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# IN VITRO TESTING OF DRUG SENSITIVITY IN TUMOR CELLS FROM EFFUSION

Carl-Olof Hillerdal



Carl-Olof Hillerdal was born in Uppsala Akademiska Sjukhuset the first of april 1983. His two baby boys were born in 2013 and 2016 respectively, to his beautiful wife Victoria. Carl-Olof graduated from Uppsala University with a bachelor degree in chemistry in 2008 and a master degree in biology in 2010.

He was registered as a PhD student in KI the spring of 2012, and remains hopeful this thesis will suffice for finally graduating.



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# IN VITRO TESTING OF DRUG SENSITIVITY IN TUMOR CELLS FROM EFFUSION

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

**Carl-Olof Hillerdal**

*Principal Supervisor:*

Prof Anders Hjerpe  
Karolinska Institutet  
Department of LabMed  
Division of Pathology

*Co-supervisor:*

Docent Katalin Dobra  
Karolinska Institutet  
Department of LabMed  
Division of Pathology

*Opponent:*

Docent Ola Brodin  
Karolinska Institutet  
Department of LabMed  
Division of Pathology

*Examination Board:*

Prof Rolf Lewensohn  
Karolinska Institutionen  
Department of OnkPat

Prof Göran Andersson  
Karolinska Institutet  
Department of Labmed  
Division of Pathology

Docent Bengt Bergman  
Sahlgrenska Universitetssjukhuset  
Department of Lungonc



*Dedicated to all the rebels out there,  
whom refuse the convenient,  
until proven true.*

*We need you now more than ever before*

*What does not kill me makes me stronger*

Friedrich Nietzsche

## ABSTRACT

Malignant mesothelioma has a mean overall survival of around 1 year and lung adenocarcinoma with pleural spread has a mean overall survival of around 5 months. Both diseases cause fluid accumulation in the pleura, which is drained to alleviate associated symptoms such as shortness of breath. This fluid oft contains exfoliated tumor cells.

All chemotherapy regiments in use against malignant mesothelioma and lung adenocarcinoma with pleural spread have an objective response rate of 30-40%, and they all increase mean overall survival with a mere 3 months. The choice of drug combinations in the chemotherapy regiments are determined based on the statistically best drug combination. However, due to tumor heterogeneity, it is unclear whether some patients would respond better to an alternative treatment rather than the gold standard.

To test this hypothesis, tumor cells were isolated from the effusions and cultured together with cytostatic drugs. After 48 or 72 h, the toxicity was measured using an automated live / dead assay, a colorimetric assay or a flow cytometer based assay and compared to an untreated control. The obtained data was then compared with patient journals, either overall survival or effect of drug treatment.

Such drug exposure assays have been performed for long, however, no drug exposure assay have seen clinical use outside of smaller studies. The work described in this thesis attempted a number of methods of improving these assays, most prominently by attempting to make the measurements tumor specific, as there is often a substantial admixture of benign inflammatory cells. Also other refinements were tested, such as increasing the concentrations of the tested drugs to above what is found in the blood of patients in order to elicit meaningful response during *in vitro* short drug exposure times.

The thesis concludes with a promising study, using the flowcytometer to make the readouts tumor cell specific and to show high variation. Initial data suggests this tumor specific assay indeed is able to predict patient response to given drugs.

## LIST OF SCIENTIFIC PAPERS

- I. **Characterization and drug sensitivity profiling of primary malignant mesothelioma cells from pleural effusions.** *BMC Cancer* 2014, 14:709.  
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- II. **Drug sensitivity profiling and molecular characteristics of cells from pleural effusions of patients with lung adenocarcinoma.** *Genes & Cancer, Vol. 6 (3-4), March 2015.* **DOI:** 10.18632/genesandcancer.56  
  
Rita Ötvös, Adam Szulkin, **Carl-Olof Hillerdal**, Aytekin Celep, Eviane Yousef-Fadhel, Henriette Skribek, Anders Hjerpe, László Székely and Katalin Dobra
  
- III. **Ex vivo evaluation of tumor cell specific drug responses in malignant pleural effusions.** *Oncotarget* 2017, issue TBA.  
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## **LIST OF ABBREVIATIONS**

MM	Malignant Mesothelioma
LAC	Lung AdenoCarcinoma
OS	Overall Survival
ORR	Objective Response Rate
RECIST	Response Evaluation Criteria In Solid Tumors
PD	Progressive Disease
SD	Stable Disease
PR	Partial Response
CR	Complete Response
SOC	Standard Of Care
GC	Gemcitabine + Carboplatin
PC	Pemetrexed + Carboplatin
CDG	Cisplatin + Doxorubicin + Gemcitabine
FACS	Fluorescence-Activated Cell Sorting
ICC	Immunocytochemistry

# 1 INTRODUCTION

## 1.1 Malignancies of the serosal cavities

### 1.1.1 General

A serosal cavity is the space between the visceral- and a parietal mesothelium, lubricated by an interstitial serosal fluid. There are four serosal cavities: the pleura that lines the lungs, the pericardium that envelops the heart, the peritoneum that lines the intestines and the tunica vaginalis, a rest of peritoneum in the testis capsule. The physiologic function of the serosal cavities is to reduce friction to allow movement between internal organs.

The mesothelium is a membrane consisting of a single layer of mesothelial cells with flat epithelioid phenotype. These cells rest on a basement membrane, beneath which lies the mesothelial stroma, a layer of fibroblast-like mesothelial precursor cells [1]. Neither cell type is terminally differentiated, and they both have the capacity to participate in healing of mesothelial defects. The serosal cavities have mesodermal origin, and mesothelial cell express N-CAM as its main adhesion molecule, thus, the mesothelium is mesenchymal by definition. Due to the dual nature of mesenchymal as well as epithelioid phenotype, these cells express a mixture of epithelial and mesenchymal markers, indicating an immunophenotype intermediate to epithelium and mesenchyme.

Like their benign precursors, malignant mesotheliomas (MM) can be either epithelioid or fibroblast-like, or even a mixture of both types. Thus, MM are subcategorized as epithelioid, sarcomatoid and biphasic. The basic biology between these growth patterns is still unclear, and certain cell lines derived from MM can be switched from epithelioid to sarcomatoid simply by changing the culture medium [2]. It should be noted, however, that it is not known to which extent this transition from one to the other phenotype is due to the environmental change causing a phenotype switch, or because the different conditions favor clonal outgrowth of either phenotype. In general, MM has a poor prognosis with an average overall survival (OS) of around 1 year. Still, a small proportion of the patients survive for 5 years or more. The sarcomatoid phenotype, however, has a substantially worse prognosis, with an average OS of 4 months [3].

