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THE CELLULAR THERMAL SHIFT ASSAY (CETSA) FOR ELUCIDATING DRUG MECHANISM OF ACTION AND RESISTANCE IN CANCER

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The cellular thermal shift assay (CETSA) for elucidating drug mechanism of action and resistance in cancer

Thesis for Doctoral Degree (Ph.D.)

By

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All that is gold does not glitter,

Not all those who wander are lost.

J.R.R. Tolkien

ABSTRACT

A complete understanding over drug mechanism of action (MoA) is important when attempting to predict treatment outcome or the presence of resistance in patients. Despite decades of scientific efforts, the MoAs of even some of the oldest and most utilized drugs in cancer therapy today remain only partially understood, while resistance continues to be a frequent and often unpredictable occurrence. The absence of a protein-focused systems-wide characterization of drug-induced changes in cellular states contributes to these gaps in knowledge, as proteins are difficult to study yet they are key players in nearly all cellular processes and the targets for most drugs. The cellular thermal shift assay (CETSA) is a recently introduced method that can directly monitor drug target engagement and drug-induced cellular changes at proteome level in intact living cells.

The research presented in this thesis focuses on the protein-centric CETSA approach to charting drug MoA and resistance development by evaluating the drug-induced changes in protein thermal stability for several important cancer drugs utilized in the clinic e.g. pyrimidine analogues, taxanes, or apoptosis-blockade releasing compounds in intact living cells or tissues. We report on an extensive set of CETSA responses that reflect on drug-target engagement or other MoA-revealing alterations in cellular processes that are either compound-specific or overlapping between some of the studied drugs. Several of the highlighted proteins or ensembles of proteins show promise for further evaluation as candidate biomarkers for drug efficacy with potential future applications in a clinical setting.

LIST OF SCIENTIFIC PAPERS

1. **CETSA interaction proteomics define specific RNA-modification pathways as key components of fluorouracil-based cancer drug cytotoxicity.**
   Liang YY*, **Bacanu S***, Sreekumar L, Ramos AD, Dai L, Michaelis M, Cinatl J, Seki T, Cao Y, Coffill CR, Lane DP, Prabhu N, Nordlund P.
   Cell Chemical Biology. 2022 Apr 21;29(4):572-585.e8.
   *Equal contribution

2. **The mechanism of action of trifluridine and TAS-102 in a colon cancer model: A proteomics study using IMPRINTS-CETSA.**
   Bacanu S, Gerault MA, Nordlund P
   Manuscript.

3. **CETSA-based target engagement of taxanes as biomarkers for efficacy and resistance.**
   Langebäck A*, **Bacanu S***, Laursen H*, Mout L*, Seki T, Erkens-Schulze S, Ramos AD, Berggren A, Cao Y, Hartman J, van Weerden W, Bergh J, Nordlund P, Lööf S.
   Scientific Reports. 2019 Dec 18;9(1):19384.
   *Equal contribution

4. **Proteome-wide CETSA reveals diverse apoptosis inducing mechanisms converging on an initial apoptosis effector stage focused at the nuclear matrix proximal region.**
   Ramos AD, Liang YY, Surova O, **Bacanu S**, Gerault MA, Mandal T, Ceder S, Langebäck A, Österroos A, Ward GA, Bergh J, Wiman KG, Lehmann S, Prabhu N, Lööf S, Nordlund P.
   Manuscript.

CONTENTS

LIST OF PROTEINS AND ABBREVIATIONS

The many proteins mentioned

ABCB1	ATP-dependent translocase ABCB1, or MDR1/Pg-p
ATR	Serine/threonine-protein kinase ATR
BCL2	Apoptosis regulator Bcl-2
BRAF	Serine/threonine-protein kinase B-raf
CALR	Calreticulin
CDK	Cyclin-dependent kinase
CHEK1	Serine/threonine-protein kinase Chk1
CTLA-4	Cytotoxic T-lymphocyte protein 4
DHFR	Dihydrofolate reductase
DNMT1	DNA (cytosine-5)-methyltransferase 1
DPYD	Dihydropyrimidine dehydrogenase
DTYMK	Thymidylate kinase
DUS1L	tRNA-dihydrouridine(16/17) synthase [NAD(P)(+)]-like
DUS3L	tRNA-dihydrouridine(47) synthase [NAD(P)(+)]-like
DUT	Deoxyuridine 5'-triphosphate nucleotidohydrolase
EGFR	Epidermal growth factor receptor
FANCD2	Fanconi anemia group D2 protein
FANCI	Fanconi anemia group I protein
FEN1	Flap endonuclease 1
HER2	Receptor tyrosine-protein kinase erbB-2
HMCES	Abasic site processing protein HMCES
IAP	Inhibitor of apoptosis
LMNB1/2	Lamin-B1/2
MATR3	Matrin-3
MDR1	Multidrug resistance protein 1, or ABCB1/P-gp
MYC	Myc proto-oncogene protein
p53/TP53	Cellular tumor antigen p53
PARP1	Poly [ADP-ribose] polymerase 1
PCLAF	Proliferating cell nuclear antigen
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death 1 ligand 1
P-gp	P-glycoprotein, or ABCB1/MDR1

