MASTER'S THESIS

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New therapeutic strategies for peritoneal carcinomatosis, a preclinical approach

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Betlehem Biratu Oljiya

OsloMet – Oslo Metropolitan University

Faculty of Health Sciences, Department of Life Sciences and Health

and

Department of Tumor Biology, Institute for Cancer Research

Norwegian Radium Hospital

Oslo University Hospital

Supervisor: Yvonne Andersson

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Abstract

About 25-35 % of patients with colorectal cancer (CRC) will develop peritoneal carcinomatosis (PC) at some point in time after initial diagnosis. PC has a very poor prognosis. The gold standard treatment for these patients is cytoreductive surgery (CRS) combined with hyperthermic intraperitoneal chemotherapy (HIPEC). HIPEC enables usage of higher chemotherapy doses, and the heated solution may increase the absorption of the drugs into tumor cells. Consequently, this could remove all microscopic tumor and free cancer cells after CRS. CRS-HIPEC have improved the overall survival (OS) of PC-CRC patients, however most patients experience relapse and treatment associated morbidity and mortality.

Relapse is caused by residual cancer cells, which may have developed resistance against chemotherapy/hyperthermia. Thermo-tolerance in cancer is usually based on the induction of heat-shock proteins (HSPs). In this master thesis, we aimed to investigate the sensitizing effect of hyperthermia on the chemotherapy drugs, Mitomycin C and Oxaliplatin, which are commonly used in the clinic HIPEC procedure to treat PC-CRC, was studied in our newly developed *in vitro* model, which closely mimic the clinical HIPEC condition. An *ex vivo* model on CRC-PC tumor tissues samples, was also tested in attempts to bring our results even closer to the clinic.

Methods: For this experiment, we used two CRC cell lines HCT116 and HT29 with different mutation profiles, and fresh PC-tumor tissues from patient-derived xenograft (PDX)-mice and one patient. Hyperthermic chemotherapy experiments were performed in temperature controlled water baths, 37°C and 42°C. HSP90 inhibitor (17-AAG) and HSP70 inhibitor (HS-72) were included in the experiments, in effort to enhance the cytotoxicity effect of the treatments. In addition, HSF1 was silenced using a short interfering RNA (siRNA). Cell viability assay was performed by MTS-assay, either 24, 48 or 72 hours post treatment. Furthermore, we investigated the effect of hyperthermia to induces immunogenic cell death (ICD) by measuring extracellular HMGB1. HSPs, HSF1 and HMAGB1 expression was measured using Western blot.

Results: Treatment response including hyperthermia was observed to be cell line and chemotherapy drug selective. We found HT29 to be less sensitive than HCT116 cells to both drugs at 37°C. HSPs inhibition did not provide additional effect on the treatment. 17AAG itself did contribute to significant cell viability reduction, but addition of hyperthermia did not further decrease in cell viability. Silencing HSF1 did not increase the effect of hyperthermic chemotherapy treatment, which was our hypothesis. Interestingly, we found hyperthermic

chemotherapy to increase a much higher HMGB1 release compared to cells treated at 37℃, indicating immunogenic cell death.

Conclusion: We conclude that hyperthermia has some form of beneficial effect, depending on the drug and/or cancer cell populations examined. Hyperthermia induces high expression of HSPs, however, inhibition of HSPs by HSP inhibitors and silencing HSF1, did not enhance the cytotoxic effect of hyperthermic chemotherapy. Interestingly, hyperthermic chemotherapy increases HMGB1 release, which indicates immunogenic cell death. This could perhaps verify the beneficial effect of hyperthermia in HIPEC treatment. However, further studies, using other cytotoxicity assay are required to validate our result.

Sammendrag

Det er anslått at ca. 25-35% av pasientene med kolorektal kreft (CRC) vil utvikle peritoneal karsinomatose (PC) noen gang i tiden etter den første diagnosen. PC har en svært dårlig prognose. Standard behandling for disse pasienter er omfattende cytoreduktiv kirurgi (CRS) kombinert med hypertermisk intraperitoneal kjemoterapi (HIPEC). HIPEC muliggjør bruk av høyere kjemoterapeutiske doser, og den oppvarmede løsningen er med på å øke absorpsjonen av legemidlene inn i tumorceller. Dette vil da kunne føre til fjerning av mikroskopisk tumorer og frie kreftceller etter CRS. CRS-HIPEC-behandling gir langsiktig overlevelse, men de fleste pasienter vil oppleve tilbakefall og behandlingsrelatert sykelighet og dødelighet. Kreftceller kan klare å utvikle mekanismer / resistans som beskytter dem mot kjemoterapi og hypertermia. Termotoleranse i kreft er vanligvis basert på induksjon av såkalte, Heat Shock Proteiner (HSP). I denne masteroppgaven, hadde vi som mål å undersøke om hypertermi kunne øke effekten av kjemoterapiene, Mitomycin C og Oxaliplatin, som oftest brukes i klinisk HIPEC-prosedyre for behandling av PC-CRC, ved å bruke vår nyutviklede *in vitro*-modell, som etterligner den kliniske HIPEC. Et *ex vivo*-eksperiment på ferske CRC tumor vev ble også brukt, for å kunne relatere våre resultater nærmere klinikken.

Metoder: Til dette formål har vi bruk to CRC-cellelinjer, HCT116 og HT29, med forskjellige mutasjonsprofiler og ferske tumorvev fra PC fra pasient deriverte xenograft (PDX)-mus modell og pasient. Hypertermisk kjemoterapi eksperimentet ble utført på temperaturregulerte vannbad, 37°C og 42°C. HSP90-inhibitor (17-AAG) og HSP70-inhibitor (HS-72) var også inkludert i denne studien for å øke effekten av behandlingen. I tillegg ble HSF1 mRNA skrudd av, ved hjelp av et kort interferens RNA (siRNA). Celleviabilitets analyse ble utført enten 24, 48 eller 72 timer post-behandling, ved hjelp av MTS-assay. Til slutt, undersøkte vi om hyperthermi induserer immunogen celledød (ICD), ved å måle ekstracellulær HMGB1. Ekspresjon av HSPs, HSF1 og HMGB1 ble målt ved bruk av Western blot.

Resultater: Behandlingsrespons fra hypertermi ble observert å varierende mellom cellelinjene og kjemoterapiene. Vi fant HT29 å være mindre følsom for begge legemidler ved 37°C. HS-72 ga ikke ytterligere effekt på behandlingen. Alene bidro 17-AAG til betydelig reduksjon av celleviabilitet, men kombinasjon med hypertermi bidro ikke til ytterligere reduksjon av celleviabilitet. Dessverre økte ikke «silencing» av HSF1 den cytotoksiske effekten av hypertermisk kjemoterapi. Men vi fant ut at hypertermisk kjemoterapi øket frigivelse av HMGB1, noe som indikerer ICD.

Konklusjon: Vi konkluderer med at hypertermi har noe fordelaktig effekt, avhengig av av legemiddel og / eller kreftcellene den brukes med. Hypertermi induserer høyt uttrykkelse av HSPs, men inhibering av HSPs, ved hjelp av HSP-inhibitorer og «silencing» HSF1, forbedret ikke den cytotoksiske effekten av hypertermisk kjemoterapi. Interessant nok, øker hypertermisk kjemoterapi HMGB1-frigjøring, noe som indikerer ICD. Dette kan kanskje verifisere den fordelaktige effekten av hypertermi ved HIPEC-behandling. Imidlertid er det nødvendig med ytterligere studier, ved bruk av annen levedyktighet og cytotoksisitets analyser, for å validere vårt resultat.

Abbreviation	Description
ADP	Adenosine diphosphate
Ag	Antigens
ATP	Adenosine triphosphate
B-actin	Beta-actin
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
ECL	Enhanced chemiluminescence
ETM	Epithelial-to-mesenchymal transformation
CC	Completeness of cytoreduction
CRC	Colorectal cancer
CRS	Cytoreductive surgery
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
DAMP	Danger associated molecular patterns
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-to-mesenchymal transition
HIPEC	Hyperthermic Intraperitoneal Chemotherapy
HMGB1	High mobility group box-1 protein
HRP	Horseradish peroxidase
HSF1	Heat shock factor 1
HSPs	Heat shock proteins
HT	Hyperthermia
ICD	Immunogenic cell death
IC50	Half maximal inhibitory concentration
IPC	Intraperitoneal chemotherapy
kDa	Kilo-Daltons (atomic mass unit)
MET	Mesenchymal-to-epithelial transition
MTS	CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay

NK	Natural killer cells
OC	Ovarian cancer
OS	Overall survival
PAGE	Sulfate- polyacrylamide gel electrophorese
PBS	Phosphate buffered saline
PC	Peritoneal carcinomatosis
PC-CRC	Peritoneal carcinomatosis from colorectal cancer
PC-OC	Peritoneal carcinomatosis from ovarian cancer
PCI	Peritoneal carcinomatosis index
PMP	Pseudomyxoma peritonei
PVDF	Polyvinylidene difluoride (membrane)
RMPI-1640 Medium	Roswell Park Memorial
SDS	Sodium dodecyl sulfate
TBST	Tris-buffered saline with Tween 20
URP	Unfolded protein response
°C	Degrees Celsius
µM	Micromolar (micromoles/liter)
ng/ml	Nanogram per milliliter

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Introduction

1.1 Cancer

Cancer is a disease characterized by out-of-control cell growth and proliferation. Cancer is a common disease worldwide, in Norway there were 32,827 new cancer cases in 2016 (Cancer Registry of Norway, 2016). According to the original hallmarks of cancer, tumor cells maintain proliferation by sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (figure 1.1) [1]. Cancer cells achieve all this, mediated via mutations in tumor suppressor genes or proto-oncogenes. In addition, epigenetic modifications, such as histone modifications and DNA methylation, can also contribute to those characteristics [2].

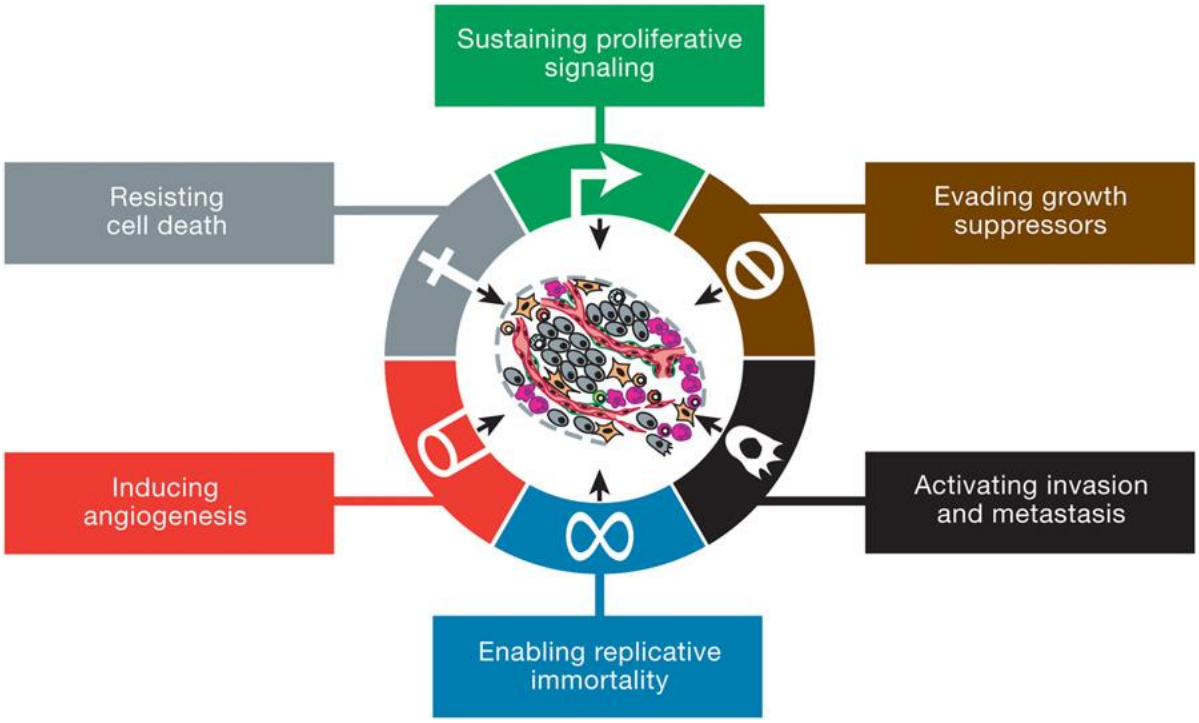


Figure 1.1: The six hallmarks of cancer proposed in 2000 by Hanahan and Weinberg. Lately, they have reported four more hallmarks.[3]

Uncontrolled cell growth leads to neoplasia (tumor), benign or malignant. Benign tumors grow slowly and are self-limiting. If left untreated, however, a benign tumor can grow large and squeeze normal tissues, which can have serious consequences depending on its location.

Benign tumors can also become malignant over time, depending on a district differentiation pathways and interactions with tumor microenvironments. Malignant tumors are commonly called cancer; they proliferate fast and can spread through the bloodstream or lymph system to secondary organs (metastasis) [2].

There are many different types of cancer, and each classified by the type of cell that is initially affected.

- Carcinoma: epithelial tissue, glands and liver; about 90% of all tumors are epithelial.
- Sarcoma: muscle, blood, connective tissue and bone.
- Leukemia and lymphomas: blood cancer
- Melanoma: melanocytes and other pigment producing cells.
- Neuroblastoma: nerve cells.

1.1.1 Metastasis

Metastasis is cancer spread to a different part of the body, from where it started as a primary tumor. Metastasis is major cause of cancer morbidity and mortality, and is responsible for 90% of cancer deaths [4]. Tumor cells can circulate using two different conduits, hematogenous (blood) vascular system and/or the lymphogenous (lymphatic) system [1]. Invasion and metastasis of the epithelial cancer is activated by a process where epithelial cells transform and adopt a mesenchymal phenotype, called epithelial-to-mesenchymal transformation (EMT). EMT can be stimulated by different signaling pathways (eg TGF-β, EGF, Wnt) [1]. During the course of invasion and metastasis, mesenchymal cells become mobile and lose "cell-cell contact", partially due to repression of the adhesion molecule E-cadherin. Additionally, expression of different proteins on the cell surface may change [5]. Consequently, the cancer cells break through the basal membrane and enter the circulatory and lymphatic system. Circulating tumor cells (CTCs) avoid immune attack, and reaches secondary organ where they undergo mesenchymal-to-epithelial transformation (MET). This allow cancer cells to re-attach and proliferate into a metastatic tumor (figure 1.2). EMT is considered an underlying characteristic of metastasis. However, not all cancer cells undergo EMT [6]. Other metastatic pathways have similar mechanisms that allow cancer cells to transform the rigid structure and provide them with features that make them mobile and invade. These tumors are often called mesenchymal-like tumors, cells that are more epithelial in their properties but which are incomplete competence in EMT transformation. [7, 8]

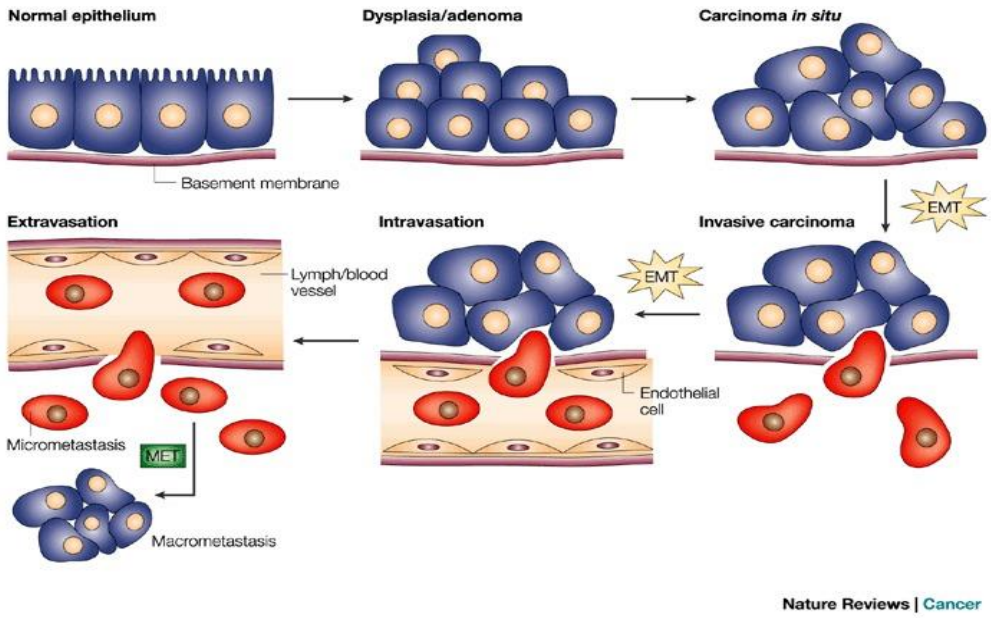


Figure 1.2: Illustration of metastatic pathway; by epithelial mesenchymal transformation (EMT) and mesenchymal epithelial transformation(MET). Figure adopted from; https://www.quizover.com/course/section/the-project-analysis-of-mirna-and-mrna-associated-with-by-openstax

1.1.2 Cancer treatment

There are many different types of cancer treatment. The choice of treatment depends on the type of cancer, and progression stage. In most cases, a combination of different treatments are used, to enhance the therapeutic effect. The most common used cancer treatments are surgery, chemotherapy and radiation. Surgery is often the first choice, if applicable, to remove the tumor. In many cases, chemotherapy is used in addition to surgery, either before (neoadjuvant) or after (adjuvant), to kill and shrink the tumor and free cancer cells. Currently, there are over 100 drugs used in cancer treatment [9, 10]. Radiation therapy is used in many types of cancer. The treatment uses high doses of radiation to induce DNA damage, to shrink or kill tumor cells.

Chemotherapy have different ways of targeting the tumors [11, 12]. Alkylating drugs damage DNA, and interfere in all phases of the cell cycle. Anti-metabolites are a group of drugs that inhibit DNA and RNA synthesis. Anti-microtubule drugs prevent microtubule functions, which is an important cellular structure composed of two proteins, α and ß tubulin. Unfortunately, treatment resistance is a major cause of treatment failure, the mechanisms behind this is not yet fully understood. Consequently, combinations of different chemotherapy drugs and other anti-cancer drugs are used to minimize the chances of treatment-resistance development [13].

Immunotherapy is now recognized as a new cancer therapy [14, 15]. The purpose of this therapy is to help the immune system to fight the cancer cells themselves. Although, cancer cells continually develop strategies to avoid recognition and elimination by the immune system. Immune checkpoint therapy, such as blocking antibodies to cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1), and chimeric antigen receptor (CAR) -T-cells are often used as immunotherapy. Regardless to the success of these treatment, immunotherapy does not work in all cancer types [14][16]. In addition, treatment such as stem cell transplant, hormone therapy and personalized medicine are playing an increasingly important role in treating several cancer types [17].

1.2 Peritoneal carcinomatosis

Peritoneal carcinomatosis (PC) is metastatic cancer that affects the abdominal cavity. PC is metastasis settled from numerous, abdominal malignancies, gastrointestinal and gynaecological and. PC is often very aggressive with a poor prognosis and low overall survival (OS) [18]. The most common symptoms for patients with PC occur late in the disease course, including abdominal pain, weight loss and abdominal distention. PC is very challenging to diagnose; because it causes symptoms that similar to common abdominal diseases. The diagnosis is therefore first obtained, when the disease has managed to spread and grow significantly. Imaging techniques, such as tomography-scan and magnetic resonance imaging, are often used for diagnosis. However, they are limited in their ability to visualize and localize PC, having low sensitivity for small tumors. The gold standard in diagnosing PC is direct peritoneal visualization, either by laparotomy or laparoscopy [19, 20].

1.2.1 PC from colorectal cancer

Colorectal cancer (CRC) is a common name of colon and rectum cancer. CRC is the third most common cancer worldwide [21]. Commonly CRC starts with cell growth, polyps, on the inner lining of the colon or rectum. There are different types of polyps; adenomas and hyperplastic and inflammatory polyps. Adenomas are pre-malignant tumors, with a high risk of progress to CRC. The stage of a CRC depends on how deeply the tumor growth into the colorectal wall and if it manages to go through the wall (figure 1.3) [22].

