New preclinical strategies for characterization and development of anticancer drugs

HENNING KARLSSON
Abstract

Increased understanding of the molecular mechanisms underlying cancer development has shifted drug discovery towards target driven drug development the last decades, but the development of effective cancer drugs has been hampered by the lack of predictive preclinical models. 3-D cultures, considered to more accurately reflect solid tumors in vivo, have been proposed as one way to increase the predictability of clinical efficacy in cancer drug discovery and development.

The aims of this thesis were to improve preclinical models for cancer drug development, with focus on colorectal cancer (CRC) and use of multicellular tumor spheroids (MCTS), and also to mechanistically characterize some potentially new anticancer drugs (papers I – IV). The most important technical improvement was the development of direct measurement of green fluorescent protein (GFP) marked cells in spheroids, simplifying live collection of viability data and enabling high-throughput screening (HTS) in the MCTS model (paper I). In paper III and IV, the 3-D model was adapted to enable studies on the interaction between drugs and radiation. Two potentially new anticancer drugs, VLX50 and VLX60, were mechanistically characterized. VLX60, a novel copper containing thiosemicarbazone, induced reactive oxygen species (ROS) formation, was selectively active against BRAF mutated colon cancer cells and exhibited anticancer activity in vivo (paper II). Furthermore, two potentially new anticancer drugs were found suitable for further development for use in combination with radiation (papers III and IV). In paper III, synergy with radiation in spheroids compared to monolayer cultured colon cancer cells was shown with the novel iron-chelating inhibitor of oxidative phosphorylation, VLX600. In paper IV, the antiprotozoal drug nitazoxanide was shown to sensitize quiescent clonogenic colon cancer cells to radiation.

In conclusion, introduction of measurement of fluorescence of GFP marked cells in spheroids makes clinically relevant 3-D models feasible for HTS experiments and characterization of candidate drugs and radiosensitizers in early cancer drug discovery and development. VLX60 has several characteristics suitable for further development into a cancer drug, notably against BRAF mutated colorectal cancer cells. VLX600 and nitazoxanide show radiosensitizing properties making them promising for further development for use as cancer drugs in combination with radiation.

Keywords: colorectal cancer, spheroids, green fluorescent protein, VLX50, VLX60, VLX600, nitazoxanide, radiation, radiosensitizer

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To my family
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>13</td>
</tr>
<tr>
<td>Cancer</td>
<td>13</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>13</td>
</tr>
<tr>
<td>Anticancer drug discovery and development</td>
<td>15</td>
</tr>
<tr>
<td>History</td>
<td>15</td>
</tr>
<tr>
<td>Drug discovery today</td>
<td>15</td>
</tr>
<tr>
<td>Preclinical <em>in vitro</em> models</td>
<td>16</td>
</tr>
<tr>
<td>Cancer immunotherapy</td>
<td>18</td>
</tr>
<tr>
<td>Drug repositioning</td>
<td>18</td>
</tr>
<tr>
<td>Radiation and radiosensitization</td>
<td>19</td>
</tr>
<tr>
<td>History</td>
<td>19</td>
</tr>
<tr>
<td>Radiation effects</td>
<td>19</td>
</tr>
<tr>
<td>Importance of oxygen</td>
<td>20</td>
</tr>
<tr>
<td>Radiosensitization</td>
<td>20</td>
</tr>
<tr>
<td>Aims</td>
<td>22</td>
</tr>
<tr>
<td>Methods</td>
<td>23</td>
</tr>
<tr>
<td>Cell lines and patient tumor cells</td>
<td>23</td>
</tr>
<tr>
<td>Cell culture models</td>
<td>24</td>
</tr>
<tr>
<td>Monolayer cultured cells</td>
<td>24</td>
</tr>
<tr>
<td>Multicellular tumor spheroids</td>
<td>24</td>
</tr>
<tr>
<td>Drugs</td>
<td>25</td>
</tr>
<tr>
<td>Cell viability assays</td>
<td>25</td>
</tr>
<tr>
<td>Fluorometric microculture cytotoxicity assay</td>
<td>25</td>
</tr>
<tr>
<td>Green fluorescent protein assay</td>
<td>25</td>
</tr>
<tr>
<td>Clonogenic assay</td>
<td>26</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>26</td>
</tr>
<tr>
<td>Hypoxia analysis</td>
<td>27</td>
</tr>
<tr>
<td>Gene expression analysis</td>
<td>27</td>
</tr>
<tr>
<td>Oxidative stress assays</td>
<td>27</td>
</tr>
<tr>
<td>Cell cycle assay</td>
<td>28</td>
</tr>
<tr>
<td>Measurement of effects on the ubiquitin-proteasome system</td>
<td>28</td>
</tr>
<tr>
<td>Apoptosis assay</td>
<td>28</td>
</tr>
<tr>
<td><em>In vivo</em> experiments</td>
<td>28</td>
</tr>
<tr>
<td>Statistics</td>
<td>29</td>
</tr>
</tbody>
</table>
Results and Discussion .......................................................................................... 31

Loss of cancer drug activity in colon cancer HCT-116 cells during spheroid formation in a new 3-D spheroid cell culture system (Paper I). .............................................. 31

Mechanistic characterization of a copper containing thiosemicarbazone with potent antitumor activity (Paper II) ........................................................................ 33

A novel tumor spheroid model identifies selective enhancement of radiation by an inhibitor of oxidative phosphorylation (Paper III) .................. 37

Selective radiosensitization by nitazoxanide of quiescent clonogenic colon cancer tumor cells (Paper IV) ................................................................. 40

Summary .................................................................................................................. 46

Concluding remarks and future perspectives .......................................................... 47

Acknowledgments .................................................................................................. 50

References ............................................................................................................. 53
Abbreviations

2-D  Two-dimensional
3-D  Three-dimensional
5-FU  5-fluorouracil
AML  Acute myeloblastic leukemia
ANOVA  Analysis of variance
APC  Adenomatous polyposis coli
BRAF  Proto-oncogene that encodes the serine/threonine-protein kinase B-Raf (see also RAF)
CI  Confidence interval
CRC  Colorectal cancer
DHE  Dihydroethidium
DMSO  Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
DSB  Double-strand break
ECM  Extracellular matrix
EGFP  Enhanced green fluorescent protein
EGFR  Epidermal growth factor receptor
ER  Endoplasmic reticulum
ERK  Extracellular signal-regulated kinase = MAPK
FAP  Familial adenomatous polyposis
FDA  Fluorescein diacetate
FLR  Fluorescence
FMCA  Fluorometric microculture cytotoxicity assay
GFP  Green fluorescent protein
GFP assay  Green fluorescent protein assay (called FLR assay in paper I)
GSEA  Gene set enrichment analysis
GTPases  Family of enzymes that can hydrolyze guanosine triphosphate (GTP)
HNPCC  Hereditary non-polyposis colorectal cancer
HRP  Hypoxia-responsive promoter
HTS  High-throughput screening
IC_{50}  Concentration resulting in a SI (or SF)-value of 50%
IHC  Immunohistochemistry
IR  Ionizing radiation
Ki67: Protein named after the location of discovery, Kiel, and the clone number in the 96-well plate. Indicator of cell proliferation.

**KRAS**: Proto-oncogene (identified in Kirsten rat sarcoma virus) that encodes the GTPase protein KRAS (see also RAS).

**LET**: Linear energy transfer.

**MAPK**: Mitogen-activated protein kinase.

**MCTS**: Multicellular tumor spheroid.

**MEK**: MAPK/ERK kinase.

**N/A**: Not applicable or not available.

**NAC**: N-acetylcysteine.

**NCI**: National Cancer Institute in the US.

**NES**: Normalized enrichment score.

**NMRI**: Naval Medical Research Institute in the US.

**NMRI nu/nu**: Mice with a mutation in the forkhead box N1 (Foxn1) gene. This causes mice with a lack of body hair, thymic aplasia and a lack of B-cells (T-cells remain) making them ideal for xenograft experiments since transplanted tumor cells are not rejected.

**NTZ**: Nitazoxanide.

**OXPHOS**: Oxidative phosphorylation.

**p53**: Gene that encodes the tumor suppressor protein p53.

**PBMC**: Peripheral blood mononuclear cells.

**PBS**: Phosphate-buffered saline.

**PCPTC**: Primary cultures of patient tumor cells.

**PDX**: Patient derived xenograft.

**RAF**: Proto-oncogene serine/threonine-protein kinase (RAF is an acronym for rapidly accelerated fibrosarcoma) (see also BRAF).

