing apoptosis and autophagy and the possible interconnection between the two cell death pathways. Hence we have explained the cross talk between apoptosis and autophagy in details for better understanding the mode of action of our drug in this study.

1.9. Cross talk between apoptosis and autophagy

For a long time, different modes of cell death were studied as mutually exclusive cellular states. However, recent advances in research have suggested that apoptosis, necroptosis and autophagy are often regulated by similar pathways. They even engage common subcellular sites and organelles, and share initiator and effector molecules. The type of cell death which a cancer cell encounters depends upon the cellular context and death trigger. The two main cell death pathways: apoptosis and autophagy involve complex cross talk between their components and will be discussed in details in this thesis (83).

Autophagy and apoptosis are both well controlled biological processes that play essential roles in development, tissue homeostasis and disease. There are several death initiator, effector molecules, signaling pathways which play a important role in both apoptosis and autophagy (Fig. 1.8). These players functions as a switch that allow the cells to decide which route to take, depending on the specific situation. The cross talk between apoptosis and autophagy by these players has been illustrated in Fig. 1.8.
1.9.1. Beclin 1

Beclin 1, the mammalian ortholog of yeast Atg6, has a critical role in autophagosome formation. It is a component of a multiprotein class III phosphatidyl inositol-3 kinase (PI3K) complex, which also includes Vps34 and Vps15 (75, 90). Beclin-1 is a novel BH3-domain only protein that has been demonstrated to bind different Bcl-2 homolog (91). Beclin-1 is an important key regulator of both autophagic and apoptotic machinery (92). Bcl-2 and Bcl-xL binding to Beclin-1 inhibits autophagy by preventing the formation of Beclin-1 and the class III PI3K complex (93). The interaction is unidirectional and maintains autophagy at a physiological level required for cellular homeostasis. This interaction does not have any reciprocal effect on apoptosis. In fact, mutations in either the Beclin-1 BH3 only domain or the Bcl-2/Bcl-xL BH3 domain prevent the interaction of Beclin-1 and Bcl-2 (94, 95). As a result, Bcl-2 is unable to inhibit autophagy resulting altogether in the stimulation of autophagy (94). Studies have also shown that other BH3-only proteins such as BAD or BH3 mimetic compounds (e.g. ABT737) could competitively break the Bcl-2/Bcl-xL and Beclin-1 complex formation thus up-regulating autophagy (95). However, it was demonstrated that only ER-localized Bcl-2/ Bcl-xL inhibits autophagy induced by starvation or by BH3 mimetics and not mitochondrial Bcl-2. Thus, only Beclin-1-Bcl-2/ Bcl-xL complexes that are located in the ER could inhibit autophagy. These data suggested that there exist an independent regulation of autophagy and apoptosis by protein sub-localization (94). Beclin-1 also exerts an anti-apoptotic role in several conditions like stimulation by TRAIL, chemotherapy, irradiation or starvation (96).
1.9.2. Caspases and other proteases.

Proteases linked to the apoptotic pathway regulate the crosstalk between apoptosis and autophagy. They can inhibit autophagy through the cleavage of autophagy-related proteins. Interestingly, caspases are important players that co-ordinate the interplay between autophagy and apoptosis (97). Emerging evidences demonstrate that caspases can also influence the non-apoptotic signaling events like autophagy (98). As discussed, caspases can regulate Beclin-1 by cell-type-specific cleavage. A C-terminal fragment of the Beclin-1, translocates to the mitochondria and induces cytochrome c release mediates this pro-apoptotic effect of Beclin-1. Moreover Beclin-1 cleavage inhibits its interaction with Bcl-2. Indeed, hetero-dimerization between Bcl-2 and a Beclin-1 isoform with a cleaved N-terminal region is completely lost, preventing Bcl-2 to inhibit autophagy (99). Also, caspase-8 can trigger ligand-induced cell death involving Atg3 cleavage. Mutation of the caspase-8 cleavage site on Atg3 abolishes Atg3 cleavage suggesting that Atg3 is a direct target of caspase-8. Hence, the pro-autophagic activity of Atg3 is abolished on its cleavage. This cleavage then inactivates autophagy during death receptor-induced apoptosis (100). Atg4D, a member of the Atg4 family that contributes to starvation-induced autophagy, is also a target of caspase-3 cleavage. This leads to the the delipidation of the LC3 (101).

Apart from caspases, other proteases like cysteine proteases called calpains also play a role in the interplay between different cell death pathways. Calpains cleave key autophagy proteins. Calpain-1 and 2 mediate cleavage of Atg5 in human neutrophils. The N-terminal truncated form translocates to the mitochondria and binds Bcl-xL to induce cytochrome c release and apoptosis without effect on autophagy induction (102).