MM is clearly linked to asbestos exposure [4], and it is generally believed that the spontaneous cancer rate of MM is very low. Asbestos have been forbidden in Sweden since 1982, with a first step taken in 1972, banning crocidolite, the most carcinogenic form of asbestos. Since the time from asbestos exposure and development of tumors is 20-50 years, the incidence of malignant mesothelioma is expected to decline [5], after reaching a plateau around the year 2020. The current incidence rate in Sweden is around 100 new patients every year since the 1990ties, owing to the rather long latency period and to immigration from countries where asbestos was banned at a later date or even have yet to be banned. Even in countries where the use of asbestos has been banned, there may exist communities that are located in areas contaminated by asbestos, either due to former mining and / or processing or because of natural presence in the superficial layers of the soil [6].

In addition to MM the serous cavities are frequent locales for metastases. These metastatic cancers reach the serous cavities by direct invasion, for example from the lungs, ovaries and gastrointestinal tract. Once in the cavity it may disseminate further along the serosal surface and enter the lymphatic system via the stomata that normally drain the serous fluid [7]. Most carcinomas in the pleura are adenocarcinomas, squamous cell carcinomas being

much less common [8]. Lung cancers and breast cancers are commonly found in the pleura [8], while ovarian and gastrointestinal cancers are common in the peritoneum. But this anatomical separation is by no means absolute, and the aforementioned cancers often appear in both the pleural and peritoneal cavities. Adenocarcinomas present in serosal cavities are by definition advanced and the prognosis is therefore poor with the mean OS limited to five months [8-10].

Of all malignancies of the serosal cavities, lung adenocarcinomas (LAC) with pleural spread are the absolutely most common [11]. Lung cancer is one of the most common cancer forms worldwide. In Sweden, preventive measures aimed at reducing tobacco smoking have abated lung cancer to the fifth most common cancer form. Even so, lung cancer remains the most common cause of cancer related deaths, highlighting the relative aggressiveness of this cancer form.

### **1.1.2 Diagnosis**

The first symptom of a malignant involvement of the serosal cavities is often the appearance of a serosal fluid accumulation, also called an effusion. The serosal fluid is drained by “stomata”, i.e., openings from the cavity directly into the lymphatics. Tumors growing in the cavity often spread and physically block these stomata, preventing fluid resorption. 80% of all patients with MM and 15% of all patients with LAC show their spread to the serosal cavity by an associated effusion [8, 12]. The pleural effusion occupies space in the thoracic cavity, causing shortness of breath. The effusion is drained to alleviate the symptoms [13].

This fluid is therefore first biological material available for demonstration of serosal cavity involvement and microscope diagnosis. Guidelines were recently published showing how the cytological diagnosis of a MM can be achieved in a majority of cases [14], still necessitating the more invasive diagnostic techniques with biopsy sampling for the remaining cases. Once the diagnosis of a malignant condition is established, modern cytology pathology and histopathology tries to establish the type and origin of the tumor, using immunocyto- and histochemistry. The diagnostic principles are the same regardless which cavity is engaged, although the tumor types and their primary locations vary.

As a metastatic manifestation, the spread of a tumor to the serous cavity is often a late event, and the primary tumor is sometimes already known [15]. Spread of such a tumor to a cavity can be traced by x-ray related techniques, also before an effusion is built up. The macroscopical changes in serous membrane morphology must, however, be examined microscopically by cyto- or histopathology in order to establish the diagnostic basis for treatment.

### **1.1.3 Treatment**

There are four principal therapy options for fighting cancer: surgery, radiotherapy, chemotherapy and immunotherapy. Treatments can be given alone, as supplements to each other or sequential. If a preferred option fails alternative options can be used, or, following remission, as maintenance therapy to decrease the risk of recurrence. The utility of each treatment option depends on the diagnosis. One factor influencing the prognosis of a cancer is the clinical stage. Once a carcinoma reaches a serosal cavity the tumor has reached an advanced stage where radical resection of the tumor is no longer possible. Similarly, the primary MM is in most cases non-resectable when detected, i.e., radical surgery seldom improve OS [16]. The therapeutic option for both primary and metastatic tumors in the

serosal cavity is therefore in most cases chemotherapy, in some cases after surgical reduction of the tumor mass.

Thus, for these tumors chemotherapy is the therapeutic option of choice. The standard of care (SOC) drug regimens used for MM and LAC with pleural spread all have an objective response rate (ORR) in the neighborhood of 30-40% [17-21], with an added mean survival benefit of a mere three months.

## **1.2 Brief summary of tumor biology**

### **1.2.1 Oncogenes**

The cell is the basic building block of all life. Cells ingest and rearrange molecules from the surrounding environment, to harvest energy from energy rich chemical bonds and then use this energy for movement and for synthesizing the molecules necessary to sustain itself. Molecules are step-wise broken down, converted and / or synthesized by macromolecular machines. Other macromolecules have structural function, signaling function or motor function, among other functions. The relationships between position, quantity and structure of macromolecules as well as the flux of metabolites in any given area inside a cell determine that area's net function. This organization of macromolecules is determined by feedback processes that up or downregulate genes. Specialized functional centers are called organelles.

The cells of complex multicellular organisms depend on each other for survival, and each individual cell must submit to the needs of the organism as a whole. This is achieved through an additional layer of feedback processes called cellular signaling. Similar to organelles, the cells in complex multicellular organisms form specialized clusters of cells called organs. An example feedback process is the release of insulin following increased blood glucose levels, which in turn signal to muscle cells to increase their glucose uptake, thus resuming normal blood glucose levels.

A gene is any segment of DNA coding for molecular machines. Genes are transcribed into either lncRNA, or into mRNA that is subsequently translated into proteins. The sequence of the gene determines which and in which order nucleotides (for RNA) and thus in turn the order for amino acids (for proteins) are synthesized, and thereby the structure and function of the molecular machine. A molecular machine can consist of one or several subunits of proteins and / or lncRNA.

Non-controlled alterations of DNA are called mutations. A mutation in the coding section for a protein might alter the function, by changing an amino acid vital for its activity or structure, or by inserting an improper stop codon. Other than the coding sequence, genes contain promoter and operator segments, and might have associated enhancer segments. Mutations in these regions will not alter the molecular machine's function, but it can alter the expression levels.

Oncogenes is a collective name for genes that, when acquiring certain mutations, in one way or another either promotes cell growth [22], such as EGFR (over-activation increase proliferation) , or promotes or inhibits checks on cell growth, such as MHC class I (pseudo-functional MHC class I can "hide" a cell from the immune defense[23]) or P53 (altered function of P53 can prevent or delay apoptosis) [24].





Fig1: Schematic representation of synthesis and delivery of a molecular machine, in this case a transmembrane protein. The picture is simplified for visual clarity, for instance, the protein is fully translated already in the ER, and there is vesicular transport also between the ER and the golgi. 1) A transcription bubble. The DNA is temporarily unwound, as the frame is transcribed into mRNA by RNA polymerase. mRNA is then feed into the endoplasmatic reticulum (ER) through pores in the nucleus. 2) The ribosome translates the mRNA sequence into an amino-acid sequence. The protein is then packaged into a vesicle by the golgi (3). The final destination of the protein can be much anywhere in the cell, dependent on "adress" "tags" that the golgi attaches to the vesicle. In this case, the vesicle is carried to its destination by a dynamin (4), and eventually fuses with the membrane (5). Image printed with permission from *Christina Hillerdal* ©.