**RAS**: Family of related proteins which are all GTPases (see also KRAS).

**RECIST**: Response evaluation criteria in solid tumors.

**RNA**: Ribonucleic acid.

**ROS**: Reactive oxygen species.

**SEM**: Standard error of the mean.

**SF**: Surviving fraction.

**SI**: Survival index.

**SPA**: Strong positive areas.

**SSB**: Single-strand break.

**TCP**: Tumor cell percentage.

**TCV**: Tumor cell volume.

**TME**: Tumor microenvironment.

**TV**: Tumor volume.

**ULA**: Ultra-low attachment.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPS</td>
<td>Ubiquitin-proteasome system</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WPA</td>
<td>Weak positive areas</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
Introduction

Cancer

Cancer is responsible for almost one third of deaths in Europe and puts a considerable burden on society [1]. It is a complex and heterogeneous group of diseases that develop as a multistep process transforming cells to acquire genomic mutations that enable them to become tumorigenic and ultimately malignant [2]. Despite the complexity on the gene-level, many mutated genes align in a limited number of molecular pathways frequently altered in cancer. Hallmark capabilities of malignant cells include self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis [3]. Also, deregulation of cellular energetics and avoidance of immune destruction have both been proposed as important cancer hallmarks. Furthermore, much research has been directed towards two processes that drive tumor progression, genome instability and tumor promoting inflammation [2, 3].

Today, chemotherapy is one of the three cornerstones, together with radiotherapy and surgery, upon which treatment of cancer is based. Although our understanding of the molecular mechanisms underlying cancer development has increased rapidly during the last decades and shifted drug discovery towards target driven drug development, the development of effective cancer medicines has been hampered by the lack of reliable predictive preclinical models. Only minor progress has been achieved in adult oncology over the past decades although recently cancer immunotherapy has shown promising results in several cancer types. Most drug treatment of cancer is still routinely administered mainly based on tumor type and stage [4-6]. Thus, three linked areas in need of development to improve drug treatment of cancer are discovery of new anticancer therapies, development of reliable predictive preclinical models and introduction in the clinic of predictors of tumor response.

Colorectal cancer

Epidemiology

Colorectal cancer (CRC) is a major cancer type in the western world and is the second most common cause of cancer-related death in Europe [1]. It has a
considerable impact on patients and healthcare systems in developed countries and despite the recent progress made in screening and in surgical, medical and radiation treatment in CRC, there are major unmet needs for improvements both in early and advanced disease [1].

Tumor biology
The vast majority of colorectal carcinomas are adenocarcinomas, most frequently originating from adenomatous polyps [7]. Colorectal carcinogenesis involves the stepwise accumulation of mutations in a number of oncogenes and tumor suppressor genes and mutations in the APC and KRAS genes are among the earliest events. Another important target is epidermal growth factor receptor (EGFR) since dependency on the EGFR pathway is prevalent in around 10% of CRC tumors and 20-30% of KRAS wild-type tumors [1, 8]. Although upregulated genes differ between tumor types, RAS/RAF/MEK/ERK is the main pathway upregulated when EGFR is activated [1, 9-11]. Loss of the tumor suppressor gene p53 is noted in the majority of colon cancers and this mutation supposedly occurs late in colorectal carcinogenesis since similar losses are infrequent in adenomas [12]. There are also hereditary forms of CRC, e.g. Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC), in which the associated genetic mutations can be found in a single or a few genes and give a lifetime CRC risk of 50% to over 90% in patients harboring these mutations [1, 7].

Treatment and management
The primary curative treatment for patients with CRC is surgery. Chemotherapy with combinations of a fluoropyrimidine, oxaliplatin and irinotecan clearly improves the outcome in advanced CRC and radiotherapy improves the outcome of locally advanced rectal cancer. Antibodies directed against vascular endothelial growth factor (VEGF) (bevacizumab), and EGFR (cetuximab, panitumumab) are routinely used in metastatic disease but predictive biomarkers for angiogenesis inhibitors are not available and the only validated biomarker used to predict therapeutic efficacy for anti-EGFR antibodies is RAS mutation status [1]. Also, important factors other than disease stage, tumor biology and drug efficacy that has to be taken into account in the treatment of CRC patients are patient performance status, drug toxicity and costs [1].

Prognosis
Tumor stage at the time of diagnosis is the single most important prognostic indicator of colorectal carcinoma with a 5-year survival rate of over 90% in patients with localized stage I CRC [1], declining to less than 10% in stage IV CRC [1, 7].
Anticancer drug discovery and development

History
The modern era of chemotherapy against cancer began in the 1940s, when a great deal of research was done on vesicant war gases [13]. Experience from the two World Wars led to the observation that in people exposed to mustard gas, both bone marrow and lymph nodes were markedly depleted [13]. Two pioneers in cancer drug development, Alfred Gilman and Louis Goodman, observed marked tumor regressions after administration of nitrogen mustard to mice bearing a transplanted lymphoid tumor. Marked regression was observed also in lymphoma patients and the use of nitrogen mustard for lymphomas spread rapidly after publication of the results in the mid-1940s [13, 14]. Also, research before and during World War II identified folic acid as important for bone marrow function and led to the development of antifolate treatment with methotrexate in children with leukemia [13].

As a result of these and other early findings, the Cancer Chemotherapy National Service Center in the United States (US) was developed in 1955 and a national program of screening of compounds in mice bearing transplantable neoplasms, mostly leukemia, started [13]. With the aim of changing the emphasis of drug discovery from leukemia to human solid tumors, the US NCI 60 human tumor cell line anticancer drug screen was developed in the 1980s and launched in 1990 [15, 16]. High throughput screening (HTS) of thousands of drugs annually on the 60 cell lines in vitro was made possible by multi-well plastic tissue-culture dishes and two-dimensional (2-D) cell culture models have dominated preclinical efforts to develop new cancer drugs [15, 17].

Drug discovery today
Our understanding of the molecular mechanisms underlying cancer development has increased rapidly during the last decades and target driven drug development of small molecules and monoclonal antibodies is now widely applied to discover new targets and to develop new anticancer drug candidates. The sequence of steps included in this target driven approach often starts with the identification of a gene or protein that is altered in cancer cells, subsequently followed by assessment of the biological activity of the target through the development of an appropriate assay, screening of compounds inhibiting the target, identification, re-testing and selection of inhibitors with adequate properties followed by in vivo evaluation of antitumor efficacy and toxicity in animal experiments and finally clinical phase I-III(IV) studies.
However, although HTS experiments, development of analogs and rational drug design has led to the discovery of many candidate agents, the development of effective cancer medicines has been hampered by the lack of predictive preclinical models [18, 19]. Whether they are hypothesis free or mechanism based, current strategies for drug discovery and development require HTS-compatible test systems [20, 21]. However, a considerable problem in cancer drug discovery is that promising preclinical activity often does not transmit to the clinic when the drug is given to cancer patients [22, 23]. Also, complexity and costs increase as standardized testing protocols go from cell free biochemical assays and 2-D cell cultures to experimental tumors in animals and finally clinical studies [21]. Therefore, the use of three-dimensional (3-D) cultures in drug discovery and development has been proposed as one way to increase the predictability of clinical efficacy of early candidate drugs [17, 20, 21, 24].

Preclinical in vitro models

Monolayer cell culture models
In cancer drug discovery, the initial screening of drugs that precedes preclinical animal studies are typically done on cells grown as a monolayer on a flat surface (2-D models) [24]. Although the continued development of this technique has resulted in the discovery of important drugs, major genotypic and phenotypic differences exist compared to tumors in vivo [24]. Since results from cell culture models often are crucial to the stop/go decisions made during drug development, models that more accurately reflect tumors in vivo would be important [16, 24-30].

Three-dimensional cell culture models
3-D cell cultures have been utilized in cancer drug discovery for half a century and are thought to more accurately reflect solid tumors in vivo with respect to drug penetration, cell interactions, hypoxia and gene expression than 2-D cultures [17, 21, 24, 31] (Fig. 1). Thus, using 3-D cultures is a way to get closer to the clinical situation when studying cancer drugs and radiotherapy. A number of different methods based on agitation, matrices and scaffolds, hanging drops, forced-floating and microfluidic cell culture platforms have been developed in efforts to produce more in vivo-like structures, make them less expensive, less labour intensive and also suitable for HTS [24, 28]. The 3-D cell culture used in this thesis is based on the multicellular tumor spheroid (MCTS) model [17, 21, 32, 33]. Since the 1970s various MCTS models have been introduced but major challenges to the use of MCTS models for HTS have been optimization of MCTS growth, data collection on intact spheroids and analysis
of data [17, 33-37]. Therefore, in this thesis, attempts were made to simplify cytotoxicity studies on spheroids.

