

1. Chapter 1

Introduction

During the past half century funding was targeted for cancer research by various funding agencies throughout the world. A major victory in the ongoing war against this frequently fatal disease however does not appear imminent. Cancer therapy has not managed to decrease cancer mortality in the last three decades(3-5). This suggests that we need new strategies to control a disease that kills over 6 million people worldwide every year(5). In the United States cancers of the lung and bronchus (30%), prostate (9%) and colon and rectum (9%) account for approximately 48% of cancer-related deaths in men and lung and bronchus (26%), breast (15%) and colon and rectum (9%) cancers account for approximately 50% of cancer-related mortality in women(5).

There are at least three ways to approach the treatment of cancer. The first way, the traditional approach, is to attempt to selectively kill the cancer cells either surgically, chemotherapeutically or via radiotherapy or a combination of the above (6). A second approach is to attempt to reprogram cancerous cells back into normal cells via epigenetic therapy and a third approach is to use treatment regimens that do not eliminate the cancer but keep it under control with low doses of anticancer drugs, for example metronomic therapy (7, 8).

A major challenge for the traditional approach of cancer chemotherapy is not to find agents that kill cancer cells, but to find agents that kill cancer cells while leaving normal cells intact. An understanding of what exactly cancer is, how it emerges and what the various causes of cancer are, is important before attempting to find new ways of treating or managing the disease.

What is Cancer?

Various textbooks define cancer differently. In the textbook “Principles of Cancer Genetics (page 2)” by Fred Bunz, cancer is described as follows (9):

“The term ‘cancer’ simply defines those tumors which have acquired the ability to invade surrounding tissues composed of normal cells. The distinction between benign and malignant tumors is solely based on this invasive capacity.”

Almeida *et al.* (2010) in their text book “Cancer: Basic Science and Clinical Aspects (page 1)” describes cancer as an abnormal growth (10):

“In the most basic sense, cancer is the abnormal, uncontrolled growth of previously normal cells. The primary characteristic of cancer cells is their ability to rapidly divide, and the resulting accumulation of cancer cells is termed a tumor.”

In “Cancer Biology (page 2)” by Raymond W. Ruddon, cancer is defined as an abnormal growth that causes significant morbidity(11):

“Cancer is an abnormal growth of cells caused by multiple change in gene expression leading to dysregulated balance of cell proliferation and cell death and ultimately evolving into a population of cells that can evade tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host.”

An ideal definition of cancer will contain the essential characteristics, the properties, of cancer. In other words, if we are asked what the essential properties of cancer are, the reply is that it possesses a property or hallmark that distinguishes it from something that is not cancer. Invasiveness, abnormal growth and malignancy are described as the essential properties of cancer in the above three definitions.

In the year 2000 Douglas Hanahan and Robert A. Weinberg identified 6 hallmarks of cancer (12). In 2011 they included four more hallmarks(13). The ten hallmarks include eight acquired features and two enabling features. The eight acquired features are self-sufficiency in growth signals, insensitivity to anti-growth signals, evading cell death, limitless replicative potential,

sustained angiogenesis, evasion of the immune system, deregulated metabolism and tissue invasion and metastasis. Unstable genomes and tumour-promoting inflammation are the two enabling features (13).

Some of these hallmarks have been questioned by Yuri Lazebnik(14). Yuri Lazebnik argues that a hallmark is “usually defined as a feature of something that distinguishes it from others”(14). A hallmark on this view is synonymous with the predicable “property”. Lazebnik proceeds to argue that self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of cell death, limitless replicative potential and sustained angiogenesis are not features that strictly satisfy the criteria for being hallmarks of cancer.

For example, somatic homeostatic cells require exogenous growth signals that are not released and transmitted by themselves before they can change from a non-proliferative state to a proliferative state(13). Cancer cells on the other hand, are known to acquire the capability to produce their own growth factors, as well as send signals to somatic cells to stimulate growth factor production(13). In this way, cancer cells are able to control their own growth and become autonomous. This is also a feature of benign tumours where autonomous growth factor signaling plays an important role in tumor development (15). Therefore, while autonomous growth factor signaling is a feature of cancer cells, it is not a feature that distinguishes it from benign hyperplasia and thus not be a hallmark or property of cancer.

Yuri Lazebnik has also questioned insensitivity to anti-growth signals as a hallmark(14). In normal tissue there are cellular signals that prevent cells from dividing in order to maintain homeostasis. Various tumor suppressor genes encode for proteins that inhibit cell proliferation. Loss of function of these genes renders cells insensitive to various anti-growth signals. Proteins that regulate or inhibit progression through specific stages of the cell cycle such as retinoblastoma (pRB) and p16 cyclin-kinase inhibitor may be activated in response to various anti-growth signals (16). Checkpoint control proteins such as tumour protein 53 (TP53) are able to block cell cycle progression in response to damaged deoxyribonucleic acid (DNA)(16). Cancer cells are capable of bypassing these anti-growth signals and continue to grow even in the presence of these anti-growth signals due to various aberrations of various tumor suppressors

(13). Tumor suppressor deregulation is also a feature of many benign tumors. For example, the tumor suppressor phosphate and tensin homologue deleted on chromosome ten (*PTEN*) is deregulated in benign hamartomas and thereby rendering the tumour cells incapable of responding to anti-growth signals (17, 18). Deregulation of the pRB protein is associated with the development of a benign tumor called retinoma(14). It is thus argued that insensitivity to anti-growth signals is merely a feature of cancer and not something to distinguish it from benign hyperplasia.

The ability of somatic cells to undergo controlled forms of cell death including autophagy and apoptosis plays a crucial role in maintaining homeostasis(13). Cancer cells are capable of evading these controlled forms of cell death and thus enable them to survive even where pro-cell death signalling events are present. This is also true for benign tumours of the thyroid and Schwann cells as well as benign atypical ductal hyperplasia in mammary cells and many other benign tumours (19-22). Evasion of cell death is thus not a hallmark of cancer, just a feature. Limitless replicative potential can be discarded as a hallmark of cancer since both benign and malignant tumours are capable of this(14).

Both benign tumours as well as malignant cancers are capable of increased angiogenesis that help sustain resource supplies such as nutrients and oxygen (23). The presence of various cytokines associated with benign as well as malignant tumours has been demonstrated (13, 24, 25). These include interleukin 6 and 10 (IL-6, IL-10), and transforming growth factor β 1 (TGF- β 1). These cytokines are capable of suppressing cancer-specific immune responses and thus enable tumour cells, benign and malignant, to evade detection by the immune system. Inflammation is a feature of malignant and non-malignant tumours since virtually every benign and malignant tumour contains immune cells that elicits an immune response (13). Such inflammation-causing responses are able to promote tumour formation and progression (13). Unstable genomes as a result of genome maintenance and repair defects have been documented in benign and malignant tumour cells(26, 27). Therefore increased angiogenesis, evasion of immune responses, tumour-promoting inflammation and unstable genomes can be characterised as features of cancer but not necessarily hallmarks.

Cancer cells undergo various modifications in their metabolism in relation to their glycolytic rate, fatty acid production and utilization of glutamine. Under conditions of normal physiological concentrations of oxygen, many types of cancer cells appear to switch from glucose metabolism by oxidative phosphorylation to mainly glycolysis. This is known as the Warburg effect (28). Another near universal feature of cancer is an increase in glucose uptake and this can be observed with [¹⁸F] fluoro-2-deoxyglucose (FDG) positron emission tomography (PET) (28). Various probes including F-choline, C-choline as well as various synthetic amino acids analogs have been successfully employed to differentiate between cancerous tissue and other benign tissue (29). Therefore an argument can be made that metabolic modifications such the Warburg effect, increased glycolysis and choline uptake may be features that distinguish cancer cells from other somatic and benign cells.

However, embryonic tissues are known to have Warburg-like metabolism (13). In addition, low glucose metabolism levels comparable to normal tissue is found in most prostate cancers, renal cell carcinomas and bronchoalveolar cell carcinoma in the lung (29). It also appears that metabolic alterations are heterogeneous and that there is no specific metabolic alteration that is universal in all cancers (30). It thus follows that metabolic alterations is a feature but not a hallmark of cancer. Of the original hallmarks of Hanahan and Weinberg as proposed in 2011, only tissue invasion and metastasis is left. Yuri Lazebnik argues that tissue invasion and metastasis are hallmarks of cancer. However, he cites an example of a reported benign tumour that is able to metastasise and remain benign (31). This is not an isolated case, there are other examples of benign metastases. Pleomorphic adenomas are neoplastic growths of the salivary glands and occasionally metastasize keeping their benign features of the primary site (32).

The Oxford English Dictionary defines it as the “transference of bodily function, disease, from one part or organ to another; transformation of chemical compounds into others in process of assimilation by an organism” (33). The Greek and Latin origins of the word translate to literally mean, change from one position to another (33). From this, it follows that not only neoplasms associated with tumours can metastasize, but other somatic cells associated with homeostatic bodily function are also able to metastasize. An example of a normal homeostatic bodily function that qualifies as metastasis is the process of haematopoiesis. Haematopoietic stem cells

reside in the bone marrow and are able to differentiate or change into various blood cells. Another example is nevogenesis. Nevogenesis is the process whereby nevus cells, a variant of melanocytes, undergo transformation that results in the migration of these cells to different systemic location (31, 34, 35). The process appears to be benign (31).

Neoplastic growths are also not the only cells that are able to be involved in tissue invasion. An example of invasion that is part of normal homeostatic function is lymphocytes that are able to invade infected tissue as part of a normal immune response. It would thus appear that tissue invasion and metastasis are not hallmarks of cancer. The definition of cancer as described by Fred Bunz in Principles of Cancer Genetics (page 2) is incomplete(9). Tissue invasion and metastasis are not features that distinguish cancer from benign hyperplasia or even other normal homeostatic activities. It is merely a common feature normally associated with various cancers.

The traditional hallmarks proposed by Hanahan and Weinberg do not appear to be features of cancer that distinguishes it from non-malignant, benign tumours as well as somatic cells or tissue. Thus, these hallmarks do not satisfy the criteria for being an actual hallmark or property of cancer. In other words, none of these so-called hallmarks captures the essence of cancer in a manner that separates it from non-malignant, benign tumours as well as somatic cells or tissue.

It may be argued that a property of cancer is related to a certain subset of genes that is causally related to the emergence of cancer phenotypes. If this view is correct then it follows that one may find a subset of genes to be modified and/or present and/or active in all cancers in a manner that is different from non-cancerous cells. This subset of genes would then constitute as hallmarks or properties of cancer. The problem with this gene-centric view is that empirical data suggests that the vast majority of gene mutations are not shared among cancer patients (33). The same is true on an epigenetic as well as on a genome or karyotype level (33). Genetic, epigenetic as well as genome heterogeneity among various cancers is the rule rather than the exception(33).

Peter Duesberg and his co-workers at the department of Molecular and Cell Biology at the University of California have proposed that cancer is a new species and the process of carcinogenesis is an evolutionary process and constitutes an example of speciation(36). Duesberg

et al. (2011) provide five common characteristics of cancer and argues why a speciation theory of cancer can explain these characteristics. Briefly, Duesberg *et al.* (2011) cite autonomy, karyotypic and phenotypic individuality, karyotypic and phenotypic flexibility within stable margins, immortality and long latent periods from pre-cancer initiation to cancer (36).