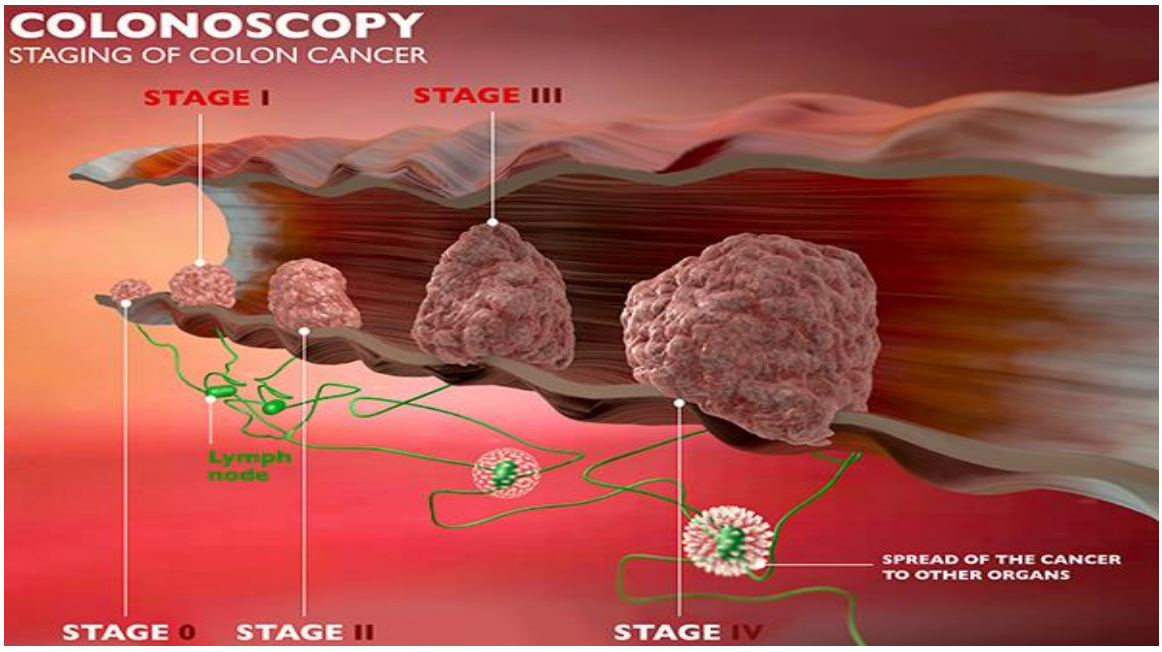


Figure 1.3: Guidelines how polyps growth on the inner lining of the colon or rectum. There are four main colorectal cancer stages depending on the extent of the polyps growth (from stage I – IV). Figure adopted from; https://www.evaidya.com/Health-Articles/colorectal-cancer-symptoms-and-treatment-guidelines/

Metastatic disease is the main cause of CRC mortality. Unfortunately, about 20-25% of CRC patients present with metastasized at the time of diagnosis[23]. Subsequently, about 4-7% of those with PC. PC-CRC is frequently associated with poor prognosis, with a median survival rates if untreated, is less than for untreated PC-CRC is less than 6 months [24]. Tumor location, size, and extent of cancer spread and the health of the patient, determines which treatment is given.

1.2.2 PC from ovarian cancer

Ovarian cancer (OC) is the seventh leading cause of death from cancer worldwide. At the time of diagnosis, 65–70% of patients have advanced disease (stage III or IV), and due to the poor anatomical barriers between the ovarian and the peritoneal cavity, peritoneal metastases are extremely common [25]. The median survival rate of treated patients with stage IV is range from 12 to 24 months. Cytoreductive surgery (CRS) with intraperitoneal chemotherapy (IPC), most commonly with a platinum (Cisplatin or Carboplatin) and Taxane combination has become the standard treatment for PC-OC. IPC is delivered through an implanted subcutaneous port that drains into the cavity of the abdomen, allowing direct access for the drug to the peritoneal cavity. However, there is still some uncertainty about IPC, regarding the optimal drugs, dose and control of the catheter delivery system. Additionally, the treatment can cause

more frequent and severe side effects, including abdominal pain, nausea, and vomiting, than systemic treatment [26]. It is therefore major request for improved treatment for OC patients.

1.3 Pseudomyxoma peritonei

Pseudomyxoma peritonei (PMP) is a rare malignant condition in the peritoneal cavity, mostly originate from appendix [27]. Only one to two individuals per million is diagnosed with PMP each year. PMP is characterized with intra-abdominal growth and progressive accumulation of mucin, referred to as "jelly belly. PMP has a slow progression, and tends to be misdiagnosed and discovered at advanced stages. Prognosis for PMP after standard treatment, CRS with Hyperthermic Intraperitoneal Chemotherapy (HIPEC), is generally among the best for PC patients, due to slow progression, nearly no risk for metastasis outside the peritoneum. The procedure was first introduced for PMP patients [28].

1.4 Cytoreductive surgery – Hyperthermic Intraperitoneal Chemotherapy

CRS-HIPEC is a relatively new treatment procedure, and uses as a standard treatment for patients with PC-CRC and PMP. Presently, there are many ongoing clinical trials evaluating HIPEC procedure in other PC prognoses [28, 29]. The term "Hyperthermic Chemotherapy" refers to the use of heated chemotherapy, to a temperature higher than normal body temperature (usually 40-42℃). The purpose of using heat is to be able to kill residual microscopic tumors and free cancer cells that remains left after surgery. The term "Intraperitoneal" in HIPEC means that the treatment is delivered directly to the abdomen. The use of heat in cancer treatment is not new. In ancient Greece, Hippocrates states in his Aphorism *"Those diseases that medicines do not cure are cured by knife. Those that knife does not cure are cured by fire. Those that fire dose not cure must be considered incurable".* Hyperthermia itself has a direct cytotoxic effect through inhibition of DNA repair mechanisms [30], denaturing of proteins and activation of anti-cancer immune response. However, heat alone as a cancer treatment is inefficient, and therefore it is used in combination with other cancer treatments, such as chemotherapy and radiation therapy. In the early 1990s researchers demonstrated that hyperthermia as well as intraperitoneal chemotherapy could be effective in killing cancer cells for PC, but only in combination with CRS. Thus, in 1995, Dr. Sugarbaker created a stepwise approach to HIPEC, in an effort to standardize and optimize this complex procedure [30, 31].

This treatment has improved the survival rate, as well as enhanced long-term 5-year survival for PC-CRC patients.[31, 32] However, HIPEC is only a possible option for a selected group of patients with PC-CRC. This is because the effectiveness of the treatment depends on Peritoneal Carcinoma Index (PCI) and Completeness of Cytoreduction (CC) score, removal of tumors after the surgery. So far it is very challenging to select patients who benefit from the treatment along with acceptable treatment-related morbidity and mortality. On average 40-50% of PC-CRC patients are eligible for this kind of treatment. Several large clinical trials have demonstrated the median time until recurrence to be approximately 20 months [33]. However, for PMP there is no upper PCI limit for a patient to proceed with HIPEC. It is usually dependent on the clinical condition of the patient. Even patients with advanced PMP may benefit from CRS-HIPEC, if all visible tumor nodules can be radically removed [34]. In Norway, CRS-HIPEC is only conducted at the Oslo University Hospital, the Norwegian Radium Hospital, as a standard treatment for PC-CRC and PMP [32].

1.4.1 Clinical CRS - HIPEC procedure

CRS-HIPEC procedure is divided into two phases. The first phase is CRS, where a surgeon opens the abdomen to get access to peritoneal cavity, and removes all visible tumor. The final stage of the procedure is meant to eliminate microscopic cancer cells that still remains after the surgery. Subsequently, four tubes are places into the abdomen and a HIPEC machine is used to circulate and heat the drug solution. HIPEC exposure time depends on which chemotherapeutic drug is used. Exposure time of Oxaliplatin is mostly shorter (30 minutes) than of Mitomycin C (often used for 90 minutes). After chemotherapy “bath”, the drug is washes out and the abdomen is stitched up. The whole procedure takes up to 10 hours, meanwhile the patient is under anesthetics. HIPEC allows use of higher dose of chemotherapy (20 to 1000 times more than the systemic dose), while the toxicity and side-effects associated with systemic chemotherapy are lower. The use of hyperthermia is believed to increases the absorption of the drugs into tumor cells, and potentiate the cytotoxicity of the drugs, mostly the drug dose and the carrier solution volume is calculated based on body surface area (BSA) [35, 36]. However, several hospitals are using concentration-based HIPEC. Recently, the first clinical study to evaluate the two dosing regimens has been started (NCT03028155, ClinicalTrials.gov).

HIPEC can be performed as an open, closed and semi-closed procedure (se figure 1.4). Closed procedure is often used, due to advantage of reduced heat loss, increased tissue penetration and

decreased contamination risk. Open HIPEC has advantage in that it makes it easy to maintain homogeneous abdominal distribution of the drugs, without triggering abdominal hypertension. In addition, it allows confirming proper circulation of the drug during the treatment. The chemotherapy used in HIPEC is high-molecular-weight hydrophilic drugs. The benefit of using this kind of drugs is that they are more or less unable to/or very slowly cross the peritoneal cavity barrier, allowing a use of higher concentration than systemically [37].

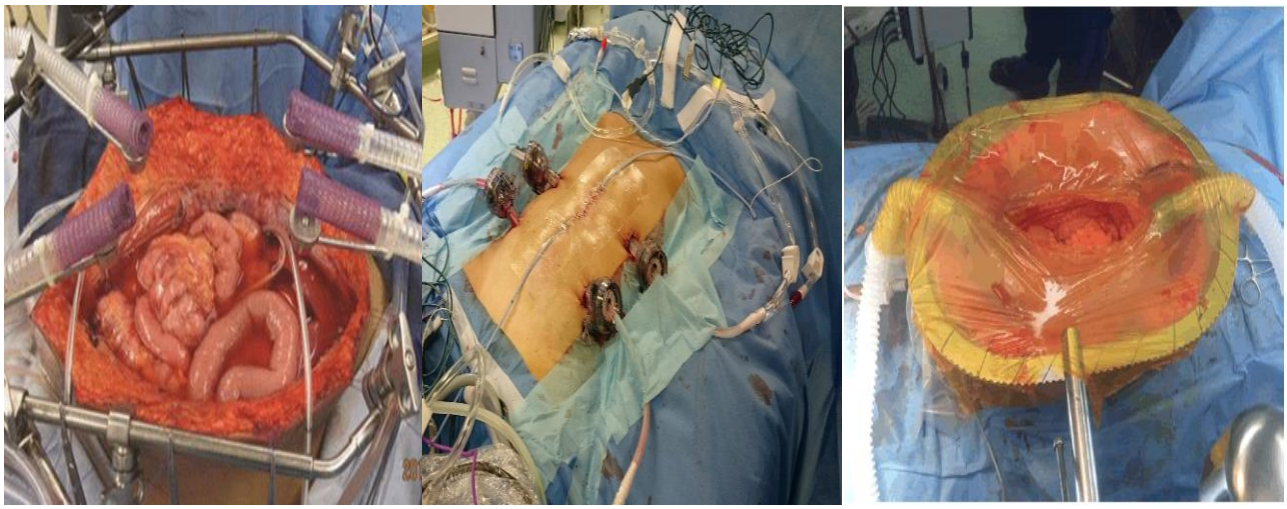


Figure 1.4: Administration of HIPEC as an open, close and semi-closed procedure. Figure adopted from; https://app.emaze.com/@AWZWTFFL#9

The success of the HIPEC treatment depends on many factors. As mentioned above, intraperitoneal administration of hyperthermia and chemotherapy is highly depended on PCI and CC. In addition, selection of drugs, their concentration and the temperature achieved in the peritoneal cavity, plays a role whether the treatment is successful or not. HIPEC is associated with significant morbidity and treatment related mortality [38]. Thus, the beneficial effects of hyperthermia and local chemotherapy need to be critically validated. Several studies have shown that cancer cells develop distinct mechanisms to protect themselves from various stress such as ischemia, heat stress, and oxidative stress. One of the mechanisms is high expression of Heat Shock Proteins (HSPs), especially during chemotherapy and hyperthermia treatment [39]. Hyperthermia induced HSPs expression, could influencing HIPEC treatment response negatively. However, the underlying role of HSPs in tumor progression and resistance to treatment remains unclear [39, 40].

1.5 Heat shock proteins

HSPs genes were first discovered in 1962 by Ritossa (Ritossa 1962; could not find the origin reference). The heat shock response is a cellular defense mechanism against physiologic and environmental stress [39, 40]. There are five main HSPs families, classified by their molecular size into large HSPs, such as HSP90, HSP70, HSP60, and small HSPs, such as HSP27. HSPs are expressed at low to moderate levels in all human cells but are abnormally high expressed in cancer cells, and increases even more after different death stimuli, including hyperthermia, chemotherapy and radiation. HSPs are proteins that have chaperone functions, and are larger hetero-complexes that are involved in protein–protein interactions, folding and assisting proper protein conformation and prevents protein aggregation. In addition, they also function as anti-apoptotic proteins, by associating with several effectors of the apoptotic machinery. HSPs also interfere with cell surviving processes and by providing proteasome degradation of selected proteins under stress conditions [40].

The high level of HSP70, HSP90 and HSP27 in cancer cells are thought to play a key role in cancer and are linked to treatment resistance and poor prognosis. Consequently, several HSP inhibitors are undergoing many clinical trials [41]. While the clinical benefits of HSP inhibitors still require validation, they could provide additional potency of HIPEC in drug combination strategies, specifically inhibitors of HSP70 and HSP90, as their high expression usually are associated with a poor clinical outcome. Clinical approaches that inhibit the expression of HSPs would be useful to sensitize cancer cells to hyperthermia.

The HSPs protein expression in response to stress is regulated by a unique transcription factor known as Heat shock factor (HSF). There are multiple isoforms of HSF. However, Heat shock factor 1 (HSF1) is the main regulator of HSP transcription [42]. HSF1 is deactivated at normal conditions by being linked with HSPs, usually with HSP90. When the cells are exposed to stress, it leads to post translational modifications, which, converts HSF1 into an active form, by dissociating HSF1 from HSPs. HSF1 then forms trimers and translocated to the nucleus and binds to 5'promotor regions of Heat Shock Sequence Elements (HSE) throughout the genome and trigger transcription of all HSPs. HSF1 is also associated with poor prognoses in many different types of human cancers and may be a further target for general and effective cancer therapy [42, 43].

1.6 Immunogenic cell death

The aim of all cancer therapy is to kill all cancer cells. Cancer cells have variety mechanisms to avoid immune system, for example by decreasing protein interleukin-33 (IL-33), increasing expression of programmed death-ligand 1 (PD-L1) or secretion of suppressive cytokines (e.g. IL-10, TGF-β), the immune system can no longer recognize cancer cells, which allows them to grow and metastasize [44]. Therefore, treatment that induces immunogenic cell death (ICD), is essential to help the immune system to recognize and react against cancer cells. ICD lead to a specific stimulus that induces the release of immunogenic proteins such as, Danger-Associated Molecular Patterns (DAMPs), that includes HSP70, HSP90, ATP or High mobility group box-1 proteins (HMGB1). Those proteins are either exposed at the cell surface or released to the cell vicinity, as an "eat-me" signals to immune cells and stimulates antigen presentation to dendritic cells (DC). Activated DCs then activates T-cell response, resulting an immune attack that eventually kills cancer cells [45, 46].

Hyperthermia in combination with other anti-cancer treatments (mostly from 40 to 42℃) induces ICD [47]. Hyperthermia leads to protein aggregation and denaturation which induces a stress response in the cells, called unfolded protein response (UPR). UPR induces transcription of HSP proteins by activating HSF1. This leads to release and exposure of HSPs on the cell surface (figure 1.5). Additionally, hyperthermia increases exposure of tumor antigens (Ag) and release of HMGB1. Finally, all this leads activation of Natural killer cells (NK effector cells) and DC, thereby induce cellular anti-tumor immunity by activating cytotoxic T lymphocytes (CTL) [48].

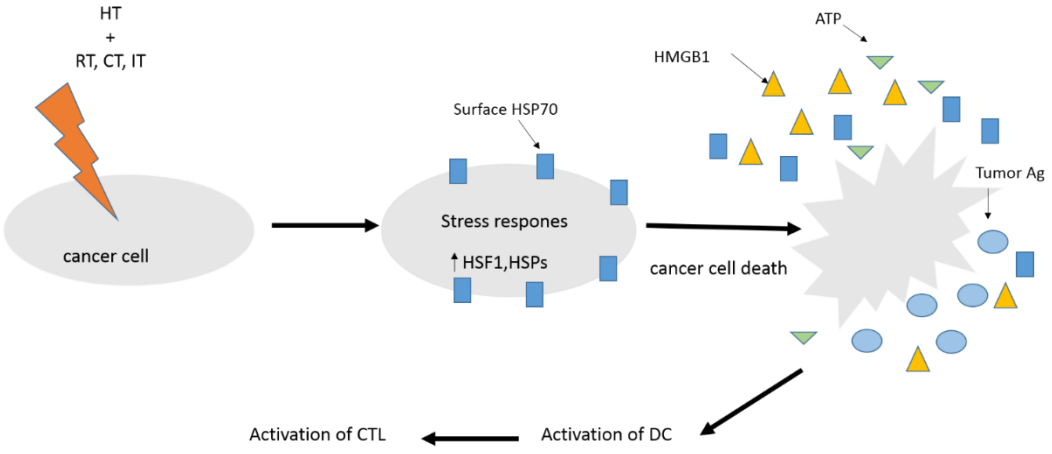


Figure 1.5: Overview of how hyperthermia induces immunogenic cell death. Hyperthermia combined with other anti-cancer therapies; chemotherapy (CT), radiation (RT) or immune therapy (IT), modifies stress response, cancer cells death and releasing of HMGB1, HSP70, ATP and Tumor antigens (Ag). Which eventually leads to immune response of cancer cells, via activation of dendritic cells (DC) and cytotoxic T lymphocytes (CTL).

2 Aim of the project

Patients with PC have poor prognosis and it is important to develop the best possible treatment. CRS-HIPEC is the gold standard treatment of PC from CRC and PMP. Nevertheless, clinical trials aiming at developing HIPEC treatment further are still ongoing. There is limited pre-clinical research on hyperthermia in combination with chemotherapy, and the current pre-clinical studies do not closely mimic clinical relevant conditions. In this master thesis, we aimed to investigate the following;

- Investigate how hyperthermia interacts with Oxaliplatin and Mitomycin C in CRC cells with different mutation profile, is it beneficial or not?
- Could HSP-inhibitors improve the cytotoxic effect of hyperthermia and chemotherapy?
- Could silencing HSF1 suppress HSPs expression and improve the cytotoxic effect of hyperthermia with chemotherapy?
- Is immunogenic cell death response an important parameter in hyperthermia plus chemotherapy induced cell death.
- Can our hyperthermic chemotherapy *in vitro* model be used on fresh tumor tissues from animals and patients?

These have been studied, using a new developed in vitro model on two different colorectal cancer cell lines. In addition, some initial experiments have been done on fresh tumor tissues from mice and a patient.

3 Methods

In this section, all the methods used for this master thesis is described. First, a general description of the methods, followed by how it was used in this research, including the protocols, techniques and materials used are presented. List of all solutions, buffer, and materials used is presented in the appendix.

3.1. Cell culture

Cell culturing is often used to study biological issues such as, cell cycle control, intra- and extra-cellular signaling and cell death. The advantage of using cell lines is that it is a steady access to cells, which makes it possible to study the cells in details. However, the cell culture integrity may change over time, due to cross-contamination with other cell lines or bacterial/mycoplasma contamination, and affect the result of the experiment. It is therefore very important to have a good sterile technique. All equipment and solutions that are used to grow and/or to split cells should be sterile to prevent infection, and should be performed on a laminar flow cell cultivation bench without turbulence (horizontal or vertical LAF benches). The bench and non-sterile equipment must be sprayed with alcohol before and after use. To avoid cross- contamination, you should never work with more than one cell type at a time. Cells growing in culture have three phases. *Lag- phase:* which could last up to several days. The cells do not divide but get used to their environment. During this period, the cells will change their cytoskeleton so that they can stick to the bottom of the flasks. *Log (*Logarithmic*)- phase:* Cell divides and cell numbers increase exponentially. *Plateau (or Stationary) Phase:* cellular proliferation slows down due to the cell population becoming confluent. The cell culture bottle is completely full (confluent) and the cells are disposed to injury.

3.1.1 Cell lines

The cell lines used for this project were; HT29 (human colon colorectal adenocacinor carcinoma homo sapiens, HTB-38^{TM}) and HCT116 (human colon colorectal carcinoma homo sapiens, CCL-247$^{TM)}$). Both cell lines are originally obtained from American Type Cell Collection (ATCC, Manassas, VA, USA). HCT116 contains wild type TP53 and APC (Adenomatous polyposis coli, a multi-functional tumor suppressor gene), while HT29 contains mutant TP53. Both cell lines are suitable as transfection host. For more detailed gene profile data, see Table 3.1.

Table 3.1: Overview of relevant wild-type and mutation profile of cell line HCT116 and HT29.

Cell line	Wild-type	Mutated
HCT116	p53, BRAF, PTEN	KRAS, PIK3CA
HT29	KRAS, PTEN	P53, BRAF, PIK3CA,

Protocol

The cell lines were cultured in Nunc ^{TM} EasyFlak^{TM} 75 cm^2 cell culturing flasks (Thermo Fisher Scientific Waltham, MA, USA) with RPMI-1640 medium (Sigma Life Science, United Kingdom), supplemented with 10% fetal bovine serum (FBS), 2% HEPES and 1% L- alanine - L-glutamine (Glutamax^{TM}). To minimize bacterial infection, 100 U/ml penicillin and 100 µg/ml streptomycin was added in the medium, since the experiments were long-lasting in time after manipulation.

The cells were incubated in a 5% CO2 incubator at 37^{0}C (Heracell™ 150i). The incubator has a water bath on the bottom, to provide moisture and to prevent the drying of cell medium due to the heat. For long-term storage, the cells were frozen at -80^{0}C, in RPMI-1640 medium containing 20% FBS and 10% dimethyl sulfoxide (DMSO; Sigma). When the cells were to be used in the experiment or split, the cells were detached from growth surface by adding 0.25% Trypsin-EDTA Solution, and incubated for maximum 5 minutes. After incubation, new medium was added to inactivate trypsin. The cell suspension was then transferred to a 50ml tube for centrifugation at 1000rpm/5 minutes. Afterwards, supernatant was discarded and medium was added to resuspending the cell pellet. Both cell lines were split twice a week, as cells should not grow to more than to 80-90% confluence. Microscope was used to assess the condition, density and confluence of cells. When reaching passage 20, a new frozen cell passage was taken up from the freezer for use. When the cells are to be thawed, it is important to quickly dilute in regular medium to get rid of the DMSO. DMSO prevents the cells from bursting when frozen but is very toxic to the cells at room temperature.

