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USING A 3-D MODEL SYSTEM TO SCREEN FOR DRUGS EFFECTIVE ON SOLID TUMORS

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TO ALL CANCER PATIENTS

ABSTRACT

There is a large medical need for the development of effective anticancer agents with minimal side effects. The present thesis represents an attempt to identify potent drugs for treatment of solid tumors. We used a strategy where 3-D multicellular tumor spheroids (cancer cells grown in three dimensional culture) were utilized as *in vitro* models for solid tumors. Drug libraries were screened using spheroids as targets and using apoptosis induction and loss of cell viability as endpoints. The hanging drop method for production of spheroids was modified to allow spheroid generation in the 96-well format. Initial studies showed that the screening of multicellular spheroids resulted in the identification of different hit compounds compared to screening of monolayer cultures. Interestingly, we found that spheroid screening enriched for hydrophobic compounds (XlogP >4), a finding of considerable interest for chemical library design and lead optimization in the field of anticancer drug development.

An approach based on the analysis of drug-induced gene profiles was used to unravel the mechanism of action of hits identified in the screen. The proposed mechanisms of action were subsequently confirmed by specific *in vitro* assays.

The generation of a caspase-cleaved product of cytokeratin 18 was used to determine apoptosis of carcinoma cells in spheroids. The same method could be used as a plasma biomarker to evaluate whether candidate compounds induced apoptosis in xenograft tumor models. The antibodies used in the assay recognize human but not mouse cytokeratin 18 – an advantage when xenograft models are used since tumor apoptosis can be specifically measured in blood samples.

The screening work resulted in the identification of a novel topoisomerase inhibitor (thaspine), a novel iron chelator (CB21) and a number of microtubuli inhibitors.

- Thaspine (an alkaloid from *Croton lechleri*) was identified in both monolayer and spheroid screening experiments. Thaspine was found to inhibit both topoisomerase I and II. Interestingly, thaspine was effective on cell lines overexpressing drug efflux transporters and showed in vivo activity.
- CB21 was of particular interest, since it was toxic to the hypoxic quiescent cell population in spheroid cores which are known to be resistant to many chemotherapeutical drugs. The compound was not toxic to quiescent immortalized cells. CB21 was shown to be a very potent iron chelator. The compound induced marked induction of autophagy both in the outer and inner layers of spheroids. Interestingly, CB21 increased glucose uptake and reduced cellular oxygen consumption. The cytotoxicity of the compound was found to be increased during low glucose conditions, known to occur in the cores of spheroids. The compound showed a significant inhibitory effect in tumor xengrafts.
- A number of novel microtubuli inhibitors were identified in the spheroid screen. This result was unexpected since such compounds are expected to be preferentially active on dividing cells.

We conclude that drug screening using multicellular spheroids is a promising approach for anticancer drug discovery. A number of novel compounds were identified by screening, and some may be possible to develop for clinical use.

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Identification of agents that induce apoptosis of multicellular tumour spheroids: enrichment for mitotic inhibitors with hydrophobic properties
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Chelation of intracellular iron as a therapeutic strategy for solid tumours
Manuscript

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LIST OF ABBREVIATIONS

3-D	Three dimensional
3-MA	3-methyl adenine
4E-BP1	4E-Binding Protein 1
5-FU	5-fluorouracil
6-MP	6-mercaptopurine
ABC-transporter	ATP-binding cassette transporter
ADME	Absorption, distribution, metabolism and elimination
ARA-C	Cytarabine
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma-2
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
CCCP	Carbonylcyanide-3- chlorophenylhydrazone
CDK	Cyclin-dependent kinase
Chk1	Checkpoint kinase 1
CK18	Cytokeratin 18
CML	Chronic myeloid leukemia
CPX	Ciclopirox olamine
CSF	Colony stimulating factor
DFO	Deferoxamine
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FasL	Fas Ligand
FDA	Food and Drug Administration
GFP	Green fluorescent protein
GIT	Gastrointestinal tract
GRP78	Glucose-regulated protein
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factors
hTERT	Human telomerase reverse transcriptase
HER2	Human epidermal growth factor receptor 2
LC3	Light chain 3
MCS	Multicellular spheroids
MDR	Multidrug resistance
MRP	Multidrug resistance-associated protein
mTOR	Mammalian target of rapamycin
NAD	Nicotinamide adenine dinucleotide
NAM	Nictotinic acid mononucleotide
NCI	National Cancer Institute
PARP	Poly ADP (Adenosine Diphosphate)-Ribose Polymerase
PDGFRβ	Platelet derived growth factor receptor-β
Pgp	P-glycoprotein
PHD	Prolyl-hydroxylases

PI3K	Phosphatidylinositol 3-kinase
poly-HEMA	Poly(2-hydroxyethyl methacrylate)
RB	Retinoblastoma protein
REDD1	Regulated in development and DNA damage responses
ROS	Reactive oxygen species
SCID	Severe combined immunodeficiency
TMRE	Tetramethyl-rhodamine ethyl ester
TNF	Tumor necrosis factor
TRAIL	TNF receptor apoptosis-inducing ligand
TSC	Tuberous sclerosis complex
VEGF	Vascular endothelial growth factor A
WHO	World Health Organization

1 CANCER AS A DISEASE

Cancer is a leading cause of death in humans, accounting for 13% of total death rates according to the WHO report 2008 (http://www.who.int/en/). It is still one of the most difficult diseases to treat. It represents a huge socioeconomic burden on nations considering billions of dollars spent per year on diagnosis, treatment, research, in addition to economic cost due to lost productivity of patients.

Cancer is a disease in which a group of cells exhibit uncontrolled division leading to a primary tumor. A malignant tumor invades surrounding normal tissues causing their destruction. It may also spread into distal organs in the body through the lymphatic system or blood vessels. Metastatic cancer leads to ~ 90% of all cancer-related deaths (Avendano and Menendez 2008).

Many years of research has revealed that cancer results from the accumulation of mutations of genes in cancer cells giving them the advantages of unlimited division and evasion of apoptosis. These genes are either oncogenes with dominant gain of function, or tumor suppressor genes with recessive loss of function. Carcinogenesis is a multistep process in which healthy cells are gradually transformed into cancer cells (Hanahan and Weinberg 2000). Genomic alterations can occur due to internal factors like point mutations which can be induced by chemical carcinogens, translocation mutations or epigenetic changes, or due to external factors like viruses, chemical carcinogens or certain kinds of radiations, or due to hereditary factors like the involvement of BRCA1 and BRCA2 hereditary mutations in breast cancer (Thurston 2007).

In a highly cited article, Hanahan and Weinberg suggested six capabilities shared by most, if not all, human tumors: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Cells must acquire all these traits to be malignant, explaining why carcinogenesis is multistage process occurring over many years (Hanahan and Weinberg 2000).

A major problem associated with cancer is *genetic instability*. Recent sequencing studies of different cancer genomes revealed great mutational heterogeneity between different types of cancers (Greenman, Stephens et al. 2007). This heterogeneous nature occurs within cancers of the same organ origin and even within different cells of the same tumor. This phenomenon represents an enormous problem for cancer treatment. It is difficult to select a chemotherapy regimen which will be effective for a certain patient. Furthermore, the heterogeneity within different tumor cells in a tumor causes the emergence of clones that are resistant to specific chemotherapeutic agents, which ultimately leads to treatment failure.

1.1 MODALITIES FOR CANCER TREATMENT

The major modalities for cancer treatment are surgery, radiotherapy, hormone therapy and chemotherapy. Others modalities are less well established like immunotherapy and gene therapy. Surgery and radiotherapy are used in the removal of the primary tumor, however complete removal of all tumor cells are not guaranteed as residual microscopic cancer cells might still remain (as micro metastases at distal sites). Accordingly, subsequent chemotherapy is frequently needed to kill any residual cancer cells. Chemotherapy and radiotherapy can also be used preoperatively to shrink the tumor and facilitate its removal. Complete removal of tumor, however, by surgery can be limited if the tumor is anatomically adjacent to vital normal tissues (DeVita, Hellman et al. 2001). In addition, it is often that metastasis happens before diagnosis and treatment are started, accordingly the prognosis of the patient is not changed though the primary tumor is treated (Tobias, Hochhauser et al. 2010). This delineates the significance of chemotherapy as it has the advantage to access residual cells from the primary tumor or metastasized cancer cells by its systemic effect (Baquiran and Gallagher 2001). Chemotherapy is used to achieve cure, control or palliation in cancer patients, it can be the primary treatment or as an adjunct to other modalities like surgery or radiotherapy (Baquiran and Gallagher 2001).

1.1.1 Chemotherapy

The word "chemotherapy" was first coined by Paul Ehrlich, at the beginning of the twentieth century, in his attempts to treat syphilis using a chemical (Ehrlich 1913). The idea was not proven until Alexander Fleming discovered penicillin as toxic agent to bacteria (Fleming 2001). This discovery opened the era of research for chemical compounds to treat various diseases including cancer. However, not yet, that any anticancer agent approached such efficacy and specificity on cancer (Perry 2001). The ideal chemotherapeutic agent is the one which is cytotoxic or cytostatic to its target cells (i.e. the tumor cells), while being tolerated by normal body cells, in other words, an agent with wide therapeutic window. In case of antibacterial agents the mission is comparatively easier as bacteria possess targets that are unique from those of their host. On the contrary, cancer cells mostly share the same biochemical machinery of normal cells, accordingly, finding chemotherapeutic agents with selective toxicity to cancer cells is a major challenge (Perry 2001; Kamb, Wee et al. 2007)

The first chemotherapeutic agent was discovered in the 1940s based on the observation that the soldiers in the world war II who were exposed to the warfare nitrogen mustard gas showed substantial myelosuppression and lymphoid hypoplasia (Lu and Mahato 2009). This observation stimulated researchers to evaluate the therapeutic efficacy of this compound in treatment of hematologic neoplasms. A patient with advanced non-Hodgkin's lymphoma was treated with nitrogen mustard. Though the remission occurred by treatment was transient, the concept of using a chemical agent to treat cancers was established (Chabner and Roberts 2005).

1.1.2 Mechanistic classification of chemotherapeutic agents

Anticancer agents can be classified according to their cellular targets. Examples of targets are DNA, RNA, enzymes necessary for cell proliferation, tubulin and the proteasome.

1.1.2.1 Antimetabolites

This group of agents blocks crucial metabolic pathways essential for cell growth. Like some other chemotherapeutic groups, they are toxic to dividing cells, explaining their well-known side effects on normal proliferating cells in bone marrow, GIT and hair follicles. This differential toxicity can be useful in case of some leukemia, but in case of older solid tumors, where they have small growth fraction, they have limited efficacy (Thurston 2007).

Most of the antimetabolites interfere with DNA synthesis. For example, methotrexate works by inhibiting the activity of the dihydrofolate reductase enzyme which is responsible for conversion of dihydrofolic acid to tetrahydrofolic acid. The latter is required for synthesis of thymine which is a constituent of DNA. Other examples are the purine synthesis inhibitors 6-mercaptopurine (6-MP), tioguanine, fludarabine and cladribine or pyrimidine synthesis inhibitors like cytarabine (ARA-C) and 5-fluorouracil (5-FU) (Huennekens, Bertino et al. 1963; Thurston 2007).

1.1.2.2 DNA interactive agents

The majority of anticancer agents exert their action through interaction with DNA. In general, proliferating cells are most sensitive to these agents due to difficulties of handling adducts or DNA breaks during DNA replication. Tumor cells frequently have damaged check-point systems and do not necessarily arrest their cell cycles to allow repair of damaged DNA. Progeny cells may be non-viable and die by apoptosis or other death modes (see further below). The mode of cell death following exposure to a DNA damaging agent is determined by many factors including cell type, the dose of the anticancer agent, type of DNA damage occurred and the genotype of the cell (e.g. p53 mutation). Apoptosis could be the primary cell death mode like in some kinds of lymphomas. In such case, loss of apoptotic pathway for example by mutation of p53 or overexpression of Bcl-2 will affect the overall cell death. However, most cancers of epithelial origin do not die primarily through apoptosis. Thus, perturbation of apoptosis pathway will not affect the overall cell death. Nevertheless, apoptosis can occur secondarily to another cell death pathways like mitotic catastrophe, in what is called "funeral mode" (Brown and Attardi 2005).

Interaction of these agents with DNA takes different forms. It can be through intercalation of the drug between the base pairs of DNA (e.g. doxorubicin and mitoxantrone) or alkylation of the DNA bases in the minor or major grooves (e.g.

dacarbazine and temozolomide). Other mechanisms are topoisomerase inhibition (e.g. irinotecan and etoposide), DNA strand cross-linking either by intrastrand or interstrand manner (e.g cisplatin and nitrogen mustards), or by DNA strand cleavage (e.g. bleomycins) (Thurston 2007; Avendano and Menendez 2008).

As observed with other anticancer agents, the DNA interactive agents are toxic to the normal proliferating cells in bone marrow and GIT. This could be an explanation for the observed preferable toxicity towards the proliferating population of cancer cells. Another explanation could be the disrupted DNA mechanisms for DNA repair in cancer cells, compared to normal cells, and thus triggering cell death (Thurston 2007). In normal cells, the cell cycle checkpoints are used to ensure DNA repair for damaged DNA caused by usual factors like metabolic activities or external exposure to radiation or UV light (Harper and Elledge 2007). There are two checkpoints; G_1 and S/G_2 checkpoints. In many cancers G_1 checkpoint is defective where they rely on functioning S/G_2 checkpoints for DNA repair. Checkpoint kinase 1 (Chk1), which is a major regulator for the S/G_2 checkpoints, currently represents an attractive target to synergize the effect of DNA damaging agents. Inhibition of S/G_2 checkpoints is believed to cause mitotic catastrophe and eventually cell death in G_1 checkpoint defective cancer cells (Xu, Cheung et al. 2010).