Duesberg *et al.* (2011) argue that the discovery that some cancers become infectious and are able to be passed on from animal to animal like bacterial and viral infections suggest that carcinogenesis is a form of speciation. The “canine venereal tumor” and the facial cancer of the Tasmanian devil are examples of cancers that can be transmitted to other animals just like bacteria and parasites (37-39). Part of the motivation for this model is that cancers exhibit karyotypic variations due to aneuploidy. They argue that speciation events occur due to change in karyotype. In other words, if the karyotype of a cell changes it would constitute an example of speciation. It follows then that the specific karyotype is a hallmark of a particular cancer and a form of speciation. One problem with the argument for karyotype change as an example of speciation is that various neuronal cells in the human nervous system exhibit various degrees of aneuploidy (and thus karyotype individuality), yet they play crucial functional roles in the nervous system (40-42). If the karyotypic view of speciation is consistent then these neuronal cells would also constitute an example of speciation.

There are other good arguments for the view that cancer is a form of speciation, however, the problem with the idea of carcinogenesis as a form of speciation is the concept of species itself. In philosophy of biology there is, what is commonly referred to as, the “Species Problem”. There are at least 24 different species concepts and none of them can be applied to all organisms that have ever lived (43). If there is no proper concept for what exactly a species is, then carcinogenesis as a form of speciation is unintelligible.

Malignancy together with the source of cancer appears to be the only standing hallmarks of cancer. The traditional hallmarks are therefore mere common features often associated with cancer. For the purpose of this study, cancer is defined as follows:

“In humans, cancer is defined as a disease that arises from somatic cells by a process of evolution whereby fitness changes over generations result in sub populations of malignant cells that has the potential to cause death. Common features associated with cancer include unstable genomes, tumour-promoting inflammation, self-sufficiency in growth signals, insensitivity to anti-growth signals, evading cell death, limitless replicative potential, sustained angiogenesis, evasion of the immune system, deregulated metabolism and tissue invasion and metastasis.”

The present study aims to generate new antimitotic anticancer compounds capable of selectively killing cancer cells and prevent tissue invasion and metastasis.

Cancer Evolution

Cell growth in normal tissue is tightly controlled by pro- and anti-growth cell signaling. During the life of somatic cells, cellular changes may occur whereby crucial regulatory mechanisms are altered. These changes may result in several alterations that result in features associated with various cancers. These features include increased aerobic glycolysis (Warburg effect), sustained proliferative signaling, evasion of growth suppressing signals, resisting cell death, replicative immortality, increased angiogenesis induction, invasion and metastasis(13). Various evolutionary models have been proposed to describe the emergence of these traits. Vineis *et al.* (2010) discusses five different models based on mutations, genome instability, non-genotoxic mechanisms, cell selection and tissue organization respectively; and they summarize that the main feature of all these models are either cellular changes or changes in the surrounding stroma or extracellular matrix of cells (44). All of the models incorporate basic evolutionary principles whereby differences in trait fitness due to cellular and/or stromal/environmental changes contribute to the propagation of these traits (45) (Figure 1.1).

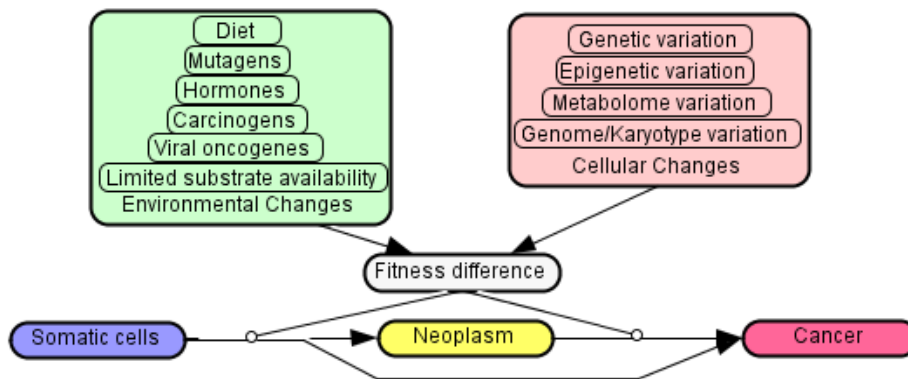


Figure 1.1: Basic model for cancer evolution. Environmental and cellular changes result in fitness differences in somatic cells. These fitness differences contribute to the propagation traits associated with cancer e.g. in features associated with various cancers. These features include increased aerobic glycolysis (Warburg effect), sustained proliferative signaling, evasion of growth suppressing signals, resisting cell death, replicative immortality, increased angiogenesis induction, invasion and metastasis.

The difference between the models is the emphasis on the role the type of change (cellular or environmental) plays in contributing towards the fitness differences during cancer evolution, for example, models that emphasize on environmental changes as the driving force. On this view, the introduction of carcinogens and mutagens to somatic cells is the cause of various genetic changes that result in different fitness traits being propagated differentially and ultimately result in the formation of cancer cells. Examples of environmental changes that are the main cause of carcinogenesis are tobacco smoke, radiation, dietary carcinogens and mutagens as well as viral oncogenes (44, 46-50).

Examples of models that focus on cellular changes as the main cause of carcinogenesis are the models based on heritable changes, genetic or epigenetic, in various oncogenes that in turn cause different fitness being propagated in a manner that results in cancer (44). Mutations that affect the retinoblastoma (pRb), breast cancer 1 early onset (BRCA1), adenomatous polyposis coli (APC) and DNA mismatch repair genes are examples of heritable cellular changes that predisposes a person to cancer (51).

The three models incorporate both cellular and environmental changes in the somatic evolution of malignancy. The gene-centered model of Fearon and Vogelstein suggests that the

accumulation of successive mutations due to environmental changes contribute to the fitness differences and adaptive advantage of neoplastic, anaplastic and cancer cells(52). The bio-energetic models proposed by Gillies and Gatenby in turn argue that the interstitial microenvironment as well as cellular and bio-energetic alterations result in fitness differences that lead to the emergence of cancerous cells (53-55). The genome/karyotype-centered view of Duesberg *et al.* (2006) and Heng *et al.* (2010) suggests that genome/karyotype heterogeneity as a result of aneuploidy result in fitness differences that play a causal role in the emergence of cancer (33, 45, 56).

Gene-centered Model

The genetic-centered model focuses on stepwise cellular changes associated with genetic and epigenetic alterations as the main drivers that result in fitness differences in populations of cells which ultimately result in the emergence of benign and malignant neoplastic growths (57). For example, somatic mutations may result in alterations of crucial regulatory mechanisms associated with cell proliferation and cell death signaling which in turn result in interstitial neoplasms. One of the first genes that was described as a human cancer gene, was the Harvey rat sarcoma virus (H-RAS) gene (58). It was discovered that mutations in this gene results in altered cell signal transduction cascades, leading to cell proliferation and differentiation associated with neoplastic growths (59). Other genetic alterations that result in increased neoplastic growth include Rb1, APC, PTEN as well as mitochondrial mutations that result in mytogenic reactive oxygen species production (60-62).

Later, genetic alterations that result in insensitivity to anti-growth signals were discovered. These include the over expression of the myelocytomatosis viral oncogene (*c-myc*), phosphatidylinositol 3-kinases (PI3K), protein kinase B (Akt/PKB) and BRCA1 (60). Because of insensitivity to anti-growth signals, neoplastic cells undergo regular cell cycling. Non-cancerous cells have a lack of telomerase, resulting in the shortening of the telomeric repeat, which results in a limited replicative life span and senescence (63). Cellular changes associated with

telomerase expression allow cells to overcome the telomere-shortening “crisis” through the constitutive reactivation of telomerase and in turn become immortalized (63, 64).

Genetic alterations resulting in resistance to cell death signaling include p53, mammalian target of rapamycin (mTOR), anti-apoptotic Bcl-2 genes and extracellular matrix-degrading proteases (13, 60). Gene products that contribute towards autonomous growth signaling include human epidermal growth factor receptor 2 (HER2) and break point cluster region- c-Abelson(BCR-ABL) genes (13). Cellular changes that increase angiogenesis include hypoxia-inducible factor 1 alpha (HIF-1 α) and vascular endothelial growth factor (VEGF) (13). Alterations in gene products such as transforming growth factor beta(TGF- β) contribute towards metastasis and invasion by activating the epithelial-mesenchymal transition (EMT) program (13). Cellular changes associated with immortalization, resistance to cell death signaling, angiogenesis, autonomous growth factor signaling, invasion and metastasis are then postulated to contribute towards fitness differences in cells that ultimately lead to the development of cancer (Figure 1.2).

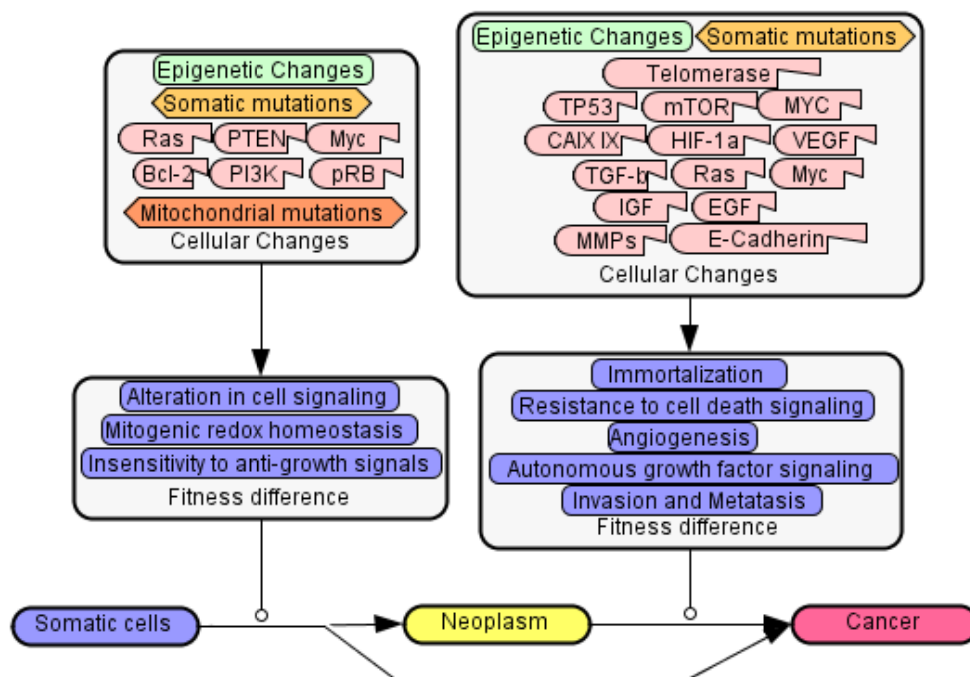


Figure 1.2: The gene-centered model: Cellular alterations as the main drivers of carcinogenesis. Cellular changes associated with epigenetic change, somatic mutations and mitochondrial mutations are the main drivers of carcinogenesis according to the gene-centered model.

One of the predictions of the gene-centric view is that a certain subset of genes is causally related to the emergence of cancer phenotypes. In other words, we should find a certain subset of genes, cancer genes, that are affected (either mutated, or over- or underexpressed, or epigenetically modified etc.) and the way these genes are affected should tell us why a certain cancer has certain characteristics(60). The problem with this gene-centric view is that empirical data suggests that the vast majority of gene mutations are not shared among cancer patients(33). Genetic heterogeneity among various cancers is the rule rather than the exception. This suggests that cellular changes associated with genetic and epigenetic changes are not the only causally relevant factors in cancer evolution.