3.2 Cell counting - Bürker chamber

Hemacytometer/Bürch Chamber was used to control the number of cells used in each experiment. By using a counting chamber and microscope, it is possible to count how many

cells there are in a cell suspension. The counting chamber consists of a thick glass plate and a thin cover glass (figure 3.1). There are two separated counting chambers on the glass plate. The counting chamber consists of nine large squares of 1 mm^2 each. Each square is subdivided by double lines (0.05 mm apart) into 16 group squares with 0.2 mm sides [49].

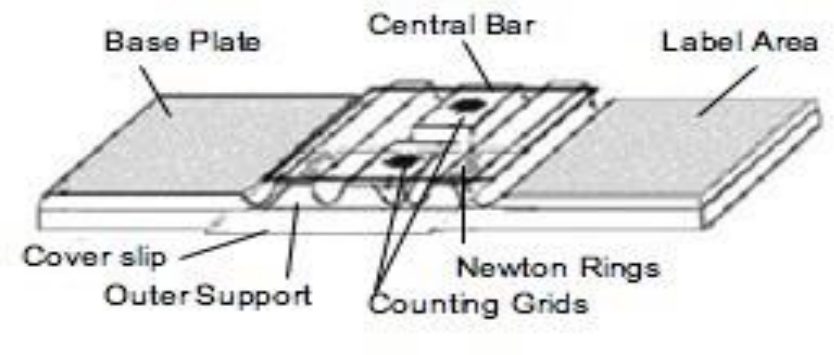


Figure 3.1: shows the different sections in Bürker chamber. The picture is adopted from: http://www.lo-laboroptik.com/information/

Protocol

When sample material (10µl) is filled in the cavity, the cells will sink and lay on the chamber. It is now possible to count the cells on the chamber, but it is important to work systematically so that the same cells are not counted more than once. All squares do not need to be counted, three to five squares are enough. When finished counting, the concentration/ number of cells in the sample, can be calculated by using the equation below:

Number of cells in 1ml = N x 10^4 /z

N = the total number of cells in all counted squares 1mm^2, z = the number of counted squares 1mm^2

3.3. Hyperthermic chemotherapy, *in vitro*

Hyperthermic drug treatment was performed to investigate how hyperthermia affect the cytotoxic effect of different chemotherapeutic drugs in CRC cells with different mutation profile *in vitro*. It is important that the experiment mimic clinical HIPEC, as much as possible. To obtain the clinical condition of HIPEC in *in vitro* experiments, there are several details that should be considered, including temperature, chemotherapy drugs and their concentrations, and exposure time (how long the cells are exposed to hyperthermia and drug). Several clinical studies have observed significant cell death, when cells are submitted to temperatures above

40ºC [36, 50, 51]. We chose to expose the cells for 90 minutes at 42ºC, as this mimic the clinical HIPEC setting used at the Norwegian Radium Hospital. All the experiments in this section were done in three biological triplicates each with three technical triplicates.

3.3.1 Chemotherapy drugs

Chemotherapy drugs used in this experiment were Mitomycin C and Oxaliplatin. Currently, both Mitomycin C and Oxaliplatin are the most clinically relevant drugs, as they are used frequently during HIPEC treatment for PC-CRC and PMP [32, 51]. Mitomycin C is an antineoplastic antibiotic that inhibits DNA-synthesis, by inducing DNA-crosslinks and by physically blocks DNA replication, recombination, and RNA transcription [52]. Oxaliplatin is a platinum drug that induces primary and secondary DNA lesions that eventually lead to cell death [51]. Mitomycin C and Oxaliplatin are suitable for this kind of treatment, because of their large molecular weight (Mitomycin C 334.3 g/mol and Oxaliplatin 397.3 g/mol), allowing a use of high concentration during HIPEC, with limited systemic absorption and toxicity. In addition, these drugs are stable at high temperatures. Therefore, which drug that is used in HIPEC, is mainly based on hospital tradition [51]. The Mitomycin C solutions used in this study, were prepared by dissolving Mitomycin C stock powder (Medac) in phosphate buffered saline (PBS; Sigma). Furthermore, the drug was diluted to the desired concentration in RPMI-1640 medium. Drug concentrations used in this study, were selected based on a commonly used clinical HIPEC drug concentration. All experiments involving chemotherapeutics were done in class 2 safety cabinets, after special training, given by Torveig Weum Abrahamsen, who is responsible for laboratory safety.

Protocol

Both cell lines were allowed to grow to 70-80% confluence at the day of the experiment. The cells were detached by adding trypsin-EDTA and incubated for 5 minutes. Five times more fresh medium were then added for the deactivation of trypsin. After centrifugation at 1000g for 5 minutes, the cells were suspended in a new medium. As mentioned above, the cells were counted using a Bürker cell counting-chamber. In order to get 15.000 cells per well in a 96 -well plate (Nunclon^{TM} Delta Surface, Thermo Fisher Scientific), a cell suspension of 300.000 cells per ml was made in RPMI-1640 medium. Afterwards, 1 ml of cell suspension was mixed with 1 ml drug suspension (figure 3.2). This were prepared in a 15 ml plastic centrifuge tube. After mixing the two suspensions together, the mixture was split equally into two 15 ml

centrifuge tubes. The tubes were then incubated in two separate temperature controlled water baths, set to 37ºC and 42ºC, for 90 minutes. The water bath was also controlled throughout the experiments with glass thermometers (Cole-Parmer®). In each experiment we included a control tube without drug: 1 ml cell suspension mixed with 1ml RPMI-1640 medium.

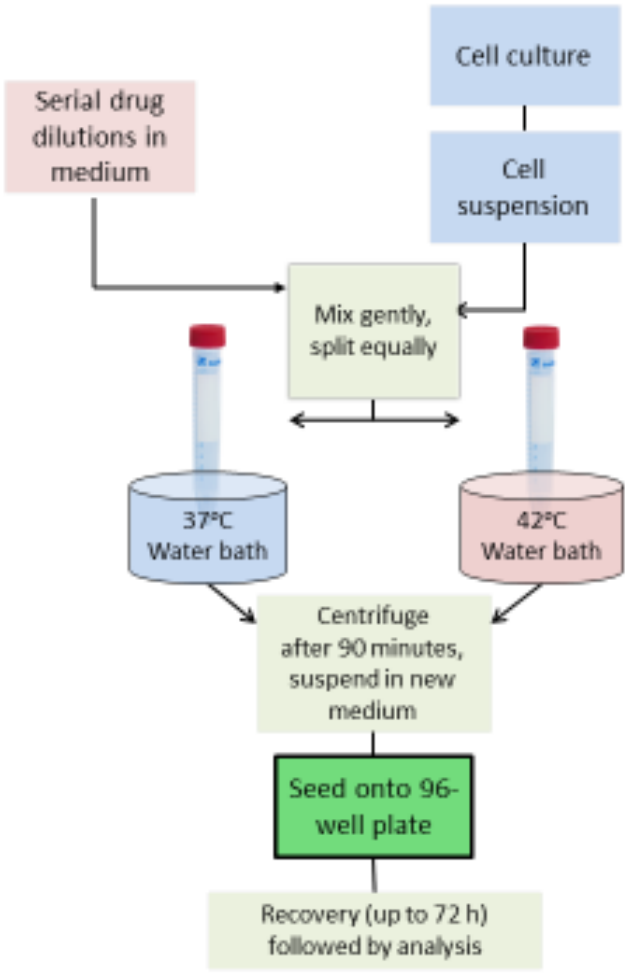


Figure 3.2: A simple overview of the different steps in the hyperthermic chemotherapy *in-vitro*. The figure is adopted and have been granted permission to use by Theodor Malmer Herud.

After hyperthermic treatment, the tubes were centrifuged at 1000g in 5 minutes and washed once with a new RPMI-1640 medium to remove remaining drug. The cells were then seeded in triplicates into 96-well plates, 15 000 cells per well (100µl/well). The plates were incubated for a period of 24 or 72 hours, before further analysis were performed.

3.4 HSP-inhibitors

In this experiment, we wanted to investigate whether combining hyperthermia with HSP inhibitors could improve the cytotoxic effect of HIPEC treatment with, Mitomycin C and Oxaliplatin. HSPs expression increases during stress conditions, such as during hyperthermia and anti-cancer therapy, and high HSPs expression correlates with treatment resistance [53]. The two most stress inducible HSPs involved in cancer are HSP70 and HSP90. Currently, inhibitors of those HSPs is emerging as a strategy of cancer therapy. We decided to use HSP70 and HSP90 inhibitors for this experiment. We used HSP90 inhibitor called 17-allylamino-17-demethoxygel-danamycin, 17-AAG (Selleckchem®), which is the best-recognized inhibitor of HSP90. 17-AAG came into clinic in 1999, and have reached phase II trials [54]. However, presently, there is no undergoing clinical trials using 17-AAG. Although, 17-AAG have improved metabolic stability and less toxicity comparing to Geldanamycin (the first HSP90 inhibitor), and induces cell arrest and apoptosis. It also inhibits the activity of oncogenic proteins such as N-ras, Ki-ras, c-Akt, and $p185^{erB2}$. 17-AAG inhibits HSP90 chaperone activity by binding to the ATP-binding site of HSP90 with higher affinity than natural nucleotides [54, 55].

For inhibition of HSP70, we used HS-72 (SIGMA-ALDRICH®), which is a selective inhibitor for the inducible form of Hsp70 (Hsp70i), also called HSP72, HSP70-1 and *HspA1A/HspA1B.* It is important selectively inhibit HSP70i, as other members of the HSP70 superfamily, such as HSC70, are constitutively active and essential for normal cellular function. The chaperon activation of HSP70i is driven by ATP hydrolysis in the N-terminal nucleotide-binding domain (NBD), which undergo ATP/ADP exchange at all times. Therefore, it is challenging to formulate specific inhibition of the protein through direct competition with bound nucleotide. Thus, HS-72 acts as an allosteric inhibitor of ATP binding, which reduces the protein's affinity for ATP. Furthermore, HS-72 is an excellent inhibitor of HSP70, because it is observed to be well tolerated and bioavailable in vivo (mice), and shows no evidence of overt toxicity at high doses [56].

Protocol

First, cell and drug suspension was made as described in section 3.3 (protocol). Then the right amount of inhibitors, 17-AAG and HS-72, was added in 2ml cell suspension +/- chemotherapeutic drugs. Afterwards, the tubes were incubated at either 37 ºC or 42 ºC water

bath, for 90 minutes. Selected drug and inhibitor concentrations for this experiment, table 3.2. After the treatment, the cells were washed ones with a new RPMI-1640 medium to remove remaining drugs and inhibitors. The cells were then seeded in triplicates into 96-well plates, 15 000 cells per well (100 µl/well). The plates were incubated for a period of 24 or 72 hours, before further analysis were performed.

Table 3.2: Selected drug and inhibitor concentrations. This table lists the different concentration of the drugs and inhibitors used for the different cell lines, based on pre-analyses of optimal concentration.

Cell line	Mitomycin C	Oxaliplatin	17-AAG	HS-72
HCT116	60 µM	73 µM	10 µM	50 µM
HT29	60 µM	144 µM	20 µM	100 µM

3.5 Hyperthermic chemotherapy *ex vivo*

Fresh tumor tissues from patients for the study of cancer progression and treatment, enhances the relevance of preclinical experiments. Patient-derived tumor xenograft (PDX) are tissue or cells from patient tumor transplanted into an immunocompromised mouse that do not reject human cells, to create microenvironment that resembles the natural growth of cancer. PDX models are used in many experiments in preclinical drug development, including CRC [57].

Tissues from PDX-animal models should maintain the original tumor heterogeneous cell population, oncogene expression profiles, and clinical response to treatment, representing the state of cancer patient [58]. In this study, we wanted to investigate treatment response in our experimental "HIPEC" model on tumor tissue samples from PDX-mice model and patient for short-term ex vivo culturing. For this experiment, we were able to obtain mucin from 4 different mice models and one from a PMP patient resembling PC.

Protocol

The consistency of the mucin sample varies. In order to dilute and dissolve the mucin, RPMI-1640 medium was added, and volume adjusted based on the amount of the mucin. Thereafter, mucin was pulled up and down several times with 14G syringe, followed by 18G and 21G syringe, to solve the mucin even more. Hyperthermic drug experiment was performed as mentioned above, with 60µM Mitomycin C and 289µM Oxaliplatin. If it was enough mucin, we did also include 17-AAG and HS-72 in the treatment. In contrast to experiment with cell

lines, we were not able to wash the cells after treatment. Cell viability was measured 48 hours post-treatment with MTS-assay.

3.6 Cell Viability Assay

Cell viability assays are used to estimate the number of viable cells by measuring factors that reflect the number of living cells in the cell culture, such as cellular metabolism and enzyme activities. The cell viability assay used in this study is called CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS-assay), a colorimetric assay that measures metabolic activity of cells. MTS-assay was used to determine cytotoxic effect of hyperthermia and drug treatment on cell proliferation [59]. Other cell viability assays are available, but the MTS-assay is one of the most use and is easy to handle.

3.6.1 The CellTiter 96® AQueous One Solution Cell Proliferation Assay

MTS-assay is based on a novel tetrazolium compound (3- (4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl) -2H-tetrazolium, inner salt; MTS), that only metabolically active cells manage to change into a colored formazan product. Subsequently, color intensity of formazan is measure by absorbance using a spectrophotometer. Thus, the amount of the absorbance is dependent on reductase enzymes present within living cells in the cell cultures [60].

Protocol

HCT116 and HT29 were seeded at 15.000 cells/well post hyperthermic drug treatment. After 24 and 72 hours of incubation, MTS-reagent was added in a 1:10 volume ratio to each well and incubated for 2 hours at 37ºC. Absorbance was then measured at 450 nm using Modulus™ Microplate-reader (Promega). Background absorbance was corrected, by measuring only RPMI-1640 medium.

3.7 Protein analysis by SDS-PAGE and Western Botting

In this study, western blot analysis was used to determine the expression of HSPs, HSF1 and HMGB1, pre or post hyperthermic drug treatment. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins based on their molecular weight. Western Blotting (WB) refers to the transfer of proteins from a gel to a membrane and their subsequent detection on the surface of the membrane. WB is also called immunoblotting,

because an antibody is used to specifically bind to protein of interest. The analysis itself has many different steps; in this section, those steps will be explained comprehensively [61].

3.7.1 Protein extraction and determination of protein concentration

The first step of a WB analysis is to prepare protein lysates, process where cellular membranes are disrupted to release their intracellular content. The cells must first be washed with PBS, to get rid of the medium, and centrifuged by cold centrifuge (4ºC) at 1000 rpm for 5 minutes. The cell containing pellet was then added boiled lysis buffer, mixed as described in appendix 1. Further denaturation of the proteins was done by incubating the sample at 100ºC for 5 minutes. To destroy DNA, the cell suspension was sheared by pull up and down in a 25G syringe, 10 to 15 times. Afterward, the sample was centrifuged (4ºC) at 13.000 rpm for 7 minutes and the protein containing supernatant was transferred to new Eppendorf tubes. Proteins that were not used immediately were stored at -20 ºC. Afterwards, Microplate BCA™ Protein Assay Kit (Thermo Fischer Scientific) was used to determine the total protein concentration of protein lysates. This is done to be able to load a specific amount on the gel, which enable comparison between samples. The assay is based on two reactions. The first reaction is "biuret" reaction, reduction of Cu^{2+} ions from copper II to Cu^{+}, by peptides from proteins in an alkaline medium. The second reaction is reduction of Cu^{+} caused by bicinchoninic acid chelate (BCA), which is found in the reagents. The last reaction produces a purple-colored complex, with a highly sensitive and selective absorbance light, that can be measured by absorbance at 595nM in a spectrophotometer. The intensity of color in the solution is directly proportional to the protein contents in the sample.

Protocol

Protein sample (3µl) replicates were placed into a 96-well plate, and a mixture of Pierce™BCA Protein Assay Reagent A (Thermo Fisher Scientific) and Pierce™BSA Protein Assay Reagent B (50:1, reagent A: B) was added (250µl) to each well and incubated for 30 minutes at 37ºC. Afterwards, absorbance was measured at 595nM using Modulus ™ Microplate-reader (Promega). Protein concentration were then determined by comparing the absorbance to a standard bovine serum albumin (BSA) curve, with the BSA-protein range from 125µg/ml to 1500µg/ml.

3.7.2 Gel electrophoresis – SDS-PAGE

SDS-PAGE is a technique used to separate proteins according to size, regarding to their ability to move in an electric field, electro-mobility. SDS-PAGE contains two gel types with different densities, stacking gel and separating gel, also called running gel. The differences between the two are in pH and gel concentration (amount of polyacrylamide). The upper part is called stacking gel and has larger pores due to lower acrylamide concentration (4%) and pH of 6.8, which is 2 units lower than the separation gel. This allows proteins to form highly defined sharp bands before they enter the separating gel. The running/separating gel has a pH of 8.8 and a higher gel concentration (4-20%), which allows proteins to separate according to their size. Determined by protein of interest, it is possible to adjust the gel's density, the pore size of the gel by adjusting the polyacrylamide and bis-acrylamide concentration. The technique uses SDS, which is an anionic detergent. SDS denatures proteins by destroying the three-dimensional structure and breaking protein-protein interaction. In addition to linearizing the proteins, SDS gives the proteins a negative charge, which causes them to migrate through the gel due to protein size (polypeptide length) regardless of the protein's charge when a voltage is applied on the gel. Selecting the proper voltage is important since too high voltage will overheat the gel and maybe deform the pores, thereby the protein bands.

Protocol

Protein lysates were first mixed with sample loading buffer, mixer of NuPAGE LDS Sample Buffer (4x) and NuPAGE Sample Reducing Agent (10x) (Invtrogen^{TM}). The lysates were then vortexed and incubated at 95ºC for 10 min, for further denaturation. 15µg of total protein lysate were then loaded per well in a NuPAGE® Novex® – 12% Bis-TrisGel (Thermo Fisher Scientific) with the running buffer 1X NuPAGE® MES SDS (Thermo Fisher Scientific). SeeBlue® Plus2 standard ladder (Thermo Fisher Scientific) was loaded next to the samples, to determine the size of proteins. The gel was set to run for 1-1½ hours at 150 Voltage (BioRad PowerPac).

3.7.3 Gel Blotting

In order to make the proteins separated by an electrophoresis available for antibody detection, they must first be transferred from the gel to a membrane by electric current, either to a nitrocellulose or Polyvinylidene difluoride (PVDF) membrane. The membrane is placed between the gel surface and the positive electrode in a “sandwich”, which also includes a fiber

pad (sponge) and filter paper at each side, to protect the gel and blotting membrane (figure3.4). The membrane is placed between the gel and the positive electrode so that the negatively charged proteins can migrate from the gel to the membrane towards the positive charge. This type of transmission is called electrophoretic transmission, and can be done in semi-dry or wet mode. Wet transmission is usually more reliable as there is less possibility of drying out gel. The advantage of semi-dry is that it is time saving, by using much shorter time than wet transfer.

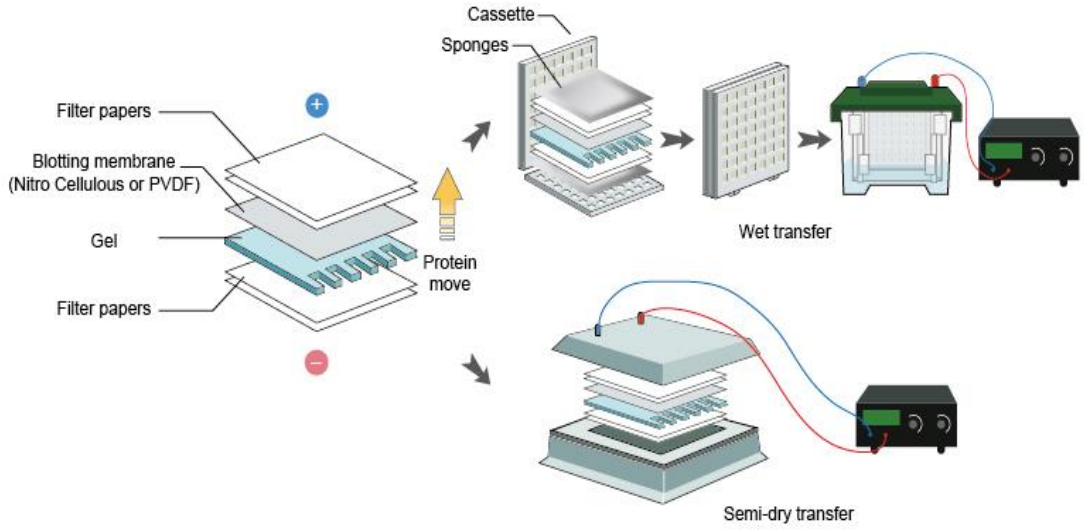


Figure 3.4: Western blot layout. Western blot can be performed in a Wet or semi-dry transfer. The figure is adopted from; https://www.creative-diagnostics.com/Electrophoresis-Protein-Transfer.htm

Protocol

The proteins were transferred to a PVDF membrane in a wet transfer method with 1x transfer buffer (mixed as described in Appendix 1) for 1 hour with a constant current of 400 Ampere. A transfer cassette was prepared in the following order, sponge pads, filter paper, gel with separated proteins, activated PVDF membrane, filter paper and sponge pads. The PVDF-membrane (Invitrogen) was methanol- activated, to allow binding of protein to membrane.