Cisplatin is an important agent in this group. Cisplatin was discovered by serendipity rather than systematic research (Rosenberg, Vancamp et al. 1965). Rosenberg and co-workers observed a cytostatic effect on Escherichia *coli* on passing an electric current through platinum electrode. The effect was eventually attributed to platinum complexes formed electrolytically. Ciplatin acts by intrastrand cross-linking of the major groove of DNA. The bis-chloro molecule of cisplatin is stable and neutral in the chloride-rich plasmatic environment which becomes activated in the low-chloride intracellular milieu. The chloride ligands are being replaced by water molecules forming a positively charged aquated form which is highly reactive with nucleophiles (Ries and Klastersky 1986).

Cisplatin is considered as a model for successful anticancer agents due to its pronounced activity particularly in testicular and ovarian cancers (Gottlieb and Drewinko 1975; Thurston 2007). Many papers of cisplatin-induced effects on tumor cells have been designed to examine short-term apoptosis. This generally occurs over 24 hours and requires high concentrations of the drug. It has been shown *in vitro* that at high concentration, cisplatin induced apoptosis in enucleated cells, indicating that cisplatin-induced apoptosis can occur independently of nuclear DNA damage (Mandic, Hansson et al. 2003; Berndtsson, Hagg et al. 2007). The importance of short-term apoptosis is therefore unclear.

Topoisomerase inhibitors are another class of DNA interactive agents. DNA topoisomerases I & II are nuclear enzymes which are responsible for the structure and topology of DNA. They play a major role in DNA replication and transcription by controlling unwinding and winding of supercoiled DNA (Sinha 1995). If unwinding of DNA fails, this causes failure of replication and transcription leading to cell death. Topoisomerase I induces single strand nicks while topoisomerase II is capable of inducing both single and double strand breaks (Sinha 1995), and after relieving the

DNA supercoiled tension by rotation, they reseal the DNA strands (Wang 2002). Topoisomerase inhibitors can act through direct inhibition of the enzyme or through intercalation with DNA. Examples of topoisomerase I inhibitors used in clinic are topotecan and irinotecan and for topoisomerase II inhibitors: etoposide, teniposide and amsacrine (Malonne and Atassi 1997)

1.1.2.3 Antitubulin agents

Antitubulin agents interfere with microtubule dynamics of spindle formation and disassembly. Tubulin is the building unit of microtubules which are the the integral components of the mitotic spindle. Microtubules are in dynamic equilibrium between polymerization and depolymerization. Antitubulin agents work either by inhibition of tubulin polymerization or by stabilization of assembled microtubules and in both cases lead to inhibition of mitosis and subsequent cell death (Pellegrini and Budman 2005)(Thurston 2007). As antitubulin agents interfere with mitosis, it is generally believed that they exhibit toxicity only on proliferating cells. However, antitubulin agents also disrupts the cellular cytoskeleton, which a has a major role in intracellular transport and signal transduction, thus it would not be surprising that they can exert toxicity to the non-proliferating cells (Gundersen and Cook 1999; Giannakakou, Sackett et al. 2000).

The major two families in this class of compounds are vinca alkaloids and taxanes. Both are of natural products origin. Vincristine and vinblastine belong to the vinca alkaloids which bind to tubulin and prevent its assembly into microtubules and thus inhibiting mitosis (Himes 1991). They have application in treatment of many kinds of cancer mainly breast cancer and certain kinds of leukemia and testicular carcinoma (Nelson 1982). Taxanes have a different mechanism of action compared to the vinca alkaloids. They stabilize the microtubule complex and inhibit its depolymerization, thus preventing mitosis. Paclitaxel is used in treatment of breast cancer, ovarian cancer and non-small-cell lung cancer. Docetaxel is a semi-synthetic taxane that was approved in 1996 for the treatment of anthracycline-refractory metastatic breast cancer and later for treating platinum-refractory stage IIIB or IV non-small-cell lung cancer (Crown and O'Leary 2000; Montero, Fossella et al. 2005). Currently it is being used in the clinic for treatment of locally advanced or metastatic breast or non-small-cell lung cancer and androgen-independent metastatic prostate cancer (Engels, Sparreboom et al. 2005).

The major side effect of microtubule inhibitors is neuropathy which can vary in onset, severity, reversibility depending on the agent used and its dose. Although the exact mechanism of neurotoxicity caused by antitubulin agents is not fully understood, preclinical physiologic and histopathologic studies indicate that it is principally a consequence of the interruption of axonal transport (Swain and Arezzo 2008).

1.1.2.4 Antihormonal therapy

Hormones play a significant role in certain kinds of cancers like breast, testicular and endometrial carcinomas. Cancers arising from hormone- dependent tissues are themselves dependent on the same hormones for growth. This is evidenced by the observation of remission of breast cancer after ovariectomy in premenopausal patients. The same was observed in prostatic cancers after orchidectomy (surgical removal of testes) (Horsley 1947). Accordingly, antihormonal therapy is beneficial in such kinds of cancers. Though, not curative, they provide extended survival in selected patients sometimes for many years. Anti-estrogens (e.g. tamoxifen) and aromatase inhibitors (aminoglutethimide) are examples of antihormonal agents used in treatment of estrogen positive breast cancers (Hughes and Burley 1970; Jordan and Tormey 1988).

1.1.3 Modern molecularly targeted agents

Recent genetic and molecular studies have unveiled cell signaling networks controlling cell activities important for cell survival and proliferation. It has been found that many of these networks are radically disrupted in cancer cells (Chabner and Roberts 2005). The identification of these targeted drugs is based on experimental inhibition of a certain target thought to be upregulated in cancer cells and that cancer cells is dependent on it for their survival and proliferation. This new trend raised the hope for identification of anticancer agents with selective toxicity to cancer cells and less side effects compared to the conventional chemotherapeutic agents.

The first successful agent belonging to the group of molecularly targeted agents is imatinib. It is a kinase inhibitor for the BCR-ABL fusion protein which is involved in the pathogenesis of chronic myeloid leukemia (CML). In addition, Imatinib also has an inhibitory effect on the KIT tyrosine kinase and platelet derived growth factor receptor-β (PDGFRβ) tyrosine kinase (Chabner and Roberts 2005). Imatinib successfully induced 97% remission in case of earlier chronic phase of CML compared with 49% in the later blast-crisis phase (Kamb, Wee et al. 2007).

A second example is the kinase inhibitor trastuzumab (Herceptin™). This is an antibody which inhibits the human epidermal growth factor receptor 2 (HER2)/neu signaling pathway with beneficial effect in breast HER2/neu positive cancer (Mannocci, De Feo et al. 2010). A further example is erlotinib (Tarceva™) which is an epidermal growth factor receptor (EGFR) inhibitor currently used in treatment of patients with non-small-cell lung cancer (Shepherd, Rodrigues Pereira et al. 2005).

Proteasome inhibition represents a novel strategy for treatment cancer. The ubiquitin-proteasome pathway is the principal route by which cells degrade proteins including signaling proteins. Thus, proteasome inhibitors retard or arrest cancer progression by interfering with the degradation of the regulatory proteins of cell activities (Myung, Kim et al. 2001). Bortezomib (Velcade™) is an example of proteasome inhibitors which proved efficacy in treatment of myeloma (Paramore and Frantz 2003).

Antiangiogenic agents are of considerable interest nowadays as a general mechanism for treatment of solid tumors. For any tumor to grow above the size of 1-2 mm^3 a process of angiogenic switch should occur where new vasculature are formed to supply the tumor with oxygen and nutrients and thus sustain tumor growth (Folkman, Long et al. 1963). Bevacizumab (Avastin™) is a humanized monoclonal anti body which targets vascular endothelial growth factor A (VEGF) which is a proangiogenic factor (Ferrara, Hillan et al. 2004). Bevacizumab is approved by FDA in treatment of metastatic colon carcinoma in combination with 5-FU.

1.1.4 Cancer growth kinetics and chemotherapy

Tumor growth kinetics and chemotherapy-induced cell death are correlated such that the rate of chemotherapy induced regression is positively related to the growth rate of unperturbed tumor (Perry 2001). In case of human solid tumors, the rate of tumor growth is not compensated with an adequate vascularization leading to the formation of gradients of oxygen and nutrients throughout the tumor (Thomlinson and Gray 1955). A parallel pattern in proliferation profile is created according to these gradients: proliferating cells proximal to blood supply, hypoxic quiescent cells adjacent to the proliferating compartment and anoxic dead cells approximately >150 µm away from the blood capillary (Perry 2001)(Tobias, Hochhauser et al. 2010). The proliferating subpopulation is called the growth fraction, which accounts for the overall growth of the tumor.

Solid tumors growth kinetics is best described by the Gompertzian model which is a sigmoid curve. The model states that the growth fraction of a tumor decreases exponentially such that the larger the tumor the lower the growth fraction and vice versa. Large tumor growth rate almost follows a plateau when it approaches a volume that can kill the patient (DeVita, Hellman et al. 2001; Perry 2001).

According to the mitoxicity hypothesis, proliferating cells have higher chemosensitivity, that is, tumors with high growth fraction are more responsive to chemotherapy. Clinically, curable cancers are those with high proliferation rate like testicular carcinoma and childhood acute lymphoid leukemia. This hypothesis can explain the toxicity of chemotherapy to rapidly dividing normal tissues like bone marrow, gastrointestinal mucosa and hair follicles (Holland 2000). On the other hand, the non-proliferating hypoxic subpopulation are significantly less sensitive to chemotherapy (Perry 2001). One reason for this insensitivity can be attributed to their quiescence. Another explanation could be that these cells are located relatively far from the blood capillaries. Systemically administered drugs will not reach this subpopulation at therapeutic concentrations (Jackson 1989).

After a chemotherapeutic dose resulting in the death of proliferating cells, these quiescent cells, upon the exposure to oxygen and nutrients, nourish and enter the cell cycle and proliferate (Jackson 1989; Mellor, Ferguson et al. 2005). This regrowth phenomenon, in addition to drug resistance, hinders the regression of the tumor aimed by chemotherapy.

1.1.5 Challenges in chemotherapy

Resistance to chemotherapy and undesirable toxicity to normal cells are the major obstacles to chemotherapy (DeVita, Hellman et al. 2001). Accessibility of the drug to various populations of tumor cells, especially in solid tumors, in effective concentrations represents another obstacle in chemotherapy. It is believed that resistance is the cause for more than 90% of therapeutic failure in patients treated with conventional chemotherapeutic agents (Neidle 2008). Drug resistance has been reported in most drug-responsive tumors and for most classes of chemotherapeutics. Resistance can be intrinsic or acquired after exposure to chemotherapy. The conventional resistance theory suggests that subsequent chemotherapy cycles lead to the selection of resistant subclones causing diminished responsiveness by time. Cancer cells develop resistance via different mechanisms such as reduction of intracellular drug concentration through drug efflux pumps (multidrug resistance-MDR), increased drug inactivation, overexpression or modification of the target, signaling with different pathways and enhanced DNA repair machinery in case of DNA damaging agents (Gottesman 2002; Thurston 2007; Neidle 2008). Another important mechanism of resistance, though not widely discussed, is multicellular drug resistance in solid tumors (Kerbel, Rak et al. 1994; Grantab, Sivananthan et al. 2006). This kind of resistance emerges from the established contact of cancer cells with their microenvironment and acquisition of the three dimensional conformation. Quiescent tumor cells contribute to such kind of resistance (Desoize and Jardillier 2000). Which kind of resistance that is responsible for clinically observed resistance is still controversial.

Another obstacle to successful therapy is the severe toxicity of chemotherapy to normal tissues. Chemotherapeutic agents are characterized by modest therapeutic windows. Nevertheless, the life threatening nature of the disease overweighs the serious side effects of chemotherapy. The most harmed tissues are the rapidly dividing like bone marrow, gastrointestinal crypt epithelial cells, hair follicle and stem cells (Baquiran and Gallagher 2001). Bone marrow toxicity is serious since it leads to anemia, leukopenia and excessive bleeding (Lu and Mahato 2009). Some strategies have been developed to combat some side effects like administration of hematopoietic growth factors like erythropoietin and colony-stimulating factors (CSFs) for treatment of chemotherapy induced anemia and neutropenia, respectively (Perry 2001). Furthermore, scheduling high doses of chemotherapy with interspersed intervals for normal tissue recovery and the use of cold cap to prevent alopecia are additional ways for reducing undesired side effects (Christodoulou, Klouvas et al. 2002). Yet, the best solution is the discovery of more selective anticancer agents with wider therapeutic index.

From a pharmacological point of view, it is not sufficient for an anticancer drug to selectively inhibit its target cultured cells in vitro. It is of crucial importance that the agent targets the majority, if not all, cancerous cells in vivo. Pharmacokinetic properties of a chemotherapeutic agent are major determinant of the antitumor effect in patients. Pharmacokinetics science is the study of the time course of drug absorption, distribution, metabolism and elimination (ADME).

Many anticancer agents are administered intravenously giving 100% bioavailability circumventing limited GIT absorption and first pass effect (Figg and McLeod 2004). In hematologic cancers, all cells are exposed to the cancer agent, however in solid tumors the situation is different as the drug should distribute in therapeutic concentration to the different tumor compartments, including those which are distal from blood supply. Many factors determine the distribution of drugs to tumors such as cardiac output, plasma protein binding, blood flow within tumor, pH of the tumor, presence of drug efflux pumps and physicochemical properties of the drug (Figg and McLeod 2004). The later determine the pharmacological and drug-like properties of a chemotherapeutic agent which have a significant role in successful treatment. For example, a drug must have a balance between aqueous and non-aqueous solubility for appropriate distribution between aqueous and non-aqueous pharmacokinetic compartments including tumors (Neidle 2008). Metabolism, in most cases, converts drugs into less pharmacologically active compounds with polar properties to facilitate their excretion. In some cases, the metabolite is more active than the parent compound as in the case of CPT-11 and its metabolite SN-38 (Kawato, Aonuma et al. 1991). Thus, metabolism and excretion generally decrease the pharmacological response but at the same time reduces toxicity to normal cells (Figg and McLeod 2004).

