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DEVELOPMENT OF THERAPEUTIC STRATEGIES FOR QUIESCENT TUMOR CELL POPULATIONS

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Stockholm 2015
Development of therapeutic strategies for quiescent tumor cell populations
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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

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

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

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. Accepted for publication in Molecular and Cellular Therapeutics

*These authors contributed equally to this work.
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.

Molecular Pharmacology 85,932–945 (2014)
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<tr>
<td>2-DG</td>
<td>2-deoxy-D-glucose</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-methoxyestradiol</td>
</tr>
<tr>
<td>3-BrPA</td>
<td>3-bromopyruvate</td>
</tr>
<tr>
<td>3-MA</td>
<td>3-methyladenine</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myelogenous leukemia</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP activated kinase</td>
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<tr>
<td>ANT</td>
<td>Adenine nucleotide translocase</td>
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<tr>
<td>ATG</td>
<td>Autophagy-related protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bHLH–ZIP</td>
<td>Basic helix–loop–helix–leucine zipper</td>
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<tr>
<td>BIP</td>
<td>Binding immunoglobulin protein</td>
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<td>C-map</td>
<td>Connectivity map</td>
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<tr>
<td>CQ</td>
<td>Chloroquine</td>
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<tr>
<td>CRD-BD</td>
<td>Coding region determinant-binding protein</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
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<tr>
<td>DARTS</td>
<td>Drug affinity responsive target stability</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Drp1</td>
<td>Dynamin-related protein 1</td>
</tr>
<tr>
<td>ECAR</td>
<td>Extracellular acidification rate</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and drug administration</td>
</tr>
<tr>
<td>FdG</td>
<td>$^{18}$F-Fluorodeoxyglucose</td>
</tr>
<tr>
<td>GC</td>
<td>Gastric carcinoma</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
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<tr>
<td>GSEA</td>
<td>Gene set enrichment analysis</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HIF1</td>
<td>Hypoxia-inducible factor 1</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IDH</td>
<td>Isocitrate dehydrogenase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>KRAS</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>LDHA</td>
<td>Lactate dehydrogenase A</td>
</tr>
<tr>
<td>IncRNA</td>
<td>Long non-coding RNA</td>
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<tr>
<td>MDR1</td>
<td>Multi-drug resistance</td>
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<tr>
<td>MHOK</td>
<td>Methyl-13-hydroxy-15-oxokaurenoate</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane permeability</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
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<tr>
<td>mTOR1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>MTS</td>
<td>Multicellular spheroids</td>
</tr>
<tr>
<td>MYC</td>
<td>Myelocytomatosis oncogene</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>nDNA</td>
<td>Nuclear DNA</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxidative consumption rate</td>
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<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
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<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PI3KCA</td>
<td>Phosphatidylinositol 3-kinase catalytic subunit</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TFAM</td>
<td>Transcription factor A of mitochondria</td>
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<tr>
<td>TME</td>
<td>Tumor microenvironment</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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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. 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). Cancer has become one of the biggest killers in world.

Records of cancer can date back to the days of the great pyramids. 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".

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.

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. 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."

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. A decade later Watson and Crick published a model for the structure of DNA. 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, the underlying cause of this abnormal phenotype is mutations in the genome. The mutations of these genes lead to defects in proteins that control signalling pathways that relate to cell growth, proliferation, metabolism.
In 2000, Hanahan and Weinberg described the six hallmarks of cancer\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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$_2$O 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. Clinical research have indicated that glycolysis-related genes are found overexpressed in almost 70% of all human cancers, The increased uptake of the glucose analogue $^{18}$F-fluorodeoxyglucose by cancer cells can be utilized for diagnostic purposes. 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. 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. 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. 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. Figure 2 is a simple overview of metabolic activities in cancer cells.

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**Figure 2:** An overview of metabolic activities in cancer cells. Glycolysis and glutaminolysis are upregulated 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. 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. 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, 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. In clinical, investigators define the regions, where \([pO_2 \text{ values}] \leq 2.5 \text{ mm Hg}\), 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.

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.

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).

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.