PUS1	Pseudouridylate synthase 1
PIK3C3 (VPS34)	Phosphatidylinositol 3-kinase catalytic subunit type 3
RNR	Ribonucleotide reductase
RPA	Replication protein A
RPUSD2	Pseudouridylate synthase RPUSD2
SAMHD1	Deoxynucleoside triphosphate triphosphohydrolase
TK1	Thymidine kinase 1
TNFα	Tumour necrosis factor alpha
TOP2α	DNA topoisomerase 2-alpha
TRMT2A	tRNA (uracil-5-)-methyltransferase homolog A
TRUB1	Pseudouridylate synthase TRUB1
TUBB3	Tubulin beta-3 chain
TYMP	Thymidine phosphorylase
TYMS	Thymidylate synthase
UMPS	Uridine 5′-monophosphase synthase
XRCC6	X-ray repair cross-complementing protein 6

Abbreviations

5-FU	5-fluorouracil
BER	Base excision repair
CCAE	Core CETSA apoptosis ensemble
CETSA	Cellular thermal shift assay
CH_2-THF	N5,N10-methylene-tetrahydrofolate
CRC	Colorectal cancer
DDA	Data-dependent aquisition
DEAR	Differential extraction accessibility by relocalisation
dNTP	Deoxynucleotide triphosphate
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
dUTP	Deoxyuridine triphosphate
ER	Endoplasmic reticulum
ESI	Electrospray ionisation
FDA	Food and drug administration
FdUMP	5-fluorodeoxyuridine monophosphate

FdUTP	5-fluorodeoxyuridine triphosphate
FNA	Fine-needle aspirate
FOLFIRI	Folinic acid, 5-fluorouracil, irinotecan
FOLFIRINOX	Folinic acid, 5-fluorouracil, irinotecan, oxaliplatin
FOLFOX	Folinic acid, 5-fluorouracil, oxaliplatin
FT-ICR	Fourier-transform ion cyclotron resonance
FUDR	5-fluorodeoxyuridine
FUR	5-fluorouridine
FUTP	5-fluorouridine triphosphate
GSH	Glutathione
IMPRINTS-CETSA	Integrated modulation of PRINTS CETSA
ITDR-CETSA	Isothermal dose response CETSA
LC	Liquid chromatography
MALDI	Matrix-assisted laser disorption/ionization
MC-CETSA	Melt curve CETSA
MoA	Mechanism of action
MS	Mass spectrometry
MS-CETSA	Mass spectrometry CETSA
OMP	Orotidine 5'-monophosphate
PDX	Patient-derived xenografts
PISA	Protein integral solubility alteration
PRINTS	Protein interaction states
PTM	Post-translational modification
QP	Quantitative proteomics
Q-TOF	Quadrupole-time of flight
RESP	Regional stabilization after proteolysis
ROS	Reactive oxygen species
TAS-102	Trifluridine-tipiracil
TE	Target engagement
TFdTMP	Trifluorothymidine monophosphate
TFdTTP	Trifluorothymidine triphosphate
TFT	Trifuorothymidine also known as trifluridine
TMT	Tandem mass tag
TOF	Time of flight
TPP	Thermal proteome profiling

TSA	Thermal shift assay
UMP	Uridine monophosphate
WB	Western blotting

1 LITERATURE REVIEW

1.1 Cancer

Despite considerable advances in therapy and survival rate improvements for many types of cancer, this disease continues to be a serious challenge and the second leading cause of death at global scale. Lung cancer, followed by breast and colorectal cancer (CRC) have the highest mortality rates worldwide (Globocan 2020).

Cancer is a particularly complex disease that can affect various organs or tissues, and stems from our own cell populations, usually after long periods of time (years or decades) in which a vast collection of DNA-related alterations can be accumulated either in the form of mutations or epigenetic changes.

With the introduction of next generation sequencing, thousands of DNA mutations that arise in cancer were identified. However, most of these mutations are not necessarily oncogenic. Mutations that provide a selective growth advantage to the cell are referred to as driver mutations and are the ones that contribute to cells becoming cancerous. Oncogenes like *EGFR*, *BRAF*, and MYC as well as altered tumour suppressor genes e.g. p53, are currently well-known and extensively studied genes in this context[1–4]. Passenger mutations are those mutations that do not contribute to tumour development, they just follow along for the trip[5]. However, mutations are not the sole culprit as cancer is often described as a collection of abnormal processes, each contributing to the overall malignancy. "The Hallmarks of Cancer", the landmark review by D. Hanahan and R. A. Weinberg published in 2000 and updated in 2011 and 2022, classifies the extensive amount of relevant scientific knowledge regarding cancer into relatively concise principles that can aid our understanding of the vast diversity of neoplastic diseases[6–8]. These include the capacity to maintain proliferative signalling and evade growth suppressors, achieving replicative immortality, resisting apoptosis, and avoiding destruction by the immune system, among others.

1.2 Cancer therapy

When applicable, surgery is a very effective strategy and can be the first-line of treatment in many cancers. Surgical resection is the main form of treatment for patients with non-metastasized CRC[9] and can represent a cure for low grade

tumours but in the case of more advanced CRCs, surgery is often accompanied by a cytostatic regimen in neoadjuvant or adjuvant setting[10].

Radiotherapy involves delivering a focused dose of high-energy electromagnetic radiation or high-energy particle beams directly to the malignant tissue while sparing the surrounding normal tissues. This approach has been proved useful for many cancers but often requires additional interventions such as chemotherapy[11].