1.1.6 Combination chemotherapy

The main principle of combination therapy is to enhance the efficacy of the chemotherapy regimen to kill cancer cells and accordingly, improvement of clinical outcome and patient's survival. The heterogeneous genomic nature of cancer cells results in the presence of resistant cells to a specific chemotherapeutic agent. This causes clonal selection for resistant cells following successive chemotherapy courses and consequently treatment failure. Combination of anticancer agents with different mechanism of actions proved to be an efficient strategy to overcome such emergence of clonal resistant cells (DeVita, Young et al. 1975; Lu and Mahato 2009).

Chemotherapeutic agents can be classified according to their activity during the cell cycle as cell cycle active or non-cell cycle active. Antimetabolites like 5-fluorouracil, methotrexate and 6-mercaptopurine are S-phase specific. Antimitotic agents such as vincristine and vinblastine are G2/M phase specific. DNA damaging agents like cyclophosphamide and doxorubicin are non-phase specific. Corticosteroids like dexamethasone and prednisone are examples for non-cell cycle active agents (Lu and Mahato 2009).

The main criteria for selecting a combination therapy can be summarized as follows:

1- Have different cell cycle specificity.
2- Have different mechanism of action.
3- To be effective as single agent.
4- Have different side effects.
5- Have different mechanisms of resistance development.

Collectively, the major goal of combining two or more chemotherapeutic agent is to achieve synergetic or additive cytotoxicity on tumor without increasing toxic side effects on normal tissues.

1.2 CELLULAR OUTCOMES OF CHEMOTHERAPY TREATMENT

It has been believed that the mechanism of cell death induced by chemotherapy is confined to solely to apoptosis. Accumulating evidences suggest that tumor response to chemotherapy includes other modes of cell death (Brown and Attardi 2005) There are four modes of cell death described that can be induced by chemotherapy; apoptosis, necrosis, mitotic catastrophe and autophagy (Brown and Attardi 2005). Senescence, a form of permanent growth arrest, is not a cell death mechanism but is considered of significant importance as a response of cancer cells to chemotherapy. These five kinds of cellular responses are classified based on biochemical and morphological features present in dying cells.

1.2.1 Apoptosis

Although the phenomenon of apoptosis was described Carl Vogt in 1842 (Peter, Heufelder et al. 1997), the term "apoptosis" was first coined by Kerr, Wyllie, and Currie in 1972 (Kerr, Wyllie et al. 1972). Apoptosis, programmed cell death, is a conserved mechanism which multicellular eukaryotic organisms rely on for tissue remodeling and maturation. It also maintains tissue homeostasis by mediating equilibrium between cellular proliferation and death (Ricci and Zong 2006). The main morphological characteristics of apoptosis are cell membrane blebbing, cell shrinkage, chromatin condensation and nucleosomal fragmentation. Cells undergoing apoptosis form apoptotic bodies which are consumed by macrophages or neighboring cells. Apoptosis is commonly believed to be the major mechanism by which chemotherapeutic agents initiate cell death. Apoptosis is recognized as an energy-requiring process. There are two distinct pathways for apoptosis execution; the intrinsic, or mitochondrial mediated pathway and the extrinsic, or extracellular activated pathway. The intrinsic pathway is activated by intracellular stress such as DNA damage (Eastman 1990), elevated levels of reactive oxygen species (Simon, Haj-Yehia et al. 2000), viral infections (Hardwick 2001) or activation of oncogenes (Liu, Chen et al. 1998). The extrinsic pathway is induced by an extracellular ligand which binds to a certain receptor on cell membrane. Tumor necrosis factor (TNF) ligands, like TNF-α, FasL and TNF receptor apoptosis-inducing ligand (TRAIL), which bind to TNF receptors are examples of stimuli of the extrinsic pathway (Gross, Yin et al. 1999). Both pathways activate caspases which are proteolytic enzymes that dismantle cellular organelles and structure (Ricci and Zong 2006).

1.2.2 Necrosis

Unlike apoptosis, necrosis is considered as a passive, random, and uncontrolled cell death due to traumatic cell destruction followed by release of intracellular components. Necrosis is characterized by induction of an inflammatory response. Cell membrane distortion, mitochondrial and cellular swelling, organelles degradation, increased vacuolation are among the main morphological features of necrosis. Necrosis can occur as a consequence of pathophysiological condition like ischemia, infection, cellular membrane physical insults, imbalance in intracellular pH and calcium levels, energy depletion, increased ROS or inflammation (Okada and Mak 2004; Ricci and Zong 2006) . Necrosis has been suggested as a cell death mechanism in response to some DNA alkylating agents, in rapidly growing cells like tumor cells. Highly proliferating cells select the aerobic glycolysis for production of ATP, since other non-glucose-derived substrates like amino acids and lipids are used for synthetic purposes. On DNA alkylation, the nuclear enzyme PARP becomes activated and degrades NAD into poly(ADP)-ribose polymers and nictotinic acid mononucleotide (NAM). This leads to the depletion of cytosolic NAD pool and consequently inhibition of glucose dependent ATP production. Shutting down ATP production, ultimately lead to necrosis (Zong and Thompson 2006).

1.2.3 Autophagy

Autophagy is an evolutionarily preserved mechanism that occurs in all eukaryotic cells from yeast to mammals. Along with the ubiquitin-proteasome system, constitutive basal autophagy plays an important role in degrading accumulated cellular proteins and unneeded/damaged organelles thus maintaining cellular homeostasis (Levine and Klionsky 2004). Autophagy can be also triggered as a response to starvation or stress where cellular protein and organelles are degraded and recycled for energy production and sustaining cellular metabolism (Marino and Lopez-Otin 2004). Although most evidence support that autophagy is a prosurvival mechanism, it has been suggested that excessive cellular consumption by autophagy can lead to cell death (Mathew, Karantza-Wadsworth et al. 2007; Meschini, Condello et al. 2011).

The morphological features elicited in autophagy include cell membrane blebbing, partial chromatin condensation with no DNA laddering, cytosolic vacuolization, and degradation of Golgi, polyribosomes and endoplasmic reticulum. Briefly, autophagy process is initiated as a flattened vesicle in the cytoplasm which grows up to a cup-shaped isolation membrane called phagophore which surrounds the selected protein or organelle. Eventually, this membrane closes to a double membrane autophagosome. The autophagosome then fuses with the lysosome forming an autolysosome which degrades the sequestered substances (Fleming, Noda et al. 2011). The genes controlling autophagosome formation are called autophagy-related (Atg) genes. *Beclin 1* is a prominent member in this group (Levine 2007). On the molecular level, the pathways which lead to autophagy involve at least the phosphatidylinositol 3-kinase (PI3K) and the mammalian target of rapamycin (mTOR) pathways (Okada and Mak 2004).

1.2.4 Mitotic catastrophe

Mitotic catastrophe is regarded as a trigger for cell death rather than a mechanism for cell death (Brown and Attardi 2005). It is proposed that mitotic catastrophe occurs due to deficient cell-cycle checkpoints (DNA structure and spindle assembly checkpoints) and cellular damage. Failure of arresting in the cell cycle, due to non-functioning checkpoints, before or during mitosis leads to an attempt for aberrant chromosomal segregation which in turn leads to induction of apoptosis (Castedo, Perfettini et al. 2004). For example, irradiated cells which undergo mitotic catastrophe, revealed by cell fusion, polyploidy and failure of mitosis, ultimately might die through secondary apoptosis (funeral mode) (Brown and Attardi 2005). Mitotic catastrophe can also be induced by DNA damaging agents and antitubulin agents (Lock and Stribinskiene 1996; Morse, Gray et al. 2005). Mitotic catastrophe is determined morphologically by the appearance of multinucleated cells and giant cells with intracellular micronuclei (Vakifahmetoglu, Olsson et al. 2008).

1.2.5 Senescence

Senescence was first described in 1961by Hayflick and Moorhead (Hayflick and Moorhead 1961). Until then, the finding of retarded and stopped division of cultured cells was thought to be a culture artifact. Human fibroblasts showed finite number of replication when grown in culture (Hayflick and Moorhead 1961) ending up in a state of growth arrest referred to as replicative senescence (Stanulis-Praeger 1987). This phenomenon was found to be due to progressive shortening of the repetitive DNA sequences found in the end of chromosome, so called telomeres (Goldstein 1990; Harley, Futcher et al. 1990).

Morphologically, senescent cells become flattened with increased granularity. On the biochemical level, senescent cells show increased levels of β-galactosidase enzyme activity. Senescence can also be induced as a result of cellular stress. Although no shortening for telomeres are observed, these cells show the phenotypical characteristics of senescent cells (Okada and Mak 2004). DNA damage is one example of stresses that can cause senescence for cells. DNA damage activates p53 which primarily arrests cell cycle through the induction of p21. p21 is a pleiotropic inhibitor for different cyclin-dependent kinases (CDKs). The induction of p21 is transient and is followed by stable activation of p16 (another CDK inhibitor). It is believed that p16 maintains cell cycle arrest after p21 shutoff. However, in tumor cells, results showed that the p53 and p21 inhibition reduces but does not abolish DNA damage induced senescence. These findings indicate the involvement of other genes in the induction of senescence in response to DNA damage (Chang, Swift et al. 2002). It has been shown that cisplatin causes premature senescence, through its DNA damaging effect on cancer cells (Berndtsson, Hagg et al. 2007). The premature senescence program activates tumor suppressor genes such as those encoding the p53, p16, p21 and retinoblastoma protein (RB). This indicates that senescence has an antitumorgenic role as it stops the proliferation of tumor cells. This was manifested *in vivo* where mutated mice which are

incapable for induction of senescence develop cancer at early stage (Sharpless, Bardeesy et al. 2001). On the other hand, the induction of premature senescence in murine mammary epithelial cells prevented tumorigenesis (Boulanger and Smith 2001)

1.3 PRECLINICAL EVALUATION OF ANTICANCER AGENTS

Two approaches: cell based assays or biochemical screens for specific targets

Systematic cancer drug discovery is based on screening of chemical libraries, either natural products or synthetic compounds, for anticancer activity *in vitro*. Cell based assay is the conventional approach where compounds are tested on cancer cell lines grown as monolayers. Such kind of screens leads to the identification of cytotoxic agents towards cancer cells but they may also be toxic to normal cells i.e. narrow therapeutic index. In order to find compounds with wide therapeutic indices, the target based approach was developed, especially after the increasing knowledge about the signaling networks in cancer cells, aiming to identify compounds that inhibit specifically upregulated proteins in cancer cells. However, the target based approach has its own shortcomings, for example, no proof that the identified hit is specific to the target (Balis 2002). In addition, cancer cells can compensate the inhibited pathway, by activation of alternative pathways, as a one form of resistance. Accordingly, target based hits may not be predictive for its effect on the whole cell (Balis 2002).

Most currently chemotherapeutic agents were identified in cell based cytotoxicity assays (Balis 2002) and it is thought that this type of screen will continue to play an important role in cancer drug discovery. However, it was found that monolayers screening is not necessarily predictive for activity *in vivo* (Chabner and Roberts 2005). As an attempt to model solid tumors *in vitro*, Sutherland *et al.* developed the multicellular spheroid model (Sutherland, McCredie et al. 1971). Spheroids were shown to be superior to monolayers in modeling solid tumors in terms of growth kinetics, gene expression, cellular heterogeneity, three dimensional structure, multicellular resistance and secretion of extracellular matrix. Thus, spheroids can be used as a model for primary screening or as a secondary screening after a primary monolayer screen, preceding the *in vivo* animal testing.

1.3.1 Spheroids as a model for preclinical evaluation of anticancer agents

Experimental models which closely mimic solid tumors will provide better estimation for anticancer activity. Most anticancer studies are performed on cancer cell lines grown as monolayers. Although monolayers retain cancer characteristics, such as unlimited division and tumorigenicity in animal models, they lack the 3-D conformation and heterogeneity in cellular kinetics found in solid tumors (Yuhas, Tarleton et al. 1979). When potential antitumor agents are tested on monolayer cultures, all cells are exposed to the compound, a condition which does not exist

clinically in a multilayered tumor. Penetrability of an anticancer agent is a major factor for its efficiency, a criterion which cannot be detected in a monolayer model. Moreover, solid tumors are composed of proliferating and quiescent hypoxic cells which are known to be resistant (Roberts, Williams et al. 2009), while all monolayer cells are exponentially growing. Thus, monolayers hits are not necessarily toxic to quiescent cancer cells.

Spheroids provide a better model for simulating solid tumors. They are produced by growing cancer cell lines three dimensionally (Inch, McCredie et al. 1970; Hamilton 1998). The spheroids are of intermediate complexity between clinically observed tumors and cancer cell lines grown as monolayers (Sutherland and Durand 1976; Sutherland, Bareham et al. 1980; Hirschhaeuser, Menne et al. 2010). Subpopulations resistant to radiation and cytotoxic agents and with altered cell cycle distribution have been shown in spheroids (Freyer and Sutherland 1980). Cancer cell lines are more resistant to antineoplastic agent when grown as multicellular compared to the corresponding monolayers (Nederman 1984; Olive, Banath et al. 1993). Moreover, there is a correlation between the sensitivity of spheroids and that of *in vivo* models (Kobayashi, Man et al. 1993). The spheroid model has been used in different applications in cancer research; radiotherapy (Qvarnstrom, Simonsson et al. 2009), immunotherapy (MacDonald and Sordat 1980), chemotherapy (Twentyman 1980), angiogenesis (Gimbrone, Leapman et al. 1972), resistance (Chen, Wang et al. 2009), invasion (Mareel, Kieler et al. 1981), metastasis (Landry, Freyer et al. 1981), growth fraction (Yuhas and Li 1978), gene expression (Chang and Hughes-Fulford 2009)and extracellular matrix studies (Hirschhaeuser, Menne et al. 2010).