## 1.2.2 Tumor heterogeneity

A cancer cell, simplified, is a cell inside a multicellular organism that, due to mutations in oncogenes, becomes insensitive to cellular regulatory mechanisms and adopts the purpose of a unicellular organism, therefore endlessly multiplying. This will eventually disrupt and destroy surrounding tissue.

Tumor heterogeneity occurs mainly as a consequence of four factors; 1) the cell type of the cancer cell origin [25], 2) the mutations developed during carcinogenesis [25, 26], 3) random mutations in the genetically unstable tumor, creating diversity even within the tumor cell population [27] and 4) the evolutionary pressure, selecting for viable cancer strains [23, 27].

All tumors start as a single cell mutating into a more rapidly proliferating cell, of which all daughter cells will carry the same genetic damage. The compact mass of daughter cells forms a tumor. In this growing mass, eventually further mutations occur, splitting the tumor lineage into two distinct phenotypes. Later, a cell of either lineage may then pick up a third (oncogenic) mutation, and so on. In a study investigating colorectal and breast cancers, it was found that the average tumor have around 80 amino-acid altering mutations, of which 15 seemed to have oncogenic importance [28]. Note however, that the distinction between a benign tumor cell and a cancerous tumor cell can be fine; premalignant lesions require only one or a few mutations to turn cancerous. Thus, a tumor burden might develop or might have developed from the same pre-malignant lesions into cancer on more than one or occasion [29]. This random creation of a multitude of lineages contributes to tumor heterogeneity.

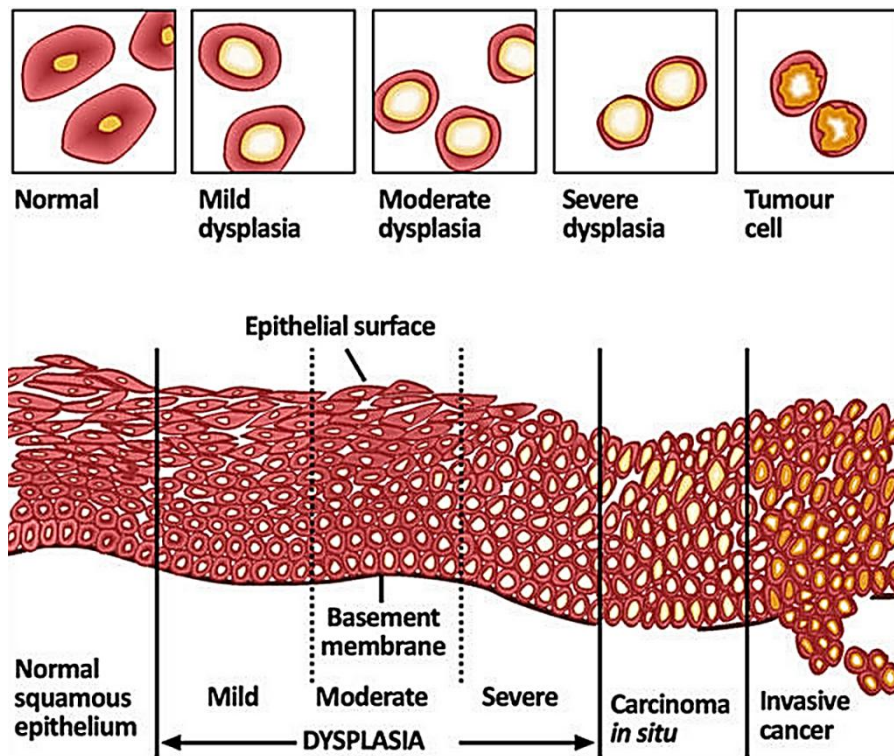


Fig2: Schematic presentation of epithelial tissue turning cancerogenous. Each shift towards a more cancerous phenotype is preceded by oncogenic mutations. Image reprinted under CC license, *Learn Oncology* © (<http://learnoncology.ca/wordpress/>).

In complex multicellular organisms, life begins as a single cell that will replicate and diversify into, for humans, more than 200 different cell types. This is a step-wise process, where parts of DNA not required for the intended function through various processes collectively known as epigenetics are made inaccessible. Each further diversification event closes the door to several “final form” cell types, creating a pyramid of sorts, with stem cells on top, with decreasing possibility for final forms the further down on the pyramid it goes, until finally reaching the base of the pyramid, the fully differentiated cells.

Thus, simplified, epigenetics determine which genes are more readily available for transcription. Consequently, the cell type of origin will determine which macromolecular machines that are active in the pre-cancerous cell, and thereby which genes that are “meaningful” to mutate [30]. A mutation in a non-expressed gene is of no relevance, and, different cancer forms differ in which genes are the most likely to be progress the tumor cells through the cancerogenesis – by providing a growth advantage, these mutations will be found in the most aggressive tumor cells, and thereby in the most prominent tumor lineages [31].

Finally, tumor cells must overcome several “barriers”. One literal barrier is the basement membrane, a thick matrix of secreted extracellular molecules, which will contain tumors until they find a way to bypass it. Another example is natural killer cells, cells of the immune system that will kill any cell that do not meet certain criteria. For instance, if MHC class I surface protein expression is downregulated it triggers natural killer cell that will kill the tumor cell. This evolutionary pressure therefore selects for cancer strains with MHC class I mimicry mutations [23].

## **1.3 Cytostatics**

### **1.3.1 General**

While cancers have been treated with herbal remedies since ancient times [32], regular systemic chemotherapy dates back almost 150 years [33] and the first properly documented modern clinical trials using chemotherapy was with mustard gas in 1943 [34], Sydney Farber is still heralded as the father of chemotherapy with his 1947 article that could show remission for patients with childhood leukemia treated with aminopterin [35]. These children would later go into recurrence, but the search for other novel drugs able to treat cancer had started, and in 1964, by the use of combination therapy, a cancer was for the first time by modern means verifiably cured using chemotherapy [36]. Since then, there has been an explosion of new cytostatics and combinations there off.

Cytostatic drugs are mostly given systemically and are not per se tumor cell specific, yet still target tumor cells. This is because the cytostatic drugs targets dividing cells and since under normal circumstances most cells in the body have relatively few cell divisions, tumor cells end up being more highly affected [37]. Tumor cell upregulation of metabolism also helps making tumor cells more susceptible [38]. But it must be noted that rapidly proliferating benign cells, for instance hair follicle cells and gastro-intestinal cells, are also more susceptible, giving raise to hair loss and bowel dysfunction, among other common side effects of chemotherapy [39]. For this reason, all cytotoxic drugs have severe side effects, and the desire to kill the cancer must be balanced with toxicity for the patient.



The therapeutic window refers to the concentration range where the drug asserts an effect on the tumor without causing unacceptable harm to the patient. Depending on the rate of clearance and inactivation of the drug by the body, primarily by the liver, and the rate of drug administration, drugs will after each administration remain at an active blood concentration level for a certain period of time. The tumor is most often not eradicated after a single administration, which is why the procedure is repeated after allowing the patient the time needed to recuperate. The number of treatments necessary depends on the drug(s) and the diagnosis [40, 41].