Bioenergetic Model

The bioenergetic model proposes that initial cellular changes (genetic and epigenetic) are the main drivers that result in fitness differences in populations of cells that ultimately result in the emergence of benign neoplastic growths. Tumour microenvironmental changes such as increased hypoxia, limited substrate availability and an acidotic environment, however, are the main drivers of carcinogenesis that result in fitness differences in populations of cells that ultimately result in bioenergetic changes associated with malignant neoplastic growths.

The motivation for this model is the observation of the Warburg effect and increased glycolysis in cancer cells (53). According to the model, stromal changes in substrate availability, pH and reactive oxygen species formation are the main drivers in somatic evolution of malignancy. After the initial formation of neoplastic growths and immortalization due to cellular changes, cell growth becomes restricted due to limited substrate availability. Oxygen is the first substrate that becomes limited during neoplastic growth (65). Oxygen concentrations decrease with distance from a capillary such that oxygenated cells are limited to a distance of less than 100 μm from a blood vessel(65). As hypoxia increases, so does the hypoxic signal and ROS-formation(66). Together, ROS and hypoxia result in the activation of hypoxic signals, causing increased glycolysis, angiogenesis, cell proliferation as well as increased insensitivity to apoptosis(67, 68). Increased glycolysis and decreased oxidative phosphorylation due to hypoxia signaling results in

the formation of excess lactic acid and results in a drop in pH in the immediate environment of the hyperproliferative neoplastic cells. HIF-1 α also induces the expression of CAIX, thereby further contributing to the immediate acidotic environment of neoplastic cells(69).

The acidotic environment serves as an evolutionary bottleneck whereby cells that are resistant to death signals caused by a low pH as well as excessive oxidative stress caused by ROS formation have increased reproductive fitness (53-55). A malignant phenotype arises whereby the cells are resistant to many forms of cell death signaling, including ROS signaling. The pro-oxidant status of cancer cells serves to increase pro-growth signals whilst being resistant to apoptotic signal, resulting in the further activation of growth factor and angiogenic signaling stimulated by the pro-oxidant redox status. Finally, copious amounts of lactic acid formation due to increases in glycolysis as well as the formation of carbonic acid catalyzed by overexpressed CAIX contributes to the breakdown of the basement membrane and breachment into the circulation, resulting in metastasis and invasion.

The end result of somatic evolution according to the bioenergetic model is the emergence of cells with features associated with cancer. Bioenergetic alterations include a pro-oxidant status as a result of altered ROS metabolism, increased glucose consumption even in the presence of oxygen and an increase in acid formation (lactic acid and carbonic acid) (28, 53, 61, 62, 70, 71) (Figure 1.3).

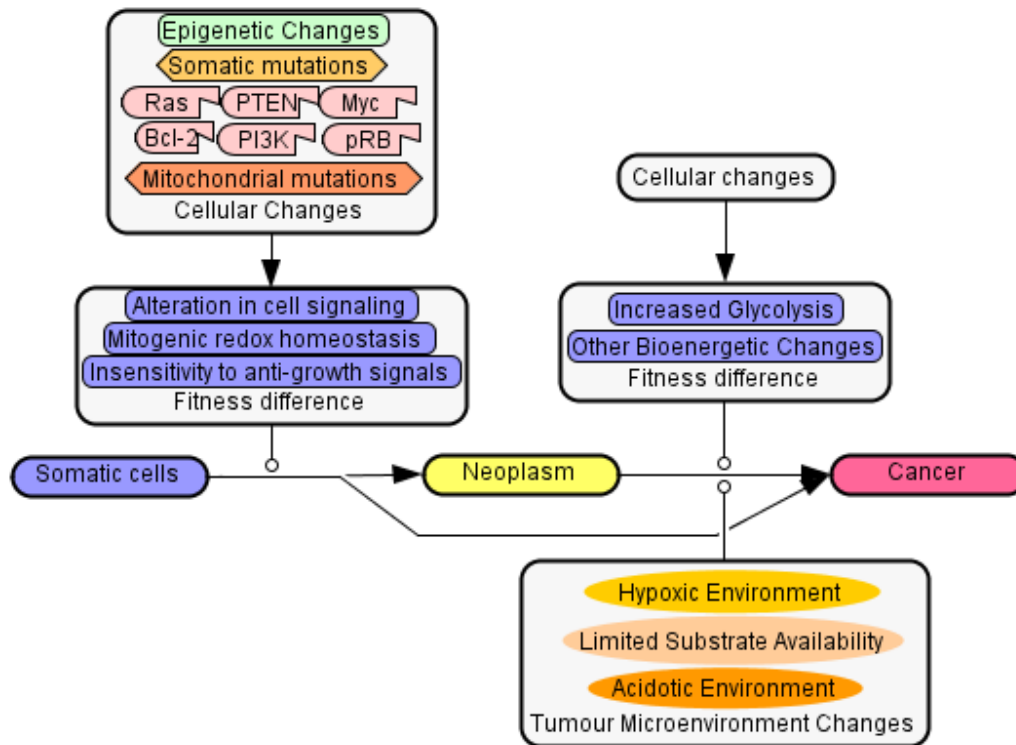


Figure 1.3: Bioenergetic model of carcinogenesis. Initial cellular changes (genetic and epigenetic) are the main drivers in the emergence of benign neoplastic growths. Tumour microenvironmental changes such as increased hypoxia, limited substrate availability and an acidotic environment, however, are the main drivers of carcinogenesis that result in fitness differences in populations of cells that ultimately result in bioenergetic changes associated with malignant neoplastic growths.

Genome/Karyotype-centered Model

The genome/karyotype model proposes that genomic or karyotypic changes such as the formation of aneuploid cells with clonal and non-clonal chromosome aberrations are the main drivers that result in fitness differences in populations of cells that ultimately result in the emergence of benign and malignant neoplastic growths. Motivations for this model include the observation that gene-centered models fail to completely explain the observations of genetic and epigenetic heterogeneity as well as why cancers carry cancer-specific aneuploidies (33, 56).

Studies have demonstrated that genetic and non-genetic factors can cause genomes to become unstable (45, 72). Genome instability and heterogeneity are also linked to carcinogenesis (45,

73). Together this suggests that genome level aberrations rather than changes in specific gene and molecular pathways are the main drivers in cancer evolution. Instead, genome heterogeneity caused by various genetic, epigenetic as well as non-genetic variables increase the diversity in cell populations. In other words, the fitness differences are due to processes that induce genome level heterogeneity and various environmental pressures serve as an evolutionary driving force for the propagation of specific traits such as metastasis, invasion, altered bioenergetics, insensitivity to apoptosis and anti-growth signaling etc. (Figure 1.4).

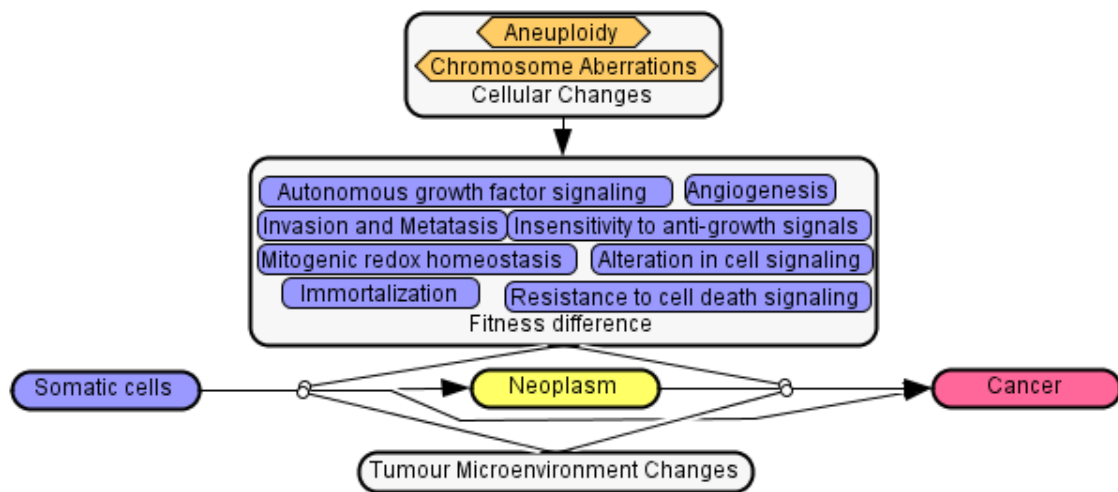


Figure 1.4: The genome/karyotype model of carcinogenesis. Genomic or karyotypic changes such as the formation of aneuploid cells with clonal and non-clonal chromosome aberrations are the main drivers that result in fitness differences in populations of cells that ultimately result in the emergence of benign and malignant neoplastic growths.

There are good arguments for each model and each model has its merits. The concept of fitness plays a causal role in the propagation of traits or features associated with cancers. On this view, features that contribute towards individual fitness are retained and those that do not, tend to be removed (53). Increased understanding of the evolutionary origins of cancer has helped researchers comprehend how resistance to anticancer treatment emerges(74). Recently it was discovered that sub-populationschemotherapeutic resistant colorectal tumors are selected and become more prominent due to anticancer treatment(75). It is therefore important to take such factors into consideration when designing new anticancer compounds. Based on these observations, treatment of cancer with combination therapies targeting at least two different

pathways is preferable(75). This can be done by treating cancers with many different kinds of chemotherapeutic agents or one agent that is capable of targeting many pathways.

It was the purpose of this study to design and synthesize novel compounds that are capable of selectively targeting molecular biological characteristics of cancer cells associated with an increased proliferation potential, as well as bioenergetic characteristics associated with adaptations to hypoxic environments in tumorigenic and metastatic cells.

Current compounds targeting molecular biological properties associated with altered cell cycle progression in cancer cells: Antimitotic agents

One of the most successful groups of chemotherapeutic compounds currently in clinical use for anticancer treatment are those that interfere with normal progression of mitosis through the interference of microtubule dynamics. Most of the chemotherapeutic anticancer drugs used in the clinic today include agents that target the cell cycle in order to inhibit the hyperproliferation of cancer cells and subsequently induce apoptosis(76-78). Microtubules play a crucial role during the cell cycle. As already mentioned, microtubules are long, hollow, cylindrical protein polymers composed of α - and β -tubulin heterodimers(76). Tubulin heterodimers are highly dynamic cytoskeletal fibres and are capable of two types of dynamics namely; treadmilling and dynamic instability (79). Microtubules polymerize at the positive end and depolymerize at the negative end. During treadmilling, polymerization and depolymerization occur at equal rates without significantly changing the average length of microtubules(79). During dynamic instability, either the positive end polymerizes at a faster rate than the negative end resulting in total elongation of the microtubule (rescue), or the negative end depolymerizes faster than the positive end can polymerize resulting in total shortening of the microtubule (catastrophe) (79).

Microtubule-interfering agents interfere with the spindle checkpoint during mitosis. The spindle assembly checkpoint ensures accurate segregation of mitotic chromosomes by delaying anaphase onset until each kinetochore has properly attached to the mitotic spindle (Figure 1.5). The kinetochore assembles on the centromere and links the chromosome to microtubule polymers

from the mitotic spindle. Accurate chromosome segregation requires bipolar attachment of sister chromatids to the mitotic spindle, mediated by connections between kinetochores and spindle microtubules (80). The segregation of sister chromatids is controlled by the anaphase-promoting complex/cyclosome complex (APC/C) and the spindle assembly checkpoint proteins converge to control the activity of the APC/C complex (81). To delay anaphase onset when there are unattached or relaxed kinetochores, the checkpoint blocks Cdh1-APC/C-mediated securin degradation (81). The segregation of sister chromatids is controlled by the APC/C complex and the spindle assembly checkpoint proteins converge to control the activity of the APC/C complex (81). To delay anaphase onset when there are unattached or relaxed kinetochores, the checkpoint blocks cadherin1 (Cdh1)-APC/C-mediated securin degradation (81). Kinetochores that are not yet attached to mitotic microtubules and chromosome pairs that lack tension across sister chromatids generated by the spindle poles activate the spindle assembly checkpoint. The mitotic checkpoint complex (MCC) is inactivated as each pair of sister kinetochores attaches to microtubules and microtubule motors generate tension that stretches them (82).