In solid tumors, the homeostatic regulation of angiogenesis breaks down as a result of more rapid proliferation of tumor cells than the cells that form blood capillaries, creating a population of cells distant >100 μm from blood vessels (Thomlinson and Gray 1955; Brown and Giaccia 1998). This results in a gradient of decreasing tumor cell proliferation with increasing distance from tumor blood vessels (Tannock 1968; Gardner, Li et al. 2001). Spheroids reflect this situation as they contain three main subpopulations which are found in solid tumors; proliferating cells, hypoxic quiescent cells and dead cells (Sutherland and Durand 1976). Outer layers are proliferating as they have sufficient nutrients and oxygen while deeper layers become quiescent due to hypoxia and malnutrition in addition to high levels of cellular waste products (Freyer and Sutherland 1980), while the deepest layers become apoptotic/necrotic because of lack of nutrients and oxygen (Sutherland, Bareham et al. 1980; Hirschhaeuser, Menne et al. 2010).

The ultimate goal of cancer therapy is to eradicate all tumor cells, as residual cells can repopulate the tumor. Clinically administered chemotherapy is believed to be toxic to proliferating cells (Holland 2000)(Valeriote and van Putten 1975; Remvikos, Mosseri et al. 1993; Siu, Arooz et al. 1999) and during recovery periods between cycles of therapy quiescent cells, which are far from blood supply, shift from quiescence to proliferation once exposed to nutrients and oxygen causing tumor regrowth (Siemann 1998). In spheroids, it was shown that these dormant cells are capable of proliferation once exposed to appropriate conditions for growth (Durand 1975). Thus, to achieve a successful cure for solid tumors, the anticancer agent should be capable of penetrating

into the tumor parenchyme and being toxic to the distal hypoxic cells in addition to the proximal proliferating cells. In another words, the rate of tumor killing should be higher than the rate of tumor regrowth until the eradication of all cancer cells during a specified chemotherapy course (Shah and Schwartz 2000)

Much of the initial preclinical characterization of anticancer agents is performed using cancer cell lines in monolayer culture (Neidle 2008). Under these conditions cells are in an exponential growth, a situation which is distinct from that occurring in 3-D tumors *in vivo*. Agents that are toxic to dividing 2-D cancer cells are not necessarily penetrating 3-D tumor tissue or toxic to non-dividing cancer cells. This, in part, might be the reason for the failure of hits from monolayer screens when tested *in vivo* and also for the unsatisfactory cure rates of currently used chemotherapeutic agents. Testicular cancer is characterized by high growth rate due to a high grow fraction (high percent of dividing cells) (Holland 2000). This might explain the exceptional success of chemotherapy in treatment of testicular carcinoma in case of solid tumors with cure rates exceeding 80% (Nakamura and Miki 2010).

Many clinically used anticancer agents showed poor activity on multicellular spheroids (Sutherland, Eddy et al. 1979; Liao, Hu et al. 2010; Yu, Chen et al. 2010). Examples of such agents are cisplatin, doxorubicin, 5-FU and vinblastine (Mellor, Ferguson et al. 2005). This might be attributed to limited penetrability of these drugs (Tunggal, Cowan et al. 1999; Tannock, Lee et al. 2002) in addition to their selective toxicity to dividing cells (Mellor, Ferguson et al. 2005).

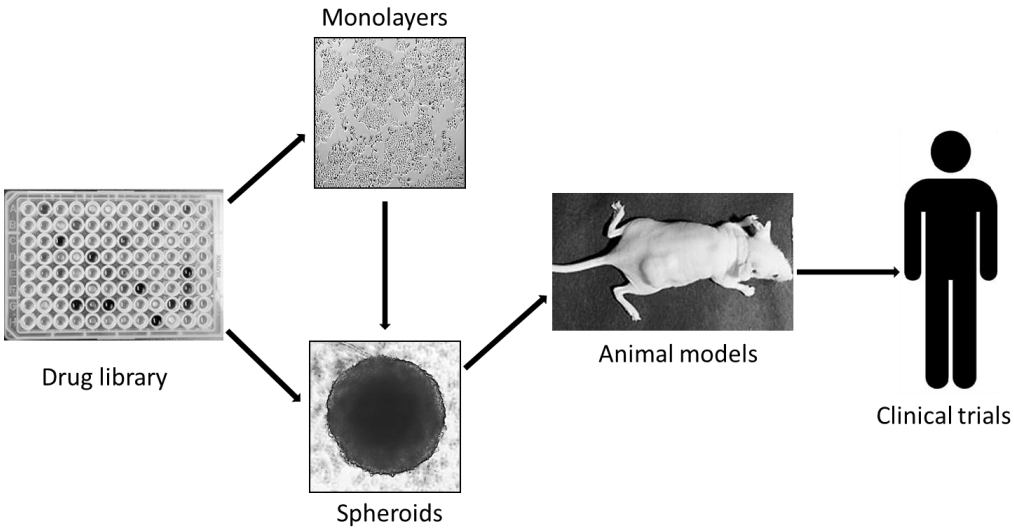

Figure (1). Schematic illustration for a cell based approach for anticancer drug discovery. Drug libraries are directly screened on spheroids or alternatively, first screened on cancer cell lines monolayers, followed by a secondary screen on spheroids. Hits are then tested on animal models and promising agents enter clinical trials.

2 AIMS OF THE THESIS

The main aim of the thesis was to identify compounds for treatment of solid tumors and to define their mechanism of action. Practically, we intended to generate uniform spheroids in the 96-well format and to find a suitable assay to screen drug libraries for cytotoxic/proapoptotic activity. We aimed also to validate animal xenograft models for testing the activity of identified hits *in vivo.* We finally asked the question of whether screening for agents active against tumor masses would enrich for specific physico-chemical or mechanistic characteristics.

3 RESULTS

3.1 PAPER I

The aim of the paper was to adapt the hanging drop method for production of spheroids into the 96 well plates thus allowing high-throughput screening. We used apoptosis induction as an endpoint for efficacy evaluation of screened compounds. Apoptosis was detected using the M30-Apoptosense® enzyme-linked immunosorbent assay method performed in 96 well plates. Spheroids were produced by adding 20 µl drops containing 1000 cells in the rings in the inner side of the lids of the 96 well plate, inverted, and then shaken for 2 hours in humidified CO_2 incubator at 37 °C and were left for three days. Drops were then centrifuged to poly-HEMA coated round bottom 96-plates with 100 µl medium with 10% serum (serum was filtered through 0.2 µm to remove particles that can deform spheroids structure) per well, and incubated for further four days. After seven days of seeding the drops, the generated spheroids were approximately 500 µm which is desirable to mimic the in vivo conditions of tumor masses. Moreover, this period (seven days after seeding) was found to be optimal time for assay assessment with regards to signal-to-noise ratio. Six out of eight epithelial cancer cell lines succeeded to produce spheroids namely, HCT116, FaDu, RT4, J82, LNCap and DU145. ACHN and A498 did not form appropriate spheroids. HCT116 was selected for further studies because of the availability of various clones of this cell line where apoptotic pathways are disrupted giving the opportunity for more comprehensive apoptosis studies. In order to quantify the suitability of the assay and the model for high-throughput screening, we calculated the Z' factor for the M30-Apoptosense® assay on HCT116 spheroids. A value of 0.52 was obtained after 24 hours treatment with 0.5 µM staurosporine (27 wells) and solvent (57 wells) which means by definition that it is an excellent model for large scale assays.

This method was later simplified to avoid the centrifugation step (paper III – VI). In the modified method, 200 µL of cell suspension with 10,000 cells were added to each well of poly-HEMA-coated 96-well plates, and the wells were overfilled by adding an additional 170 µL media to acquire a convex surface curvature. Plasticine spacers (3 mm) were placed in the corners of each plate to prevent the lids from touching the media. The plates were then inverted to allow the cells to sediment to the liquid–air interface. Plates were returned to normal position after 24 hr incubation allowing formed aggregates to settle in the bottom of the wells, excess media and spacers were removed. Plates were then incubated for further 4 days to produce spheroids with ~500 µm diameter.

After optimization and validation of our model, we tested the NCI COMBO Set, from the Developmental Therapeutics Program of the US National Cancer Institute, for apoptosis induction in HCT116 spheroids. The plate contains 77 compounds, 23 of which are anticancer drugs approved by the Food and Drug Administration (FDA). Various degrees of induction of caspase cleavage of CK18 were observed by these agents after 24 hours of treatment. 10 compounds were selected for more detailed studies. 24 and 48 hours treatments of these compounds were comparatively tested on

HCT116 monolayers and spheroids for apoptosis induction, and parallel assessment of viability after 24 hours treatments by counting Trypan blue negative (viable) cells. In case of spheroids, the 10 compounds showed correlation between apoptosis induction and loss of viability, while in monolayers correlation was found in 8 compounds. 2 compounds, tentrandrin and miconazole, showed low apoptosis and low viability, presumably, the death mode shifted from apoptosis to necrosis at such concentrations. Various degrees of induction of caspase cleavage of CK18 by these agents were observed. Piroxicam was not significantly apoptotic for either monolayers or spheroids. Other compounds were apoptotic for both monolayers and spheroids like tamoxifen and pimozide. Cisplatin, a standard DNA-damaging agent, induced relatively more apoptosis in monolayers than in spheroids.

A detailed description for culture, treatment and analysis of multicellular spheroids has also been published by Friedrich et al. (Friedrich, Seidel et al. 2009). These authors listed human carcinoma cell lines that produce spheroids under identical culture conditions. Assay end points were spheroid integrity and cell survival measured by the acid phosphatase assay.

3.2 PAPER II

In this paper we showed that we can detect apoptosis in the sera of mice with certain human epithelial xenografts using the M30-Apoptosense® ELISA independently of host toxicity.

The M30-Apoptosense® ELISA assay detects caspase cleaved cytokeratin 18 (CK18) in human epithelial cells. It uses two antibodies: M5 as capture antibody and M30 as the HRP-conjugated deecting antibody. M30 is a monoclonal antibody which recognizes a neo-epitope formed after internal caspase-cleavage of human CK18 at Asp396 (Leers, Kolgen et al. 1999). Competition experiments were performed using a recombinant human $CK18_{284–396}$ polypeptide and peptides corresponding to the C-terminal caspase-cleavage site from different species. It was observed that the human, macaca mulata and bovine peptides competed more effectively with the $CK18_{284–396}$ polypeptide than those of mouse, rat, dog and xenopus. We concluded that the M30 antibody recognizes the human, chimpanzee, macaca mulata and bovine caspase cleaved CK18, but not that of mouse, rat, dog or xenopus. We also observed that the M5 capture antibody does not detect mouse CK18.

Different human epithelial cancer cells lines were grown as xenografts in mice and treated with different cytotoxic agents. M30-Apoptosense® ELISA assay was used to detect caspase cleaved CK18 in mice plasma. Three SCID mouse models have been successful: FaDu head neck carcinoma, NCI-H146 small cell lung carcinoma and HT29 colon carcinoma. In addition, nude rat model with colon carcinoma SW620 has also been successful.

Time course studies to examine the release of cleaved CK18 in treated animals were performed. Two models were used: SCID mice with FaDu tumours and nude rats with

SW620. SCID mice with FaDu were treated with 5mg/kg doxorubicin intravenously. Increases of CK18-Asp396 from pre-treatment levels were observed in plasma of treated mice at 48, 72, 96 hours after treatment. In case of SW620 model, rats were injected intravenously with 25 mg/kg of the anti-mitotic agent AZ1152 (an aurora kinase inhibitor) for four consecutive days. Increases of CK18-Asp396 were observed in rat plasma at 3–5 days after treatment. However, the amplitudes of the increases induced by AZ1152 were much smaller compared to those found using DNA damaging agents, but were statistically significant.

Thereafter, a dose response study was done in the SCID mice FaDu model using doxorubicin at doses of 0, 0.1, 2.5 and 12.5 mg/kg and 48 hour time point. Significant increases in the CK18-Asp396 levels were observed at the 2.5 and 12.5 mg/kg doses.

We conclude from the experiments done that the M30-Apoptosense® ELISA assay is a useful tool for quantitative detection of apoptotic response induced by cytotoxic agents in xenograft rodent models bearing human epithelial cancer cell lines. The method is able to detect apoptotic response independent from animal toxicity. The results showed that time course and dose response studies can be performed using M30-Apoptosense® ELISA. The assay can be used in preclinical evaluation of anticancer agents.

3.3 PAPER III

In this paper we investigated the effect of the standard chemotherapeutic agent cisplatin on HCT116 spheroids. We modified the method for production of spheroids as described above (see paper I).

First we attempted to have deeper insight on growth characteristics of the HCT116 spheroids. We found that the S-phase fraction dropped from ~40% at day 0 to ~10% 5-7 days after seeding. This indicates significant reduction in the growth fraction consistent with that observed in tumors in vivo. By Ki-67 staining, it was shown that only the outer layers of the spheroids were dividing. Cells in the central portion of the spheroids stained positive for p27Kip1, a cyclin-dependent kinase inhibitor associated with quiescence (Eblen, Fautsch et al. 1995).