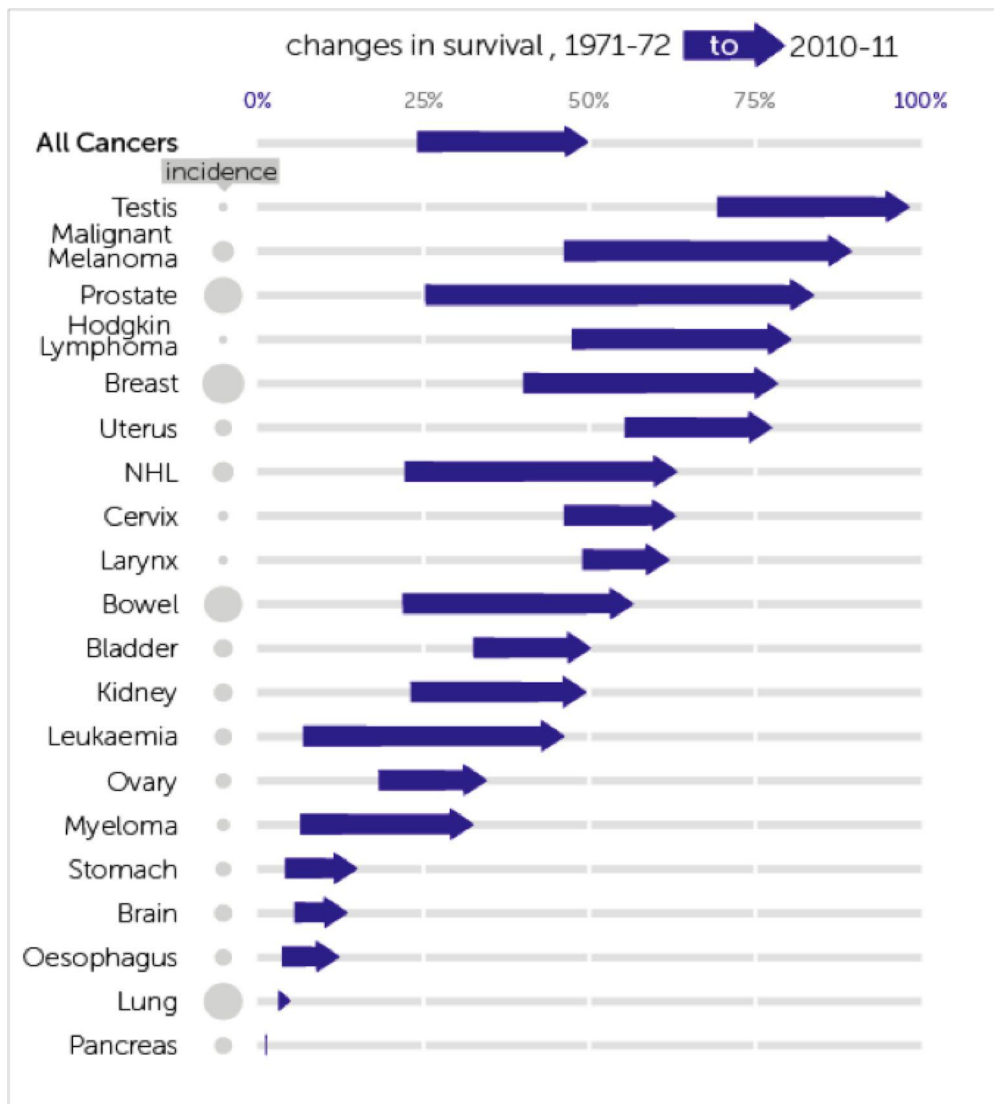


Fig3: Age-standardized ten-year net survival trends, in adults (aged 15–99) of selected cancers. Patients from England and Wales were included, spanning from 1971 to 2011. Reprinted from “Repositioning approved drugs for the treatment of problematic cancers using a screening approach” under *PlusOne*© CC.

### 1.3.2 Classes of cytostatics

Most cytostatic drugs target the replication process. In order to replicate, the cell needs to synthesize the nucleotides that will be incorporated as complementary strands to both original DNA strands. Anti-metabolites are a class of drugs that mimic metabolites critical for dNTP synthesis. The consequence is that enzymes, unable to distinguish between its natural metabolite and the anti-metabolite, randomly uses or attempts to use the anti-

metabolite instead of the metabolite. This can lead to inhibition of the enzyme activity, as the anti-metabolite have a structure that cannot be processed, or, if the metabolite is properly processed and inserted into DNA, downstreams DNA damaging effects, as the anti-metabolite will make the DNA unstable or unreadable. Pemetrexed is an example of a folate acid mimicry. By competing with the intended substrate during TS (thymidylate synthase), DHFR (DiHydroFolateReductase) and GARFT (glycinamide ribonucleotide formyltransferase) activity, dNTP synthesis is inhibited, therefore hampering progression through the S-phase [42].