Figure 1. Features of tumor cells growing as 2-D monolayers vs. 3-D spheroids. Compared to traditional 2-D monolayers (upper left panel), 3-D spheroids (upper right panel) accurately mimic many features of solid tumors in patients, e.g. cellular heterogeneity, internal structure (different cell layers), cell-cell signalling and physical interactions, extracellular matrix (ECM) deposition, ECM-cell signalling and physical interactions, growth kinetics, gene expression, gradients of oxygen, pH and nutrients, drug penetration and drug resistance [21, 24, 28, 32, 38, 39]. The lower panel (from Sant et al) illustrates that multicellular tumor spheroids (MCTS) resemble tumor nodules, micro-metastases or intervascular parts of large solid tumors with respect to many features [31].
Cancer immunotherapy

Our understanding of cellular immunology and tumor-host immune interactions has resulted in the recent success in cancer immunotherapy [6, 40]. Although evidence for tumor recognition by cells of the immune system was obtained from murine tumor experiments in the 1940s, it is in the last few years that the understanding and utilization of nonspecific immune stimulation for the treatment of metastatic cancer have made cancer immunotherapy successful in some tumor types [6, 40]. However, most data available on the interactions between the immune system and tumors has been obtained in conventional 2-D experiments and the mode of action of these new therapies is debated [25]. Development of predictive preclinical models for evaluation of cancer immunotherapies is dependent on models that reflect the interaction between cancer cells and the immune system and are therefore often more complex than those used for chemotherapeutics or targeted drugs that interfere with intracellular signalling. However, it is now recognized that the immune system is probably important also in the outcome of many conventional treatments, e.g. chemo- and radiotherapy [25]. Therefore, the routine inclusion of simplified and more clinically relevant preclinical in vitro models that better reflect the interaction between the tumor microenvironment and immune system would be of substantial value in the discovery and development of immunotherapy as well as chemo- and radiotherapy.

In vivo evaluation of cancer immunotherapies has been associated with difficulties. Firstly, the use of inappropriate animal models has confused the preclinical evaluation since model systems that use e.g. artificially introduced foreign antigens do not appear to be relevant to the clinical situation with bulky metastatic disease in patients [6]. In addition, evaluation of the effectiveness of cancer immunotherapies in patients has been hampered by the use of standard oncologic criteria, e.g. Response Evaluation Criteria in Solid Tumors (RECIST), that have not taken into consideration that lymphocyte infiltration and delayed responses can be radiologically misclassified as progressive disease [6, 41, 42]. Therefore, new guidelines, e.g. iRECIST, are emerging for evaluation of cancer immunotherapy [43].

Drug repositioning

Drug repositioning, or drug repurposing, is the identification of new clinical applications of well-known and approved drugs [44]. In general, the molecular targets of a compound are not specific and its off-target effects can be utilized for medical uses different from the ones originally considered [44, 45]. Since de novo anticancer drug discovery is both expensive and time consuming and most early clinical trials of novel anticancer drugs fail during development, drug repositioning in oncology offers a promising opportunity to faster and
cheaper drug development [45]. Importantly, since the toxicity and safety profile of an approved drug is already known, promising non-oncological drugs with new therapeutic applications in oncology can bypass early stages of drug development [45].

Radiation and radiosensitization

History

The era of radiotherapy against cancer dates back to the discovery of X-rays, in 1895, by Wilhelm Conrad Röntgen in Germany. Clinical radiotherapy was born less than 60 days after the discovery, when Emil Grubbé, in January 1896, treated an ulcerated breast cancer with X-rays in the United States (US) [6, 46]. Although little was known at that time about the mechanisms of X-ray action, discoveries in radiation physics, chemistry and biology have led to a more accurate, less harmful and more efficient radiotherapy [46].

Radiation effects

Ionizing radiation (IR) is the type of radiation that is commonly used in cancer treatment and is defined as “radiation that has sufficient energy to ionize molecules by displacing electrons from atoms” [6]. IR is either electromagnetic (e.g. X-rays and gamma rays) or consists of particles (e.g. protons, electrons, neutrons, alpha-particles or carbon ions). The dominant mode of action of IR with high linear energy transfer (LET), e.g. particles, is direct deposition of energy to a target molecule whereas about two thirds of the damage induced by the more commonly used low-LET electromagnetic radiation is due to indirect radiolysis of water molecules which generates free oxygen radicals that target molecules [6].

After a molecule has been hit and ionized, scavengers (e.g. glutathione) can protect the damaging effects by donation of a hydrogen atom to the radical (termed chemical repair) whereas oxygen molecules solidify the damage by peroxidation (termed the oxygen effect) [6]. Although IR can target proteins and lipids and affect membrane signalling, common belief today is that damage to DNA molecules is the critical mode of action of radiotherapy [6, 47]. Base lesions and damage to sugar moieties in the backbone of DNA can result in single-strand breaks (SSB) and double-strand breaks (DSB). IR can also induce DNA-DNA- and DNA-protein cross-links [6]. The number of DSBs is thought to be the major determinant of radiation induced damage [48].
In general, proliferating cells are more sensitive to radiation than non-dividing cells and certain phases of the cell cycle are more sensitive (e.g. G2). Checkpoint molecules, e.g. p53, are important to radiation response which can result in cell cycle arrest, DNA repair, apoptosis, mitotic catastrophe, autophagy or necrosis depending on the type and extent of the damage [6]. Fractionated radiotherapy utilizes the impaired DNA repair often present in tumor compared to normal tissue. Since normal tissue recover better than tumor tissue after each fraction, the difference in cell survival in the two tissues increases with the number of fractions. Also, reoxygenation is an important event that is utilized with fractionation, as discussed below.

Importance of oxygen

Elevated oxygen levels make tumors more sensitive to radiation. Although both normal tissue and tumor tissue vary in oxygenation, only tumors are thought to be sufficiently hypoxic to influence radiation induced killing [6, 49]. Since hypoxia has been associated with resistance to radiotherapy and non-proliferating stem-like cells have been proposed to be located in hypoxic parts of tumors, much effort has been devoted to achieve reoxygenation, e.g. by fractionation, hyperbaric oxygen treatment or drugs. However, except fractionation, which is standard use in radiotherapy, this approach has not been successful [6, 49-52]. In this thesis, spheroids were used in early preclinical evaluation of possible reoxygenating drugs since spheroids are thought to more accurately reflect solid tumors in vivo with respect to hypoxia, compared to the more commonly used monolayers.

Radiosensitization

The last decades, drugs and radiation have been administered concurrently, called chemoradiotherapy, with the aim to maximize the antitumor effects. When drugs act synergistically with radiation, i.e. the observed cytotoxic effect of drug + radiation is larger than the product of the two treatments alone, the combined effect is called radiosensitization and the drugs are called radiosensitizers. If local tumor control by radiation is combined with the systemic effect of drugs, the combined effect is called spatial additivity. For radiosensitization, concurrent administration of drugs and radiation is theoretically better than sequential administration whereas for spatial additivity concurrent and sequential treatment effects are theoretically more equal. Abscopal effects are increasingly discussed in the field of cancer immunotherapy, where irradiation of a tumor might release antigens that can trigger the immune system to recognize tumors also in other sites of the body [6].
The common view that the cytotoxic drugs used as radiosensitizers in the clinic today result in an improved therapeutic ratio, compared with radiotherapy alone, has been questioned recently. Although the combined treatment may provide improved tumor control, increased normal tissue toxicity is also often observed [53-57]. Thus, novel radiosensitizers that more selectively sensitize cancer cells to radiation would be of substantial value in oncology. In this thesis, a new spheroid model was used to identify radiosensitizers that selectively potentiate the effect of radiation in tumor spheroids compared to monolayers. Such radiosensitizers may potentially provide improved tumor control and less normal tissue toxicity compared to the chemotherapeutic drugs now used in combination with radiotherapy.
Aims