Five days old spheroids were treated with 40µM cisplatin. This is a very high concentration, at least one magnitude higher than what can be achieved in vivo (Berndtsson, Hagg et al. 2007), but commonly used in *in vitro* studies of the effects of cisplatin on tumor cells. We aimed to address whether – even at such a high concentration – cisplatin induces significant apoptosis of HCT116 cells in spheroids. Clonogenicity was determined for both cisplatin-treated and untreated spheroids at days 0, 2, 4, 6 and 8 after treatment. Spheroids were trypsinized, counted and 2000 viable cells were plated in 6-well plates. Untreated controls showed ~12.5% to ~15% clonogenicity in the different time points, while treated spheroids showed 0% clonogenicity at day 0 and gradually increased to ~2.5% at day 8. We stained sectioned treated spheroids for active caspase-3 to check whether acute apoptosis has a role in such reduced clonogenicity. Results showed that apoptosis was confined to the outer

layers (0–30 μm) at detected time points (0, 24, 48, 72 and 96 hr) where an increase in percent of positive cells was observed (~1.1% after 24 hr to ~3.8% after 96 hr). Increased caspase-3 activation was not observed in these cell layers in untreated spheroids during the same time span. A similar pattern of peripheral positivity was observed on staining for p53, p21Cip1 and p27Kip1 in sectioned treated spheroids, while untreated spheroids were negative.

Although cisplatin caused significant reduction in clonogenicity, it was noticed that it did not eradicate spheroids even in long term. We examined the spheroids after 8 days of treatment. Treated spheroids diameters were similar to those observed at the onset of treatment and equal to ~75% of that of untreated spheroids. On counting, treated spheroids contained ~2×10^4 viable cells compared to ~10^5 cells in untreated controls. Approximately 5 % of treated spheroids cells stained positive for the senescence marker β-galactosidase while, untreated controls were < 5% positive. We concluded that a major fraction of cells are rendered senescent when spheroids are treated with 40 μM cisplatin. Senescence was also observed after six days on treating HCT116 monolayers with 10 μM cisplatin, >70% of the surviving cells were positive to β-galactosidase staining and growth arrested. Growth arrest was assessed by staining with the PKH2 dye prior cisplatin treatment. The PKH2 signal is diluted in dividing cells, but not diluted in growth arrested cells and was determined by flow cytometry. Double staining with propidium iodide was used to determine the % of dying cells. Treated growth arrested cells showed ~45% dead cells compared to ~15% in the respective untreated cells. These data show that treated growth-arrested cells have a decreased long-term viability.

Next, we investigated cisplatin effect in two in vivo xenograft models; SCID mice with HCT116 and FaDu cell lines. A dose of 2.5 mg/kg was used, and thaspine (Fayad, Fryknas et al. 2009) was used as a positive control in a dose of 10 mg/kg. Two parameters were considered; measuring of caspase-cleaved cytokeratin 18 in mouse serum after 48 hr as a marker for apoptosis and tumor size measurement. In the HCT116 model, cisplatin did not induce significant increase in cleaved cytokeratin 18 and showed weak growth retardation in tumors. In FaDu model, we got different results, where cisplatin caused significant increase in cleaved cytokeratin 18 levels and no effect on tumors growth. It is noteworthy to mention here that cisplatin did not induce apoptosis in FaDu spheroids at 48 hr, revealed by immunostaining for active caspase-3. Therefore, it seems that apoptosis induction in tumors after 48 hr does not reflect cisplatin response.

We conclude from the study that acute apoptosis is not the major mechanism by which cisplatin response occurs. In the HCT116 spheroid model, apoptosis was limited to the outer layers (mainly at 72-96 hr) and a major fraction of the cells were senescent after 8 days of treatment. These senescent cells showed high spontaneous death compared to the untreated proliferating cells.

3.4 PAPER IV

About two-thirds of all approved anticancer agents are natural products or structurally related to them (Neidle 2008). We here screened a natural products library for apoptosis induction in a colon carcinoma cell line. The NCI natural products set (221 compounds) was screened at 25µM on HCT116 cell line monolayers for proapoptotic activity after 24 hr treament. 20 hits were identified, 14 of which were with known targets. Thaspine (taspine; NSC76022) was selected for further investigation, as it had no known target and it is derived from the cortex of the tree *Croton lechleri* which is used in folk medicine.

Thaspine induced apoptosis at 10 µM measured by M30 CytoDeath ELISA (a version of the M30 Apoptosense ELISA recently developed for in vitro use). Thaspine was found to induce the mitochondrial apoptotic pathway evidenced by the decrease in the mitochondrial membrane potential measured by flow cytometry of the fluorescent probe tetramethyl-rhodamine ethyl ester (TMRE). Bak and Bax are two key regulators in the mitochondrial apoptotic pathway (Hsu and Youle 1998; Griffiths, Dubrez et al. 1999; Wei, Zong et al. 2001). Thaspine was here found to induce a conformational activation of Bak and Bax. We furthermore found increased levels of cytochrome c in cytosol after thaspine treatment, as determined by Western blotting. In order to investigate which BH3 only protein has a role in apoptosis execution after thaspine treatment, an siRNA approach was used. Out of 9 tested siRNAs, Bid and Bik significantly reduced apoptsis, suggesting their role in thaspine-induced apoptosis.

In order to elucidate the mechanism of action of thaspine, we used the Connectivity Map (CMAP) which is a method that compares the gene expression signature of a tested compound on certain cell line to a data base of gene expression patterns of drug-treated cell lines (Lamb, Crawford et al. 2006). Thaspine was tested on MCF-7 cell line as the 1309 compounds in CMAP were tested on this cell line. Thaspine induced gene expression matched with ellipticine, a topoisomerase II inhibitor, and with camptothecin which is a topoisomerase I inhibitor. These data gave a hypothesis that thaspine is most likely a topoisomerase inhibitor. For proving this hyposthesis, we tested the inhibitory effect of thaspine on topoisomerase I&II in an *in vitro* assay. Indeed, thaspine inhibited both enzymes at a concentration of 10 µM. The assay in brief is based on measuring the inhibitory effect of tested compound on topoisomerase activity on a supercoiled plasmid. The topoisomerase enzyme converts the plasmid into its nicked form, while in case of inhibition of the enzyme, the supercoiled plasmid remains unchanged (as was noticed with thaspine). Different samples were detected by gel electrophoresis and staining with ethidium bromide. Furthermore, thaspine was found to to have a reduced cytotoxic effect on the viability on CEM/VM-1, a cell line selected for resistance to the topoisomerase II inhibitor teniposide compared to the parental cell line CCRF-CEM, adding more evidence that thaspine is a topoisomerase inhibitor.

We then tested the efficacy of thaspine on some forms of resistance to chemotherapy. It was found that thaspine toxicity was marginally affected in two cell lines

overexpressing the membrane-associated drug efflux transporters P-glycoprotein (Pgp, ABCB1) and the multidrug resistance-associated protein (MRP, ABCC1). Interestingly, parallel testing using HCT116 spheroids showed that thaspine also induced apoptosis of 3-D cultured cells. We found that 20 µM of thaspine caused wide-spread apoptosis in HCT116 spheroids after 16 hr treatment as revealed by staining with active caspase-3 in sectioned spheroids. This was paralleled by almost total loss of clonogenicity of thaspine treated spheroids (0.006% of untreated control). 24 hr treatment with 20 µM doxorubicin and 40 µM cisplatin caused limited induction of active caspase-3 in HCT116 spheroids.

Next, we investigated the effect of thaspine in vivo. Three parameters were measured; immunostaining of HCT116 xenograft tumors sections for active caspase-3, caspase-cleaved cytokeratin 18 in sera of mice bearing HCT116 and FaDu xenografts and tumor volume of FaDu xenograft. A single dose of 10 mg/kg thaspine was used in both tumor models. Treatment started when tumors were ~400 mm^3. Positivity for active caspase-3 was observed in treated HCT116 tumors sections after 48 hr treatment. At the same time point, a significant increase in cleaved cytokeratin-18 was observed in the sera of the treated mice both in HCT116 and FaDu xenografts. Apoptosis was paralleled by a significant, but transient, reduction of tumor size in the FaDu model. We conclude that thaspine is capable of induction of apoptosis in vivo.

In summary, we identified thaspine as a novel dual topoisomerase inhibitor, from **a** screen of natural products. Thaspine induced apoptosis in HCT116 monolayers, spheroids and mice xenografts. It also induced apoptosis in FaDu xenografts with significant, but transient, tumor size reduction.

3.5 PAPER V

In this paper we used the multicellular spheroid model to screen for apoptotic/cytotoxic compounds. In addition to our previous characterization of HCT116 spheroids, we here showed that they contained a core of hypoxic cells as evidenced by staining for pimonidazole adducts. Killing this hypoxic subpopulation is essential for successful treatment. We aimed in this paper to identify compounds which are cytotoxic to both proliferating and hypoxic non-proliferating cells. Two distinct screens were performed, one where the library was screened both on HCT116 spheroids and monolayers and apoptosis was used as read-out and in a second where spheroids were screened for induction of apoptosis and loss of viability. In the second screen, the library was first screened on monolayer cells at a high drug concentration and continuous exposure to select active compounds.

In the first screen, the NCI mechanistic set and the natural product set (999 compounds in total) were screened on HCT116 spheroids at 5 µM and 25 µM respectively. The two sets were in parallel screened on HCT116 monolayers at the same concentrations. 24 hours exposure was used and apoptosis was the readout using the M30 CytoDeath ELISA. The 40 strongest hits were selected, and it was found that only 5 compounds were common in the identified spheroids and monolayers hits. We asked if the spheroid

hits enrich for certain chemical properties. We examined the molecular weight and XLogP of both sets of hits. It was found that the molecular weight did not differ significantly in monolayers and spheroids hits compared to libraries. On the other hand, it was found that spheroids hits selected against hydrophilicity, i.e. selects for compounds with higher XLogP values. The median XlogP of spheroids screening hits was 4.34 compared to a median XlogP of 3.16 in the libraries. The 10^{th} percentile of the XlogPs of 3-D hits was 1.27 compared to -0.55 of the libraries. In contrast, the distribution of XlogP values of 2-D active compounds did not differ significantly from the libraries. The strongest monolayer hit (NSC285116, siomycin A) was essentially inactive in 3-D culture. Siomycin A is a hydrophilic molecule (XLogP -3), likely to explain its poor penetration into spheroids. The hydrophobicity of 3-D active compounds was much higher compared to that observed in 18 standard chemotherapeutical drugs used as reference (median XlogP = 0.95).
In the second screen, we screened the DIVERSet library form Chembridge (10,000 compounds) on HCT116 monolayers at 25 µM with continuous exposure for 72 hours and assayed for general toxicity using the FMCA assay (Lindhagen, Nygren et al. 2008). 382 hits were selected and rescreened on HCT116 spheroids at 25 µM with 6 hours exposure followed by further 66 hours incubation in drug free medium. This time, both apoptosis and toxicity were used as readout. Apoptosis was detected by M30 CytoDeath ELISA while toxicity was measured by the acid phosphatase assay as described by Friedrich et al. (Friedrich, Eder et al. 2007). The screen yielded 40 compounds which were retested at different drug concentrations, and the 11 most potent compounds were selected for further studies. Again with regards to XLogP properties, similar to the first screen, these compounds showed a higher distribution of XlogP values compared to the library (median 4.40 versus 3.65) and there was a selection against compounds with low XlogP values (10th percentile = 2.70). We conclude that two independent screening procedures resulted in hits with very similar median XlogP values (4.34 and 4.40) and selected against hydrophilic compounds.

Afterwards, we considered the 11 hits from the second screen for further mechanistic investigation. We started with cell cycle analysis, and it was found that seven of the 11 compounds induced statistically significant increases in the number of cells in G2/M. Furthermore, visual inspection of the DNA flow cytometry profiles suggested that an eighth compound (5248881) also induced G2/M arrest, but that the profile was masked by dying cell debris. Three of 8 compounds that induced G2/M arrest (5268231, 5346277 and 5350849) are sulphonamides, a class of compounds with reported microtubule inhibitory activity. This stimulated us to test the tubulin polymerization inhibition activity of these compounds *in vitro*. A number of the compounds showed some degree of inhibitory activity with 5248881 showing the strongest inhibition of polymerization with potency similar to colchicine. For adding more confirmation to the mechanism of action of these compounds, we used the CMAP technique (Lamb, Crawford et al. 2006) for comparison of the gene expression of our tested compounds to that of known compounds. Seven compounds showed a similar pattern to mitotic inhibitors including 5248881, 5276937 and 5350849. Then we attempted to investigate whether these compounds are selectively toxic to cancer spheroids and not toxic to normal non proliferating compounds. We set up spheroids from hTERT-RPE1, a normal human immortalized retinal epithelial cell line. Cell cycle analysis of RPE1 spheroids showed that > 99% of the cells accumulated in the G1 phase indicating their

quiescence. 5 compounds with antimitotic transcriptional response were tested on both HCT116 and RPE1 spheroids. Spheroids were treated for 6 hours, washed, and then incubated for 5 days. Spheroids were trypsinized and tested for clonogenicity. Four of 5 compounds reduced HCT116 spheroid cell clonogenicity of > 70% while the clonogenicity of hTERT-RPE1 cells was reduced by 20% or less, indicating a promising therapeutic window. Vinblastine, the known mitotic inhibitor showed selective toxicity towards cancer spheroids, while staurosporine and doxorubicin were 100% toxic to the normal spheroids while showed 75% and 45% reduction in HCT116 spheroids clonogenicity, respectively.

