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Drug Repositioning for Cancer Treatment

Novel Candidate Identification Strategies

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Abstract

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Regardless of the enormous investments in cancer research and drug development, the proportion of approved drugs in oncology is low compared to other indications, and new avenues are needed. One attractive approach in this regard is drug repositioning where new uses outside the scope of the original medical indications for existing drugs are identified. It offers the advantages of reduced development risks, time and cost over de novo drug discovery pathways.

The main focus of this thesis was to explore and employ different strategies to identify repurposable drug candidates for treatment of cancer. Aiming for this, in the first project we followed a bioinformatics approach to evaluate PDE3A as a drug target and biomarker. We showed that subgroups of tumors, within many different cancer types, overexpress PDE3A (mRNA and protein) and that PDE3A can predict sensitivity to the clinically tested phosphodiesterase inhibitors zardaverine and quazinone (Paper I). In the second project, a novel automated image based microscopy assay was developed and used for detection of apoptotic cells. In a screen the method was successfully used to identify apoptosis inducing compounds. Two of these apoptosis inducers were found to have repurposing potential (Paper II). Moreover, high-throughput combination screening was performed using different cell models. In paper III, monolayer cell cultures were used as cell model to search for combination partners for the anti-parasitic compound mebendazole (a repurposing candidate). As a result, the antipsychotic drug thioridazine was found to have synergistic effect when combined with mebendazole. Finally, a novel drug-combination platform for three-dimensional cell culture based screening, in 384 well formats, was developed. This assay was used to search for combination partners to the anti-parasitic compound nitazoxanide (a repurposing candidate), which was previously reported to specifically target quiescent cancer cells. The screen identified the antifungal agent ketoconazole as selectively toxic to hypoxic and nutrient deprived cancer cells when combined with nitazoxanide (Paper IV). Thus, we have developed/explored several methodological approaches and identified drugs that potentially can be repurposed for treatment of cancer.

Keywords: cancer treatment, drug repositioning, Phosphodiesterase 3A (PDE3A), apoptosis, combination screening

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*To the pillars of my strength,
my parents and my loving husband*

List of Papers

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

- I **Nazir M**, Senkowski W, Nyberg F, Blom K, Edqvist PH, Andersson C, Gustafsson MG, Nygren P, Larsson R and Fryknäs M, (2017) Targeting tumor cells based on Phosphodiesterase expression (PDE3A). *Experimental Cell Research*, 361(2):308-315
- II Aftab O, **Nazir M**, Fryknäs M, Hammerling U, Larsson R and Gustafsson MG, (2014) Label free high throughput screening for apoptosis inducing chemicals using time-lapse microscopy signal processing. *Apoptosis*,19(9):1411-8
- III **Nazir M**, Senkowski W, Gustafsson MG, Nygren P, Larsson R and Fryknäs M, (2017) Drug combination screening for Mebendazole for the treatment of colorectal cancer. *Manuscript*
- IV Senkowski W, **Nazir M**, Gustafsson MG, Nygren P, Larsson R and Fryknäs M, (2017) Drug combination screening in multicellular tumor spheroids identifies synthetic lethality in quiescent cancer cells. *Manuscript*

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Abbreviations

2D	Two-Dimensional
3D	Three-Dimensional
AIDS	Acquired Immunodeficiency Syndrome
ALK	Anaplastic Lymphoma Kinase
ATP	Adenosine Triphosphate
BRAF	v-Raf murine sarcoma viral oncogene homolog B
CAR	Chimeric Antigen Receptor
CML	Chronic Myeloid Leukemia
CNS	Central Nervous System
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EML4	Echinoderm Microtubule-associated protein-like 4
FMCA	Fluorometric Microculture Cytotoxicity Assay
GFP	Green Fluorescent Protein
GI tract	Gastrointestinal tract
GIST	Gastrointestinal Stromal Tumors
HT	High Throughput
IF	Immunofluorescence
IH	Immunohistochemical
KIT	Tyrosine Kinase Receptor
LFAD	Label Free Apoptosis Detector
LINCS	Library of Integrated Network-based Cellular Signatures
MCTS	Multicellular Tumor Spheroids
MDR	Multiple Drug Resistance
MET	Mesenchymal to Epithelial Transition
mRNA	messenger Ribonucleic Acid
mTOR	mechanistic Target of Rapamycin
NSCLC	Non-Small Cell Lung Carcinoma
OXPPOS	Oxidative phosphorylation
PCM	Phase Contrast Microscopy
PD	Programmed Death
PDE	Phosphodiesterase
PDE3A	Phosphodiesterase 3A
PDGFR	Platelet Derived Growth Factor Receptor
PNET	Pancreatic Neuroendocrine Tumors
Q-MCTS	Quiescent Multicellular Tumor Spheroids

SD	Standard Deviation
SEM	Standard Error of the Mean
SUI	Stress Urinary Incontinence
TSGs	Tumor Suppressor Genes
VEGFR	Vascular Endothelial Growth Factor Receptor

1. Introduction

1.1 Cancer

The second leading cause of human death is cancer [1]. According to world cancer report 2014, there were about 14 million new cancer cases and 8.2 million cancer related death cases reported in 2012 that are expected to be around 11.5 million until 2030 [2].

Cancer progression is multistep process where different molecular events lead to abnormal properties of cells. According to Hanahan and Weinberg, almost all human cancers share six crucial changes that result in malignancy. They are described as self-sufficiency in growth signals, insensitivity to growth inhibitory signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evasion of apoptosis [3]. These hallmarks of cancer were subsequently updated with two additional alterations; reprogramming of energy metabolism and avoiding immune destruction [4]. Genetic instability of cancer cells and inflammation by immune cells contribute towards acquiring these above mentioned changes [4].

1.2 Treatment of cancer

Several different treatment strategies are currently in use or under development, and these approaches can be grouped in five general categories; surgery, radiation, chemotherapy, targeted and immunotherapy [5].

Surgery is the most common treatment method for solid tumors. Surgery is often the best treatment option when the tumor is small and without spread to lymph nodes, and it is the preferred method just to relieve pain even if complete cure is not possible. Surgery is often combined with radio or chemotherapy to get maximum effect. When surgery is not feasible, for example in many lung cancer cases, radiation therapy can be used as an alternative. Radio therapy can be used before surgery to shrink the tumor or after surgery to eliminate remaining cancer cells.

Ionizing radiation damages genetic material of cancer cells that effects their division and proliferation resulting in cell death. Radiotherapy can be used alone for treatment of some early stage cancers e.g. some skin cancers, cervical and head and neck carcinomas. It is performed in combination with other modalities for treatment of breast carcinomas, advanced cervical carci-

nomas, lymphomas, CNS tumors, pediatric tumors and many more types of cancers [6].