The overall aims of the thesis were to improve and develop models for pre-clinical development of cancer drugs, with focus on CRC, and to use these models to identify and investigate potentially new cancer drugs. The specific aims of the studies were to:

- Optimize MCTS growth, data collection and analysis of data in clinically relevant 3-D models (papers I-IV).
- Develop direct measurement of fluorescence of green fluorescent protein (GFP) marked cells in spheroids as a method to search for new anticancer drugs (papers I and II).
- Investigate the mechanisms responsible for the antitumor effects of the novel copper containing thiosemicarbazone VLX60 (paper II).
- Adapt the GFP based spheroid model in the search for new anticancer drugs suitable for use in combination with radiation (papers III and IV).
- Investigate the radiosensitizing effects of two candidate anticancer drugs, VLX600 and nitazoxanide (papers III and IV).
Methods

Cell lines and patient tumor cells

The colon cancer cell line HCT116 GFP was used to investigate the cytotoxic effects of candidate drugs (papers I-IV) and radiation (papers III and IV) throughout the thesis, since measurement of fluorescence of GFP labelled cells simplifies cytotoxicity measurements on spheroids and enables kinetic evaluation of viability during an ongoing experiment. HCT116 WT, without GFP, was used for comparison with HCT116 GFP and the hypoxia reporter cell line HCT116 HRP EGFP was used to study hypoxia in paper I. Cell lines originating from a monocytic cell line (U937-GTB), kidney cancer (ACHN, Caki-2 and 786-O), ovarian cancer (A2780), colon cancer (HCT116 WT) and normal colon (CCD 841 CoN) were used to investigate the cytotoxic effect of VLX50 and VLX60 and three pairs of colon cancer cell lines were used to study the relationship between drug effect and \textit{KRAS/BRAF} mutation status in paper II: HCT116 WT, HCT116 \textit{KRAS} (+/-), RKO, RKO \textit{BRAF} (+/-/-), DLD and DLD \textit{KRAS} (+/-). Also, in this paper, the colon cancer cell line HCT116 \textit{p53}/- was used to study the relationship between drug effect and \textit{p53}-status and the melanoma cell line MelJuSoUb-YFP (yellow fluorescent protein) was used to study effects on the ubiquitin-proteasome system (UPS). All cell lines were kept at -150°C, thawed prior to use and cultured at 37°C in a humidified incubator containing 5% CO₂ in medium supplemented with fetal calf serum, with change of medium as recommended.

Patient tumor samples were used in paper II and obtained from patients diagnosed with acute myeloblastic leukemia (AML), ovarian-, colorectal- or kidney cancer by bone marrow/blood sampling, surgery or diagnostic biopsy. Peripheral blood mononuclear cells (PBMC) and tumor cells from bone marrow/blood (AML) were collected by centrifugation and isolated by Ficoll-Paque and/or Percoll density gradient centrifugation. Samples from solid tumors were finely minced and digested with collagenase and tumor cells were isolated with Percoll density gradient centrifugation. Pathology reports in the patient files were used to retrieve \textit{KRAS-} and \textit{BRAF} mutation status of the colorectal cancer patient samples. The patient sampling was approved by the regional ethical committee, Uppsala University (file Dnr 2007/237).
Cell culture models

Monolayer cultured cells
Monolayers were used in papers I-IV. In most cases, a cell suspension with medium and cells from cell lines or patient tumor samples were seeded into 96- or 384-well plates and allowed to pre-incubate overnight. The next day, drug was added. Also, in some experiments, monolayer plates were prepared with drug before the cell suspension was added to the plates. With both methods, monolayers were incubated with drug at 37°C in 5% CO₂ and/or irradiated before analysis of mechanistic features or assessment of cell viability as specified in papers I-IV. Of note, 72 h is a well-established drug incubation time in our laboratory for assessment of drug induced cytotoxicity with the fluorometric microculture cytotoxicity assay (FMCA) and endpoints for mechanistic studies were therefore evaluated at earlier time points when the cells were still viable.

Multicellular tumor spheroids
In papers I and II, HCT116 GFP cells were seeded into NanoCulture® 96-well plates and the plates were incubated at 37°C in 5% CO₂. Drug exposure started when the spheroids had formed for 3 or 6 days. Half of the medium was changed to fresh medium at start of drug exposure (and 72 h after cell seeding for 6 days old spheroids). The spheroids were incubated with drug for 72 h in the IncuCyte fluorescence (FLR) system and then dissociated and washed in phosphate-buffered saline (PBS) before cell viability was assessed in the FMCA. The IncuCyte monitoring of the GFP labelled cells allows kinetic visual and functional quality control of spheroid growth through automated data acquisition of phase contrast and fluorescent images.

In papers III and IV, HCT116 GFP cells were seeded into Corning® black clear bottom ultra-low attachment (ULA) 384-well plates and the plates were placed on a Vari-Mix™ Platform Rocker in an angled position for 4 days and then fixed in a second angled position, as described previously [58], and incubated at 37°C in 5% CO₂. Drug exposure started when the spheroids had formed for 7 days and the plates were irradiated with an external low dose-rate gamma radiation source (GammaCell 40 Exactor) 4-6 h after drug was added. Following exposure to drug and/or radiation, cell viability was assessed in the FMCA, GFP- and clonogenic assays. Culture medium was not changed during the experiment.
Drugs

Standard cytotoxic drugs and the experimental drugs acriflavine and VLX50 were used in paper I. In paper II, the copper containing thiosemicarbazone VLX60 and its original compound VLX50 were chemically characterized and the impact of iron and copper on the activity of the two drugs were examined. In paper III, experimental compounds were compared to standard cytotoxic drugs and drugs previously considered to have a potential role as radiosensitizers and focus was on VLX600, a novel iron-chelating inhibitor of oxidative phosphorylation. The radiosensitizing properties of the anti-protozoal drug nitazoxanide were examined in paper IV. 5-fluorouracil (5-FU) is the backbone therapy for CRC chemotherapy regimens and is also in clinical use as a radiosensitizer. Therefore, since focus on the thesis was on CRC, 5-FU was included for comparison and as a reference compound in papers I-IV.

Cell viability assays

Fluorometric microculture cytotoxicity assay

The FMCA was used as the standard outcome measurement to evaluate cytotoxic effects of drugs and/or radiation in this thesis. It is based on conversion of fluorescein diacetate (FDA) to fluorescent fluorescein by viable cells with intact plasma membrane and has previously been described in detail [59]. The results are presented as survival index (SI) defined as fluorescence in experimental wells in percent of that in unexposed control wells, with fluorescence of blank wells subtracted.

Green fluorescent protein assay

The direct measurement of fluorescence of GFP marked cells simplifies cytotoxicity analysis, especially in the spheroid experiments, since dissociation of spheroids are not needed and viability data can be extracted continuously on intact spheroids during the experiment (Fig. 2). Object confluence in each well, based on fluorescence of GFP marked cells, was measured in the IncuCyte and was used as read out in both monolayer and spheroid experiments. In papers III and IV, mean spheroid fluorescence was measured in the Arrayscan. Both outcome measurements are presented as SI, defined as above. In papers III and IV, an AUTO SI was calculated in the GFP assay, and defined as the spheroid fluorescence in experimental wells in percent of that in the same wells immediately before addition of drug/radiation 7 days earlier.
Figure 2. Spheroids formed in NanoCulture® 96-well plate for 6 days and exposed to PBS (left) or 50 µM VLX60 (right) for 72 h. Direct measurement of GFP marked cells enables kinetic live analysis of cell viability continuously during spheroid formation and drug exposure since dissociation of spheroids is not needed. Scale bar 100 µm.

Clonogenic assay
The clonogenic assay is a more sensitive assay than the FMCA and GFP assay and shows effects on the clonogenicity of cells, i.e. the ability of single cells to form clones, after exposure to drugs and/or radiation. Therefore, induction of cell cycle arrest and cell senescence, which can be missed with total cell kill assays such as the FMCA and GFP assay, can be detected as well as direct cell kill mechanisms. Cells exposed to drug and/or radiation were transferred to fresh medium in 6-well plates, and then incubated at 37°C in 5% CO₂. Spheroids were dissociated into single cells before being transferred. After 10 days cells were fixed, stained, rinsed and dried as previously described [60] and the colonies that had formed were photographed and counted. Cell survival data is presented as surviving fraction (SF) defined as number of colonies in percent of that in unexposed control wells. Since plating efficiency was not assessed, this is a slightly modified definition of SF.