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.\textsuperscript{62} Drugs that target proliferating tumor cell will not be effective on these quiescent cancer cells.\textsuperscript{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.\textsuperscript{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.\textsuperscript{49}

1.1.3.2 *Extracellular acidosis*

Intensive glycolysis in tumor cells will result in extracellular acidosis. Under glycolysis, glucose does not form H\textsubscript{2}O and CO\textsubscript{2} from OXPHOS, but lactate from pyruvate. The production of lactic acid leads to acidification in tumor microenvironment.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{83-86}

Hypoxia, extracellular acidosis and nutrient deprivation associate with each other and the relations between them are addressed in *Figure 3*.\textsuperscript{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 \textit{CMYC} proto-oncogene, which was first discovered as an inducer of retrovirally mediated tumorigenesis, belongs to the myelocytomatosis oncogene family (MYC) that also contains \textit{MYCN} (N-myc) and \textit{MYCL} (L-myc),\textsuperscript{88-90} which evolve from myc-like genes found in drosophila and hydra.\textsuperscript{91,92} In human, the \textit{c-MYC} oncogene is overexpressed in at least 40\% of cancers and serves as a "master regulator" of cellular metabolism and proliferation.\textsuperscript{89,93,94}

\textit{C-MYC} is an early response gene to growth factor stimulation. As \textit{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, \textit{c-MYC} gene expression is down regulated in normal cells in order to stop proliferation. In contrast, when \textit{c-MYC} is oncogenically activated, for example, by chromosomal translocation or gene amplification, \textit{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.\textsuperscript{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. The MYC-Max complex is involved in the control of a wide array of cellular activities (Figure 4), like cell mass regulation, cell survival and is a master regulator of metabolic processes including glycolysis, oxidative phosphorylation and mitochondrial biogenesis.

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. Mitochondrial generation of acetyl-CoA is dependent on c-MYC. 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. Interestingly, c-MYC recently has been shown to be able to alter glutamine metabolism by transcriptionally repressing miRNAs, like miR-23a and miR-23b.

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 through different approaches. For example, JQ1, which is a selective inhibitor of bromodomain protein, leads to downregulation of c-MYC. Researchers also have attempted to use c-MYC antisense oligonucleotides, which has been encouraging in vitro but failed to translate into effective clinical treatments. Moreover, in some experiments, c-MYC suppression has initially resulted in a tumor regression but subsequently recurred. 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). 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.](image-url)
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.\textsuperscript{71,124-126} Recent studies have revealed the importance of autophagy in development,\textsuperscript{127,128} aging,\textsuperscript{129} and pathophysiology such as neurodegenerative disease, obesity and cancer.\textsuperscript{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.\textsuperscript{135-137} Decreases of their phosphorylation termed a sign of mTOR inhibition and autophagy activation in cells.\textsuperscript{138,139}

Autophagy starts from the formation of a double membrane-bound autophagosome mediated by autophagy-related protein (ATG) proteins.\textsuperscript{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.\textsuperscript{126,141-143}

![Figure 5: The process of autophagy in mammalian cells.](image-url)

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.\textsuperscript{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.\textsuperscript{71,133,134} Because of this, autophagy is viewed as a druggable process in cancer treatment.\textsuperscript{142,144-149} For example, 3-methyladenine (3-MA), which is the most widely used autophagy inhibitor for \textit{in vitro} experiments.\textsuperscript{150,151} Chloroquine (CQ) which was firstly used for malaria also has shown an anti-cancer effect as an autophagy inhibitor.\textsuperscript{73,152-155} The latest autophagy inhibitor lys05, a dimeric chloroquine, produces more potent antitumor activity as a single agent both \textit{in vitro} and \textit{in vivo} in several human cancer cell lines and xenograft models.\textsuperscript{156,157}

1.1.4.3 Aerobic glycolysis in tumor cells

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

$$\text{Glucose} + 2\text{NAD}^+ + 2\text{ADP} + 2\text{P} \rightarrow \text{2pyruvate} + 2\text{ATP} + 2\text{NADH} + 2\text{H}^+$$ \textsuperscript{158}