The above mentioned standard cancer therapies have the potential to treat cancer efficiently but there is still need to prevent metastasis and to eliminate disseminated tumor cells. Cancer immunotherapy is being used specifically to target these problems. Recent developments in immunotherapy aim to induce continuing tumor antigen specific immune response using for example vaccines [7]. One type of immune therapy involves genetic modification of the patients own T cells to target tumors through the expression of a chimeric antigen receptor (CAR). This treatment is known as CAR-T cell therapy [8]. The discovery of agents targeting at the anti-programmed death 1 (PD1) pathway has started a new era of promising immunotherapeutic drugs. PD1 inhibitors have great potential for treatment of immunogenic and non-immunogenic cancers [9].

1.3 Chemotherapy

Chemotherapy is the use of medicines or drugs to treat a disease, such as cancer. The use of chemotherapy to treat cancer started in early 1900s but for a long time surgery and radiotherapy were more in practice until nitrogen mustard derivatives were discovered and proved to be effective against lymphoma in the 1940s [10]. This gave the hope that drugs also could be used to treat cancer patients. Now chemotherapy is an important treatment modality and has been developed into adjuvant chemotherapy, combination chemotherapy and targeted chemotherapy. The idea of using all three treatment modalities i.e. surgery, radiation and chemotherapy to get maximum effect against cancer has become standard clinical practice [10].

Most chemotherapeutic agents target actively replicating malignant cells. They interact with DNA in different ways and interfere at different steps of cell division leading to cell death. Others interfere with DNA indirect via microtubules and spindle formation leading to mitosis inhibition and cell death [11]. Some immune cells produce inflammatory proteins toxic to tumor cells. Thus, immune stimulators in combination with conventional chemotherapeutic agents can be used to target tumors [12].

1.4 Targeted chemotherapy

Despite its numerous benefits, one major problem associated with chemotherapy is lack of selectivity where normal cells are also damaged by the drugs. Besides, tumor cells often have a genomic instability and they become resistant to anticancer drugs. This resistance can already exist in cancer cells genetically or can be acquired during chemotherapy treatment. Tumors are

known to have a mix of drug sensitive and drug resistant cells and during chemotherapy treatment, drug sensitive cells are gradually removed, leaving only the drug resistant cells [13]. To overcome these problems, there is a need of some kind of targeted action of drugs that do not damage normal cells and tissues. The tremendous increase in knowledge in genetics, pathology, cancer development and in tumor biology have paved the way for more specific treatment alternatives. For targeted chemotherapy antitumor agents are formulated in a way that they specifically act on their targets for example receptors, ligands, enzymes and tumor environment [14]. Targeted therapy can also be treatment option for those patients who do not respond to traditional anticancer therapy e.g. elderly patients with non-Hodgkin's lymphoma [15].

For some cancers like chronic myeloid leukemia (CML), targeted chemotherapy has been very successful e.g. imatinib targeting the Bcr-Abl tyrosine kinase. However, resistance to imatinib and tumor relapse have been observed [16]. This resistance is developed due to mutations resulting in amino acid substitutions at ATP binding site rendering patients non-responsive for further treatment [17]. Other mechanisms of resistance to imatinib include increased level of Bcr-Abl protein [18], gene amplification [19] and overexpression of multidrug resistance P-glycoprotein [17]. New combination treatment regimens with imatinib can be a possible option to address these challenges.

Another successful targeted therapy example is BRAF inhibitor vemurafenib for melanoma. More than 90% of BRAF mutations in melanoma make it a therapeutic target for BRAF inhibitors [20]. However, resistance to vemurafenib also inevitably develops over time, for instance by MAPK pathway reactivation due to up-regulation of tyrosine kinases [21] contributing to relapse and acquired BRAF inhibitor resistance in patients.

1.5 Limitations of chemotherapy

Cancer chemotherapy is one of the most important therapeutic methods to combat cancer but it has some limitations as well. One of the major obstacles to chemotherapy is drug resistance. There are several mechanisms through which tumor cells acquire resistance e.g. alterations in drug metabolism, modification of drug targets, drug inactivation, cell death inhibition, drug efflux and epigenetics etc. [22]. One example of drug resistance through drug inactivation is treatment of myelogenous leukemia with cytarabine (AraC). This drug should be metabolically activated in order to be effective. It is activated through a series of phosphorylation events that convert it to AraC-triphosphate. Alterations in enzymes involved in this metabolic pathway e.g. deoxycytidine kinase can lead to inactivation of AraC resulting in drug resistance [23]. Second, non specific cytotoxicity of chemotherapeutic

agents where normal tissues with rapidly dividing cells for example bone marrow can also be affected along with tumor cells. Bone marrow cytotoxicity can lead to immunosuppression, anemia and excessive bleeding [24]. Another obstacle can be poor penetration and non-effective distribution of the drug. Due to abnormal blood vascular system in solid tumors, drugs cannot penetrate and achieve the required concentrations [25]. Tumor location is another factor limiting the effect of chemotherapy. Some tumors e.g. those located in CNS are not well suited for treatment with drugs due to poor penetrance (although some drugs cross the blood brain barrier). Also, proper drug dosing is very important to achieve maximum effect of drug because of the pharmacokinetic variability between patients [26].

1.6 Combination chemotherapy

To prevent drug resistance, it is not an option to just increase the dose of a single chemotherapeutic agent since the toxicity will be dose-limiting. The most successful approach is often to combine multiple anticancer drugs with different toxicities and different mode of actions. Combination therapy can work for many types of aggressive cancers that are sensitive to chemotherapy [26]. For example, 5-fluorouracil based regimen combined with oxaliplatin is in practice for stage III colorectal cancer patients. Also, combination of oxaliplatin and capecitabine (a pro-drug to 5-fluorouracil) is found to improve disease free survival in metastatic colon cancer patients [27]. Advantages are that drug combinations can result in reduced chances of drug resistance and can work on multiple pathways [28]. Combination therapy can result in drug interaction effects which can be either as expected (according to predefined assumptions) or synergistic/antagonistic [29].

1.7 Repositioning of drugs

In oncology, the approval rate for new drugs is about fifty percent lower than for other indications [30]. New drug development approaches are required to address this issue. One such approach can be drug repositioning where a new indication for an existing drug is identified. The advantages of repositioning/repurposing are that for repurposed drug, substantial information on pharmacology, formulation and toxicity is already available and that reduces the development time as well as the cost [31].