Immunotherapies rely on reactivating the immune system against tumour cells. Novel treatment strategies in the form of immune checkpoint inhibitors (CPIs) are able to re-engage the immune responses towards even advanced cancers and have given new hope to numerous cancer patients[12,13]. Therapies such as antagonistic monoclonal antibodies that target programmed cell death protein 1 (PD-1), programmed cell death ligand 1 (PD-L1), and T-lymphocyte associated antigen 4 (CTLA-4) are being explored for several cancers, including subtypes of metastatic CRC[14].

Targeted therapies are typically directed towards oncogenic proteins that drive tumour growth such as cell-surface receptor tyrosine kinases (HER2, EGFR)[15]. Many of these therapies rely on monoclonal antibodies that efficiently bind and inhibit these receptors. Biomarkers such as receptor expression levels[16] or the presence of specific mutations, can help identify the patients that are more likely to respond to these types of therapy.

Chemotherapy involves the administration of cytotoxic compounds meant to eliminate cancer cells with the minimum possible adverse effects on healthy cells. There is a wide variety of drugs currently in use today with many more still in pre-clinical development or clinical trials. Many of them attempt to exploit characteristics that cancer cells exhibit e.g. fast division rate and metabolism. Overall, while being one of the main forms of cancer therapy, the delivery of chemotherapy to patients has evolved to be a rather complex and challenging venture due to the many disease-related intricacies. Since the majority of the work presented in this thesis revolved around targeting cancer with drugs, a more in-depth discussion follows in the next section.

1.3 Chemotherapy approaches

One of the hallmarks of cancer is the ability to proliferate at an uncontrolled and seemingly faster rate than the cells of origin. To accommodate the increased need of DNA replication and protein production, tumour cells rely on *de novo* synthesis of the necessary "building blocks" for these macromolecules, but can also obtain them via salvage pathways[17]. The abovementioned aspects constitute a core vulnerability that has become central to numerous chemotherapies currently in use. Examples are drugs that target key enzymes in the nucleotide metabolism and aim to introduce disruptions in dNTP or other nucleotide pools such as analogues of uracil, deoxyuridine, or thymidine e.g. 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (FUDR), and trifluorothymidine (TFT) respectively, that target thymidylate synthase (TYMS)[18,19]. Other examples are certain folate analogues such as pemetrexed that not only inhibit TYMS but also disrupt the N5,N10-methylene-tetrahydrofolate (CH_2-THF) pools by inhibiting dihydrofolate reductase (DHFR) in the folate metabolism[20]. Analogues of deoxycytidine (gemcitabine) or deoxyadenosine (cladribine, clofarabine) inhibit the M1 (large) subunit of ribonucleotide reductase (RNR) while hydroxyurea targets the M2 subunit of RNR[21].

DNA can also be seen as a chemotherapy target when the triphosphate form of all the abovementioned purine and pyrimidine deoxynucleoside analogues are misincorporated into DNA leading to replicative stress and DNA damage, which are exacerbated by the disruptions in dNTP pools occurring upstream. Other notable examples of such DNA-damaging drugs are alkylating agents e.g. cyclophosphamide which damage DNA by introducing intrastrand and interstrand cross-links[22], while anthracyclines like doxorubicin intercalate into DNA and inhibit topoisomerase II α (TOP2A)[23]. Platinum-based compounds e.g. cisplatin and oxaliplatin function via mechanisms that are not fully understood but believed to be similar to those of alkylating agents[24]. The introduced DNA abnormalities interfere with DNA replication and DNA damage repair strategies until cell death mechanisms are ultimately triggered.

Other classes of drugs target components of the cytoskeleton e.g. microtubules[25]. Microtubule-binding agents like taxanes can disrupt microtubule dynamics in different ways that lead to e.g. mitotic catastrophe[26], although other causes for their cytotoxicity have also been proposed[27].

One important consideration for all these drugs is that their mechanism of action (MoA) is only partly understood to this day. They are designed or believed to target certain proteins or cellular processes but some aspects and intricacies of their MoA regarding the effects on other cellular events are still to be explored. This contributes to many of the current challenges encountered in the treatment of cancer e.g. personalized treatment selection for patients. Moreover, when the administration of single compounds has not produced sufficiently robust therapeutical responses or has been limited by acquired drug resistance, combinations of drugs from different classes have been employed for achieving an enhanced effect. Still, our knowledge regarding the MoA of drugs in such combination therapies and how they synergise also remains fragmented.

Several chemotherapeutical agents are described in the different papers included in this thesis. However, the focus of my research has been on fluoropyrimidines and taxanes and therefore, a more in-depth discussion of these drug classes follows.

Targeting the nucleotide metabolism with pyrimidine analogues

Targeting the pyrimidine nucleotide metabolism is a strategy employed by both existing and emerging cancer drugs. Thymidylate synthase (TYMS) plays an essential role in the *de novo* deoxypyrimidine metabolism by converting deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) using CH_2-THF as cofactor. This reaction is the only way in which cells synthesize dTMP anew.

