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DEVELOPMENT OF THERAPEUTIC STRATEGIES FOR QUIESCENT TUMOR CELL POPULATIONS

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Development of therapeutic strategies for quiescent tumor cell populations
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博学而笃志，切问而近思，仁在其中矣。

——《论语·子张》

To my family

ABSTRACT

Phenotypic screening is an effective approach to the discovery of small-molecule drugs. In this thesis, I have used cancer cells grown as 3D microtissues (multicellular spheroids) to study the effect of anticancer drugs and as drug discovery tools. Most clinically used cytotoxic drugs have only modest efficacy on spheroids, possibly explaining their limited efficacy on many solid tumors. We therefore used spheroids as targets for screening campaigns aimed to discover novel anti-cancer drugs. This work resulted in the identification of compounds that showed preferential cytotoxicity to spheroids compared to monolayer cultures. We also used multicellular spheroids to study regrowth of cells after cytotoxic therapy.

The compound VLX600 was identified in our spheroid screening work. This drug was found to induce autophagy and upregulation of glycolysis in tumor cells. Further work showed that VLX600 is an inhibitor of mitochondrial oxidative phosphorylation. The work has lead to the hypothesis that cells in the deep tumor parenchyme are sensitive to disturbances of energy metabolism due to lack of metabolic plasticity. Our studies demonstrated that VLX600 also decreased the levels of c-MYC. This phenomenon was also observed after treatment with other mitochondrial inhibitors. An additional screen was performed using glucose-deprived spheroids. This screen identified five molecules that showed selectivity to spheroids. All five drugs were found to inhibit mitochondrial respiration. The FDA-approved antiprotozoal drug nitazoxanide was chosen for further studies and may be a candidate for future clinical trials.

Increased glycolysis in tumor cells leads to the generation of metabolic acids and a subsequent acidification of the microenvironment of solid tumors. We found that tumor acidosis leads to induction of autophagy and present evidence that autophagy is a mechanism for cancer cells to adapt to an acidic environment.

We conclude from this work that multicellular spheroids can be used to screen for novel anticancer agents. Several drugs identified by this approach were found to be inhibitors of mitochondrial function. This finding suggests a therapeutic strategy to target quiescent tumor cells in metabolically compromised microenvironments.

LIST OF SCIENTIFIC PAPERS

I. The PI3K/mTOR inhibitor NVP-BEZ235 is effective in inhibiting regrowth of tumor cells after cytotoxic therapy

Emma Hernlund, Maria Hägg Olofsson, Walid Fayad, Mårten Fryknäs, Karolina Lesiak-Mieczkowska, **Xiaonan Zhang**, Slavica Brnjic, Vivien Schmidt, Padraig D'Arcy, Tobias Sjöblom, Angelo De Milito, Rolf Larsson & Stig Linder.
European Journal of Cancer 48, 396–406 (2012)

II. Chemical biology identifies induction of mitochondrial dysfunction as a strategy for targeting tumor cells in hypoxic and nutrient limited microenvironments

Xiaonan Zhang*, Mårten Fryknäs*, Emma Hernlund, Walid Fayad, Angelo De Milito, Maria Hägg Olofsson, Vladimir Gogvadze, Long Dang, Sven Påhlman, Leoni A. Kunz Schughart, Linda Rickardson, Padraig D'Arcy, Joachim Gullbo, Peter Nygren, Rolf Larsson & Stig Linder.
Nature Communications DOI: 10.1038/ncomms4295 (2014).

III. Autophagy is a protective mechanism for human melanoma cells under acidic stress

Maria Lucia Marino, Paola Pellegrini, Giuseppe Di Lernia, Mojgan Djavaheri-Mergny, Slavica Brnjic, **Xiaonan Zhang**, Maria Hägg, Stig Linder, Stefano Fais, Patrice Codogno and Angelo De-Milito.
Journal of Biological Chemistry 287, 30664–30676 (2012).

IV. Effects of mitochondrial inhibitors on c-Myc expression: consequences for drug discovery projects

Xiaonan Zhang, Padraig D'Arcy, Ganna Oliynykc, Marie Arsenian-Henriksson and Stig Linder
Manuscript

V. Three-dimensional cell culture-based screening identifies the anthelminthic drug nitazoxanide as a candidate for treatment of solid tumors

Wojciech Senkowski, **Xiaonan Zhang**, Maria Hägg Olofsson, Ruben Isacson, Urban Höglund, Mats Gustafsson, Peter Nygren, Stig Linder, Rolf Larsson and Mårten Fryknäs.
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OTHER PAPERS, NOT INCLUDED IN THE THESIS

I. Induction of tumor cell apoptosis by a proteasome deubiquitinase inhibitor is associated with oxidative stress

Slavica Brnjic, Magdalena Mazurkiewicz, Mårten Fryknäs, Chao Sun, **Xiaonan Zhang**, Rolf Larsson, Pádraig D'Arcy and Stig Linder.
Antioxidants & Redox Signaling 21, 2271-2285 (2014)

II. Label-free detection and dynamic monitoring of drug-induced intracellular vesicle formation enabled using a 2-dimensional matched filter

Obaid Aftab, Mårten Fryknäs, **Xiaonan Zhang**, Ulf Hammerling, Stig Linder, Rolf Larsson and Mats G Gustafsson
Autophagy 10, 57–69 (2013)

III. The 19S deubiquitinase inhibitor b-AP15 is enriched in cells and elicits rapid commitment to cell death.

Xin Wang, William Stafford, Magdalena Mazurkiewicz, Mårten Fryknäs, Slavica Brjnic, **Xiaonan Zhang**, Joachim Gullbo, Rolf Larsson, Elias S. J. Arnér, Padraig D'Arcy, and Stig Linder.
Molecular Pharmacology 85,932–945 (2014)

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LIST OF ABBREVIATIONS

2-DG	2-deoxy-D-glucose
2-ME	2-methoxyestradiol
3-BrPA	3-bromopyruvate
3-MA	3-methyladenine
ADP	Adenosine diphosphate
AML	Acute myelogenous leukemia
AMPK	AMP activated kinase
ANT	Adenine nucleotide translocase
ATG	Autophagy-related protein
ATP	Adenosine triphosphate
bHLH–ZIP	Basic helix–loop–helix–leucine zipper
BIP	Binding immunoglobulin protein
C-map	Connectivity map
CQ	Chloroquine
CRD-BD	Coding region determinant-binding protein
CSC	Cancer stem cell
DARTS	Drug affinity responsive target stability
DNA	Deoxyribonucleic acid
Drp1	Dynamin-related protein 1
ECAR	Extracellular acidification rate
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
ETC	Electron transport chain
FDA	U.S. Food and drug administration
FdG	^{18}F-Fluorodeoxyglucose
GC	Gastric carcinoma
GFP	Green fluorescent protein
GPx	Glutathione peroxidase
GSEA	Gene set enrichment analysis
GSH	Glutathione
HIF1	Hypoxia-inducible factor 1
HSP	Heat shock protein
IDH	Isocitrate dehydrogenase

KRAS	Kirsten rat sarcoma viral oncogene homolog
LDHA	Lactate dehydrogenase A
lncRNA	Long none-coding RNA
MDR1	Multi-drug resistance
MHOK	Methyl-13-hydroxy-15-oxokaurenoate
MMP	Mitochondrial membrane permeability
mtDNA	Mitochondrial DNA
mTOR1	Mammalian target of rapamycin complex 1
MTS	Multicellular spheroids
MYC	Myelocytomatosis oncogene
NADPH	Nicotinamide adenine dinucleotide phosphate
nDNA	Nuclear DNA
OCR	Oxidative consumption rate
OXPHOS	Oxidative phosphorylation
P-gp	P-glycoprotein
PET	Positron emission tomography
PI3KCA	Phosphatidylinositol 3-kinase catalytic subunit
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SOD	Superoxide dismutase
TEM	Transmission electron microscopy
TFAM	Transcription factor A of mitochondria
TME	Tumor microenvironment
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 CANCER—A COMPLEX AND CHRONIC DISEASE

1.1.1 What is cancer?

A report from the nation's leading cancer organization has shown that, in the United States only, there were ~ 1,665,000 new cases (not including non-melanoma skin cancers) and ~ 585,000 cancer-related deaths in 2014.[1,2] Cancer Research UK has reported that nearly 76,000 people die from cancer each year in the UK (more than 40 per cent of the total individuals who die early), compared to 28,000 who die from heart disease (15 per cent) and 17,000 from respiratory diseases (9 per cent).[3-5] Cancer has become one of the biggest killers in world.

Records of cancer can date back to the days of the great pyramids.[6] The first description about cancer can be traced back to 3000 B.C., although the word "cancer" was not used. In an ancient Egyptian text, 8 cases of tumors were described and the conclusion about the disease was sadly made as "There is no treatment".[7]

The word "cancer" was initially used by the Greek physician Hippocrates (460-370 BC), the person who is regarded as the "Father of Medicine". Hippocrates used the terms *carcinos* and *carcinoma,* which were referred to a crab in Greek and later translated into *cancer,* to describe non-ulcer forming and ulcer-forming tumors.[7,8]

As late as 30-40 years ago, our understanding of cancer was limited (the "riddle of cancer"). We could only see tumors when they were already big enough, weigh them, and calculate its size, but few tools could be used to really know what had happened inside of cancer cells. Some talent researchers were ahead of their periods, for example Rudolf Virchow, a German doctor and known as the "People of medicine", used microscope firstly observed the blood disease and now named as leukemia as well as firstly brought up the theory that cancers came from the over-active dormant cells.[9] Stephen Paget, the scientist who in 1889 brought up the seed-and-soil hypothesis of metastasis. According to his theory, tumor cells were compared to seeds and distant organs were compared to soil: "When a plant goes to seed, its seeds are carried in all directions but they can only live and grow if they fall on congenial soil."[10,11] The comprehension of biology and genetics was for many years too primitive to allow an understanding of the causes of cancer. Oswald Avery showed in 1944 that genetic information was transmitted by deoxyribonucleic (DNA) and not by proteins.[12] A decade later Watson and Crick published a model for the structure of DNA.[13] Research in the 1970s and 1980s subsequently showed that cancer is a disease induced by gene mutations.

Cancer is now recognized as a complex disease where abnormal cells divide out of control and may acquire the ability to invade and to spread to other tissues,[1] the underlying cause of this abnormal phenotype is mutations in the genome.[1,8,14,15] The mutations of these genes lead to defects in proteins that control signalling pathways that relate to cell growth, proliferation, metabolism.[14,16-18]

In 2000, Hanahan and Weinberg described the *six hallmarks of cancer*[19] to describe the multistep development of human tumors: self-sufficiency in growth signalling, insensitivity to anti-growth signals, evading apoptosis, sustained angiogenesis, endless proliferation and tissue invasion metastasis.[19] In 2011, Hanahan and Weinberg further developed their theory: their acquisition is made possible by *the enabling characteristics*—the development of genomic instability and tumor-promoting inflammation. And *the emerging hallmarks*: reprogramming energy metabolism and avoiding immune destruction.[20] An overview of the hallmarks of cancer is shown in *Figure 1*.

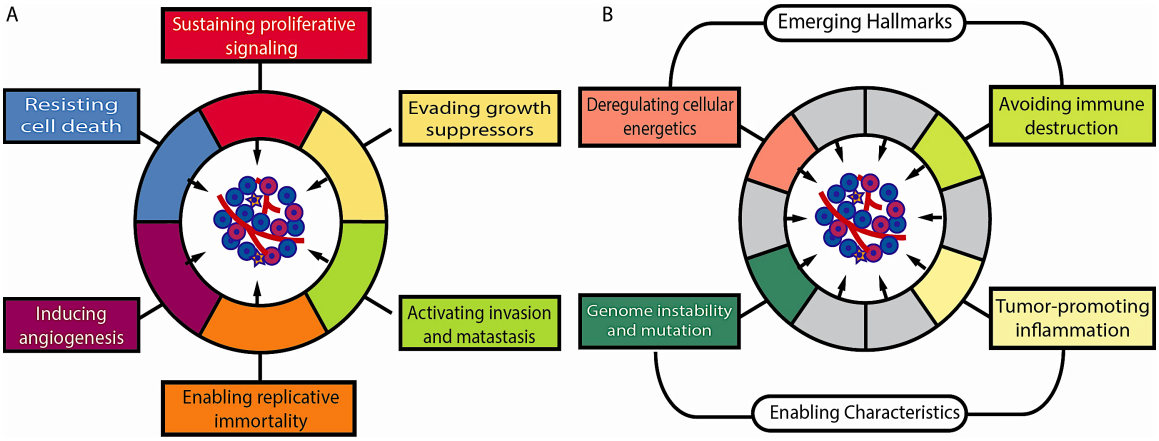

Figure 1: The hallmarks of cancer and enabling characteristics A) Illustration of the six hallmark capabilities originally proposed in 2000 perspective. B) Further illustration of enabling characteristics and emerging hallmarks in 2011.[19,20]

Cancer **types** can be grouped into broader categories. The main categories of cancer are *Carcinoma* – a malignant tumor composed of epithelia cells. *Sarcoma* - cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue; *Leukemia* - cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood; *Lymphoma and myeloma* - cancers that begin in the cells of the immune system and *Central nervous system cancers* - cancers that begin in the tissues of the brain and spinal cord. Cancer **names** can always be based on where they start, for example, cancer that begins in the breast is called breast cancer; cancer that starts in lung is lung cancer.[1,21-25]

1.1.2 Cancer metabolism

Cancer cells have for many years been noted to have alterations in their metabolism. This phenomenon, observed by researchers nearly 100 years ago, now is termed one of the fundamental hallmarks of cancer.[26] Normal cells produce adenosine triphosphate (ATP) mainly by glycolysis together with oxygen consumption from the highly efficient mitochondrial oxidative phosphorylation (OXPHOS). Already in the 1920s Otto Warburg

demonstrated that tumor cells had elevated glucose consumption compared to normal cells, but glucose did not form H_2O and CO_2 that were generated from OXPHOS, but instead formed lactate from pyruvate. This increase in lactate formation occurred no matter whether there was sufficient oxygen or not. This phenomenon was termed the "Warburg effect" or aerobic glycolysis.[27] Clinical research have indicated that glycolysis-related genes are found overexpressed in almost 70% of all human cancers,[28] The increased uptake of the glucose analogue ^{18}F-fluorodeoxyglucose by cancer cells can be utilized for diagnostic purposes.[29] The enhanced glycolytic flux may confer a growth advantage in tumor cells. Recent findings suggest a more complex situation where cancer cells take up more glucose but at the same time they still depend on functional mitochondria and OXPHOS to meet their high energy demands.[30-35] I will return to this theme later in this thesis.

In addition to up-regulation of glycolysis, glutaminolysis also increases in order to sustain nicotinamide adenine dinucleotide phosphate (NADPH) regeneration. Glutamine carbons replenish the Krebs cycle, yet produces metabolites that can be used for macromolecular biosynthesis, like nucleic acids, proteins, and carbohydrates.[36-39] It has also been shown that in addition to glucose and glutamine, which are involved in supporting energy metabolism and anabolic processes, one-carbon molecules, which are mainly refueled by the chemical reactions of folate compounds, also play important roles in integrating cellular nutrient status.[40,41] Reactions carry out in a cyclical path and during reactions, a carbon unit is transferred to other metabolic pathways and eventually generated diverse outputs, including redox maintenance and cellular biosynthesis.[28,42,43 44,45] *Figure 2* is a simple overview of metabolic activities in cancer cells.[46]

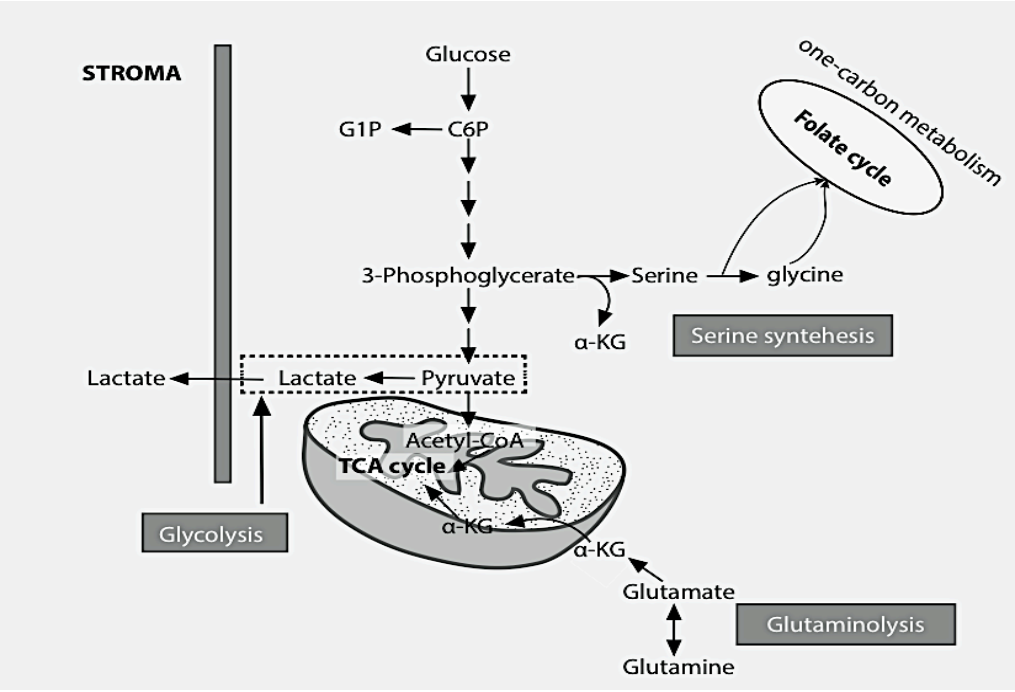

Figure 2: An overview of metabolic activities in cancer cells. Glycolysis and glutamiolysis are up regulated in tumor cells. One-carbon molecules that can be from serine and glycine metabolism also play important roles in integrating cellular nutrient status.

The underlying reasons for the metabolism changes of cancer cells have been pursued by many researches. Metabolic reprogramming may be a consequence of "survival of the fittest". First, in solid tumors, cancer cells distant from blood vessels will not be able to obtain sufficient oxygen for OXPHOS to generate enough ATP to sustain their increased energy. In addition to energy production, tumor cells also require various building block molecules for anabolic processes, which can be fulfilled by glycolysis and glutaminolysis.[14,16-18,38,47,48] Last but not the least, lactate, the product of glycolysis, plays a key role in tumor progression, tumor metastasis, immune escape and resistance to cancer therapy.[29,49-52] All these factors may be the reasons for the metabolic changes of cancer cells.

1.1.3 Tumor microenvironment (TME) (solid tumor)

The microenvironmental physiology of tumors can be characterized by hypoxia, extracellular acidosis and nutrient deprivation,[53] which are quite different from normal cells.

1.1.3.1 Hypoxia

Hypoxia is a characterization of solid tumors that can be classified into *hypoxic hypoxia* and *chemical hypoxia*.

Hypoxic hypoxia

The presence of hypoxic hypoxia in human solid tumors, which is due to an imbalance between the supply and consumption of oxygen, was firstly proved by Thomlinson and Gray some 50 years ago.[54] In clinical, investigators define the regions, where [pO2 values] $\leq$ 2.5 mm Hg, as hypoxic areas in solid tumors. The major causes involved in the process of hypoxic hypoxia are:

(1) The tumor microvessels have severe structural and functional abnormalities (perfusion-limited O_2 delivery), which are often transient.[53,55]

(2) A degeneration of the diffusion geometry (diffusion-limited O_2 delivery), which can be induced by an increase of the diffusion distance to blood vessels.[55,56]

(3) The decreased surface to volume ratio S/V (S: surface area, V: the volume of the viable rim, $S=4\pi r^2$ and $V=4/3\pi r^3$ (r= radius), the ratio of S/V is 3/r) thus restricting the area through which oxygen diffuse into solid tumors (this conclusion was from the spheroids study).[57,58]

Tumor hypoxic hypoxia causes a severe problem in radiation therapies, because the low pO_2 value reduces the ROS-mediated fixation efficacy of DNA damage.[59-61]

Tumor cells in hypoxic regions are also considered to be resistant to most anticancer drugs. Firstly, the distance from blood vessels and the limited drug permeabilization ability (i.e. doxorubicin) make tumor cells not adequately exposed to anticancer reagents. Secondly, cancer cell proliferation is often impaired in hypoxic regions, due to the growth arrest by

HIF-1α dependent induction of p21, p27 and p53.[62] Drugs that target proliferating tumor cell will not be effective on these quiescent cancer cells.[63,64]

Chemical hypoxia

The definition of chemical hypoxia is that the accumulating of reductive equivalents caused inhibition of respiration chain in mitochondria and it is independent of the microenvironmental oxygen level.[65,66,49]

Such reducing equivalents include *endogenous molecules*, like glutathione (GSH) or pyruvate, as well as *exogenous pharmacological substances*, like inhibitors of mitochondrial respiration chain. A common effect of chemical hypoxia is the shifting cellular redox state towards a strongly reduced cellular environment by reductive molecules, which is also able to interrupt ROS-mediated fixation of DNA damage.[49]

1.1.3.2 Extracellular acidosis

Intensive glycolysis in tumor cells will result in extracellular acidosis. Under glycolysis, glucose does not form H_2O and CO_2 from OXPHOS, but lactate from pyruvate. The production of lactic acid leads to acidification in tumor microenvironment.[17,53,67-70]

The acidic pH of the tumor microenvironment is believed to play an important role in tumor metastasis, genetic instability and malignant progression.[71-76] Data from clinical studies has shown that high lactate concentrations are associated with high incidence of distance metastasis and low survival probability in both human cervix carcinoma and human head & neck cancer.[52,77-79] Moreover, some anticancer drugs that are always work at physiological pH (≈7.4) can be ineffective at low pH. For example, chloroquine (CQ), which is currently evaluated in clinical trials, shows anticancer effect in neutral pH, yet, does not work well in acidic environment.[73]

1.1.3.3 Nutrient deprivation

Tumors are usually characterized by nutrient deprivation. This happens both in rapidly proliferating areas and quiescent tumor cells that are far from blood vessels, due to inefficacy of nutrition diffusion.[63,80-82] Experiments using the multicellular spheroids (MTS) model with quantitative bioluminescence and single photon imaging have shown that there is a strong decrease of glucose concentrations from the outer-rim of spheroids towards the core, from > 21 μmol/g to ≈ 0 μmol/g.[83-86]

Hypoxia, extracellular acidosis and nutrient deprivation associate with each other and the relations between them are addressed in *Figure 3*.[87]

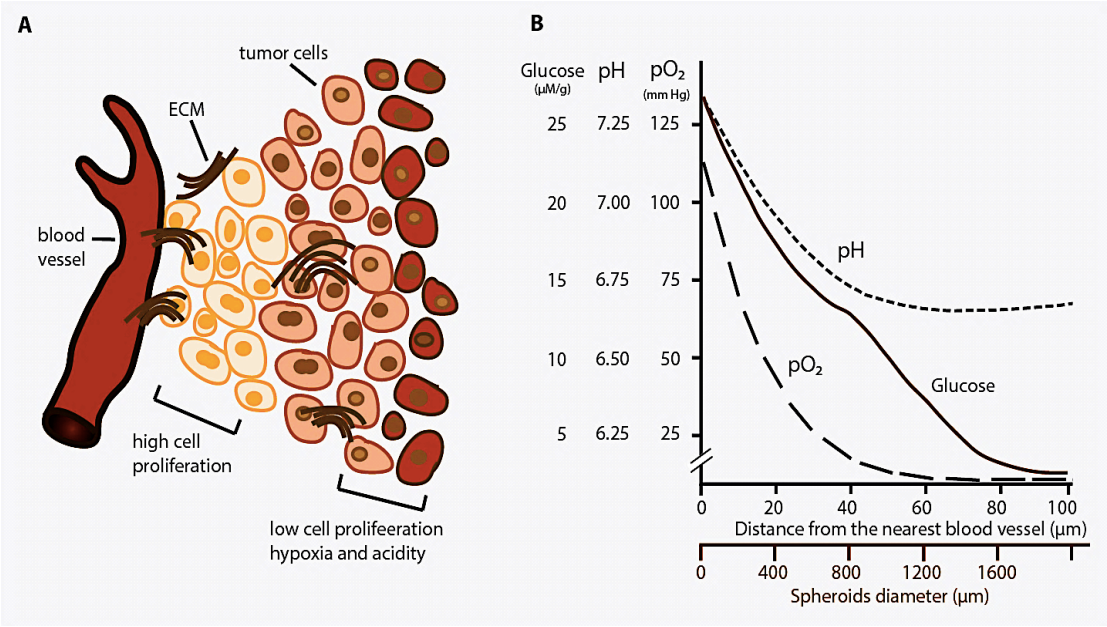

Figure 3: An overview of the tumor microenvironment. A) Diagrammatic representation of tumor cells surrounding a capillary. B) Schematic representation of the gradient of oxygen concentration, pH and glucose concentration.