1.8 Current approaches to drug repositioning

Approaches to search for new drug repositioning candidates can be divided into two main categories: Experimental and computational. The experimental approach employs experimental procedures in laboratory whereas the computational is using the information about drugs, diseases or pathways to predict drug interactions computationally.

Experimental approaches include imaging techniques, in-vivo disease animal models and high throughput screening of approved drug libraries to identify potential repositioning compounds that can be subsequently used in clinical trials [32, 33]. In recent years, predictive computational approaches have gained a lot of attention and many studies have been published based on computational approaches of drug repositioning. These approaches mainly rely on large amount of experimental data in different public databases. Most of these are based on chemical structure and assume that drugs with similar chemical structure have same targets. Thus, from a database of interest, one can search for structurally related approved compounds for repositioning [32]. For example, recently a Japanese group of scientists, have constructed highly applicable and accurate statistical models to predict new indications for various drugs based on chemical structure similarities [34]. Among many biologically meaningful examples of newly predicted drug targets and indications predicted by this group, some related to cancer are as: imdometacin sodium originally used for barter syndrome treatment is predicted for renal cell carcinoma, folate sodium already used for treating hereditary spherocytosis found to have new indications for breast cancer and edatrexate used for treatment of dihydrofolate reductase deficiency have potential to treat small cell lung cancer. These predictions are supported by literature as well. Similarly, protein structure and molecular docking based methods also fall in this category and compounds that interact with more than one protein can be identified and repositioned [33].

Traditional approaches to identify compounds for drug repositioning are summarized in figure 1. Drugs for repositioning can be identified through serendipitous observations as in case of Path 1 in the figure. It can be exemplified by Thalidomide (see below). High throughput screening can be used to identify drugs with specific biological activity or target as in case of path 2 and 3. If a new role of some target proteins is discovered, relevant drugs can be repositioned (Path 4). For example, ALK (membrane receptor tyrosine kinase) was first discovered as drug target in anaplastic lymphoma but then it was identified as drug target in NSCLC as well and this led to repositioning of the ALK inhibitor crizotinib. If signalling pathways are discovered to be common in more than one disease, the drug can be repositioned (Path 5). Also if one drug shows unexpected side effects during clinical trials, it can be repositioned (Path 6) for new indications e.g. repositioning of sildenafil [32].

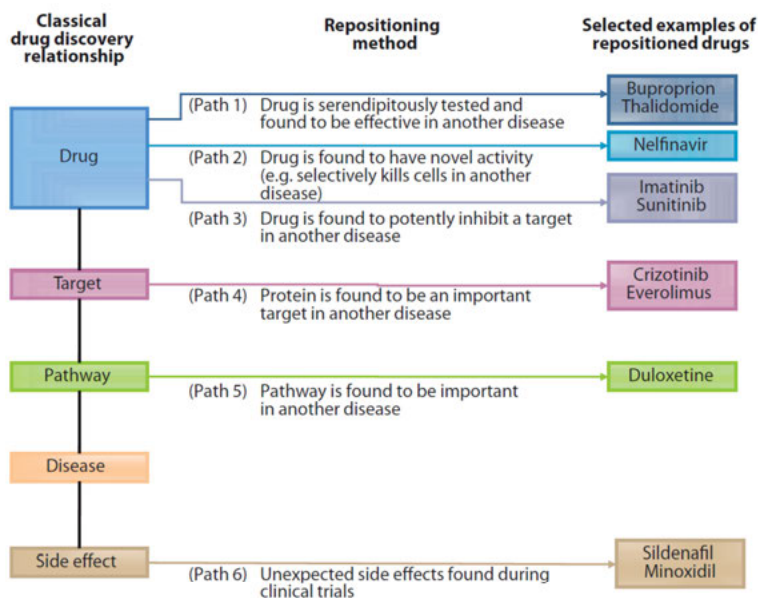


Figure 1. Potential avenues of drug repositioning. Different paths adapted for drug repositioning and examples of repositioned drugs for each method are listed here. Figure adapted with the permission [32].

1.8 Examples of repurposed drugs

Bupropion and thalidomide: Bupropion was originally developed for treatment of depression but later found to be effective for the treatment of obesity. However, it was rejected by FDA afterwards owing to its adverse side effects [35]. Thalidomide is a classic example of repurposed drug that was initially developed as sedative and for the treatment of nausea in pregnant women in late 1950s but resulted in children born with malformed limbs along with other birth defects and was therefore banned. After some years it was reported to be effective against leprosy as it resulted in improvement of skin lesions of the patient with erythema nodosum leprosum [36] and was approved and repurposed for treatment of leprosy. In 1994, it was found that thalidomide has anti-angiogenic activity and can inhibit angiogenesis induced by fibroblast growth factor and vascular endothelial growth factor [37]. For this property it was tested in myeloma cancer patients and proven effective [38] and successfully repurposed for treatment of multiple myeloma [39].

Nelfinavir: Nelfinavir was originally developed for treating AIDS, later it was found to inhibit AKT pathway when tested against a panel of 60 cancer cell lines and now it is in clinical trials for multiple cancers [40].

Imatinib and sunitinib: Imatinib targets BCR-ABL tyrosine kinase and was used to treat CML initially. In addition to inhibiting ABL tyrosine kinase, it was identified that imatinib also inhibit PDGFR and KIT tyrosine kinases. KIT and PDGFRA activating mutations are observed in 85% of GISTs [41, 42] that made it suitable repurposing candidate for treatment of GISTs. Sunitinib is an antiangiogenic agent and was primarily developed to target multiple kinases and was used to treat GIST and renal cell carcinoma. The receptor tyrosine kinases that it inhibits have been described in a number of other tumors as well including pancreatic neuroendocrine tumors (PNET)[43]. That's why it was evaluated for treatment of PNET and found effective in inhibition of VEGFR and PDGFR [44]. After this finding it was repurposed for treatment of pancreatic neuroendocrine tumors [45].

Crizotinib and everloimus: Original target of Crizotinib was MET kinase and it was in clinical trials for anaplastic large cell lymphoma until it was found to target echinoderm microtubule associated protein like 4 (EML4) gene and anaplastic lymphoma kinase (ALK) [46]. EML4-ALK fusion protein is active in a small subset of non-small cell lung cancer (NSCLC) and this discovery lead to repositioning of crizotinib for treatment of NSCLC [47]. Everolimus initially used as immunosuppressant was found to act on mammalian target of rapamycin (mTOR) signalling pathway that has a key role in neuroendocrine tumor cells [48] and therefore was repurposed for treatment of pancreatic neuroendocrine tumors [49].