In most mammalian cells, glycolysis is inhibited by the presence of oxygen, which allows mitochondria to oxidize pyruvate to CO\textsubscript{2} and H\textsubscript{2}O 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.\textsuperscript{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\textsuperscript{27,160} and has later been confirmed by numerous investigators.\textsuperscript{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.\textsuperscript{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,\textsuperscript{167-169} actives the transcription of genes encoding glycolytic enzymes (such as phosphoglycerate kinase 1 and pyruvate kinase M).\textsuperscript{168-171} The upregulation of glycolysis will have significant negative consequences, due to extracellular acidosis. Tumor cells upregulate Na\textsuperscript{+}–H\textsuperscript{+} exchange and vacuolar H\textsuperscript{+}-ATPases, undergo mutations or epigenetic changes to adapt extracellular acidification.\textsuperscript{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 (\textit{Figure 6}).\textsuperscript{18,47,53,176-178} At the same time, these biological changes also make tumor cells more resistance to cancer therapies.\textsuperscript{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 flow183,185-187 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. 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. 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. 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%. 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 (rho₀) by growing tumor cells in medium with ethidium bromide (EtBr). The rho₀ cancer cells show a dropped growth rate, decreased colony formation and markedly reduced tumor formation in nude mice.

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 assist cancer cells accommodating to the constantly changing bioenergetic environments.

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 H₂O₂ to water. The increased level of H₂O₂ can contribute
to neoplastic transformation.\textsuperscript{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, HIF1\textgreek{a} is stabilized\textsuperscript{215} and causes a shift from oxidative phosphorylation to glycolytic energy metabolism.\textsuperscript{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.\textsuperscript{183,216-221} It is also true that mitochondria are controlled or regulated by other proteins (like CMYC, Hsp90 and VEGF),\textsuperscript{222-231} cellular biological process (mitophagy and proteasome degradation)\textsuperscript{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,\textsuperscript{232-234} 3-bromopyruvate (3-BrPA), a lactate/pyruvate analogue, is an alkylating agent and a potent inhibitor of glycolysis,\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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-strand 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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, Aurantricarboxylic 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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).\textsuperscript{234,265,266} Metformin, used in diabetes before, recently has been reported as a mitochondrial complex I inhibitor.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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. 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.

One possible reason to explain the intrinsic drug resistance of tumor cells is their high levels of expression of p-glycoprotein (P-gp). 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. Recent study has indicated that, in solid growing tumors, extracellular acidosis leads to an increased P-gp activity, which is pH-dependent.

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. 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.

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. Acquired drug resistance is a more complex cell response to anticancer drugs and a result of constantly selection for tumor cell survival and proliferation.

Reasons of tumor cell acquired drug resistance include a) decreased drug uptake and increased drug efflux, b) mutation or alteration of the expression of a drug target, c) DNA repair, d) deregulation of apoptosis and e) autophagy upregulation.

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. 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) and drug affinity responsive
target stability (DARTS)\textsuperscript{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 \textit{in vivo}, but does not influence drug activity on monolayer cultures. Today, these shortcomings increase the risk of failures \textit{in vivo}.\textsuperscript{300-307} Multicellular spheroids represent an \textit{in vitro} tumor model in which cancer cells are supplied by the diffusion of substrates from the surrounding growth medium.\textsuperscript{58} Multicellular spheroid model was adapted to cancer researches in the early 1970s by Sutherland and colleges\textsuperscript{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.\textsuperscript{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.\textsuperscript{58,304,310} Figure 8 is an overview of the spheroid characterizations and how structure influences the function in spheroids.

\textbf{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.\textsuperscript{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,\textsuperscript{316} the role of E-cadherin in the induction of apoptosis,\textsuperscript{317} and the role of HIF1-1α on the malignant phenotype have been studied in the multicellular spheroid model.\textsuperscript{318} Multicellular spheroids model have been widely used in studies of cancer therapeutic drugs and more recently in cancer drug discover.\textsuperscript{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. Nutrients, oxygen, and waste products do not diffuse freely in and out of spheroids. Tumor cell lines are in general more resistant to anticancer agents when the cells are grown as 3-dimensional spheroids rather than monolayer cultures. 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.

The gene expression patterns of cells grown in monolayer cultures and as spheroids differ, and some differences can be attributed to intrinsic drug sensitivity. For these reasons, MTS model-based drug development is regarded as an important step before in vivo experiments (Figure 9).

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, 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).

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 p27Kip1 (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 to determine cell viability and the M30 Apoptosence method 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.