Microtubule-interfering drugs bind to microtubules at diverse sites. Vinblastine binds at the plus end and inhibits microtubule polymerization (83). Colchicine forms complexes between the α - and β -tubulin dimers to suppress microtubule dynamics and Paclitaxel binds along the interior surface of the microtubule, thereby interfering with the dynamics of the microtubules (83). Various agents that bind to the colchicine binding site of microtubules are in various stages of clinical trials. These include combretastatins and its various analogs, as well as 2-methoxyestradiol (2ME)(84, 85).

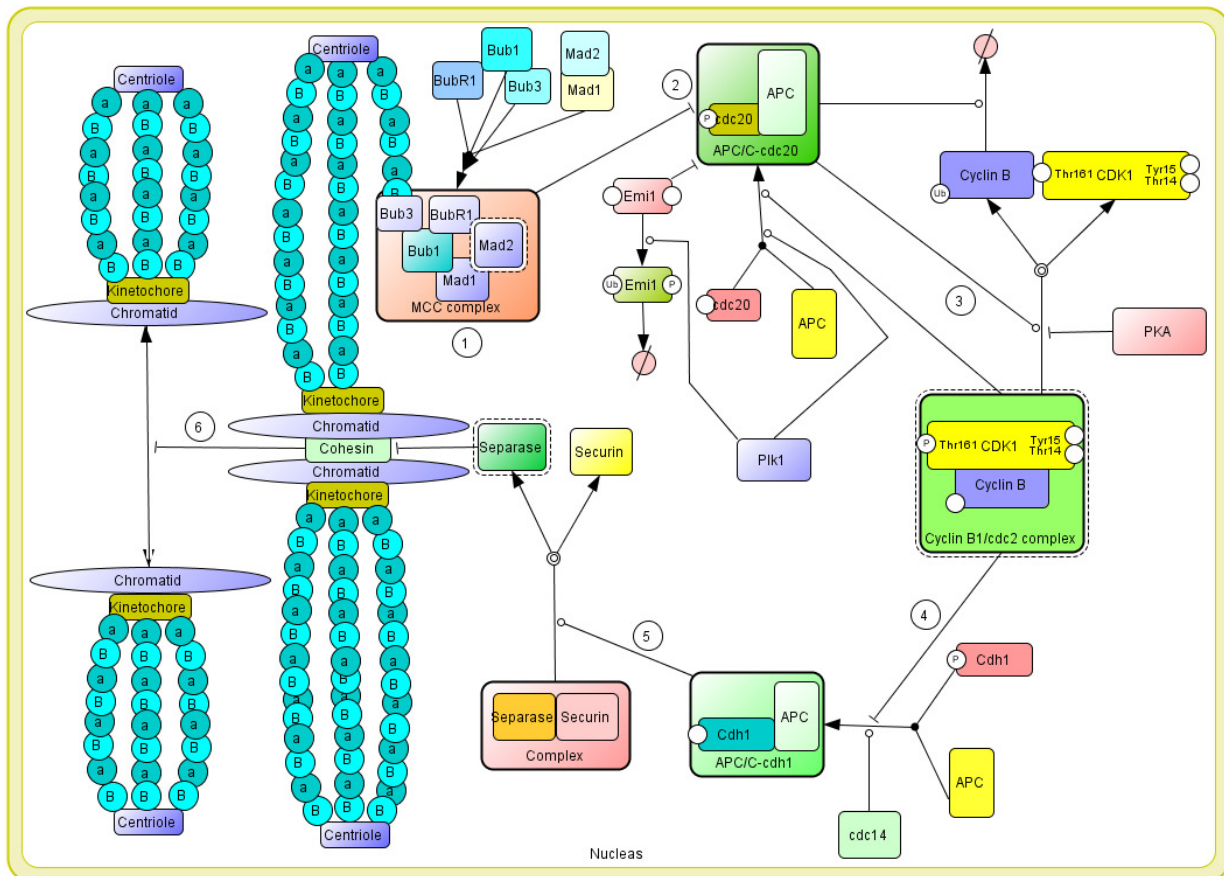


Figure 1.5: APC/C regulation and the spindle assembly checkpoint. 1) The mitotic checkpoint complex (MCC) assembles to mitotic microtubules unattached to kinetochores, as well as chromosome pairs that lack tension across sister chromatids and inhibits the APC/C-cell division cycle 20 complex. 2) Activation of APC/C is regulated by Emi1 and the MCC complex. 3) Cyclin B is ubiquitinated and degraded and CDK1 activity abolished once the APC/C-cdc20 complex is activated. 4) CDK1 inhibition of Cdh1 is removed after Cyclin B degradation and Cdh1 dephosphorylation by cdc14 activates the APC/C-Cdh1 complex. 5) Active APC/C-Cdh1 targets securin for ubiquitination and activates securin. 6) Activated securin cleaves cohesin and allows chromatid segregation (Image generated with CellDesigner 3.5.1)(1).

2ME is an endogenous metabolite of 17 β -estradiol exerting both antiangiogenic and antimetastatic effects *in vitro* and *in vivo* (86). Abrogation of microtubule dynamics is one of the mechanisms of action of 2ME and it is proposed that 2ME interacts with the colchicine binding site of microtubules (84, 87). 2ME has both antiproliferative, antitumor and antiangiogenic properties and is currently in phase I and II clinical trials (Panzem[®]) for the treatment of multiple myeloma, glioblastoma multiforme and carcinoid, prostate, and breast tumors (84). Limited oral

bioavailability and a short half life as a result of degradation and conjugation are issues that need to be addressed for it to be successful (88, 89). It is thus important to develop novel 2ME formulations or derivatives that are able to improve bioavailability and potency.

Reactive oxygen species and cell signaling in cancer cells

Reactive oxygen species (ROS) and specifically hydrogen peroxide play important roles in many biological processes and they function as signaling agents as part of signal transduction pathways. The redox status within cells is determined by the concentration of the various ROS and the activity of the antioxidant systems. The redox status affects the activity of various signaling proteins and cell signaling cascades and doing so bring about changes in cellular and enzymatic activity as well as gene expression. ROS act as second messengers and control the action of several signaling pathways including non-receptor cytoplasmic protein tyrosine phosphatases (cPTPs), receptor protein tyrosine phosphatases (RPTP), non-receptor cytoplasmic protein tyrosine kinases (cPTKs), receptor-like protein tyrosine kinases (RPTKs), mitogen-activated protein kinases (MAPKs), PKB/Akt, protein kinase C (PKC) and nuclear factor-kappa Beta (NF- κ B) (Figure 1.6)(90, 91).

Kinases and phosphatases are complementary in function and have been shown to control signaling responses. Kinases determine the amplitude of the signaling response, whereas phosphatases have a role in the control of the rate and duration of the response (92). The most abundant protein kinases are the ones that transfer a phosphate group from the ATP molecule to the amino acids Ser, Thr, and Tyr(92). PTPs dephosphorylate phosphotyrosine residues and are involved in the control of cell proliferation, adhesion, and migration. All members of the PTP family contain a highly conserved sequence HCXXGXXRS/T, which contains a Cys residue that is essential for catalysis(92). The Cys residue of PTPs is predominantly present as a thiolate anion at physiologic pH and is very susceptible to oxidation because of its nucleophilic nature (92). Oxidation of PTPs results in inhibition of their activity and can consequently result in increased levels of proteins phosphorylated on tyrosine(92, 93). Trx, Grx, and GSH are essential

in the recovery and reversal of oxidized PTP activity(93). Thus, a pro-oxidant redox status in cells caused by increased levels of ROS and/or abrogated antioxidant systems may lead to increased levels of phosphotyrosine and consequently increased tyrosine kinase signaling of PTKs(93). However, pro-oxidants and ROS can stimulate tyrosine phosphorylation in a PTP independent manner as well. Pro-oxidants and ROS cause activation cPTKs and RPTKs independent of the presence of its ligand. Examples of affected RPTKs include the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR). Hydrogen peroxide strongly stimulates EGFR tyrosine phosphorylation levels on carcinoma cell lines overexpressing the EGF receptor(92). ROS-induced nitration of Tyr residues in PDGFR has been observed in mild oxidant-treated cells, causing downstream activation of Src dependent activation of the ERK1/2 MAPK pathway and PKB/Akt(94). The Src cPTK is also redox regulated and undergoes oxidation/activation due an S-S bond formation caused by ROS (Figure 1.6) (93).

Upon activation of cPTKs and RPTKs, various downstream signaling pathways are activated that control cell metabolism, growth, migration, and differentiation(92). V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (Src) is one such signal mediator, and upon activation through phosphorylation either through RPTKs or reactive oxygen species, ROS can phosphorylate and activate Src homology 2 domain containing protein (Shc) and the Ras (GTPase activating protein) G-protein subfamily, especially p21R (Figure 1.6)(92). Shc can also activate Ras (92). Active p21Ras promotes cell survival and proliferation and mediates its proliferative activities via the activation of v-raf-1 murine leukemia viral oncogene homolog 1 (raf-1) and receptor-mediated phosphoinositide-3 kinase I (PI3K I). Raf-1 activation leads to the activation of the extracellular signal regulated kinase (ERK1/2) MAPK pathway which in turn activates several mitogenic transcription factors e.g. Elk-1, *c-fos*, *c-myc*, AP-1 and E2F(95). Ras-activated PI3K I leads to the downstream activation of Ras-related C3 botulinum toxin substrate 1 (Rac), another small G protein, which in turn activates p21cdc42/rac1-activated Ser/Thr kinase (PAK), a positive regulator of the cell cycle as well as Raf-1 (Figure 1.6) (92). Ras-activated PI3K I also leads to the downstream activation of PKB/Akt, another mitogenic and proliferative kinase. PKB/Akt is able to phosphorylate the transcription factor forkhead box O3 (Foxo3A) and thereby suppresses its ability to induce transcription of pro-apoptotic and anti-proliferative proteins(96). PKB/Akt also phosphorylates and activates inhibitor κ B kinase (IKK), which in turn activates

anti-apoptotic, pro-survival effects NF- κ B(Figure 1.6)(96). ROS do not exclusively activate the PKB/Akt kinase through the Ras-PI3K I pathway, but can also act through another upstream regulator, phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual- specificity protein phosphatases (e.g. PTEN) (93). ROS inhibits the PKB/Akt inhibiting activities of PTEN through the reversible formation of disulfide bonds on crucial Cys residues (93). To summarise, ROS has the ability of activating pro-growth, pro-survival and proliferative signals through the Ras-Raf-ERK1/2, PKB/Akt and NF- κ B pathways among others (Figure 1.6).