Duloxetine: It acts through serotonin and norepinephrine reuptake pathway and was used to treat depression. Later it was discovered that same pathway is involved in spinal cord activation of external urethral sphincter and now it is used to treat stress urinary incontinence (SUI) and chronic musculoskeletal pain as well [50, 51].

Sildenafil and minoxidil: Another well-known example of a repurposed drug is sildenafil that was initially developed to relax the heart blood vessels and treat angina pectoris but trials were not successful. However the patients experienced erections as side effects and it shifted the research towards erectile dysfunction treatment by sildenafil. Later on it was successfully repurposed for treatment of erectile dysfunction and pulmonary hypertension as both conditions can be treated by Phosphodiesterase 5 inhibition [31]. Minoxidil was developed for hypertension but unexpected hair growth in patients was observed and it was tested for hair loss treatment. After successful trials it was approved for hair loss treatment [32].

2. Aims

The overall aim of the projects in this thesis was to develop and use innovative strategies to repurpose clinically used drugs into new cancer therapies. Another overarching aim was to discover specific molecular targets and rare vulnerabilities in cancer cells and target those using specific drugs. The specific goals of the studies were:

Paper I: To use a bioinformatics approach to investigate if phosphodiesterase3A (PDE3A) expression can be used as biomarker and drug target in cancer patients.

Paper II: To develop and apply a computationally fast and label free detection method for detection of apoptotic cells from phase-contrast time-lapse microscopy images for high-throughput screening.

Paper III. To develop and apply a screening approach to identify drug candidates that potentiate the effect of the anthelmintic drug mebendazole for treatment of colorectal cancer, using monolayer cell cultures.

Paper IV. To develop and apply a screening approach to identify drug candidates that potentiate the effect of nitazoxanide using three-dimensional (3D) cell cultures (multicellular tumor spheroids).

3. Material and Methods

Material and methods will be briefly outlined here. For further details, see the individual papers.

3.1 Cell lines

Twelve Cell lines of different cancer origins were used for paper I. Among these HeLa (cervix adenocarcinoma), Colo741 (colon carcinoma), A2058 (melanoma), H2122 (lung adenocarcinoma), A2780 (ovarian carcinoma), SKOV-3 (ovarian adenocarcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma), HT-29 (colorectal adenocarcinoma) and HCT 116 (colorectal adenocarcinoma) are adherent whereas CCRF-CEM (acute lymphoblastic leukemia) and HL-60 (acute promyelocytic leukemia) are suspension cells. For paper II, colorectal cancer cell line, HCT116 was used. For paper III, HCT 116 was used for screen. Various other cell lines were used for validation experiments. In paper IV, HCT116 GFP and HT-29 GFP were used. Details of cell line origin and culture conditions are presented in the papers.

3.2 Drug libraries and screening formats

The compounds used in Paper I were Phosphodiesterase (PDE) inhibitors; zardaverine and quazinone as well as the cytotoxic drug oxaliplatin. For paper II, compounds from two libraries were used. The Pharmakon 1600 library that encompasses 1600 compounds and all have been tried in humans was obtained from Micro source Discovery Systems (Gaylordsville, CT, USA) and the LOPAC¹²⁸⁰ library from Sigma-Aldrich (Stockholm, Sweden) contains 1266 annotated compounds distributed over 56 pharmacological classes. In paper III, Pharmakon 1600 library was used and in paper IV, pharmacologically active compound library which contains 1650 molecules with previously reported biological activity, was used. All compounds were prepared as high concentration stock solutions, dissolved in dimethyl sulphoxide (DMSO), transferred to experimental plates using Echo Liquid Handler 550 (Labcyte), an acoustic liquid dispenser that allows precise and rapid

liquid transfer. The final solvent concentration did not exceed 1%. All cell culture reagents were purchased from Sigma Aldrich, unless stated otherwise.

3.3 Patient tumor samples

In paper I, solid tumor samples from ovarian cancer patients were used. Details about sample collection and preparation are described in the material and methods section of the paper. The sampling of patient tumors was approved by the regional ethical committee in Uppsala (Dnr2007/237).

3.4 Immunofluorescence (IF) staining and Immunohistochemistry (IHC) for PDE3A expression

Assessment of PDE3A protein expression was evaluated with both immunofluorescence and immunohistochemistry staining using primary antibody PDE3A (HPA014492) according to standard protocols. For details, material and methods section of paper I.

3.5 Gene expression analysis and protein expression database

PDE3A mRNA gene expression data was downloaded from Oncomine [52]. All data in Oncomine are median center-normalized. The following data datasets were used in the analysis; Wagner et al. [53] (Affymetrix probe ID 206389_s_at), All samples from primary tumor in dataset by Bittner et al. (Gene Expression Omnibus Series GSE2109, Affymetrix probe ID 206389_s_at and 206388_at), Segal et al. [54] (probe 34650_at) and Cho et al. [55] (probe ILMN_1805415,.)

Images of immunohistochemical *PDE3* stainings on cancer tissues (using the same antibody as in this study, HPA01492) were retrieved from The Human Protein Atlas [56, 57] together with clinical data.

3.6 Cell proliferation and viability assays

3.6.1 Measurement of cytotoxicity (The Fluorometric Microculture Cytotoxicity Assay FMCA)

Fluorometric Microculture Cytotoxicity Assay, FMCA, described in detail previously [58] was used for measurement of cytotoxicity of drugs in mono-layer cell cultures and primary patient cells used in paper I- III. The FMCA is based on measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes. As preparation for the FMCA, 2500-5000 cells per well for different cell lines were seeded in 384-well plates using the Biomek 4000 Laboratory Automation Workstation (Beckman Coulter) and cultured over night before drugs were added. In each plate, two columns without drugs served as controls and one column with medium only served as blank. The end point of assay is survival index that is calculated after 72 hours of treatment with drugs. It is the fraction of surviving cells relative to unexposed control and is calculated as

$$SI\% = 100 \times \frac{f_{sample} - f_{blank}}{f_{control} - f_{blank}}$$

where f_{sample} denotes the fluorescence signal from the well of the sample, f_{blank} denotes the fluorescence signal from the well of the blanks (average) and $f_{control}$ denotes the fluorescence signal from the control wells (average). Quality criteria for a successful assay included a mean coefficient of variation of less than 30% in the control wells and a fluorescence signal in control wells of more than 5 times the blank (10 times for cell lines). Data from the FMCA was presented using the GraphPad Prism software.