![Figure 9: An overview of drug development processes.](image)
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.\textsuperscript{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\textsuperscript{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\textsuperscript{wt/-} cells showed a similar rate of spheroid growth as the KRAS\textsuperscript{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.\textsuperscript{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.\textsuperscript{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. KRAS is one isoform of RAS family (H-RAS, N-RAS and K-RAS are R-RAS). It has been noticed that different RAS family members play different roles in tumor progression and invasion. For example, K-RAS was found to lead to a loss of cell–cell and cell–matrix adhesion. 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. 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. 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. And similar results have been also reported in other publications. 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. The oral pan-PI3K inhibitor buparlisib (BKM120), that has been tested in clinical phase I, has shown positive result in advanced solid tumors. 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{354,355} The altered phenotype of quiescent cells enables them to survive chemotherapeutic regimes and reseed nascent tumors following secession of chemotherapy.\textsuperscript{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,\textsuperscript{356-358} and consistent with previous reports,\textsuperscript{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 \(\mu M\)) 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-1α (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. 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 (Figure 10).

<table>
<thead>
<tr>
<th>High glucose</th>
<th>Low glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proliferating tumor cell with high energy demand</strong></td>
<td><strong>Non-proliferating normal cell with low energy demand</strong></td>
</tr>
<tr>
<td>Rib. reduct.</td>
<td>ATP</td>
</tr>
<tr>
<td>DNA synth.</td>
<td>ATP</td>
</tr>
<tr>
<td>Glycolysis</td>
<td>HIF-1α</td>
</tr>
<tr>
<td>ATP</td>
<td>OXPHOS</td>
</tr>
<tr>
<td>Autophagy</td>
<td>ATP &lt; demand</td>
</tr>
<tr>
<td>Cell death</td>
<td>ATP = demand</td>
</tr>
<tr>
<td>High ATP demand not met by glycolysis and autophagy</td>
<td>Lower ATP demand met by glycolysis, autophagy not required</td>
</tr>
</tbody>
</table>

**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, drugs can be preferentially effective in 3D but not in 2D (like nitazoxanide). As the spheroids have gradient structures, the outer 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 outer 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. 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 trials. Lys05, Autophagy inhibitor, has shown a good effect alone both in vitro and in vivo. 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.

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, a lead-like chemical should have MW < 300, log P < 3, H-bond donors and acceptors <3 and rotatable bonds <3. 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 IC50 on HCT116 monolayer cells is 0.63µM, 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. 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.
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, 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. 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 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 that are obviously overexpressed in tumor cells, have been used as anti-cancer and anti-metastasis agents. This indicates
that targeting pH regulation in tumor cells represents a novel and attractive therapeutic strategy, as acidic pH is an essential positive regulator of autophagy.
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. 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, and depletion of MYC leads to proliferative arrest of tumor cell lines. The MYC protein is a relatively weak transcriptional activator and is believed to function as a general enhancer of transcription. Genes encoding proteins involved in cell growth, metabolism, ribosome biogenesis, protein synthesis and mitochondrial function are overrepresented as MYC targets. The work of several groups has established an important role of MYC in supporting mitochondrial biogenesis. MYC affects mitochondrial mass by influencing both mitochondrial fusion and fission, favouring the latter process.

**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. 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 (IncRNA-GHET1) is high expressed in gastric carcinoma.\(^ {408}\) Possible mechanisms are summarized below in *Figure 11.*

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**Figure 11:** 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. 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. 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, and nitazoxanide (described in paper V), which is a FDA approved anthelmintic drug, has shown an effect of mitochondrial inhibition and c-MYC reduction in spheroids. Both drugs can be continuously taken by patients. 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. 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 heterogeneity 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.
4 ACKNOWLEDGEMENTS

Time always goes fast especially when I find something I really love and get along with people I appreciate during my whole life.