1.1.4 Cancer characterizations

Cancer cells reprogram their biological functions at different levels in order to meet demanding on their high proliferation and overcome cell death after cancer treatments. Here, several different characters, which are also important considerations in my study, are described below:

1.1.4.1 c-MYC is de-regulated in many tumors

The *CMYC* proto-oncogene, which was first discovered as an inducer of retrovirally mediated tumorigenesis, belongs to the myelocytomatosis oncogene family (MYC) that also contains *MYCN* (N-myc) and *MYCL* (L-myc),[88-90] which evolve from myc-like genes found in drosophila and hydra.[91,92] In human, the *c-MYC* oncogene is overexpressed in at least 40% of cancers and serves as a "master regulator" of cellular metabolism and proliferation.[89,93,94]

C-MYC is an early response gene to growth factor stimulation. As *c-MYC* is able to cause tumorigenesis, its activation in many normal cells is always restrained through multiple genetic and epigenetically controlled checkpoint mechanisms, including proliferative arrest, apoptosis, and cellular senescence. For example, in the absence of nutrients, *c-MYC* gene expression is down regulated in normal cells in order to stop proliferation. In contrast, when *c-MYC* is oncogenically activated, for example, by chromosomal translocation or gene amplification, *c-MYC* bypasses these restrained checkpoints and enforces many of the “hallmark” features of cancer, including unlimited cellular proliferation, a constitutive biomass accumulation, altered cellular metabolism and a condition addictive to nutrients.[95-101]

C-MYC, the oncoprotein encoded by *c-MYC* gene, is a basic helix–loop–helix–leucine zipper (bHLH–ZIP) transcription factor whose known biological activities require interactions with the bHLH–ZIP protein Max.[102,103] The MYC-Max complex is involved in the control of a wide array of cellular activities (*Figure 4*),[94] like cell mass regulation, cell survival and is a master regulator of metabolic processes including glycolysis, oxidative phosphorylation and mitochondrial biogenesis.[92,104,105]

The first suggestion that c-MYC has a great importance in regulation of glycolysis is the observation that lactate dehydrogenase A (LDHA), which converts pyruvate to lactate as part of the glycolytic pathway, is a c-MYC target.[106,107] Mitochondrial generation of acetyl-CoA is dependent on c-MYC.[108,109] Moreover, it has been reported that c-MYC directly regulates mitochondrial transcription factor A (TFAM), which encodes a key factor involved in mitochondrial transcription and mitochondrial DNA (mtDNA) replication.[110] Interestingly, c-MYC recently has been shown to be able to alter glutamine metabolism by transcriptionally repressing miRNAs, like miR-23a and miR-23b.[111-113]

Since c-MYC oncoprotein takes part in different kinds of important cellular processes and biological functions in tumor cells, researchers have attempted to inhibit its activity trough different approaches.[104] For example, JQ1, which is a selective inhibitor of bromodomain protein, leads to downregulation of c-MYC.[111,114-118] Researchers also have attempted to use *c-MYC* antisense oligonucleotides, which has been encouraging *in vitro* but failed to translate into effective clinical treatments.[119-121] Moreover, in some experiments, c-MYC suppression has initially resulted in a tumor regression but subsequently recurred.[122] The c-MYC-Max complex has also been reported to be a target for pharmacological inhibitors. The small molecule 10058-F4 has been reported to specifically inhibit the c-MYC-Max complex formation and the growth of c-MYC overexpressed cells *in vitro* (>50μM),[92,123] No drug that targets c-MYC has been shown a strong antitumor effect in animal models, and no such drug has to my knowledge been tested in clinical trials.

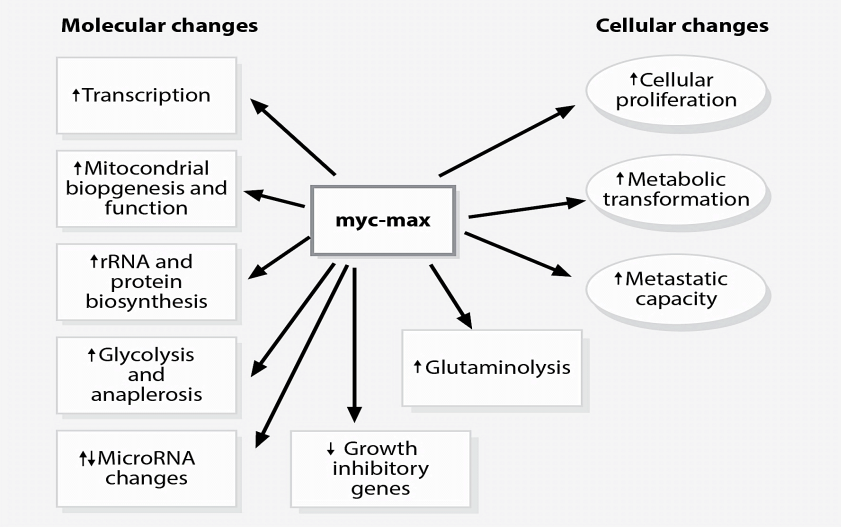

Figure 4: Myc-max complex affects almost every activity of cell life.

1.1.4.2 Autophagy is up-regulated in tumors

Autophagy, which is a catabolic process used by eukaryotic cells to recycle long-lived proteins, lipids, and clear up protein aggregates and organelles, is up-regulated for tumor cell survival in acidic and nutritionally deprived environments.[71,124-126] Recent studies have revealed the importance of autophagy in development,[127,128] aging,[129] and pathophysiology such as neurodegenerative disease, obesity and cancer.[130-134] The mammalian target of rapamycin complex 1 (mTORC1) is a major checkpoint in signalling pathways and regulates autophagy. It integrates signalling through the PI3K/Akt pathway and cellular nutrient status or energy levels, which are sensed by AMP-activated kinase (AMPK). 4E-BP1 and p-70-S6 kinase, which regulate protein synthesis, are the key downstream effectors of mTORC1.[135-137] Decreases of their phosphorylation termed a sign of mTOR inhibition and autophagy activation in cells.[138,139]

Autophagy starts from the formation of a double membrane-bound autophagosome mediated by autophagy-related protein (ATG) proteins.[130,140] and the whole process can be simply described as *Figure 5*: A portion of cytoplasm, including organelles, is enclosed by a phagophore to form an autophagosome. The outer membrane of the autophagosome first fuses with the endosome and then with the lysosome. The internal material is then degraded in matured autolysosome.[126,141-143]

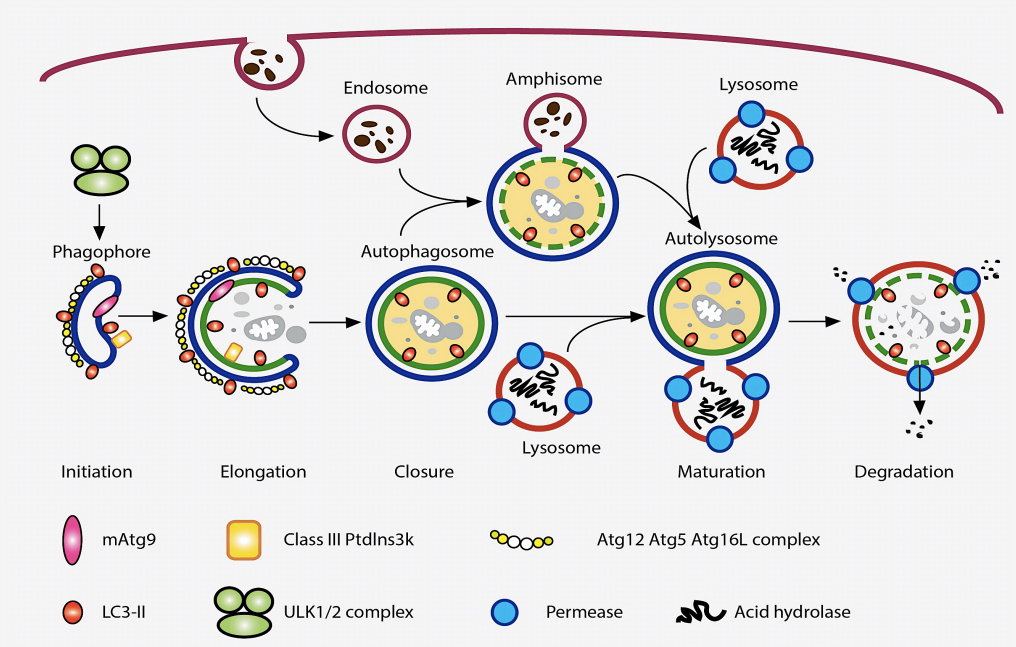

Figure 5: The process of autophagy in mammalian cells. A portion of cytoplasm, including organelles, is enclosed by a phagophore to form an autophagosome. The outer membrane of the autophagosome first fuses with the endosome and then with the lysosome. The internal material is then degraded in matured autolysosome.

In cancer progression, autophagy plays a complex role at different stages. At the tumor initiation stage, autophagy is a tumor suppressor in order to limit the production of reactive oxygen species (ROS) and DNA damage.[125,132] However, autophagy is used as a survival

pathway by tumor cells to overcome metabolic stress and cell death in response to the changing microenvironment and cancer therapies.[71,133,134] Because of this, autophagy is viewed as a druggable process in cancer treatment.[142,144-149] For example, 3-methyladenine (3-MA), which is the most widely used autophagy inhibitor for *in vitro* experiments.[150,151] Chloroquine (CQ) which was firstly used for malaria also has shown an anti-cancer effect as an autophagy inhibitor.[73,152-155] The latest autophagy inhibitor lys05, a dimeric chloroquine, produces more potent antitumor activity as a single agent both *in vitro* and *in vivo* in several human cancer cell lines and xenograft models.[156,157]

1.1.4.3 Aerobic glycolysis in tumor cells

Glycolysis (from *glycose* [an older term for glucose] + *-lysis* degradation) is a metabolic pathway that converses per glucose to two pyruvates molecules, generating 2 molecules of ATP and 2 molecules of NADH.

Glucose + 2NAD$^+$ + 2ADP + 2P --> 2pyruvate + 2ATP + 2NADH + 2H$^+$ [158]

In most mammalian cells, glycolysis is inhibited by the presence of oxygen, which allows mitochondria to oxidize pyruvate to CO_2 and H_2O by OXPHOS system. This inhibition is termed the **Pasteur effect**, named after Louis Pasteur, who first demonstrated that glucose flux was reduced by the presence of oxygen.[159] Rapidly growing tumor cells typically have glycolytic rates that are up to 200 times higher than those of their normal cells and after glycolysis, pyruvate was finally fermented to lactate. This phenomenon was first described in 1927 by Otto Warburg and is referred to as the **Warburg effect**[27,160] and has later been confirmed by numerous investigators.[161-165]

As we know, glycolysis can only produce 2 ATP by digesting per glucose, whereas between 30 and 36 ATPs are produced by the oxidative phosphorylation.[166] And lactate, the production of glycolysis, causes an acidification of the extracellular space, which might result in cellular toxicity. So, why do tumor cells still prefer glycolysis?

With increased tumor size, cancer cells, gradually far from blood vessels, are not able to obtain sufficient oxygen and oxidative phosphorylation (OXPHOS) pathway will be impaired. The hypoxia-inducible factor 1 (HIF1), which is a hypoxia-generated signal and termed the key regulator of oxygen homeostasis,[167-169] actives the transcription of genes encoding glycolytic enzymes (such as phosphoglycerate kinase 1 and pyruvate kinase M).[168-171] The upregulation of glycolysis will have significant negative consequences, due to extracellular acidosis. Tumor cells upregulate Na^+–H^+ exchange and vacuolar H^+-ATPases, undergo mutations or epigenetic changes to adapt extracellular acidification.[172-175] When tumor cells bypass this crucial evolutionary selection, they are able to take advantage of the local acidic microenvironment, compete with normal cells and transit from pre-malignant lesions to invasive cancer (*Figure 6*).[18,47,53,176-178] At the same time, these biological changes also make tumor cells more resistance to cancer therapies.[68,70,179,180]

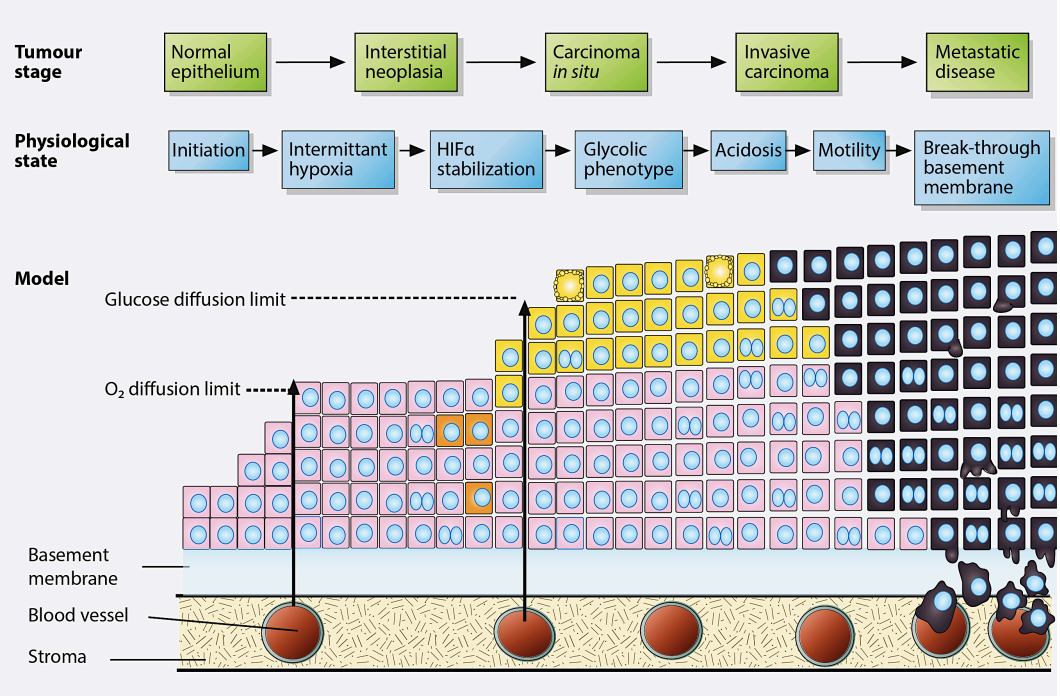

Figure 6: Function of glycolysis on cancer progression. Normal epithelial cells (pink) become hyper proliferative following induction (orange). As they reach the oxygen diffusion limit, they become hypoxic (yellow) and either lead to cell death or adaptation of a glycolytic phenotype, which allows cells to survive. As a consequence of glycolysis, lesions become acidotic, which selects for motile cells (black) that eventually breach the basement membrane.[47]

1.1.4.4 Mitochondrial function —not really impaired

The mitochondrial bacterium was engulfed around 1.5 billion years ago since then lived within eukaryotic cells as a symbiotic partner (endosymbiotic theory).[181] The mitochondrion (*Figure 7A*) is sometimes referred to as the ″powerhouse of the cells″.[182] The mitochondrial DNA (mtDNA) encodes the 13 most important OXPHOS genes, and the nuclear DNA (nDNA) contains all of the remaining OXPHOS genes, as well as the genes responsible for mitochondrial metabolism and biogenesis.[183,184]

The mitochondrion is a double-membrane organelle (*Figure 7B*). The electron transport chain (ETC) (*Figure 7C)* locates in the inner membrane. The ETC contains four components, complexes I, II, III, and IV, together with substrates, cofactors, and the paths of electron flow[183,185-187] to generate a proton gradient. This gradient is applied to produce ATP, which is later used as a source of chemical energy in mammalian cells.[188,189]

In addition to supplying cellular energy, mitochondria also are involved in other tasks such as signalling, cellular differentiation, cell death, as well as maintaining the control of cell growth. Until today, mitochondria have been implicated in several human diseases (e.g. Parkinson's, Alzheimer's, Lou Gehrig's disease) and may play a role in the aging process and termed one of the important organelles in mammalian cells.[190,191]

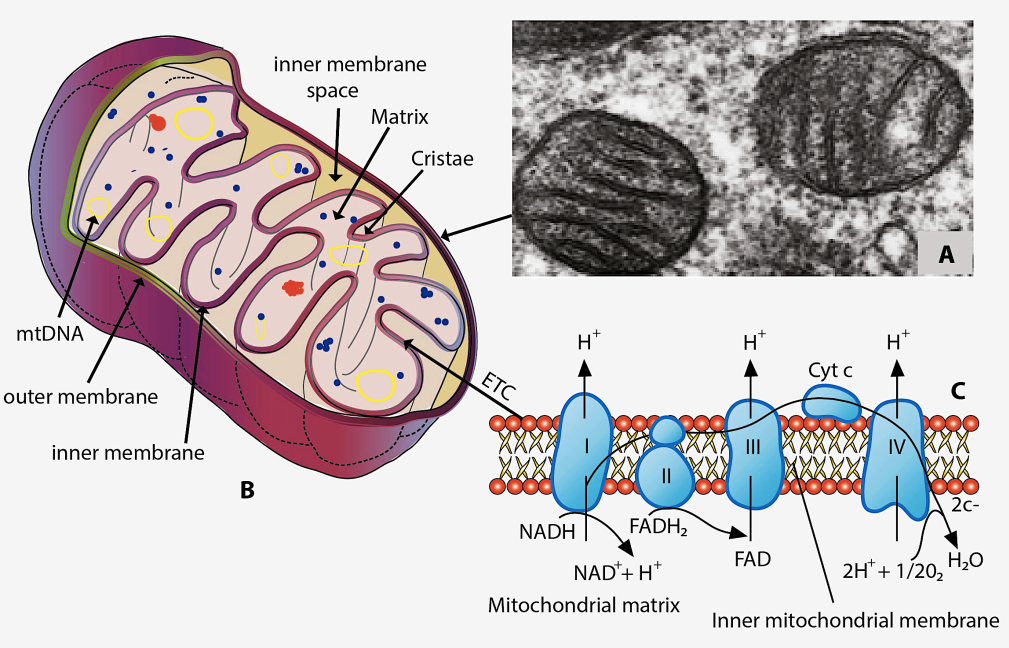

Figure 7: The structure and electron transport chain of mitochondria. A: Mitochondrial structure under transmission electron microscopy B: Basic structure of the mitochondrion. C: Basic components on the mitochondrial electron transport chain.

Warburg observed that glycolysis is up-regulated in tumor cells because of the impaired mitochondrial metabolism.[27] The result is true, but the reason now has been proven incorrect. In the pioneering studies, mitochondrial function was determined by measuring the products both from oxidative phosphorylation and glycolysis.[192-194] But it is worth noting that the glycolytic rate is usually determined by measuring the lactate production, whereas, during the reaction, the concentrations of the substrates and variations of oxygen concentrations *in vitro* and *in vivo* can both affect result of the measurements.[195-197] Some recent reports have shown that despite the accelerated glycolysis in many fast-growth tumor cells, its total contribution to the cellular ATP supply only reaches 10%.[196] This might indicate that the mitochondrial oxidative phosphorylation still take an important role in tumor cells. Another strong argument to mitochondrial impairment in cancer cells is from experiments of generating various mtDNA eliminated cancer cells (rho_0) by growing tumor cells in medium with ethidium bromide (EtBr). The rho_0 cancer cells show a dropped growth rate, decreased colony formation and markedly reduced tumor formation in nude mice.[198-204]

Mitochondrial mutations are observed in tumors, presumably in order to better meet altered metabolic demands. There are two classes of mutations in mtDNA of cancer cells: a) mutations that serve to stimulate neoplastic transformation and b) mutations that assistant cancer cells accommodating to the constantly changing bioenergetic environments.[205-208]

In addition to mtDNA mutations, many mitochondrial proteins or enzymes that are coded by nuclear DNA also become mutated in cancer cells. For example: the mutant isocitrate dehydrogenase (IDH) proteins decrease NADPH levels and then inhibit glutathione peroxidase (GPx) that can reduce H_2O_2 to water. The increased level of H_2O_2 can contribute

to neoplastic transformation.[209-214] The succinate dehydrogenase (SDH), which is a complex II protein on mitochondrial electron transport chain, is inhibited due to the mutation of its subunits. Subsequently, HIF1α is stabilized[215] and causes a shift from oxidative phosphorylation to glycolytic energy metabolism.[209]

It is likely that glycolytic and oxidative metabolisms undergo dynamic changes in proliferated and transformed cancer cells, as a result of varying selective pressures in terms of nutrients and oxygen availability. Even if OXPHOS becomes impaired, mitochondria still have a great importance in cell signalling, cell cycle and cell death regulation.[183,216-221] It is also true that mitochondria are controlled or regulated by other proteins (like CMYC, Hsp90 and VEGF),[222-231] cellular biological process (mitophogy and proteasome degradation)[228-231] in tumors.

In present researches, targeting mitochondria or its related substrates is regarded as a therapeutic strategy in cancer treatment. For example lonidamine, which is already used in clinic phase trial.[232-234] 3-bromopyruvate (3-BrPA), a lactate/pyruvate analogue, is an alkylating agent and a potent inhibitor of glycolysis.[235,236] 2-deoxy-D-glucose (2-DG), not targeting mitochondria, but is a glucose analogue that worked as a competitive inhibitor of glucose metabolism and can be combined with radiotherapies in cancer treatment.[237-239]

In Paper II, we show that a compound, which is identified in a screen for drugs and decreases tumor spheroids viability, is a mitochondrial inhibitor. In paper IV, we found that targeting mitochondria in tumor cells decreases CMYC levels. In paper V, we demonstrated that a number of drugs identified by screening multicellular spheroids are all mitochondrial inhibitors. These findings indicate that functional mitochondria are potential targets for cancer therapeutics.