3.7.4 Blocking

Blocking is an important step for WB. This can be done with a blocking agent or nonionic detergents. Blocking prevent unspecific antibody binding to free binding sites on the membrane, which reduces background image. It also gives a weak protein bond because the primary antibodies that were intended to bind to protein of interest will instead bind to free bindings on the membrane. Blocking is often made with 5% BSA or fat-free dry milk in Tris-Buffered Saline Tween (TBST, mixed described in Appendix 1). BSA is mostly used when

studying phosphoproteins, as milk contains the phosphoprotein casein. This can give a high background because the antibody can detect the casein in milk.

Protocol

After removing the membrane from the transfer sandwich, it was incubated with blocking buffer, 5% milk-TBST, for 1 hour at room temperature on a shaking plate. The buffer contains Tween 20, which is a detergent that prevents unwanted protein-protein interaction, and dry milk to block non-specific protein binding to the membrane.

3.7.5 Immunoblotting and ECL detection

Immunoblotting is the detection of proteins on the membrane, by adding specific primary antibody, which specifically binds to the protein of interested. Furthermore, secondary antibody coupled with either fluorescence or enzymes such as horseradish peroxidase (HRP), binds specifically to the primary antibody, to visualize the protein band on the membrane. The primary antibodies used are most commonly mouse or rabbit antibodies, and secondary antibodies from sheep or goat that are anti-mouse or anti-rabbit specific. The antibodies may be monoclonal (formed by one clone and directed against one type of epitope) or polyclonal (produced by different clones and directed to multiple epitopes on the protein). Enhanced Chemiluminescence (ECL) is used to visualize the membrane when using secondary antibodies with HRP. The reagent contains hydrogen peroxide (H2O2) and luminol. HRP enzymes oxidize the ECL substrate luminol in the presence of H2O2 and emit light. The light can be detected as a dark bond, corresponding to the amount of protein on the membrane.

It is very important to be aware that the result of a WB is usually considered semi-quantitative. This is because it provides a relative comparison of protein levels, but not an absolute measure of protein concentration. There are two reasons for this; first, there could be variations in loading and transfer rates between samples. These differences must be normalized using a house keeping protein, proteins that are expressed in all cells under normal and most often under patho-physiological conditions. Secondly, the signal generated by detection is not linear over the concentration range of samples.

Protocol

The blocked membrane was further incubated in blocking buffer containing primary antibody at 4ºC overnight on a shaking plate. The membrane was then washed with washing buffer (TBST), three times á 5 minutes, before incubated in a blocking buffer containing secondary antibodies conjugate with HRP for 1 hour at room temperature. All antibodies, used in this study are listed in Appendix 1. The washing procedure was repeated after incubation with secondary antibodies, to remove excess antibody solution. For detection, the membrane was incubated for 5 minutes with Super Signal™ Western Plus Substrate (Thermo Fisher, mixed according to manufacturer's protocol). The proteins are visualized using ChemiDoc™ Imaging System (Bio-Rad). All proteins expression levels are standardized to ß-actin, which is in most cells a housekeeping protein.

3.8 Silencing HSF1 with siRNA

HSF1 plays an important role in tumorigenesis, by being the master regulator of HSPs [62]. In this study, we wanted to investigate if silencing of HSF1 could improve cytotoxic effect of hyperthermia drug treatment. For this purpose, we used HSF1- specific short interfering RNA (siRNA). siRNA is double-stranded RNA molecules, 20-25 base pairs in length, and interferes with the expression of specific genes with complementary nucleotide sequences by degrading mRNA. Thereby, preventing translation of specific protein. HSF1siRNA™ (Thermo Fisher Scientific) was transfected using Lipofectamine™ RNAiMAX, which is a proprietary formulation specifically developed for highly efficient delivery of siRNA. The effect of HSF1siRNA transfection was controlled by WB, 24 and 48 hours post transfection (Appendix 5), using primary HSF1 polyclonal antibody (Thermo Fisher Scientific). Non-silencing siRNA was used as a negative control. After transfection of HSF1siRNA, we performed hyperthermic drug treatment to investigate the effect of silencing HSF1 on cell viability, using MTS-assy. In addition, expression level of HSP90, HSP70 and HSP27 were analyzed by WB, to confirm whether silencing of HSF1 contributed to reduce expression level of HSP proteins.

Protocol

Silencing of HSF1 was performed by transfecting HCT116 cell lines with siRNA oligonucleotide using Lipofectamine® RNAiMAX (Thermo Fisher Scientific). 24 hours prior to transfection, cells were seeded in triplicate in 6 or 96-well plates and incubated under standard conditions. At the day of transfection, 1.25µl RNAi duplex (20µM) and 7µl

Lipofectamine RNAiMAX was diluted in 125µl RPMI-1640 medium without serum and antibiotics. Both solutions were then mixed gently and incubated for 5 minutes at room temperature to allow for the transfection complexes to form (figure 3.5). Meanwhile the medium in the plates was replaced with new RPMI-1640 medium without antibiotics. Subsequently, RNAi duplex-Lipofectamine RNAiMAX complexes were added dropwise to each wells.

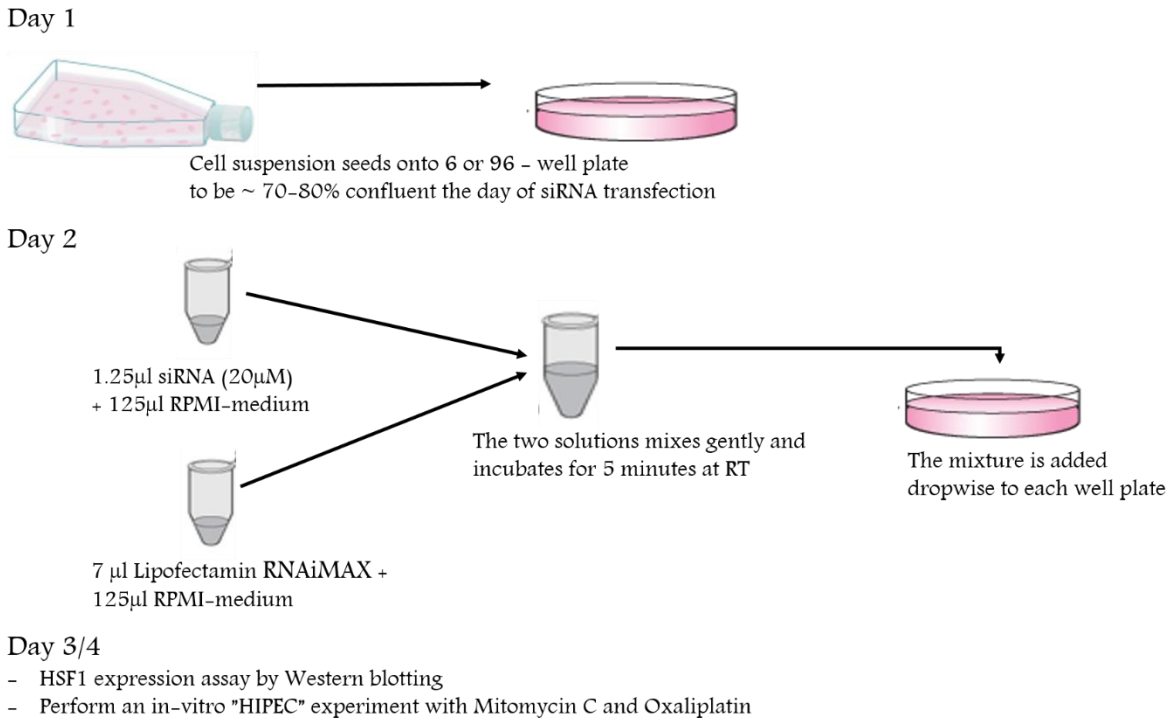


Figure 3.5: General guideline of silencing HSF1 with HSF1siRNA. This image is self-made.

3.8.1 Hyperthermic drug treatment after HSF1 transfection

The first experiment was performed in a 96-well plate, while the cells were attached to the plate. After 48 hours of HSF1siRNA transfection, HCT116 cells were treated with Mitomycin C and Oxaliplatin, before the whole plate was incubated either at 37℃ or 42℃ water bath for 90 minutes. Afterward, new medium was added and the plate was incubated for 48 hours before MTS was performed. MTS data of this experiment turned out to be unreliable. Consequently, we decided to carry out the hyperthermia treatment in 15 ml tube, as we did in previous experiments. After the cells were transfected with siRNA, they were detached by trypsin-EDTA. Hyperthermic chemotherapy treatment was then performed, as described in section 3.3. MTS was added after 48 hours of incubation. The MTS-values in this experimental setting, were more reliable, as they were comparable to earlier experiments. In each experiments, we

had control cells that underwent the same treatment without being transfected by siRNA. Cells treated with the transfection reagent Lipofectamine RNAiMAX without siRNA were also included in the first experiments as an additional control (Appendix 4). Concentration of Mitomycin C and Oxaliplatin used for this experiment was the same as previous experiment with HSPs inhibitors, this can be seen at table 3.2.

3.9 Statistical analysis

All data obtained in this study were statistically analyzed using Microsoft® Excel software. As mentioned above, all experiments on cell viability are analyzed in two or three biological experiments, each with three technical triplicates. The average MTS-value (signal) of triplicates from each treatment (including the untreated control) was first calculated, before the average background signal of "medium only" was subtracted from each samples. Cell viability of each treatment, was then calculated, by dividing the average signal of each samples with the average signal of control (untreated control, without drugs at 37℃). Cell viability was expressed as percentage of the control (the control was set to 100%). Error bars on the graph represent standard deviations (SD) of three biological experiments (n=3), each with three technical triplicates. A 2-tailed unpaired Student's t-test was used to evaluate significant differences in cell viability. For our analyses, the threshold of statistical significance p-value was set to 0.05, and is marked in the results with asterisks to indicate the level of significance: * = $p<0.05$ and ** = $p<0.01$.

4 Results

4.1 Chemotherapy treatment response with or without hyperthermia *in vitro*

We investigated whether hyperthermia (42℃) for 90 minutes, could sensitized HCT116 and HT29 to chemotherapy *in vitro*. The two most common intraperitoneal chemotherapy agents used in HIPEC for PC-CRC, Mitomycin C and Oxaliplatin were chosen. To examine the dose-response effect on cell viability, a clinical relevant concentration of the drugs and two other concentrations were selected. The clinical relevant concentration of Mitomycin C is 60µM, the ranged from 30µM to 120µM were therefore selected. The clinical relevant concentration of Oxaliplatin is 289µM, at first concentrations ranged from 144µM to 579µM were selected. However, in a pilot experiment the result showed that concentration above 289µM was toxic for both cell lines. Consequently, it was decided to use 289µM and two lower concentrations (73 and 144µM). Cell viability was determined using MTS-assay, 72 hours post-treatment. Based on the results from this experiment, the best suitable concentration for each cell line was selected to be used in experiment 4.3 (Hyperthermic drug treatment with HSP inhibitors).

4.1.1 Mitomycin C

Mitomycin C treated HCT116 showed a dose-dependent decrease of cell viability (figure 4.1). The half maximal inhibitory concentration (IC50) of the drug was around 60µM at both temperatures, which is the most common used clinical HIPEC dose of Mitomycin C. However, there was nearly no additional effect of hyperthermia compared to normothermia.

In contrast, HT29 was not as sensitive to Mitomycin C as HCT116. Only a reduction in cell viability of 11% at 37℃ and 15% at 42℃ was observed with 60µM, relatively to the control cells (untreated cells). IC50 of the drug was not achieved even with the highest concentration (120µM). Importantly, hyperthermia increased the Mitomycin C (120µM) induced decrease in cell viability from 25% at 37℃ to 40% at 42℃ ($p=0.019$). Similarly, to HCT116, hyperthermia alone did not appear to have any effect on cell viability.

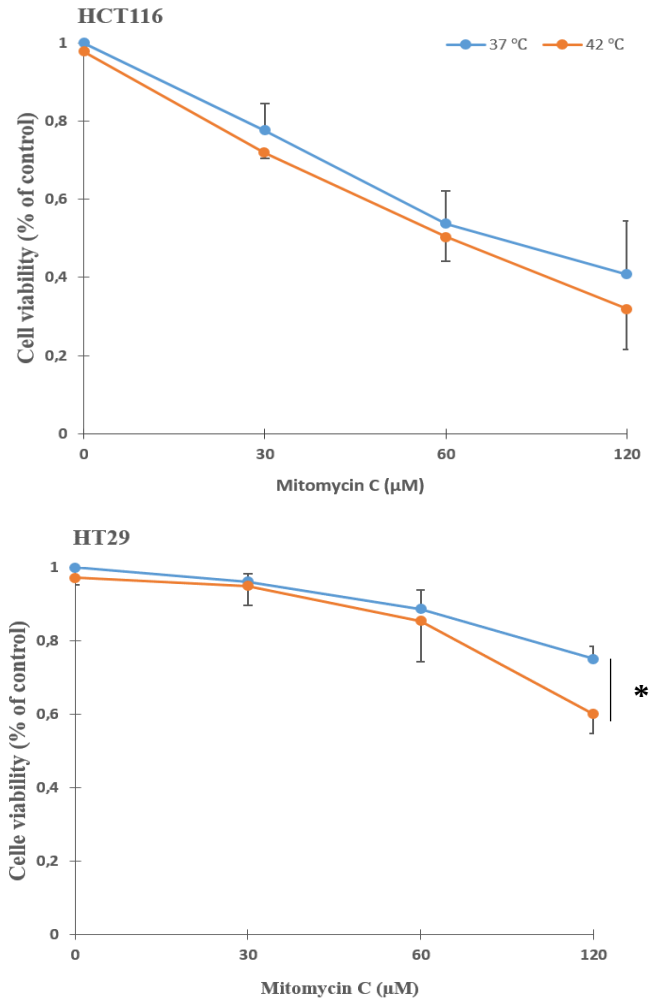


Figure 4.1: Cell viability measured 72 hours after treatment with Mitomycin C by MTS assay. Both HCT116 and HT29 cell lines show a dose-dependent cell viability decrease at both temperatures. HT29 was less sensitive to Mitomycin C than HCT116. Hyperthermia (42℃) did contribute to a significant reduction of cell viability vs normothermia (37℃) at the highest concentration in HT29 (*p<0.05). The absorbance was measured at 450nm and normalized to control-treated cells (cell without drug at 37℃). Error bars represent standard deviations of three biological experiments (n=3), each with three technical triplicates.

4.1.2 Oxaliplatin

HCT116 also showed a dose-dependent decrease in cell viability when treated with Oxaliplatin at both temperatures (figure 4.2). Cell viability was reduced with 27% at 37℃ vs 48% at 42℃ with Oxaliplatin 73µM, compared to control. The IC50 is approximately 73 and 144µM, as more specific determination is not possible based on these 3 experiments performed. Importantly, hyperthermia decreases cell viability significantly at the highest concentration (289µM), compared to at 37℃ (p= 0.007).

In contrast to HCT116, HT29 cells were less sensitive to Oxaliplatin at 37℃. However, hyperthermia seems to have a potentiating cytotoxic effect, with a cell viability reduction of 20% at 37℃ and 55% at 42℃ (p=0.002). IC50 for Oxaliplatin were not obtained at any of the concentrations measured with 37ºC, but was around 289µM with hyperthermia. In contrast to the results from treatment with Mitomycin C, HT29 was more sensitized to Oxaliplatin by hyperthermia.

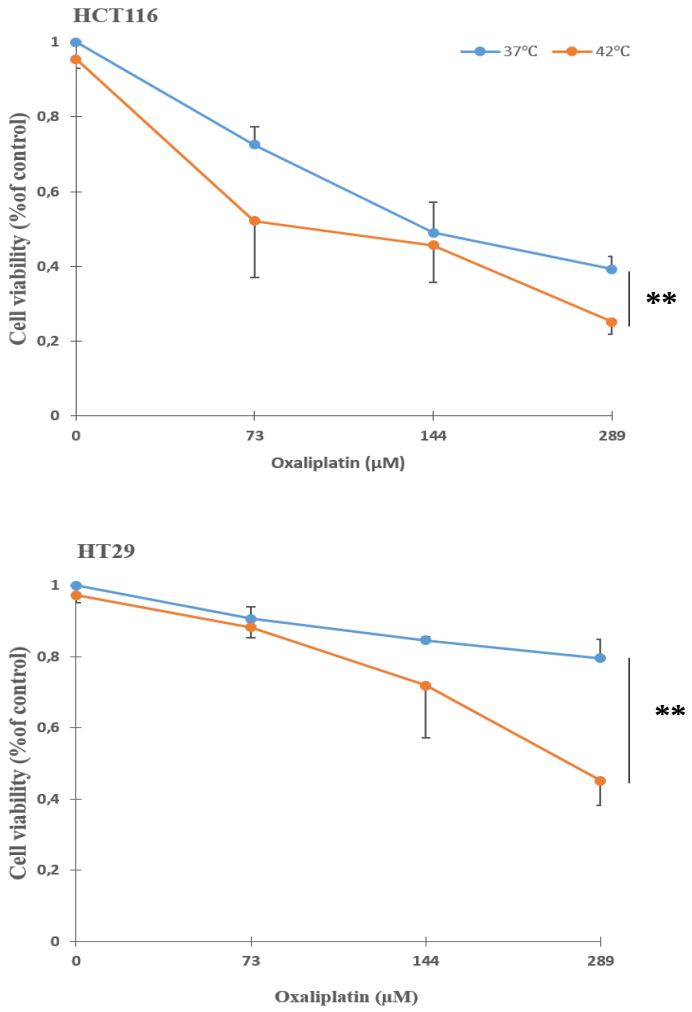


Figure 4.2: Cell viability measured 72 hours after treatment with Oxaliplatin by MTS assay. Both cell lines show a dose-dependent cell viability inhibition. At the highest concentrations hyperthermia (42℃) contributed significant cell reduction with both HCT116 (**p<0.01) and HT29 (**p<0.01). The absorbance from MTS-assay was measured at 450nmM and normalized to control-treated cells (cell without drug at 37℃). Error bars represent standard deviations of three biological experiments (n=3), each with three technical triplicates.

4.2 Heat shock protein expression

Before testing the efficacy of HSP inhibitors in our hyperthermic chemotherapy experiments, we first sought to determine whether hyperthermia induces a higher expression of HSP proteins. HSPs expression levels are known to be higher in cancer cells than in healthy cells. Several experiments have shown that the level of those proteins get even higher, when cells are exposed

to e.g. hyperthermia and chemotherapy. In this study, we examined the expression profiles of HSP27, HSP70 and HSP90 after exposure to 37℃ and 42℃ for 90 minutes in HCT116 and HT29 cell lines *in vitro*.

Both HCT116 and HT29 displayed a higher expression of HSP70 when treated with 42℃, compared to 37℃ (see figure 4.3). In contrast, expression level of HSP90 was moderately increased by hyperthermia. The expression of HSP90 and HSP70 increased by 24% and 68% respectively, at 42℃ compared to 37℃ in HCT116 cells. Similar, increased expression of HSP90 and HSP70 by 15% and 54% respectively, at 42℃ compared to 37℃, was observed in HT29. The results of this experiment, indicate that 90 minutes of treatment with 42℃, is enough to increase the expression of HSP proteins, particularly HSP90 and HSP70. However, the expression pattern of HSP27 was different between the cell lines. HTC116 had an increased expression of HSP27 when treated with hyperthermia, while HT29 had almost no additional increase.

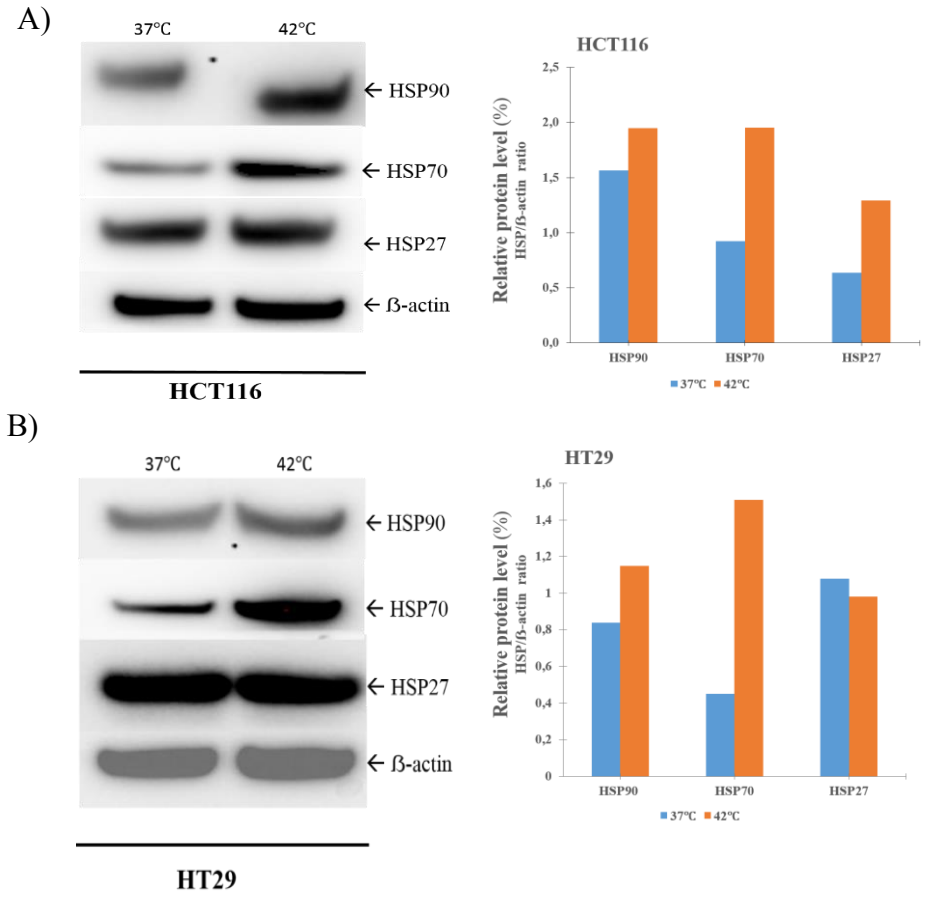


Figure 4.3: Protein expression of HSPs in HCT116 and HT29 cell lines, after exposure to 37 and 42℃. Protein expression of HSPs in A) HCT116 and B) HT29 cell lines were analyzed by Western blot (left panel), showing expression of HSP90, HSP70 and HSP27. ß- actin was included as a protein loading control to normalize HSP-expressions. Right panel shows quantifications of the protein bands done by the ImageJ program. Western blot samples were run with two biological experiments, with similar results.