Developed in the 1950s, 5-FU is one of the oldest drugs utilized for cancer treatment today. 5-FU is a pyrimidine analogue that contributes cytotoxic effects partly via the inhibition of TYMS. With a structure closely resembling that of uracil, 5-FU rapidly enters the cell and is converted to several metabolites with different active roles[18]. 5-fluorodeoxyuridine monophosphate (FdUMP) is a potent inhibitor of TYMS, while 5-fluorodeoxyuridine triphosphate (FdUTP) and 5-fluorouridine triphosphate (FUTP) are misincorporated into DNA- and RNA- respectively[18,28]. TYMS inhibition leads to a deoxythymidine triphosphate (dTTP) level decrease in the cell, as well as considerable imbalances in other deoxynucleotide pools, in particular an accumulation of deoxyuridine triphosphate (dUTP) that, alongside FdUTP, is misincorporated into DNA. This causes an array of DNA replication and repair abnormalities which ultimately lead to the cell's death. FUTP

misincorporation into RNA has been reported to deliver several levels of toxicity e.g. interference with rRNA maturation[29,30] or with RNA post-transcriptional modifications like pseudouridylation[31]. Despite the extensive research on 5-FU (more than 60 000 published papers), many of the intricacies of its MoA remain unclear to this day.

In the clinic, 5-FU has been broadly used in the treatment of many cancers for the past decades[18,32] and continues to be a cornerstone of CRC therapy, often in combination with e.g. folinic acid as supplement and either oxaliplatin (FOLFOX), irinotecan for inhibiting DNA topoisomerase I (FOLFIRI), or all these compounds together (FOLFIRINOX)[33,34]. Capecitabine, a prodrug that is metabolized to 5-FU was also evaluated in the clinic and was reported to display milder toxicities compared to 5-FU[35].

When administered to patients, 5-FU suffers considerable degradation to dihydrofluorouracil in the liver by dihydropyrimidine dehydrogenase (DPYD) with more than 80% of the administered dose reported to be catabolized[36]. Hence, caution is needed when dosing 5-FU or capecitabine as DPYD-related deficiencies have been linked to severe toxicities in patients[37] as further discussed in section 1.6.

As is the case with many drugs in the clinic today, drug resistance to 5-FU is a significant impediment. Alternative fluoropyrimidines have been evaluated in clinical trials for use in advanced CRCs. Among them, the thymidine analogue trifluridine (TFT)[38], which will be further discussed in section 1.5.

Targeting the cytoskeleton

Microtubules are important components of the cytoskeleton and are assembled from α- and β-tubulin heterodimers. Arguably one of the most important roles of microtubules is the formation of the mitotic spindle, a structure whose function relies greatly on the ability of microtubules to rapidly polymerize and depolymerize. Tubulin-binding agents can hamper microtubule dynamics in different ways that lead to cellular disruptions, most notably of antimitotic nature[25].

Taxanes (e.g. docetaxel, paclitaxel) and epothilones are microtubule-stabilizing agents i.e. they prevent microtubule depolymerisation by binding to different but partly overlapping taxoid-binding sites located on β-tubulin and oriented towards

the interior microtubule surface (Fig. 1.1). Microtubule-destabilizing agents interfere with tubulin assembly by binding β-tubulin e.g. at vinca-domains located on microtubule ends, as is the case for vinca-alkaloids like vincristine and vinorelbine[25].

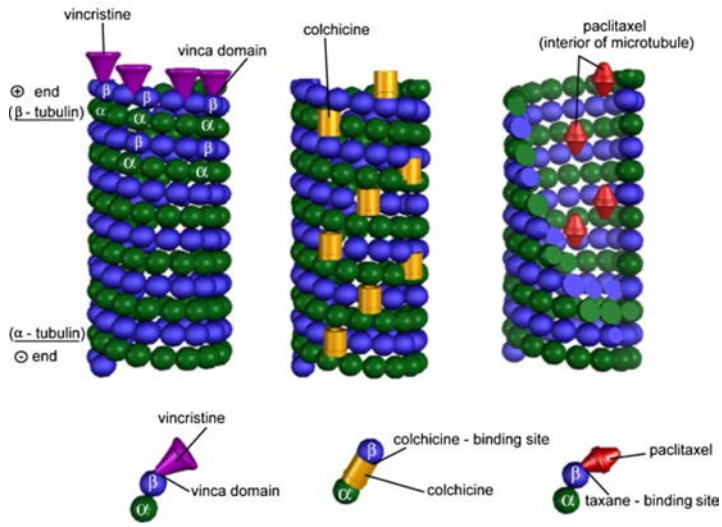

Figure 1.1. Microtubule-binding drugs target the tubulin heterodimers and bind to different sites. *Reprinted from Biochimica et Biophysica Acta* 1785(2):96-132*, McGrogan et al.,* Taxanes, microtubules and chemoresistant breast cancer, 2008, *with permission from Elsevier*.

By inhibiting microtubule depolymerization, taxanes consequently induce mitotic spindle-dependent cell cycle arrest in G_2/M-phase, which has been considered the major component of their MoA. However, recent studies have also proposed non-mitotic cell-killing mechanisms where rigid taxane-stabilized microtubules would physically pull nuclei apart leading to the formation of micronuclei and ultimately to cell death[27].

Microtubule-binding drugs have been in use for decades in the treatment of solid tumours but also haematological cancers[25]. Taxanes are part of the standard therapy regimens for primary and metastatic breast cancer, usually in combination with an anthracycline (epirubicin, doxorubicin) and different other agents like 5-FU or, when applicable, HER2-directed monoclonal antibodies or endocrine therapy[39,40].