3.6.2 Spheroid GFP fluorescence intensity assay

This assay was used in paper IV where we used spheroids as cell model. Spheroids formed from GFP expressing cell lines were treated with experimental compounds and then the mean spheroid GFP fluorescence intensity was measured using an automated fluorescence microscope, Arrayscan VTI reader (Cellomics Inc) [59].

3.6.3 Spheroid based clonogenic assay

In paper IV, it was used as final viability assay. It directly determines the potential of cells from spheroids to resume growth. Following drug treatment, spheroids are dispersed into single-cell suspensions. Then, cells are

seeded into 6-well culture-treated plates and left for regrowth for 10 days. Then, colonies are fixed and stained using Giemsa dye. The treatment effects are determined by comparing the number of colonies formed by treated cells with untreated controls [59].

3.6.4 Assay for quantification of apoptosis specific caspase activity

In paper II and III, Essen BioScience, CellPlayer™ 96-Well Kinetic Caspase-3/7 assay was used for apoptosis detection. Caspase-3/7 reagent was used at a final concentration of 5 μ M according to manufacturer instructions. Caspase-3 substrate consists of a highly negatively charged DEVD peptide that is attached to a DNA-binding dye to make the dye unable to bind to DNA and thus unable to produce fluorescence in the presence of DNA. The substrate rapidly crosses cell membrane to enter the cell cytoplasm, where it is cleaved by caspase-3 to release the high-affinity DNA dye. The released DNA dye migrates to the cell nucleus to stain the nucleus brightly green. Cells were monitored in IncuCyte FLR and images were acquired using a 20X objective in the fluorescence channel.

3.7 Combination analysis and statistics

For paper III, the analysis of Fluorescence data was performed according to Bliss Independence model (Bliss,1939) using the MacSynergy II software (<https://www.uab.edu/medicine/peds/macsynergy>) [60]. A synergy score for each individual drug concentration combination was calculated according to Bliss Independence model [61].

3.8 Drug combinations and therapeutic synergy in MCTS

In paper IV, combinations of different drugs in MCTS were evaluated. Briefly, drugs were added into 384-well spheroid plates in 3 \times 3 and 3 \times 4 matrix. The spheroid viability was assessed using GFP-based assay. Bliss independence model was used to characterize the type of interaction between two drugs. According to the Bliss model, we can predict the cell toxicity effect caused by a combination when drugs act independently using following formula:

$$T_p = T_A + T_B - T_A T_B$$

Where T_p = predicted toxicity, T_A = toxicity of drug A used alone and T_B = toxicity of drug B used alone.

Based on predicted cytotoxicity, we can characterize the interaction between drugs A and B using observed toxicity (T_o) as follows:

$T_o > T_p$ synergistic

$T_o < T_p$ antagonistic

$T_o = T_p$ independent (expected)

4. Summary of the publications

4.1 Paper I

Targeting tumor cells based on Phosphodiesterase 3A (PDE3A) expression

Madiha Nazir, Wojcieh Senkowski, Frida Nyberg, Kristin Blom, Per Henrik Edqvist, Claes Andersson, Mats G Gustafsson, Peter Nygren, Rolf Larsson and Mårten Fryknäs. *Experimental Cell Research*, 2017 Dec 361(2); 308-315.

4.1.1 Background

We have previously reported a link between high Phosphodiesterase 3A (PDE3A) expression and selective sensitivity to Phosphodiesterase inhibition [62] indicating PDE3 has the potential to be both a biomarker and drug target. To expand on the previous findings, we performed experiments that are described in this study.

4.1.2 Results

We previously showed that high *PDE3A* mRNA expression in HeLa cells was associated with sensitivity to PDE inhibitors [62]. To further investigate this phenomenon, we searched public microarray data to identify cell lines with high *PDE3A* mRNA expression and chose three cell lines from various diagnoses [53] with the highest mRNA expression of *PDE3A*: COLO741 (colon carcinoma), A2058 (lung adenocarcinoma) and H2122 (malignant melanoma), as shown in Fig 2A. The selected cell lines were tested for sensitivity to two different PDE inhibitors, zardaverine and quazinsonone, as well as standard cytotoxic drug oxaliplatin (Fig. 2B-D). The cell lines with high *PDE3A* mRNA expression showed pronounced sensitivity to PDE inhibitors when compared with the other cell lines in the panel (Fig. 2B and C) The cell lines with high sensitivity towards PDE inhibition were not generally more sensitive to standard drugs than the other cell lines in the panel.

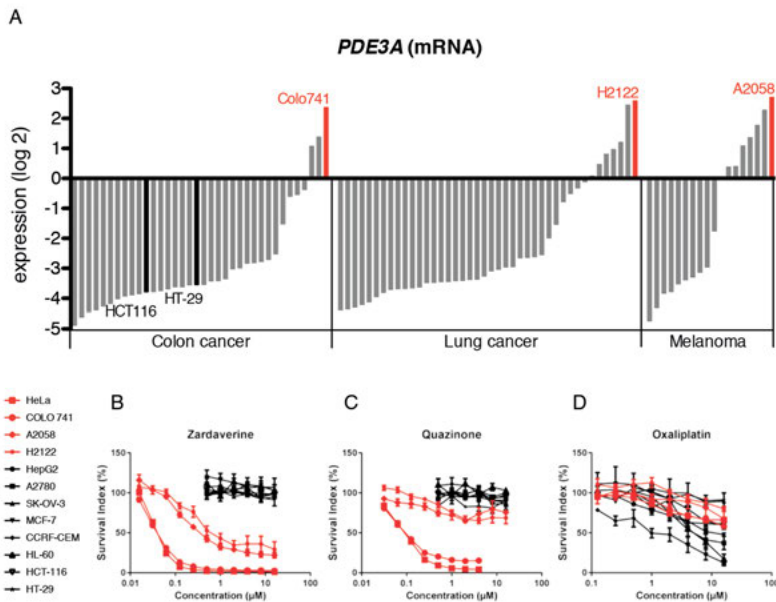


Figure 2. *PDE3A* mRNA expression is associated with phosphodiesterase inhibitor sensitivity. (A) Publically available *PDE3A* gene expression (probe ID 206389_s_at) data from a cell line panel [53]. Three cell lines, COLO741, H2122 and A2058) were selected based on high *PDE3A* expression (red), and two cell lines, HCT 116 and HT-29, based on low expression (black). (B-D) Cell lines were exposed to indicated concentrations of zardaverine, quazinsonone and oxaliplatin and viability was assessed (FMCA) after 72 hours of treatment. HeLa, H2122, A2058 and COLO741 (red), showed higher sensitivity to zardaverine and quazinsonone compared to the other cell lines. All concentrations were tested in triplicate; experiments repeated three times, graphs indicate mean \pm S.E.M.