ROS do not only activate pro-growth signals, but also activate several stress-activated pathways. The apoptosis signal-regulating kinase 1-Trx (ASK1–Trx) signaling complex has been implicated in redox signaling. ASK1 is an upstream regulator of the MAPK kinase 4 and 7 (MKK4 and 7)-c-Jun-N-terminal kinase (JNK) and MAPK kinase 3 and 6 (MKK3 and 6)-p38 MAPK signaling cascades respectively and plays crucial roles in cellular responses to various types of stresses, including ROS (97). Rac1, a downstream effector of activated Ras is also able to induce JNK activation and subsequent cell death(98). Redox status and ASK1 signaling is connected by the redox status of Trx. Reduced Trx binds to and inhibits the activity of ASK1 by preventing it from associating with tumor necrosis factor receptor-associated factor 2 and 6 (TRAF2 and 6)(97). An activated ASK1–Trx signalosome caused by excessive ROS (oxidized Trx) results in the downstream activation of stress activated protein kinases (SAPKs) JNK and p38, resulting in the transcription and activation of pro-apoptotic genes including Bcl2-interacting mediator of cell death(Bim), BH3-interacting domain death agonist(Bid), activating transcription factor 2 (ATF2), p53, and *c-jun* (Figure 1.6) (99, 100). Downstream of ASK1-Trx mediated ROS signaling at the JNK junction, glutathione S-transferases (GST) in conjunction with GSH binds to and inhibits JNK-related apoptotic signaling. ROS and a pro-oxidant intracellular status cause transient dissociation of GST from JNK as a result of ROS-related GSH inhibition through glutathione disulfide (GSSG) formation(101).SAPKs do not exclusively activate pro-apoptotic signaling as they are also capable of inducing pro-survival transcription factors including Elk-1 and *c-jun*(91, 99, 100). The pro-apoptotic effect of activated SAPKs is dependent on the strength and duration of the signal. Low, transient stimulation of SAPKs by ROS results in pro-growth signaling through the activation of Elk-1 and *c-jun*, while excessive

and prolonged stimulation of SAPKs by ROS results in apoptotic signaling and ultimately cell death (Figure 1.6) (91).

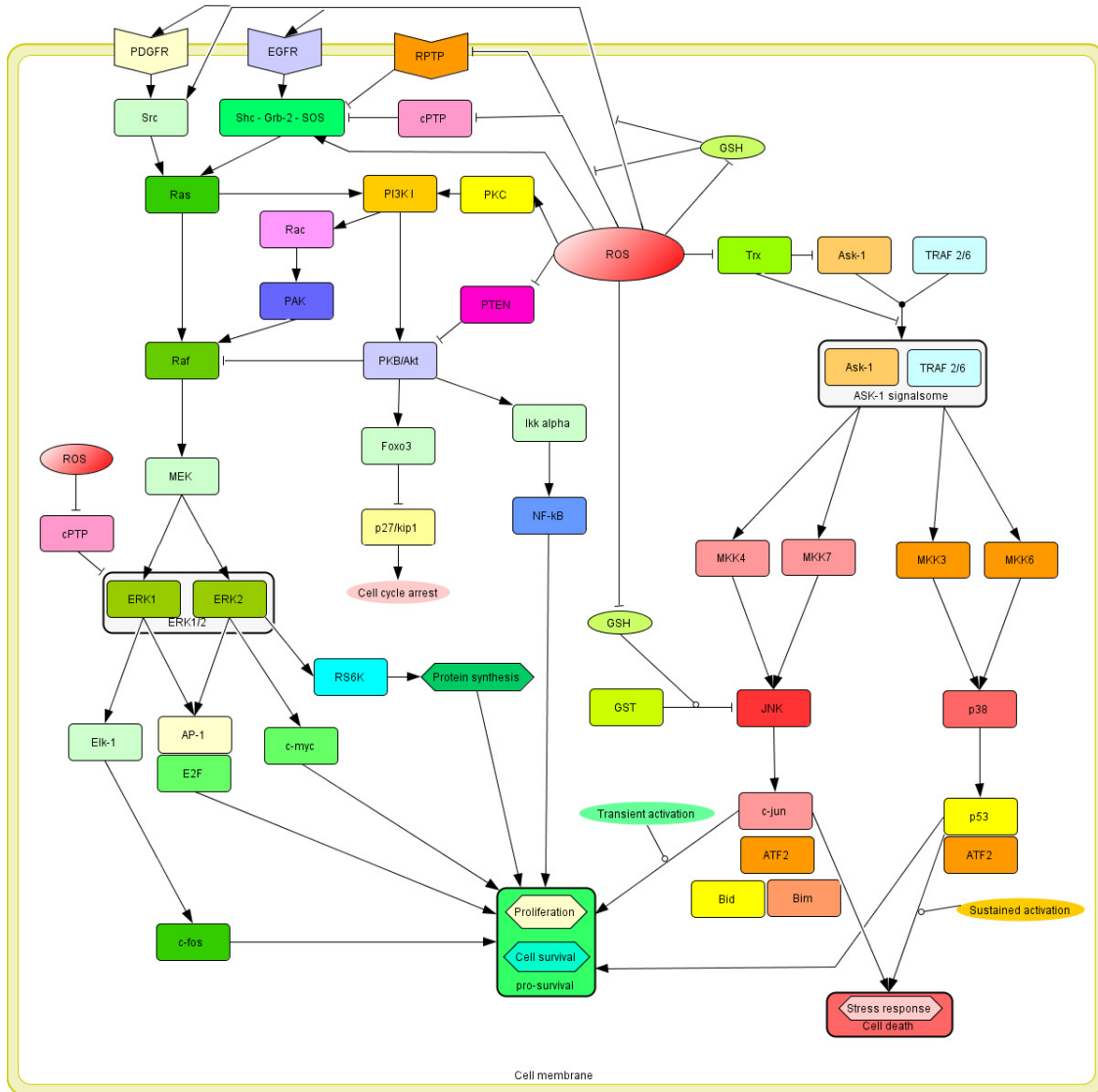


Figure 1.6: Reactive oxygen species signaling pathways. ROS are capable of both pro-survival and pro-death signaling. The outcome of ROS signaling depends on strength and duration of the ROS signal and the redox status of cells.

The redox state of cells is determined by the balance between the rate of ROS production and the rate of their removal by various antioxidants. ROS are molecules with free, unpaired electrons and cause oxidative damage to biomolecules, including nuclear and mitochondrial DNA, lipids and proteins(102). These include superoxide- ($O_2^{\cdot-}$), hydroxyperoxyl- ($\cdot OH$), carbonate- ($CO_3^{\cdot-}$),

peroxyl- ($\text{RO}_2^{\cdot-}$) and alkoxyl radicals(103). The NADPH complex and the electron transport chain are the two main sources of intracellular ROS (103). The electron transport chain is one of the major contributors of superoxide and ROS generation in mitochondria and is considered to be a major source ROS generation(104, 105). It is estimated that between 0.15% - 3% of total oxygen consumption is reduced to superoxide during oxidative phosphorylation (104, 105). Superoxide is unstable and either undergoes dismutation, resulting in the formation of hydrogen peroxide (H_2O_2) or forms peroxyl radicals, a conjugate acid of superoxide, by reacting with hydrogen ions. The dismutation reaction is catalyzed by manganese superoxide dismutase (MnSOD) within the mitochondrial matrix and intracellular superoxide is catalyzed by copper-zinc superoxide dismutase (Cu/Zn-SOD)(106). Superoxide dismutation results in the formation of H_2O_2 . Neither superoxide nor H_2O_2 are strong oxidizing molecules, but are the major sources for the downstream formation of ROS(107).

Superoxide converts ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions ($\text{Fe}^{3+} + \text{O}_2^{\cdot-} \rightarrow \text{Fe}^{2+} + \text{O}_2$). Ferrous ions in turn react with hydrogen peroxide in the Fenton reaction, yielding the highly reactive hydroxyperoxyl radicals ($\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-$)(102). This process is described as the Haber-Weiss reaction ($\text{O}_2^{\cdot-} + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \cdot\text{OH} + \text{OH}^-$)(102). The majority of hydroxyperoxyl radicals generated *in vivo* are formed during the Fenton reaction from hydrogen peroxide(102). Hydroxyperoxyl radicals are highly reactive and cause oxidative damage to biomolecules, including nuclear and mitochondrial DNA, lipids and proteins (103). Superoxide is primarily generated at two components of the electron transport chain, complex I and complex III (104). Superoxide does not directly contribute to oxidative stress within cells as it is efficiently detoxified initially on both sides of the mitochondrial inner membrane to H_2O_2 by superoxide dismutase. H_2O_2 in turn is the major source of hydroxyl radicals generated *in vivo* as a result of the Fenton reaction and contributes to the pro-oxidant balance within cells. H_2O_2 is also able to diffuse freely through mitochondrial and cellular membranes.

Cells maintain an oxidant-antioxidant balance termed “redox homeostasis” (103). Abrogation of intracellular redox homeostasis has been implicated in various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, ageing and other diseases (108). Cells employ various antioxidant systems to defend against ROS-induced

oxidative stress and the concentrations of ROS are determined by the balance between their generation and removal by these antioxidant defense-systems. Antioxidant defenses are enzymatic and non-enzymatic. Enzymatic antioxidant defenses include superoxide dismutase(SOD), catalase(CAT), the glutathione peroxidase (GPx) system, thioredoxin reductase (Trx) system, glutathione S-transferases (GST), glutaredoxins (Grx) and peroxiredoxins (Prx)(108-110). Non-enzymatic antioxidants include ascorbic acid (Vitamin C), α -tocopherol (Vitamin E), glutathione (GSH tripeptide), thioredoxin (Trx), carotenoids, flavonoids, and other antioxidants (Figure 1.7) (108-110).

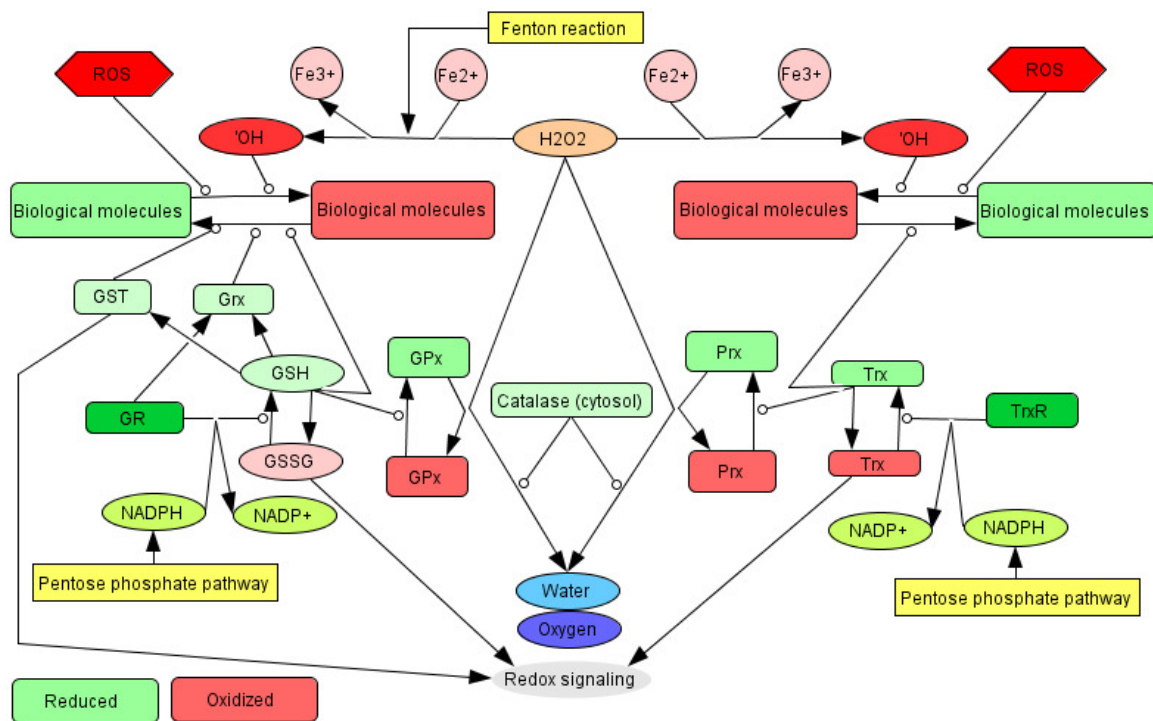


Figure 1.7: Hydrogen peroxide metabolism. Hydroxyl radicals form during the Fenton reaction, which in turn causes protein disulfide bond formation, lipid peroxidation and also damages both the purine and pyrimidine bases and also the deoxyribose backbone of DNA. Glutathione peroxidase (GPx) and peroxiredoxins (Prx) in both the cytosol and mitochondrial matrix reduce hydrogen peroxide to water and oxygen with glutathione (GSH) and thioredoxin (Trx) as substrates respectively. Catalase reduces hydrogen peroxide primarily in peroxisomes. Glutaredoxins (Grx), GSH and Trx are able to reverse disulfide bond formation and lipid peroxidation. Glutathione reductase (GR) and thioredoxin reductase (TrxR) are able to regenerate GSSG and oxidized Trx respectively at the cost of one NADPH molecule. NADPH molecules are generated through the pentose phosphate glucose catabolic pathway.