1.4 Challenges in cancer therapy

Cancer research has contributed countless improvements to the currently available therapies, but there are still challenges to overcome. Among the limiting factors, drug-related toxicities and the lack of treatment efficacy are arguably the most acute.

Attempts to select treatment based on different markers

Drug efficacy integrates several levels of systemic and cellular processes (drug transport, metabolism, target engagement, downstream pathway activation or inhibition etc.), aspects that can vary significantly in tumours between individual patients[41]. Extensive scientific efforts have been dedicated to classifying the various cancer types and subtypes followed by attempts to correlate them to successful treatment regimens. This gave rise to opportunities to improve treatment efficacy by developing drugs targeting subtype specific vulnerabilities[42,43]. Several subtypes have been defined for e.g. breast cancer where patients diagnosed with HER2+ luminal B (ER+ and PR+/-) breast cancer are more likely to respond to HER2 targeted monoclonal antibodies like trastuzumab and endocrine therapy with e.g. tamoxifen. These approaches can be complemented with chemotherapy, surgery, or radiotherapy during the course of the disease.

Treatment-associated toxicity

While anticancer drugs aim to selectively target and eliminate cancer cells, they often exert unintended toxic effects on healthy cells and tissues, leading to a range of adverse reactions that are a major source of concern and often the limiting consideration in therapy. Severe adverse effects that may lead to treatment discontinuation are e.g. bone marrow suppression, cardiotoxicity, and neurological complications[44]. Toxicities also arise at times due to pharmacokinetic considerations. As mentioned in section 1.3, one notable example involves 5-FU treatment in patients with deficiencies in DPYD, a key enzyme in pyrimidine catabolism.

Despite considerable advances in treatment selection strategies and precision oncology, many patients don't respond to treatment[45]. Drug resistance often arises following repeated treatment administration which further limits treatment

options. Tackling drug resistance is a major challenge and will be covered in the next section.

1.5 Drug Resistance

Drug resistance, innate or acquired, is the main culprit when it comes to the inability of current cancer therapies to achieve a full curative effect. Many tumours, despite promising initial response to drugs, can quickly become resistant and so disease relapse occurs.

The risk of drug resistance occurring encompasses multiple factors such as tumour growth rate, tumour heterogeneity, tumour burden, selective pressure applied by therapeutic approaches, immune system infiltration, and characteristics of the tumour microenvironment. Combinations of these factors are linked to tumours often displaying either an innate resistance, a quick adaptive response, or a prolonged acquired resistance to therapy, all types presenting complexities that are challenging to decipher[46].

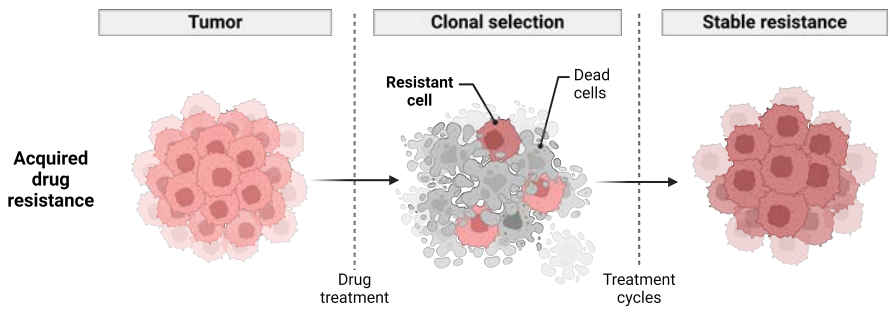

Figure 1.2. Acquired drug resistance can develop due to selective pressure exerted by therapeutic approaches. *Image generated with BioRender.*

Mutational processes resulting in tumour heterogeneity play an important role in the development of drug resistance and are very complex and extensively studied aspects of cancer[47]. Tumour responses to selective therapeutic pressure encompass the reduction or disappearance of the more sensitive cellular clones and the acquiring of more resistance-related mutations and adaptations in cellular processes by the remaining clones, ultimately leading to a complete change in the tumour phenotype (Fig. 1.2)[48].

There are many ways in which cancer cells can adapt at a molecular level to evolve to escape the toxic action of a drug and become resistant. These can include adaptations that influence the effective drug concentration at the site of the target protein such as: altered drug cellular import or export mechanisms e.g. overexpression of efflux pump ABCB1/MDR1/P-gp[49], increased drug degradation, alterations in drug activation pathways e.g. changes in key protein expression levels, and target protein alterations[50,51]. The activation of parallel pathways that counter or bypass the effects of the drug e.g. increased DNA damage repair[52] or metabolite salvage pathways[53] are also cellular strategies for escaping the action of the drug. Furthermore, drug resistance is likely to be a multifaceted phenomenon where multiple resistance mechanisms can be employed simultaneously.

In the case of CRC, current therapies encounter a major setback due to innate or acquired drug resistance[54]. Response rates to 5-FU therapies are at best ~30% when administered alone[55] and while typically higher in combination therapies[56], resistance is developed in many cases. Several mechanisms of 5-FU resistance have been reported by many studies and involve e.g. the expression levels of TYMS or the activity of DPYD[57,58]. Due to the high incidence of 5-FU resistance, other fluorinated pyrimidine analogues are being evaluated in clinical trials. Trifluorothymidine (TFT) also known as trifluridine, has produced encouraging results in refractory advanced CRC when administered in combination with the thymidine phosphorylase (TYMP) inhibitor tipiracil[38,59,60]. TFT-tipiracil, also known as TAS-102, was FDA approved in 2015 for the treatment of advanced CRCs for which treatment options are limited by drug resistance.