The strongly tubulin depolymerizing compound 5248881 was studied in further detail. Staining spheroid sections for active caspase-3 showed that this compound induced apoptosis in the outer layers of the spheroids at 48 hours of treatment. The extent of caspase-3 activation by compound 5248881 was stronger than that observed using different standard tubulin inhibitors at tested concentrations. The volume of the area responding by apoptosis was estimated to be < 40% of total spheroid volume which could not account for the reduction of clonogenicity which was in the order of 80%. The antiproliferative effect of the compound on the cells of the spheroids can therefore not only be accounted for by induction of apoptosis. The possibility that the inner core cells are not clonogenic (contributing for the reduced clonogenicity) can be excluded in the light of NSC620358 results. NSC620358 is a compound identified in the screen and showed similar pattern of apoptosis induction (confined to outer layers) to that of 5248881 compound. However, the clonogenicity of NSC620358 treated spheroids was only reduced by ~10%.

We conclude from the work done that spheroid screening enriches for hydrophobic compounds. Mechanistic analysis revealed that the majority of the spheroid screening hits were microtubuli inhibitors. We identified the tubulin inhibitor 5248881 having a therapeutic window with selective toxicity to cancer spheroids and not to normal spheroids. The compound was found to be cytotoxic towards non-proliferating tumour cells in the spheroids but not to non-proliferating normal hTERT-RPE1 cells.

3.6 PAPER VI

In this paper, we identified CB21as a cytotoxic compound against HCT116 spheroids. CB21 was selected in the second screen outlined in paper V. As previously outlined, outer layers of the spheroids stained positive for Ki67 while inner layers were negative. Furthermore, the inner layers stained positive for p27 (cyclin dependent kinase inhibitor) and pimonidazole (hypoxia marker). We additionally show here that the core cells stain positive for the ER stress marker GRP78. All of these markers indicate that the inner spheroids cells are quiescent, hypoxic and stressed, all are characteristics of resistant cells in solid tumors.

CB21 scored as cytotoxic to HCT116 spheroids both in apoptosis induction using the M30 assay and reduction of viability using the acid phosphatase assay. While, in non-confluent untransformed hTERT-RPE1 epithelial cell line, the compound caused

growth arrest but no cytotoxicity and for confluent cells it did not induce cell loss. Sectioned CB21 treated spheroids (6hr treatment followed by 96 hr incubation) were small and exhibiting a dead core.

CMAP experiments suggested that CB21 is an iron chelator, as it induced similar gene experession as that of the known iron chelator ciclopirox olamine (CPX). Incubation of CB21 with iron chloride caused complete loss of activity, confirming that CB21 acts through iron chelation. Iron chelators are known to inhibit ribonucleotide reductase and thus inhibiting DNA synthesis (Nordlund and Reichard 2006). We found that CB21 completely inhibited DNA synthesis at 24 hr treatment using BrdU incorporation assay. In addition, CB21 was equally toxic to HCT116 cells with wt p53 and disrupted p53. CB21 was the most potent in cytoxicity when compared to other known iron chelators; VLX50, deferasirox, ciclopirox olamine and deferoxamine (DFO).

We observed formation of multible cytoplasmic vesicles in HCT116 cells treated with CB21. These cells stained positive with an antibody against LC3 protein indicating the induction of autophagy. Western blot confirmed this finding where both LC3-I and LC3-II were upregulated in CB21 treated HCT116 monolayers. LC3-II levels were also increased in CB21 treated HCT116 spheroids. In RPE1 cells, LC3-II was induced but to a lesser extent compared to HCT116 cells. This observation indicated that CB21 toxicity is associated with autophagy.

Different iron chelators induced LC3-I and LC3-II after 24 hr treatment on HCT116 monolayers at toxic concentrations. This indicated that autophagy induction is a common mechanism caused by iron chelators. Electron microscopy for sectioned CB21 spheroids showed autophagic vesicles both in outer layers and in deeper layers. We can conclude from this that CB21 is a penetrating compound which is able to induce autophagy in spheroids both in outer layers and deeper resistant cells.

It is controversial whether autophagy is a rescue mechanism or a death mode (Kondo, Kanzawa et al. 2005). In order to answer the question if CB21 induced autophagy is a mechanism by which cells rescue themselves or dye with, we tested different autophagy inhibitors co-treated with CB21. We used 3MA, Bafilomycin A and siRNA against Beclin/Atg6. In all experiments, autophagy inhibition enhanced CB21 cytotoxicity. We can conclude from this that autophagy is induced as a rescue mechanism as a result of certain metabolic insult caused by CB21.

Results from CMAP showed that the hypoxia induced protein HIF-1α was upregulated by CB21. This was confirmed by Western blotting and by induction of GFP in a reporter cell line where GFP is regulated by HIF-1 α promotor. In addition, it is known that several proteins containing Fe complexes are involved in energy metabolism including oxygen consumption (Schieke, Phillips et al. 2006). This suggested the hypothesis that iron chelation affects oxygen consumption. We found two evidences of reduced oxygen consumptions. The first one by direct measurement of V3 (state 3) and Vu (uncoupled) respiration which were found to be significantly ($p < 0.05$) decreased after 6 hours of CB21 treatment. The second evidence was indirect using immunohistochemistry by staining for the hypoxia marker pimonidazole. CB21 treated sections showed ~50% decrease in hypoxic area. This means that cells' consumption of

oxygen decreased on treatment with CB21 leading to abundance of oxygen and thus increase in the area of normoxic cells (Arteel, Thurman et al. 1998)**.** As a control, HCT116 were treated with a mitochondrial uncoupling agent (carbonylcyanide-3-chlorophenylhydrazone, CCCP) known to increase oxygen consumption. CCCP treated sections showed larger area of hypoxia compared to untreated controls.

BNIP3is a BH3-only protein which is regulated by HIF-1α (Bruick 2000). BNIP3 is reported to be involved in induction of autophagy (Vande Velde, Cizeau et al. 2000). Western blot showed that BNIP3 is upregulated by CB21, however silencing BNIP3 did not inhibit LC3-II induction or cell death in HCT116 monolayers. BNIP3 was induced in RPE1 as well, indicating that BNIP3 is not responsible for cytotoxicity of CB21.

mTOR is a serine/threonine kinase which regulates cell growth in response to nutrient status as well as mitochondrial oxygen consumption. We examined if CB21 reduced oxygen consumption is associated with mTOR inhibition. CB21 treatment caused the inhibition of the mTOR substrate 4E-BP1paralleled by increased Akt phosphorylation. The mTOR inhibitor rapamycin caused a reduction in the pimonidazole staining in sectioned spheroids though weaker than CB21. Although the dual PI3K/mTOR inhibitor NVP-BEZ235 was able to inhibit the phosphorylation of 4E-BP1 in spheroids, it did not exhibit cytoxicity on the inner layers of them.

Decreased oxygen consumption is expected to increase the dependence on glucose for anaerobic energy production. We found that the hypoxic cells in the core of HCT116 spheroids, but not the normoxic outer layers, died when grown in glucose deprived medium. Accordingly, the reduced oxygen consumption caused by CB21 is expected to increase the dependence of treated cells on glucose. This is what we found as the CB21 treated cells showed higher death rate and apoptosis induction in glucose free medium compared to cells grown in usual glucose concentrations.

Finally, we tested CB21 in mice xenografts. SCID mice with ~200 mm^2 HCT116 tumors were injected with a single i.v. dose (16 mg/kg) of CB21. The compound showed a significant growth inhibition in treated tumors until 15 days after treatment. These data are preliminary, studies are ongoing.

In summary, we identified CB21 as a novel permeable iron chelator toxic to HCT116 spheroids. The compound was non-toxic to untransformed human epithelail cells. CB21 induced autophagy associated cell death in the outer layers of the spheroids as well as the core cells known to be resistant to chemotherapeutic agents. The cytotoxicity of CB21 was enhaced by autophagy inhibitors. CB21 significantly inhibited tumor growth in a HCT116 xenograft model.

4 DISCUSSION

4.1 MULTICELLULAR SPHEROIDS AS A MODEL FOR SOLID TUMOURS

The main scope of the thesis was to identify compounds with curative potential to solid tumors. Alhough improvements in cancer treatment modalities have been achieved, current cure rates are not satisfactory for many forms of advanced cancer diseases (Kamb, Wee et al. 2007). Only a few forms of solid tumors can be treated with curative intent when diagnosed at advanced stages, notably testicular carcinoma (Carbone 1990; Holland 2000). Accordingly, there is a large medical need for novel, efficient chemotherapeutic agents.

We have chosen the multicellular spheroid model to screen for compounds which could potentially eradicate solid tumors. The model we chose was HCT116 colon carcinoma. Characterization of HCT116 spheroids revealed they are composed of outer proliferating layers and inner hypoxic quiescent cells. In immunohistochemistry staining, the outer layers stained positive for the proliferation marker Ki67 while the inner layers did not. In addition, the inner layers stained positive for p27, pimonidazole and GRP78 indicating their quiescence, hypoxia and ER stress, respectively. It has been shown that spheroids > 200 µm consume ~1/4 of oxygen compared to the exponentially growing spheroids of < 200 µm diameter (Freyer, Tustanoff et al. 1984). The hypoxic non-proliferating subpopulation is of particular interest as this cell population is known to be therapy resistant, and thought to be responsible for tumor regrowth. Finding compounds which are toxic to this subpopulation of cells provides a promising strategy for identification of effective drugs for treatment of solid tumors. Additionally, the three dimensional nature of the spheroids allows the identification of penetrating compounds, which is an essential characteristic for an agent effective on solid tumors. We have shown that apoptosis induction of a high dose of 40 µM cisplatin was limited to the outer layers of the spheroids, and did not eradicate the spheroids even after eight days of incubation, most probably because of its poor penetrability.

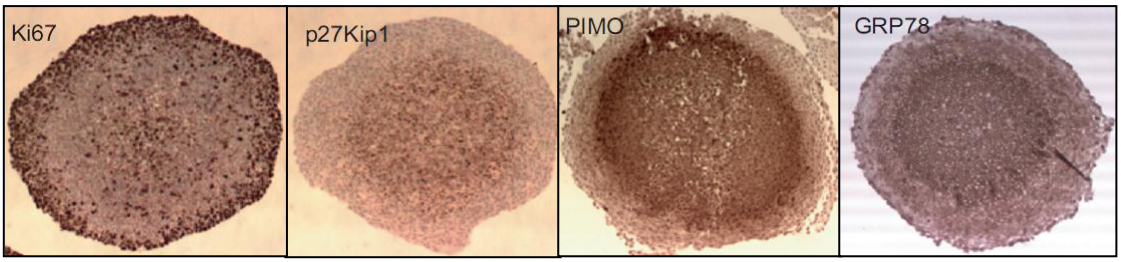

Figure (2). Immunohistochemical characterization for HCT116 colon carcinoma spheroids. Outer layers are stain positive for the proliferation marker Ki-67, while the spheroids core is stained positive for the cyclin dependent kinase inhibitor p27, the hypoxia marker pimonidazole and ER stress marker GRP78.

This triggered us (in papers V & VI) to use the spheroid model for screening drug libraries aiming to find compounds able to penetrate the tumor mass and being toxic to the inner hypoxic quiescent spheroid cells as well as the outer proliferating cells.

In our second screen (paper V and paper VI), we used a six hours exposure time, followed by washing and further incubation a step to mimic clinical situation where the compounds are washed away from the body through metabolism and excretion. This step also allows the identification of penetrating compounds, excluding the possibility that the activity of the drug is attributed to killing the spheroids layer by layer in case of continuous exposure. In this screen also we introduced a counter screening step where we used the normal human immortalized retinal epithelial RPE1 cell line, in an attempt for early identification of compounds with wide therapeutic window.

4.1.1 Spheroids as a model for studying combination therapy

Spheroids can be multiply treated owing to their three dimensional nature, making it possible to use them as a model for studying complex combination therapy modalities where drugs are used at separate times. It seems reasonable to assume that alternating treatment using cytotoxic agent and cytostatic agent can lead to decreases in tumor mass. An illustration is shown in the figure below. HCT116 spheroids were treated with a cytotoxic agents (NSC620358, 20 µM) identified in our screen. NSC620358 is a proteasome inhibitor which shows strong cytotoxicity to the peripheral cell layers of HCT116 (actually "peeling" these cells off, leaving inner cell layers intact) (manuscript in preparation). Following treatment with the cytotoxic agent, there is a slow increase in the size of the spheroids (associated with an increase in Ki67 and cyclin A positive cells). Whether this combination of compounds will be effective *in vivo*, and whether the cytotoxic treatment can be repeated until the tumor mass is eradicate, is not clear.

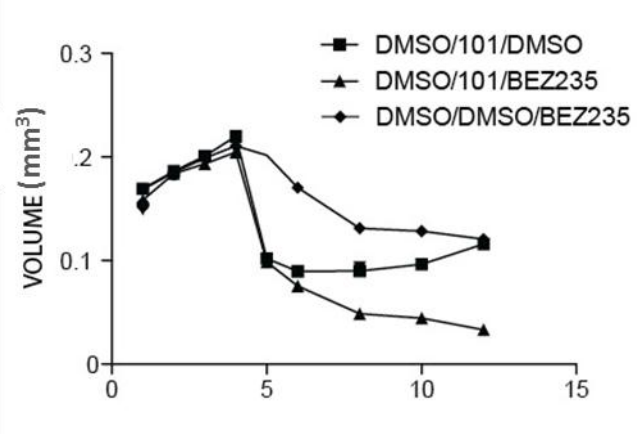

Figure (3). Effect on HCT116 spheroids of the combination of the cytotoxic agent NSC620358 (comp.10) (20 µM) and the PI3K/ mTOR inhibitor NVP-BEZ235 (0.2 µM). Spheroids were treated with NSC620358 for 24 hours on Day 4. After treatment, spheroids were incubated with or without NVP-BEZ235 as indicated. Diameters were measured in quadruplicate wells.