1.2 CANCER TREATMENT—STILL ON THE WAY

1.2.1 Cancer treatment

In the early 20th century, only small and localized tumors could be completely removed by surgery. Later, radiation was used to treat tumors that could not be removed by surgery. The period of cancer chemotherapy began in the 1940s with the first use of nitrogen mustards to treat lymphomas and folic acid antagonist drugs on leukemia. [240,241] Later, chemotherapy was used to treat colon cancer, testicular cancer, and other tumors. Nowadays, cancer therapies include *surgery, radiation, chemotherapy, hormone therapy, immunotherapy and targeted therapies*. In clinic, a combination of therapies is frequently used in order to achieve optimal results.[21,242,243]

Chemotherapies generally target the process of DNA replication or target the cell division process. This results in toxicity to proliferating normal cells and cause side effects on patients. Targeted therapies are being developed which are more specific to tumor cells

(mutant receptors, activated kinases etc.). Targeted therapies work by influencing the processes (mostly proteins) that control growth, division, and spread of cancer cells, as well as the signals that cause cancer cells to die naturally, for example the proteasome inhibitor bortezomib (Velcade®) and the BCR-ABL inhibitor imatinib (Gleevec®) and etc.[242]

Over the past 25 years, more and more researches have indicated that many signalling pathways are involved together in tumor cell formation and progression. This leads to questioning the strategy that only target individual signalling molecules. More and more researchers start to agree that targeting metabolic differences between normal cells and tumor cells can be an applicable strategy in cancer therapy.[17]

1.2.2 Chemotherapeutic drugs

Chemotherapy has been used in anticancer treatment for about seventy years.[21] During the last 20 years, around 180 anticancer drugs have been approved by FDA and used in clinic.[244] Based on their chemical structure and mechanism of action, these drugs are classified as:

- Alkylating agents and platinum compounds
- Antibiotics
- Antimetabolites
- Topoisomerase I and II inhibitors
- Mitosis inhibitors
- Others

This kind of classification serves two main objectives: obtaining an overview of the available drugs and designing new combination therapies.[245-247]

1.2.2.1 Alkylating agents and platinum agents

Alkylating agents work by covalent modification of intracellular macromolecules. They may have one or two reactive groups (monofunctional or bifunctional agents). Monofunctional agents modify only one base in DNA and cause DNA single-stand breaks. Bifunctional agents form crosslinks between biological molecules and may cause DNA interstrand crosslinking. Alkylating agents directly damage DNA and prevent cancer cells from reproducing. These agents are not cell phase specific, since they damage DNA, tissues characterized by rapid proliferation, such as the bone marrow that can get long-term injury after alkylating agents treatment.[247,248] There are different classes of alkylating agents, including：

- Nitrogen mustards: mechlorethamine, chlorambucil, cyclophosphamide (Cytoxan®), ifosfamide, and melphalan
- Nitrosoureas: streptozocin, carmustine (BCNU), and lomustine
- Alkyl sulfonates: busulfan
- Triazines: dacarbazine (DTIC) and temozolomide (Temodar®)

- Ethylenimines: thiotepa and altretamine (hexamethylmelamine)

1.2.2.2 Antibiotics

This type of drugs interferes with enzymes involved in DNA replication and work in all phases of the cell cycle. They are widely used for a variety of cancers. A major consideration is that antibiotics can cause permanent damage to the heart if high doses is given.[248] Examples of antibiotics:

- Daunorubicin
- Doxorubicin (Adriamycin®)
- Epirubicin
- Idarubicin

1.2.2.3 Antimetabolites

Antimetabolites have structures that are similar to natural existing substances, such as amino acid or nucleosides. They usually compete with natural substrates for the active sites on key enzymes or receptors.[249,250] Antimetabolites can interfere with DNA replication and/or RNA transcription. These agents damage cells during the S phase. They are commonly used to treat leukemia, cancers of the breast, ovary, and the intestinal tract.[15,248,250] Examples of antimetabolites include:

- Fludarabine
- Gemcitabine (Gemzar®)
- Hydroxyurea
- Methotrexate
- Pemetrexed (Alimta®)
- Pentostatin
- Thioguanine
- 5-fluorouracil (5-FU)
- 6-mercaptopurine (6-MP)
- Capecitabine (Xeloda®)
- Clofarabine
- Cytarabine (Ara-C®)
- Floxuridine
- Cladribine

1.2.2.4 Topoisomerase I and II inhibitors

This type of inhibitors interact with enzymes called topoisomerases and are involved in a number of DNA-related events, such as DNA replication, transcription, recombination, chromosome condensation and segregation.[251-256] Topoisomerase inhibition results in DNA double strand breaks, inhibition of cell proliferation and/or apoptosis. These drugs are phase-specific and prevent cells from entering mitosis. Examples of topoisomerase inhibitors:

- Topoisomerase I inhibitors: Topotecan, Camptosar, Irinotecan and etc.
- Topoisomerase II inhibitors: Teniposide, Etoposide (VP-16), Doxorubicin, Daunorubicin, Amsacrine, Aurintricarboxylic acid, Mitoxantrone and etc.

1.2.2.5 Mitosis inhibitors (tubulin inhibitor)

Mitotic inhibitors are often plant alkaloids and other compounds derived from natural products. They can stop mitosis or inhibit enzymes from making proteins that are needed for cell reproduction.[257,258]

These drugs work during the M phase of the cell cycle but can damage cells in all phases. They are used to treat many different types of cancer including breast cancer, lung cancer, myelomas, lymphomas, and leukemias. Mitotic inhibitors have a potential to cause peripheral nerve damage, which can be a dose-limiting side effect.[248,259-261]

1.2.2.6 Mitochondrial inhibitors

The mitochondrion is the energy supplier in cells; it generates 80%–90% ATP for cell survival and plays an essential role in the integration of pro-apoptotic and anti-apoptotic stimuli. Cancer cells reprogram their mitochondrial function in order to meet their different energy requirements.[245,262,263]

Normally, there are two kinds of mitochondrial inhibitors in cancer therapies. *The first class* contains the drugs that directly target mitochondria and induce mitochondrial impairment or dysfunction. For example, 2-methoxyestradiol (2-ME), which is used in photodynamic anticancer therapy, causes an accumulation of ROS by inhibition of the superoxide dismutase (SOD) activity and ensuing cytotoxic effects.[245,264] Lonidamine is another anti-cancer drug that already used in Phase II pilot study of chemotherapy in patients with metastatic breast adenocarcinoma. It has a direct effect on adenine nucleotide translocase (ANT) that localized in the mitochondrial inner membrane and cause mitochondrial membrane permeability (MMP).[234,265,266] Metformin, used in diabetes before, recently has been reported as a mitochondrial complex I inhibitor.[267-269] *The second class* includes drugs that mitochondrial dysfunction is their second effects. For example, inhibiting topoisomerase II, which is extracted from mitochondria, induces mtDNA breaks.[270,271]

1.2.2.7 Targeted therapies

Nowadays, researchers also have begun to synthesize new drugs that attack cancer cells more specifically than traditional chemotherapy drugs. The new generation of chemotherapy drugs will specifically target mutant versions of certain genes or cells that express too many copies of a particular gene. These drugs can be used as part of the main treatment, or they may be used after treatment to maintain remission or decrease the chance of recurrence.[272-275] Examples of targeted therapies include:

- Imatinib (Gleevec®)— BCR-ABL inhibitors
- Cetuximab (Erbitux®)—EGFR inhibitors
- Vemurafenib (Zelboraf®)— BRAF inhibitors
- Bortezomib(Velcade®)— Proteasome inhibitors

1.2.3 Cancer resistance and relapse

Chemoresistance is a major problem and one main reason of failure in cancer treatment.[276] Normally, chemoresistance includes two different categories: intrinsic and acquired.

Intrinsic drug resistance is a term used to describe the fact that tumor cells may show natural resistance to certain therapies.[277]

One possible reason to explain the intrinsic drug resistance of tumor cells is their high levels of expression of p-glycoprotein (P-gp).[278-282] P-gp, an efflux pump, is the product of the *MDR1* (multi-drug resistance) gene in human. This protein can detect and bind many hydrophobic natural-product drugs (doxorubicin, vinblastine and etc.) when these drugs enter the plasma membrane and release them back into the extracellular space.[283-285] Recent study has indicated that, in solid growing tumors, extracellular acidosis leads to an increased P-gp activity, which is pH-dependent.[286]

Another possible reason is their high extracellular acidosis. It has been reported that lactate concentrations in term of μmol/g which can be considered mM in viable tumor regions.[83] And high extracellular acidosis results in the enhanced tumor anti-oxidant capacity that might impair anticancer drug efficacy, which works mainly by inducing ROS-mediated fixation of DNA in tumor cells.[49]

Acquired drug resistance means that tumor cells initially are sensitive to drugs but due to mutations and various adaptive responses during drug treatment, tumor cells gradually gain resistance and become insensitive to drugs.[277] Acquired drug resistance is a more complex cell response to anticancer drugs and a result of constantly selection for tumor cell survival and proliferation.[280,287]

Reasons of tumor cell acquired drug resistance include a) decreased drug uptake and increased drug efflux,[280,287,288] b) mutation or alteration of the expression of a drug target,[16,270,289] c) DNA reparation,[290] d) deregulation of apoptosis[291] and e) autophagy upregulation.[292-294]

1.2.4 Models for new anti-cancer drug discoveries

1.2.4.1 Traditional approaches

Traditionally, there are two major approaches to discover new anticancer drugs, one is the biochemical screen for drugs that target specific molecules and the other is cell-based phenotypic screening.[295] The biochemical screen for specific target is able to screen hundreds of thousands of compound within a relative short time. Cell-based assays have the advantage that a pharmacological effect is known to be elicited by a hit compound. A problem with cell based screens is that the target(s)/mechanism of action of the hit compounds must be identified. Approaches such as connectivity map (Cmap)[296] and drug affinity responsive

target stability (DARTS)[297-299] have been developed in recent years and test methods are quite useful for target identification/finding molecular mechanism of action.

1.2.4.2 Multicellular spheroids (MTS) model

Monolayer cultures are not able to mimic the real tumor hypoxic and nutrient-deprived microenvironments of solid tumors. Furthermore, penetration of anticancer agents into the parenchyma of solid tumors limits their efficacy *in vivo*, but does not influence drug activity on monolayer cultures. Today, these shortcomings increase the risk of failures *in vivo*.[80,300-307] Multicellular spheroids represent an *in vitro* tumor model in which cancer cells are supplied by the diffusion of substrates from the surrounding growth medium.[58] Multicellular spheroid model was adapted to cancer researches in the early 1970s by Sutherland and colleges[307,308] and is in use in many laboratories throughout the world today.

One major advantage of multicellular spheroids is their well-defined geometry, which makes it possible to directly relate structure to function.[309] The other advantage is that its biological pattern (like microenvironment and proliferation gradient) and other activities (metabolism, cell death, drug resistance) are more close to that in living organisms.[58,304,310] *Figure 8 is* an overview of the spheroid characterizations and how structure influences the function in spheroids.

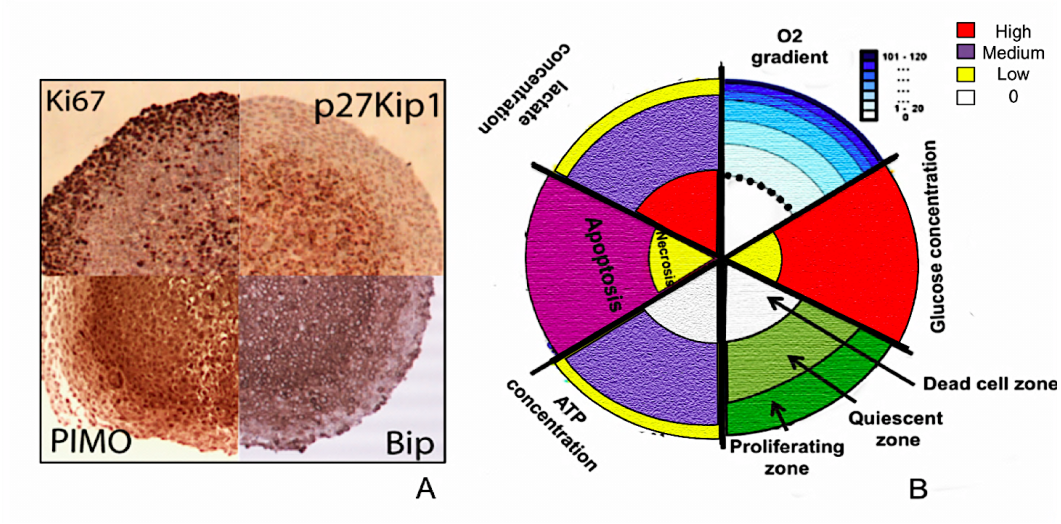

Figure 8: An overview of multicellular spheroids. A) Sections of HCT116 colon cancer cells, stained for Ki67, p27Kip1, pimonidazole adducts or Bip. B) Tumor cells are in different status and the concentrations of glucose, lactate, ATP and O_2 are different from the out-rim of spheroids towards the core.[58,193,311,312-315]

Multicellular spheroids have been used as models to investigate the impacts of different factors, such as pH, oxygen and glucose levels, on cell metabolism, proliferation and viability. A number of important biological questions such as cell cycle checkpoints,[316] the role of E-cadherin in the induction of apoptosis,[317] and the role of HIF1-1α on the malignant phenotype have been studied in the multicellular spheroid model.[318] Multicellular spheroids model have been widely used in studies of cancer therapeutic drugs and more recently in cancer drug discover.[71,302,304,319,320]

1.2.4.3 High-throughput multicellular spheroids screening

Spheroid cultures are known to more closely mimic the properties of tumor tissue than monolayer cultures with regard to growth kinetics and metabolic rates.[300,303] Nutrients, oxygen, and waste products do not diffuse freely in and out of spheroids.[304,309] Tumor cell lines are in general more resistant to anticancer agents when the cells are grown as 3-dimensional spheroids rather than monolayer cultures.[305,321] The resistance of spheroids to anticancer drugs appears to reflect both limited drug penetration into the inner regions of the 3-dimensional cell populations as well as acquired resistance at the multicellular level.[305,322] The gene expression patterns of cells grown in monolayer cultures and as spheroids differ, and some differences can be attributed to intrinsic drug sensitivity.[301,323] For these reasons, MTS model-based drug development is regarded as an important step before *in vivo* experiments (*Figure 9*).

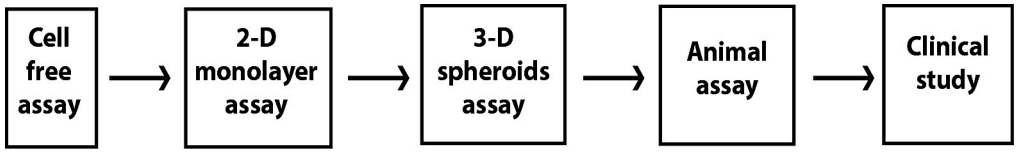

Figure 9: An overview of drug development processes.

The traditional method to generate multicellular spheroids was to use spinner cultures. The resulting spheroids are heterogeneous in size and cannot be used for drug screening. A method that is capable of generating individual and uniformly sized spheroids in hanging drops has subsequently been described. Spheroids are generated in inverted microwell trays and then transferred by pipette to agar-coated 48-well plates,[322,323] this method is time-consuming, and other methods have been developed more suitable for screening. Our group uses a procedure based on the principle of hanging drop but spheroids are generated directly in 96-well plates (avoiding pipetting steps to transfer the spheroids).[304]

In most of our experiments we use high glucose medium (4.5 g/L; 25 mM). These conditions are unphysiological but allow the production of 500 μm spheroids after 5 days culturing containing a large central core of hypoxic cells. Cells in the core area are quiescent, based on negative staining for the proliferation marker Ki67 and positive staining for $p27^{Kip1}$ (*Figure 1* in paper I). The presence of a significant proportion of hypoxic/quiescent cells in spheroids is expected to improve the sensitivity of screening for drugs that act on these cell populations. We initially used the method acid phosphatase assay[324] to determine cell viability and the M30 Apoptosence method[325] to measure apoptosis of drug-treated spheroids. These methods do not rely on the addition of exogenous substrates that need to penetrate into spheroids. In Paper V we used cells expressing green fluorescent protein (GFP) in a spheroid screen in a 384-well format.

2 AIM OF THE THESIS

The aim of the thesis is to develop therapeutic strategies for quiescent tumor cell populations, which are resistant to chemotherapy and a major problem in clinical oncology.

The specific aim of each paper included in this thesis is described below:

Paper I: We here found that the PI3KCA (phosphatidylinositol 3-kinase catalytic subunit) mutation in HCT116 colon cancer cells is essential for spheroid growth. This observation prompted us to examine the effect of pharmacological inhibition of the PI3K/mTOR pathway on spheroid viability.

Paper II: The aim of this study was to screen a chemical library for compounds that reduce the viability of spheroids. The work resulted in the identification of the compound VLX600. This drug was found to decrease mitochondrial OXPHOS, induce metabolism shift and to trigger the death of both proliferating and quiescent tumors.

Paper III: The aim of this paper was to contribute to the understanding of the role of tumor autophagy. We found that autophagy is a survival mechanism of tumor cells in acidic conditions.

Paper IV: The aim of this study was to provide insight in the ability of VLX600 to induce growth inhibition of cells cultured in monolayer (paper II). We found that VLX600 decreases the level of c-MYC protein in tumor cells and this effect appears to be associated with mitochondrial impairment.

Paper V: The aim of this paper was to examine to extend the screen of paper II to get more information on the mechanism of action of drugs that affect spheroid viability. Several promising compounds, which also target mitochondria, were identified.

2 RESULTS AND DISCUSSION

2.1 PAPER I:

Studies of tumor cell regrowth after cytotoxic therapy: using an mTOR/PI3K inhibitor to prevent tumor cell regrowth.

Background: Regrowth of tumor cells between cycles of chemotherapy is a significant problem in cancer therapy.[326-328] Regrowth is believed to occur from populations of cells in the deep tumor parenchyme that are slowly proliferating and comparatively insensitive to many clinically used drugs. Strategies to control regrowth of these cell populations may improve the results of treatment of solid tumors.

Methods: Colon carcinoma cells with wild type, mutant phosphatidylinositol 3-kinase catalytic subunit (PI3KCA) or Kirsten rat sarcoma viral oncogene homolog (KRAS) alleles were allowed to form multicellular spheroids. The effects of different pharmacological inhibitors on the growth of colon carcinoma spheroids were examined.

Results: HCT116 colon carcinoma multicellular spheroids were found to have hypoxic and quiescent tumor cells in the core, and proliferating cells in outer layers. A number of clinically used chemotherapeutical agents had limited cytotoxic effect on these spheroids, consistent with published data.

Secondly, our data suggest that the PI3KCA mutation in HCT116 cells is required for the growth of these cells as spheroids. In contrast, disruption of the $PI3KCA^{mut}$ allele had only a minor influence on the rate of proliferation of HCT116 cells in monolayer culture. In contrast to the strong effect on MTS growth by disruption of the mutant PI3KCA allele, $KRAS^{wt/-}$ cells showed a similar rate of spheroid growth as the $KRAS^{wt/mut}$ parental cells. In fact, the difference in the proliferation rate of these cells was larger in monolayer culture.

Thirdly, we used methyl-13-hydroxy-15-oxokaurenoate (MHOK) to study tumor cell regrowth after treatment. Regrowth after exposure to most drugs was difficult to study since MTS contain an outer rim of dead cells, which made it difficult to estimate the real size of the viable portion of the spheroids. MHOK was identified in a drug screen for compounds effective on multicellular spheroids[304,329] Treatment with MHOK resulted in activation of caspase-3 in outer cell layers as early as after 4 h, followed by detachment of these cells. The remaining MTS cell masses have a defined border of viable cells, facilitating accurate determination of MTS size after drug exposure and further incubation. When MHOK-treated spheroids were monitored over time, MTS volumes started to increase after a lag period. We conclude from these experiments that the hypoxic cells of the HCT116 spheroid cores were able to reinitiate cell proliferation within 18 h after detachment of the outer cell layers and subsequent re-exposure to oxygen and nutrients.

Finally, our findings suggest that inhibition of PI3K signalling might be effective in inhibition of regrowth of HCT116 MTS after cytotoxic therapy. We examined the effect of the dual pan-class PI3K/ mTORC1/mTORC2 inhibitor NVP-BEZ235.[330] We found that

NVP-BEZ235 was effective in blocking regrowth of MTS after MHOK treatment. Sequential treatment with MHOK and NVP-BEZ235 reduced the volume of HCT116 MTS to <5% of the initial volume.

Discussion: The results showed that PI3KCA mutation is required for growth of HCT116 colon cancer cells in multicellular spheroids, whereas the KRAS mutation in these cells was not necessary for growth in the 3-D in vitro model. RAS genes are the most frequently mutated oncogenes detected in human cancer and interact with various effectors, in order to stimulate various downstream signalling pathways and regulate cell proliferation, differentiation, survival and death.[331,332] KRAS is one isoform of RAS family (H-RAS, N-RAS and K-RAS are R-RAS).[333] It has been noticed that different RAS family members play different roles in tumor progression and invasion.[334-336] For example, K-RAS was found to lead to a loss of cell–cell and cell–matrix adhesion.[336-338] And this kind of loss might enhance the potential for invasion and metastasis. In contrast, H-RAS was found to be a much more effective activator of PI3Kinase.[339] PI3Kinase family has a pivotal role in translating extracellular stimuli (e.g. growth factors, cytokines, hormones) into a broad range of cellular functions, such as cell cycle progression, cell growth, survival and apoptosis.[340] Paper has indicated that growth was restricted significantly ($p<0.0001$) in a dose-dependent response in spheroids following treatment of PI3K inhibitors, NVP-BKM120 and NVP-BEZ235.[341] And similar results have been also reported in other publications.[342-345] These findings support our result that inhibition PI3K, not KRAS, leads to tumor cell growth arrest in MTS.

Our data suggested that mTOR inhibitors together with PI3K inhibitor might achieve better outcome and PI3K inhibitors can be used between chemotherapies and prevent tumor regrowth in clinic. Rapamycin was the first available mTOR inhibitor. Presently, FDA has also approved temsirolimus and tverolimus, which also target mTOR. Metformin, first used in diabetes, also has been reported a PI3K inhibitor and has anticancer activity.[346] The oral pan-PI3K inhibitor buparlisib (BKM120), that has been tested in clinical phase I, has shown positive result in advanced solid tumors.[342,343,347,348] All these results predict that treatment of rapamycin/temsirolimus/tverolimus combines/sequential with metformin/ buparlisib might be worth testing in MTS for the further clinic application.