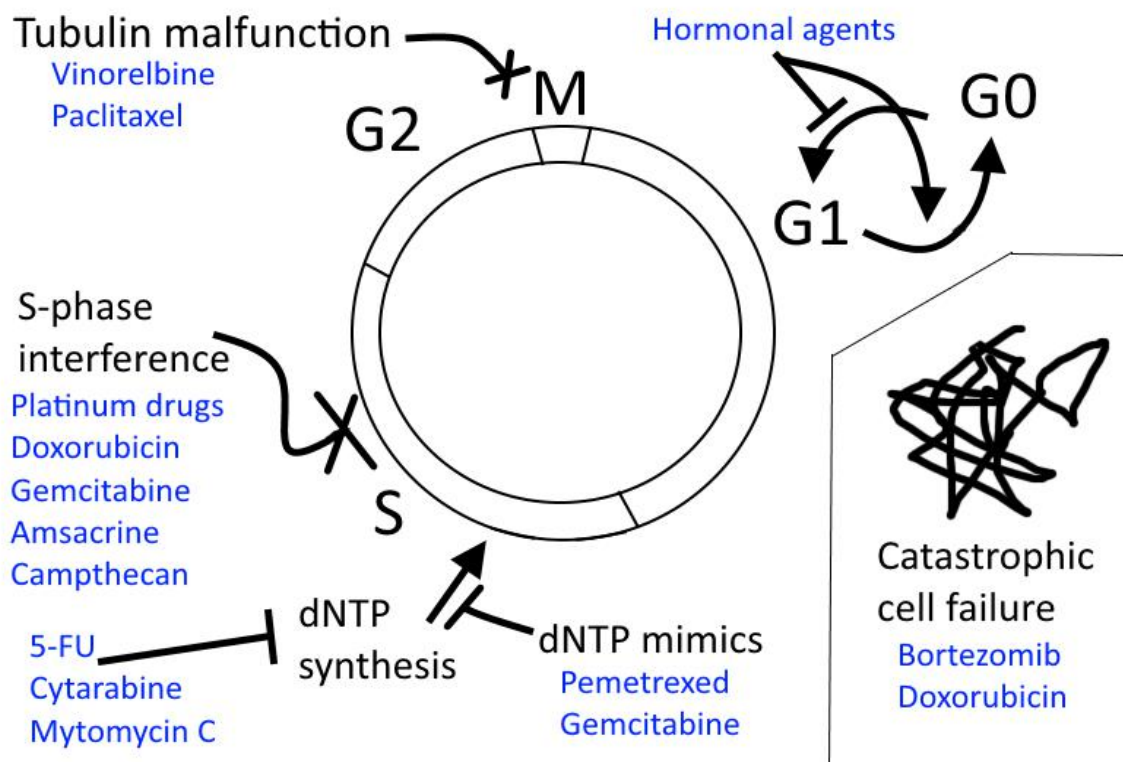


Fig4: Schematic representation of the cell cycle overlaid with a selection of cytostatics and their respective targets. Note how most of these drugs directly or indirectly targets the S-phase.

Gemcitabine is an example of an antimetabolite that is inserted into DNA during replication [43]. Once there, it prevents further elongation of the complementary strand, causing replication failure and DNA shearing, which will trigger apoptosis. Moreover, gemcitabine also irreversibly bind the active site of RNR (ribonucleotide reductase) [44], which, like pemetrexed, inhibits dNTP synthesis.

Carboplatin creates DNA adducts [45], and although not adding alkyl groups to DNA, it carries out the same function as alkylating agents and is classified as such. In LAC, carboplatin is combined with gemcitabine (GC) [20] and in MM with pemetrexed (PC) [46, 47]. Failure to exit S-phase triggers DNA repair mechanisms, such as ERCC1 (excision repair cross-complementation group 1), an enzyme that cuts away the damaged strand, followed by incorporating dNTPs to the complementary strand [48]. An acute shortage of dNTPs will lead to this process failing [49] – importantly, the DNA will thus at every instance where ERCC1 have bound remain single-stranded until more dNTPs are located. It is well known that platinum drugs cause shearing of DNA, and one could speculate whether this is the process by which that occurs [50]. In either case, once the DNA repair

mechanisms are triggered, and no “the DNA is rescued” signal follows, S-phase arrest will eventually trigger apoptosis [51].

Doxorubicin is an anti-tumor antibiotic, a group of cytostatics so called because they were first discovered in unicellular organisms, much like penicillin. Common for all anti-tumor antibiotics is that they target DNA replication. Doxorubicin is a multi-functional drug with several acutely toxic effects [52], which includes triggering ROS, but the most important function is DNA intercalation [53] – it inserts itself between the layers of DNA. Once there, it will interfere with the function of topoisomerase II, a protein that stabilizes double-stranded DNA, hampering helicase function. This interference leads to replication failure, and trigger apoptosis. Doxorubicin is therefore also an example of a topoisomerase inhibitor. The drug combination of cisplatin (a platinum drug, like carboplatin) [54], doxorubicin and gemcitabine (CDG) have previously been used as a SOC for MM [18].

Once synthesis is completed, the cells enter the mitosis phase. During this phase, DNA is condensed into chromatin, and pulled apart by microtubules. Microtubules consist of spiraling loops of tubulins (a dimer of alpha and beta tubulin), 13 for each rotation,

Quickly assembling and disassembling, in a process known as dynamic instability [55]. Mitosis inhibitors either bind tubulins, making assembly difficult, or stabilize the microtubules, making disassembly difficult. Interfering with this dynamic instability cause failure to exit M-phase, which triggers apoptosis [56]. Vinorelbine is a mitosis inhibitor used in LAC and MM [19, 57, 58]. It can be used in combination with cisplatin or gemcitabine, although their proposed combinatory effect is unclear; cells that due to carboplatin and gemcitabine cannot exit S-phase also cannot enter M-phase, where vinorelbine exerts its effect. Thus, this could be a clear-cut example of covering more bases rather than the combined effect pushing the tumor cell to its limit that we could appreciate for the combinations GC, PC and CDG.

On top of these drug classes, there are also hormonal agents, and drugs with unknown mechanism of action. Hormonal agents generally work by restoring function of mutated proteins or even by inducing differentiation, which puts the cancer cells into G0-phase, thus not killing them but subverting them [59]. This may inhibit tumor growth, by restoring the normal levels of proliferate signaling, which, due to the DNA damage tumor cells have suffered, tips the scale towards apoptosis signaling [60].

### **1.3.3 Drug sensitivity profile**

Drug resistance is a serious concern for treating physicians; solid tumors tend to relapse following chemotherapy, and when they do, they may have evolved into a more aggressive phenotype. This can be explained by clonal selection of cancer cells with increased resistance to given drugs. Each tumor is genetically unstable. Every replication adds a risk for further mutations, where clones with growth advantage will expand. Thus, in theory, the larger amount of tumor at the initiation of treatment, the more likely are subpopulations of resistant cells that will survive and become predominant under the pressure of chemotherapy [61].

Cytostatics can be classified based on their mechanism of action. Tumor cells, on the other hand, are not only different depending on the diagnosis, but also inter-individually. These differences may include variations in the pathways that can be targeted by the cytostatics used. On top of picking the right drug target for the right tumor, resistances also develop over time following chemotherapy [62]. For instance, the drug target can be further mutated

so that the drug can no longer bind or otherwise exert its function, or the tumor cells can bypass the drug target itself, by instead inhibiting or hyper activating a function downstream of the target [63]. Even if the vulnerability remain, secondary resistances can render the drug ineffective, for instance by inactivating the drug, relocating drugs from the area where they exert their effect or by developing resistance towards the damaging effect of the drugs. An example of bypassing a function is hyper activation of KRAS following gefitinib treatment, a drug that targets EGFR, since KRAS is downstream of EGFR [64]. An example of a secondary resistance is upregulation of ERCC1 [65], a molecular machine which can excise and replace DNA adducts created by platinum drugs. Further, resistances might have already developed inadvertently in chemotherapy naïve patients, as a consequence of tumor heterogeneity [66].

Novel mutations causing resistance to cytostatics often do not confer full resistance to the drugs; as is shown by cell line experiments, increasing the drug concentrations will eventually overpower the defenses [67] – but you cannot increase concentrations in patients without unwanted side effects. Most drug regimens are a combination of two or more cytostatic drugs that oftentimes also includes non-cytostatic drugs that mitigate the side effects of the cytostatic drugs. Combination therapies are often designed so that the first drug potentiates the second one, i.e., the combination synergistically turns the tumor cell non-viable. In other cases drugs in combinations target different functions of the tumor cell, thereby increasing the likelihood of effect.