I would like to especially thank:

Stig Linder, my dear and lovely main supervisor, thank you so much for all your guidance, encouragements and supports during my four-year PhD study. Every time when I feel disappointed about the failed experiments or do not know how to continue, you always give me smiles and helpful suggestions. It is you to make me gradually feel confident in myself and make me believe I can do well in researches. But the most important thing I have learnt from you is how to be a better person. You spare no effort to keep balance between work and family. You always give a hand whenever it is needed. You always see things on the bright side and seldom complain. You always work a lot for others but say little… You have deeply influenced me, perhaps you have not noticed. Dear Stig, without you I can never be a person I wished to be during theses years! Billions of thanks and best wishes! ☺

Maria Hägg: my dear co-supervisor thanks so much for all your help during these years. You are so calm and considerate, no matter what kind of troubles I meet, I always feel safe when I come to you. You help me find stuff, arrange schedule and order reagents… You also help me a lot beside work. Every time when I need suggestions, the first thing comes to my mind is that-I can ask Maria tomorrow! Thanks for your delicious cakes for fika and dinners for our group during these years. The taste of the food has already become part of my memory. ☻

Angelo De Milito: my dear co-supervisor, it is you to make me start to love Italy! Thanks for all your help during these years. It is always enjoyed to discuss with you, your suggestions for projects are always professional and practical. Most importantly, following your suggestions always come to good results!☺ Your passion on science influences me; I have never found a person can keep reading scientific paper the whole day before we sited in the same office. And thank you for all the food: mussels, homemade dry tomato and even bread, they are so delicious. I always feel I have four stomachs when I eat at your place! ☺

Pádraig Darcy: my dear co-supervisor. Now I still remember that you made me a cup of tea when I first came to CCK. You can never know how nervous I was at that moment and your smile was like a light in the dark. Thanks! You are kind and patient to every one. You always have fantastic ideas and generously share with others. You are like a ‘moving Wikipedia’; every question can have an answer from you! You always save me from totally failed experiments and encourage me to continue. Thanks for all your advices on growing flowers and all the muffins you prepared for us. ☻

Ingrid Holmberg: Thanks for all your organization for us and all your help for my thesis, I can never image the figures in my thesis can be so beautiful! Best wishes for everything. ☻
Henrik Duid: Four years ago, without your recommendation, I might not have the chance to come to Karolinska, my dreamed place. It is you to help me open the door and make my life different. Thanks is already not enough, I will keep it in mind in my life! 😊

Wolfgang Mueller-Klieser, thank you so much for your acceptance to be my opponent! 😊
Ewa Ehrenborg, Agneta Jansson and Hakan Mellstedt, thank you for sparing your time to be my committee members.

I also would like to thank my lovely group members, previous and present:

Xin, thanks so much for all your companies during my PhD study, we have shared so many stories, we have travelled together, we have faced difficulties at the same time. You are like my older sister and give me so many valuable advices when I am in the face of choice. Thanks so much and best wishes for everything in your brilliant future! 😊 Paola, you are like a happy bird in our lab and it is really my pleasure to work with you. When I ask you questions, you always stop what you are doing and answer patiently. Thanks for all the packages of Taralli from Italy. They make my stomach satisfied every time. Best wishes for your PhD study and your lovely future. 😊 Magda, it is so nice to have you in our group, you are very humour and open-minded, it is really enjoyed to talk with you. You are such a beautiful girl and wish you a beautiful life! 😊 Ellin, welcome to this new family and thanks for your cakes! Best wishes for PhD study, I am sure you will be a star! 😊 And I would say thank you to David, it is nice to talk with you. Best wishes for your future!

Thanks to my previous group members: Slavica, thanks for all your help in lab.😊 Walid, it is really nice to know you and I wish I could know you earlier. Best wishes. Emma, thank you so much Emma. Without you, I cannot get familiar with all the things in lab in a short time. You gave me your protocols and they are still on my bench. You can never know how important of your warm smile, it gave me a lot of encouragements and confidence during my first year of study. Wish you a wonderful life! 😊 Karolina, I really cherish the time we spent together in lab, we had dinner together, drank together and worked together. I have learnt a lot from you. Thanks for all your help and pleased also say hi to your husband. You are such a wonderful couple! Hope to see you again. 😊 Giuseppe: too much would like to say, you are very positive, kind and easy going. It is lucky to meet you here. Good luck and come to Sweden again and let us have a cup of drink! Angela, I am missing your smile, hope to see you again! Best wishes. 😊 Chiara, hope I can pronounce your name exactly next time when I see you, you are such a warm girl and make everyone feel conformable. I am sure you will be very successful in the future! 😊 Hanif, Chitralekha, Maria B, Feli and Francesca, it is really nice to work with you. Hope to see you and talk with you again! 😊

It is my honour to know you all in my life and we are members of Stig Linder group forever!
I would like to thank my collaborators:

Mårten Fryknäs, Rolf Larsson and Wojciech Senkowski from Uppsala University, thanks for all your efforts on our collaborated projects! ☺ Vladimir Gogvadze, thank you for providing me so many suggestions and help on mitochondrial analysis.