Redox homeostasis abrogation in cancer cells is characterized by an intracellular pro-oxidant status as a result of the cumulative production of ROS and the diminished capacity of antioxidant systems to balance the excess production of ROS (108). ROS activate pro-growth, pro-survival and proliferative signals through the Ras-Raf-ERK1/2, PKB/Akt and NF- κ B pathways (95, 96, 108). ROS also activate stress-activated pathways through the ASK1–Trx signaling complex (91, 111). The pro-apoptotic effect of activated SAPKs is dependent on the strength and duration of the signal. Low, transient stimulation of SAPKs by ROS results in pro-growth signaling through the activation of Elk-1 and *c-jun*, while excessive and prolonged stimulation of SAPKs by ROS results in apoptotic signaling and ultimately cell death (91).

The altered redox status in cancer cells is as a result of many contributing factors. Mitochondrial DNA mutations are associated with various cancers it is possible that they are not only symptomatic but also causal in the development and somatic evolution of malignancy by contributing to a pro-oxidant state and thereby activate pro-growth signaling through ROS signaling (62). During the somatic evolution of malignancy, oxygen supply becomes limited, leading to hypoxic conditions near neoplastic growths (65). Hypoxia in turn results in the increased formation of ROS and the Q_o site of complex III of the electron transport chain is responsible for ROS production during hypoxia (112). This increase in ROS production in response to hypoxia in turn activates pro-growth signaling and also contributes to the activation of HIF-1 α . Excessive ROS (hypoxia-induced or otherwise) results in a pro-oxidative intracellular redox status, resulting in ferrous iron depletion because of the Fenton reaction. Depletion of intracellular ferrous iron as a result of excessive ROS in turn diminishes the activity of the prolyl hydroxylase domain (PHD) and in turn results in HIF-1 α stabilization and activity (67, 68).

Another factor contributing to the altered redox status in cancer cells is an impaired antioxidant defense system. Catalase promotes the conversion and detoxification of H₂O₂ to water and oxygen and the capacity of catalase to do this is significantly decreased in a variety of tumors (102). The most important antioxidant system within mitochondria is the GPx system (113). GPx, in conjunction with GSH, reduces H₂O₂ to water and lipid hydroperoxides to their corresponding alcohols (113). GSH does not react directly with hydrogen peroxides, but is used as a substrate for GPx and is oxidized to GSSG during the reaction (Figure 1.7) (113). Low levels of GSH thus

impair the activity of GPx and it has been observed that the GSH/GSSG ratio is significantly decreased in the blood of patients with colon and breast cancer(114). Elevated levels of Trx have been reported in a wide variety of cancers(102). Trx inhibits the ASK1-signalosome and thus protects cells from SAPKs while simultaneously stimulating the growth of cancer cells (109).

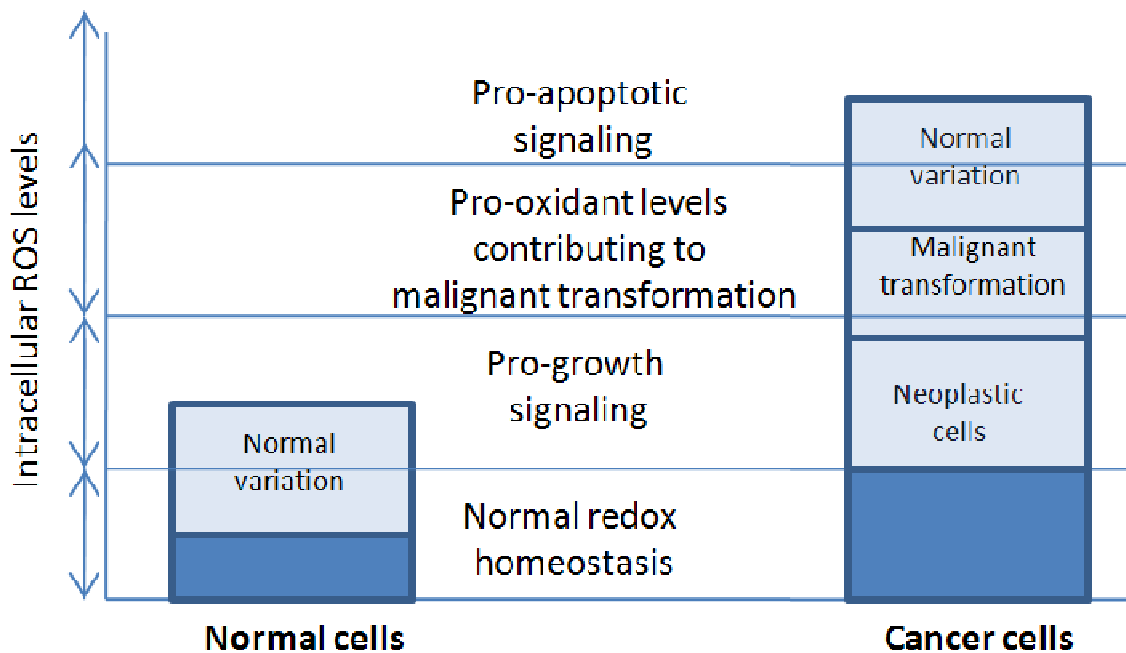


Figure 1.8: Intracellular ROS levels and cell signaling in normal and cancer cells. The pro-oxidant status of cancer cells allows for the selective killing through therapeutic increases in ROS levels, leaving normal cells unaffected (adopted from Lo'pez-La'zaro (2007) (115)).

The progressive increase in the pro-oxidant status of cells during the somatic evolution of malignancy results in the activation of pro-growth signaling and contributes to the malignant transformation of cells. This pro-oxidant status in turn contributes to the increase in somatic and mitochondrial mutations because of the damaging effects of ROS on biomolecules, thereby further contributing to the malignant transformation of cells. The increased pro-oxidant status of cancer cells does however provide an opportunity for the selective killing of cancer cells (Figure 1.8). ROS and an intracellular pro-oxidant status above a certain level selectively activate pro-apoptotic SAPK pathways (Figure 1.8)(91). The development of compounds specifically aimed

at sufficiently increasing the pro-oxidant status of cancer cells and thereby selectively killing them can thus be therapeutically useful (115).

Generation of H_2O_2 as a result of 2ME treatment has been demonstrated in various cell lines and has been implicated as mediators of apoptosis induction(86).One possible source of H_2O_2 is the inactivation of peroxiredoxin 1 (Prx 1). During mitosis, active cdc2 is responsible for the phosphorylation and inactivation of the Prx 1 enzyme (116).It has been shown that 2ME significantly increased cdc2 activity in 2ME-treated MCF-7 and Jurkat cells as a result of cell cycle arrest (117, 118).Thus, prolonged cdc2 activity as a result of a mitotic block and subsequent elevation of H_2O_2 due to Prx 1 inhibition provides a mechanism for selectively killing hyper-proliferative cells with an elevated pro-oxidant status. Designing more potent and selective analogs of 2ME capable of interfering with the normal progression of mitosis as well as targeting the pro-oxidant status of cancer cells is warranted.

Cell stress and cell death

The development and maintenance of multicellular organisms depends on the proper control of programmed cell death (PCD). Various forms of the process of PCD include apoptosis, autophagy and mitotic catastrophe. These pathways are executed by active cellular processes that can be intercepted by interfering with intracellular signaling (119). Other forms of cell death include necrosis and metabolic catastrophe.

Apoptosis

The morphological features of apoptosis include changes in plasma membrane asymmetry and attachment, condensation of cytoplasm, nucleus and internucleosomal cleavage of DNA (120). Externalization of phosphatidylserine, another characteristic of apoptotic cell death, governed by activation of a calcium-dependent phospholipid scramblase activity in concert with inactivation of the aminophospholipid translocase at the cell surface (120).

Apoptotic cell death is mediated by caspase-dependent and caspase independent proteins. Mediators of caspase independent cell death include cathepsins, calpains, granzymes and apoptosis inducing factor (121). The cathepsin family of proteases consists of cysteine, aspartate, and serine proteases. Cathepsin B and cathepsin L, both cysteine proteases, and cathepsin D, an aspartate protease, are most frequently linked to apoptosis (121). Cathepsin activity is associated with mitochondrial membrane permeability, chromatin condensation, the degradation of the intracellular matrix, the processing of procaspases, and the externalization of PS on the plasma membrane of apoptotic cells. The calpain family of cysteine proteases resides in the cytosol and is activated by irregular increases in intracellular free Ca^{2+} (122). Calcium activated M-calpain cleaves and activates caspase 12 and thereby activating downstream apoptotic pathways (Figure 1.9). Granzyme B promote caspase-independent DNA fragmentation by directly cleaving inhibitor of CAD (ICAD) allowing CAD to trigger nucleosomal DNA fragmentation (123). Granzymes are also able to cleave the proapoptotic Bcl-2 family members Bid and Bax, thereby inducing mitochondrial membrane permeabilization (124). Apoptosis-inducing factor (AIF) is a mitochondrial flavoprotein that is released from the intermembrane space during apoptosis. Once liberated from the mitochondria, AIF translocates to the nucleus where it induces chromatin condensation and DNA fragmentation (125).

Mitochondrial EndoG is a mitochondrial nuclease that assists with the maintenance of the mitochondrial genome by participating in mitochondrial DNA duplication and repair (126). After EndoG is released from the mitochondria into the cytoplasm as a result of apoptotic stimuli, it is translocated to the nucleus where it induces DNA fragmentation that is similar to CAD-induced DNA fragmentation (127). However, unlike CAD, EndoG does not require caspase processing to be activated.

Caspase-dependent apoptotic cell death is mediated by the caspase family of enzymes comprising of caspase 2, caspase 3, caspase 4, caspase 6, caspase 7, caspase 8, caspase 9 and caspase 10 (128). Caspases 2, 4, 8, 9, and 10 are initiator caspases that activate the executioner or downstream caspases 3, 6, and 7 which are responsible for the many of the morphological aspects of apoptosis (128).