The elevated concentration of lysosomal cathepsin proteases in autophagolysosomes makes them potentially harmful and causes cell death. The principal role of lysosomal
proteases is to maintain cellular homeostasis by recycling cellular content. However, lysosomal cathepsins, such as cathepsin B, cathepsin D and cathepsin L are implicated in cell death affecting mitochondria and/or caspases (103).

On the other hand, the cytoprotective autophagy counter-balances apoptosis by continuous sequestration of active caspase-8 into autophagosomes for its subsequent degradation in Bax-/- Hct116 colon carcinoma cells (104). However, autophagosomal membrane also serves as a platform for intracellular death-inducing signaling complex (DISC)-mediated caspase-8 activation and apoptosis (105). Therefore, studying these unexpected functions of caspases, and their possible link in the autophagic and apoptotic signaling pathways will help decipher the molecular basis underlying autophagy-apoptosis crosstalk. Manipulation these two important processes will surely help in therapeutic development.

1.9.3. TOR kinase pathways

mTOR is a serine/threonine kinase which signals to downstream effectors and regulates several anabolic and catabolic process. It promotes cell growth, protein synthesis and inhibits autophagy (106). Low amounts of nutrients or energy limitation and stress induce mTOR inactivation leading to autophagy induction. mTOR activity is regulated by numerous kinases such as AMP-activated kinase (AMPK), phosphatidylinositol-3-kinases (PI3Ks) or mitogen-activated protein kinases (MAPKs). mTOR is a downstream mediator in the PI3K/Akt signaling pathway, which plays a critical role in regulating the cellular metabolism. The PI3K/Akt pathway is deregulated in cancer cells and the Akt hyper-activation phosphorylates and activates mTOR with the consequent inhibition of autophagy (106, 107). mTOR has been demonstrated to have a pleiotropic effect on apoptosis depending on the cellular context by affecting several targets such as Bcl-2 protein family members. A recent study showed that mTOR inhibition after nutrient
deprivation causes degradation of the anti-apoptotic Mcl-1 that play a role in the induction of autophagy or apoptosis (108).

In this thesis we have tried to explore the role of an anticancer agent Piroxicam (Px), a traditional painkiller and anti-inflammatory agent, which modulates the ROS/Akt pathway to cause cell death by apoptosis. The other anticancer agent Resveratrol (Res) however induces both apoptosis and autophagy and there is causes complex cross talk with apoptosis and autophagy. The cross talk however differs between the Res and its analog C1. Hence it is essential to understand the mechanism of modulation of the programmed cell death pathways by anticancer agents in general.

Figure. 1.8. Cross talk between apoptosis and autophagy through mTor, Beclin-1 and caspases.

![Diagram showing cross talk between apoptosis and autophagy through mTor, Beclin-1 and caspases.](image-url)
1.10. Anticancer agents modulating programmed cell death pathways.

Fundamental knowledge in programmed cell death has generated tremendous information and insights on the development of cancer targets. Both apoptosis and autophagy signaling pathways have been frequently found to be impaired in many human cancers. Thus modulating apoptosis and autophagy might be an important strategy to fight against the cancer. Cancers which are resistant to the apoptotic effects may be sensitive to drugs which induces autophagic cell death and vice versa. Thus designing the therapeutic strategies based on the autophagy activities of the tumors may be another good option. This is in view that autophagy may have different effects in different stages of cancer progression. Studies have suggested that tumors which have normal autophagy response and activities can be sensitive to the combination of standard chemotherapy drugs and autophagy inhibitors. This combinational approach may lead to re-activation of apoptosis (109). In case of highly resistant tumors, utilizing both autophagy and apoptosis inducers may prove effective. As discussed earlier, both apoptosis and autophagic cell death pathways may be interlinked to each other. Studies have demonstrated that certain compounds have to ability to trigger both apoptosis and autophagy cell deaths simultaneously in cancer cells (110, 111). Studies have also showed that blocking one of the pathways will trigger the activation of another. Researchers have shown that there are several factors that may activate preferential biochemical cascades that will ultimately result in either apoptosis or autophagic deaths (112). Thus understanding the complex relationships between these two cell death mechanisms may bring about a new paradigm towards the fight against cancer.

Our study aims to explore the role of two different anticancer agents in inducing different cell death pathways in breast cancer cells. The two anticancer agents which we have used
in our study are: Piroxicam, a traditional NSAID and Resveratrol, a natural product and its synthetic analog.