When it comes to taxanes, several mechanisms have been reported to confer resistance. For instance, taxane resistance has been linked to the overexpression of efflux pumps e.g. ABCB1/MDR1/P-gp in several breast cancer models[61]. These efflux pumps can be inherently expressed in cancer cells, likely contributing to some initial and not necessarily detected resistance and often become overexpressed following treatment leading to even more decreased drug efficacy. Still, efforts aimed at pharmacologically inhibiting these transporters using specific drugs have not resulted in satisfactory results in the clinic[62]. Another suggested mechanism for taxane resistance is the increased expression of tubulin isoforms that exhibit decreased interaction with taxanes e.g. the upregulation of β-III-tubulin (TUBB3)[63].

Even immunotherapies face many of the same challenges as chemotherapies do. Although highly effective in some cancer forms such as malignant melanoma, in most cancers, few patients experience long-lasting responses[64].

Several considerations can make an impact on combating the occurrence of drug resistance. These include earlier disease detection, treatment strategies aimed at deepening the responses from drugs, therapeutic monitoring, and adaptive interventions in regard to treatment selection[46]. One approach to decreasing resistance to therapy would be the administration of combinations of drugs with non-overlapping mechanisms of action. This approach has shown improved chances of success compared to single-agent treatment regimens and therefore combination therapies have become the preferred approach of administering chemotherapy in both primary and advanced cancers. In some cases, these combinations are increasingly complex, containing several drugs and supplements e.g. FOLFIRINOX in CRC treatment. Administering drugs with different MoAs in an alternate manner (intermittent therapy) to avoid continuous selective pressure being applied to tumour cells, is a strategy for avoiding the occurrence of long running resistance and unbearable toxicities[65].

Biomarkers or ensembles of biomarkers that can reliably and robustly monitor drug efficacy during the course of therapy would enable clinicians to make informed decisions and treatment adjustments in real-time. However, considerable challenges and discrepancies in study results have been encountered in the quest for drug efficacy biomarkers.

1.6 The quest for drug efficacy biomarkers

Personalized cancer therapy is one of the most pursued areas in cancer research at the present time, and considering all the levels of complexity and the challenges that cancer encompasses, it is to be expected that many solutions in the treatment of this disease would have to be "tailored" to each individual patient. However, reliable predictive biomarkers for treatment efficacy or toxicity are often lacking, notable exceptions are some targeted therapies as mentioned in section 1.2. This is arguably in part a consequence of the fragmentary knowledge base concerning drug MoA and resistance mechanisms.

Numerous preclinical and clinical studies have been directed at investigating the levels of basic cellular components e.g. proteins or mRNA – what we refer to as the vertical cell biology aspect – and making sense of these levels in disease

contexts[66]. Other studies investigated the link between the presence of mutations in specific genes with the outcome to certain treatments e.g. EGFR mutations and the response to EGFR inhibitor gefitinib in advanced lung cancer[67]. Such data has provided important contributions to our understanding of cancer and in some cases has helped narrow down the search for predictive biomarkers. However, for many drugs, current biomarkers are at best partly correlative to therapeutic response. Furthermore, most cellular processes are to a large extent triggered and modulated by interactions made by proteins with other molecules in the cell i.e. cellular biochemistry – what we refer to as horizontal cell biology. A deeper understanding of this protein-centric dimension is likely a crucial component to unlocking the many cellular unknowns that contribute to the low success rates that many cancer therapies encounter.

In the case of the two drug classes utilized mostly in my work, fluoropyrimidines and taxanes, no single biomarker (to the best of my knowledge) has been able to reliably and robustly report on drug efficacy in patients (further discussed below). Given that resistance to these classes of compounds most likely is a complex and multifaceted phenomenon and based on the vast literature available regarding this matter, it is improbable that the efficacy of a therapeutic intervention could be attributed to a singular biomarker. The search continues and more and more incorporates omics-level studies that offer a broader view over the cellular landscape and enhance the likelihood of impactful biomarker ensemble discoveries.

Candidate biomarkers for predicting 5-FU efficacy

TYMS as a 5-FU efficacy biomarker has been studied extensively, with many publications addressing this question in one way or another. Studies have reported TYMS protein levels increases to be associated with limited response to 5-FU in patients with e.g. CRC or gastric cancer[68–70] or breast cancer[71]. However, other studies reported not so straight forward or even conflicting results[72,73]. Overall, the reliability of TYMS expression levels as a predictive biomarker for 5-FU efficacy remains in question[74].

DPYD, has also been investigated for a potential use in predicting 5-FU efficacy as well as toxicity. DPYD polymorphisms are linked to deficiencies in this enzyme and consequently with the occurrence of severe and sometimes deadly 5-FU- or fluoropyrimidine-related toxicities[75,76]. Genotyping has been employed to detect

the presence of DPYD deficiency-linked polymorphisms and the results were utilized in tailoring the doses administered to patients in clinical trials. Utilizing this approach greatly decreased the occurrence of dangerous toxicities. Therefore, DPYD deficiency testing prior to fluoropyrimidine administration is encouraged in clinical practice[75,76]. With this in mind, studies have also been directed to investigate the correlation between DPYD expression and 5-FU and other fluoropyrimidine drug responses but, no general consensus was reached regarding treatment outcome[77].