2.2 PAPER II:

Induction of mitochondrial dysfunction is a strategy for targeting quiescent tumor cells in metabolically compromised microenvironments

Background: Abnormal vascularization of solid tumors leads to the generation of tissue microenvironments that are chronically starved of oxygen and nutrients.[349,350] Tumor cells living in such environment are slowly growing or quiescent and display altered phenotypic characteristics when compared with cells located in more vascularized regions.[351-353] Such non-dividing cells are often resistant to mainstay standard chemotherapies that rely on DNA replication and cell division to elicit their antitumor effect.[354,355] The altered phenotype of quiescent cells enables them to survive chemotherapeutic regimes and reseed nascent tumors following secession of chemotherapy.[328]

Results: In this paper, we used MTS of HCT116 colon cancer cells to screen a diverse chemical library with the aim to find compounds with cytotoxic activity in core, hypoxic, regions. And a small molecule compound, VLX600, was identified from this screening and shown to decrease mitochondrial oxidative phosphorylation (OXPHOS).

1. Multicellular spheroids model

Firstly, we have shown again that multicellular spheroids model can mimic real tumor microenvironment and be used in drug screening. Comparison of gene set enrichment analysis (GSEA) of microarray data between HCT116 cells grown as monolayers and MCS showed upregulation of genes associated with hypoxia and glycolysis (*Supplementary Fig. 1a*). Hypoxia was confirmed by staining for pimonidazole adducts (*Fig.1a*). Cells in MCS core regions stained positively for BiP/Grp78 (*Fig.1a*), an endoplasmic reticulum (ER) chaperone. Similar to solid tumor tissue,[356-358] and consistent with previous reports,[84,85,359,360] MCS contain decreased levels of glucose per cell (*Supplementary Fig.1c*). Furthermore, glucose was found to be an essential nutrient for MCS viability since decreasing glucose concentrations in the culture medium resulted in death of cells in inner areas (*Supplementary Fig.1d*).

2. VLX600 reduces multicellular spheroid viability

VLX600 was the most effective compound found in this screen VLX600 exposure (6 μM) resulted in the appearance of central necrotic areas after 72–96 h and modest induction of active caspase-3 in stained sections (*Fig.1C*). Importantly, exposure of MCS to VLX600 resulted in a strong decrease in clonogenicity of dispersed cells. In contrast to the cancer cell lines, immortalized epithelial hTERT-RPE1 cells became growth arrested in the absence of detectable cytotoxicity (*Fig.1h; Supplementary Fig.1g*). Furthermore, when non-proliferating, confluent hTERT-RPE1 cells were exposed to VLX600, no loss of cell viability was observed (*Fig.1i*). These results suggest that VLX600 displays selective cytotoxic activity against malignant cells.

3. VLX600 induces glycolytic and autophagic responses

Thirdly, data indicates that VLX600 induces glycolytic and autophagic responses. HCT116 MCS were exposed to VLX600 or vehicle for 6h followed by microarray-based gene expression analysis. GSEA of genes induced by VLX600 showed a strong positive correlation to genes associated with hypoxia, glycolysis. Consistent with the induction of a hypoxic gene signature, expression of the hypoxia-inducible factor (HIF-1α) transcription factor was upregulated by VLX600 in monolayer cells (*Fig.2b*). Increase of cellular lactate production indicated the induction of glycolysis, which was proved dependent on HIF-1a (*Fig.2a; Supplementary Fig.2b*). HIF-1α knocked out cells grew much slower and appeared more sensitive to VLX600 both on monolayer and spheroids.

VLX600-exposed HCT116 cells increased in size and also showed the presence of large cytoplasmic vesicles, which were proved to be autolysosomes. And this phenomenon was due to VLX600 inhibition of mTOR downstream effectors 4EBP1 and p70-S6K under a HIF-1α independent way. The cytotoxic effect of VLX600 was indeed found to be potentiated by different autophagy inhibitors. We conclude that VLX600 induced HIF-1α-dependent glycolytic and autophagic responses play protective roles on tumor cell survival.

4. VLX600 induces mitochondrial dysfunction and leads to larger glucose dependence.

Fourthly, our results have indicated that mitochondrial dysfunction leads to larger glucose dependence. We added FCCP to HCT116 cells at different times of VLX600 exposure and measured OCR. Already after 90min, uncoupled respiration was impaired (*Fig.6a*), and no stimulation by the uncoupler was observed after 4 h (*Fig.6b*). Importantly, no effects on p70 phosphorylation were observed at 90 min, showing that the effect on mitochondria appeared earlier than mTOR inhibition. Using digitonin-permeabilized HCT116 cells, we found partial inhibition of complex I, II and IV (*Fig.6e,f*). If tumor cells grew in a glucose-starved condition, their sensitivity to VLX600 would increase, especially HCT116 HIF-1α knock out cells (*Fig.5g,h*). We conclude from these results that VLX600 has profound effects on mitochondrial OXPHOS by inhibiting the function of complexes in the electron transport chain and made tumor cells more glucose dependent.

5. VLX600 has shown an antitumor activity in human tumor xenografts

Finally, VLX600 has shown an antitumor activity in human tumor xenografts. Using the maximally tolerated dose (16mg/kg), antitumor activity was observed in both HCT116 and HT29 colon cancer xenografts (*Fig.7b–e*). Importantly, minimal systemic toxicity was observed as evidenced by no loss of body mass and no or minor changes in plasma parameters such as liver alanine aminotransferase, blood glucose and total protein (*Supplementary Fig.7a,b*). Moreover, synergetic affect was observed with both irinotecan and oxaliplatin *in vivo* (*Supplementary Fig.7c*).

Discussion: VLX600, an inhibitor of mitochondrial OXPHOS system, decreases the viability of quiescent cells in 3-D tumor spheroids. This might suggest that tumor cells in nutritionally compromised microenvironments are sensitive to disturbances of mitochondrial function.

Coincidentally, another paper published after us has also shown that drugs toxic to MTS are all targeting mitochondria.[361] In paper V of my thesis, we have introduced several FDA approved reagents that have significant cytotoxic effects on MTS and are mitochondrial uncouplers. All these support the hypotheses that, in MTS, tumor cells, especially the inner core of the spheroids, are more dependent on functional mitochondria and extracellular nutrient concentrations (*Figure10*)

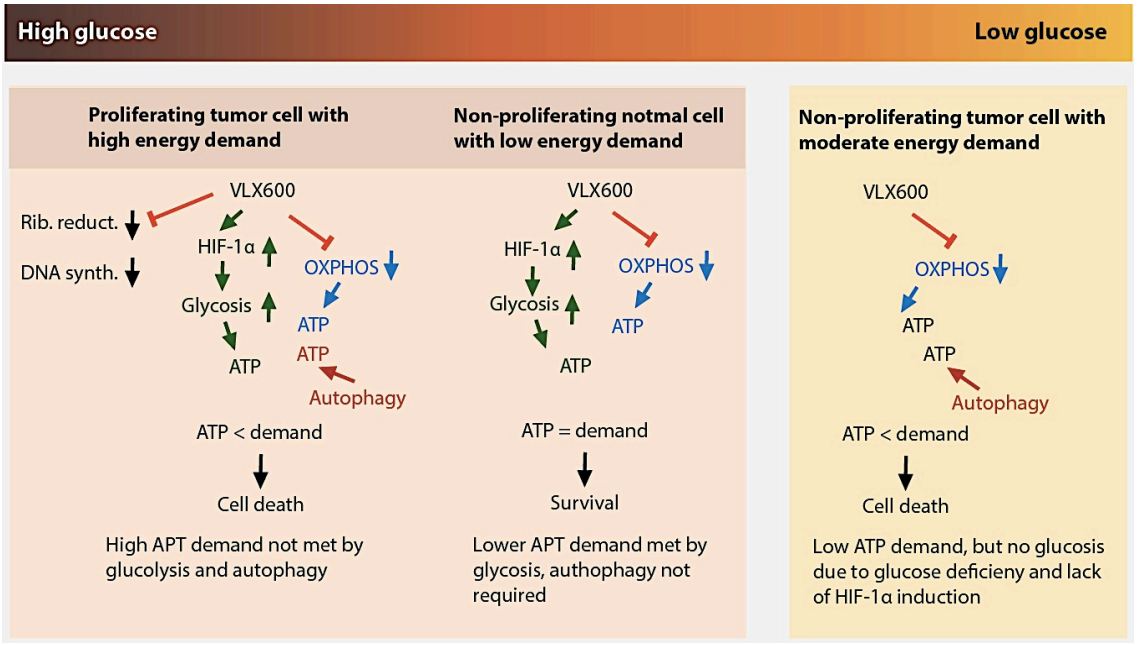

Figure 10: Model for the cytotoxic effects of VLX600 on tumor cells. VLX600 impairs OXPHOS and induces a HIF-1α-dependent shift to glycolysis. This shift protects tumor cells and normal cells in microenvironments where glucose is available. Tumor cells cannot meet their energy demands solely by glycolysis, leading to induction of autophagy. In metabolically compromised microenvironments, the ability to shift to glycolysis is limited because of glucose unavailability and lack of HIF-1α-stabilization.

And our results might also suggest more that can be considered in further studies.

Because of the low supply of oxygen and nutrients in the core of MTS, OXPHOS already slows down, if mitochondrial dysfunction only impairs OXPHOS activity and reduces ATP production, core cells might not show such a significant sensitivity to VLX600. VLX600 might also interrupt cell signalling, cell death, as well as maintaining the control of cell growth that are also dependent on functional mitochondria. Since it has been shown that the gene expression pattern of cells cultured in 3D is much different from that in 2D,[310,361,362] drugs can be preferentially effective in 3D but not in 2D (like nitazoxanide). As the spheroids have gradient structures, the out rim of viable cells will average some significant differences. It will be really cool, if we can trypsin or cutting out the periphery of spheroids and do gene expression analysis or proteomics for the core. We might be able to find some potential targets. Of course, we could also use MHOK to mimic the trypsin effect (cells in the out rim of the spheroids are dead and off spheroids), which has been shown in paper I, but this might also induce changes in gene expression.

Spheroids formed *in vitro* by culturing tumor cells from patients contain cancer stem cells (CSC) in the cores. These cells show characters of quiescence and ability of re-proliferation under certain conditions.[363-367] For the development of novel types of anticancer therapy, only those applications that can more efficiently eliminate cancer stem cells will improve outcomes. Due to this reason, it is worth testing VLX600 and other drugs, which are more toxic to quiescent tumor cells, on cancer stem cell models.

The mechanism of action of VLX600 is under investigation. Analysis of gene expression profiles has suggested that the drug is a metal chelator (Mårten Fryknäs, personal communications). The exact spectrum of metals that are chelated is under investigation, but iron belongs to this group. The effect on mitochondrial OXPHOS may be due to depletion of heme, resulting in inhibition of electron transport. The decrease in COX-1 expression would be secondary to heme depletion in the scheme.

Clinical trials have been initiated with VLX600 and it is worth exploring drugs that will be more effective to combine with VLX600. We have mentioned that after VLX600 treatment, autophagy and glycolysis up-regulation are rescued responses, so drugs that can inhibit these two responses might give better results. Lonidamine (Phase III) and TLN-232 (Phase II) are glycolysis inhibitors and already used in clinic trails.[368] Lys05, Autophagy inhibitor, has shown a good effect alone both *in vitro* and *in vivo.*[156] Proteasome is another organelle in cells that are also responsible for protein degradation and recycling in cells, its inhibitor can also be combined with VLX600, For example, velcade and b-AP15.[369,370]

Last, but not least, one of the advantages to use 3D models for novel drug development is that it takes the penetration ability of the drug candidates into account. Based on the Lipinski's lead-like rules of three, which is a revolution of the rules of five (RO5) and generally followed in small molecule library screens,[371-374] a lead-like chemical should have MW < 300, log P < 3, H-bond donors and acceptors <3 and rotatable bonds <3.[374] The logP of VLX600 is ≈2.85, this physical character could also contribute to its cytotoxicity to tumor spheroids and hard to be considered by using monolayer based screening. The clinically used agent cisplatin has a logP of 0.041. Its 6-day IC_{50} on HCT116 monolayer cells is 0.63μM, [301] but based on data from Paper I and the previous, even 40μm used on HCT116 spheroids, caspase-3 was limited induced around the out-rim of spheroids and did not result in eradication of spheroids after 6-days treatment.[301,319] These results both strongly suggest the necessity of using spheroids for pre-clinical drug discovery. MTS based screens can be time-consuming and expensive at beginning but it does give higher chance of success *in vivo* and *in clinic.*

In summary, since conventional cytotoxic anticancer agents are significantly less effective under conditions of nutrient depletion, so it is necessary to use MTS model for preclinical drug screening and testing, at the same time, targeting mitochondrial and induce energy catastrophe could be an interesting strategy for therapy.[375]

2.3 PAPER III:

Autophagy is a protective mechanism for human melanoma cells under acidic stress and feasible to be a target in the anticancer therapy.

Background: Cancer cells are characterized by aerobic glycolysis. An inevitable result of up-regulated glucose metabolism is tumor acidosis.

Results: All melanoma cell lines tested were able to slowly proliferate at pH 6.5. All cell lines cultured at pH 6.5 continued to slowly proliferate during 72 hours culturing. Even in acute exposure to the pH 6.5 medium, some melanoma cells died but surviving cells still were able to continue to proliferate. Analysis of LC3 and p62, two indicators for autophagy, indicated the activation of autophagy in acidic conditions. We hypothesized that autophagy may be a protective mechanism for cancer cells.

Autophagy upregulation is generally induced as a response to impairment of energy supplies,[131,142,376-378] it therefore seemed reasonable to hypothesize exposure to acidic conditions results in a problem of energy metabolism. Therefore, we measured glucose and amino acid uptake in human melanoma cells after exposure to acidic medium. Glucose uptake was measured using the fluorescent non-metabolizable glucose analogue 2-NBDG. Melanoma cells cultured at acidic pH (pH 6.5) showed a time-dependent reduction in the uptake of glucose compared with cells cultured at neutral pH. In line with this observation, both ATP and extracellular lactate concentrations were significantly reduced in cells cultured at acidic pH, likely as a result of decreased glucose consumption and inhibition of glycolysis.

Functional autophagy represents a survival strategy for cells under metabolic stress.[379-385] To determine the role of autophagy in acidic stress-induced cell death we used siRNA to knockdown the expression of ATG5, a crucial protein involved in the build-up of autophagosomes. Under pH 6.5, cell death was ~19% in cells treated with scrambled siRNA and ~51% in cells treated with ATG5 siRNA ($p<0.05$). The difference in survival was even more pronounced at pH 6.2, where cell death was ~36% in control cells and ~80% under conditions of autophagy inhibition.

Discussion: Autophagy plays a protective role or a suppressed role[126,386,387] might much depend on tumor stages, so the role of autophagy requires to be checked before using autophagy inhibitors or inducers as a combination in experiments or treatments. Based on our study, at least in human melanoma, autophagy is a protective mechanism in tumor cells. This study might provide support to further therapeutic strategies that inhibits autophagy to kill tumor cells, directly or in combination with chemotherapy.

PH has been gradually believed to be an important factor for keeping tumor cells special characters (like drug resistance and metastasis), nowadays treatments based on the pH regulation has been proven impactful, for example, Girentuximab (phase III) and Tasigna (in clinic use), the inhibitors of carbonic anhydrases 9 that are obviously overexpressed in tumor cells, have been used as anti-cancer and anti-metastasis agents.[388,389] This indicates

that targeting pH regulation in tumor cells represents a novel and attractive therapeutic strategy,[390-393] as acidic pH is an essential positive regulator of autophagy.[124,125]

2.4 PAPER IV

Mitochondrial dysfunction induces reduction in c-MYC expression.

Background: In paper IV, we have continued our studies on VLX600. We are interested in the consequences after VLX600 caused mitochondrial dysfunction in tumor cells.

MYC and Max proteins form heterodimers that bind to promoter E-box sequences to regulate gene expression.[90,102] MYC overexpression is associated with malignant transformation and MYC is necessary for the proliferation of tumor cells. Thus, continuous c-Myc expression is required for tumor proliferation *in vivo,*[394] and depletion of MYC leads to proliferative arrest of tumor cell lines.[395] The MYC protein is a relatively weak transcriptional activator and is believed to function as a general enhancer of transcription.[396,397] Genes encoding proteins involved in cell growth, metabolism, ribosome biogenesis, protein synthesis and mitochondrial function are overrepresented as MYC targets.[398] The work of several groups has established an important role of MYC in supporting mitochondrial biogenesis.[110,220,399,400] MYC affects mitochondrial mass by influencing both mitochondrial fusion and fission, favouring the latter process.[220]

Results: Firstly, we found inhibition of c-MYC expression by VLX600. During our studies we noticed that this compound affected the expression of c-MYC protein in HCT116 colon carcinoma cells and in TGR-1 rat fibroblasts. C-MYC expression increased for several hours, followed by a decrease. We also examined the effects of VLX600 on the rat cell line HO-MYC3 where c-MYC is expressed under the control of a retrovirus promoter.[401] Interestingly, the same pattern of an initial increase followed by a decreased MYC expression was observed in these cells. This indicates that MYC expression after VLX600 is not regulated by transcription promoters.

Secondly, the decreased c-MYC mRNA level was observed after exposure to VLX600. We examined the effect of VLX600 on c-MYC mRNA levels and found a reduction of c-MYC mRNA levels to ~50% after 24 hours of drug exposure.

Thirdly, destabilization of c-MYC mRNA by VLX600 was found in our experiments. We examined the effects of VLX600 on c-MYC mRNA levels in the presence and absence of α-amanitin, an inhibitor of RNA polymerase II. While VLX600 reduced c-MYC mRNA levels ~2-fold over 16 hours in the absence of α-amanitin, > 5-fold decreases were observed in the presence of VLX600 and the RNA polymerase inhibitor. These observations are consistent with VLX600 decreasing c-MYC mRNA stability.

Fourthly, inhibition of c-MYC expression was observed in other standard mitochondrial inhibitors. We compared other standard mitochondrial inhibitors, like oligomycin, rotenone and antimycin, and all of them presented similar effects on c-MYC in both HCT116 colon carcinoma cells and in TGR-1 rat fibroblasts.

Finally, the c-MYC-Max interaction inhibitor 10058-F4 was noted to reduce OXPHOS. As it was reported that 10058-F4 was a c-MYC-Max inhibitor, then we compared VLX600 with it.

As expected, 10058-F4 reduced c-MYC levels over 24 hours. Exposure of HCT116 cells to 50 μM 10058-F4 resulted in reduction of oxidative consumption rate (OCR). The decrease in OCR by 10058-F4 was rapid and unlikely to be mediated by c-MYC. To further address this question we examined the effect of 10058-F4 on the *c-MYC*$^{-/-}$ cell line HO15.19. We found that 10058-F4 affected OXPHOS also in these cells, which was showing that the effect is not c-MYC-mediated.

Discussion: Many publications have discussed a lot about mitochondrial function regulated by c-MYC, but the mechanism of mitochondria regulating c-MYC has not been stressed enough. One original paper, has mentioned that alterations in c-MYC phenotypes resulting from dynamin-related protein 1 (Drp1)-mediated mitochondrial fission.[402] Here, our data has showed that mitochondrial dysfunction leads to down-regulation of c-MYC expression and this is a general effect of mitochondrial inhibitors (Rotenone and Antimycin). These results might indicate that the mechanism of mitochondria regulating c-MYC is worth exploring.

How to address the decrease of c-MYC mRNA level after VLX600 will be one issue in our following study. It has been reported that if the RNA-binding protein, coding region determinant-binding protein (CRD-BP) is attacked, mRNA transcription will be interrupted.[403] This might be one possible explanation of decrease of c-MYC mRNA level after VLX600. Refer to our data of destabilization of mRNA after VLX600, we assume it might be regulated by miRNA, since it has been shown that miRNA-494 targets c-MYC[404,405] and locates in mitochondria. If this can be evidenced, mitochondrial dysfunction reduced c-MYC expression after VLX600 exposure could also be explained.

Besides reduction of mRNA level, the c-MYC decrease at protein level should also be a possibility to explain c-MYC de-regulation after VLX600. It has been reported that mTOR inhibition and p70-S6K reduction control c-MYC translation;[406] USP37 regulates c-MYC[407] and long none-coding RNA transcript 1 (lncRNA-GHET1) is high expressed in gastric carcinoma.[408] Possible mechanisms are summarized below in *Figure 11*.

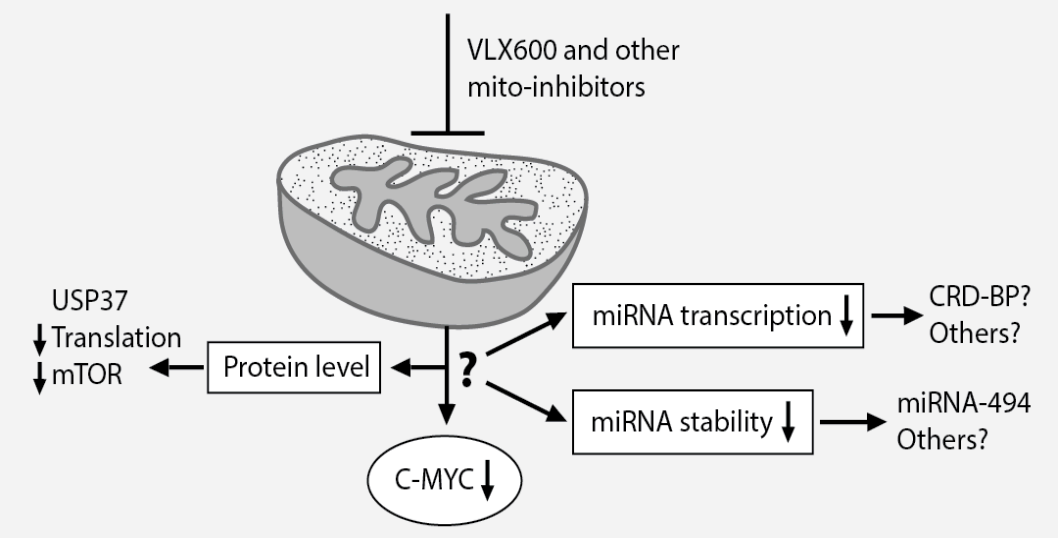

Figure11: Possible mechanisms involved in c-MYC reduction after mitochondrial inhibitors

In human, the *c-MYC* oncogene is overexpressed in at least 40% of cancers and serves as a "master regulator" of cellular metabolism and proliferation.[89,93,94] But, there is still No drug that targets c-MYC has been shown a strong antitumor effect in animal models, and no such drug has to my knowledge been tested in clinical trials. Our finding has suggested that targeting mitochondria could be an alternative strategy of indirectly regulating c-MYC.

Some review about c-MYC has mentioned that the ability of cells to grow under hypoxic conditions partly because of the crosstalk between hypoxia-inducible factors (HIFs) and the c-MYC. And when O_2 levels return to normal, c-MYC can help tumor cells return to rapid proliferation.[409] In Paper II, our results have shown that even withdrawing VLX600 after 3 days treatment, the effect of VLX600 still exists and spheroids are unable to grow back. This possibly indicates that inhibiting c-MYC is another strategy to prevent cells growing back in solid tumors. As mentioned above, metformin that has already been used in clinic is a mitochondrial complex I inhibitor,[269,410] and nitazoxanide (descried in paper V), which is a FDA approved anthelmintic drug, has shown an effect of mitochondrial inhibition and c-MYC reduction in spheroids.[411] Both drugs can be continuously taken by patients.[412-414] So c-MYC inhibition by these two drugs might able to be used between cycles of chemotherapy in order to inhibit tumor cells growing back. But this assumption needs to be verified firstly by spheroids based study *in vitro.*

In Summary, the mechanism of mitochondria regulating c-MYC is worth exploring. Targeting mitochondria can be a therapeutic strategy in c-MYC overexpressed tumors and metformin/ nitazoxanide might be used in sequential chemotherapy to inhibit cancer relapse.