### **1.3.4 Estimation of the tumor cell drug sensitivity profile ex vivo**

As the tumor increase its burden on the patient, the total number of replications increase, thereby increasing the likelihood of acquiring new mutations eventually resulting in a subpopulation of drug resistant cells. The risk of recurrence decreases with the effectiveness of the given chemotherapy [61]. The different SOC for MM and LAC used have an ORR of 30-40% [17-21]. Both patients that are multi-resistant and those multi-sensitive to chemotherapeutic options would receive only limited benefit from an individualized choice of drugs. However, it is also likely that many patients are resistant to one class of drugs but not another. Drug sensitivity profiles have been attempted since long, using tumor biomarkers, genetic analyze and *ex vivo* drug exposure of patient own tumor cells. In theory, perhaps the chaotic reshuffling and mutations of DNA and high heterogeneity can result in a phenotype or mix of phenotypes perhaps difficult to appreciate with genetic tests alone. Better then to test the phenotypes directly by *ex vivo* drug exposure. Studies of the survival benefit of selecting the most appropriate drugs over the SOC have shown promising results [68-70]; however, effectiveness varying from one study to another and with the way drug effects are estimated [71, 72]. Tumor cells from effusions have been used for this purpose, however, without considering the effects of a varied tumor cell proportion.

## 2 AIMS

The aim of this thesis is to determine the *in vitro* sensitivity profile of malignant cells obtained from effusions. The rationale for such an *in vitro* testing is that the response rate is currently only 30-40% patients with malignancies in the serosal cavities respond to the standard of care cytostatic drug regimens. Previous studies attempting drug response predictions using tumor cells isolated from effusions have had a varying degree of success, yet none have found their way into regular clinical practice. The overall aim of this thesis is therefore to further refine the methods used for drug response prediction by increasing the prediction accuracy.

Specific aims:

<b>Paper I and II</b>	To establish drug sensitivity profiles against a battery of 32 drugs, and compare this data to patient overall survival
<b>Paper III</b>	To determine to which degree benign cell presence mask activity of the tumor cells, and to find ways of performing tumor cell specific assays. Secondary aims included finding working concentrations, investigating the combined effect of using two or more drugs compared to drugs as singles and to investigate the difference between dissociated cells and cells in 3D-structures.

## **3. Methodology and Results**

### **3.1 General methods**

The basis for this work was tumor cells isolated from malignant effusions of the pleura, pericardium or peritoneum. The cells were separated from the effusion liquid using centrifugation followed by growing them in cell culture medium. In general, there is a mix of benign and malignant cells in these isolates. The proportion of tumor cells in the samples was determined by immunocytochemistry (ICC) or flow cytometry (FACS). Both ICC and FACS used antibodies against the tumor markers mesothelin, EpCAM and / or EMA and inflammatory cell marker CD45.

Attempts to increase the proportion of tumor cells in the isolates (paper I, II and III) were made using overnight cell culturing, a technique whose outcome depends on the tumor and benign cells propensity to adhere or remain in the suspension. Additional methods used for tumor cell enrichment comprised, filters, manual removal of macroscopic tissue fragments and magnetic beads labelled with tumor marker EpCAM or with inflammatory cell marker CD45. Tissue fragments will adhere and spontaneously release dissociated cells. To decrease dissociation times, the area to volume ratio was increased by shearing larger tissue fragments into smaller fragments using treatment with trypsin or collagenase under heavy vortexing.

To determine the sensitivity to different drugs *in vitro* the isolates were exposed to batteries of cytostatics for 48 or 72 h, followed by the measuring of their relative survival compared to an untreated control. The isolates from malignant effusions were also compared to cells isolated from benign effusions (paper I-III). Finally, the results from the *in vitro* tests were compared to outcomes of given treatment as presented in patient journals. Of interest were overall survival (paper I and II) or RECIST assessment of tumor response (paper III). RECIST classifies the response as one of four possible outcomes: progressive disease (PD), when the tumor mass has increased more than 20%, stable disease (SD), when the change was too small to quantify for PD or PR, partial response (PR), when there was more than 30% decrease in tumor mass and complete response (CR) was there was no evidence of disease. RECIST is generally evaluated as the change from before initiation of treatment to the situation 8-12 weeks following initiation of treatment.

### **3.2 Drug screening using a large battery (Paper I and II)**

Cells were isolated in short term cultures from malignant effusions caused by malignant mesothelioma (Paper I) or from lung adenocarcinoma (Paper II) and from 4 benign effusions (paper I and II). The cells were seeded in 384-well plates and exposed to a battery of 32 cytostatics for 72 hours. Each drug was tested at 4 different concentrations, followed by a colorimetric live-dead assay. The results were recorded using an automated laser confocal microscope that measured the color of each cell individually. Images were analyzed through automated software that calculated the number of live and dead cells.

While some mesothelioma cell isolates were sensitive to a broad spectrum of drugs, other appeared resistant to all tested cytostatics. The sensitivity profile differed, i.e., some isolates were sensitive to one set of drugs while being killed by other drugs. We were able to find a

correlation of *in vitro* drug susceptibility and patient overall survival, as well as the relative expression of two proteins involved in general drug resistance (ERCC1 and RRM1) for malignant mesotheliomas (Paper I) but not for lung adenocarcinomas (Paper II).

Mathematical compensation for the benign admixture, by assuming benign cells from effusions of benign origin would behave similarly to benign cells from effusions of malignant origin, showed an increased correlation of our *in vitro* determined general drug susceptibility to patient overall survival.

### 3.3 Tumor specific drug screening (Paper III)

Based on experiences from paper I and II, we identified two limitations with the employed sensitivity testings:

1. The admixture of benign cells may have a significant impact on survival data from tumor isolates as a whole. More precisely, the mathematical compensation suggested that tumor cells and benign cells have different response to cytostatics, possibly resulting in an error related to the relative ratio between the different cell types.
2. Many of the drugs were at given concentrations ineffective in more cases than described response rates *in vivo*.

At the Karolinska University Hospital, Sweden, drugs are given as combination therapies for lung adenocarcinomas involving the pleural, pericardial or peritoneal cavities (carboplatin plus gemcitabine) and for malignant mesotheliomas (pemetrexed plus carboplatin). To clarify if both used drugs were effective and to reveal possible synergistic effects, we found it relevant also to test the effect of administrating the drugs in combination.

This approach, however, necessitated a downscaling from 32 to 6 tested drugs in order to allow optimization of the methodology. We included the drugs that are relevant in the Swedish clinical setting (carboplatin, gemcitabine and pemetrexed). For comparison, we also included drugs from common alternative first line chemotherapy (vinorelbine, as a single, or in combination with carboplatin or gemcitabine) as well as an alternative regimen (doxorubicin, in combination with carboplatin and gemcitabine). Finally, even though carboplatin today is more commonly used, carboplatin can be exchanged with cisplatin in all combinations described above, and this drug was therefore also included.

The issue of insensitivity to clinical drug concentrations was solved by using stepwise alterations of the concentrations until we could identify “working” concentrations; levels at which the inter-isolate variation would distinguish sensitive from insensitive ones. Pemetrexed only rarely showed effect at 48 hours, even at high concentrations. Therefore, pemetrexed was measured using cell cycle histograms, comparing the cells in early S-phase of sample and control, instead of comparing survival.