UMP synthase (UMPS), the bifunctional protein that converts orotate first to orotidine monophosphate (OMP) then to uridine monophosphate (UMP) as well as 5-FU first to 5-fluorouridine (FUR) then to 5-fluorouridine monophosphate (FUMP), has also been evaluated as a potential biomarker for 5-FU efficacy with studies that address protein levels, gene expression, or enzyme activity. Some studies reported a correlation between UMPS gene expression or enzymatic activity and the response to 5-FU in patient tumour material from different cancers[78–80] but its role as an efficacy biomarker is still uncertain.

Other proteins that have been considered as candidate biomarkers for efficacy are additional enzymes with roles in the nucleotide metabolism e.g. TYMP[81], but again, no conclusion regarding their applicability for predicting 5-FU and other fluoropyrimidine efficacy has emerged.

p53 overexpression was reported to be linked to 5-FU treatment outcome by a number of clinical studies[82,83]. However other studies have found no such link[84,85]. The applicability of p53 for predicting 5-FU and fluoropyrimidine efficacy therefore requires further evaluation.

Candidate biomarkers for taxane efficacy

The expression of ABC transporters e.g. ABCB1/MDR1/P-gp has been linked to resistance to multiple drugs including taxanes in breast cancer patients[61]. However, due to reasons such as heterogeneity in the cell composition of samples, sensitivity-related inconsistencies or other limitations in the detection techniques employed in different studies, no clinically validated method for assessing the contribution of these transporters to lack of drug efficacy in patient tumours exists at the present time.

The increased expression levels of tubulin isoforms that do not so readily interact with taxanes has also been associated with resistance[63]. For example, the overexpression of β-III-tubulin (TUBB3) has been linked to decreased sensitivity to paclitaxel in breast cancer patients[86]. Studies have however produced conflicting results which is likely a consequence of the complexity of the cellular processes involved[63]. Therefore, further evaluation is required regarding the utility of tubulin isoforms as candidate biomarkers for taxane efficacy.

1.7 Why proteins?

Among all cellular constituents, proteins stand out as key components, while also being the targets for most therapeutic drugs. Even though proteins are involved in most cellular processes, with the introduction of high-throughput DNA or RNA sequencing technologies an increased research emphasis has been on exploring cellular contexts through the analysis of genomic or transcriptomic data. However, these approaches to understanding cell biology, while generating valuable information in many cases, have proven to rarely be able to accurately reflect on the state of the proteome when a strong overall correlation between e.g. mRNA levels and protein expression levels can seldom be made[87–89]. Contributing to the observed low mRNA-protein correlation are several aspects, from cellular processes e.g. post-transcriptional regulation, translation efficiency, and RNA or protein degradation rates to methodological challenges such as sampling conditions, extraction methods, sensitivity and dynamic range limitations within the methodologies employed for quantification. Furthermore, protein function and activity are not solely dictated by sequence or abundance (however important these are) but also by post-translational modification (PTM) status, structure, the concentration of substrate or products, and the interactions with other cellular components e.g. other proteins, metabolites, or nucleic acids.

Seen from this point of view, genomic and transcriptomic data only provide indirect views on cellular states. In order to fully understand the cell and the complex processes that take place within, comprehensive functional proteomic analyses are needed to complement insights provided by genomics and transcriptomics. However, proteins are very challenging to study, especially in an unmodified and unperturbed living cell context.

1.8 Target engagement and the cellular thermal shift assay (CETSA)

The large majority of drugs, from classical cytotoxic agents to most immunotherapies, are designed to bind key proteins in the cell and interfere with their function. In order for a drug to engage its target, it must first reach the cell, escape metabolic degradation, access the cellular environment of the target, and then bind (Fig. 1.3 A). Target engagement (TE) is an essential aspect of drug efficacy and one of the three pillars set to determine the likelihood for a candidate drug to perform well in phase II clinical trials and proceed to phase III [90].

However, monitoring TE and other functional aspects of proteins *in situ* is challenging. For an extended period of time, the only ways to determine whether TE takes place was to either perform ligand-binding studies in purified proteins and cell lysates or to investigate effects downstream of TE. Many of the methods employed for the assessment of ligand-target interaction involve in one way or another the modification of proteins or ligands with e.g. tags that could be used for detection, immobilization, or pull-down[91]. Such methods can often shed some light on TE and assist in target identification, but they also risk providing artefactual information due to binding specificity or affinity issues linked to the introduced ligand or protein modifications typically utilized. Furthermore, the simple fact that experiments are performed in an environment that is different to the one found in the living cell i.e. cell lysates, exacerbates these issues. Therefore, many of the affinity capture approaches have turned out to be insufficient for reliable TE evaluation in complex systems.

Thermal shift assays (TSAs) have been extensively used for performing ligand binding studies on purified proteins[92–94]. TSAs operate on the principle that when proteins bind a ligand, they typically become more thermally stable. When exposed to increasing temperatures, proteins will melt (denature/ unfold) and many will quickly aggregate after melting. By quantifying the extent of aggregate formation during heating, protein melt curves (MCs) can be generated and any changes (i.e. shifts) in thermal stability that occur e.g. when a ligand binds, can be measured and evaluated.