I would like to thank Administrative staff:

Erika, Susanne, Sören, Monica, Ann-Gitt, Eva-Lena, Elisabeth and Elle, thanks for your always help. It is you to make our working environment friendly and harmony! ☺

I would like to thank all my friends and colleagues from CCK:

Ran & Xinsong, we have shared so many things together and it is so nice to meet you and become fiends with you. Best wishes! Lisa, Tack! You gave me so many useful advices and you were together with me when I needed help. Best wishes for you and your families. Wilma is so cute! LiMin & Björn, nice dinner with you, and hope to play the game together again! Limin, thanks for your help in and out of work, you rescued my curtain! Best wishes for you and Björn! Lidi & Peng, Thanks for your every invitation, I would say you both are the core of our Chinese friends. I have learnt a lot from you, Lidi, especially how to become an elegant lady. ☺ Qiongzi & Kjell, thanks for your sharing with my happiness and sadness, we played Poker together, we ate together and it was your wedding making me start to expect mine. Best wishes for everything! ☺ Qiang, I bother you a lot, but you are always glad to help. Thanks and best wishes. ☺

Bertha, Svetlana, thanks for all your help in my PhD study. Juan, Lotta, Jelena, Isabel, Rouknuddin, Wang Na & Jia Kelin, Zhaojian, Huiyuan & Liu Tong, Yuanjun & Lu Changwu, Xie Hong, Sara Corvigno, Xianli, Liang Shuo, Karthik, Yumeng, Yuanyuan, Yu Rong, Zhuochun, it is so nice to meet you and work with you in CCK! Du Juan, thanks for your host in USA, it was really a good time to talk with you and your friends in Boston! Yanli, Dali, Barry, Seema, miss you all and hope you enjoy your wonderful life in different countries! Daniel, Katarzyna, Aravindh and Soniya, it was really a good time to be together with you all in 2014 Winterviken committee! ☺

I would like to thank my friends outside of CCK:

Jing, we have been known each other for nearly 7 years! We became friends, worked together in Göteborg. And our friendship continues in Stockholm! Thanks for all your help, encouragements and sharing with all the things happened in my life. I can never be so strong without your company. My dear friend, I wish you dreams come true and happiness comes together with luckiness. We are best fiends forever! ☺ Yue, Ziyang, Xue & Huaqing, Peter Gu & Tanglin, Jialiang, Feng, Bing, Akram and Andreas, how appreciated I am to meet you all in Goteborg, it is you to make my life abroad happy and colorful! Thanks! ☺

I also would like to thank Jiangnan, Chengjun, Yaoyao & Qian, Yuning, Hu Wei, Hongya & Xinyan, Xintong, Songhuan, Zhuang Ting & Zhu Jian, Li Xin & Yao Liqun, Hou Chen, Xuan Yang, and Lixin & Pelle, no matter where I am, I will never forget the happy time we have had together!
I would like to thank my parents:
感谢你们让我来到这个世界，并教会我爱与感恩，坚持与奋斗，乐观和坚强，我永远爱你们，祝愿你们健康长寿！

I would like to thank my parents-in-law:
感谢你们像疼爱女儿一样的疼爱我，接纳我。我是怎样的福份又拥有了一双爱我的父母，谢谢你们，祝愿你们健康长寿！

I would like to thank my husband:
帆，那么多的巧合让我们相遇并相爱，谢谢你这么多年的等待，等待这么一个倔强又有些幼稚的我。谢谢你对我的宽容和忍让，忍让我不时的小性子和急脾气。人生中最浪漫的事，莫过于和你一起慢慢变老。

感谢上天的眷顾，给予平凡的我如此厚重的爱与美好.
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