4.1.2 Methods for generating multicellular spheroids

Different methods have been described for making spheroids. The common concept of these methods is to grow cancer cells in a non-adherent substratum like agar, agarose or Poly(2-hydroxyethyl methacrylate) (poly-HEMA) (Folkman and Moscona 1978). The main methods used for generation of spheroids are spinner flask method (Moscona 1957; Moscona 1968), liquid overlay method (Rofstad, Wahl et al. 1986; Enmon, O'Connor et al. 2001; O'Connor and Venczel 2005), gel encapsulation (Kupchik, Langer et al. 1983; Kupchik, Collins et al. 1990; O'Keane, Kupchik et al. 1990; Hoffmann, Schirner et al. 1997) and hanging drop method (Kelm, Timmins et al. 2003; Del Duca, Werbowetski et al. 2004; Timmins and Nielsen 2007). With exception to the hanging drop method, the other methods have the drawback of production of spheroids of heterogeneous sizes. This makes it unsuitable for high throughput screening. One method for generation of spheroids in 96-well plates has been described based on the liquid overlay method combined with a centrifugation step (Ivascu and Kubbies 2006) but on trying it in our lab it did not produce single spheroid per well. The hanging drop method is characterized by generation of homogenous spheroids but the problem with it is the need for transferring procedure making it tedious for screening purpose.

In the present study, we introduced two new methods for production of spheroids in the 96-well plates. The two methods are based on combination of hanging drop principle and liquid overlay method. These methods produced single spheroid per well, with uniform sizes. For HCT116 colon carcinoma, we obtained a Z' factor (Zhang, Chung et al. 1999) of ~0.5 for both methods using the M30-Apoptosense® assay indicating that these models are excellent for high throughput screening. One method, which is based on flipping the plate, succeeded to produce spheroids from the FaDu cell line and the immortalized human epithelial retinal RPE1 cell line. The introduction of a shaking step for the inverted plate significantly improved the ability of the cell lines to form spheroids and the homogeneity of generated spheroids.

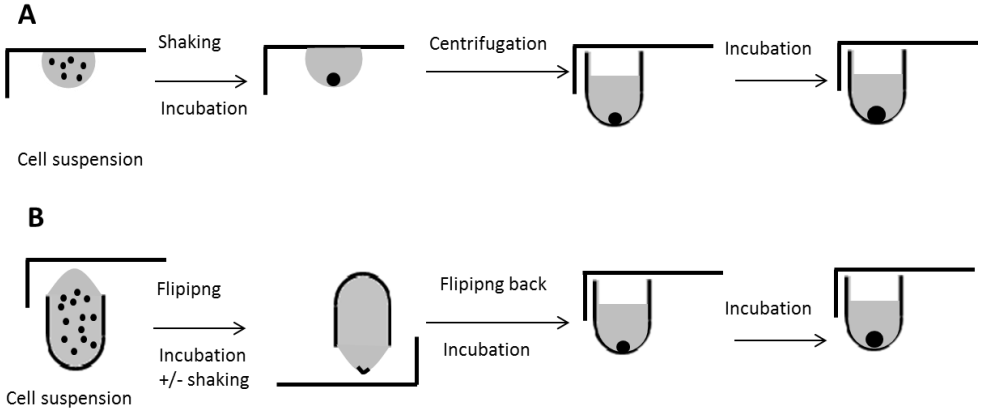

Figure (4). Two methods for generation of spheroids in 96-well plate were developed based on the hanging drop method. (A). 20 µl cell suspension drops were added to the inner side of the 96-well plate lids which were shaken, incubated, centrifuged to poly-HEMA coated 96-well plates, then incubated further till formation of spheroids with the required size. (B). 96-well plate wells were overfilled with cell suspension to obtain convex surface. The plates were then flipped, shaken (if required) and incubated for 1-2 days. Media is kept in wells by surface tension. Plates are then flipped back, allowing aggregates to settle down in the poly-HEMA coated bottom. Aggregates were incubated further until forming the desirable size spheroids.

4.2 THE M30 ASSAY FOR DETECTION OF APOPTOSIS

Throughout the present study, we used the M30 Apoptosense® assay for detection of apoptosis induction *in vitro* and *in vivo* by different tested agents (Linder 2007). It is available in 96-well format. The assay detects caspase cleaved CK18 in epithelial cells. The advantage of this assay that it is applicable to cell cultures *in vitro* as monolayers (Hägg, Biven et al. 2002; Erdal, Berndtsson et al. 2005), spheroids (Herrmann, Fayad et al. 2008). In addition, the method can be used to determine tumor cell death using plasma/serum samples from human xenografts and patients (Ueno, Toi et al. 2003; Kramer, Erdal et al. 2004) .

Available cytotoxicity assays for monolayer cultures are not necessarily suitable for performance on the spheroid model. Standard methods such as MTT and XTT assays rely on exogenous substrates, which may not diffuse into the depth of the spheroids. It is therefore possible that such assays only measure the viability of a subpopulation in the spheroids. The M30 ELISA assay overcomes this obstacle as it measures the endogenously produced caspase cleaved CK18. Both intracellular and extracellular cleaved CK18 are detected, by the aid of freezing and thawing the spheroids and by addition of non-ionic detergent. Another advantage of the assay that it is accumulative in nature, as it measures the accumulated produced cleaved CK18 which is stable in the culture medium. This is in contrast with other apoptosis assays, like caspase-3 measurement, which require an optimum time point for detection of apoptosis.

In addition to the M30 assay, we used the acid phosphatase assay, for measurement of general toxicity (Friedrich, Eder et al. 2007). Similar to the M30 assay, this assay measures an endogenous substrate which is the acid phosphatase enzyme, explaining its suitability for the spheroids model. One problem we faced with acid phosphatase assay is the presence of background levels in spheroids where all cells were dead as determined by clonogenic assay. We believe that this background is from dead cells in the middle of the spheroids where acid phosphatase may be entrapped and could not be washed away during the washing steps. In many experiment we subtracted the background levels from all samples including the untreated controls.

We here showed the applicability of the M30 assay in rodents' xenografts (Paper II). The assay does not detect mouse/rat caspase cleaved CK18 – making the M30 Apoptosense ELISA a useful tool for determining whether drugs induce apoptosis in human xenograft tumors in rodents. We considered two main possible sources for the elevated levels of cleaved CK18 in blood. Both possibilities are related to tumor cells which are adjacent to blood vessels. The first explanation is that apoptotic bodies, which are not engulfed by neighboring cells and macrophages due to overload, undergo secondary necrosis and disintegration. This is followed by the release of the contents of these bodies into circulation (Chang, di Tomaso et al. 2000). The second possibility is based on the fact that cancer cells which form part of the vessel wall (mosaic vessels) are shed into circulation. It is estimated that around one million cancer cells are shed into circulation per day for one gram tumor. These cells are known to have high apoptotic index (Larson, Moreno et al. 2004). Hou et al (2009) showed that plasma M30 concentrations correlated to the number of apoptotic circulating tumor cells (Hou,

Greystoke et al. 2009), consistent with this interpretation. We studied the effect of cisplatin in two mice xenograft models bearing HCT116 colon carcinoma and FaDu head and neck carcinoma cell lines (Paper III) (Xu, Cheung et al. 2010). Despite the very limited apoptosis detected in the spheroid model, we did detect some caspase-cleaved CK18 in the plasma of treated mice. The induction of caspase-cleaved CK18 by cisplatin was not, however, correlated with the tumor volume reduction. This observation is consistent with a model where the cancer cells which are close to the blood vessels are the source of the apoptotic signal while the bulk of the tumor is not affected by cisplatin causing undetectable decrease in tumor volume. This agrees with what we found in restriction of acute apoptosis induced by cisplatin to the outer layers of the HCT116 spheroids.

This observation raises the question if apoptosis induction is a relevant screening target. Most of anticancer agents are thought to exert their efficacy through induction of apoptosis. Paradoxically, evasion of apoptosis is a hallmark for most if not all types of cancers. Recent studies support the involvement of other modes of cell death/arrest like necrosis, mitotic catastrophe, autophagy and senescence as a response to chemotherapy. We believe that apoptosis induction will remain an interesting mechanism for discovery of new and more effective antineoplastic agents. However exploration for compounds which induce other types will be beneficial in the light of the fact that apoptosis mechanisms are disrupted within cancer cells.

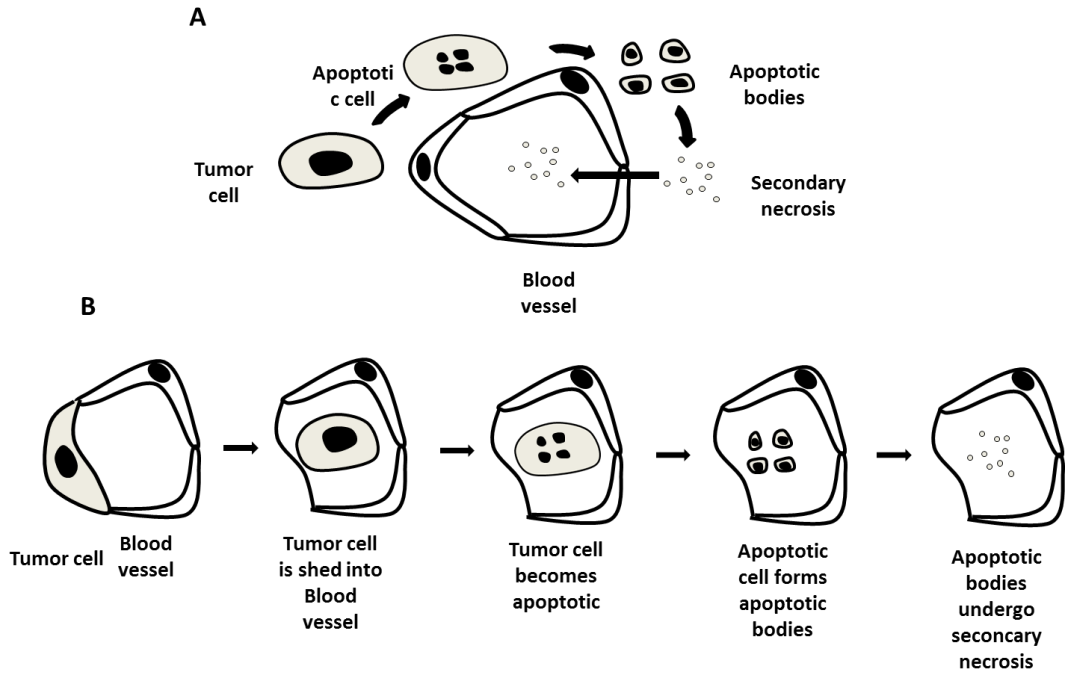

Figure (5). Two hypotheses explaining elevated levels of M30 in blood of cancer patients. (A). Tumor cells first undergo apoptosis, rupture, and followed by releasing of M30 into circulation. (B). Tumor cells which that are part of the vessel walls of mosaic vessels are shed into blood where they undergo apoptosis.

4.3 CHEMICAL PROPERTIES OF SPHEROIDS SCREENING HITS

In the present thesis we report the first relatively large scale screening of drug libraries for cytotoxicity/apoptosis induction on cancer spheroids. We validated the screening procedure by testing the COMBO plate (77 compounds) from the NCI then we performed two main screens. The first one was for the mechanistic set (827 compounds) and the natural product set (221 compounds) both provided by the NCI, and the second screen for 382 hits from a monolayer screen of 10000 compounds from the DIVERSet library purchased from Chembridge company. The screens were performed on the colon carcinoma cell line HCT116. The total number of compounds screened on spheroids is 1458 taking in consideration the overlap of some compounds between the natural products and mechanistic sets.

A total of 999 compounds from the mechanistic set and the natural products set were screened on both HCT116 monolayers and spheroids for apoptosis induction. Interestingly, the screens yielded different sets of hits. Only 5 compounds were common in the strongest 40 hits. This finding indicates that the selection of the experimental model affects significantly the path of the research and consequently the usefulness of the obtained results.

It has been reported that resistance to anticancer agents can be overcome by increasing their lipophilicity (Fry and Jackson 1986; Seiler 2005; Verma 2006). This could be attributed to the enhanced penetrability into the tumor parenchyme. In our first screen we studied the chemical properties of both sets of monolayers and spheroids hits, with regards to molecular weight and lipophilicity and addressed the question whether the spheroids model enriches for compounds with certain characteristics. The molecular weight distribution did not differ between monolayers hits and spheroids hits. However, the spheroids screen selected for hydrophobic compounds with high XlogP values. The same finding was observed in the second screen. These results suggest that hydrophobic compounds are more capable of penetrating the tumor mass than hydrophilic compounds. The median XlogP of 18 standard chemotherapeutic agents was significantly lower than that of the spheroids hits, a possible explanation for their limited efficacy on spheroids and solid tumors. The most potent monolayer hit was siomycin A, this compound was inactive on spheroids in the M30 assay and in caspase-3 induction in the immunostained sectioned spheroids. Siomycin A is a hydrophobic compound with XlogP of -3, adding more evidence for the assumption that hydrophobicity is a major factor for the efficacy of anticancer agents on 3-D tumors.

One point to be outlined here is that the hydrophobic hits are not soluble in aqueous solvents. This necessitates an additional effort for formulating such compounds for *in vivo* testing. We faced this problem with the iron chelator CB21 which precipitated in the mice when injected subcutaneously. Considerable work had to be performed to obtain a proper formulation where no precipitation of the compound occurred.

To summarize, our findings have implications for drug design and lead compounds optimization. The findings suggest that screens aimed to identify compounds effetive

on solid tumors should use chemical libraries where the XlogPs of the compounds are in the order of 3 – 5.

4.4 MECHANISTIC PROPERTIES OF SPHEROIDS SCREENING HITS

In the current study, we identified 13 compounds cytotoxic to cancer spheroids. The mechanism of action of majority of the hits was inhibition of tubulin polymerization: seven compounds were microtubuli inhibitors, one was a dual topoisomerase inhibitor and one was an iron chelator.