Immunofluorescence and immunohistochemical staining was performed to test whether high *PDE3A* mRNA expression is correlated with high *PDE3A* expression at the protein level and results show that cell lines selected based on their high *PDE3A* mRNA expression (HeLa, COLO741, H2122 and A2058) all stained positive for *PDE3A* protein expression, whereas the other cell lines from the panel stained negative. Quantification from immunofluorescence images also showed that *PDE3A* protein expression is comparably higher in sensitive cell lines. Therefore, there is a good correlation between mRNA and protein expression in the investigated cell lines. Moreover, the primary antibody used in this experiment is suitable to evaluate *PDE3A* expression at the protein level.

Subsequently, we were interested to see if we can observe differential responses to PDE inhibition in the patient-derived material from cancer biopsies. Therefore, we tested eight different patient-derived primary ovarian

carcinoma cultures for PDE inhibitor sensitivity and the standard cytotoxic compound oxaliplatin and results indicated that cells from three of the primary samples demonstrated pronounced sensitivity to zardaverine and quazinone while the remaining five samples were resistant to the treatment. We did not observe major differences in the response of the eight clinical specimens to oxaliplatin. Immunohistochemical staining for PDE3A of all eight samples revealed that the three samples sensitive to PDE inhibition expressed PDE3A protein at high levels whereas, the five resistant samples showed only minor to intermediate levels of PDE3A expression. Thus, these observations indicate that patient-derived cells with high PDE3A expression are more sensitive to treatment with PDE inhibitors. Also, Vulnerability to PDE3 inhibitors is associated with co-expression of PDE3A and Schlafen family member 12 (SLFN12).

The observations above suggest that the level of PDE3A expression is a predictive of PDE inhibition sensitivity *in vitro*, including experiments involving clinical cancer biopsy material. However, the prevalence of PDE3A expression across different tumor types is not known. In order to, at least preliminarily, address this question, we took advantage of the Human Protein Atlas database [56], which contains a number of immunohistochemical stainings of normal/cancer tissue specimens for a wide range of molecular markers. Notably, the primary antibody that is used in the Atlas (HPA014492) is the same antibody that we used for the stainings in this report. Searching the Human Protein Atlas database revealed that differential PDE3A expression, as observed in ovarian cancer specimens, is also prevalent in many other cancer types, including colorectal, melanoma, endometrial, testis and urothelial cancers. Importantly, the question whether the PDE3A-expressing samples from various human tumors would also be sensitive to PDE inhibition has yet to be addressed. However, our observations provide a rationale for further detailed studies of the PDE3A overexpression as a biomarker for PDE inhibitor sensitivity in a wide range of different cancer types.

4.1.3 Conclusion

We have demonstrated that there is a correlation between high PDE3A expression and selective sensitivity to PDE inhibitors. Cell lines with high PDE3A expression showed striking sensitivity to PDE inhibitors zardaverine and quazinone. Also, in cell lines, high PDE3A mRNA expression can be corroborated at protein level as well. Differential PDE3A mRNA and protein expression can be observed in patient tumor cells from different solid cancer diagnoses and sensitivity to PDE inhibitors can also be observed in primary tumor cells from cancer patients. These observations suggest that PDE3A has the potential to be both a biomarker of PDE sensitivity and drug target for cancer treatment.

4.2 Paper II

Label free high throughput screening for apoptosis inducing chemicals using time-lapse microscopy signal processing

Obaid Aftab, **Madiha Nazir**, Mårten Fryknäs, Ulf Hammerling, Rolf Larsson and Mats G Gustafsson. *Apoptosis*, 2014 Sep; 19(9):1411-8.

4.2.1 Background

In Paper II, a computationally fast method for identification of apoptotic cells from plain phase contrast microscopy (PCM) images was developed and evaluated. Apoptosis is an evolutionarily-conserved cell death and growth regulating pathway with great therapeutic potential [4]. Apoptotic cells are characterized by cell shrinkage, highly condensed chromatin, membrane blebbing and loss of adhesion [63]. Apoptosis is currently detected using various molecular labelling techniques [64] but these methods are costly and time consuming for high throughput (HT) analyses. Moreover, molecular labelling techniques may affect the biological processes of interest and/or the effects of the compounds under investigation. Therefore, access to methods offering label free detection would be useful for drug discovery and repositioning.

4.2.2 Methods and results

The feasibility of a label-free method to successfully detect and quantify apoptotic cells by using an image processing algorithm applied to PCM images is presented. Very few approaches along this direction have been reported previously. A very limited but similar study has been performed in-house using the open source image analysis software CellProfiler [65]. However, this software is not designed for phase contrast images and should not be able to compete in terms of computational speed (complexity) compared with the simple linear filtering approach evaluated here. Based on characteristic features of apoptotic cells, a 2D linear matched filter was designed to detect objects from phase-contrast images. In a first experiment, the human colorectal carcinoma cell line HCT116 exposed to mitomycin, an apoptosis inducing drug was labelled with caspase-3/7 reagent to monitor caspase activity as a function of time. Phase contrast images were recorded in parallel and then used for detection of apoptotic cells using our designed filter. The detections made showed a pattern similar to the fluorescence readout from caspase activity over time. The proposed method was then tested on a large time-lapse microscopy dataset for identification of compounds leading to apoptosis. Two compound libraries, LOPAC¹²⁸⁰ and Pharmakon 1600 (consisting of 1266 and 1600 pharmacologically active compounds, respectively)

were screened against HCT116 cell line. Two practical advantages of our linear filter approach are the low computational complexity and that there is no need for manual selection of many training examples needed for machine learning based tuning. In order to show the accuracy of detection made a colocalization between caspase-3/7 signal and detection made through our detector is displayed in Figure 3.

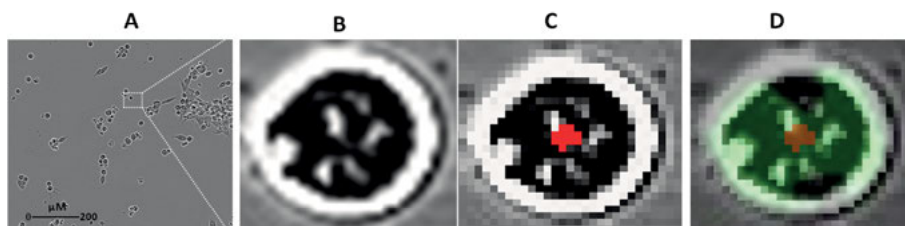


Figure 3. Detection of apoptotic cells from phase contrast images obtained from HCT116 cells exposed to Mitomycin ($10 \mu\text{M}$) at time point 20 h. **(A)** Phase contrast image obtained from IncuCyte FLR. **(B)** An enlarged image to show morphology of an apoptotic cell. **(C)** Image scanned through Apoptotic detector representing identification of candidate apoptotic object as indicated by red mark in the center. **(D)** Colocalization between caspase-3/7 signal and cells undergoing apoptosis identified by detector.