Immunohistochemistry
Assessment of protein expression was evaluated with immunohistochemistry (IHC). After monolayers and spheroids were established and exposed to drug and/or radiation, cells and spheroids were harvested, washed, embedded in paraffin, sectioned and stained with specific antibodies against each protein of interest as specified in papers I, III and IV, according to standard protocols. Immunohistochemical staining was quantified manually in a light microscope.
Hypoxia analysis

In paper I, cells from the hypoxia reporter cell line HCT116 HRP EGFP were seeded into NanoCulture® plates and monitored in the IncuCyte FLR system. HCT116 HRP EGFP contains cDNA coding for EGFP under the control of an artificial hypoxia-responsive promoter (HRP). Therefore, hypoxic cells will become fluorescent.

Gene expression analysis

Gene expression analysis was performed in papers I and II, were the procedure is described in detail. Briefly, RNA from cells grown as monolayers or spheroids were isolated using RNeasy Mini Kit and immediately stored at -70°C until further use. An ND 1000 spectrophotometer and a Bioanalyzer 2100 were used to measure RNA purity and quality respectively. Genome U133 Plus 2.0 Arrays were used to analyse gene expression, starting from 2 µg of total RNA. Gene expression ratios for spheroids vs. monolayer (paper I) or drug vs. vehicle exposed cells (paper II) were calculated to generate a list of regulated genes. Average fold change was used as rank metric to establish rank lists that were compared to a priori defined and curated gene sets in the Gene Set Enrichment Analysis (GSEA) software with the purpose to find out whether the a priori defined gene sets were significantly enriched toward the upper or lower end of the ranked lists [61]. Supplementary gene lists are published online (see links in papers I and II).

Oxidative stress assays

Two commercially available kits were used to study oxidative stress in paper II. The Cellomics® Oxidative Stress I kit provides a fluorescence readout of the superoxide indicator dihydroethidium (DHE) oxidation of reactive oxygen species (ROS) generation after cells have been exposed to drug. The assay was analysed in the Arrayscan. Rotenone was used as positive control. Induction of other ROS than superoxide was assessed with Oxidative Stress Detection Reagent (Green) from the Cellular ROS/Superoxide Detection Assay Kit, where fluorescence provides a readout of the probe which has a low sensitivity for superoxide and reacts directly with a wide range of reactive species, e.g. hydrogen peroxide, peroxynitrite, hydroxyl radicals, nitric oxide and peroxy radicals. The assay was analysed in the IncuCyte. Pyocyanin and N-acetylcysteine (NAC) were used as positive and negative controls respectively. Also, the effect of ROS on cell viability was evaluated in ROS inhibitor experiments in paper II, where measurement of drug induced cytotoxicity was performed.
with FMCA in monolayers as described above with 5 mM NAC added 30 min before drug.

Cell cycle assay
The commercially available Two-step cell cycle analysis kit was used to study effects on the cell cycle after drug exposure, in paper II. The assay was analysed in the NucleoCounter® NC-250™, which allows rapid quantification of DNA content in cells and enables determination of G0/G1, S and G2/M cell cycle phases.

Measurement of effects on the ubiquitin-proteasome system
In paper II, cells from the melanoma cell line MelJuSoUb-YFP were seeded into monolayer plates, exposed to drug and monitored in the IncuCyte FLR system. MelJuSoUb-YFP is transfected with a plasmid encoding the reporter substrate Ub\(^{G76V}\)-YFP. Therefore, proteasome inhibition, endoplasmic reticulum (ER) stress or other stress factors that compromise the UPS cause accumulation of the Ub\(^{G76V}\)-YFP substrate, which results in an increase in cellular fluorescence.

Apoptosis assay
In paper II, cells were exposed to the Essen CellPlayer™ Kinetic Caspase-3/7 Apoptosis reagent drug immediately before experimental drug exposure, for assessment of apoptosis. Cells were monitored in the IncuCyte FLR system. The assay provides a kinetic readout of apoptotic signalling based on activation of Caspase-3/7.

In vivo experiments
Tumor xenografts in mice were used to assess the effects of drug and/or radiation in papers II and IV. Female NMRI nu/nu mice were injected subcutaneously at the right flank with HCT116 GFP cells. When the majority of the tumors had reached 0.1 cm\(^3\) (day 0), the animals were randomly assigned into treatment groups. In paper II, experimental animals were administered a daily intraperitoneal dose of VLX60 for 28 days and compared to the control used
In paper IV, experimental animals were administered nitazoxanide or vehicle control twice daily for 3 days, starting at day 0 (only one administration was given on day 2). Drug was given by oral gavage and the animals were then irradiated with 6 Gy to the tumor 3-4 h after the last drug administration. A caliper was used to measure the length and width of each tumor twice weekly and tumor volume (TV) was calculated with the formula length (cm) x width (cm) x width (cm) x 0.44.

In paper IV, a new method to evaluate antitumor effects *in vivo* was introduced. Since xenograft tumors only partially consist of tumor cells alongside considerable parts with necrosis and also mice tissue, it was considered important to evaluate the fraction of HCT116 GFP cells in the tumors. The animals were euthanized at stop of study on day 28 and the tumors were then dissected, put into 4% buffered formaldehyde solution for subsequent histopathological examination, embedded in paraffin, sectioned, evaluated for hematoxylin and eosin staining and electronically scanned according to standard protocols. The electronically scanned images were analysed in Aperio ImageScope, using a slightly modified version of the software algorithm Positive Pixel Count V9. Strong positive areas (SPA) corresponded well to areas visually classified as HCT116 GFP cells in the microscope. A surrogate marker for the fraction of HCT116 GFP cells in the tumor is referred to as tumor cell percentage (TCP) and was calculated as following: SPA/(weak positive areas (WPA) + SPA) x 100. Tumor cell volume (TCV) was calculated as TCP x TV/100. Mice experiments had ethical approval by regional animal experimental ethics committee in Stockholm (North; approval numbers N37/15 and N88/15).

**Statistics**

For *in vitro* studies, the concentration resulting in a SI (or SF)-value of 50% (IC$_{50}$) was obtained using non-linear regression. Results are presented as means ± SEM or means ± 95% confidence interval (CI). Comparisons between mutated and parental cell lines in paper II were done with unpaired Student’s *t*-test. In accordance with independent Bliss interaction [62], the interaction between drug and radiation (papers III and IV) was characterized as follows: the mean SI (or SF) for wells treated with drug only (SI$_{d}$ or SF$_{d}$) and the mean SI (or SF) for wells irradiated only (SI$_{r}$ or SF$_{r}$) were used in the calculation of an expected combination SI (or SF) (SI$_{e}$ = SI$_{d}$ x SI$_{r}$ or SF$_{e}$ = SF$_{d}$ x SF$_{r}$). To get an interaction ratio for each experiment, the SI (or SF) actually observed for the combination (SI$_{o}$ or SF$_{o}$) was then divided by SI$_{e}$ (or SF$_{e}$). One-sample *t*-test was used to calculate interaction ratios (SI$_{o}$/SI$_{e}$ or SF$_{o}$/SF$_{e}$) different from 1. A SI$_{o}$/SI$_{e}$ (or SF$_{o}$/SF$_{e}$) ratio <1 was considered to indicate synergy.
For *in vivo* studies, tumor volumes (TVs) were recorded at set intervals during the experiment and results are presented as means ± SEM. Differences in TVs between the groups were calculated using repeated measures two-way ANOVA followed by Tukey’s multiple comparisons test. In paper IV, differences in TCP and TCV were calculated using one-way ANOVA followed by Tukey’s multiple comparisons test.

GraphPad Prism was used for result calculations and graphical presentation in both *in vitro* and *in vivo* experiments.
Results and Discussion

Loss of cancer drug activity in colon cancer HCT-116 cells during spheroid formation in a new 3-D spheroid cell culture system (Paper I)

A new 3-D spheroid cell culture system well suited for identification and characterization of cancer drugs for solid tumors

The routine inclusion of 3-D spheroid cell culture systems in early drug discovery and development has been proposed to increase the predictability of clinical efficacy of early candidate drugs but has been hurdled by the higher complexity, cost, labour intensiveness and time consumption associated with these models, compared with cell free or monolayer based models [17, 24, 28, 31, 33]. The aim of this paper was to investigate the properties of a new 3-D spheroid cell culture system and to optimize spheroid growth, data collection and analysis of data in this model.