4.3 Hyperthermic chemotherapy with HSP inhibitors *in vitro*

Increased expression of HSP proteins in cancer cells has been reported to be associated with malignant features and poorer prognosis of cancer patients. HSP proteins are therefore promising targets for cancer treatment, and inhibitors of HSP proteins are currently used in many clinical trials [63].

In this study, we wanted to investigate if inhibiting HSP protein expression could improve the effectiveness of hyperthermia treatment *in vitro*. For this purpose, we used 17-AAG (HSP90 inhibitor) and HS-72 (HSP70 inhibitor). In order to determine which concentration was optimal for our analysis, a serial concentration dilution range was performed for each inhibitor (Appendix 2), the cells were treated with the inhibitors for 90 minutes, and 72 hours post-treatment MTS-assay was performed. Inhibitor and drug concentrations used for this experiment is presented in table 3.2.

HCT116 and HT29 cells were treated with HSP inhibitors in combination with Mitomycin C and Oxaliplatin, with or without hyperthermia for 90 minutes. Cell viability was then assessed with MTS-assay 24 and 72 hours post-treatment. The purpose of cell viability measured at two different time points, was to see how early we could detect any effect of the treatment. MTS data at 24 hours post-treatment, can be seen at Appendix 3.

4.2.1 HCT116

Our results indicate that 17-AAG has a better effect than HS-72 on HCT116 cells. 17-AAG alone gave a significant cell viability reduction compared to control cells ($p= 0.001$). It had the same cytotoxic effect as Mitomycin C and Oxaliplatin, and it appears to give further decrease of cell viability when combined with each drug. HCT116 had a cell viability reduction of 43% at 37℃, relative to the control cells, when treated with 17-AAG alone. Mitomycin C and Oxaliplatin induced a cell viability reduction of 40% and 30%, respectively. Interestingly, as shown in figure 4.4, 17-AAG and Mitomycin C resulted in a cell viability reduction of 66%, which is significant vs treatment with only Mitomycin C ($p=0.02$). Hyperthermia gave an additional reduction of 10%.

Inhibition of HSP70 by HS-72 without addition of drugs had no effect on cell viability. Indeed, HS-72 seems to inhibit Oxaliplatin cytotoxic effect at 37℃, a cell viability reduction of only 12% was obtained in combination compared to 30% with Oxaliplatin alone (figure 4.5).

However, HS-72 effects on Oxaliplatin seems to be abolished at 42℃. HS-72 with Mitomycin C had no additional reduction of cell viability vs Mitomycin C alone.

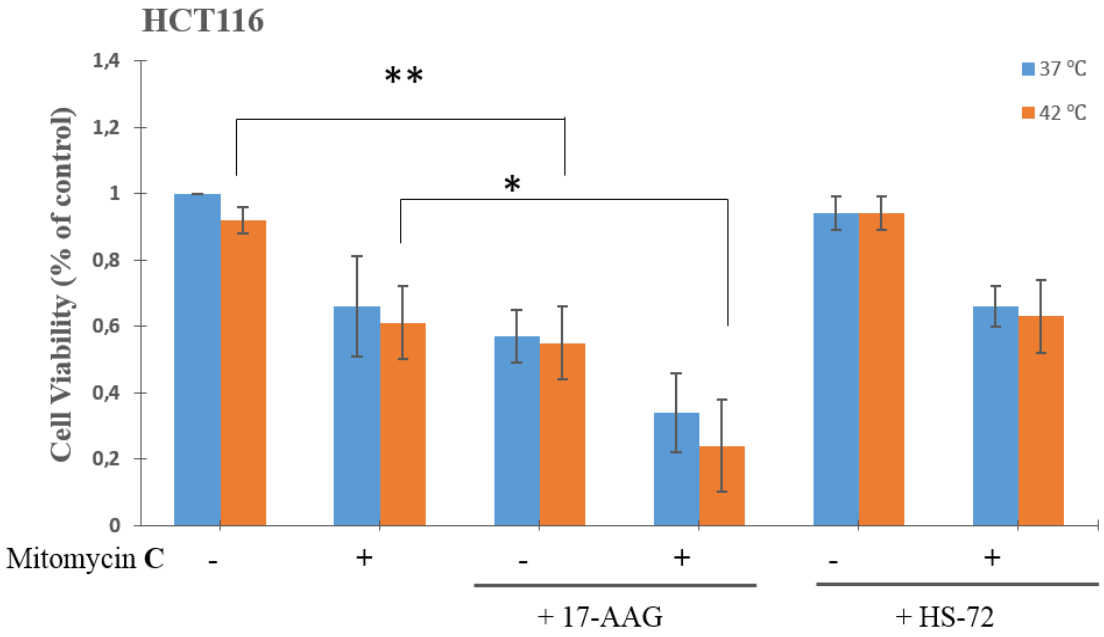


Figure 4.4: Hyperthermic drug treatment with Mitomycin C and HSP-inhibitors of HCT116 cell line. The graph shows cell viability estimated by MTS-assay, 72 hours post-treatment with Mitomycin C in combination with hyperthermia, 17-AAG and HS-72. Absorbance was measured at 450nmM and normalized to control-treated cells (cell without drug incubated for 90 minutes at 37℃). Error bars represent standard deviations of three biological experiments (n=3), each with three technical triplicates. *p<0.05, **p<0.01.

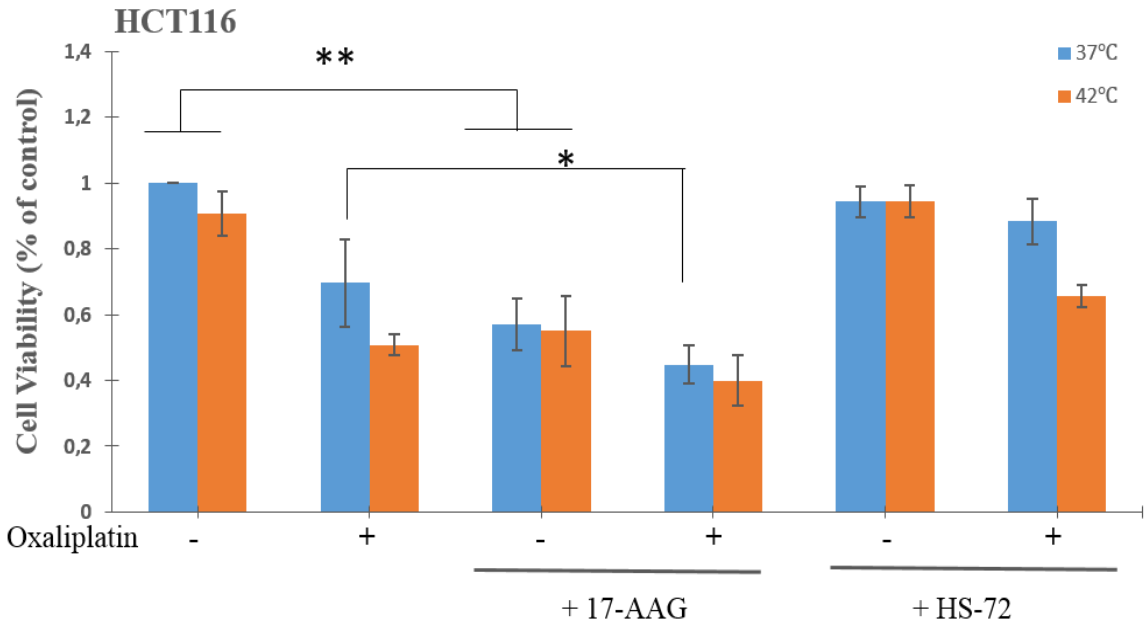


Figure 4.5: Hyperthermic drug treatment with Oxaliplatin and HSP-inhibitors of HCT116 cell line. The graph shows cell viability estimated by MTS-assay, 72 hours post-treatment with Oxaliplatin in combination with hyperthermia, 17-AAG and HS-72. Absorbance was measured at 450nM and normalized to control-treated cells (cell without drug incubated for 90 minutes at 37℃). Error bars represent standard deviations of three biological experiments (n=3), each with three technical triplicates. *p<0.05, **p<0.01.

4.2.2 HT29

HT29 cells were less sensitive to Mitomycin C than HCT116 in the previous experiment (section 4.1). It was therefore of interest to see if addition of HSP inhibitors would affect the drug-effectiveness of Mitomycin C in combination with hyperthermia.

With HT29 cells almost the same results were seen as for HCT116 cells. However, 17-AAG had more effect on cell viability than either of the chemotherapeutic drugs. Cell viability reduction of 65% at 37℃ was achieved by 17-AAG alone, compared with Mitomycin C (7%) and Oxaliplatin (11%). Significant cell viability reduction was obtained comparing to control cells (p=0.001). 17-AAG did not seem to increase the effect any further when combined with Mitomycin C, at both temperatures (figure 4.6). Interestingly, 11% additional decrease of cell viability was obtained when combined with Oxaliplatin at 42℃ (figure 4.7).

Unlike 17-AAG, HS-72 inhibitor alone had no effect on HT29 at 37 and 42℃. However, significant cell viability reduction of 44% at 42℃ was observed in combination treatment with Mitomycin C, compared to Mitomycin C alone (16%). This effect was not seen in combination with Oxaliplatin. HS-72 even inhibits the cytotoxic effect of Oxaliplatin, similar as showed previously with HCT116 (figure 4.7).

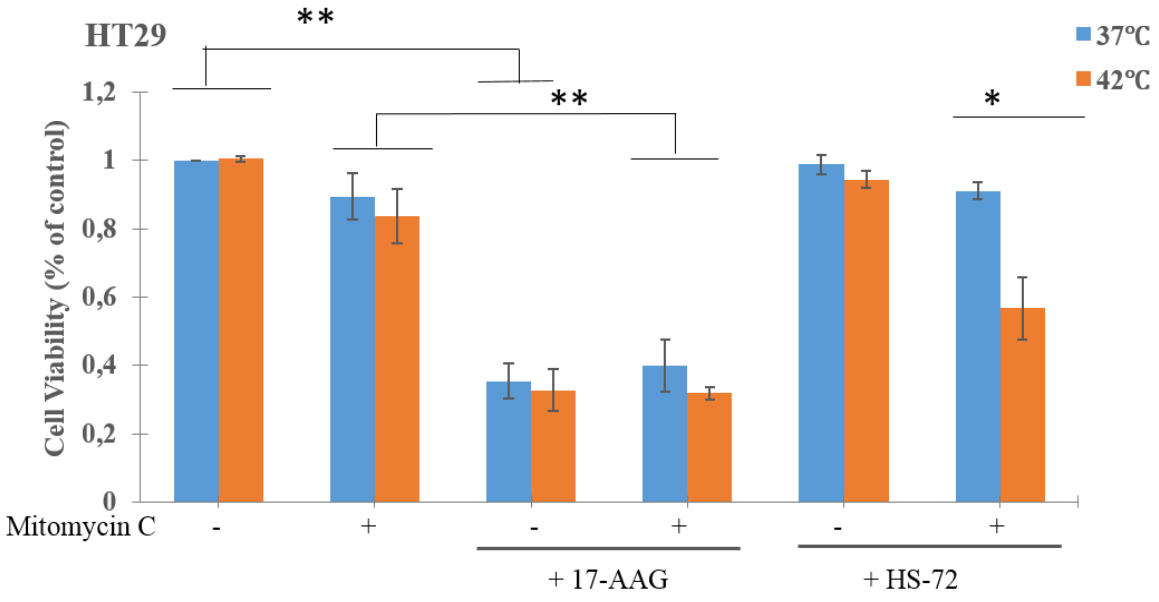


Figure 4.6: Hyperthermic drug treatment with Mitomycin C and HSP-inhibitors in H29 cell line. The graphs show cell viability estimated by MTS-assay, 72 hours post-treatment with Mitomycin C in combination with hyperthermia, 17-AAG and HS-72. Absorbance was measured at 450nM and normalized to control-treated cells (cell without drug incubated for 90 minutes in 37℃). Error bars represent standard deviations of three biological experiments (n=3), each with three technical triplicates. *p<0.05, **p<0.01.

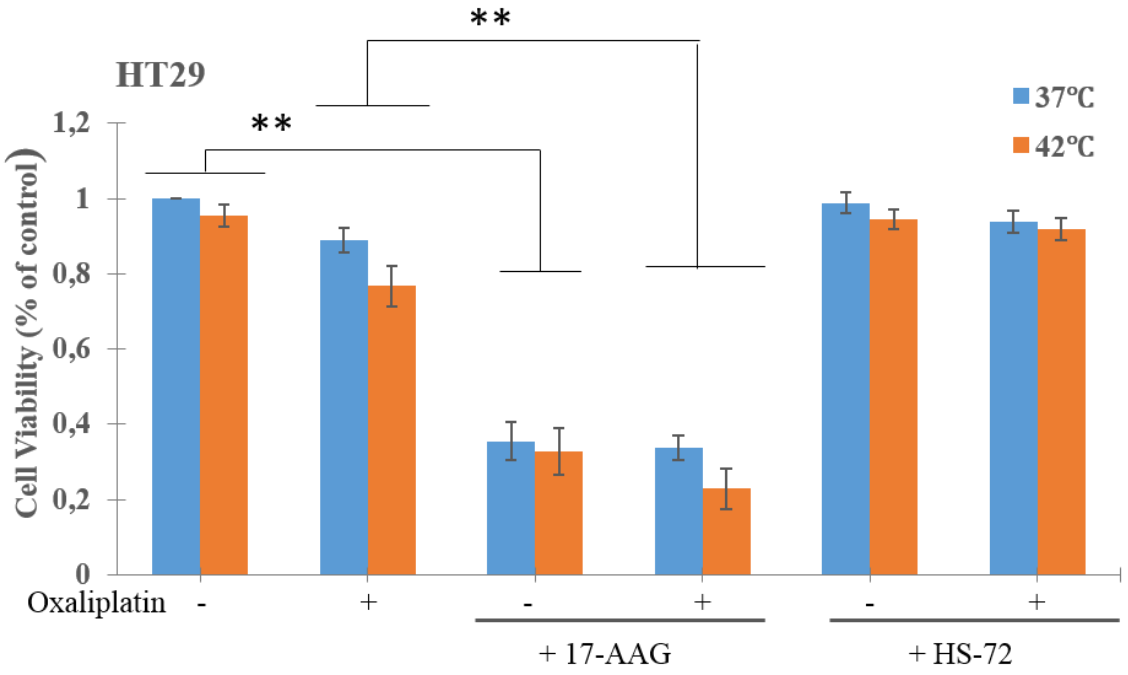


Figure 4.7: Hyperthermic drug treatment with Oxaliplatin and HSP-inhibitors of HT29 cell line. The graphs show cell viability estimated by MTS-assay, 72 hours post-treatment with Oxaliplatin in combination with hyperthermia, 17-AAG and HS-72. Absorbance was measured at 450nM and normalized to control-treated cells (cell without drug incubated for 90 minutes in 37℃). Error bars represent standard deviations of three biological experiments (n=3), each with three technical triplicates. *p<0.05, **p<0.01.

4.3 HSF1 siRNA

To investigate whether silencing of the heat shock transcription factor 1 (HSF1) could enhance the cytotoxic effect of the drugs, small-interfering RNA (siRNA) based gene silencing technology was applied. HSF1 regulates HSP proteins at the transcriptional level. Thus, similar to HSP proteins, HSF1 has also been discovered to be well expressed in numerous cancers. Silencing HSF1 has therefore been shown to prevent the progression of tumors and to enhance sensitivity to different anti-cancer treatments, particularly treatments which includes hyperthermia. [64]. This experiment was based on my previous master thesis results, and due to time shortage, only the HCT116 cell line was selected for investigation.

Before we could proceed with the experiment, we had to confirm that transfection of HSF1 siRNA could really silence HSF1. This was done by performing a protein expression analysis by Western blot, 24 and 48 hours post transfection of HSF siRNA. The results of this analysis can be seen at Appendix 5. Reduction of HSF1 protein expression was clearly observed by 24 hours post transfection. It was therefore decided to transfect the cells for 24 hours, before proceeding to the next part of the treatment, which was to include the hyperthermia drug

treatment itself. Consequently, 24 hours post transfection of HSF1 siRNA, the cells were incubated at 37℃ and 42℃ for 90 minutes, either in combination with Mitomycin C or Oxaliplatin. Further, we analyzed expression of HSP and HSF1 proteins by western blot, post-hyperthermia chemotherapy. Importantly, expression levels of HSPs were clearly suppressed in the HSF1 siRNA-transfected cells (figure 4.8). However, HSF siRNA transfected cells after treatment with Oxaliplatin in combination with hyperthermia, increased expression of HSP70 and HSP27. Due to the time limitation, this was done with only one biological experiment.

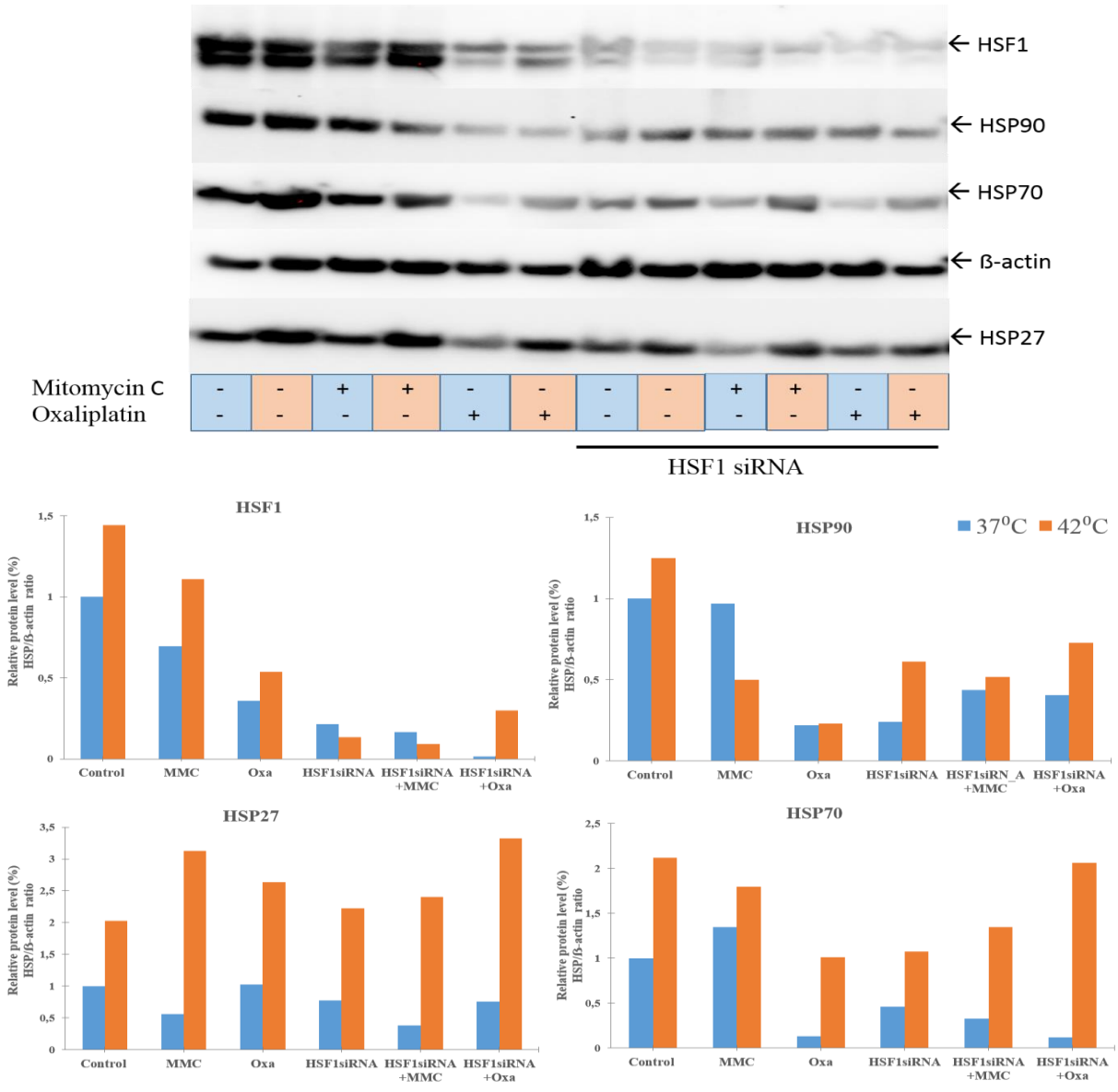


Figure 4.8: Effects of HSF1 siRNA transfection on HSP protein expression post hyperthermic chemotherapy. HCT116 cells were first transfected with HSF1siRNA for 24 hours. Following treatment with either Mitomycin C (MMC) or Oxaliplatin (Oxa) for 90 minutes at 37 and 42℃. 48-hours post drug treatment, expression of HSPs was analyzed by Western blotting. ß- actin were used to normalize HSP-expressions. The graphs show quantifications of the bands, done by the ImageJ program. Western blot is performed with one biological experiment.