Image based screening for detection of apoptosis

The human colorectal carcinoma cell line HCT116 was used to screen the Pharmakon 1600 and LOPAC¹²⁸⁰ chemical libraries. All drugs were tested at a concentration of $10 \mu\text{M}$ in complete cell culture medium and imaged every 2nd hour over three consecutive days, using IncuCyte HD (see Material & Methods section of the paper). Images from each of the drug treated wells were transferred to a database and a detector was then applied to screen for drugs capable of inducing apoptosis. For the current analysis a filter of size 30×30 pixels was adopted to match cell size, see Figure 4. A flow chart of the procedure is displayed in Figure 5.

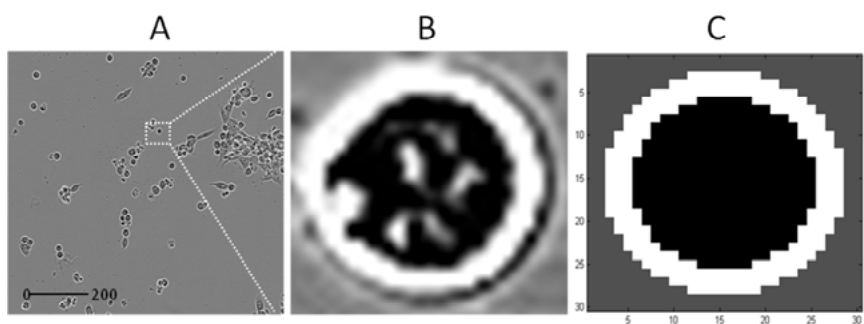


Figure 4. (A) Image displaying HCT116 cells treated with Mitomycin. (B) An enlarged image to show the apoptotic cell. (C) Illustration of one vesicle prototype in the form of a $M \times M$ dimensional matrix for $M=30$. Thus the prototype consists of a circular centre consisting of low intensity pixel values (black) surrounded by a relatively thin ring of high intensity pixels (white) which in turn are surrounded by an area of mid intensity pixel values (gray).

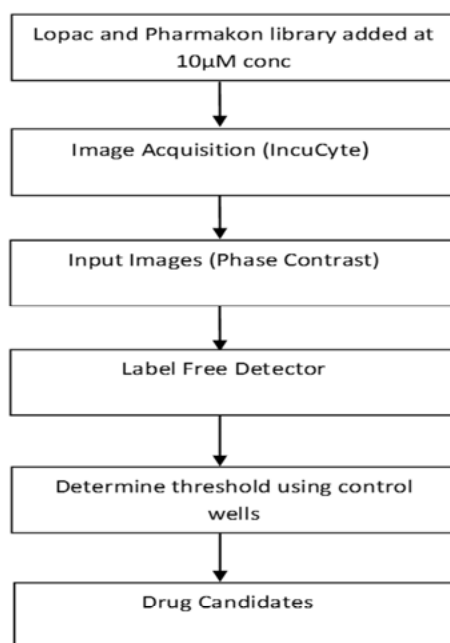


Figure 5. Flow chart of the automated microscopy-based screen. Drugs were added to HCT116 cells at a final concentration of 10 μM and incubated for 72 hours. Phase contrast images were taken every 2 hours and moved to a database. Detection thresholds corresponding to 5% false alarms were determined by applying them to control wells.

Validation of apoptosis inducing compounds

Among the 32 statistically most significant hits ($p = 0.05$) detected through automated microscopy screen, 27 have already been reported as apoptosis modulators. The remaining 5 hits, which have not been reported as apoptosis inducing compounds, were subjected to an independent validation using the caspase assay kit. The results indicate that two of the hits tested, 2, 3-dimethoxy- 1, 4-naphthoquinone (cyan curve) and Dipiperodon Hydrochloride (green curve), showed significant apoptosis modulating activity (Fig. 6). For the remaining three hits the corresponding compounds did indeed induce apoptosis as evident from looking at the caspase signal but the signals were not sufficiently strong to reach above the significance threshold used. Thus only 2, 3-Dimethoxy-1, 4- naphthoquinone and Dipiperodon Hydrochloride were formally confirmed as positive hits at the significance level (5 %) employed. Dipiperodon hydrochloride is a local anaesthetic and 2, 3-dimethoxy-1, 4-naphthoquinone is a redox cycling agent.

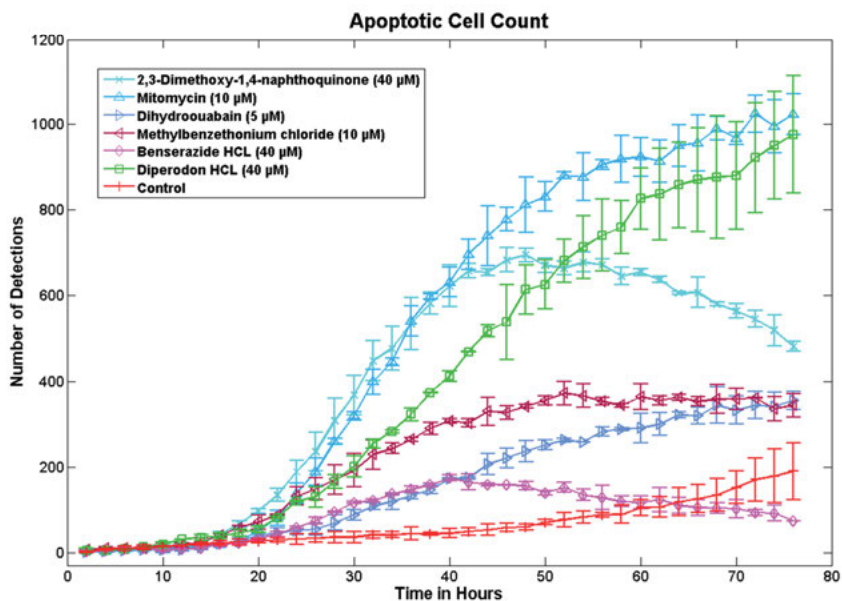


Figure 6. Results of validation experiment using caspase assay kit for five top hits not having any known relationship to apoptosis. Compounds were added at following concentrations: 2, 3-dimethoxy- 1, 4-naphthoquinone(40µM), dihydroouabain (5µM), methylbenzethonium chloride (10µM), benserazide hydrochloride (40µM) and dipiperodon hydrochloride (40µM). Kinetic measure of the number of caspase-3/7 positive cells is recorded over time using IncuCyte™ adaptive segmentation algorithm. Mitomycin (10µM) was used as positive control and cells with medium only served as negative control. For each concentration, the number of detections is presented as the mean \pm SEM from 3 different experiments.