2.5 PAPER V

GFP-labeled HCT116 cells cultured in 384-well plates were used for high-throughput screening. Nitazoxanide, an FDA-approved antihelminthic drug, was identified as a potential candidate for treatment of solid tumors.

Background: The 3D model was developed to mimic the tumor microenvironment, which is hypoxic, nutrient deprived and extracellular acidic. Multicellular tumor spheroids (MCTS) were formed in 384-well plates using GFP-labelled HCT116 colon cancer cells. Based on this model, 1600 clinically active compounds were screened and followed by hit-validation.

Results: After comparing the activities in our MTS model with that in 2D-cultures, five compounds with 3D-specific activity were identified, nitazoxanide, niclosamide, closantel, pyrvinium pamoate and salinomycin. It is noteworthy that, the five most active hits identified were all compounds reported to target mitochondria, supporting our previous finding that respiration is an attractive target in solid tumors.[415] Then we verified the five compounds on HCT116 cells and the mitochondrial oxygen consumption rates were measured using Seahorse XF analyzer. Nitazoxanide, niclosamide and closantel, which have similar effect as FCCP, increased OCR at low concentrations while as the concentration increased, the period of elevated OCR became shorter and was followed by shutdown of mitochondrial respiration, indicated by a rapid decrease of OCR. The effect of the pyrvinium pamoate and salinomycin was different from what was observed for the uncouplers, but both of them also cause mitochondrial dysfunction after several hours treatment.

We further demonstrated that mitochondria are able to recover from exposure to the tested OXPHOS inhibitors. It was evident from assessment of OCR in drug-free medium following exposure of HCT116 to nitazoxanide for 24 h. OCR gradually increased after removal of the drug and returned to normal levels after 7 days. Thus, continuous drug exposure is required to eliminate tumor re-growth potential. This is an important finding considering an optimal treatment schedule for compounds targeting mitochondria.

Among the five compounds, nitazoxanide, which is a FDA-approved antiprotozoal drug, has an excellent pharmacokinetic and safety profile. This character makes it suitable to be continuously treated on patients. So we finally selected it for further evaluation and demonstrated strong anti-tumor activity *in vivo* when combined with a standard chemotherapeutic agent.

In paper V, we tried to explain the reason of the nitazoxanide toxicity to cancer cells that agreed with our previous finding (paper II). This made us believe that anticancer drugs, which target mitochondria, might have better clinical effects on solid tumors treatment. Moreover, different tolerations to energetic deficiency between tumor cells and normal cells can be a therapeutic target in cancer treatment.

3 CONCLUSION

The 3-D multicellular spheroid model can be used to discover new drugs that target quiescent cancer cells.

- 3-D multicellular spheroids are characterized by its cellular hetergeneity that is able to mimic the *in vivo* tumor microenvironment where large proliferating tumor cells are in the periphery and smaller quiescent tumor cells are in the core of the spheroids.
- 3-D multicellular spheroids can be used to study tumor cell regrowth after cytotoxic therapy. This application enables us to find new drug combinations that might have higher chance to obtain satisfied effects in clinic.
- Screening in the 384-well format using GFP-labelled multicellular spheroids is reliable, reproducible and time saving, and promises to be useful for future drug screening projects.

Induction of mitochondrial dysfunction as a strategy for targeting quiescent tumor cells in metabolically compromised microenvironments.

- Compounds screened by 3-D multicellular spheroids are likely to be mitochondrial inhibitors (paper II and paper V).
- Mitochondrial dysfunction induces tumor cell energy catastrophe in both proliferating and quiescent tumor cells but not normal cells.
- Mitochondrial dysfunction makes both proliferating and quiescent cells more addicted to glucose by up-regulation of glycolysis. Quiescent tumor cells are likely to be more sensitive to mitochondrial inhibitors due to low glucose availability.
- Tumor cells have rescue responses (like autophagy and glycolysis up-regulation) after treatment in order to survive.
- Mitochondrial dysfunction can decrease c-MYC levels: targeting mitochondria can be an indirect method to inhibit c-MYC in tumor cells.

VLX600 combination therapy is worth testing on 3-D multicellular spheroids model.

- VLX600 will be combined with other compounds that are already used in clinic trial. Combining VLX600 with compounds that target proliferating cells may be expected to result in improved tumor control.
- VLX600 would be interesting to combine with compounds that inhibit autophagy or disturb glucose uptake.
- VLX600 is currently being evaluated in combination with radiotherapy on solid tumors, due to its ability of reducing hypoxia in spheroids.

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5 REFERENCES

1 Institute, N. C. What Is Cancer. (2014).
2 DePinho, R. A. The age of cancer. *Nature* (2000).
3 Key changes to cancer care in the UK. *Br J Nurs* **24**, S3, doi:10.12968/bjon.2015.24.Sup4.S3 (2015).
4 Slade, I., Riddell, D., Turnbull, C., Hanson, H. & Rahman, N. Development of cancer genetic services in the UK: A national consultation. *Genome medicine* **7**, 18, doi:10.1186/s13073-015-0128-4 (2015).
5 Torjesen, I. Half of the UK population can expect a diagnosis of cancer. *BMJ* **350**, h614, doi:10.1136/bmj.h614 (2015).
6 Mieroop, M. V. D. *A History of Ancient Egypt* (August 30, 2010).
7 Sudhakar, A. History of Cancer, Ancient and Modern Treatment Methods. *Journal of cancer science & therapy* **1**, 1-4, doi:10.4172/1948-5956.100000e2 (2009).
8 Margaret M. Olszewski. Concepts of Cancer from Antiquity to the Nineteenth Century. *History of medcine* (2010).
9 Wagner, R. P. Anecdotal, historical and critical commentaries on genetics. Rudolph Virchow and the genetic basis of somatic ecology. *Genetics* **151**, 917-920 (1999).
10 Paget, S. The distribution of secondary growths in cancer of the breast. 1889. *Cancer metastasis reviews* **8**, 98-101 (1989).
11 Meek, E. S. The cellular distribution of deoxyribonucleic acid in primary and secondary growths of human breast cancer. *The Journal of pathology and bacteriology* **82**, 167-176 (1961).
12 Avery, O. T., Macleod, C. M. & McCarty, M. Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types : Induction of Transformation by a Desoxyribonucleic Acid Fraction Isolated from Pneumococcus Type Iii. *The Journal of experimental medicine* **79**, 137-158 (1944).
13 Watson, J. D. & Crick, F. H. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* **171**, 737-738 (1953).
14 Weinberg, D. H. a. R. A. the hallmarks of cancer. *Cell* (2000).
15 Bishop, J. M., and Weinberg, R.A., eds. . *Molecular Oncology (New York: Scientific American, Inc.* (1996).
16 Patrick S. Ward1, a. C. B. T., *. Metabolic Reprogramming A Cancer Hallmark Even Warburg Did Not Anticipate. *Cancer cell*, doi:10.1016/j.ccr.2012.02.014 (2012).
17 Rob A. Cairns*, I. S. H. a. T. W. M. Regulation of cancer cell metabolism. *Nature Rev.*, doi:10.1038/nrc2981 (2011).
18 Susana Romero-Garcia, J. S. L.-G., 1 José Luis Báez-Viveros,2 Dolores Aguilar-Cazares1 and Heriberto Prado-Garcia1,*. Tumor cell metabolism. *Cancer Biology & Therapy* doi:10.4161/cbt.12.11.18140 (2011).
19 Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
20 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:10.1016/j.cell.2011.02.013 (2011).
21 society, T. A. C. The History of Cancer. (2014).
22 Konig, J. *et al.* Radiotherapy effects on early breast cancer survival in observational and randomized studies: a systematic analysis of advantages, disadvantages and differences between the two study types. *Breast Cancer*, doi:10.1007/s12282-014-0579-2 (2015).
23 Prcic, A., Aganovic, D. & Hadziosmanovic, O. Impact of complications and bladder cancer stage on quality of life in patients with different types of urinary diversions. *Med Arch* **67**, 418-422, doi:10.5455/medarh.2013.67.418-422 (2013).
24 McAlpine, H., Joubert, L., Martin-Sanchez, F., Merolli, M. & Drummond, K. J. A systematic review of types and efficacy of online interventions for cancer patients. *Patient education and counseling* **98**, 283-295, doi:10.1016/j.pec.2014.11.002 (2015).
25 Ye, Y., Shen, Z. & Wang, S. [Three types of abdominoperineal excision procedures for the rectal cancer based on anatomic landmarks classification]. *Zhonghua wei chang wai ke za zhi = Chinese journal of gastrointestinal surgery* **17**, 1170-1174 (2014).
26 Rous, P. A Transmissible Avian Neoplasm. (Sarcoma of the Common Fowl.). *The Journal of experimental medicine* **12**, 696-705 (1910).
27 Warburg, O. On the Origin of Cancer Cells. *Science* (1956).
28 Altenberg, B. & Greulich, K. O. Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes. *Genomics* **84**, 1014-1020, doi:10.1016/j.ygeno.2004.08.010 (2004).
29 Hirschhaeuser, F., Sattler, U. G. & Mueller-Klieser, W. Lactate: a metabolic key player in cancer. *Cancer research* **71**, 6921-6925, doi:10.1158/0008-5472.CAN-11-1457 (2011).

30 Sanchez-Arago, M., Formentini, L. & Cuezva, J. M. Mitochondria-mediated energy adaption in cancer: the H(+)-ATP synthase-geared switch of metabolism in human tumors. *Antioxidants & redox signaling* **19**, 285-298, doi:10.1089/ars.2012.4883 (2013).

31 Cirri, P. & Chiarugi, P. Tumors and their stroma: mitochondria at the crossroad. *Cell Cycle* **12**, 204-205, doi:10.4161/cc.23421 (2013).

32 Chintha, R., Kaipa, P. R., Sekhar, N. & Hasan, Q. Mitochondria and tumors: a new perspective. *Indian journal of cancer* **50**, 206-213, doi:10.4103/0019-509X.118732 (2013).

33 Berneburg, M. [Mitochondria: not only involved in aging, but also important in tumors]. *Der Hautarzt; Zeitschrift fur Dermatologie, Venerologie, und verwandte Gebiete* **59**, 598-600, doi:10.1007/s00105-008-1595-y (2008).

34 Welter, C., Reichert, G. & Zang, K. D. Increased amount of a mitochondria-associated 21 x 10(3) dalton protein in human gastrointestinal tumors. *Journal of cancer research and clinical oncology* **114**, 411-414 (1988).

35 Ahn, C. S. & Metallo, C. M. Mitochondria as biosynthetic factories for cancer proliferation. *Cancer & metabolism* **3**, 1, doi:10.1186/s40170-015-0128-2 (2015).

36 Chin, R. M. *et al.* The metabolite alpha-ketoglutarate extends lifespan by inhibiting ATP synthase and TOR. *Nature* **510**, 397-401, doi:10.1038/nature13264 (2014).

37 Newsholme, E. A., Crabtree, B. & Ardawi, M. S. The role of high rates of glycolysis and glutamine utilization in rapidly dividing cells. *Bioscience reports* **5**, 393-400 (1985).

38 Semenza, G. L. *et al.* 'The metabolism of tumours': 70 years later. *Novartis Foundation symposium* **240**, 251-260; discussion 260-254 (2001).

39 Vander Heiden, M. G., Cantley, L. C. & Thompson, C. B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324**, 1029-1033, doi:10.1126/science.1160809 (2009).

40 Stipanuk, M. H. Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. *Annual review of nutrition* **24**, 539-577, doi:10.1146/annurev.nutr.24.012003.132418 (2004).

41 Stover, P. J. Physiology of folate and vitamin B12 in health and disease. *Nutrition reviews* **62**, S3-12; discussion S13 (2004).

42 Locasale, J. W. & Cantley, L. C. Genetic selection for enhanced serine metabolism in cancer development. *Cell Cycle* **10**, 3812-3813, doi:10.4161/cc.10.22.18224 (2011).

43 DeBerardinis, R. J. Serine metabolism: some tumors take the road less traveled. *Cell metabolism* **14**, 285-286, doi:10.1016/j.cmet.2011.08.004 (2011).

44 Amelio, I., Cutruzzola, F., Antonov, A., Agostini, M. & Melino, G. Serine and glycine metabolism in cancer. *Trends in biochemical sciences* **39**, 191-198, doi:10.1016/j.tibs.2014.02.004 (2014).

45 Locasale, J. W. Serine, glycine and one-carbon units: cancer metabolism in full circle. *Nature reviews. Cancer* **13**, 572-583, doi:10.1038/nrc3557 (2013).

46 Schulze, A. & Harris, A. L. How cancer metabolism is tuned for proliferation and vulnerable to disruption. *Nature* **491**, 364-373, doi:10.1038/nature11706 (2012).

47 Gatenby, R. A. & Gillies, R. J. Why do cancers have high aerobic glycolysis? *Nature reviews. Cancer* **4**, 891-899, doi:10.1038/nrc1478 (2004).

48 Gillies, R. J., Robey, I. & Gatenby, R. A. Causes and consequences of increased glucose metabolism of cancers. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine* **49 Suppl 2**, 24S-42S, doi:10.2967/jnumed.107.047258 (2008).

49 Sattler, U. G. & Mueller-Klieser, W. The anti-oxidant capacity of tumour glycolysis. *International journal of radiation biology* **85**, 963-971, doi:10.3109/09553000903258889 (2009).

50 Schmid, S. A. *et al.* Lactate adversely affects the in vitro formation of endothelial cell tubular structures through the action of TGF-beta1. *Experimental cell research* **313**, 2531-2549, doi:10.1016/j.yexcr.2007.05.016 (2007).

51 Goetze, K., Walenta, S., Ksiazkiewicz, M., Kunz-Schughart, L. A. & Mueller-Klieser, W. Lactate enhances motility of tumor cells and inhibits monocyte migration and cytokine release. *International journal of oncology* **39**, 453-463, doi:10.3892/ijo.2011.1055 (2011).

52 Walenta, S. & Mueller-Klieser, W. F. Lactate: mirror and motor of tumor malignancy. *Seminars in radiation oncology* **14**, 267-274, doi:10.1016/j.semradonc.2004.04.004 (2004).

53 Vaupel, P. Tumor microenvironmental physiology and its implications for radiation oncology. *Seminars in radiation oncology* **14**, 198-206, doi:10.1016/j.semradonc.2004.04.008 (2004).

54 Thomlinson, R. H. G., L. H. The histological structure of some human lung cancers and the possible implications for radiotherapy. *Br. J. Cancer* (1955).

55 Yasuda, H. Solid tumor physiology and hypoxia-induced chemo/radio-resistance: novel strategy for cancer therapy: nitric oxide donor as a therapeutic enhancer. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **19**, 205-216, doi:10.1016/j.niox.2008.04.026 (2008).

56 Liu, Y. *et al.* Radiation-/hypoxia-induced solid tumor metastasis and regrowth inhibited by hypoxia-specific upconversion nanoradiosensitizer. *Biomaterials* **49**, 1-8, doi:10.1016/j.biomaterials.2015.01.028 (2015).

57 Mueller-Klieser, W., Freyer, J. P. & Sutherland, R. M. Influence of glucose and oxygen supply conditions on the oxygenation of multicellular spheroids. *Br J Cancer* **53**, 345-353 (1986).

58 Mueller-Klieser, W. F. & Sutherland, R. M. Oxygen tensions in multicell spheroids of two cell lines. *Br J Cancer* **45**, 256-264 (1982).

59 Hockel, M. & Vaupel, P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *Journal of the National Cancer Institute* **93**, 266-276 (2001).

60 Vaupel, P., Thews, O. & Hoeckel, M. Treatment resistance of solid tumors: role of hypoxia and anemia. *Med Oncol* **18**, 243-259, doi:10.1385/MO:18:4:243 (2001).

61 Gray, L. H., Conger, A. D., Ebert, M., Hornsey, S. & Scott, O. C. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *The British journal of radiology* **26**, 638-648, doi:10.1259/0007-1285-26-312-638 (1953).

62 Carmeliet, P. *et al.* Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* **394**, 485-490, doi:10.1038/28867 (1998).

63 Durand, R. E. The influence of microenvironmental factors during cancer therapy. *In Vivo* **8**, 691-702 (1994).

64 Durand, R. E. & Raleigh, J. A. Identification of nonproliferating but viable hypoxic tumor cells in vivo. *Cancer research* **58**, 3547-3550 (1998).

65 Hoffmann, A., Gloe, T. & Pohl, U. Hypoxia-induced upregulation of eNOS gene expression is redox-sensitive: a comparison between hypoxia and inhibitors of cell metabolism. *Journal of cellular physiology* **188**, 33-44, doi:10.1002/jcp.1092 (2001).

66 Jeong, J. I., Lee, Y. W. & Kim, Y. K. Chemical hypoxia-induced cell death in human glioma cells: role of reactive oxygen species, ATP depletion, mitochondrial damage and Ca2+. *Neurochemical research* **28**, 1201-1211 (2003).

67 Kuin, A. *et al.* Potentiation of anti-cancer drug activity at low intratumoral pH induced by the mitochondrial inhibitor m-iodobenzylguanidine (MIBG) and its analogue benzylguanidine (BG). *Br J Cancer* **79**, 793-801, doi:10.1038/sj.bjc.6690127 (1999).

68 Rottenberg, H. & Moreno-Sanchez, R. The proton pumping activity of H(+)-ATPases: an improved fluorescence assay. *Biochimica et biophysica acta* **1183**, 161-170 (1993).

69 Teicher, B. A., Linehan, W. M. & Helman, L. J. Targeting cancer metabolism. *Clinical cancer research : an official journal of the American Association for Cancer Research* **18**, 5537-5545, doi:10.1158/1078-0432.CCR-12-2587 (2012).

70 Gluck, S. L. The vacuolar H(+)-ATPases: versatile proton pumps participating in constitutive and specialized functions of eukaryotic cells. *International review of cytology* **137C**, 105-137 (1993).

71 Marino, M. L. *et al.* Autophagy is a protective mechanism for human melanoma cells under acidic stress. *The Journal of biological chemistry* **287**, 30664-30676, doi:10.1074/jbc.M112.339127 (2012).

72 Tavakol, S. Acidic pH derived from cancer cells may induce failed reprogramming of normal differentiated cells adjacent tumor cells and turn them into cancer cells. *Medical hypotheses* **83**, 668-672, doi:10.1016/j.mehy.2014.09.014 (2014).

73 Pellegrini, P. *et al.* Acidic extracellular pH neutralizes the autophagy-inhibiting activity of chloroquine: implications for cancer therapies. *Autophagy* **10**, 562-571, doi:10.4161/auto.27901 (2014).

74 Navratilova, J., Hankeova, T., Benes, P. & Smarda, J. Acidic pH of tumor microenvironment enhances cytotoxicity of the disulfiram/Cu2+ complex to breast and colon cancer cells. *Chemotherapy* **59**, 112-120, doi:10.1159/000353915 (2013).

75 Min, K. H. *et al.* Tumoral acidic pH-responsive MPEG-poly(beta-amino ester) polymeric micelles for cancer targeting therapy. *Journal of controlled release : official journal of the Controlled Release Society* **144**, 259-266, doi:10.1016/j.jconrel.2010.02.024 (2010).

76 Lan, A., Lagadic-Gossmann, D., Lemaire, C., Brenner, C. & Jan, G. Acidic extracellular pH shifts colorectal cancer cell death from apoptosis to necrosis upon exposure to propionate and acetate, major end-products of the human probiotic propionibacteria. *Apoptosis : an international journal on programmed cell death* **12**, 573-591, doi:10.1007/s10495-006-0010-3 (2007).

77 Mariappan, R. *et al.* Serum lactate as a potential biomarker of malignancy in primary adult brain tumours. *Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia* **22**, 144-148, doi:10.1016/j.jocn.2014.06.005 (2015).

78 Gerolymatos, A. *et al.* Evaluation of lactate dehydrogenase activity as an index of cervical malignancy. *European journal of gynaecological oncology* **12**, 471-476 (1991).

79 Singh, S. K., Hasan, S. A., Sharma, S. C., Chandra, K. & Ali, R. Serum lactate dehydrogenase in head and neck malignancy patients. *Indian journal of physiology and pharmacology* **37**, 93-94 (1993).

80 Sutherland, R. M. Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science* **240**, 177-184 (1988).

81 Marion MacFarlane, G. L. R. a. K. C. Glucose—a sweet way to die. *Cell Cycle*, doi:10.4161/cc.21804 (2012).

82 Gatenby, R. A. & Frieden, B. R. Information dynamics in carcinogenesis and tumor growth. *Mutation research* **568**, 259-273, doi:10.1016/j.mrfmmm.2004.04.018 (2004).

83 Mueller-Klieser, W. Tumor biology and experimental therapeutics. *Critical reviews in oncology/hematology* **36**, 123-139 (2000).

84 Walenta, S., Doetsch, J., Mueller-Klieser, W. & Kunz-Schughart, L. A. Metabolic imaging in multicellular spheroids of oncogene-transfected fibroblasts. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* **48**, 509-522 (2000).

85 Weinlich, M. & Acker, H. Flavoprotein-fluorescence imaging for metabolic studies in multicellular spheroids by means of confocal scanning laser microscopy. *Journal of microscopy* **160**, RP1-2 (1990).

86 Teutsch, H. F., Goellner, A. & Mueller-Klieser, W. Glucose levels and succinate and lactate dehydrogenase activity in EMT6/Ro tumor spheroids. *European journal of cell biology* **66**, 302-307 (1995).

87 Tredan, O., Galmarini, C. M., Patel, K. & Tannock, I. F. Drug resistance and the solid tumor microenvironment. *Journal of the National Cancer Institute* **99**, 1441-1454, doi:10.1093/jnci/djm135 (2007).

88 Walz, S. *et al.* Activation and repression by oncogenic MYC shape tumour-specific gene expression profiles. *Nature* **511**, 483-487, doi:10.1038/nature13473 (2014).

89 Gabay, M., Li, Y. & Felsher, D. W. MYC activation is a hallmark of cancer initiation and maintenance. *Cold Spring Harbor perspectives in medicine* **4**, doi:10.1101/cshperspect.a014241 (2014).

90 Wahlstrom, T. & Arsenian Henriksson, M. Impact of MYC in regulation of tumor cell metabolism. *Biochimica et biophysica acta*, doi:10.1016/j.bbagrm.2014.07.004 (2014).

91 Hartl, M. *et al.* Stem cell-specific activation of an ancestral myc protooncogene with conserved basic functions in the early metazoan Hydra. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 4051-4056, doi:10.1073/pnas.0911060107 (2010).