The human colon cancer cell line HCT116 WT and HCT116 WT transfected with the green fluorescent protein (HCT116 GFP) reproducibly formed spheroids in NanoCulture® plates (Fig. 1). The spheroids were cultured for 3 or 6 days and morphology, hypoxia, protein expression and gene expression were assessed to characterize the spheroids. Six days old spheroids were larger, consisted of more cells and the cells were more tightly attached to each other, compared with those 3 days old. The development of a hypoxic center over time in the spheroids was clearly evident from experiments with the hypoxia reporter cell line HCT116 HRP EGFP. Immunohistochemistry showed that proliferation, as indicated by Ki67 expression, was high in monolayer cultured cells and in 3 days old spheroids but substantially lower in those 6 days old, indicating slower cell proliferation by time in spheroids. The stem cell marker CD44 was absent in monolayer cultured cells but increasingly expressed by time of spheroid age. Gene expression analysis indicated that genes associated with hypoxia and cell adhesion were up-regulated in 6 days old spheroids compared to cells grown as monolayers whereas genes involved in DNA replication and cell cycle were suppressed. All drugs tested were highly active in
monolayer cultures but gradually lost most of their activity in spheroids, indicating that especially the 6 days old spheroids represent a very resistant and challenging model for investigational drugs. Since the spheroids formed generally showed geno- and phenotypical properties well in correspondence with solid tumors in patients, they provide a model well suited for preclinical cancer drug discovery and development.

Direct measurement of fluorescence of GFP marked cells simplifies collection and analysis of viability data on spheroids

GFP-labelling did not seem to affect the cellular phenotype of HCT116 WT cells and fluorescence of GFP labelled cells as a read out was compared with the laborious and time consuming FDA based method when analyzing cytotoxicity. Closely mimicking the pattern seen using FDA, 3 days old spheroids were substantially more resistant than monolayer cells for all drugs tested. In general, lower SI-values were reported by the GFP assay compared to FDA, suggesting that an earlier sign of cell death based on decline of gene expression is measured in the GFP assay. The introduction of assessment of cytotoxicity based on fluorescence of GFP marked cells in spheroids, simplifies HTS with spheroids and shows interesting features of potential utility in the early phases of cancer drug development.
Mechanistic characterization of a copper containing thiosemicarbazone with potent antitumor activity (Paper II)

A novel copper containing thiosemicarbazone

A thiosemicarbazone, 3-(3-methoxypropyl)-1-[[pyridin-2-yl)methylidene]amino]thiourea (CD 02750, subsequently denoted VLX50), was recently identified as a hit in a phenotype based drug screen and found to be active against ovarian carcinoma cells both *in vitro* and *in vivo* [63]. Based on previous knowledge that copper complexes of mono- and bis-thiosemicarbazones have been associated with many fold enhanced antitumor activities [64-70], a copper complex (Copper (II) chloride complex of 3-(3-methoxypropyl)-1-[[pyridin-2-yl)methylidene]amino]thiourea) of VLX50, the copper complex subsequently denoted VLX60, (Fig. 3) was synthesized by Gunnar Westman (Vivolux AB) and was investigated regarding its antitumor and mechanistic properties both *in vitro* and *in vivo*.

![Suggested structural formulae of VLX50 (left) and VLX60 (right).](image)

**Figure 3.** Suggested structural formulae of VLX50 (left) and VLX60 (right).

Anticancer activity and mechanistic characterization of VLX60

The cytotoxic effect of VLX60 was investigated in six different cell lines of various origins as well as in primary cultures of patient tumor cells (PCPTC) and was compared with the effect of VLX50. Whereas all cell lines tested showed steep drops in cell viability well below 10 µM VLX60, the three kidney cancer cell lines and the ovarian cancer cell line were highly resistant to VLX50. VLX60 was several fold more active than VLX50 in both PCPTC and the non-proliferative and very resistant tumor spheroid model.

The colon cancer cell line HCT116 WT was the most sensitive cell line for both drugs and therefore colon cancer models able to associate the activity to the *KRAS* and *BRAF* mutation status, established to have predictive and/or prognostic importance in this tumor type, were included [71, 72]. Interestingly, the effect of VLX60 was significantly higher in RKO cells with mutant *BRAF*, compared to RKO cells with knock-out of mutant *BRAF*. Also, VLX60 showed a trend towards higher activity against *KRAS*- and *BRAF* mutated
compared with wild-type tumor cells from patients. The presence of a \textit{BRAF} mutation in colorectal cancer is associated with poor prognosis [72] and a drug that selectively targets \textit{BRAF} mutated colorectal cancer cells would be of considerable benefit in the clinic, which makes further evaluation of VLX60 in this condition of interest. Furthermore, the compromise of the UPS seen with VLX60, in contrast to VLX50, is interesting since proteasome inhibition has recently been suggested to represent a valuable target strategy in \textit{BRAF} mutated colorectal cancer [73].

Gene expression analysis was used to explore the mechanistic properties of VLX60 in drug exposed tumor cells and demonstrated that among 4,431 a priori defined gene sets, the most substantial finding was that genes associated with oxidative stress were enriched in cells exposed to VLX60 (Fig. 4). Assessment of the superoxide indicator DHE indicated that there was an increase in ROS formation by time (2-24 h) after exposure to VLX60, in contrast to VLX50. However, the use of the Oxidative Stress Detection Reagent demonstrated early (2 h) ROS formation other than superoxide also after exposure to VLX50 and pre-incubation with NAC reduced the cytotoxic activity of both VLX50 and VLX60, an observation that might be explained by the ability of VLX50 to scavenge copper in the cell culture and induce ROS production through formation of VLX60. Also, VLX50 can theoretically interact with intracellular copper and induce ROS formation at levels able to interfere with intracellular signalling but non-detectable by microarray analysis or assessment of DHE.
An excessive copper load through pharmacological insults might be a promising strategy to selectively eliminate cancer cells since cancer cells have been shown to exhibit higher copper levels than normal cells \cite{74, 75} and increased ROS production, leading to cell death, has previously been proposed as one of the primary ways in which redox active copper complexes exert their effects \cite{64, 70, 74, 76}. The results suggest that a very high and non-physiological extracellular concentration of copper would probably need to be available for VLX50 to accumulate copper intracellularly in a similar way as VLX60. No substantial differences between VLX50 and VLX60 were seen in the cell cycle analysis and both compounds were associated with apoptotic cell death.

**In vivo activity of VLX60**

The antitumor activity of VLX60 was evaluated in HCT116 GFP xenograft tumors in female NMRI nu/nu mice. Intraperitoneal administration of 0.6 mg/kg/day of VLX60 significantly inhibited tumor growth after 28 days compared to control, whereas the effect of 0.4 mg/kg/day was not significant (Fig.
5). VLX60 was well tolerated at both doses. This finding of a therapeutic window, *i.e.* a drug dose interval that is active against tumors while sparing normal tissue, is fundamental for drugs to be used for cancer treatment and further supports the development of VLX60 into an anticancer drug.

*Figure 5.* Antitumor activity of daily intraperitoneal treatment of VLX60 from day 0 to day 28 in HCT116 GFP xenograft tumors in mice. ** = \(P = 0.0035\) for VLX60 0.6 mg/kg vs. control at day 28. × = \(P = 0.0968\) for VLX60 0.4 mg/kg vs. control at day 28. See paper II for details.
A novel tumor spheroid model identifies selective enhancement of radiation by an inhibitor of oxidative phosphorylation (Paper III)

Direct measurement of fluorescence of GFP marked cells in spheroids as a feasible method in the search for novel radiosensitizers

A major problem in the search for novel radiosensitizers is to study the interplay between drugs and radiation in clinically relevant high-throughput models. Therefore, in this study, the aim was to introduce a clinically relevant high-throughput preclinical model well suited for investigation of the interaction between drugs and radiation. Since cell lines grown as spheroids are thought to more closely mimic solid tumors in vivo with respect to drug penetration, hypoxia/necrosis, metabolism, stem cell characteristics, proliferation, cell interaction and gene expression compared to monolayer cultures of human cell lines [21, 24, 28, 32, 38, 39], the use of spheroid models is reasonably a way to better reflect the clinical situation when studying radiosensitizers.