1.11. Piroxicam

Non-steroidal anti-inflammatory drugs (NSAIDs) are traditionally used as anti-inflammatory and analgesic agents (113). NSAIDs are commonly known to be inhibitors of the Cyclooxygenase/ prostaglandin-endoperoxide synthase (COX/PTGS) enzymes, which are involved in arachidonic acid metabolism and the production of eicosanoids. There are two types of the COX enzyme (COX1/PTGS1 and COX2/PTGS2) and NSAIDs belonging to the oxicam group inhibit both COX1 and COX2. COX 1 is expressed constitutively and is required for many normal physiological processes. However, COX 2 is inducible in cells including endothelial cells, macrophages, and intestinal epithelial cells (114). Piroxicam [4-hydroxy-2-methyl-N-(pyridin-2-yl)-2H-1,2-benzothiazine-3-carboxamide1,1 dioxide] is a drug belonging to the oxicam group of Non-steroidal anti-inflammatory drug (NSAIDs) (115). Findings suggest that NSAIDs also show their antitumor and anticarcinogenic effect in different cell lines by COX independent mechanisms (116, 117). Piroxicam (Px) apart from being a good anti-inflammatory agent also has chemopreventive and chemosuppressive effects as observed in different canine cancer cell lines and animal models (118-120). Px has been shown to induce apoptosis in human malignant mesothelioma cell line in combination with cisplatin and also alone in head and neck cancer cell lines (121, 122). Px have shown its anti-proliferative effect (123) in feline, canine as well as human oral squamous cell carcinoma cells (OSCC) in combination with Masitinib, AB1010 (a novel receptor tyrosine kinase inhibitor). Simultaneous application of Px and c-phycocyanin was effective as chemopreventive in 1,2-dimethylhydrazine (DMH) induced colon cancer through inhibition of the PI3-K/Akt
pathway (124). On the other hand, the oxicam group of NSAIDs like Meloxicam, Tenoxicam and Piroxicam have been reported to act as potent disease modifying drugs which can halt progressive dopaminergic neurodegeneration in Parkinson’s disease. The mechanism by which they exert the neuroprotection is by activation of PI3K/ Akt kinase pathway (125, 126). NSAIDs like, celecoxib have also shown to induce the alternative cell death pathway, autophagy in human glioblastoma cells (127) and colon cancer cells (128). Preliminary data on the cytotoxic effects of Px on human breast cancer cell lines are available but thorough explorations of the upstream mechanisms have not been done (129). Thus studying the new functions associated with these conventional drugs will help in better understanding of the signalling cascades activated in breast cancer. We have tried to establish the role of Px as effective anticancer agent in some some specific type of breast cancer cells in the first part of the thesis.

1.12. Resveratrol

Resveratrol, trans-3,5,4’-trihydroxystilbene, was first isolated in 1940 as a constituent of the roots of white hellebore (Veratrum grandiflorum) (130), but has since been found in various plants, including grapes, berries and peanuts. The anticancer potential of resveratrol was identified many years later. Resveratrol was found to act as an antioxidant and antimitugen and to induce phase II drug-metabolizing enzymes (anti-initiation activity). Resveratrol also exhibited anti-inflammatory effects and inhibited cyclooxygenase and hydroperoxidase functions (antipromotion activity). Resveratrol induced human promyelocytic leukemia cell differentiation (antiproggression activity) and demonstrated chemotherapeutic effects against various cancers including lymphoid and myeloid cancers; multiple myeloma; cancers of the breast, prostate, stomach, colon, pancreas, and thyroid; melanoma; head and neck squamous cell carcinoma; ovarian
carcinoma; and cervical carcinoma (131). Since then the scientific community became really interested in resveratrol and reports on the effects and properties of this compound started accumulating exponentially.

Extensive in vitro studies revealed multiple intracellular targets of resveratrol, which affect cell growth, inflammation, apoptosis, angiogenesis, and invasion and metastasis. These include tumor suppressors p53 and Rb; cell cycle regulators, cyclins, CDKs, p21WAF1, p27KIP and INK and the checkpoint kinases ATM/ATR; transcription factors NF-κB, AP-1, cJun, and c-Fos; angiogenic and metastatic factors, VEGF and matrix metalloprotease 2/9; cyclooxygenases for inflammation; and apoptotic and survival regulators, Bax, Bak, PUMA, Noxa, TRAIL, APAF, survivin, Akt, Bcl2 and Bcl-XL. In addition to its well-documented anti-oxidant properties, there is increasing evidence that resveratrol exhibits pro-oxidant activity under certain experimental conditions, causing oxidative DNA damage that may lead to cell cycle arrest or apoptosis (132).