92 Zirath H1, F. A., Oliynyk G, Segerström L, Westermark UK, Larsson K, Munksgaard Persson M, Hultenby K, Lehtiö J, Einvik C, Påhlman S, Kogner P, Jakobsson PJ, Henriksson MA. MYC inhibition induces metabolic changes leading to accumulation of lipid droplets in tumor cells. *PNAS* (2013).

93 Dang, C. V., Le, A. & Gao, P. MYC-induced cancer cell energy metabolism and therapeutic opportunities. *Clinical cancer research : an official journal of the American Association for Cancer Research* **15**, 6479-6483, doi:10.1158/1078-0432.CCR-09-0889 (2009).

94 Miller, D. M., Thomas, S. D., Islam, A., Muench, D. & Sedoris, K. c-Myc and cancer metabolism. *Clinical cancer research : an official journal of the American Association for Cancer Research* **18**, 5546-5553, doi:10.1158/1078-0432.CCR-12-0977 (2012).

95 Yuneva, M., Zamboni, N., Oefner, P., Sachidanandam, R. & Lazebnik, Y. Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. *The Journal of cell biology* **178**, 93-105, doi:10.1083/jcb.200703099 (2007).

96 Zitterbart, K. *et al.* Low-level copy number changes of MYC genes have a prognostic impact in medulloblastoma. *Journal of neuro-oncology* **102**, 25-33, doi:10.1007/s11060-010-0289-3 (2011).

97 Calado, D. P. *et al.* The cell-cycle regulator c-Myc is essential for the formation and maintenance of germinal centers. *Nature immunology* **13**, 1092-1100, doi:10.1038/ni.2418 (2012).

98 Swarnalatha, M., Singh, A. K. & Kumar, V. The epigenetic control of E-box and Myc-dependent chromatin modifications regulate the licensing of lamin B2 origin during cell cycle. *Nucleic acids research* **40**, 9021-9035, doi:10.1093/nar/gks617 (2012).

99 Sundarraj, S. *et al.* gamma-Sitosterol from Acacia nilotica L. induces G2/M cell cycle arrest and apoptosis through c-Myc suppression in MCF-7 and A549 cells. *Journal of ethnopharmacology* **141**, 803-809, doi:10.1016/j.jep.2012.03.014 (2012).

100 Taira, N. *et al.* DYRK2 priming phosphorylation of c-Jun and c-Myc modulates cell cycle progression in human cancer cells. *The Journal of clinical investigation* **122**, 859-872, doi:10.1172/JCI60818 (2012).

101 Akiba, J. *et al.* N-myc downstream regulated gene1/Cap43 overexpression suppresses tumor growth by hepatic cancer cells through cell cycle arrest at the G0/G1 phase. *Cancer letters* **310**, 25-34, doi:10.1016/j.canlet.2011.05.034 (2011).

102 Luscher, B. & Larsson, L. G. The basic region/helix-loop-helix/leucine zipper domain of Myc proto-oncoproteins: function and regulation. *Oncogene* **18**, 2955-2966, doi:10.1038/sj.onc.1202750 (1999).

103 Bahram, F., Wu, S., Oberg, F., Luscher, B. & Larsson, L. G. Posttranslational regulation of Myc function in response to phorbol ester/interferon-gamma-induced differentiation of v-Myc-transformed U-937 monoblasts. *Blood* **93**, 3900-3912 (1999).

104 Dang, C. V. Therapeutic targeting of Myc-reprogrammed cancer cell metabolism. *Cold Spring Harbor symposia on quantitative biology* **76**, 369-374, doi:10.1101/sqb.2011.76.011296 (2011).

105 Alfredo Csibi, G. L., Sang-Oh Yoon,Haoxuan Tong,Didem Ilter, Ilaria Elia,Sarah-Maria Fendt,Thomas M. Roberts,and John Blenis. S6K1 Pathway Regulates Glutamine Metabolism through the eIF4B-Dependent Control of c-Myc Translation. *Current biology* (2014).

106 Shim, H. *et al.* c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 6658-6663 (1997).

107 Ramanathan, A., Wang, C. & Schreiber, S. L. Perturbational profiling of a cell-line model of tumorigenesis by using metabolic measurements. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 5992-5997, doi:10.1073/pnas.0502267102 (2005).

108 Morrish, F. *et al.* Myc-dependent mitochondrial generation of acetyl-CoA contributes to fatty acid biosynthesis and histone acetylation during cell cycle entry. *The Journal of biological chemistry* **285**, 36267-36274, doi:10.1074/jbc.M110.141606 (2010).

109 Morrish, F. & Hockenbery, D. Myc's mastery of mitochondrial mischief. *Cell Cycle* **2**, 11-13 (2003).

110 Li, F. *et al.* Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis. *Molecular and cellular biology* **25**, 6225-6234, doi:10.1128/MCB.25.14.6225-6234.2005 (2005).

111 Schreiber, S. L. & Bernstein, B. E. Signaling network model of chromatin. *Cell* **111**, 771-778 (2002).

112 Li, X. *et al.* c-MYC-regulated miR-23a/24-2/27a cluster promotes mammary carcinoma cell invasion and hepatic metastasis by targeting Sprouty2. *The Journal of biological chemistry* **288**, 18121-18133, doi:10.1074/jbc.M113.478560 (2013).

113 Gao, P. *et al.* c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* **458**, 762-765, doi:10.1038/nature07823 (2009).

114 Yang, Z. *et al.* Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. *Molecular cell* **19**, 535-545, doi:10.1016/j.molcel.2005.06.029 (2005).

115 Jang, M. K. *et al.* The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription. *Molecular cell* **19**, 523-534, doi:10.1016/j.molcel.2005.06.027 (2005).

116 Brannon, A. R., Maresca, J. A., Boeke, J. D., Basrai, M. A. & McBride, A. A. Reconstitution of papillomavirus E2-mediated plasmid maintenance in Saccharomyces cerevisiae by the Brd4 bromodomain protein. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 2998-3003, doi:10.1073/pnas.0407818102 (2005).

117 Shao, Q. *et al.* BET protein inhibitor JQ1 attenuates Myc-amplified MCC tumor growth in vivo. *Cancer research* **74**, 7090-7102, doi:10.1158/0008-5472.CAN-14-0305 (2014).

118 Kumar, K. *et al.* GLI2-dependent c-MYC upregulation mediates resistance of pancreatic cancer cells to the BET bromodomain inhibitor JQ1. *Scientific reports* **5**, 9489, doi:10.1038/srep09489 (2015).

119 Harel-Bellan, A., Ferris, D. K., Vinocour, M., Holt, J. T. & Farrar, W. L. Specific inhibition of c-myc protein biosynthesis using an antisense synthetic deoxy-oligonucleotide in human T lymphocytes. *J Immunol* **140**, 2431-2435 (1988).

120 Holt, J. T., Redner, R. L. & Nienhuis, A. W. An oligomer complementary to c-myc mRNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation. *Molecular and cellular biology* **8**, 963-973 (1988).

121 Wechsler, A. S. Cost-effective management of high-risk patients: a case-map approach. *The Annals of thoracic surgery* **64**, S76-79; discussion S80-72 (1997).

122 D'Cruz, C. M. *et al.* c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous Kras2 mutations. *Nature medicine* **7**, 235-239, doi:10.1038/84691 (2001).

123 Yin, X., Giap, C., Lazo, J. S. & Prochownik, E. V. Low molecular weight inhibitors of Myc-Max interaction and function. *Oncogene* **22**, 6151-6159, doi:10.1038/sj.onc.1206641 (2003).

124 Papackova, Z. & Cahova, M. Important role of autophagy in regulation of metabolic processes in health, disease and aging. *Physiological research / Academia Scientiarum Bohemoslovaca* **63**, 409-420 (2014).

125 Meijer, A. J. & Codogno, P. Autophagy: regulation and role in disease. *Critical reviews in clinical laboratory sciences* **46**, 210-240, doi:10.1080/10408360903044068 (2009).

126 Yang, Z. & Klionsky, D. J. Eaten alive: a history of macroautophagy. *Nature cell biology* **12**, 814-822, doi:10.1038/ncb0910-814 (2010).

127 Cecconi, F. & Levine, B. The role of autophagy in mammalian development: cell makeover rather than cell death. *Developmental cell* **15**, 344-357, doi:10.1016/j.devcel.2008.08.012 (2008).

128 Cuervo, A. M. Autophagy and aging: keeping that old broom working. *Trends in genetics : TIG* **24**, 604-612, doi:10.1016/j.tig.2008.10.002 (2008).

129 Rubinsztein, D. C., Marino, G. & Kroemer, G. Autophagy and aging. *Cell* **146**, 682-695, doi:10.1016/j.cell.2011.07.030 (2011).

130 Mizushima, N. & Komatsu, M. Autophagy: renovation of cells and tissues. *Cell* **147**, 728-741, doi:10.1016/j.cell.2011.10.026 (2011).

131 Singh, R. Autophagy in the control of food intake. *Adipocyte* **1**, 75-79, doi:10.4161/adip.18966 (2012).

132 Mizushima, N., Levine, B., Cuervo, A. M. & Klionsky, D. J. Autophagy fights disease through cellular self-digestion. *Nature* **451**, 1069-1075, doi:10.1038/nature06639 (2008).

133 White, E. & DiPaola, R. S. The double-edged sword of autophagy modulation in cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **15**, 5308-5316, doi:10.1158/1078-0432.CCR-07-5023 (2009).

134 Janku, F., McConkey, D. J., Hong, D. S. & Kurzrock, R. Autophagy as a target for anticancer therapy. *Nature reviews. Clinical oncology* **8**, 528-539, doi:10.1038/nrclinonc.2011.71 (2011).

135 Wullschleger, S., Loewith, R. & Hall, M. N. TOR signaling in growth and metabolism. *Cell* **124**, 471-484, doi:10.1016/j.cell.2006.01.016 (2006).

136 Chang, Y. Y. & Neufeld, T. P. An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. *Molecular biology of the cell* **20**, 2004-2014, doi:10.1091/mbc.E08-12-1250 (2009).

137 Yang, Z. & Klionsky, D. J. Mammalian autophagy: core molecular machinery and signaling regulation. *Current opinion in cell biology* **22**, 124-131, doi:10.1016/j.ceb.2009.11.014 (2010).

138 Kim, Y. C. & Guan, K. L. mTOR: a pharmacologic target for autophagy regulation. *The Journal of clinical investigation* **125**, 25-32, doi:10.1172/JCI73939 (2015).

139 Jiang, L. B. *et al.* Activation of autophagy via Ca-dependent AMPK/mTOR pathway in rat notochordal cells is a cellular adaptation under hyperosmotic stress. *Cell Cycle* **14**, 867-879, doi:10.1080/15384101.2015.1004946 (2015).

140 Weidberg, H., Shvets, E. & Elazar, Z. Biogenesis and cargo selectivity of autophagosomes. *Annual review of biochemistry* **80**, 125-156, doi:10.1146/annurev-biochem-052709-094552 (2011).

141 Meijer, A. J. & Codogno, P. Autophagy: a sweet process in diabetes. *Cell metabolism* **8**, 275-276, doi:10.1016/j.cmet.2008.09.001 (2008).

142 Mizushima, N. Autophagy: process and function. *Genes & development* **21**, 2861-2873, doi:10.1101/gad.1599207 (2007).

143 Yoshimori, T. Autophagy: a regulated bulk degradation process inside cells. *Biochemical and biophysical research communications* **313**, 453-458 (2004).

144 Kroemer, G. Autophagy: a druggable process that is deregulated in aging and human disease. *The Journal of clinical investigation* **125**, 1-4, doi:10.1172/JCI78652 (2015).

145 Wang, Z. *et al.* MicroRNA-25 regulates chemoresistance-associated autophagy in breast cancer cells, a process modulated by the natural autophagy inducer isoliquiritigenin. *Oncotarget* **5**, 7013-7026 (2014).

146 Cheng, C. Y., Chi, P. I. & Liu, H. J. Commentary on the regulation of viral proteins in autophagy process. *BioMed research international* **2014**, 962915, doi:10.1155/2014/962915 (2014).

147 Loos, J. A., Caparros, P. A., Nicolao, M. C., Denegri, G. M. & Cumino, A. C. Identification and pharmacological induction of autophagy in the larval stages of Echinococcus granulosus: an active catabolic process in calcareous corpuscles. *International journal for parasitology* **44**, 415-427, doi:10.1016/j.ijpara.2014.02.007 (2014).

148 Comincini, S. *et al.* microRNA-17 regulates the expression of ATG7 and modulates the autophagy process, improving the sensitivity to temozolomide and low-dose ionizing radiation treatments in human glioblastoma cells. *Cancer Biol Ther* **14**, 574-586, doi:10.4161/cbt.24597 (2013).

149 Yang, X. *et al.* mir-30d Regulates multiple genes in the autophagy pathway and impairs autophagy process in human cancer cells. *Biochemical and biophysical research communications* **431**, 617-622, doi:10.1016/j.bbrc.2012.12.083 (2013).

150 Wu, Y. *et al.* Synthesis and screening of 3-MA derivatives for autophagy inhibitors. *Autophagy* **9**, 595-603, doi:10.4161/auto.23641 (2013).

151 Li, J. *et al.* Inhibition of autophagy by 3-MA enhances the effect of 5-FU-induced apoptosis in colon cancer cells. *Annals of surgical oncology* **16**, 761-771, doi:10.1245/s10434-008-0260-0 (2009).

152 Nsimba, S. E. How sulfadoxine-pyrimethamine (SP) was perceived in some rural communities after phasing out chloroquine (CQ) as a first-line drug for uncomplicated malaria in Tanzania: lessons to learn towards moving from monotherapy to fixed combination therapy. *Journal of ethnobiology and ethnomedicine* **2**, 5, doi:10.1186/1746-4269-2-5 (2006).

153 Mohapatra, P. K. *et al.* Evaluation of chloroquine (CQ) and sulphadoxine/pyrimethamine (SP) therapy in uncomplicated falciparum malaria in Indo-Myanmar border areas. *Tropical medicine & international health : TM & IH* **10**, 478-483, doi:10.1111/j.1365-3156.2005.01401.x (2005).

154 Talisuna, A. O. *et al.* Role of the pfcrt codon 76 mutation as a molecular marker for population-based surveillance of chloroquine (CQ)-resistant Plasmodium falciparum malaria in Ugandan sentinel sites with high CQ resistance. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **96**, 551-556 (2002).

155 Ekanem, O. J. *et al.* Treatment of malaria in north-eastern and south-eastern Nigeria: a population study of mefloquine, sulphadoxine, pyrimethamine combination (MSP) vs chloroquine (CQ). *West African journal of medicine* **19**, 293-297 (2000).

156 Amaravadi, R. K. & Winkler, J. D. Lys05: a new lysosomal autophagy inhibitor. *Autophagy* **8**, 1383-1384, doi:10.4161/auto.20958 (2012).

157 McAfee, Q. *et al.* Autophagy inhibitor Lys05 has single-agent antitumor activity and reproduces the phenotype of a genetic autophagy deficiency. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 8253-8258, doi:10.1073/pnas.1118193109 (2012).

158 Vaccari, F. & Rossanda, M. Blood glycolysis during the alarm reaction. *Nature* **169**, 327-328 (1952).

159 Racker, E. History of the Pasteur effect and its pathobiology. *Molecular and cellular biochemistry* **5**, 17-23 (1974).

160 Warburg, O., Wind, F. & Negelein, E. The Metabolism of Tumors in the Body. *The Journal of general physiology* **8**, 519-530 (1927).

161 Hawkins, R. A. & Phelps, M. E. PET in clinical oncology. *Cancer metastasis reviews* **7**, 119-142 (1988).

162 Weber, W. A., Avril, N. & Schwaiger, M. Relevance of positron emission tomography (PET) in oncology. *Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft ... [et al]* **175**, 356-373 (1999).

163 Gambhir, S. S. Molecular imaging of cancer with positron emission tomography. *Nature reviews. Cancer* **2**, 683-693, doi:10.1038/nrc882 (2002).

164 Pantuck, A. J. *et al.* CL1-SR39: A noninvasive molecular imaging model of prostate cancer suicide gene therapy using positron emission tomography. *The Journal of urology* **168**, 1193-1198, doi:10.1097/01.ju.0000026576.46595.61 (2002).

165 Czernin, J. & Phelps, M. E. Positron emission tomography scanning: current and future applications. *Annual review of medicine* **53**, 89-112, doi:10.1146/annurev.med.53.082901.104028 (2002).

166 Rich, P. R. The molecular machinery of Keilin's respiratory chain. *Biochemical Society transactions* **31**, 1095-1105, doi:10.1042/ (2003).

167 Semenza, G. L. & Wang, G. L. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Molecular and cellular biology* **12**, 5447-5454 (1992).

168 Iyer, N. V., Leung, S. W. & Semenza, G. L. The human hypoxia-inducible factor 1alpha gene: HIF1A structure and evolutionary conservation. *Genomics* **52**, 159-165, doi:10.1006/geno.1998.5416 (1998).

169 Sitkovsky, M. & Lukashev, D. Regulation of immune cells by local-tissue oxygen tension: HIF1 alpha and adenosine receptors. *Nature reviews. Immunology* **5**, 712-721, doi:10.1038/nri1685 (2005).

170 Gultice, A. D., Kulkarni-Datar, K. & Brown, T. L. Hypoxia-inducible factor 1alpha (HIF1A) mediates distinct steps of rat trophoblast differentiation in gradient oxygen. *Biology of reproduction* **80**, 184-193, doi:10.1095/biolreprod.107.067488 (2009).

171 Semenza, G. L., Roth, P. H., Fang, H. M. & Wang, G. L. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *The Journal of biological chemistry* **269**, 23757-23763 (1994).

172 Dewhirst, M. W. *et al.* Microvascular studies on the origins of perfusion-limited hypoxia. *The British journal of cancer. Supplement* **27**, S247-251 (1996).

173 Fearon, E. R. & Vogelstein, B. A genetic model for colorectal tumorigenesis. *Cell* **61**, 759-767 (1990).

174 Gatenby, R. A. & Vincent, T. L. An evolutionary model of carcinogenesis. *Cancer research* **63**, 6212-6220 (2003).

175 Gilead, A. & Neeman, M. Dynamic remodeling of the vascular bed precedes tumor growth: MLS ovarian carcinoma spheroids implanted in nude mice. *Neoplasia* **1**, 226-230 (1999).

176 Vincent, T. L. & Gatenby, R. A. An evolutionary model for initiation, promotion, and progression in carcinogenesis. *International journal of oncology* **32**, 729-737 (2008).

177 Waliszewski, P. Controversies about the genetic model of colorectal tumorigenesis. *Polish journal of pathology : official journal of the Polish Society of Pathologists* **46**, 239-243 (1995).

178 Wykoff, C. C. *et al.* Expression of the hypoxia-inducible and tumor-associated carbonic anhydrases in ductal carcinoma in situ of the breast. *The American journal of pathology* **158**, 1011-1019, doi:10.1016/S0002-9440(10)64048-5 (2001).

179 Park, H. J., Lyons, J. C., Ohtsubo, T. & Song, C. W. Acidic environment causes apoptosis by increasing caspase activity. *Br J Cancer* **80**, 1892-1897, doi:10.1038/sj.bjc.6690617 (1999).

180 Shrode, L. D., Tapper, H. & Grinstein, S. Role of intracellular pH in proliferation, transformation, and apoptosis. *Journal of bioenergetics and biomembranes* **29**, 393-399 (1997).

181 Keeling, P. J. & Archibald, J. M. Organelle evolution: what's in a name? *Current biology : CB* **18**, R345-347, doi:10.1016/j.cub.2008.02.065 (2008).

182 Siekevitz, P. powerhouse of the cell. *Scientific American*, 131 - 144 (1957).

183 Wallace, D. C. Mitochondria and cancer. *Nature reviews. Cancer* **12**, 685-698, doi:10.1038/nrc3365 (2012).

184 Falk, M. J. *et al.* Mitochondrial disease genetic diagnostics: optimized whole-exome analysis for all MitoCarta nuclear genes and the mitochondrial genome. *Discovery medicine* **14**, 389-399 (2012).

185 Shrotriya, S. *et al.* Grape seed extract targets mitochondrial electron transport chain complex III and induces oxidative and metabolic stress leading to cytoprotective autophagy and apoptotic death in human head and neck cancer cells. *Molecular carcinogenesis*, doi:10.1002/mc.22246 (2014).

186 Xiao, F. *et al.* Role of mitochondrial electron transport chain dysfunction in Cr(VI)-induced cytotoxicity in L-02 hepatocytes. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* **33**, 1013-1025, doi:10.1159/000358672 (2014).

187 Doherty, E., Oaks, Z. & Perl, A. Increased mitochondrial electron transport chain activity at complex I is regulated by N-acetylcysteine in lymphocytes of patients with systemic lupus erythematosus. *Antioxidants & redox signaling* **21**, 56-65, doi:10.1089/ars.2013.5702 (2014).

188 Henze, K. & Martin, W. Evolutionary biology: essence of mitochondria. *Nature* **426**, 127-128, doi:10.1038/426127a (2003).

189 McBride, H. M., Neuspiel, M. & Wasiak, S. Mitochondria: more than just a powerhouse. *Current biology : CB* **16**, R551-560, doi:10.1016/j.cub.2006.06.054 (2006).

190 Sack, G. H. Introduction to the minireviews series on mitochondrial matters in amyotrophic lateral sclerosis, Lou Gehrig's disease. *Journal of bioenergetics and biomembranes* **43**, 565-567, doi:10.1007/s10863-011-9391-2 (2011).

191 Chaussenot, A. & Paquis-Flucklinger, V. An overview of neurological and neuromuscular signs in mitochondrial diseases. *Revue neurologique* **170**, 323-338, doi:10.1016/j.neurol.2014.03.007 (2014).

192 Warburg, O. The Chemical Constitution of Respiration Ferment. *Science* **68**, 437-443, doi:10.1126/science.68.1767.437 (1928).

193 Crabtree, H. G. Observations on the carbohydrate metabolism of tumours. *The Biochemical journal* **23**, 536-545 (1929).

194 Warburg, O. On respiratory impairment in cancer cells. *Science* **124**, 269-270 (1956).

195 Haridas, V. *et al.* Avicins, a novel plant-derived metabolite lowers energy metabolism in tumor cells by targeting the outer mitochondrial membrane. *Mitochondrion* **7**, 234-240, doi:10.1016/j.mito.2006.12.005 (2007).

196 Moreno-Sanchez, R., Rodriguez-Enriquez, S., Marin-Hernandez, A. & Saavedra, E. Energy metabolism in tumor cells. *The FEBS journal* **274**, 1393-1418, doi:10.1111/j.1742-4658.2007.05686.x (2007).

197 Krutmann, J. & Schroeder, P. Role of mitochondria in photoaging of human skin: the defective powerhouse model. *The journal of investigative dermatology. Symposium proceedings / the Society for Investigative Dermatology, Inc. [and] European Society for Dermatological Research* **14**, 44-49, doi:10.1038/jidsymp.2009.1 (2009).

198 Desjardins, P., Frost, E. & Morais, R. Ethidium bromide-induced loss of mitochondrial DNA from primary chicken embryo fibroblasts. *Molecular and cellular biology* **5**, 1163-1169 (1985).