Caspase activation can proceed via the extrinsic pathway whereby death receptors on the surface of cells transmit apoptotic signals initiated by specific death ligands (129, 130). The receptors belong to the tumor necrosis factor gene super family and include CD95 (also called Fas, Apo1), tumor necrosis factor receptor-1 (TNFR-1, also called p55, CD120a), death receptor-3 (DR3, also called Apo3, TRAMP, LARD), and death receptor-4 and 5 (DR4, DR5, also called Apo TRAIL-r2, killer). (135). These receptors contain a death domain in their intracellular region to recruit downstream apoptotic proteins (129, 130). These receptors can activate the caspase cascade within seconds of ligand binding and converge in activating the Fas associated death domain (FADD) (129). FADD recruits procaspase 8 by protein–protein interaction via a homologous death effector domain (DED) to form a death inducing signal complex (DISC) (129, 130). During DISC formation procaspase 8 is autolytically cleaved to yield caspase 8. Active caspase 8 is rapidly released from DISC to the cytoplasm and serves as an enzyme for downstream effector caspase 3, 6, and 7 (Figure 1.9).

Caspase activation may also occur via intrinsic pathways. Mitochondria and the endoplasmic reticulum play important roles in responding to cell stress and activating caspases. Mitochondria are comprised of a matrix surrounded by inner membrane, the intermembrane space and the outer membrane. The inner membrane contains molecules such as ATP synthase, electron transport chain, and adenine nucleotide translocator (130). Under normal physiological conditions these molecules allow the respiratory chain to create an electrochemical gradient or membrane potential. The intermembrane space contains cytochrome *c*, certain procaspases, adenylate kinase-2, Endo G, Dialbo/Smac, and apoptosis inducing factor (AIF) (131). The permeabilization of the outer membrane results in the release of these molecules in the cytoplasm. The release of cytochrome *c* is one of the major events in apoptosis associated with permeabilization of the mitochondrial outer membrane (131). Cytoplasmic cytochrome *c* promotes the formation of an apoptosome which in turn orchestrates apoptosis (Figure 1.9). The components of an apoptosome are cytochrome *c*, an adapter molecule apoptotic protease activating factor (Apaf-1) and procaspase 9. The binding of cytochrome *c* to Apaf-1 leads to the activation of procaspase 9. Active caspase 9 cleaves executioner caspases to induce apoptosis mainly through the activation of caspase 3 (130).

The B-cell lymphoma (Bcl) family members consist of anti-apoptotic proteins including Bcl-2, Bcl-w, Bcl-xL, Mcl-2 and Bcl-A1, and pro-apoptotic proteins including Bax, Bak and Bok (132). Bcl-2 homology (BH)3-only proteins are Bcl-2 related pro-apoptotic proteins with Bad, Bid, Bik/NBK, Bmf, Hrk/DP5, Noxa, and PUMA/BBC3 as members (132). The Bcl-2 family of proteins plays an important part in modulating mitochondrial permeability. Bax or Bak are necessary for mitochondrial outer membrane permeabilization as upon activation they are able to translocate from the cytoplasm and insert into the mitochondrial membrane as dimers via oligomerization which in turn promotes the formation of pores, resulting in mitochondrial depolarization (133). Anti-apoptotic Bcl-2 members interact with Bax and Bak that prevents their activation (134). BH-3 proteins are able to inhibit the effect of anti-apoptotic Bcl-2 members on Bax and Bak and therefore indirectly promote apoptosis (134). The intrinsic and extrinsic apoptosis signaling pathways communicate with each other through truncated Bid (tBid). Caspase 8 has been shown to cleave the pro-apoptotic Bcl-2 family member Bid (135).

The endoplasmic reticulum (ER) is the site of assembly of polypeptide chains destined for secretion or routing into various subcellular compartments. The ER represents the primary storage site for calcium (Ca^{2+}) within the cell (136). Within the ER, Ca^{2+} functions in protein folding and when improperly regulated, Ca^{2+} has been shown to play a role in several cell death pathways (137). Increases in intracellular and extra-ER calcium levels are able to trigger several pathways that are able to activate caspase activity. Calcineurin, a Ca^{2+} /calmodulin-dependent phosphatase, can dephosphorylate and activate the pro-apoptotic BH3-only protein, Bad, inducing cell death through the mitochondrial pathway (138). The cytoplasmic protease, M-calpain, is activated by calcium. Once activated, it cleaves and activates caspase 12 (138). Caspase 12 plays the central role in the ER-mediated pathway of apoptosis induction in murine cells (139). Once activated, caspase 12 can initiate downstream apoptotic pathways by inducing the activation of caspase 9 independent of Apaf-1 (140). Caspase 4, the human homolog of murine caspase 12, is localized on the ER membrane and is specifically activated by m-Calpain and required for ER stress-induced apoptosis. DNA-damage-inducible transcript 3 (DDIT/CHOP/GADD153) activity is up regulated by extra-ER Ca^{2+} and is able to inhibit Bcl-2 activity and promote apoptosis via the mitochondrial pathway (Figure 1.9) (141).

Macroautophagy is the major lysosomal route for the turnover of cytoplasmic components and is commonly referred to as autophagy (142). In mammals, cells undergo autophagy during short-term starvation. By degrading non-essential components, cells obtain nutrients for vital biosynthetic reactions. Autophagy also contributes to cell homeostasis in muscle, liver and pancreas, as well as development and growth regulation. The down-regulation of macroautophagy observed in cancer cells can be associated with tumor progression (142).

The mTOR kinase is a major sensor in the mammalian autophagy signaling pathway(143, 144). Once activated, mTOR can activate pathways that promote mRNA synthesis, protein synthesis, cell cycle progression, nutrient uptake and glycolysis, while inhibiting apoptosis and autophagy(143). The microtubule-associated proteins 1A/1B light chain 3B membrane protein (LC3) is required for the formation process of autophagosomes(145). Two distinct mTOR kinase complexes exist, mTOR complex 1 and 2 (mTORC1/2)(146). mTORC1 contains regulatory associated protein of mTOR (Raptor) and mTORC2 contains rapamycin-insensitive companion of mTOR (Rictor) (Figure 1.10). Both mTORC1 and mTORC2 contain the protein GβL(146). Another protein that is associated with mTORC1 is the small GTPase Ras homolog enriched in brain protein (Rheb)(147). GTPase Rheb in its active, GTP-bound form, positively regulates mTORC1 activity by direct interaction(147). Rheb's activity is regulated by the tuberous sclerosis complex tumor suppressors TSC1 and TSC2, which form a GTPase activating protein (GAP), converting Rheb from its active to its inactive (GDP-bound) form (Figure 1.10)(147). Growth factor and insulin signaling through receptor-mediated phosphoinositide-3 kinase (PI3K) activation leads to phosphorylation and activation of the oncogenic kinase, Akt/PKB, which in turn inactivates TSC2, thereby activating Rheb and mTORC1(147). The energy sensor AMP-activated protein kinase (AMPK) inhibits mTORC1 by activating TSC2 in response to high AMP/ATP ratios, therefore AMPK serves as a sensor for energy (ATP) status within the cell (Figure 1.10)(148). High levels of AMP (low-energy status) activate AMPK which leads to the downstream inactivation of mTOR and ultimately upregulation of autophagy in order to regain energy through catabolic means. mTORC2 is insensitive to Rapamycin and phosphorylates PKB, PKC, serum and glucocorticoid induced kinase 1 (SGK1)(149). Both mTORC1 and mTORC2 are activated by growth factors such as insulin and insulin growth factor 1 (IGF-1) via

the PI3K pathway, however, the signaling steps beyond PI3K are distinct for mTORC1 and mTORC2 and the exact mechanism of control of mTORC2 needs to be clarified(146, 149).

The TSC1/TSC2 complex integrates amino acid signaling upstream of mTOR(145). Amino acids have also been shown to mediate mTORC1 signaling by activating class III PI3K(145). Beclin1, the mammalian homolog of Atg6 involved in autophagosome formation, is found in a complex with PI3K class III (Figure 1.10)(150). In order to be able to bind to PI3K class III and to stimulate autophagy, Beclin 1, which is found in association with the antiapoptotic protein Bcl-2, must first dissociate from the inhibitory Beclin 1-Bcl-2 complex(150). The Beclin 1-PI3K class III complex activity is also inhibited by the availability of amino acids (Figure 1.10)(150, 151). Ras-dependent activation of the MAP-Erk1/2 pathway has a stimulatory effect on starvation-induced autophagy in colon carcinoma cells(152). However, amino acids stimulate the phosphorylation of Ser259 of Raf-1 kinase and thereby inactivate the Erk1/2 MAPK kinase Raf-1 and down regulate autophagy(152).

The tumor suppressor genes PTEN and p53 act in the mTOR signaling network to stimulate autophagy by inhibiting mTOR through TSC1/2 activation (Figure 1.10)(153, 154). PTEN inhibits autophagy by hydrolyzing phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) to phosphatidylinositol (4,5)-bisphosphate (PIP2) and p53 inhibits mTOR activity through activation of AMPK. c-Myc, a proto-oncogene that controls cell division and cell growth, increases autophagic activity when overexpressed in rat 3Y1 fibroblasts and is not dependent on its apoptogenic or tumorigenic functions(155).

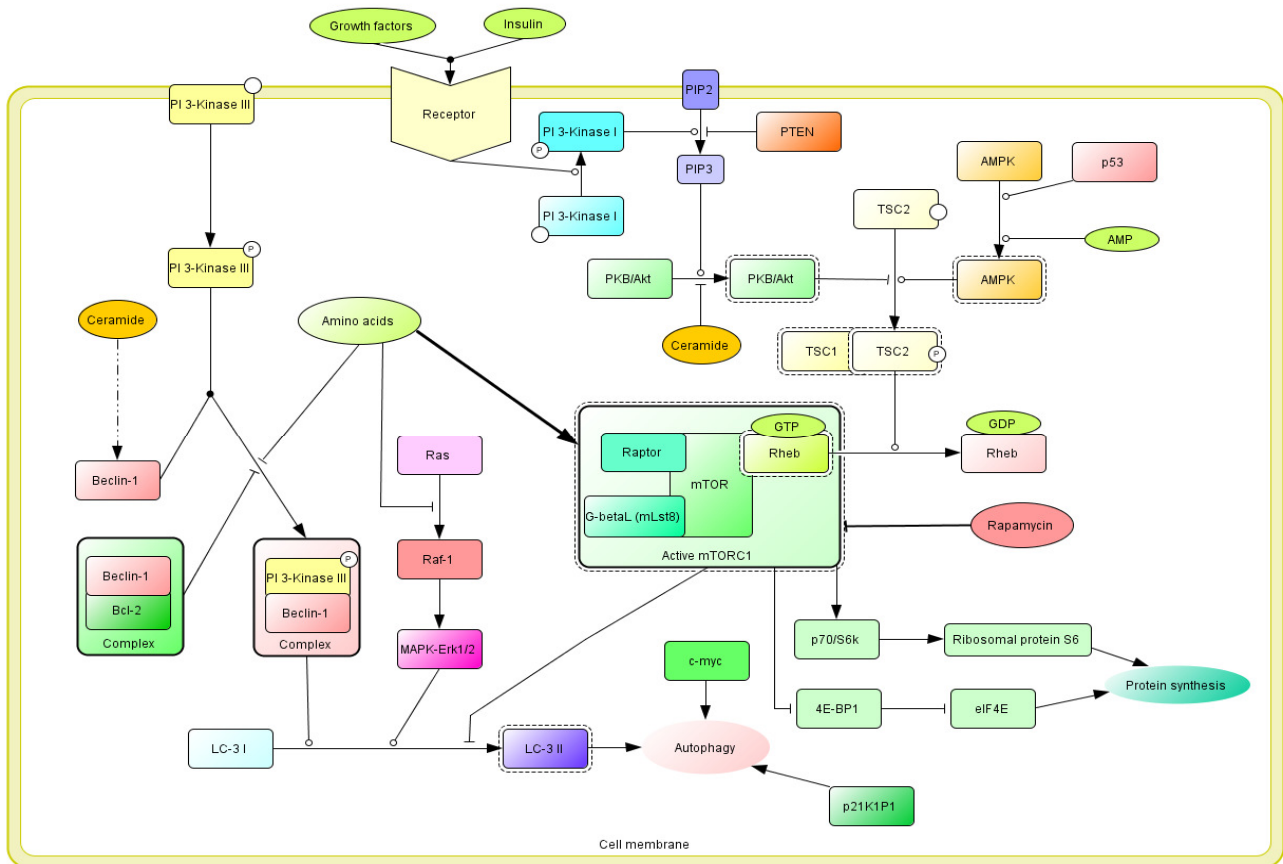


Figure 1.10: Signaling and the regulation of autophagy. Amino-acid-dependent activation of mTOR plays a crucial role in repressing autophagy. Insulin suppresses autophagy through the PI-3K-I/Akt/TSC1 and 2 mTOR pathway. Amino acid signaling controls autophagy in various ways; including mTOR stability, TSC1/2 pathway, the PI-3k-III pathway to inhibit Beclin1 activity and by inhibiting Ras-Raf1 signaling. AMPK serves as an energy sensor and is able to induce autophagy when the intracellular energy status is low. mTOR plays a crucial role in the initiation of ribosomal activity and protein synthesis.