Figure 4.9 shows, cell viability measured by MTS-assay, 48 hours post hyperthermic chemotherapy. The result of this experiment was not consistent with our hypothesis, that siRNA transfection could provide additional effect on reduction of cell viability. HSF siRNA transfected cells had almost the same cell viability as non-transfected and negative siRNA treated cells. Importantly, the results of the chemotherapy drug treatment, Mitomycin C and Oxaliplatin, were the same as in previous experiments. In addition, this experiment also shows hyperthermia have no significant cytotoxic effect compared to 37℃. MTS data of this experiment is from two biological experiments, each with three technical triplicates. Importantly, we have achieved what was the most important task, to reduce HSPs transcriptional level to an expected extent.

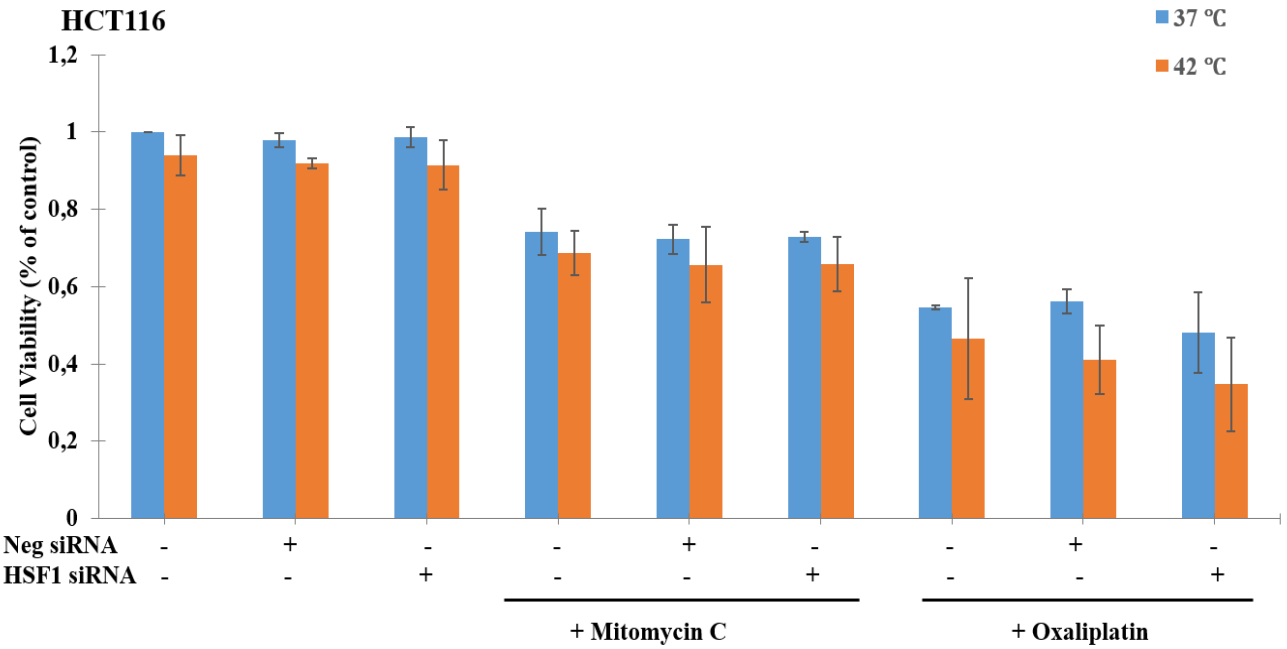


Figure 4.9: The effects of HSF1siRNA and hyperthermia with Mitomycin C and Oxaliplatin on cell viability. The cells were treated with either HSF1siRNA or negative siRNA for 24 hours and were then treated with Mitomycin and Oxaliplatin at 37℃ and 42℃ for 90 minutes. Cell viability was determined by MTS-assay 48h post- hyperthermic chemotherapy. Absorbance was measured at 450nM and normalized to control cells (cells without drug at 37℃). Error bars represents standard deviations of two biological experiments (n=2), each with three technical triplicates.

4.4 Hyperthermic drug treatment of tumor tissue from patient and mice

In this part of the study, we were interested in investigating the possibility of using our hyperthermia drug treatment model, on fresh tumor tissue from a patient and mice. We used tumor tissues (mucin) from PC-CRC PDX-mice models and from a PMP patient. Hyperthermia drug response was measured by analyzing cell viability, 48 hours post-treatment. We received a total of five tumor tissue samples, mucin from four mice and one patient. The samples were

largely dissimilar with regards to, consistency and the amount of material. It is therefore difficult to compare the results between the samples.

Figure 4.10 shows treatment response of two mice mucin samples. The sample from mouse 1 was treated with 60µM Mitomycin C and 289µM Oxaliplatin. Significant cell viability reduction of 43% at 37℃ and 53% at 42℃, was observed when treated with Mitomycin C ($p=0.001$), compared to control cells without drugs. With Oxaliplatin a cell viability reduction of 30% at 37℃ was achieved. Importantly, hyperthermia did increase the drug effect significantly, by a cell viability reduction of 64% ($p=0.001$). Due to the little amount of tissue sample, we were not able to use HSP inhibitors on the mouse 1 sample.

In contrast, sample from mouse 2 was only treated with Oxaliplatin. The result shows no additional effect of hyperthermia when treated with Oxaliplatin, but a cell viability reduction of approximately 40% was observed at both temperatures, at 37℃ ($p=0.006$) and 42℃ ($p=0.06$). Fortunately, we were able to treat the sample with both 17-AAG and HS-72, in combination with Oxaliplatin. 17-AAG did not contribute to additional cell viability reduction vs Oxaliplatin alone. When combined with HS-72, we observed the same result as we have seen with CRC cell lines. HS-72 did inhibit the cytotoxic effect of Oxaliplatin at 37℃, the cell viability was the same as for control cells. Cell viability did decrease with 26% with hyperthermia, but found not to be significant from that at 37℃.

Two more mucin samples from animals, mouse 3 and mouse 4, were examined and the results can be seen at Appendix 6. Due to, higher standard deviation between the replicas, we have chosen not to include the results in this section. To exclude disturbance from the mucin, we improved the assay by including a centrifugation step of the 96-well plates before reading the absorbance from MTS-assay.

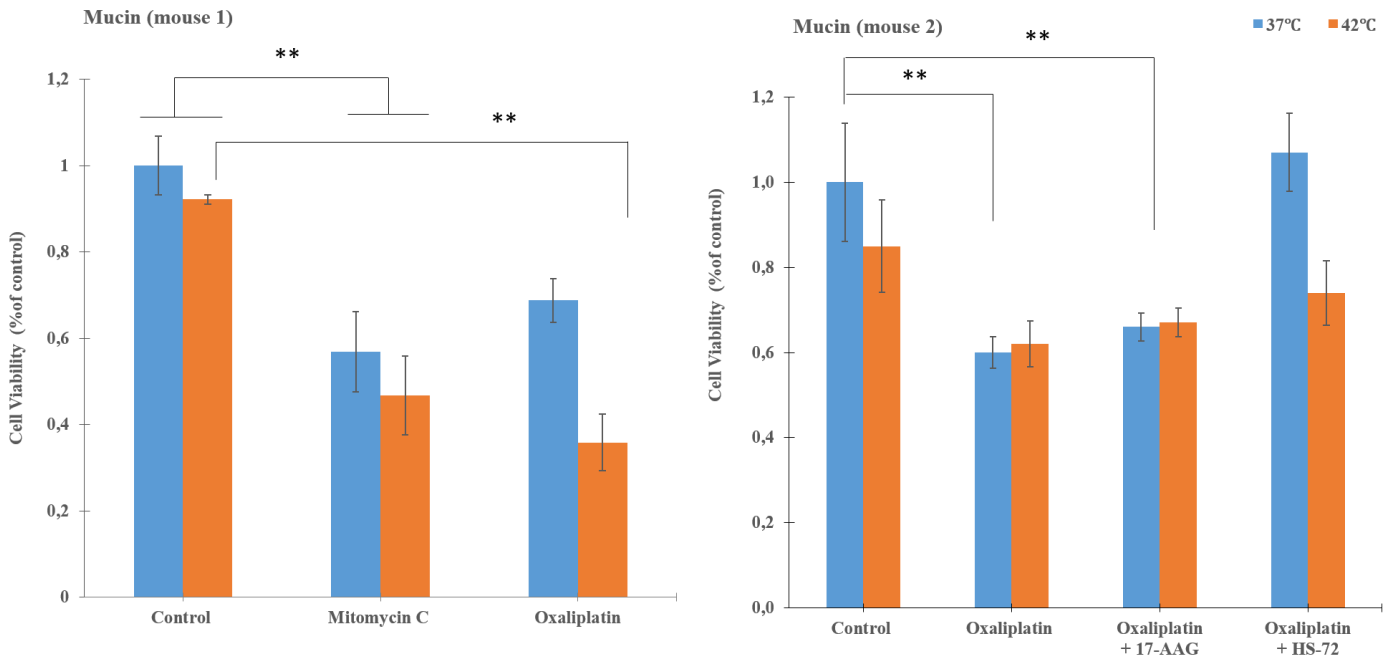


Figure 4.10: Hyperthermic drug treatment of PC-CRC mucin from mmice The graphs show cell viability analyzed by MTS-assay, 48 hours post hyperthermic drug treatment of tumor tissues (mucin) from mice. Sample from mouse 1 was treated with both Mitomycin C and Oxaliplatin in combination with hyperthermia. Sample from mouse 2 was treated with Oxaliplatin in combination with hyperthermia, 17-AAG and HS-72. Absorbance was measured at 450nmM and normalized to control-treated cells (cell without drug incubated for 90 minutes at 37℃). Error bars represent standard deviations of one biological sample, with four technical triplicates. *p<0.05, **p<0.01.

Fortunately, we received mucin from PMP a patient, that we managed to treat with both drugs and 17-AAG (figure 4.11). There was significant different in cell viability between the drugs and control cells. Hyperthermia significantly decreased cell viability when combined with Mitomycin C, a reduction of 46% vs 26% at 37℃ (p= 0.01). Oxaliplatin had a significant reduction in cell viability at both temperatures (p=0.008 at 37℃ and p=0.04 at 42℃) compared with control cells, viability reduction of 39% at 37℃ and 41% at 42℃, indicating no additional effect of hyperthermia.

17-AAG gave no additional cell viability reduction when combined with Mitomycin C. However, compared with control cells it had a significant effect (p<0.001). Oxaliplatin plus 17-AAG treated cells resulted in additional cell viability reduction vs Oxaliplatin alone, 50% at 37℃ and 66% at 42℃. Significant cell viability reduction was observed at 42℃ compared with Oxaliplatin alone (p=0.002). Unfortunately, we did not have enough mucin to analyze the single treatment effect of 17-AAG. Therefore, the result from this data, cannot be used to predict the combination treatment, because cell viability reduction may have been initiated with only 17-AAG.

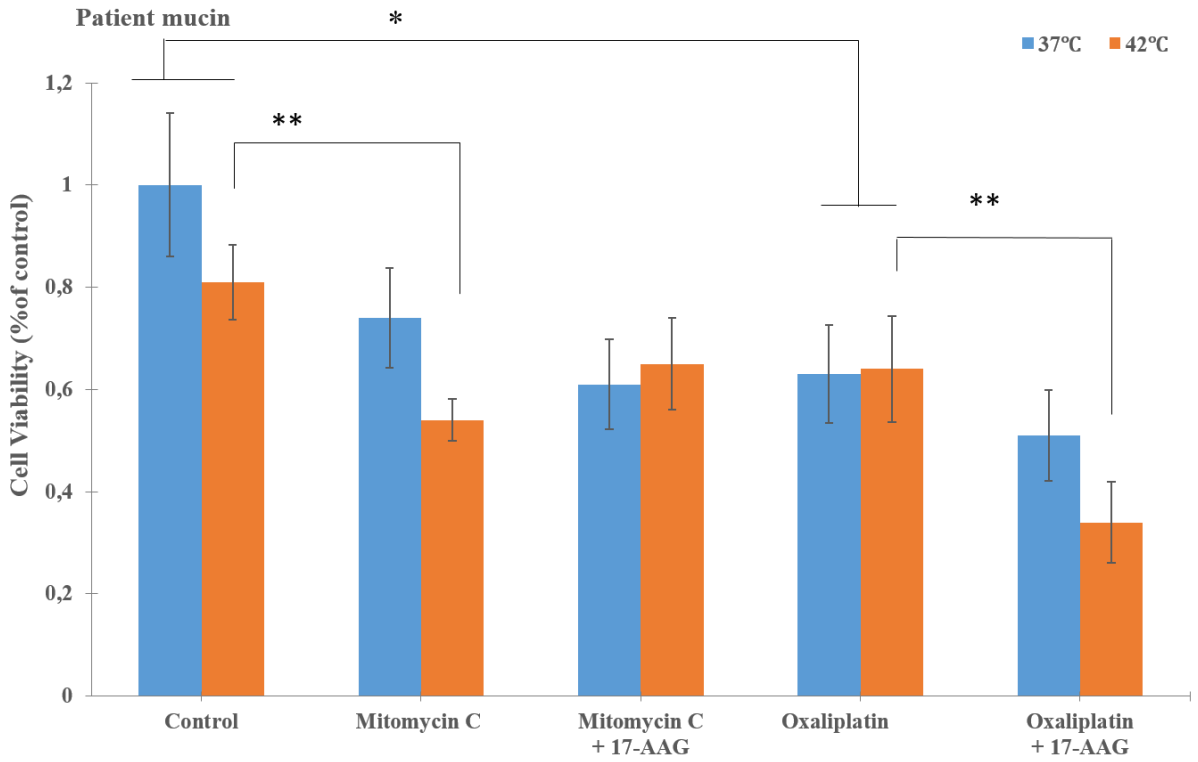


Figure 4.11: Hyperthermic drug treatment of mucin from patient. The graphs show cell viability determined using MTS-assay, 48 hours post hyperthermic treatment in combination with Mitomycin C, Oxaliplatin and 17-AAG, of tumor tissues (mucin) from PMP patient. Absorbance was measured at 450nmM and normalized to control-treated cells (cell without drug incubated for 90 minutes at 37℃). Error bars represent standard deviations of one biological sample, with five technical triplicates. *p<0.05, **p<0.01.

4.5 HMGB1

Cancer treatment that induces immunogenic death of tumor cells, leads to activation of several antitumor immune responses. Hyperthermia is known to induces several danger-associated molecular patterns (DAMPs), such as HSP70, HSP90, ATP and HMGB1 [65, 66]. In this study, we wanted to see if hyperthermic chemotherapy induces the release of HMGB1. Subsequently, HMGB1 released from the treated cells into the cell medium was analyzed by Western blot, 72 hours post hyperthermic chemotherapy. The relative level of HMGB1 to a control loading protein could not be measured, since there is no known such extracellular protein to our knowledge. Our result shows an increased level of HMGB1 on cells treated with hyperthermia vs cells treated with 37℃ (figure 4.12). Treatment with hyperthermia in combination with Mitomycin C and Oxaliplatin gave an additional increased release of HMGB1. HCT116 had a significant increased level when treated with Mitomycin C and hyperthermia. Meanwhile, the results with HT29 showed a significant increased level with Oxaliplatin and hyperthermia. Oxaliplatin is recognized as one of the chemotherapy drugs that induces immunogenic cell

death.[67] Thus we have achieved to confirm that, by both of our two cell lines, with and without hyperthermia.

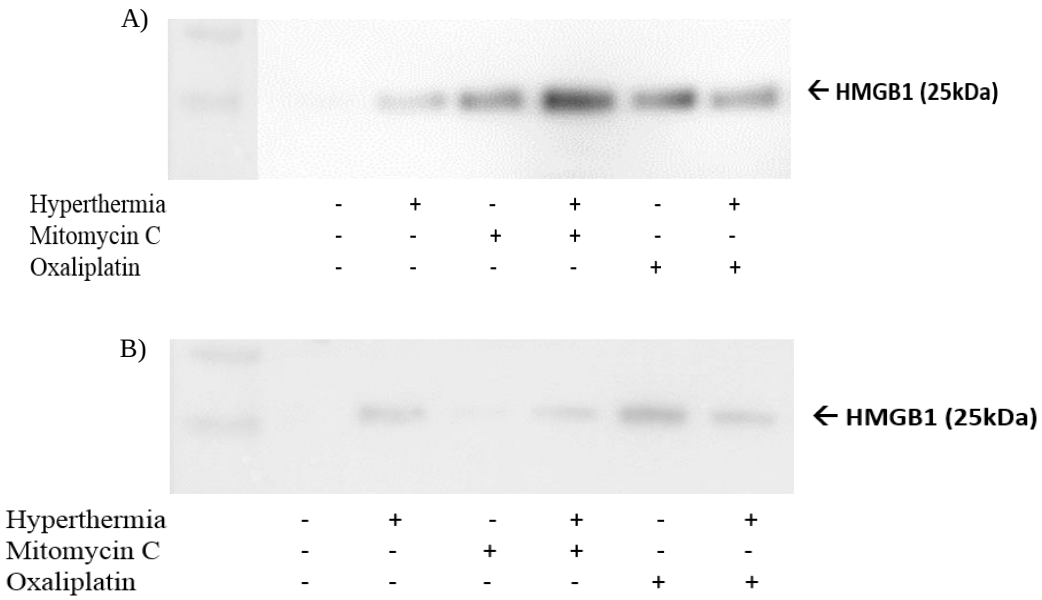


Figure 4.12: HMGB1 level visualized by western blot; 72 hours post hyperthermic chemotherapy on A) HCT116 and B) HT29. The cells were treated with 60µM Mitomycin C and 289µM Oxaliplatin, and allowed to recover for 72 hours before western blot analyzes was performed. HMGB1 protein level were visualized, using ChemiDoc™ Imaging System (Bio-Rad). Furthest to the left, is the molecular weight of standard (SeeBlue®Plus2 Pre-Staind Standard, 17kDa and 28kDa) Western blot was performed with one biological experiment; new round of samples is in the freezer to be used in the further.

5 Discussion

CRS-HIPEC treatment provides a long-term survival to PC-CRC patients, however most patients experience relapse and treatment associated morbidity and mortality.[68] Tumor cells manage to develop mechanisms/resistance that protect them against chemotherapy and hyperthermia [69]. Thermo-tolerance in cancer is usually based on the induction of heat-shock proteins (HSP) [70]. In this master thesis, we aimed to investigate the sensitizing effect of hyperthermia on chemotherapeutic drugs, Mitomycin C and Oxaliplatin, which are commonly used in the clinical HIPEC procedure to treat PC-CRC.For this purpose we used our newly developed *in vitro* model, which closely mimic the clinical HIPEC condition, with the HCT116 and HT29 CRC cell lines. Treatment response varied greatly between the cell lines as well as between the drugs. Consequently, HSPs inhibitors and HSFsiRNA were included in the experiment, in efforts to enhance the cytotoxic effect of the treatment. In addition, an *ex vivo* experiment on CRC tumor tissues was used to in order to translate our finding as close as possible to the clinical situation. Furthermore, we investigated the beneficial effect of hyperthermia to immunogenic cell death (ICD), by measuring extracellular HMGB1, post-treatment.

5.1 Is hyperthermia beneficial to use for PC-CRC treatment?

In many European hospitals, CRS-HIPEC using Mitomycin C or Oxaliplatin has become a gold standard treatment of PC-CRC and PMP [71]. CRS-HIPEC has shown to have an overall survival benefit compared with palliative treatment only. At the Norwegian Radium Hospital, Mitomycin C is used in the HIPEC procedures and Frøysnes, *et al*., reported long-term outcome with acceptable morbidity after HIPEC treatment in PC-CRC patients. [32]. Subsequently, they concluded that CRS-HIPEC as the best treatment option for PC-CRC patients. In addition, one randomized trial has reported a survival benefit for gastric cancer patients treated with HIPEC, comparing intraperitoneal perfusion (IPC) with Mitomycin C and Cisplatin under normothermic and hyperthermic conditions [72]. However, the clinical benefit of hyperthermia during HIPEC and its importance for the reported improved survival is yet unclear. An in vitro experiment of PC-CRC, concluded that the effectiveness of CRS-HIPEC treatment depends on the chemotherapeutic drugs and not on hyperthermia [73]. Importantly, the most determining factor for all PC-CRC patients is the completeness of CRS.

Firstly, our result shows dose-dependent cell viability reduction on both cell lines, when treated with Mitomycin C and Oxaliplatin, either at hyperthermia (42°C) or normothermia (37°C). However, hyperthermia as an inducer of additional cell viability reduction was cell line and chemotherapy drug selective. Interestingly, we found HT29 to be less sensitized by both Mitomycin C and Oxaliplatin, compared to HCT116, which is in line with published reports. Richard, *et al*., demonstrated that HT29 to be more resistance to Oxaliplatin than HCT116 [74] and Gaur, *et al*., reported similar result [75]. However, we found hyperthermia to have significant potentiated cytotoxic effect when combined with Mitomycin C (120µM) and Oxaliplatin (289µM) compared normothermia. Consequently, this indicate that sensitizing effect of hyperthermia is dependent on selected drug(s) at different concentrations. Indeed, it is few systematic data available, that provide evidence for the underlying hypothesis of a positive cytotoxic effect of hyperthermia in combination with chemotherapy.