Resveratrol can also activate alternative cell death pathways like autophagy. In some cancer cells, the autophagy caused by resveratrol helped in inducing apoptosis (133). However simultaneous induction of apoptosis and autophagy by resveratrol has also been reported (134). Autophagy inhibition have shown to increase the cytotoxicity of resveratrol in human melanoma and rat tumour cells (135, 136). Thus cell death pathways induced by resveratrol and their interconnection depends upon the cellular state and the stress trigger.

1.13. Resveratrol analogs: the need of the hour

Resveratrol has been proposed as a potential drug for cancer chemoprevention, treatment and cardioprotection. But it has suffered from recurrent criticism by the physicians and pharmacologists due to several drawbacks. Low bioavailability, poor absorption and
rapid metabolism of resveratrol in mammals have impeded the clinical application of resveratrol (Fig. 1.9). But one cannot overlook the enormous biological activities exhibited by resveratrol (137). To enhance the bioavailability in humans, different approaches are being carried out, keeping the beneficial role of resveratrol intact. Combination with agents (CYP inhibitors like piperine) that can inhibit in vivo metabolism of resveratrol, nano-particle mediated delivery and development of natural or synthetic analogs of resveratrol are the need of the hour (138).

Bakuchiol, an analog of resveratrol have shown to cause ROS dependent apoptosis human lung adenocarcinoma cell line (139). The analog of resveratrol, pterostilbene has been proposed to have properties like anticancer, anti-inflammation, antioxidant, apoptosis, antiproliferation and analgesic potential. Many studies have confirmed that pterostilbene could inhibit tumor growth both in vitro and in vivo. Notably, following equimolar oral dosing in rats, plasma levels of pterostilbene and pterostilbene sulfate were markedly greater than plasma levels of resveratrol and resveratrol sulfate. The greater bioavailability of pterostilbene indicated that pterostilbene could be potentially developed for clinical applications. Pterostilbene sulfate were markedly greater than plasma levels of resveratrol and resveratrol sulfate (140). The greater bioavailability of pterostilbene indicated that pterostilbene could be potentially developed for clinical applications. Morover, pterostilbene have also shown to induce autophagy along with cell cycle arrest and apoptosis in breast cancer cells (141). Studies showed that combination with autophagy inhibitors sensitzes lung cancer cells to pterostilbene induced apoptosis and cell death (142). Trans-3,4-dimethylstilbene (3,4-DMS), a methylated derivative of resveratrol, showed its potential as a novel angiogenic agent by inducing apoptosis in endothelial cells along with autophagy induction (143). Hence several connections between the apoptosis and autophagy as induced by resveratrol its analogs exist. We have tried to establish the
preliminary indication of the apoptosis and autophagy cross talk by the Res and its analog in the second part of this thesis.

Figure. 1.9. Resveratrol: from bench to bedside.
1.14. Objective of the thesis

Breast cancer is a heterogeneous disease. Apart from the molecular classification, two breast cancer cell lines differ from each other in metastatic potential, invasive behaviour, status of tumor suppressor like p53 and sensitivity to drugs. This makes the possible therapy for breast cancer difficult. So different breast cancer cell lines were included in our study. The thesis aims to study the effect of two completely different anti–cancer agents on different breast cancer cells.

Piroxicam is a traditional NSAID which has shown its cytotoxic effect in breast cancer. But the detailed molecular mechanism has not been studied so far. Identifying the molecular mechanism by which Px exerts its action on breast cancer cell lines will allow the use of this drug either in isolation or as an adjunct therapy against breast cancer. As has been pointed out, Akt pathway acts as ‘survival crutch’ by the cancer cells and hence it can serve as a good target for chemotherapy. Moreover many NSAIDs like Celecoxib are known to exert their anticancer effect by affecting the Akt pathway. We have therefore studied the effect of Px in inducing cell death by the Akt pathway in MCF-7 cell line and have also extended the study to other breast cancer cell lines as well. The first part of the thesis aims to identify the novel signalling pathway responsible for Piroxicam mediated apoptosis induction in breast cancer.

Res have an established role in cancer chemoprevention and chemotherapy. However it suffers from several limitations of poor bioavailability and low metabolism which impedes its success at the clinical trials. Thus exploring the novel synthesized analogs of Res will allow to exploit its potential benefits in cancer studies. We have therefore tried to compare the potential role of Res and its analog C1 in inducing apoptosis and autophagy in MCF-7 and MDA-MB-468 breast cancer cell lines.
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