4.3 Paper III

Drug combination screening for Mebendazole for the treatment of colorectal cancer

Madiha Nazir, Wojciech Senkowski, Mats Gustafsson, Peter Nygren, Rolf Larsson and Mårten Fryknäs. *Manuscript* (2017).

4.3.1 Background

The anthelmintic drug mebendazole has been reported to be a promising drug for treatment of advanced colorectal cancer and a strong candidate for drug repositioning [66, 67]. So in this project we aimed to find out suitable combination partners for mebendazole to enhance its effects.

4.3.2 Methods

A compound library Pharmakon consisting of 1600 compounds previously used in different clinical studies was screened. This screen was done alone and in combination with two different concentrations of mebendazole i.e. 1 μ M and 5 μ M. Colon cancer cell line HCT116 was used primarily for this screen and different other cell lines from various origins were used for validation experiments. FMCA was used to quantify cell viability and Bliss synergy [60] was used for combination analysis.

4.3.3 Results and conclusions

Six compounds: thioridazine, acriflavin, pimozide, maprotiline, loperamide and prochlorperazine were identified as hits. They seemed to be effective when used in combination with mebendazole. Four of these hits share structural similarities. For validation experiments, mebendazole was used at nine 2-fold dilutions ranging from 0.0625 -16 μ M and hit compounds at 2-fold dilution series at a range of 0.5-32 μ M. After 72-hr exposure, viability of cells was measured using FMCA. Subsequently, a synergy score for each individual drug concentration combination was calculated according to Bliss Independence model [61] using the MacSynergy II software. All three compounds identified as potential hits seem to have synergistic effect when using HCT116 cell line and this confirms results obtained from initial screen. However, in other cell lines, we could not identify the same synergistic phenomena. To conclude, validation experiments using different cell lines for three of these compounds (thioridazine, pimozide and acriflavin) showed that thioridazine and mebendazole combination is quite effective for colorectal cancer cell line HCT116. Apoptosis analysis using caspase 3/7 kit showed that thioridazine and mebendazole combination induces apoptosis in

HCT116 cells. Mebendazole and thioridazine combination works very well for targeting HCT116 cells. Since HCT116 contains mainly cancer stem cells [68] and thioridazine is also reported to kill cancer stem cells [69] it could be speculated that this combination actually is stem cells specific.

4.4 Paper IV

Drug combination screening in multicellular tumor spheroids identifies synthetic lethalties in quiescent cancer cells

Wojcieh Senkowski, **Madiha Nazir**, Mats G Gustafsson, Peter Nygren, Rolf Larsson and Mårten Fryknäs. *Manuscript* (2017).

4.4.1 Background

We have previously reported that anthelmintic drug Nitazoxanide can be a candidate for treatment of colorectal cancer [59]. We found that nitazoxanide is selectively toxic to quiescent cells in spheroids and that this toxicity is a result of uncoupling of mitochondrial membrane potential, thereby inhibiting oxidative phosphorylation (OXPHOS) a process where most of cell's ATP is produced. Therefore nitazoxanide, with its excellent clinical pharmacokinetic profile and virtually no side effects [70-72], may be a suitable candidate for drug repositioning against colorectal cancer. In this project we aimed to identify suitable combination partners for this drug to increase its anticancer effect.

4.4.2 Methods

We have performed combination drug screening using Q-MCTS (quiescent spheroids formed without medium change over the culture period) as a cellular model and HCT116 GFP cell line that constitutively express green fluorescent protein (GFP). For the screen, we have used Pharmacologically Active Compound Library (Selleckchem) that contains 1650 bioactive compounds, including both approved drugs and experimental molecules with reported biological activity. We used GFP-based measurement of spheroid viability. For each library compound, we calculated a ratio of the combination treated spheroid viability to the viability of the spheroid treated with compound alone. Spheroid based clonogenic assay was also performed to identify the molecules with pronounced synergistic activity in combination.

4.4.3 Results and conclusion

64 molecules were identified as outstanding in combination with nitazoxanide. After validation experiments, this number reduced to 14 molecules identified as synergistic hits and 6 as antagonistic hits. In order to search for compounds that generally target tumor quiescent cells rather than the specific cell line used, one additional colon cancer cell line, HT-29, was used. These experiments resulted in 10 synergistic and 5 antagonistic hits. In order to identify the molecules with most pronounced synergistic activity in Q-MCTS, all hits have been tested in spheroid-based clonogenic assay. All ten of selected synergistic hit compounds caused inhibition of clonogenicity in combination with nitazoxanide in HCT116 Q-MCTS. In conclusion, here we applied MCTS for high throughput screening for potent drug combinations and identified novel promising drug interactions.

5. Conclusion and final remarks

Referring to the aims, we can summarize the results of the thesis as:

- We showed that subgroups of tumors, within many different cancer types, overexpress PDE3A (mRNA and protein) and that PDE3A expression can predict sensitivity to phosphodiesterase inhibitors (paper I).
- The phosphodiesterase inhibitors zardaverine and quazinone seem to have repurposing potential.
- A new automated image based microscopy assay was developed and used to identify apoptosis inducing chemicals.
- Two new compounds (2, 3-dimethoxy-1, 4-naphthoquinone and dipiperdon hydrochloride) have been identified that can induce apoptosis and they have not been previously reported as apoptosis inducing compounds (papers II).
- Combination screening was used to identify new candidates to work in combination with anthelmintic drug mebendazole (previously identified as a repurposing candidate) for treatment of colorectal cancer (paper III).
- From this combination screen, the antipsychotic drug thioridazine was identified as potential combination partner for mebendazole that have synergistic effect and is capable of inducing apoptosis in colon cancer cells, when combined with mebendazole.
- A novel 3D culture based combination screening assay was developed and used to identify combination partners for 3D specific antiparasitic drug nitazoxanide (paper IV).
- Ketoconazole and nitazoxanide combination was identified to have synergistic antitumor effect on colorectal cancer cell lines when grown as spheroids.

In conclusion, the work presented in this thesis adds knowledge about how to follow different pathways for finding new drug candidates for repositioning. Also screening of the compounds can not only be important first step in drug discovery but also for drug repositioning.

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