There have been several clinical trials, to evaluate which one of the two drugs are the most beneficial to use in HIPEC treatment. In a clinical trial, HIPEC was performed either with Oxaliplatin or Mitomycin C to evaluate differences in toxicity profile and long-term outcome between these two drugs, and no significant difference between the drugs was observed [76]. However, clinical studies have reported higher morbidity for Oxaliplatin, compared to Mitomycin C- treated groups, assuming Mitomycin C as the best chemotherapy drug to use in HIPEC [77, 78]. Although, both drugs are suitable for HIPEC, because of their ability of high cytotoxicity even at short exposure time, have metabolic transformation, do slowly penetrate through peritoneum, and have effective synergy with hyperthermia [79]. Interestingly, one clinical Phase 1 experiment demonstrated that combining those two drugs, plus 5-fluorouracil, appears to be safe for patients at high risk of PC-CRC. They also demonstrated, *in vitro*, that the combination strategy contributed to growth inhibition of CRC cells under hyperthermic conditions [80]. Perhaps we could have enhanced the effectiveness of hyperthermia by combining Mitomycin C and Oxaliplatin in our model.

Additionally, chemo-sensitization effect of hyperthermia, depends on a combination of temperature and time of exposure, referred to as "thermal dos". Thermal dose have been observed to affect the treatment response [70]. In this study, we used an exposure time of 90 minutes, as it mimics the clinical setting at our hospital. However, we are aware of that increasing the incubation time most likely would had increased cell cytotoxicity, but it is not

clinical relevant. This also applies to the choice of temperature; many *in vitro* experiments have used higher temperature than we have, monitoring significant reduced cell viability. This will of obvious reasons not be relevant for use in the clinic, because temperature above 43°C causes cell death in normal cell [70]. Additionally, we are aware that viability assay provides limited data, and must be supplemented with other methods of analysis to draw conclusions on treatment response in HTC116 and HT29.

5.2 Hyperthermia treatment induces a Heat Shock Response *in vitro*

It is important to investigate targets and other strategies, which could enhance the effect of hyperthermia plus chemotherapy. High expression of HSPs have been observed in many cancer cells [81]. Beside their chaperon function, HSPs interacts with several ant-apoptotic and survival pathways, which contributes to cytoprotective and ant-apoptotic effect, resulting in thermo-tolerance and chemo- resistance [82]. Hyperthermia is known to induce a high HSPs expression [83]. Indeed, we found that hyperthermia alone induces a constitutively higher expression of HSP70 and HSP90 on both cell lines, comparing to cells treated with normothermia, which has been observed previously [84]. Particularly, HSP70 expression was found highly increased in HTC116, the cell line that was found less sensitized to hyperthermia in combination treatment. This could perhaps explain its cytoprotective and anti-apoptotic function during the treatment. Meanwhile, HT29 had less expression of HSPs than HCT116, which can clarify its sensitivity to hyperthermia. Do to time constraints; we investigated post-treatment expression level of HSPs, only in HCT116. Consistent to our result, expression of HSPs have been observed to be much higher after hyperthermic chemotherapy treatment, by Mitomycin C and Oxaliplatin [85, 86]. Additionally, high up-regulated gene expression of HSPs, have also been reported in patient tumor tissue after HIPEC treatment compared to samples before treatment [87]. Determining HSPs expression after treatment, could reveal the thermo-tolerance behavior is due to an increased expression of HSPs. In order to confirm this statement, we need a better quantitative expression analysis of HSPs at several time points, pre- and post- treatment. Because of observations like our study, targeting HSPs in cancer treatment have become interesting. Presently, several HSPs inhibitors are undergoing pre-clinical and clinical trials in combination with different cancer treatment, including hyperthermia [88].

5.3 HSP inhibitors in hyperthermic drug treatment

To verify our hypothesis, that inhibition of HSPs could enhance the effect of hyperthermic chemotherapy, HSP90 inhibitor (17-AAG) and HSP70 inhibitor (HS-72) were included in this experiment. 17-AAG is the first to enter into clinical trials, due to the fact that it is found to be less toxic than first HSP90 inhibitor, Geldanamycin [55]. 17-AAG alone had a high cytotoxic effect in both HCT116 and HT29. However, when combined with hyperthermia and chemotherapy, it did not contribute to additional cell viability reduction. Contrary to our result, another experiment, where 17-AAG was used in combined with Oxaliplatin and Capecitabine (anti-metabolite which is often used with Oxaliplatin to enhance the drugs effect), shows a significantly cell viability decrease [89]. Nevertheless, cell viability assay was done after the cells were treated with the drugs and 17-AAG for 24 hours. Rakitina, *et al*., also investigated the effects of 17-AAG and Oxaliplatin, using both HT29 and HCT116 cell lines. They found HT29 cells to be more sensitized to the treatments, than HCT116 [90]. However, hyperthermia was not used in this experiment.

HS-72 inhibitor, on the other hand, did not appear to have any cytotoxic effect in both cell lines, even when combined with hyperthermia and chemotherapy drugs. Previous experiment using HS-72 inhibitor across multiple tumorigenic cell lines, has shown anti- proliferative effect [56]. However, the cells were incubated with HS-72 for 24, 48 and 72 hours, which is different to our experiment (90 minutes). Interestingly, another improved HSP inhibitor, Pifithrin-μ, (2-phenylethynesulfonamide (PES), has been reported and showed to enhance the cytotoxic effect of Oxaliplatin in HT29 cell line [91]. The effective way to enhance the effect of HSPs inhibitors has been reported to be inhibition of two or more HSPs at the same time. Subsequently, inhibition of HSP90 induces high expression of HSP70 and HSP27 [92]. The combination treatment of 17-AAG (HSP90 inhibitor) and VER155008 (HSP70 inhibitor) *in vitro*, has shown a significantly cell viability reduction compared to cells treated without inhibitors [85]. Unlike our experiment, the cells were exposed to hyperthermic chemotherapy for 60 minutes at 43°C. Consequently, this incoherence between pervious experiments and the results in our study may be due to the different dose regimens and exposure times. In our case, 90 minutes of treatment might have been too short with HS-72. In further investigations, we should look for a more effective dose and exposure time of the inhibitors in the CRC cell lines. Treatment of 17-AAG and HS-72 for 24 hours, after/before hyperthermic chemotherapy could perhaps contribute to additional cell kill. In addition, further studies on hyperthermic chemotherapy, where HSP

inhibitors are included, may use a combination of HSP70/HSP90 inhibitors to be able to successfully inhibit both HSPs.

5.4 Effective silencing of HSF1 contributes to suppression of HSP70, HSP90 and HSP27

HSF1 is the main regulatory of HSP expression, and therefor promotes indirectly, tumor cell survival [93]. In this study, HSF1 was silenced to prevent the expression level of HSPs. Certainly, we have accomplished a successful reduction of HSF1 protein level in HCT116 cell line, using HSF1siRNA. HSF1 protein level was kept low for at least 48 hours after transfection of HSF1siRNA in HCT116 cell lines. Interestingly, silencing HSF1 did in fact contributed to efficient suppression of HSP70, HSP90 and HSP27 expression. Study using siRNA to target HSF1 in HeLa cell lines, have shown to be effective to suppress several HSPs, also post-hyperthermic treatment [94]. Importantly, we found the siRNA transfection procedure does not have a toxic effect, by observing no differences in cell viability between negative-siRNA treated and control cells (appendix 4). The fact that we manage to suppress HSF1 for 48 hours post-hyperthermia, shows that HSF1siRNA interference is not reversed or inhibited by heat. This could be important observation in view of possible clinical application on HIPEC treatment.

5.5 HSF1 silencing did not sensitizes HCT116 cells to hyperthermic drug treatment *in vitro*

HSF1 could be a promising target in many cancer treatments, especially with treatment that includes hyperthermia [95]. In this study, silencing HSF1 could be a more efficient strategy to enhance the effect of hyperthermic chemotherapy treatment, than targeting specific HSPs as with the inhibitors. During this experiment, we were able to improve our *in vitro* model, making it suitable for use of siRNA transfection. The first experiments were done on the 96-well-plate, which eventually turned out to be technically challenging. First of all, adding a small amount of drug dilution on a 96-well-plate makes it difficult to mix the drug properly, and secondly, incubating 96-well-plate in a water bath was found to be complicated. Therefore, we aimed to transfect the cells on a 6-well plate, before the cells were detached by trypsin the day of treatment. Consequently, we could perform the treatment in a tube, as in previous experiments.

Surprisingly, the result of this study shows no additional difference in response to Mitomycin C and Oxaliplatin between HSF1siRNA treated cells and non-transfected cells, under both

hyperthermic and normothermia conditions (figure 4.9). Previous *in vitro* experiment on melanoma cells (MeWo) has shown that silencing HSF1 with adenovirus-expressing short hairpin RNA (shRNA), enhanced the sensitivity of hyperthermia, but did not affect their sensitivity to Dacarbazine [96]. Additionally, silencing HSF1 by stabile transfection (HSF1i) in cervical carcinoma (HeLa cells), did not enhance cytotoxic effect of Cisplatin treatment, but when combined with hyperthermia, ~95% of HeLa-HSF1i cells underwent apoptosis, 24 hours post- treatment [94]. However, unlike our experiment where the cells were treated with drug and hyperthermia (42°C) at the same time for 90 minutes, HeLa-HSF1i were treated with Cisplatin for 2 hours and then treated with hyperthermia for 1 hour at 43°C. Temperature and exposure time could be the reason to the different effect. To our knowledge, no other hyperthermic chemotherapy studies have been carried out, on silenced HSF1 CRC cell lines in an *in vitro* model mimicking clinical HIPEC. Therefore, it is impossible to compare our data with previous experiments.

5.6 Hyperthermic chemotherapy induces releases of HMGB1

Immunotherapy is a treatment that uses the body's own immune system to help fight cancer, resulting in a good outcome and prolonged survival in some cancer types. In this study, we wanted to investigate if hyperthermic chemotherapy induces ICD, by the releasement of HMGB1. Subsequently, hyperthermia alone was found to increase HMGB1 release, making hyperthermia an excessive ICD inducer. HMGB1 is a DNA-binding protein originally known as a nuclear non-histone chromatin-binding protein, released from cells undergoing apoptosis and necrosis, and acts as danger signal and induce inflammation by binding to different receptor on the surface of antigen-presenting cell (APCs) and dendritic cells (DCs) [97, 98]. In consistent to our result, previous experiments, have also reported hyperthermia induced ICD processes, such as increased extracellular HMGB1 and/or HSPs, in different type of cancers [99, 100]. Combination studies of hyperthermia and radiation have also showed increased released of HSP70 and HMGB1, after treatment in melanoma cell line (B16) [101, 102]. Similar results have been observed in CRC cell lines (HCT15 and SW480) [103]. This may confirm the benefit of hyperthermia, in combination treatment with different type of anti-cancer agents.

In addition, we found increased level of HMGB1 after combination treatment of hyperthermia and chemotherapy in both HCT116 and HT29 cell lines. To our knowledge, no previous experiments done in this type of combination to support these result. However, previous

experiments, *in vivo* and *in vitro*, have showed that chemotherapy alone induce release of HMGB1, such as Paclitaxel, Doxorubicin and Oxaliplatin [104-107]. Furthermore, we found Oxaliplatin to trigger more release of HMGB1, than Mitomycin C, during both hyperthermic and normothermia conditions. [108]. Oxaliplatin is a known inducer of ICD, by a massive release of immunomodulation factors, which has been demonstrated in CRC cells and in post treated CRC patients [109]. The result of this study could verify the beneficial effect of hyperthermia in HIPEC treatment. However, further investigation of how hyperthermia induces ICD should be considered, by using other analyzes, such as examining the extracellular HSPs and ATP.

5.7 Hyperthermic drug model was successfully used on mucin from PDX-mice and patient, *ex vivo*.

One of the major problems in oncology drug or treatment development is the low success of converting preclinical results to clinical use. To be able to translate our experiment closer to clinic use, we used our experimental model to examine fresh tumor tissues from patient and PDX-mice, so-called *ex vivo* experiment. Consequently, *ex vivo* experiments, provides a relative heterogeneous reflection of the original tumor. Importantly, the results of this study shows significant response to hyperthermic chemotherapy, with both drugs. Additionally, hyperthermia did in fact increase the effect of Mitomycin C in sample tissues from mice. Meanwhile, hyperthermia increased the effect of Oxaliplatin in the patient sample. To our knowledge, no other ex vivo studies have been carried out testing the same type of cancers as we have done, to mimicking clinical HIPEC. However, there are data from experiments done with only chemotherapy [110-112]. In addition, no other *ex vivo* experiments, have been done using HSPs inhibitors in this kind of treatment. Although, 17-AAG and HS-72 in our experiment, showed no beneficial effect of combination treatment of hyperthermia and chemotherapy.

Consequently, the use of our model could facilitate drug screening or personalized medicine strategies, for PC-CRC, but also for PC from other origins as PMP and OC. However, it is required to improve our model, when using mucin tissues. Mucin is very difficult to dissolve in medium; how good the mucin is dissolved in solution varies from sample to sample, depending on its thickness and complexity. In addition, we were not able to wash out the drugs after the treatment, resulting an exposer time, different from experiments with cell lines. All this could

perhaps have an impact on our result. In addition, the use of MTS-assay, to inspect cell viability of mucin sample, also needs critical evaluation. Mucin- sample consistence, possibly affect reflection or transmission of MTS absorbance in spectrophotometer.

5.8 Methodological discussion

Human cancer cell lines are frequently used in cancer research, including for development of cancer treatment, drug screening, and to study molecular mechanisms of tumor cell biology and metastasis [113]. Cell line culturing contributes to a series of advances in cancer research. A collection of CRC cell lines have been used for several experiments, many of them are available from ATCC, including HCT116 and HT29 [114]. However, there are several disadvantage of using cell lines. In many cases, drug effects in preclinical experiments can not be considered to give a promising clinical response. Due to that several factors can change over time, as introduction of new mutations, contamination and change in cell line characteristics (morphology), and changes in protein expression profiles in the a cell line [115, 116]. In addition, culture conditions represent simplified physiological condition, and do not resemble tumor microenvironment and heterogeneity of cancer cells in the cancer patient [117]. In our study, we have used two different CRC cell lines, with different genetic profiles, to assume that each cell line represents homogenous cell population in a patient, in a way to predict a sort of heterogeneity. However, including several cell lines is needed to support our data and eventually translate to the clinic. To further support a translation to the clinical situation, we used our *in vitro* HIPEC model on patient and PDX-mice tissue. The use of PDXs and ex vivo experiment on fresh patient tissue, have improved preclinical evaluation of treatment response, and enhance the ability to predict a clinical trial procedure [118].

In cancer research, cytotoxic assay or cell viability assay are often used to investigate drug response *in vitro*. Many cell viability assays, including MTS, measured the number of metabolic active cells, whereas viability assay should actually measure the correlation between 'living' and 'dead' cells, and gave an expression of the proportion that remain viable. The "cell viability" we observed using MTS, is a decrease in metabolic activity, and not necessarily cell viability. It estimates growth inhibitory effect of drug treatment, rather than cell viability with in each treated cell-suspension [119, 120]. Therefore, we suggest further experiment, different analyses, such as ATP, TUNEL, DNA fragmentation or caspase assay.

As we discussion above, many factors can affect hyperthermic chemotherapy response, such as concentration of drugs, temperature and exposer time. Several *in vitro* experiments have continuous incubation of cells with drug, and uses different time point before cell viability assays. This should therefore be taken into account when comparing data with previous experiments. Moreover, many experiments performed in hyperthermic condition uses different temperatures, which also prevent data comparison. In this study, we decided to mimic the clinic condition of HIPEC, as much as possible to be able to refer the data for clinic use.

6 Conclusion

CRS-HIPEC is recognized as the best therapeutic option for patients with PC-CRC, and has improved the survival rate, as well as enhanced long-term 5-year survival. However, few pre-clinical studies have provided evidence for the underlying hypothesis of a positive cytotoxic effect of hyperthermia in combination with chemotherapy. Consequently, there is a need for increase knowledge and new therapeutic strategies to improve the effect of the treatment. In this study, the effect of hyperthermia was observed to be cell line and chemotherapeutic drug selective. Hyperthermia enhanced the cytotoxic effect of Oxaliplatin at the clinical relevant concentration in both cell lines, compared to treatment at 37℃. Interestingly, the HSP inhibitor 17-AAG had stronger cytotoxic effect than the other inhibitor HS-72. However, neither 17-AAG nor silencing HSF1 did enhance the cytotoxic effect of hyperthermic chemotherapy. This indicates that there are several mechanisms making cancer cells resistance to this kind of treatment. Maybe the use of a combination of HSP70 and HSP90 inhibitors could contribute to a positive outcome in future experiments. Of note, the initial experiments on fresh CRC tumor tissues from PDX mice, hyperthermia did increase the effect of Mitomycin C, whereas, hyperthermia increased the effect of Oxaliplatin on a fresh patient PC-tissue sample. In conclusion, hyperthermia in combination with chemotherapy has some beneficial effects, depending on the drug and/or cancer cell population examined. Interestingly, we found hyperthermic chemotherapy to increase a higher HMGB1 release, than the treatments alone. This indicates immunogenic cell death, but further studies are needed to verify to what extent this might explain the beneficial effect of hyperthermia in HIPEC treatment.

Appendix1

Reagents and buffers

Buffer/Reagents	Provider	Catalog number
Cell culture		
RPMI-1640 Medium	Sigma-Aldrich	R0883
Fetal Bovine Serum (FBS)	Sigma-Aldrich	F7524
Glutamax^{TM}Supplement	Gibco ® by Life Technologies	35050-038
Pencillin Streptomycin 10mg/ml	Sigma-Aldrich	P4458
Tripsin – EDTAmixture (1X)	Sigma-Aldrich	T3924
Dulbecon's Phosphate- Buffered Saline (PBS)	Sigma-Aldrich	D8537
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	D2650
Nunc^{TM} Cell Culture Treated EasYFlaks, 75 cm^2	Thermo Fisher Scientific	156499
Nunc^{TM} Cell Culture Treated EasYFlaks, 25 cm^2	Thermo Fisher Scientific	156367
Etanol (96%)	VWR Chemicals	20824.296
MTS-assay		
MTS	Promoga	G3582
Nunclon^{TM} Delta suface 96-Well plate	Thermo Fisher Scientific	167008
Celltiter 96 Aqueous One Solution MTS	Promega	G3581
SDS-PAGE and Western blotting		
Lysis buffer; 2xIP-buffer • 500 µl 10% SDS • 500 µl Na_3VO_4 • 50 µl 1M Tris-HCL pH 7.5 • 3,95 ml ddH_2O	 Abcam Thermo Fisher Scientific	 142227 15567-027

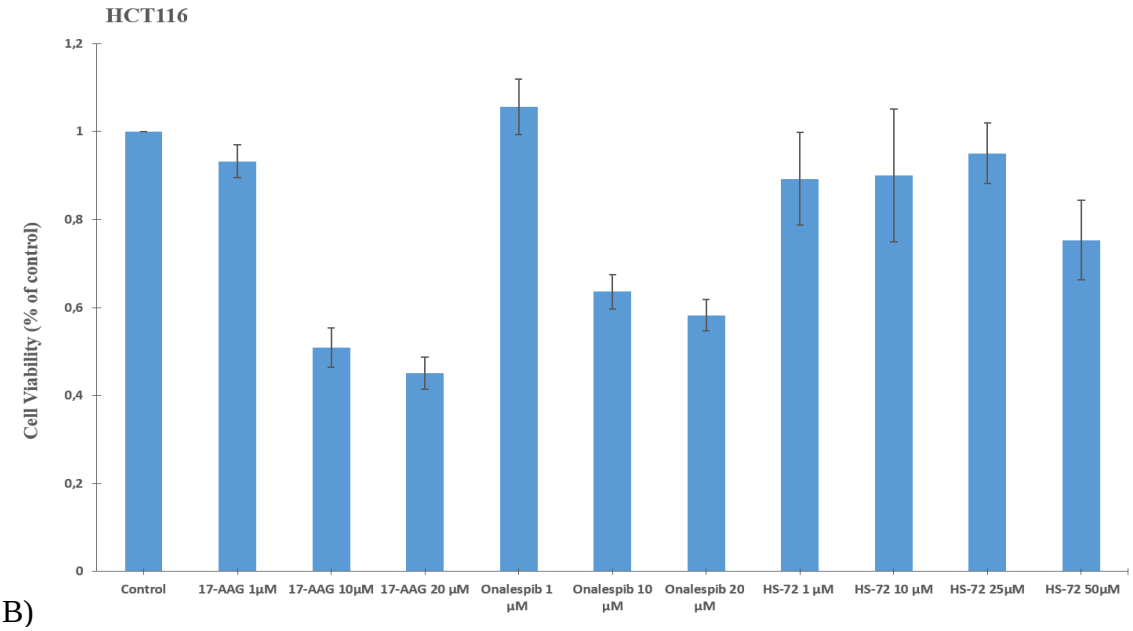
1X running buffer • 50mL MES SDS running buffer (20X) • 0.95 L ddH_2O	Thermo Fisher Scientific	NP0002-02
10X Transfer buffer • 30.3g Tris • 144g Glycine • 1L ddH_2O	Merck, Millipore VWR Chemicals	108382 1.04201.1000
1X transfer buffer 200 mL Metanol (99.8) 100 mL 10x transfer buffer 700 mL ddH_2O	VWR Chemicals	20847.307
TBST buffer • 5ml Tween-20 (20%) • 27ml 5M NaCl • 18ml Tris-HCL pH 7.5 • ddH_2O (up to 1L)	Fisher BioReagents™ Sigma-Aldrich Thermo Fisher Scientific	BP 337-500 433209 15567-027
Blocking buffer • 2.5g dry milk • 50 ml of 0.1% TBST	Tine®, Norway	
PVDF membrane	Whatman®	3030-335
NuPAGE® 4-12% Bis –Tris gel 1.0mm x 10 well	Novex® by life technologies	NP0331BOX
NuPAGE® 4-12% Bis –Tris gel 1.0mm x 15 well	Novex® by life technologies	NP0323BOX
NuPAGE® LDS Sample buffer (4X)	Thermo Fisher Scientific	NP0008
NuPAGE® sample Reducing Agent (10X)	Thermo Fisher Scientific	NP0009
Pierce™ BCA protein Assay Kit	Thermo Fisher Scientific	23225
SeeBlue®Plus2 Pre-Staind Standard	Thermo Fisher Scientific	LC5925
Super Signal West Dura Extended Duration Substrate	Thermo Fisher Scientific	34075