After an induction signal, autophagy commences when a flat membrane cistern wraps around a portion of cytosol and organelles to form a closed double-membrane bound vacuole containing cytosol and organelles. Such a vacuole is also known as an autophagosome and does not contain lysosomal proteins(156). Autophagosomes are formed by phagophores or isolation membranes(156). Autophagosomes undergo a stepwise maturation process by fusing the segregated cytosol and organelles with endosomal and/or lysosomal vesicles. Autophagosomes that have fused with endosomes are called amphisomes(156). Autophagosomes and/or

amphisomes that have fused with lysosomes are called autolysosomes. Collectively autophagosomes, amphisomes and autolysosomes are called autophagic vacuoles (Figure 1.11)(156). Both the segregated cytosol and organelles are then degraded by lysosomal hydrolases and the degradation products are transported back to the cytoplasm to be used for metabolic purposes.

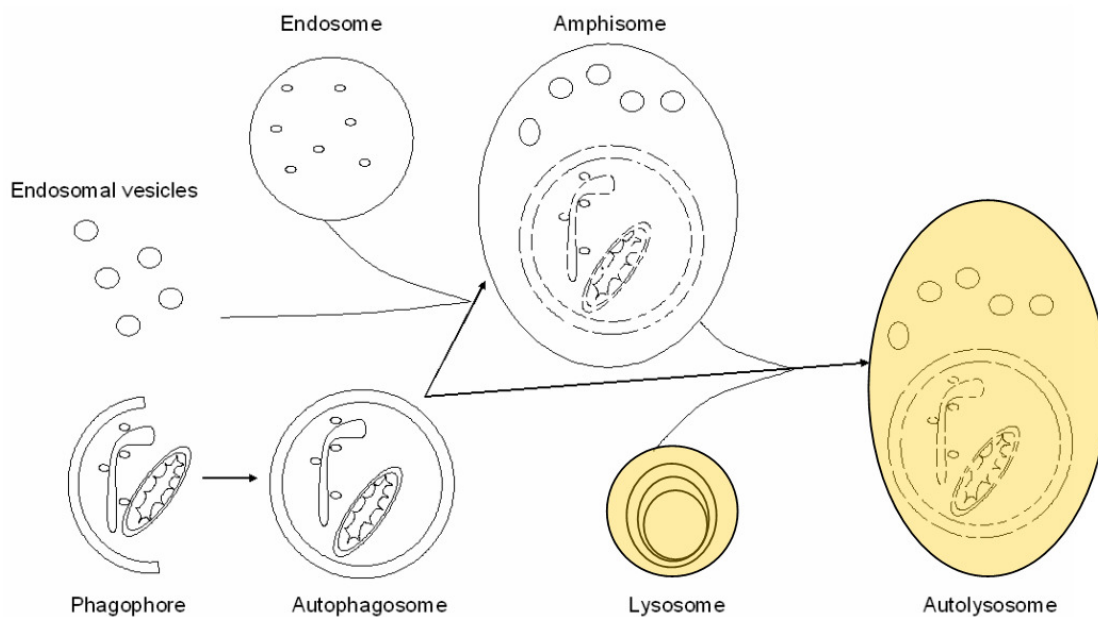


Figure 1.11: Schematic representation of the formation of autophagic vacuoles (Adapted from Eskelinen 2005(156)).

Cells in the early stages of autophagy contain several autophagic vacuoli, and both the nucleoplasm and the cytoplasm appear slightly darkened while the nuclear structure still appears normal. Mitochondria and the endoplasmic reticulum are sometimes dilated, and the Golgi apparatus is often enlarged. The plasma membrane loses specializations such as microvilli and junctional complexes, and blebbing can occur. During late stages, both the number and size of vacuole increase, and many of them contain myelin figures or are filled with lipids, which appear as pale gray inclusions in the cytoplasm under electron microscopy(156). The nucleus of a cell undergoing autophagic cell death can become pyknotic and identifiable as such by light and

fluorescence microscopy. However, nuclear condensation is not as common or as remarkable as that of apoptosis(156).

Interactions between apoptotic and non-apoptotic cell death pathways are common and may inhibit each other or act in synergy to induce cell death. It is therefore important to understand whether autophagic as well as apoptotic processes are activated in response to treatment with anticancer compounds in an effort to understand the mechanism of action and improve the efficacy of the treatment.

Current compounds targeting the bioenergetic alterations of cancer cells: Increase in acid formation and carbonic anhydrase IX as a target.

The metabolic environment in solid tumors has several characteristics including acidosis (157). The mechanisms responsible for the acidotic microenvironmental conditions include abnormal vasculature leading to poor circulation, increased hypoxia leading to the activation of glycolysis and the formation of lactic acid and the increased formation of carbonic acid as a result of increased expression of extracellular membranous CAIX and CAXII (157). Carbonic anhydrases are zinc enzymes that catalyze the conversion of carbon dioxide and water to carbonic acid (158). CAIX is overexpressed in a variety of tumors (69). The CAIX gene contains a hypoxia-response element (HRE) that binds active HIF-1 α and induces the transcription of CAIX (69). The characteristic hypoxic microenvironment of tumors together with the HRE of the CAIX gene explains the over expression of CAIX in a variety of tumors (69).

The over expression of CAIX contributes to the acidification of the extracellular microenvironment. Acidic extracellular pH in turn contributes the breakdown of the basement membrane as well as the induction of the expression of proteinases which facilitate invasion and metastasis(159). Selective inhibition of CAIX as well as other cancer-associated carbonic anhydrases provides a valuable strategy for curtailing the development of metastatic processes associated with acidotic microenvironmental conditions in tumors.

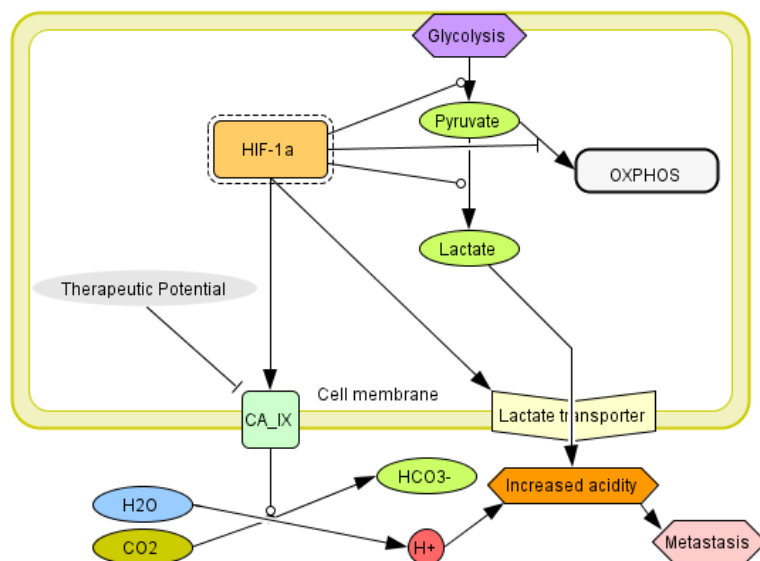


Figure 1.12: Targeting CAIX to prevent metastasis. By blocking CAIX-mediated pH regulation, reduction in extracellular acidosis could decrease tumor survival and metastatic processes associated with acidotic microenvironmental conditions in tumors.

Utilizing virtual screening docking software to identify potential new compounds targeting molecular biological and bioenergetic alterations of cancer cells

Virtual screening (VS) is a method that ranks a set of molecules through the use of a scoring method (160). Various docking methods have been developed for VS and it is one of the most common methods used to attempt to predict the binding modes of novel ligands to protein structures (161). In order for VS to be successful, a protocol needs to be developed that assigns good scores to active ligands and bad scores to inactive ligands. The successful docking of a ligand into a protein relies on two complementary components of the docking software (161). Firstly, a method to successfully explore all the possible conformational changes a ligand and protein can undergo is needed. Secondly, a scoring function is needed to accurately measure the binding energy of a particular pose. A successful docking run would thus assign the best score to the native pose observed in an accurate crystal structure. The accuracy of a docking pose is quantified by calculating the root mean square deviation (RMSD) between the docked ligand and the ligand in the crystal complex structure.

The best indicator of the accuracy of a VS protocol is the ability to identify novel compounds that are experimentally confirmed and AutoDock4, DOCK, GOLD, FlexX and Glide have been very successful (161). The accuracy of VS in comparative studies have shown that there is not yet a single program that is universally accurate for extracting active compounds from a library of compounds (161). The accuracy of VS is protein dependent and no VS docking program outperforms another consistently (161). However, VS does provide enrichments of known active compounds over known inactive compounds and it is still worth running a VS as a guide to obtain leads in a library of novel ligands (161). Two docking software suites, DOCK and AutoDock4, will be used for VS.

Many new medicinally relevant compounds have been successfully identified with these programs (162). Utilizing a VS protocol by making use of freely available docking software is a commercially viable and sensible method to identify novel, more potent compounds that are able to target molecular biological (microtubule dynamics interferences) and bioenergetic (carbonic anhydrase) alterations associated with cancer cells.

Relevance and aim of the study

The clinical usefulness of antimetabolic compounds that interfere with microtubule dynamics via the colchicine binding site, including 2ME, chalcones and combretastatins, are currently under investigation (163). Mediocre biopharmaceutical properties such as short half-life and low bioavailability of 2ME have prompted the research and development of estradiol analogues with improved *in vivo* efficacy (164-168).

The aim of this research is three-fold:

- 1) To utilize academically available bioinformatics software to develop an *in silico* protocol in order to identify potential inhibitors of microtubule dynamics and carbonic anhydrase II and IX.
- 2) To synthesize the identified novel compounds with potential inhibitory properties towards microtubule dynamics and carbonic anhydrase II, IX and XII.

3) To evaluate and elucidate the mechanism of action of these newly synthesized antimetabolic compounds on three cell lines.

Information generated from this study will also provide a basis for future research projects in not only testing various hypotheses for the mechanism of these newly synthesized compounds but also motivate further pre-clinical research on these compounds.