One possible explanation why we got many tubulin inhibitors as hits is that tubulin polymerization is a sensitive process that is easily disrupted by chemical agents. These agents target tubulin, the evolutionary conserved building unit of microtubule (Buiret, Combe et al. 2010). Most of the clinically available microtubuli inhibitors like taxol, vinorelbine, colchicine, vincristine and vinblastine are natural products obtained from different species indicating that tubulin has been selected as target at different evolutionary occasions (Hamel 1996). As spheroids contain high fraction of non-dividing cells, it was surprising to identify agents which are toxic to proliferating cells like the antimitotic microtubuli inhibitors. However, it has been reported that microtubuli inhibitors can be toxic to non-dividing cells (Leoni, Hamel et al. 2000). This toxicity can be due to the disorganization of the cytoskeleton caused by the tubulin agents, which will result in disturbances of cellular organization, intracellular transport and signal transduction (Baranello, Bertozzi et al. 2010).

4.5 IDENTIFICATION OF THASPINE AS A NOVEL DUAL TOPOISOMERASE INHIBITOR EFFECTIVE ON SPHEROIDS

Topoisomerase inhibitors are important agents in cancer therapy. Irinotecan and topotecan are topoisomerase I inhibitors (Pommier 2006) while doxorubicin and etoposide are topoisomerase II inhibitors (Baranello, Bertozzi et al. 2010). There are no dual topoisomerase inhibitors being in use in the clinic. Dual topoisomerase inhibitors have the benefit of targeting the two enzymes which are activated at different cell cycle time points. Additionally, they have the advantage of overcoming topoisomerase-dependent drug resistance which might occur in either of the enzymes. Using combination of specific inhibitors of topoisomerase I & II gave encouraging preclinical results, however clinical trials suffered from additive toxicities. Accordingly, single dual topoisomerase compounds might represent promising agents for cancer treatment (Stewart, Mistry et al. 2001).

Resistance is the major factor for cancer treatment failure. There are different forms of resistance found in solid tumors either in the cellular or the multicellular levels. One important mechanism of resistance to clinically used DNA damaging anticancer drugs is the expression of ABC transporters such as Pgp and MRP (Ling 1997). It is therefore interesting that thaspine cytotoxicity was marginally affected by such transporters. In

addition to being a top hit in the monolayer screen, we found that thaspine induced widespread apoptosis in HCT116 multicellular spheroids.

Thaspine induced apoptosis *in vivo*, and induced a short period of growth retardation of xenograft tumors *in vivo*. It is possible that thaspine can be developed into an effective drug for clinical use. Formulation work is required since the compound is lipophilic with XlogP value of 2.8. It is possible that a better formulation of the compound will generate more sustained growth retardations of tumors compared to those observed to date. Thaspine should perhaps also be combined with a cytostatic agent to inhibit tumor growth between treatment cycles as discussed above.

4.6 IDENTIFICATION OF CB21 AS A NOVEL IRON CHELATOR EFFECTIVE ON SPHEROIDS

CB21 showed an interesting profile of activity by being cytotoxic to HCT116 spheroids but not to non-proliferating immortalized hTERT-RPE1 cells. Our experiments showed that the compound works through depleting intracellular iron. Iron is essential for cell proliferation and viability (Le and Richardson 2002). Its presence in ribonucleotide reductase is vital for the enzyme activity and therefore necessary for DNA synthesis (Nordlund and Reichard 2006)This is evidenced by the occurrence of G_1-S arrest on iron depletion (Yu, Wong et al. 2006). Accordingly, cancer cells have a higher demand for iron because of their high proliferation rate compared to their normal counterparts (Yu, Wong et al. 2006). This fact can be exploited to use iron chelators as effective anticancer agents with acceptable therapeutic window.

Iron plays an important role in cellular energy production (Sariban-Sohraby, Magrath et al. 1983) and is required as a cofactor for oxygen-binding proteins (Le and Richardson 2002). About 50% of cellular ATP in cancer cells is produced by oxidative phosphorylation (Sariban-Sohraby, Magrath et al. 1983) . The iron chelator deferoxamine was found to inhibit the citric acid cycle enzymes aconitase, citrate synthase, isocitric dehydrogenase, and succinate dehydrogenase (Oexle, Gnaiger et al. 1999). Consequently, exposure to deferoxamine resulted in reduced oxygen consumption, decreased ATP production and increased glycolysis and thus increased glucose utilization (Oexle, Gnaiger et al. 1999). We found that CB21 reducted oxygen consumption both by direct measurements and by staining sectioned treated spheroids by pimonidazole as a hypoxia marker. One possible explanation for reduced oxygen consumption is the inhibition of mTOR observed after CB21 treatment. It has been shown that the iron chelator deferasirox inhibits mTOR signaling through the induction of the hypoxia induced gene REDD1 (also referred to RTP801) and its down-stream protein, tuberin (TSC2) (Ohyashiki, Kobayashi et al. 2009). The mTOR pathway is known to regulate the mitochondraial oxygen consumption (Schieke, Phillips et al. 2006) . However, the PI3K/mTOR inhibitor NVP-BEZ235 did not show cytotoxicity in the spheroids core cells, meaning that mTOR inhibition is not the only mechanism for CB21 cytotoxicity. Interestingly, CB21 lead to increased dependence on glucose revealed by slight increase in glucose uptake and enhanced drug-toxicity in glucose free medium. We observed death of core cells in spheroids when grown in glucose depleted

medium indicating the dependence of this subpopulation on glucose. Possibly, available glucose, in the core cells, which are already subjected to lower concentration than outer cells, is not enough to keep cells viable after the oxidative phosphorylation inhibition caused by CB21.

The ability of the iron chelator CB21 to inhibit cell proliferation was expected and can be explained by ribonucleotide reductase inhibition. In contrast, the toxicity to the non-proliferating cells subpopulation in spheriods was unexpected. CB21 induced pronounced autophagy both in HCT116 monolayers and spheroids. In contrast, autophagy was not as pronounced in immortalized hTERT-RPE1 cells. Autophagy is a self digestion process mediated by HIF-1 (Jiang, Semenza et al. 1996). During normal conditions, autophagy functions as a process by which damaged proteins and organelles accumulate in autophagosomes which fuse with endo/lysosomes for degradation (White, Karp et al. 2010). However, during conditions of nutrient limitation, autophagy is induced to catabolize degraded constituents for ATP production (Brahimi-Horn, Bellot et al. 2011). We did not observe decreases in cellular ATP levels after treatment with CB21, and it is possible that ATP production might have been compensated by autophagy. Then, we can understand that autophagy induction is a mechanism by which cells try to rescue themselves from an energy problem caused by CB21. It is worthwhile here to note that the mTOR inhibitors NVP-BEZ235 and rapamycin did not induce autophagy to a similar extent as CB21, indicating that mTOR inhibition is not the main mechanism by which CB21 toxicity occurs.

Inhibiting autophagy by treatment with 3-MA (3-methyl adenine), Bafilomycin A or by silencing Beclin/Atg6 caused increased toxicity by CB21. This observation is consistent with the hypothesis that autophagy is a rescue mechanism in CB21-treated cells. Recent studies showed that autophagy inhibitors potentiated the cytotoxic effect of anticancer agents (Livesey, Tang et al. 2009). For example, chloroquine, the antimalarial agent, which is an autophagy inhibitor as well, increased the cytotoxic effect of 5-FU in HT29 cell line (Sasaki, Tsuno et al. 2010). Another example, chloroquine, 3-MA and siRNA targeting Atg7 enhanced cell killing of Src family kinase (SFK) inhibitors (Wu, Chang et al. 2010). The strong induction of autophagy in response of CB21 raises the possibility that autophagy inhibition in combination with the iron chelator could be a powerful treatment for solid tumors. One may speculate that autophagy may be particularly important for the viability of cells in the hypoxic core of tumors, and that these cells are very sensitive to CB21 in the presence of autophagy inhibitors.

The main aim for using spheroids for drug screening is that such screen identifies compounds with high probability to be active *in vivo*. Indeed, CB21 showed promising activity when tested on a mice xenograft model bearing HCT116 tumors, the same cell line in which the original screen was performed.

Iron chelators are expected to induce very complex effects on cells. We observed both an induction of p53 and of HIF-1α. HIF-1α is known to be a central mediator for cell adaptation to hypoxia conditions (Jiang, Rue et al. 1996). In presence of oxygen, the regulatory subunits of HIF including HIF-1α are hydroxylated in two prolines by prolyl-hydroxylases (PHDs) leading to degradation of HIF through ubiquitination and

proteasomal degradation (Semenza 2007; Peyssonnaux, Nizet et al. 2008). In hypoxic conditions PHDs are inhibited leading to stabilization of the non-hydroxylated active form of HIF. Not only hypoxia causes inactivation of PHD but also iron depletion (Peyssonnaux, Nizet et al. 2008). This explains the upregulation of HIF-1α on treatment with CB21 inspite of the increase in normoxic area in sections of CB21 treated spheroids stained with the hypoxia marker pimonidazole. We do not at present understand the respective roles of the various responses to CB21, but believe that the main effects are inhibition of ribonucleotide reductase, induction of autophagy (due to an underlying metabolic alteration), and inhibition of oxygen consumption possibly due to mTOR inhibition**.**

CB21 was found to be more potent in cytotoxicity assays compared to other iron chelators, including deferoxamine. Though deferoxamine showed some antitumor activity in leukemia and neuroblastoma, its efficacy is limited by its poor membrane penetration. Triapine is considered one of the most studied iron chelators for antitumor activity and already entered clinical trials (Richardson 2002). Though showed some activity, it was not sufficiently effective and was limited by toxic side effects at a dose of 160 mg/m2/d (Yu, Wong et al. 2006). Whether CB21, alone or in combination with autophagy inhibitors, will be more effective is not known but it would be quite interesting to perform clinical trials.

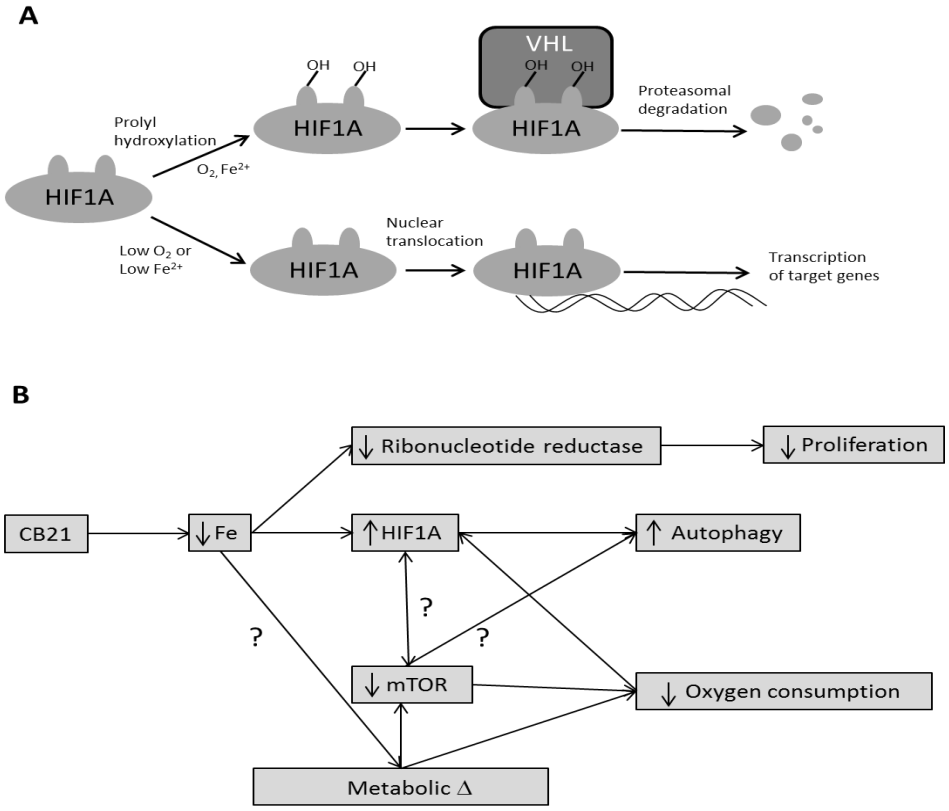

Figure (6). (A). Regulation of HIFA by oxygen and iron. (B). Proposed cellular effects of the iron chelator CB21.

4.7 SUMMARY

Several factors contribute for an efficient chemotherapeutic agent for treatment of solid tumors. Both pharmacokinetic and pharmacodynamic properties are determinant elements for a successful anticancer agent. We should also take in consideration the severe side effects of chemotherapy which limit the dosage and continuation of treatment. The new targeted anticancer agents provide a hope for a milder chemotherapy. However, the heterogeneous genomic nature of cancers, within the same tumor or between different tumors, provides and significant challenge for such agents. Accordingly, we believe that cytotoxic agents hitting vital targets in cancer cells would provide more efficiency in treatment of cancer, nevertheless, it is expected to be toxic to normal cells as well.

In the present study, we tried to optimize a cell based approach to identify cytotoxic agents against tumor masses grown as spheroids. This model allows the selection of penetrating cytotoxic agents against proliferating and hypoxic non-proliferating resistant cancer cells. In other words, compounds possessing favorable pharmacokinetic and pharmacodynamic characteristics. In order to exclude the identification of compounds toxic to normal cells, we set up spheroids from a normal human cell line as a counter screen. This system resulted in the identification of an iron chelator and antitubulin agents. The iron chelator indeed showed promising *in vivo* activity and was tolerated by mice.

Another important finding was the enrichment of the spheroid screen for lipophilic compounds. Thus, the physicochemical properties of an anticancer agent should be taken into consideration in drug design and not to be overlooked.

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