Primary Antibodies		
HSP27 (1:1000)	Cell Signaling	95357S
HSP70 (1:1000)	Cell Signaling	46477S
HSP90 (1:1000)	Cell Signaling	4875S
B-actin (1:2000)	Sigma-Aldrich	A5316
HSF1 (1:1000)	Thermo Fisher Scientific	PA3-017
HMGB1 (1:1000)	R&D SYSTEMS®	MAB1690
Secondary antibodies		
Polyclonal Goat anti-Rabbit Immunogloulin/HRP (1:2000)	Dako	P0448
Polyclonal Rabbit anti-Mouse Immunogloulin/HRP (1:2000)	Dako	P026002-2
siRNA Transfection		
Lipofectamine^{TM} RNAiMAX 0.75mL	Thermo Fisher Scientific	13778075
HSF1 silencer® Pre-designed siRNA	AMBION® The RNA Company	AM16708
Silencer® Negative Control siRNA#1	AMBION® The RNA Company	AM4611
Nunclon^{TM} Delta suface 6-Well plate	Thermo Fisher Scientific	140675
HSP inhibitors		
177-AAG (Tanespimycin)	Selleckchem	S1141
HS-72	Sigma-Aldrich	SML1325-25MG

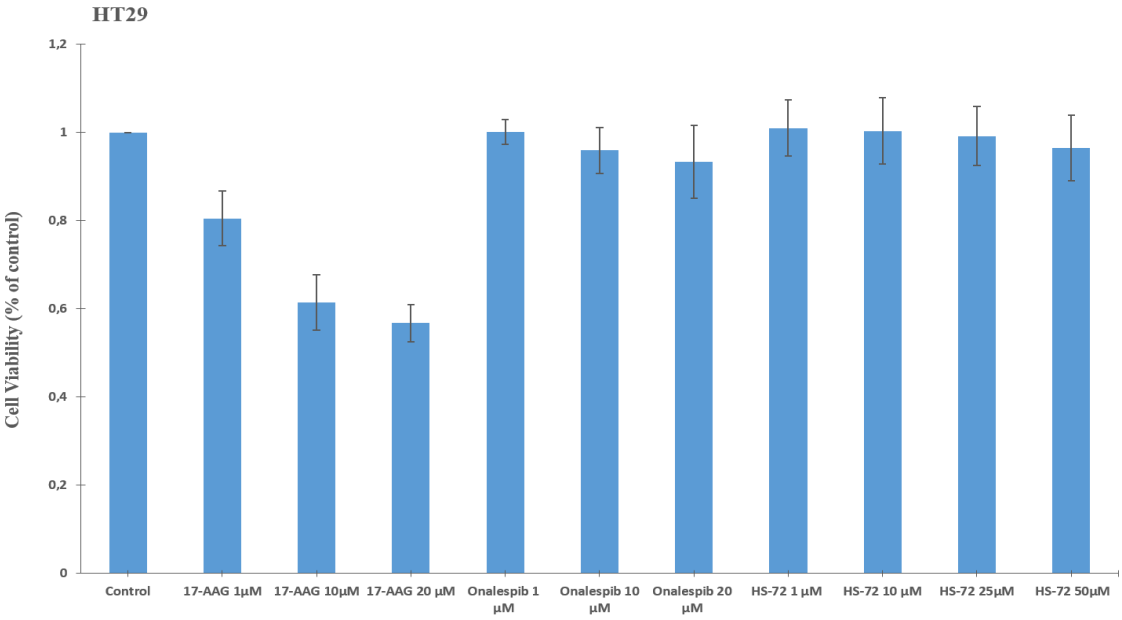
Appendix 2

Inhibitor concentration range for HSP inhibitors – 90 minutes at 37℃.

A)



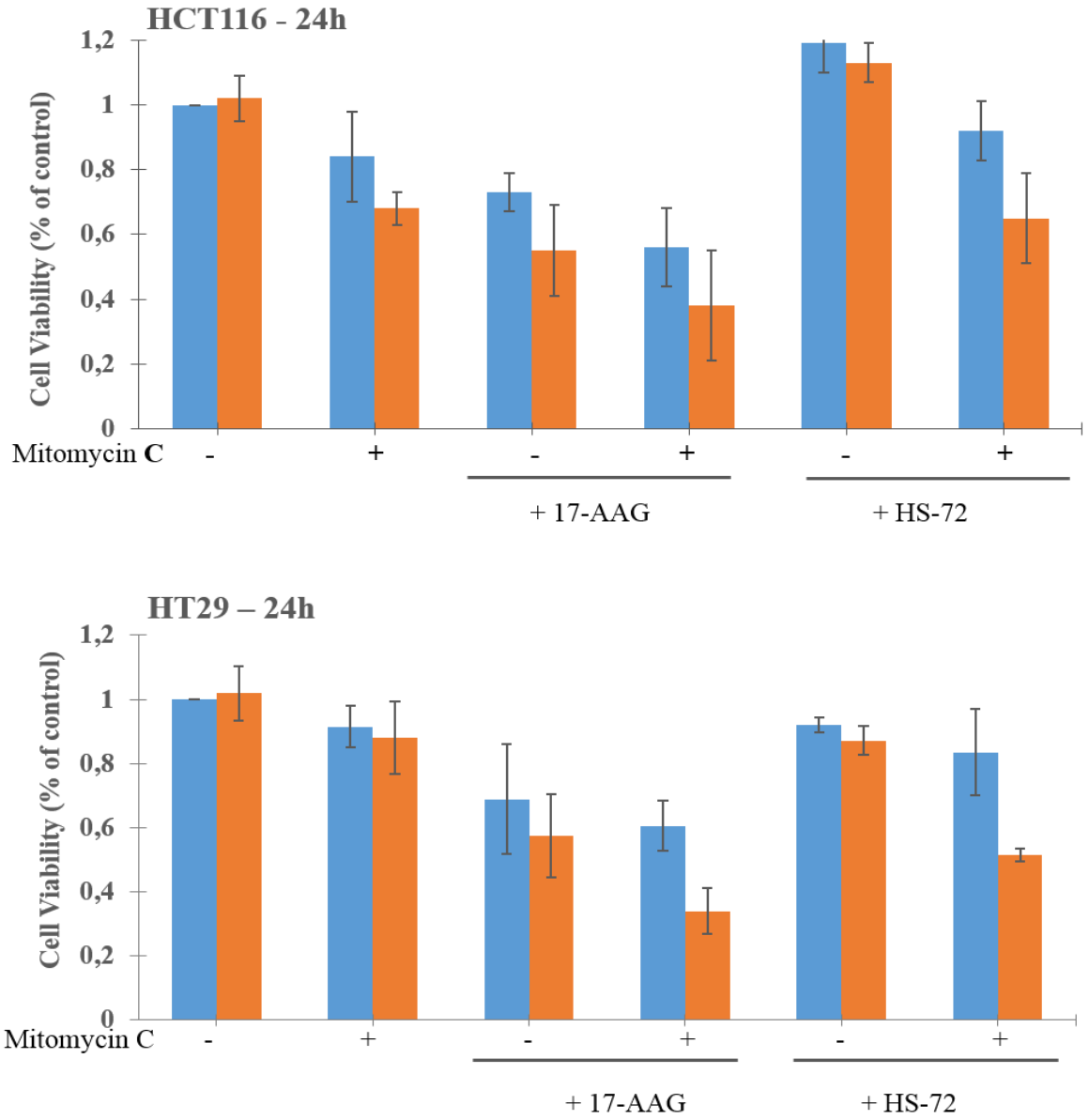
B)



Appendix 2: Inhibitor concentration range of HSP inhibitors, 17AAG, HS-72 and Onalespip (HSP90 inhibitor) in A) HCT116 and B) HT29 cell lines. The graphs show cell viability estimated by MTS-assay, 72 hours post-treatment for 90 minutes at 37℃. Absorbance was measured at 450nmM and normalized to control-treated cells (cell without drug incubated for 90 minutes at 37℃ water bath). Error bars represent standard deviations of three biological experiments (n=3), each with three technical triplicates.

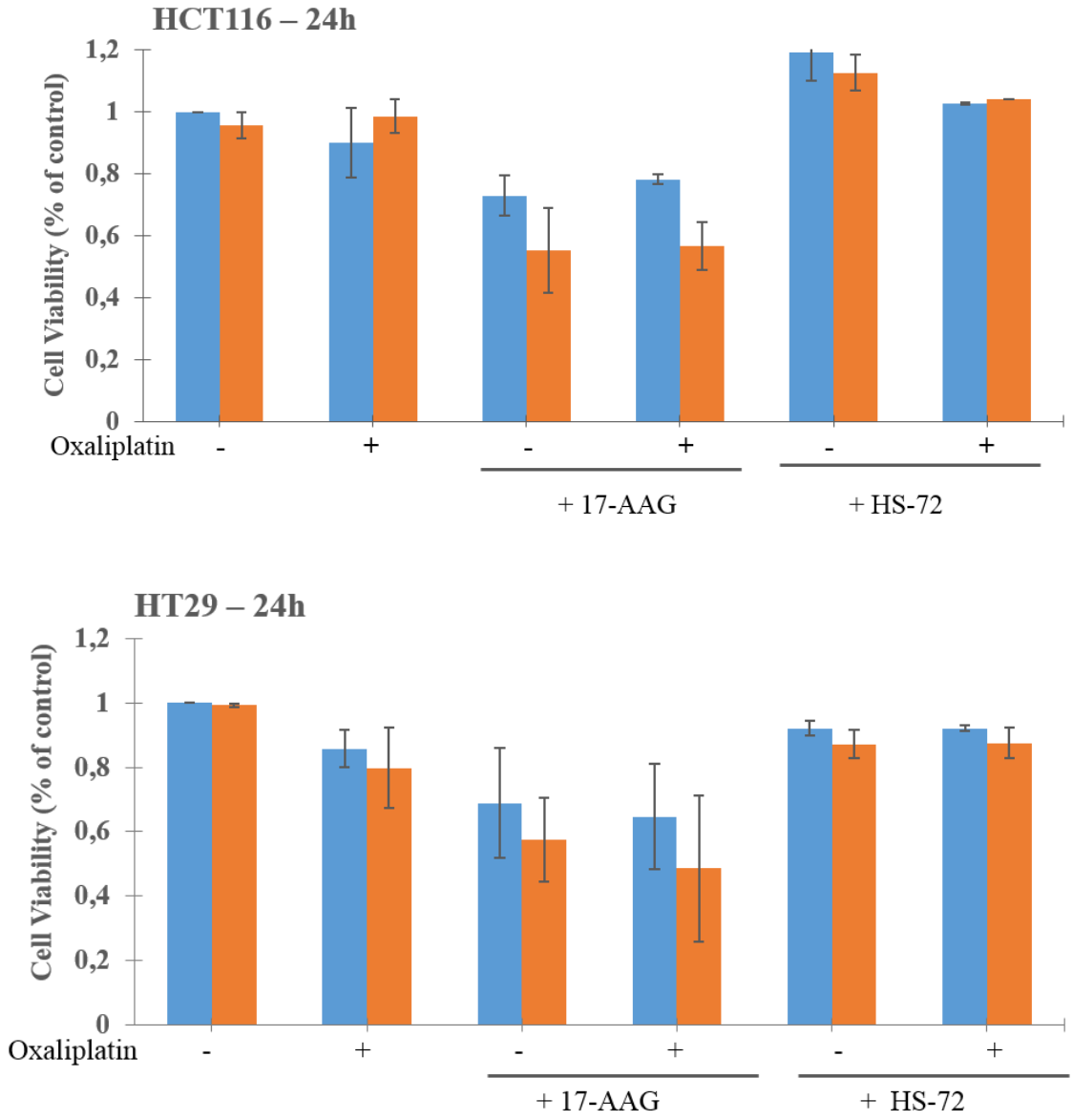
Appendix 3

MTS-data of HCT116 and HT29, 24 hours post-hyperthermic chemotherapy, with Mitomycin C.



Appendix 3.1: Hyperthermia –drug treatment with Mitomycin C and HSP-inhibitors of HCT116 and HT29 cell line. The graphs show cell viability estimated by MTS-assay, 72 hours post-treatment with Mitomycin C in combination with hyperthermia, 17-AAG and HS-72. Absorbance was measured at 450nmM and normalized to control-treated cells (cell without drug incubated for 90 minutes at 37℃ water bath). Error bars represent standard deviations of three biological experiments (n=3), each with three technical triplicates.

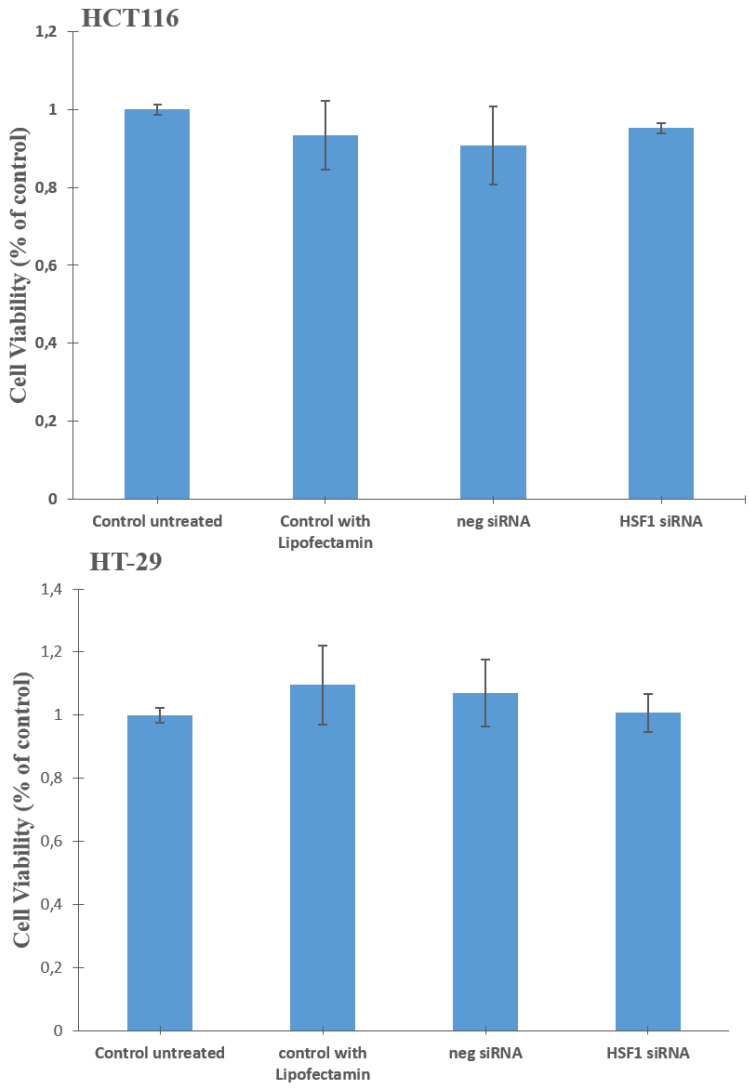
MTS-data of HCT116 and HT29, 24 hours post-hyperthermic chemotherapy, with Oxaliplatin.



Appendix 3.2: Hyperthermia –drug treatment with Oxaliplatin and HSP-inhibitors of HCT116 and HT29 cell line. The graphs show cell viability estimated by MTS-assay, 72 hours post-treatment with Oxaliplatin in combination with hyperthermia, 17-AAG and HS-72. Absorbance was measured at 450nM and normalized to control-treated cells (cell without drug incubated for 90 minutes at 37℃ Error bars represent standard deviations of three biological experiments (n=3), each with three technical triplicates.

Appendix 4

MTS data, 48 hours post-transfection, of HSF1siRNA and negative siRNA

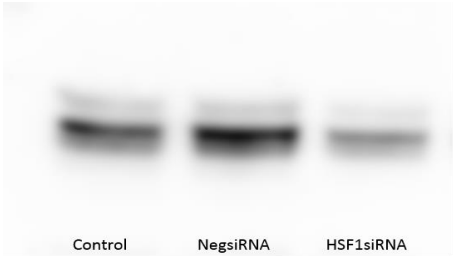


Appendix 4: Effects of transfection of HSF1 siRNA on cell viability. The cells were transfected with either HSF1siRNA, negative siRNA or Lipofectamine. Cell viability was determined by MTS-assay 48h post-transfection. Absorbance was measured at 450nM and normalized to control cells, without siRNA transfection. Error bars represents standard deviations of three technical triplicates (n=1).

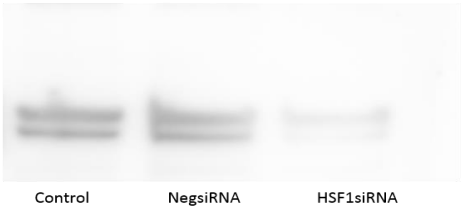
Appendix 5

HSF1 protein level analyzed by WB, 24 and 48 hours, post-transfection of HSF1siRNA in HCT116.

A)



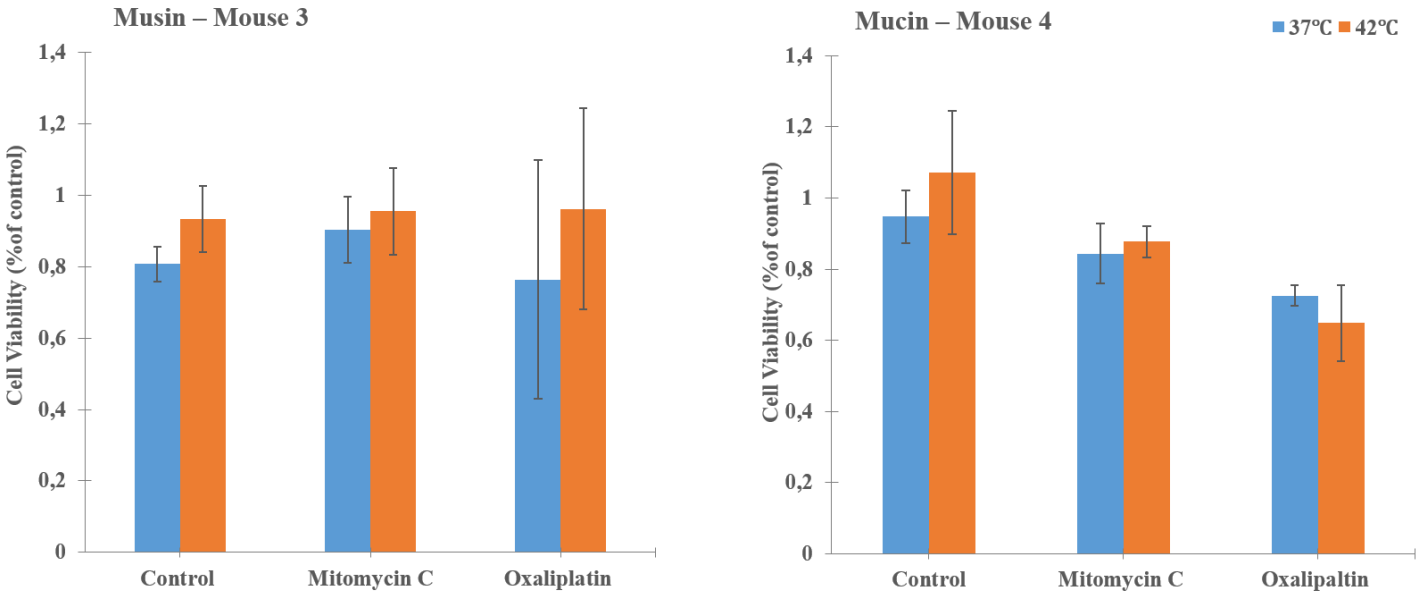
B)



Appendix 5: HSF1 protein expression after transfection of HSFsiRNA. HCT116 cells were transfected with HSF1siRNA for A) 24h and B) 48h, before expression of HSF1 was analyzed by Western blotting.

Appendix 6

MTS data from 48 hours post-hyperthermic chemotherapy of mucin from CRC-PC PDX-mice.



Appendix 6: Hyperthermic drug treatment of CRC-PC mucin from mice. The graphs show cell viability analyzed by MTS-assay, 48 hours post hyperthermic drug treatment of tumor tissues (mucin) from mouse. Sample from mouse 3 and 4 was treated with both Mitomycin C and Oxaliplatin in combination with hyperthermia. Absorbance was measured at 450nmM and normalized to control-treated cells (cell without drug incubated for 90 minutes at 37℃ water bath). Error bars represent standard deviations of one biological sample, with four technical triplicates.

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