199 Desjardins, P., de Muys, J. M. & Morais, R. An established avian fibroblast cell line without mitochondrial DNA. *Somatic cell and molecular genetics* **12**, 133-139 (1986).

200 King, M. P. & Attardi, G. Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* **246**, 500-503 (1989).

201 Magda, D. *et al.* mtDNA depletion confers specific gene expression profiles in human cells grown in culture and in xenograft. *BMC genomics* **9**, 521, doi:10.1186/1471-2164-9-521 (2008).

202 Morais, R. *et al.* Tumor-forming ability in athymic nude mice of human cell lines devoid of mitochondrial DNA. *Cancer research* **54**, 3889-3896 (1994).

203 Cavalli, L. R., Varella-Garcia, M. & Liang, B. C. Diminished tumorigenic phenotype after depletion of mitochondrial DNA. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* **8**, 1189-1198 (1997).

204 Weinberg, F. *et al.* Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 8788-8793, doi:10.1073/pnas.1003428107 (2010).

205 Kassauei, K. *et al.* Mitochondrial DNA mutations in pancreatic cancer. *International journal of gastrointestinal cancer* **37**, 57-64, doi:10.1007/s12029-007-0008-2 (2006).

206 Baysal, B. E. Role of mitochondrial mutations in cancer. *Endocrine pathology* **17**, 203-212 (2006).

207 Chatterjee, A., Mambo, E. & Sidransky, D. Mitochondrial DNA mutations in human cancer. *Oncogene* **25**, 4663-4674, doi:10.1038/sj.onc.1209604 (2006).

208 Brandon, M., Baldi, P. & Wallace, D. C. Mitochondrial mutations in cancer. *Oncogene* **25**, 4647-4662, doi:10.1038/sj.onc.1209607 (2006).

209 Wallace, D. C. & Fan, W. Energetics, epigenetics, mitochondrial genetics. *Mitochondrion* **10**, 12-31, doi:10.1016/j.mito.2009.09.006 (2010).

210 Wallace, D. C., Fan, W. & Procaccio, V. Mitochondrial energetics and therapeutics. *Annual review of pathology* **5**, 297-348, doi:10.1146/annurev.pathol.4.110807.092314 (2010).

211 Kongara, S. & Karantza, V. The interplay between autophagy and ROS in tumorigenesis. *Frontiers in oncology* **2**, 171, doi:10.3389/fonc.2012.00171 (2012).

212 Woo, D. K. *et al.* Mitochondrial genome instability and ROS enhance intestinal tumorigenesis in APC(Min/+) mice. *The American journal of pathology* **180**, 24-31, doi:10.1016/j.ajpath.2011.10.003 (2012).

213 Burdon, R. H. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free radical biology & medicine* **18**, 775-794 (1995).

214 Lander, H. M. An essential role for free radicals and derived species in signal transduction. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **11**, 118-124 (1997).

215 Bardella, C., Pollard, P. J. & Tomlinson, I. SDH mutations in cancer. *Biochimica et biophysica acta* **1807**, 1432-1443, doi:10.1016/j.bbabio.2011.07.003 (2011).

216 Calabrese, C. *et al.* Respiratory complex I is essential to induce a Warburg profile in mitochondria-defective tumor cells. *Cancer & metabolism* **1**, 11, doi:10.1186/2049-3002-1-11 (2013).

217 Frezza, C. & Gottlieb, E. Mitochondria in cancer: not just innocent bystanders. *Seminars in cancer biology* **19**, 4-11, doi:10.1016/j.semcancer.2008.11.008 (2009).

218 Galluzzi, L., Kepp, O. & Kroemer, G. Mitochondria: master regulators of danger signalling. *Nature reviews. Molecular cell biology* **13**, 780-788, doi:10.1038/nrm3479 (2012).

219 Ramsay, E. E., Hogg, P. J. & Dilda, P. J. Mitochondrial metabolism inhibitors for cancer therapy. *Pharmaceutical research* **28**, 2731-2744, doi:10.1007/s11095-011-0584-5 (2011).

220 Graves, J. A. *et al.* Mitochondrial structure, function and dynamics are temporally controlled by c-Myc. *PloS one* **7**, e37699, doi:10.1371/journal.pone.0037699 (2012).

221 Dong, L. F. *et al.* Mitochondrial targeting of vitamin E succinate enhances its pro-apoptotic and anti-cancer activity via mitochondrial complex II. *The Journal of biological chemistry* **286**, 3717-3728, doi:10.1074/jbc.M110.186643 (2011).

222 Xu, J. *et al.* Colorectal cancer cells refractory to anti-VEGF treatment are vulnerable to glycolytic blockade due to persistent impairment of mitochondria. *Molecular cancer therapeutics* **12**, 717-724, doi:10.1158/1535-7163.MCT-12-1016-T (2013).

223 Wang, R. *et al.* The Hsp90 inhibitor SNX-2112, induces apoptosis in multidrug resistant K562/ADR cells through suppression of Akt/NF-kappaB and disruption of mitochondria-dependent pathways. *Chemico-biological interactions* **205**, 1-10, doi:10.1016/j.cbi.2013.06.007 (2013).

224 Kang, B. H. *et al.* Preclinical characterization of mitochondria-targeted small molecule hsp90 inhibitors, gamitrinibs, in advanced prostate cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **16**, 4779-4788, doi:10.1158/1078-0432.CCR-10-1818 (2010).

225 Didelot, C. & Garrido, C. [Mitochondria HSP90: the target to inactivate in cancer therapy?]. *Medecine sciences : M/S* **24**, 363-364, doi:10.1051/medsci/2008244363 (2008).

226 Nieminen, A. I., Partanen, J. I., Hau, A. & Klefstrom, J. c-Myc primed mitochondria determine cellular sensitivity to TRAIL-induced apoptosis. *The EMBO journal* **26**, 1055-1067, doi:10.1038/sj.emboj.7601551 (2007).

227 Hotti, A., Jarvinen, K., Siivola, P. & Holtta, E. Caspases and mitochondria in c-Myc-induced apoptosis: identification of ATM as a new target of caspases. *Oncogene* **19**, 2354-2362, doi:10.1038/sj.onc.1203567 (2000).

228 Carelli, V. *et al.* Mitochondria: Biogenesis and mitophagy balance in segregation and clonal expansion of mitochondrial DNA mutations. *The international journal of biochemistry & cell biology*, doi:10.1016/j.biocel.2015.01.023 (2015).

229 Di Sante, G. *et al.* Loss of Sirt1 promotes prostatic intraepithelial neoplasia, reduces mitophagy, and delays PARK2 translocation to mitochondria. *The American journal of pathology* **185**, 266-279, doi:10.1016/j.ajpath.2014.09.014 (2015).

230 Figueiredo-Pereira, M. E., Rockwell, P., Schmidt-Glenewinkel, T. & Serrano, P. Neuroinflammation and J2 prostaglandins: linking impairment of the ubiquitin-proteasome pathway and mitochondria to neurodegeneration. *Frontiers in molecular neuroscience* **7**, 104, doi:10.3389/fnmol.2014.00104 (2014).

231 Mookerjee, S. A. & Brand, M. D. Characteristics of the turnover of uncoupling protein 3 by the ubiquitin proteasome system in isolated mitochondria. *Biochimica et biophysica acta* **1807**, 1474-1481, doi:10.1016/j.bbabio.2011.07.011 (2011).

232 Nath, K. *et al.* Effects of hyperglycemia on lonidamine-induced acidification and de-energization of human melanoma xenografts and sensitization to melphalan. *NMR in biomedicine* **28**, 395-403, doi:10.1002/nbm.3260 (2015).

233 Nath, K. *et al.* Lonidamine induces intracellular tumor acidification and ATP depletion in breast, prostate and ovarian cancer xenografts and potentiates response to doxorubicin. *NMR in biomedicine* **28**, 281-290, doi:10.1002/nbm.3240 (2015).

234 Macchioni, L., Davidescu, M., Roberti, R. & Corazzi, L. The energy blockers 3-bromopyruvate and lonidamine: effects on bioenergetics of brain mitochondria. *Journal of bioenergetics and biomembranes* **46**, 389-394, doi:10.1007/s10863-014-9577-5 (2014).

235 Ubah, O. C. & Wallace, H. M. Cancer therapy: Targeting mitochondria and other sub-cellular organelles. *Current pharmaceutical design* **20**, 201-222 (2014).

236 Xu, R. H. *et al.* Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. *Cancer research* **65**, 613-621 (2005).

237 Brown, J. Effects of 2-deoxyglucose on carbohydrate metablism: review of the literature and studies in the rat. *Metabolism: clinical and experimental* **11**, 1098-1112 (1962).

238 Kang, H. T. & Hwang, E. S. 2-Deoxyglucose: an anticancer and antiviral therapeutic, but not any more a low glucose mimetic. *Life sciences* **78**, 1392-1399, doi:10.1016/j.lfs.2005.07.001 (2006).

239 Singh, D. *et al.* Optimizing cancer radiotherapy with 2-deoxy-d-glucose dose escalation studies in patients with glioblastoma multiforme. *Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft ... [et al]* **181**, 507-514, doi:10.1007/s00066-005-1320-z (2005).

240 Gilman, A. The initial clinical trial of nitrogen mustard. *American journal of surgery* **105**, 574-578 (1963).

241 Wright, J. C., Prigot, A., Wright, B., Weintraub, S. & Wright, L. T. An evaluation of folic acid antagonists in adults with neoplastic diseases: a study of 93 patients with incurable neoplasms. *Journal of the National Medical Association* **43**, 211-240 (1951).

242 Sawyers, C. targeted cancer therapy. *Nature* (2004).

243 Vincent T. DeVita, J., M.D., and Steven A. Rosenberg. Two Hundred Years of Cancer Research. *The new england journal of medicine* (2012).

244 FDA. FDA Approved Drugs by Medical Condition | CenterWatch. (2015).

245 Dias, N. & Bailly, C. Drugs targeting mitochondrial functions to control tumor cell growth. *Biochemical pharmacology* **70**, 1-12, doi:10.1016/j.bcp.2005.03.021 (2005).

246 Warwick, G. P. the mechanism of action of alkylating agent *Cancer research* (1963).

247 Hall, A. G. a. M. J. T. Mechanisms of action of, and modes of resistance to, alkylating agents used in the treatment of haematological malignancies. *Blood* (1992).

248 society, A. c. Different types of chemotherapy drugs : Print Preview. (2013).

249 Davey, P. Anticancer chemotherapy. *British Medical Journal* (1983).

250 Kaye, S. New antimetabolites incancer chemotherapy and their clinical impact. *British Journal of cancer* (1998).

251 Bertrand, R., Solary, E., Jenkins, J. & Pommier, Y. Apoptosis and its modulation in human promyelocytic HL-60 cells treated with DNA topoisomerase I and II inhibitors. *Experimental cell research* **207**, 388-397, doi:10.1006/excr.1993.1206 (1993).

252 Duan, Y. T., Wang, Z. C., Sang, Y. L., Tao, X. X. & Zhu, H. L. Exploration of structure-based on imidazole core as antibacterial agents. *Current topics in medicinal chemistry* **13**, 3118-3130 (2013).

253 Holm, C., Covey, J. M., Kerrigan, D. & Pommier, Y. Differential requirement of DNA replication for the cytotoxicity of DNA topoisomerase I and II inhibitors in Chinese hamster DC3F cells. *Cancer research* **49**, 6365-6368 (1989).

254 Kurmasheva, R. T. *et al.* Initial testing (stage 1) of the topoisomerase II inhibitor pixantrone, by the pediatric preclinical testing program. *Pediatric blood & cancer* **61**, 922-924, doi:10.1002/pbc.24800 (2014).

255 Verschraegen, C. F. *et al.* A phase I study of the combination of temsirolimus with irinotecan for metastatic sarcoma. *Cancers* **5**, 418-429, doi:10.3390/cancers5020418 (2013).

256 Yao, B. L. *et al.* Design, synthesis and biological evaluation of novel 7-alkylamino substituted benzo[a]phenazin derivatives as dual topoisomerase I/II inhibitors. *European journal of medicinal chemistry* **92**, 540-553, doi:10.1016/j.ejmech.2015.01.024 (2015).

257 Jackson, H. W. *et al.* Expansion of stem cells counteracts age-related mammary regression in compound Timp1/Timp3 null mice. *Nature cell biology*, doi:10.1038/ncb3118 (2015).

258 George Rosenker, K. M. *et al.* Synthesis and biological evaluation of 3-aminoisoquinolin-1(2H)-one based inhibitors of the dual-specificity phosphatase Cdc25B. *Bioorganic & medicinal chemistry*, doi:10.1016/j.bmc.2015.01.043 (2015).

259 Munch, C. *et al.* Therapeutic polo-like kinase 1 inhibition results in mitotic arrest and subsequent cell death of blasts in the bone marrow of AML patients and has similar effects in non-neoplastic cell lines. *Leukemia research*, doi:10.1016/j.leukres.2015.01.007 (2015).

260 Ogden, A., Rida, P. C., Reid, M. D., Kucuk, O. & Aneja, R. Die-hard survivors: heterogeneity in apoptotic thresholds may underlie chemoresistance. *Expert review of anticancer therapy*, 1-5, doi:10.1586/14737140.2015.1016425 (2015).

261 Vinod, P. K. & Novak, B. Model scenarios for switch-like mitotic transitions. *FEBS letters*, doi:10.1016/j.febslet.2015.02.007 (2015).

262 Zhou, H., Sun, L., Yang, X. L. & Schimmel, P. ATP-directed capture of bioactive herbal-based medicine on human tRNA synthetase. *Nature* **494**, 121-124, doi:10.1038/nature11774 (2013).

263 Hattori, M. & Gouaux, E. Molecular mechanism of ATP binding and ion channel activation in P2X receptors. *Nature* **485**, 207-212, doi:10.1038/nature11010 (2012).

264 Golab J, N. D., Skrzycki M, Czeczot H, Baranczyk-Kuzma A, Wilczynski GM, et al. . Antitumor effects of photodynamic therapy are potentiated by 2-methoxyestradiol. A superoxide dismutase inhibitor. *The Journal of biological chemistry* (2003).

265 Li, N. *et al.* Development of targeting lonidamine liposomes that circumvent drug-resistant cancer by acting on mitochondrial signaling pathways. *Biomaterials* **34**, 3366-3380, doi:10.1016/j.biomaterials.2013.01.055 (2013).

266 Schmeisser, S., Zarse, K. & Ristow, M. Lonidamine extends lifespan of adult Caenorhabditis elegans by increasing the formation of mitochondrial reactive oxygen species. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme* **43**, 687-692, doi:10.1055/s-0031-1286308 (2011).

267 Wang, S. S. *et al.* Destabilization of MYC/MYCN by the mitochondrial inhibitors, metaiodobenzylguanidine, metformin and phenformin. *International journal of molecular medicine* **33**, 35-42, doi:10.3892/ijmm.2013.1545 (2014).

268 Luengo, A., Sullivan, L. B. & Heiden, M. G. Understanding the complex-I-ty of metformin action: limiting mitochondrial respiration to improve cancer therapy. *BMC biology* **12**, 82, doi:10.1186/s12915-014-0082-4 (2014).

269 Wheaton, W. W. *et al.* Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *eLife* **3**, e02242, doi:10.7554/eLife.02242 (2014).

270 Aneja, R. *et al.* Multidrug resistance-associated protein-overexpressing teniposide-resistant human lymphomas undergo apoptosis by a tubulin-binding agent. *Cancer research* **68**, 1495-1503, doi:10.1158/0008-5472.CAN-07-1874 (2008).

271 Tepper, C. G. & Studzinski, G. P. Teniposide induces nuclear but not mitochondrial DNA degradation. *Cancer research* **52**, 3384-3390 (1992).

272 Pu, Q. H., Wu, Q. Q., Jin, X. B. & Wang, W. Z. [Gleevec induces apoptosis in K562 cells through activating caspase-3]. *Yao xue xue bao = Acta pharmaceutica Sinica* **49**, 1124-1129 (2014).

273 Gordon, J. *et al.* Imatinib mesylate (Gleevec) in the treatment of diffuse cutaneous systemic sclerosis: results of a 24-month open label, extension phase, single-centre trial. *Clinical and experimental rheumatology* **32**, S-189-193 (2014).

274 Procaccia, V., Nakayama, H., Shimizu, A. & Klagsbrun, M. Gleevec/imatinib, an ABL2 kinase inhibitor, protects tumor and endothelial cells from semaphorin-induced cytoskeleton collapse and loss of cell motility. *Biochemical and biophysical research communications* **448**, 134-138, doi:10.1016/j.bbrc.2014.04.063 (2014).

275 Patrizi, A. *et al.* Cutaneous adverse reactions linked to targeted anticancer therapies bortezomib and lenalidomide for multiple myeloma: new drugs, old side effects. *Cutaneous and ocular toxicology* **33**, 1-6, doi:10.3109/15569527.2013.787086 (2014).

276 Holohan, C., Van Schaeybroeck, S., Longley, D. B. & Johnston, P. G. Cancer drug resistance: an evolving paradigm. *Nature reviews. Cancer* **13**, 714-726, doi:10.1038/nrc3599 (2013).

277 Shapira, A., Livney, Y. D., Broxterman, H. J. & Assaraf, Y. G. Nanomedicine for targeted cancer therapy: towards the overcoming of drug resistance. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy* **14**, 150-163, doi:10.1016/j.drup.2011.01.003 (2011).

278 Juliano, R. L. & Ling, V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et biophysica acta* **455**, 152-162 (1976).

279 Ueda, K., Cardarelli, C., Gottesman, M. M. & Pastan, I. Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 3004-3008 (1987).

280 Nooter, K. *et al.* Expression of the multidrug resistance-associated protein (MRP) gene in human cancers. *Clinical cancer research : an official journal of the American Association for Cancer Research* **1**, 1301-1310 (1995).

281 Goldstein, L. J. *et al.* Expression of a multidrug resistance gene in human cancers. *Journal of the National Cancer Institute* **81**, 116-124 (1989).

282 Gottesman, M. M. Mechanisms of cancer drug resistance. *Annual review of medicine* **53**, 615-627, doi:10.1146/annurev.med.53.082901.103929 (2002).

283 Chen, C. J. *et al.* Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* **47**, 381-389 (1986).

284 Bogman, K., Peyer, A. K., Torok, M., Kusters, E. & Drewe, J. HMG-CoA reductase inhibitors and P-glycoprotein modulation. *British journal of pharmacology* **132**, 1183-1192, doi:10.1038/sj.bjp.0703920 (2001).

285 Ramachandra, M. *et al.* Human P-glycoprotein exhibits reduced affinity for substrates during a catalytic transition state. *Biochemistry* **37**, 5010-5019, doi:10.1021/bi973045u (1998).

286 Sauvant, C. *et al.* Acidosis induces multi-drug resistance in rat prostate cancer cells (AT1) in vitro and in vivo by increasing the activity of the p-glycoprotein via activation of p38. *International journal of cancer. Journal international du cancer* **123**, 2532-2542, doi:10.1002/ijc.23818 (2008).

287 Dean, M. ABC transporters, drug resistance, and cancer stem cells. *Journal of mammary gland biology and neoplasia* **14**, 3-9, doi:10.1007/s10911-009-9109-9 (2009).

288 Labhasetwar, V. Drug resistance in cancer therapy. *Drug delivery and translational research* **1**, 407-408, doi:10.1007/s13346-011-0047-x (2011).

289 Oerlemans, R. *et al.* Molecular basis of bortezomib resistance: proteasome subunit beta5 (PSMB5) gene mutation and overexpression of PSMB5 protein. *Blood* **112**, 2489-2499, doi:10.1182/blood-2007-08-104950 (2008).

290 Beretta, G. L., Gatti, L., Perego, P. & Zaffaroni, N. Camptothecin resistance in cancer: insights into the molecular mechanisms of a DNA-damaging drug. *Current medicinal chemistry* **20**, 1541-1565 (2013).

291 Baguley, B. C. Multiple drug resistance mechanisms in cancer. *Molecular biotechnology* **46**, 308-316, doi:10.1007/s12033-010-9321-2 (2010).

292 Elion, G. B. The purine path to chemotherapy. *Science* **244**, 41-47 (1989).

293 Tong, J., Yan, X. & Yu, L. The late stage of autophagy: cellular events and molecular regulation. *Protein & cell* **1**, 907-915, doi:10.1007/s13238-010-0121-z (2010).

294 Glick, D., Barth, S. & Macleod, K. F. Autophagy: cellular and molecular mechanisms. *The Journal of pathology* **221**, 3-12, doi:10.1002/path.2697 (2010).

295 Balis, F. M. Evolution of anticancer drug discovery and the role of cell-based screening. *Journal of the National Cancer Institute* **94**, 78-79 (2002).

296 Lamb, J. *et al.* The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. *Science* **313**, 1929-1935, doi:10.1126/science.1132939 (2006).

297 Pai, M. Y. *et al.* Drug affinity responsive target stability (DARTS) for small-molecule target identification. *Methods Mol Biol* **1263**, 287-298, doi:10.1007/978-1-4939-2269-7_22 (2015).

298 Lomenick, B., Jung, G., Wohlschlegel, J. A. & Huang, J. Target identification using drug affinity responsive target stability (DARTS). *Current protocols in chemical biology* **3**, 163-180, doi:10.1002/9780470559277.ch110180 (2011).

299 Lomenick, B. *et al.* Target identification using drug affinity responsive target stability (DARTS). *Proceedings of the National Academy of Sciences of the United States of America* **106**, 21984-21989, doi:10.1073/pnas.0910040106 (2009).

300 Bates, R. C., Edwards, N. S. & Yates, J. D. Spheroids and cell survival. *Critical reviews in oncology/hematology* **36**, 61-74 (2000).

301 Fayad, W. *et al.* Restriction of cisplatin induction of acute apoptosis to a subpopulation of cells in a three-dimensional carcinoma culture model. *International journal of cancer. Journal international du cancer* **125**, 2450-2455, doi:10.1002/ijc.24627 (2009).

302 Fayad, W. *et al.* Identification of agents that induce apoptosis of multicellular tumour spheroids: enrichment for mitotic inhibitors with hydrophobic properties. *Chemical biology & drug design* **78**, 547-557, doi:10.1111/j.1747-0285.2011.01170.x (2011).

303 Hagg, M. *et al.* A novel high-through-put assay for screening of pro-apoptotic drugs. *Investigational new drugs* **20**, 253-259 (2002).

304 Herrmann, R., Fayad, W., Schwarz, S., Berndtsson, M. & Linder, S. Screening for compounds that induce apoptosis of cancer cells grown as multicellular spheroids. *Journal of biomolecular screening* **13**, 1-8, doi:10.1177/1087057107310442 (2008).

305 Kunz-Schughart, L. A., Freyer, J. P., Hofstaedter, F. & Ebner, R. The use of 3-D cultures for high-throughput screening: the multicellular spheroid model. *Journal of biomolecular screening* **9**, 273-285, doi:10.1177/1087057104265040 (2004).

306 Santini, M. T. & Rainaldi, G. Three-dimensional spheroid model in tumor biology. *Pathobiology : journal of immunopathology, molecular and cellular biology* **67**, 148-157, doi:28065 (1999).