Homogenous and equally formed spheroids were formed when HCT116 GFP cells were cultured as spheroids in Corning® Ultra Low Attachment (ULA) 384-well plates, as previously described [58]. FMCA and the GFP assay were used as outcome measurements of total cell kill and the results were then further explored in the clonogenic assay. Radiation at 6 Gy had very little effect on cell survival in spheroids in total cell kill assays (SI 92 % and 93 % in the GFP assay and FMCA, respectively). However, the clonogenicity of cells from spheroids was clearly affected by radiation only; SF 36.1 ± 5.16 % and 8.13 ± 1.94 % (mean ± SEM) at 4 and 6 Gy, respectively. Results retrieved with the new molecule VLX600 (see below) were qualitatively similar with both outcome measurements and argue for the use of the faster and less labour intense total cell kill as a simpler read out, although the slower and more labour intense clonogenic assay probably is a more sensitive assay for identification of induction of cell cycle arrest and cell senescence. Thus, total cell kill assays might miss marginally active drugs that would be identified as hits in the clonogenic assay, which was seen with 5-FU in this paper, but could be used in HTS experiments to sort out the most promising radiosensitizers for further evaluation. GFP and FMCA readouts in total cell kill assays in spheroids were qualitatively similar. However, the GFP assay allows kinetic evaluation throughout the experiment and is both faster and less laborious than the FMCA.
Identification of selective enhancement of radiation by VLX600

VLX600, a novel iron-chelating inhibitor of oxidative phosphorylation [77, 78], was the only drug that showed consistent and significant synergistic effects with radiation in both the GFP assay and FMCA in spheroids when compared to experimental drugs, known radiosensitizers and cytotoxic drugs clinically used in combination with radiotherapy. This effect was specific to spheroids and could not be observed in monolayer cell cultures (Table 1). Immunohistochemistry was used for the assessment of DNA DSBs, as judged by gamma-H2AX expression, in spheroids after exposure to drug and/or radiation. Expression of gamma-H2AX increased in the hypoxic, centrally located, parts of spheroids after exposure to the combination of 3 µM VLX600 + 6 Gy compared to either treatment alone. This finding, the selective enhancement of radiation in spheroids, compared to monolayer cultured cells, and the fact that VLX600 has been shown to decrease oxygen consumption and reduce the hypoxic fraction of spheroids through inhibition of oxidative phosphorylation [77, 78], strengthens the hypothesis that one of the major ways in which VLX600 exerts its radiosensitization effect is through reduced tumor hypoxia. Thus, VLX600 shows interesting characteristics suitable for further development into a novel radiosensitizer. Notably, VLX600 is now in phase I clinical development in solid tumors (ClinicalTrials.gov Identifier: NCT02222363).
Table 1. Interaction ratios for drug and radiation in HCT116 GFP cells cultured as spheroids or monolayers as described in Methods and then irradiated (6 Gy) 4-6 h after addition of drug. Data are presented as the mean interaction ratio (SIo/SIe) for 3-7 individual experiments. Duplicate wells were used for each drug concentration. One-sample t-test was used to calculate interaction ratios different from 1. SIo/SIe ratios <1 are considered to indicate synergy and are shown in bold whereas ratios >1 indicate subadditive or antagonistic interactions. A two-tailed p-value of <0.05 was used to indicate interaction ratios significantly different from 1. In spheroid experiments, drug concentrations for all drugs except salinomycin ranged 100-0.5 µM with 2-fold dilution steps. Drug concentrations for salinomycin ranged 50-0.25 µM. In monolayer experiments, drug concentrations for 5-FU, oxaliplatin, deferoxamine, tirapazamine and VLX50 ranged 100-0.5 µM, for salinomycin and VLX600 3-0.015 µM and for ciclopirox and VLX60 10-0.05 µM with 2-fold dilution steps. * = P = <0.05. N/A, SIₜ ≤25 %.

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<td>Lowest conc.</td>
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Selective radiosensitization by nitazoxanide of quiescent clonogenic colon cancer tumor cells (Paper IV)

Nitazoxanide selectively radiosensitizes quiescent clonogenic colon cancer tumor cells

Nitazoxanide, formally known as 2-(acetyloxy)-N-(5-nitro-2-thiazolyl) benzamide NTZ, is a non-toxic, antiprotozoal drug approved by the Food and Drug Administration in the US, with several characteristics theoretically suitable for selective radiosensitization of solid tumors [79-81]. It was recently shown to be selectively active against quiescent and glucose-deprived tumor cells and exposure of colon cancer cells to nitazoxanide resulted in oxidative phosphorylation (OXPHOS)-inhibition and reduced hypoxia in spheroids [58]. Therefore, with the hypothesis that nitazoxanide would enhance the number of DNA double-strand breaks (DSBs) caused by radiation induced formation of free oxygen radicals in hypoxic tumors, the aim of this study was to explore the theoretically beneficial radiosensitization effect of nitazoxanide in the clinically relevant models described in paper III.

As previously shown [58], total cell kill assays in monolayer- and spheroid cultures confirmed that nitazoxanide is more selectively active against spheroids (IC₅₀ 1.44 µM and 2.41 in the GFP assay and FMCA, respectively), compared to monolayers (IC₅₀ 5.35 µM in the FMCA). In monolayer cultures, radiation was modestly active with a higher cell survival after 7 days compared to 3 days, indicating that the radiation doses used in this experiment (2, 4 and 6 Gy) induce transient inhibition of cell growth rather than cell death. Unexpectedly, in monolayer experiments nitazoxanide seemingly protected the cells from the effect of radiation after 7 days, but not after 3 days, which might be explained by the selective effect of nitazoxanide against the quiescent and glucose-deprived cells in wells not irradiated due to faster cell growth in these wells secondary to lack of inhibitory radiation. This would theoretically cause less toxicity toward normal cells, which are exposed to normal glucose levels, and, thus, be clinically beneficial.

Nitazoxanide showed no statistically significant synergistic interaction with radiation in the total cell kill assays for monolayer- or spheroid cultured cells. However, the total cell kill assays might miss effects secondary to cell cycle arrest and cell senescence as well as in small subpopulations of cells. Importantly, when the nitazoxanide radiation interaction was investigated in the clonogenic assay in spheroids, nitazoxanide clearly interacted synergistically with radiation in a radiation dose-dependent manner (Fig. 6). In contrast, there was no synergy in cells cultured as monolayers in the clonogenic assay (Fig.
6), as expected from the known specific effect against spheroids. There was no effect of nitazoxanide alone in the clonogenic assay in spheroids, which was expected from the short incubation time in this assay [58].

![Figure 6. Clonogenic assay.](image)

IHC for assessment of DSBs, as judged by the IHC gamma-H2AX expression, showed that nitazoxanide and radiation resulted in higher gamma-H2AX expression than after each exposure alone (Fig. 7). The mechanisms responsible for the induction of DSBs in spheroids by nitazoxanide itself were beyond the scope of this study, but is not unexpected since nitazoxanide can induce cell growth inhibition through formation of free radicals [80, 82]. The hypothesis that reduced hypoxia and formation of free radicals are important mechanisms behind the radiosensitization effect of nitazoxanide was strengthened by the
increase in DSBs in centrally located, quiescent and hypoxic, parts of spheroids exposed to the combination of nitazoxanide and radiation compared to spheroids exposed to nitazoxanide or radiation only, and is important since clonogenic tumor driving stem cells are thought to be located in quiescent and hypoxic parts of tumors [51, 83-87].

<table>
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<tr>
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<th>0 Gy</th>
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<td>DMSO</td>
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<tr>
<td>Nitazoxanide 3 μM</td>
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<td><img src="image" alt="Nitazoxanide 3 μM 6 Gy" /></td>
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<tr>
<td>Nitazoxanide 6.5 μM</td>
<td><img src="image" alt="Nitazoxanide 6.5 μM 0 Gy" /></td>
<td><img src="image" alt="Nitazoxanide 6.5 μM 6 Gy" /></td>
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*Figure 7.* Immunohistochemical expression of gamma-H2AX in HCT116 GFP cells cultured as spheroids for 7 days, then irradiated (6 Gy) 4-6 h after addition of drug and harvested 24 h later.
**In vivo activity of nitazoxanide**

When tested in HCT116 GFP xenograft tumors in mice, combination treatment with nitazoxanide + radiation resulted in no further inhibition of tumor growth compared to radiation only, whereas animals treated with nitazoxanide or radiation individually both developed smaller tumors compared to vehicle control. However, when the tumor xenografts were examined histopathologically, only nitazoxanide + radiation showed a TCP significantly lower compared to control (Figs. 8 and 9), indicative of a selective radiosensitizing effect of nitazoxanide against the tumor driving HCT116 GFP mass of the tumor. This finding is compatible with a synergistic interaction between nitazoxanide and radiation only in quiescent clonogenic cells and might necessitate serial orthotopic transplantation of tumors to provide proof of principle evidence of the long term radiosensitization effects of nitazoxanide [87].
Figure 8. Upper panel| Tumor cell percentage (TCP) for HCT116 GFP xenograft tumors, exposed to radiation and/or nitazoxanide as indicated, and defined as described in Methods. Lower panel| Differences in tumor cell percentage (TCP) were calculated using one-way ANOVA followed by Tukey’s multiple comparisons test in GraphPad Prism. A p-value of <0.05 was used to indicate statistical significance in all experiments. ** = P = <0.01. ns = not statistically significant.