The interfering effects of benign cells present in the effusion could be demonstrated by comparing survival of MACS-beads enriched and depleted fractions of the same isolate and by comparing the benign and tumor specific apoptosis in unaltered isolates using FACS annexin-V / PI apoptosis assays. Tumor specific measures were ensured by using fluorescence-labeled antibodies against tumor markers. This disturbing effect of benign

cells was substantial when the tumor cells amounted to less than 75% of cells present, which was the case for most isolates. The FACS viability assays were found superior, because the MACS-beads often failed to increase the tumor proportion to 75% or above.

With this study design it was also possible to show that drugs, even when they as singles did not elicit any response, could have a profound effect when combined. The *in vitro* results of pemetrexed and carboplatin of nine isolates were compared to patient outcome, estimated as RECIST scores at 8-12 weeks after initiation of treatment and described in the patients' journals. There are four possible such scores: progressive disease (PD) when the tumor mass has increased  $\geq 20\%$  on evaluation, stable disease (SD) when the change was too small to qualify for PD or PR, partial response (PR) when there was  $\leq 30\%$  decrease in tumor mass and complete response (CR) where there was no evidence of disease when evaluating effect of treatment. Out of the nine isolates, seven could correctly predict the scores. However, no conclusions can be drawn regarding sensitivity and specificity of the prediction based on this limited number of patients.



## **4. SUMMARY**

### **4.1 Discussion**

The mean overall survival for malignant mesothelioma is around a year, and around five months for metastatic lung adenocarcinoma with pleural involvement. For both situations chemotherapy is in most cases the only available treatment. The standard of care first line of chemotherapy has varied historically, and from hospital to hospital. Common for all these treatments is a roughly 30-40% objective response rate, resulting in similar overall survival.

Cytostatics can be classified based on their general mechanism of action. Still, drugs within the same class may differ in their specific mechanism of action, including their specific target(s). The biologic differences in tumors from different patients influence the susceptibility to cytostatics, depending on the drug specific mechanisms of action. Therefore, a guided choice of cytotoxic drugs based on the analysis of drug sensitivity would increase OS by virtue of increasing the response rate. This working hypothesis is supported by the results from in vitro testing, with highly variable patterns for the different isolates (paper I-III).

There was no single patient isolate that was sensitive to only a single microtubule agent (paper I). All patient isolates but one with sensitivity to docetaxel also reacted to paclitaxel, while more than half of the isolates sensitive to at least one taxane were sensitive to at least one vinca alkaloid too. Likewise, sensitivity to actinomycin D is often telling of sensitivity to daunorubicin. Some isolates were multiresistant and some were multisensitive, while other isolates were clearly sensitive to one class of cytostatics and as clearly resistant to another (cf paper II, Figure 2). Thus, resistance to one class of cytostatics does not necessarily mean resistance to another class.

There are many specific mutations, that alters expression or function in a way causing resistance or sensitivity towards certain cytostatic drugs. One example is gain of function mutation in some lung adenocarcinomas is the epidermal growth factor receptor deficiency (EGFR), an oncogene. The mutation is an event early during carcinogenesis, and all tumor cells will therefore carry this same genetic deficiency. Specific EGFR inhibitors will then have a good effect on these tumors, although a later second mutation in the same gene will block the effect of the EGFR inhibitor, thereby causing relaps of the disease. Other lung cancers show mutated KRAS, which is another protein in this replication activating pathway, downstreams of EGFR. Such a gain of function mutations in KRAS will then not be influenced by targeting the upstream EGFR inhibitor.

An alternate way of predicting sensitivity or resistance to certain drugs is to demonstrate the expression of factors related to gene expression or DNA repair. These compounds can be shown on either as RNA expression or as proteins by immunocytochemistry. Two substances that have been suggested as such prediction markers for chemoresistance are ERCC1 and RRM1. ERCC1 is involved in DNA repair mechanisms and RRM1 in RNA synthesis. When analysed on the mesothelioma samples there seemed to be a correlation between general drug resistance and high RRM1 expression, while no such tendency could be seen in lung cancers. The simultaneous analyses of ERCC1 showed no correlation to the drug sensitivity pattern. It may well be that the synthesis and repair of nucleic acids, counteracting a drug's mode of action, can be rescued also by other factors and alternate pathways, which makes this way of predicting response to therapy uncertain.

These studies are based on primary tumor cell isolates obtained from effusions, most of them from the pleura, but also some from the peritoneum and the pericardium. These cells can be assumed to reflect the "current" situation of the cancer, representing a part of the tumor with metastatic capacity and perhaps also representing more aggressive clones of the tumor mass. Malignant effusions also contain a mixture of benign immune cells and macrophages together with benign mesothelial cells. The mathematical compensation for the benign contribution (paper I and II) was based on the drug sensitivity profile of benign cells in four effusions from patients without malignant diagnoses.

The influence of such benign cells could better be shown when their proportion was altered experimentally (Paper III). Surprisingly the effect was unpredictable. While in some cases tumor enrichment increased drug sensitivity, in other isolates the effect was reversed. This effect of the benign "noise" can thus not be accurately determined by comparing with cells from benign effusions, but should be considered in each single case. The use of FACS circumvents this problem, since this allows the specific analysis of tumor cells. This "noise"-effect became minute, when the tumor cells represented >75% of the cellular yield. Effusions with such richness in tumor cells often, however, represent a more advanced stage of the cancer, with less effects of cytostatics. Effusions with less tumor cells are therefore more interesting in this context, and in these cases FACS is a clearly advantageous alternative.

It is well known that the biology and function of cancer cells not only depend on intrinsic factors but also on their microenvironment. Thus when growing them on a hydrophobic support, they attach less and spread less on the plastic surface compared to growing them in hydrophilic flasks. Instead they tend to form spheroids, with numerous desmosomes with some similarities to the situation in dense cultures. Such confluent cultures as well as spheroids decrease their sensitivity to cytostatics. As expected, growing the cells on the hydrophobic support increased the drug resistance in an unpredictable way. However, it was difficult to create uniform spheroids, therefore, better protocols or some manner of

mathematical compensation would have been necessary for inter-isolate comparisons.

The drug concentrations used for the broad screening (paper I and II) were calculated based on area of curve calculations of patient drug blood levels; corresponding concentrations in the extracellular microenvironment around the tumor cells is not known. While clinical responses in the 30-40% range is seen with drugs, such as cisplatin, carboplatin, gemcitabine and pemetrexed, there was complete or almost complete resistance to these drugs in the experimental setting (Paper I and II). In order to find a concentration where the test discriminated between more and less sensitive isolates during the 48 hours exposure, the drug concentration levels were elevated to beyond that of the previously used concentrations (paper III). The risk with this approach is that supraclinical concentrations might activate other apoptosis pathways than what occurs in the patient.