307 Sutherland, R. M., McCredie, J. A. & Inch, W. R. Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. *Journal of the National Cancer Institute* **46**, 113-120 (1971).

308 Inch, W. R., McCredie, J. A. & Sutherland, R. M. Growth of nodular carcinomas in rodents compared with multi-cell spheroids in tissue culture. *Growth* **34**, 271-282 (1970).

309 Mueller-Klieser, W. Three-dimensional cell cultures: from molecular mechanisms to clinical applications. *The American journal of physiology* **273**, C1109-1123 (1997).

310 Abbott, A. Cell culture: biology's new dimension. *Nature* **424**, 870-872, doi:10.1038/424870a (2003).

311 Weinhouse, S. The Warburg hypothesis fifty years later. *Zeitschrift fur Krebsforschung und klinische Onkologie. Cancer research and clinical oncology* **87**, 115-126 (1976).

312 Groebe, K. & Mueller-Klieser, W. On the relation between size of necrosis and diameter of tumor spheroids. *International journal of radiation oncology, biology, physics* **34**, 395-401 (1996).

313 Mueller-Klieser, W. F. & Sutherland, R. M. Influence of convection in the growth medium on oxygen tensions in multicellular tumor spheroids. *Cancer research* **42**, 237-242 (1982).

314 Mueller-Klieser, W., Vaupel, P. & Manz, R. Tumour oxygenation under normobaric and hyperbaric conditions. *The British journal of radiology* **56**, 559-564, doi:10.1259/0007-1285-56-668-559 (1983).

315 Hirschhaeuser, F. *et al.* Multicellular tumor spheroids: an underestimated tool is catching up again. *Journal of biotechnology* **148**, 3-15, doi:10.1016/j.jbiotec.2010.01.012 (2010).

316 Laurent, J. *et al.* Multicellular tumor spheroid models to explore cell cycle checkpoints in 3D. *BMC cancer* **13**, 73, doi:10.1186/1471-2407-13-73 (2013).

317 Haga, T., Uchide, N., Tugizov, S. & Palefsky, J. M. Role of E-cadherin in the induction of apoptosis of HPV16-positive CaSki cervical cancer cells during multicellular tumor spheroid formation. *Apoptosis : an international journal on programmed cell death* **13**, 97-108, doi:10.1007/s10495-007-0132-2 (2008).

318 Tian, X. *et al.* Hypoxia-inducible factor-1alpha enhances the malignant phenotype of multicellular spheroid HeLa cells in vitro. *Oncology letters* **1**, 893-897, doi:10.3892/ol_00000159 (2010).

319 Hernlund, E. *et al.* The phosphoinositide 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235 is effective in inhibiting regrowth of tumour cells after cytotoxic therapy. *Eur J Cancer* **48**, 396-406, doi:10.1016/j.ejca.2011.11.013 (2012).

320 Schwachofer, J. H. Multicellular tumor spheroids in radiotherapy research (review). *Anticancer research* **10**, 963-969 (1990).

321 Davidson, P., Bigerelle, M., Bounichane, B., Giazzon, M. & Anselme, K. Definition of a simple statistical parameter for the quantification of orientation in two dimensions: application to cells on grooves of nanometric depths. *Acta biomaterialia* **6**, 2590-2598, doi:10.1016/j.actbio.2010.01.038 (2010).

322 Kelm, J. M., Timmins, N. E., Brown, C. J., Fussenegger, M. & Nielsen, L. K. Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. *Biotechnology and bioengineering* **83**, 173-180, doi:10.1002/bit.10655 (2003).

323 Timmins, N. E., Dietmair, S. & Nielsen, L. K. Hanging-drop multicellular spheroids as a model of tumour angiogenesis. *Angiogenesis* **7**, 97-103, doi:10.1007/s10456-004-8911-7 (2004).

324 Friedrich, J. *et al.* A reliable tool to determine cell viability in complex 3-d culture: the acid phosphatase assay. *Journal of biomolecular screening* **12**, 925-937, doi:10.1177/1087057107306839 (2007).

325 Biven, K. *et al.* A novel assay for discovery and characterization of pro-apoptotic drugs and for monitoring apoptosis in patient sera. *Apoptosis : an international journal on programmed cell death* **8**, 263-268 (2003).

326 Preisler, H. D. & Venugopal, P. Regrowth resistance in cancer: why has it been largely ignored? *Cell proliferation* **28**, 347-356 (1995).

327 Preisler, H. D. & Gopal, V. Regrowth resistance in leukemia and lymphoma: the need for a new system to classify treatment failure and for new approaches to treatment. *Leukemia research* **18**, 149-160; discussion 161 (1994).

328 Kim, J. J. & Tannock, I. F. Repopulation of cancer cells during therapy: an important cause of treatment failure. *Nature reviews. Cancer* **5**, 516-525, doi:10.1038/nrc1650 (2005).

329 Baker, M. L., M, W. F. & Goldman, B. Hypertrophic cardiomyopathy with features of left ventricular non-compaction: how many diseases? *International journal of cardiology* **148**, 364-366, doi:10.1016/j.ijcard.2010.09.026 (2011).

330 Maira, S. M. *et al.* Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. *Molecular cancer therapeutics* **7**, 1851-1863, doi:10.1158/1535-7163.MCT-08-0017 (2008).

331 Barbacid, M. ras genes. *Annual review of biochemistry* **56**, 779-827, doi:10.1146/annurev.bi.56.070187.004023 (1987).

332 Malumbres, M. & Barbacid, M. RAS oncogenes: the first 30 years. *Nature reviews. Cancer* **3**, 459-465, doi:10.1038/nrc1097 (2003).

333 Giehl, K. Oncogenic Ras in tumour progression and metastasis. *Biological chemistry* **386**, 193-205, doi:10.1515/BC.2005.025 (2005).

334 Apolloni, A., Prior, I. A., Lindsay, M., Parton, R. G. & Hancock, J. F. H-ras but not K-ras traffics to the plasma membrane through the exocytic pathway. *Molecular and cellular biology* **20**, 2475-2487 (2000).

335 Bergo, M. O. *et al.* Inactivation of Icmt inhibits transformation by oncogenic K-Ras and B-Raf. *The Journal of clinical investigation* **113**, 539-550, doi:10.1172/JCI18829 (2004).

336 Bar-Sagi, D. & Hall, A. Ras and Rho GTPases: a family reunion. *Cell* **103**, 227-238 (2000).

337 Yan, Z. *et al.* Oncogenic c-Ki-ras but not oncogenic c-Ha-ras up-regulates CEA expression and disrupts basolateral polarity in colon epithelial cells. *The Journal of biological chemistry* **272**, 27902-27907 (1997).

338 Yan, Z., Chen, M., Perucho, M. & Friedman, E. Oncogenic Ki-ras but not oncogenic Ha-ras blocks integrin beta1-chain maturation in colon epithelial cells. *The Journal of biological chemistry* **272**, 30928-30936 (1997).

339 Laezza, C., Di Marzo, V. & Bifulco, M. v-K-ras leads to preferential farnesylation of p21(ras) in FRTL-5 cells: multiple interference with the isoprenoid pathway. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 13646-13651 (1998).

340 Song, G., Ouyang, G. & Bao, S. The activation of Akt/PKB signaling pathway and cell survival. *Journal of cellular and molecular medicine* **9**, 59-71 (2005).

341 Serra, V. *et al.* NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. *Cancer research* **68**, 8022-8030, doi:10.1158/0008-5472.CAN-08-1385 (2008).

342 Bedard, P. L. *et al.* A phase Ib dose-escalation study of the oral pan-PI3K inhibitor buparlisib (BKM120) in combination with the oral MEK1/2 inhibitor trametinib (GSK1120212) in patients with selected advanced solid tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research* **21**, 730-738, doi:10.1158/1078-0432.CCR-14-1814 (2015).

343 Bendell, J. C. *et al.* Phase I, dose-escalation study of BKM120, an oral pan-Class I PI3K inhibitor, in patients with advanced solid tumors. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **30**, 282-290, doi:10.1200/JCO.2011.36.1360 (2012).

344 Tabe, Y. *et al.* Class IA PI3K inhibition inhibits cell growth and proliferation in mantle cell lymphoma. *Acta haematologica* **131**, 59-69, doi:10.1159/000353164 (2014).

345 Vona-Davis, L. *et al.* MAPK and PI3K inhibition reduces proliferation of Barrett's adenocarcinoma in vitro. *The Journal of surgical research* **127**, 53-58, doi:10.1016/j.jss.2005.03.013 (2005).

346 Liu, Y., Zhang, Y., Jia, K., Dong, Y. & Ma, W. Metformin inhibits the proliferation of A431 cells by modulating the PI3K/Akt signaling pathway. *Experimental and therapeutic medicine* **9**, 1401-1406, doi:10.3892/etm.2015.2220 (2015).

347 Rodon, J. *et al.* Phase I dose-escalation and -expansion study of buparlisib (BKM120), an oral pan-Class I PI3K inhibitor, in patients with advanced solid tumors. *Investigational new drugs* **32**, 670-681, doi:10.1007/s10637-014-0082-9 (2014).

348 Ando, Y. *et al.* Phase I dose-escalation study of buparlisib (BKM120), an oral pan-class I PI3K inhibitor, in Japanese patients with advanced solid tumors. *Cancer science* **105**, 347-353, doi:10.1111/cas.12350 (2014).

349 Harris, A. L. Hypoxia--a key regulatory factor in tumour growth. *Nature reviews. Cancer* **2**, 38-47, doi:10.1038/nrc704 (2002).

350 Mellor, H. R. & Callaghan, R. Resistance to chemotherapy in cancer: a complex and integrated cellular response. *Pharmacology* **81**, 275-300, doi:10.1159/000115967 (2008).

351 Sahin, A. A. *et al.* Tumor proliferative fraction in solid malignant neoplasms. A comparative study of Ki-67 immunostaining and flow cytometric determinations. *American journal of clinical pathology* **96**, 512-519 (1991).

352 St Croix, B. *et al.* Impact of the cyclin-dependent kinase inhibitor p27Kip1 on resistance of tumor cells to anticancer agents. *Nature medicine* **2**, 1204-1210 (1996).

353 Gardner, L. B. *et al.* Hypoxia inhibits G1/S transition through regulation of p27 expression. *The Journal of biological chemistry* **276**, 7919-7926, doi:10.1074/jbc.M010189200 (2001).

354 Davis, A. J. & Tannock, I. F. Tumor physiology and resistance to chemotherapy: repopulation and drug penetration. *Cancer treatment and research* **112**, 1-26 (2002).

355 Tannock, I. F. Tumor physiology and drug resistance. *Cancer metastasis reviews* **20**, 123-132 (2001).

356 Hirayama, A. *et al.* Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry. *Cancer research* **69**, 4918-4925, doi:10.1158/0008-5472.CAN-08-4806 (2009).

357 Gribbestad, I. S., Sitter, B., Lundgren, S., Krane, J. & Axelson, D. Metabolite composition in breast tumors examined by proton nuclear magnetic resonance spectroscopy. *Anticancer research* **19**, 1737-1746 (1999).

358 Chan, E. C. *et al.* Metabolic profiling of human colorectal cancer using high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) spectroscopy and gas chromatography mass spectrometry (GC/MS). *Journal of proteome research* **8**, 352-361, doi:10.1021/pr8006232 (2009).

359 Venkatasubramanian, R., Henson, M. A. & Forbes, N. S. Incorporating energy metabolism into a growth model of multicellular tumor spheroids. *Journal of theoretical biology* **242**, 440-453, doi:10.1016/j.jtbi.2006.03.011 (2006).

360 Sacks, P. G., Racz, T., Schantz, S. P. & Rosenblum, M. G. Growth inhibition by interferon beta and gamma of MDA 886Ln monolayer cells and multicellular tumor spheroids. A differentiation therapy model for squamous cell carcinoma. *Archives of otolaryngology--head & neck surgery* **120**, 1267-1272 (1994).

361 Wenzel, C. *et al.* 3D high-content screening for the identification of compounds that target cells in dormant tumor spheroid regions. *Experimental cell research* **323**, 131-143, doi:10.1016/j.yexcr.2014.01.017 (2014).

362 Desoize, B. & Jardillier, J. Multicellular resistance: a paradigm for clinical resistance? *Critical reviews in oncology/hematology* **36**, 193-207 (2000).

363 Cho, R. W. & Clarke, M. F. Recent advances in cancer stem cells. *Current opinion in genetics & development* **18**, 48-53, doi:10.1016/j.gde.2008.01.017 (2008).

364 Mimeault, M., Hauke, R. & Batra, S. K. Stem cells: a revolution in therapeutics-recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. *Clinical pharmacology and therapeutics* **82**, 252-264, doi:10.1038/sj.clpt.6100301 (2007).

365 Mimeault, M. & Batra, S. K. Concise review: recent advances on the significance of stem cells in tissue regeneration and cancer therapies. *Stem Cells* **24**, 2319-2345, doi:10.1634/stemcells.2006-0066 (2006).

366 Dalerba, P. *et al.* Phenotypic characterization of human colorectal cancer stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 10158-10163, doi:10.1073/pnas.0703478104 (2007).

367 Lee, J. *et al.* Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer cell* **9**, 391-403, doi:10.1016/j.ccr.2006.03.030 (2006).

368 Tennant, D. A., Duran, R. V. & Gottlieb, E. Targeting metabolic transformation for cancer therapy. *Nature reviews. Cancer* **10**, 267-277, doi:10.1038/nrc2817 (2010).

369 Wang, X. *et al.* The 19S Deubiquitinase inhibitor b-AP15 is enriched in cells and elicits rapid commitment to cell death. *Molecular pharmacology* **85**, 932-945, doi:10.1124/mol.113.091322 (2014).

370 D'Arcy, P. *et al.* Inhibition of proteasome deubiquitinating activity as a new cancer therapy. *Nature medicine* **17**, 1636-1640, doi:10.1038/nm.2536 (2011).

371 Lipinski, C. A. Lead- and drug-like compounds: the rule-of-five revolution. *Drug discovery today. Technologies* **1**, 337-341, doi:10.1016/j.ddtec.2004.11.007 (2004).

372 Lipinski, C. A., Lombardo, F., Dominy, B. W. & Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced drug delivery reviews* **46**, 3-26 (2001).

373 Fichert, T., Yazdanian, M. & Proudfoot, J. R. A structure-permeability study of small drug-like molecules. *Bioorganic & medicinal chemistry letters* **13**, 719-722 (2003).

374 Congreve, M., Carr, R., Murray, C. & Jhoti, H. A 'rule of three' for fragment-based lead discovery? *Drug Discov Today* **8**, 876-877 (2003).

375 Lu, J., Kunimoto, S., Yamazaki, Y., Kaminishi, M. & Esumi, H. Kigamicin D, a novel anticancer agent based on a new anti-austerity strategy targeting cancer cells' tolerance to nutrient starvation. *Cancer science* **95**, 547-552 (2004).

376 Izumi, M. *et al.* Establishment of monitoring methods for autophagy in rice reveals autophagic recycling of chloroplasts and root plastids during energy limitation. *Plant physiology*, doi:10.1104/pp.114.254078 (2015).

377 Kim, K. H. & Lee, M. S. Autophagy as a crosstalk mediator of metabolic organs in regulation of energy metabolism. *Reviews in endocrine & metabolic disorders* **15**, 11-20, doi:10.1007/s11154-013-9272-6 (2014).

378 Li, Z. L. & Lerman, L. O. Impaired myocardial autophagy linked to energy metabolism disorders. *Autophagy* **8**, 992-994, doi:10.4161/auto.20285 (2012).

379 Mathew, R. & White, E. Autophagy in tumorigenesis and energy metabolism: friend by day, foe by night. *Current opinion in genetics & development* **21**, 113-119, doi:10.1016/j.gde.2010.12.008 (2011).

380 Wu, J. J., Quijano, C., Wang, J. & Finkel, T. Metabolism meets autophagy. *Cell Cycle* **9**, 4780-4781 (2010).

381 Rabinowitz, J. D. & White, E. Autophagy and metabolism. *Science* **330**, 1344-1348, doi:10.1126/science.1193497 (2010).

382 Song, J. *et al.* Autophagy in hypoxia protects cancer cells against apoptosis induced by nutrient deprivation through a Beclin1-dependent way in hepatocellular carcinoma. *Journal of cellular biochemistry* **112**, 3406-3420, doi:10.1002/jcb.23274 (2011).

383 Marino, M. L. *et al.* Proton pump inhibition induces autophagy as a survival mechanism following oxidative stress in human melanoma cells. *Cell death & disease* **1**, e87, doi:10.1038/cddis.2010.67 (2010).

384 Yang, S. *et al.* Pancreatic cancers require autophagy for tumor growth. *Genes & development* **25**, 717-729, doi:10.1101/gad.2016111 (2011).

385 Amaravadi, R. K. *et al.* Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *The Journal of clinical investigation* **117**, 326-336, doi:10.1172/JCI28833 (2007).

386 Qu, X. *et al.* Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *The Journal of clinical investigation* **112**, 1809-1820, doi:10.1172/JCI20039 (2003).

387 Cairns, R. A., Harris, I. S. & Mak, T. W. Regulation of cancer cell metabolism. *Nature reviews. Cancer* **11**, 85-95, doi:10.1038/nrc2981 (2011).

388 Chiche, J. *et al.* Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH. *Cancer research* **69**, 358-368, doi:10.1158/0008-5472.CAN-08-2470 (2009).

389 Neri, D. & Supuran, C. T. Interfering with pH regulation in tumours as a therapeutic strategy. *Nature reviews. Drug discovery* **10**, 767-777, doi:10.1038/nrd3554 (2011).

390 Robey, I. F. *et al.* Bicarbonate increases tumor pH and inhibits spontaneous metastases. *Cancer research* **69**, 2260-2268, doi:10.1158/0008-5472.CAN-07-5575 (2009).

391 Supuran, C. T. Carbonic anhydrase inhibitors and activators for novel therapeutic applications. *Future medicinal chemistry* **3**, 1165-1180, doi:10.4155/fmc.11.69 (2011).

392 Supuran, C. T. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nature reviews. Drug discovery* **7**, 168-181, doi:10.1038/nrd2467 (2008).

393 Fais, S. Proton pump inhibitor-induced tumour cell death by inhibition of a detoxification mechanism. *Journal of internal medicine* **267**, 515-525, doi:10.1111/j.1365-2796.2010.02225.x (2010).

394 Jain, M. *et al.* Sustained loss of a neoplastic phenotype by brief inactivation of MYC. *Science* **297**, 102-104, doi:10.1126/science.1071489 (2002).

395 Wang, H. *et al.* c-Myc depletion inhibits proliferation of human tumor cells at various stages of the cell cycle. *Oncogene* **27**, 1905-1915, doi:10.1038/sj.onc.1210823 (2008).

396 Lin, C. Y. *et al.* Transcriptional amplification in tumor cells with elevated c-Myc. *Cell* **151**, 56-67, doi:10.1016/j.cell.2012.08.026 (2012).

397 Nie, Z. *et al.* c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. *Cell* **151**, 68-79, doi:10.1016/j.cell.2012.08.033 (2012).

398 Dang, C. V. *et al.* The c-Myc target gene network. *Seminars in cancer biology* **16**, 253-264, doi:10.1016/j.semcancer.2006.07.014 (2006).

399 Fan, Y., Dickman, K. G. & Zong, W. X. Akt and c-Myc differentially activate cellular metabolic programs and prime cells to bioenergetic inhibition. *The Journal of biological chemistry* **285**, 7324-7333, doi:10.1074/jbc.M109.035584 (2010).

400 Kim, J., Lee, J. H. & Iyer, V. R. Global identification of Myc target genes reveals its direct role in mitochondrial biogenesis and its E-box usage in vivo. *PloS one* **3**, e1798, doi:10.1371/journal.pone.0001798 (2008).

401 Mateyak, M. K., Obaya, A. J., Adachi, S. & Sedivy, J. M. Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* **8**, 1039-1048 (1997).

402 Sarin, M. *et al.* Alterations in c-Myc phenotypes resulting from dynamin-related protein 1 (Drp1)-mediated mitochondrial fission. *Cell death & disease* **4**, e670, doi:10.1038/cddis.2013.201 (2013).

403 Noubissi, F. K. *et al.* CRD-BP mediates stabilization of betaTrCP1 and c-myc mRNA in response to beta-catenin signalling. *Nature* **441**, 898-901, doi:10.1038/nature04839 (2006).

404 He, W. *et al.* miR-494 acts as an anti-oncogene in gastric carcinoma by targeting c-myc. *Journal of gastroenterology and hepatology* **29**, 1427-1434, doi:10.1111/jgh.12558 (2014).

405 Lim, L. *et al.* MicroRNA-494 within an oncogenic microRNA megacluster regulates G1/S transition in liver tumorigenesis through suppression of mutated in colorectal cancer. *Hepatology* **59**, 202-215, doi:10.1002/hep.26662 (2014).

406 Hu, G., Lou, Z. & Gupta, M. The long non-coding RNA GAS5 cooperates with the eukaryotic translation initiation factor 4E to regulate c-Myc translation. *PloS one* **9**, e107016, doi:10.1371/journal.pone.0107016 (2014).

407 Pan, J. *et al.* USP37 directly deubiquitinates and stabilizes c-Myc in lung cancer. *Oncogene*, doi:10.1038/onc.2014.327 (2014).

408 Yang, F. *et al.* Long non-coding RNA GHET1 promotes gastric carcinoma cell proliferation by increasing c-Myc mRNA stability. *The FEBS journal* **281**, 802-813, doi:10.1111/febs.12625 (2014).

409 Gordan, J. D., Thompson, C. B. & Simon, M. C. HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. *Cancer cell* **12**, 108-113, doi:10.1016/j.ccr.2007.07.006 (2007).

410 Brunmair, B. *et al.* Thiazolidinediones, like metformin, inhibit respiratory complex I: a common mechanism contributing to their antidiabetic actions? *Diabetes* **53**, 1052-1059 (2004).

411 Di Santo, N. & Ehrisman, J. A functional perspective of nitazoxanide as a potential anticancer drug. *Mutation research* **768**, 16-21, doi:10.1016/j.mrfmmm.2014.05.005 (2014).

412 Rodriguez-Garcia, R., Rodriguez-Guzman, L. M. & Cruz del Castillo, A. H. [Effectiveness and safety of mebendazole compared to nitazoxanide in the treatment of Giardia lamblia in children]. *Revista de gastroenterologia de Mexico* **64**, 122-126 (1999).

413 Liu, C., Wu, D., Zheng, X., Li, P. & Li, L. Efficacy and safety of metformin for patients with type 1 diabetes mellitus: a meta-analysis. *Diabetes technology & therapeutics* **17**, 142-148, doi:10.1089/dia.2014.0190 (2015).

414 Liu, X. *et al.* The long-term efficacy and safety of DPP-IV inhibitors monotherapy and in combination with metformin in 18,980 patients with type-2 diabetes mellitus--a meta-analysis. *Pharmacoepidemiology and drug safety* **23**, 687-698, doi:10.1002/pds.3586 (2014).

415 Zhang, X. *et al.* Induction of mitochondrial dysfunction as a strategy for targeting tumour cells in metabolically compromised microenvironments. *Nature communications* **5**, 3295, doi:10.1038/ncomms4295 (2014).