<table>
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<th>TCP</th>
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<td>Control (vehicle) vs. Control (vehicle) + 6 Gy</td>
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<tr>
<td>Control (vehicle) vs. nitazoxanide</td>
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<td>Control (vehicle) vs. nitazoxanide + 6 Gy</td>
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<td>Control (vehicle) + 6 Gy vs. nitazoxanide</td>
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<tr>
<td>Control (vehicle) + 6 Gy vs. nitazoxanide + 6 Gy</td>
<td>ns</td>
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<tr>
<td>Nitazoxanide vs. nitazoxanide + 6 Gy</td>
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Figure 9. HCT116 GFP xenograft tumors embedded in paraffin, sectioned, evaluated for hematoxylin and eosin staining and scanned. The analysis algorithm “Positive Pixel Count v9” in Aperio ImageScope [v12.3.2.8013] was used to calculate TCP. A typical example from the control group is shown. The dark red rim of cells in the right panel corresponds to viable HCT116 GFP cells. More central parts consist of pyknotic HCT116 GFP cells, necrosis and mice tissue.

<table>
<thead>
<tr>
<th>Control</th>
<th>Hematoxylin and Eosin</th>
<th>Positive Pixel Count v9</th>
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Summary

- Collection and analysis of viability data on spheroids in a new 3-D spheroid cell culture system, well suited for preclinical cancer drug discovery and development, were simplified through direct measurement of fluorescence of GFP marked cells.
- The spheroids formed showed geno- and phenotypical properties well in correspondence with solid tumors in patients and represent a very resistant and challenging model for investigational drugs.
- A novel copper containing thiosemicarbazone, VLX60, was synthesized and characterized, and showed promising antitumor activity both in vitro and in vivo.
- Direct measurement of fluorescence of GFP marked cells in spheroids was found to be a feasible method in the search for new radiosensitizing drugs.
- A potential new radiosensitizer, VLX600, was found to selectively enhance the effect of radiation in spheroids but not in monolayer cultured cells.
- Nitazoxanide was tested in vitro and in vivo and selectively radiosensitized quiescent clonogenic colon cancer tumor cells.
In this thesis, one important aim was to develop clinically relevant preclinical models to be utilized in drug discovery and development. Although the common use during the last decades of 2-D monolayer cell line cultures have provided a wealth of information about tumor biology and identified active anti-cancer drugs, the low predictive value of drug effect in these 2-D based assays has resulted in a >90% failure rate of candidate drugs in clinical trials [31, 88]. Therefore, cancer drug development is in dire need of preclinical models with a considerable higher predictive value that can translate basic laboratory findings into clinically relevant results and sort out drugs with a higher probability of success in clinical trials. Larger panels of cell lines (>1000) have been suggested as a way forward to better capture genetic cancer heterogeneity, but is not a realistic option for most laboratories [16]. Also, although driver mutations are often retained in cell lines when compared to their primary tumor origin, transcriptomic changes are substantial when selection for rapid growth and extended cell culture precedes the establishment of a new cell line. It has been suggested that cell lines from different origins are often phenotypically more similar to each other than to their tissues of origin [16, 89]. Furthermore, the interindividual tumor heterogeneity and intratumor genetic heterogeneity are not well represented in cell line based monolayer cultures.

Although no in vitro model will be able to fully replace or mimic the complexity that exists in vivo, improvements in cell culture growth, data collection and analysis of data from clinically relevant in vitro models would be of considerable value in cancer drug discovery and development. In comparison with traditional 2-D monolayers, 3-D spheroids accurately mimic many features of solid tumors in patients (Fig. 1) [21, 24, 28, 32, 38, 39]. Three-dimensional cultures are now becoming increasingly utilized but low cost, large scale amenability, reproducibility, fastness and automatization are fundamental for routine inclusion of these models in drug discovery and development [17, 24, 28, 31, 33].

A cell line cultured as 2-D monolayer normally represents only a rapidly proliferating subpopulation of its original tumor, whereas the same cell line cultured as 3-D spheroids better reflects tumor architecture with cell heterogeneity, including both proliferating cells and non-proliferating cells with more stem like properties [21, 24, 28, 32] (paper I). Therefore, with the use of 3-D
spheroids, cell line dependency and the need to use numerous cell lines to reflect tumor heterogeneity are less pronounced in the search for novel drugs or combination treatments that are effective against tumors in the clinic, including the important tumor driving stem cells. To further develop this model, 3-D co-cultures with tumor cells, fibroblasts, endothelial cells, immune cells etc. have been used with the aim to better reflect the tumor microenvironment (TME) [25, 26, 28, 90-97]. Such more complex models have been proposed to better evaluate and characterise new therapies, especially cancer immunotherapy, in vitro but are more laborious and time dependent.

Due to technical advancement during the last decades, spheroids can now easily be reproducibly formed at high speed and low cost in large scale. With the use of simplified assessment of drug response, e.g. the direct measurement of fluorescence of GFP marked cells used in this thesis (papers I-IV), spheroids are well-suited for automatization in HTS experiments. However, there is a substantial number of outcome measures available for the in vitro evaluation of drug effect against spheroids based on morphology, size, growth, gene expression, protein expression, cellular organisation, cell viability, drug penetration etc. [98] and the future will show which of these that have the greatest potential for clinically relevant applications.

Interindividual tumor heterogeneity is better represented by primary cultures of tumor cells from patients than cell lines cultured as monolayers or spheroids or used in xenografts in vivo. However, since much of the complexity and important characteristics of solid tumors are lost when patient tumor samples are used as monolayers, there has been an interest in developing patient derived xenograft (PDX) models over the last decade. These in vivo models are now becoming an integral part of many drug development programs and can also be used for personalized cancer medicine and assist in clinical trial designs but cost, take rate, time to model generation, issues concerning human stroma and immune-related elements etc. are hurdles that have to be overcome before PDX models can be seen as standard in drug development [99]. In early drug discovery and development, PDX models need to be preceded by HTS amenable in vitro models to avoid cost, legal and ethical concerns. Therefore, using patient tumor samples to establish spheroids in vitro, co-cultured with e.g. fibroblasts, endothelial cells, immune cells etc., might be a promising strategy in future drug development and personalized cancer medicine. Importantly, such assays will rely on standardization of all procedures from tumor sampling and handling to culturing conditions and data analysis to ensure extraction of clinically relevant results.

Most drugs are more active in monolayers compared to spheroids (papers I-III). However, since tumor driving stem like cells might be important drug
targets in quiescent parts of tumors, the use of spheroids will probably facilitate identification of novel drugs and combination treatments with the ability to effectively and specifically target these tumor driving parts. VLX600 and nitazoxanide have both been shown to target quiescent parts of spheroids and the enhanced effect of nitazoxanide and other drugs in spheroids compared to monolayers have been shown previously [58, 100]. Also, selective radiosensitization with other compounds, in 3-D cell culture models compared to monolayers, has been demonstrated earlier [101]. In papers III and IV, it was demonstrated that the new 3-D spheroid models used in this thesis can be used to identify radiosensitizers with ability to selectively target spheroids (VLX600) or quiescent clonogenic cancer cells (nitazoxanide) compared to proliferating cells cultured as monolayers. However, to validate these findings proof of principle has to be demonstrated in future in vivo experiments.

Finally, further development and standardization of more clinically relevant models, outcome measures and imaging techniques in preclinical in vitro and in vivo experiments are clearly needed before basic laboratory findings more reliably can be translated to the clinic. Future will tell which of the new and emerging techniques that will prove efficient and reliable enough to be widely applied in drug discovery and development.
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References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)