The short time for culturing the cell isolates (48 hours) was selected for several reasons. First, with longer times it may become necessary to change medium during exposure. The mechanical handling of the culture when changing medium may influence the cells in an unpredictable manner and there will be a random factor depending on how many supernatant and attached cells are lost. Then, a clinically useful predictive test preferably provides the results within a reasonable time. For this test, isolation, two days culturing and analyzing the results, can be performed within a week. It should also be considered that longer culturing may result in expansion of certain tumor clones that may give misleading result.

We managed to find “working concentrations”, concentrations that at 48 hours gives rise to differences in response (paper III). Pemetrexed is a drug that in general will not elicit apoptosis during the short exposure, even at high concentrations. Effects of the drug could instead be shown by analyzing the content of DNA by FACS, estimating the proportion of cells in S-phase arrest using a cell cycle distribution histogram. Variations thereof were able of distinguishing sensitive and non-sensitive isolates.

Attempts to predict drug effect often correlated with the clinical outcome (paper III), although the number of patients should be expanded for a final evaluation of the predictive possibilities. Therefore, before personalized medicine based on drug sensitivity assays can become a reality, these assays must prove their reliability in predicting the patient response to standardized regimens.

## 4.2 Conclusions

- \* There is a great diversity in *in vitro* drug sensitivity profiles.
- \* As with cell lines, pemetrexed is best measured as S-phase arrest at short exposure assays
- \* Interference of benign cells is substantial in isolates with < 75% tumor cells
- \* Cell sorting techniques employing filters and MACS-beads can be used to increase the tumor proportion at least 2-fold (for isolates with <45% tumor cells) for at least 70% of all isolates.
- \* FACS can measure the tumor specific apoptosis using fluorescence labeled annexin-V, PI and antibodies against tumor markers
- \* FACS seem to outperform MACS-beads and filters when tumor proportions are low
- \* Growing tumor cells into artificial spheroids increase their resistance to drugs

## 4.3 Future perspectives

This thesis describes a work aimed at optimization of experiment conditions for patient-own tumor cells derived from malignant effusions in short term cultures. The purpose is to establish a drug sensitivity profile for said tumor, with an ultimate purpose of predicting patient drug response. When these predictions become sufficiently accurate, they can be used to guide a personalized choice of drugs, instead of the statistically most likely effective drug according to standardized programs. Established regimens have objective response rates in the ranges of 30-40%, and it has been shown that drug responders have an improved overall survival over drug non-responders [73]. It was also found (papers I, II and III) from experiments with *ex vivo* cultures that resistance to one drug regimen does not necessitate resistance to another, there is reason to believe that guided choice of therapy could have a survival advantage over conventional regimens.

Survival index (SI), comparing the proportion of live and dead cells of a treated sample with an untreated control is the most commonly used measure to evaluate the cytostatic effect on tumor cells *in vitro*. However, different studies using SI as outcome are difficult to compare if different drug concentrations are used. Paper III showed that short term (48 h) primary tumor cell isolates should (a) be tumor specific and (b) use an extended range of concentrations. All cytostatic drugs tested in paper III, with the possible exception of pemetrexed, functions as standard inhibitors, where the maximal efficiency is complete death of the entire cell population. Therefore, for these drugs and at 48 h incubation, the concentration which yields an SI of 0.5 is always equal to the half maximum effective concentration (EC50), and further, the drugs have near linear dose-response relationship shortly following minimal inhibition and shortly before maximal inhibition (see figure 5, below).

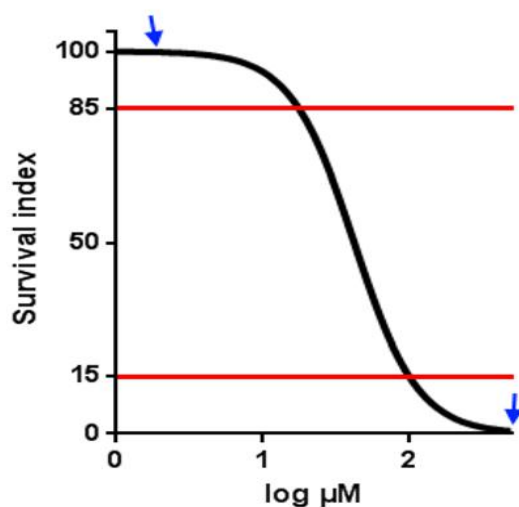


Figure 5: Celline M-14-K tested at 24 different concentrations of gemcitabine and analyzed as a standard inhibitor curve in prism v6.0. Note the blue arrows indicating minimal and maximum efficiency, and the near linear relation of survival index and concentration between the red lines.

Thus, EC50 values are readily calculated using a straight-line equation, as long as there are at least two concentrations with sufficient distance from each other in the linear range. Using cell line data, we could determine that the accuracy of that calculation is within 0.85 to 1.15 fold compared to the 24 data point standard inhibitor curve for gemcitabine, and within 0.95 to 1.05 fold for carboplatin.

Thus, assuming sufficiently high concentrations are used to land within the effective range and assuming appropriate distance between the tested concentrations, SI can instead be reported as EC50 with minimal effort. This simple adaption would produce higher quality meta-studies in the field of cancer research this thesis concerns.

Of course, the relevance of EC50, i.e., the predictive value, still need confirmation through correlation with the clinical routine, by comparing obtained *ex vivo* drug sensitivity profiles with patient outcome. The obvious continuation of the optimization work herein would be a large cohort study using the methods and strategies just presented. Assuming a positive outcome, that future study could then be presented before an ethics committee, hopefully

resulting in a follow up clinical trial of guided choice of therapy as one arm and the conventional regimens as the other arm.

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For humans, the concept of adulthood is a strange one. The more social a creature becomes, the more childhood is prolonged. Childhood is a time of learning all the skills of the trade one would need to survive, once it is time to leave the safety of the parental nest. Evolution sugarcoats this training for adulthood as games and play, and the need for parents to fend for their offspring until they can fend for themselves is sugarcoated in feelings of love and pride. An adult wolf might become wiser as the years pass, but he is, none-the-less, not so different once adulthood is reached – he need not learn more, and thus shed his interest in games and play. Humans on the other hand, perhaps especially those of us involved in science and research, constantly need to update our set of skills. Even as we do leave the parental nest, we need to maintain our intellectual curiosity.

When I finished school, I thought I had become an adult, yet, university proved there were more to learn. Then I graduate university and am convinced that now, finally, I am an adult. Yet, I register as a PhD student, and I found out that I know so very little. Am I an adult then, finally, now that I graduate? Building on my previous experience, I would have to say that scientists never grow up, because there is never a point in time when we can consider ourselves fully trained, and can safely shed our intellectual curiosity to simply focus on being an adult by applying a constant skill set. Or perhaps we become adult several times? Whatever the truth is, I must conclude I am wiser now, than when I started, for this have indeed been a learning experience, an exposure to a world you could not fathom, as you take your master degree.

Now then, on to the task at hand, recognizing those that helped me climb this particular rock. First off, of course, a big thank you to my supervisors Anders Hjerpe and Katalin Dobra, first of all for welcoming me to this group, and secondly for steering the project course clear.

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