Based on the principles of TSAs, the cellular thermal shift assay (CETSA) was introduced as the first biophysical method for monitoring TE in living cells and tissues[95.] By performing the heating step in intact cells (e.g. drug-treated or untreated), isolating the soluble protein fraction following cell lysis, and analysing the amount of target protein remaining in the soluble fraction, MCs can now reveal thermal shifts (CETSA shifts) that directly

represent binding events to proteins in their unaltered environment (Fig 1.3 B). One key advantage of CETSA is that the pivotal step in the experimental workflow, the heating, is performed in intact cells in a non-destructive and label-free manner – the protein itself reports whether ligand binding occurs or not.

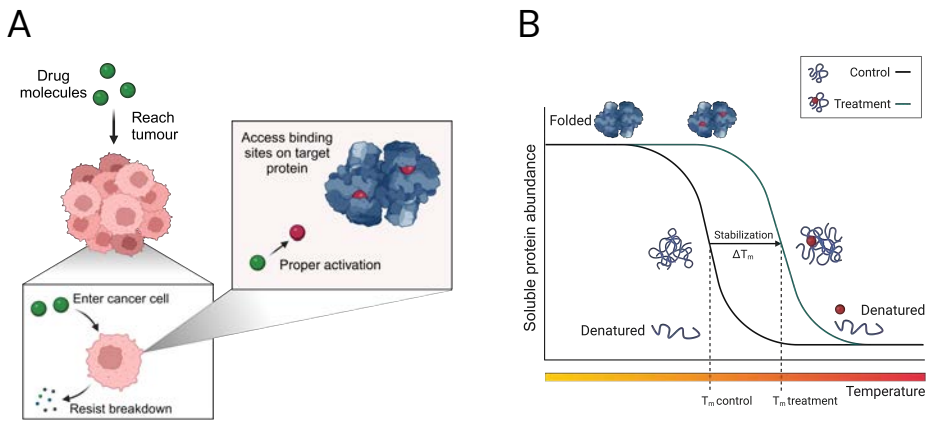

Figure 1.3 A. Drug target engagement (TE) inside living cells encompasses several steps a drug would need to navigate to effectively bind to its target protein. B. The principle of thermal shift assays and CETSA. *Images generated with BioRender.*

The CETSA proof-of-principle publication in 2013[95] explored the applicability and versatility of the method in monitoring TE for multiple chemotherapeutic agents in a set of human cell lines and tissues from mice. In addition to MCs, where the extent of protein melting is recorded over a temperature gradient, this initial publication also introduced an isothermal dose-response (ITDR)-CETSA format, where the changes in thermal stability are monitored at constant temperature over a compound concentration gradient. ITDR-CETSA provides another dimension of analysis that allows e.g. evaluating target occupancy, ranking of affinities of several compounds directed at the same protein target[96,97] and investigating whether resistance mechanisms that affect TE are present in the cell[96].

CETSA has evolved significantly over the years, becoming a widely adopted technique and contributing to many areas such as drug discovery and TE studies[97–99] as well as the exploration of drug MoA and resistance[96,100], both in the academia and the industry. With the implementation of mass spectrometry as a protein detection method for CETSA, extensive horizontal cell biology aspects became accessible at proteome level,

therefore increasing the method's potential for novel discoveries (further discussed in section 1.9).

1.9 Proteomics

A large part of the work presented in this thesis revolved around utilizing MS-based proteomics for investigating the proteome-wide cancer drug induced changes in protein interaction states (PRINTS) i.e. the differential interactions of proteins with ligands or other cellular components following exposure to cytotoxic compounds. Therefore, the focus of the following section will be on these applications for proteomics studies.

A brief history of mass spectrometry

Mass spectrometry (MS) could be seen as a science on its own and has its origins around the turn of the 20th century. The first MS experiment was performed in 1913 by Joseph John Thomson using a parabola spectrograph and provided the first evidence for the existence of two neon isotopes[101]. A few years later, in 1919 Francis Aston constructed the first mass spectrometer. At the heart of any mass spectrometer is the mass analyser, which uses electric or magnetic fields to separate ionized analytes based on their mass and charge. The resulting data is recorded as a ratio (m/z), which forms the basis for further analysis.

The technological advancement of MS happened gradually over time and was marked by several notable moments. The time of flight (TOF) mass analyser, introduced in 1946, exceeded the capabilities of its predecessors by performing with higher mass accuracy and increased resolution in a wider mass range[102,103]. The quadrupole was introduced in 1953 and became an important element for ion manipulation and mass selection[103,104]. Both the quadrupole and TOF are still used to this day, usually in tandem (Q-TOF). Around the same time in the 1950s chromatographic separation techniques began to be linked to MS for the first time, which allowed the separation and identification of species in more complex mixtures.

The development of the soft ionization techniques, electrospray ionization (ESI) in 1984[105] and matrix-assisted laser desorption/ionization (MALDI) in 1985[106], revolutionized the field of MS. In time, these breakthroughs addressed the longstanding challenges of ionizing large biomolecules e.g. peptides and proteins

with minimal fragmentation, opening up new possibilities in proteomics and structural biology[107,108]. They are still the dominant forms of macromolecule ionization to this day.

The introduction of the Fourier-transform ion cyclotron resonance (FT-ICR), proposed in 1974[109] and available in 1990s, along with the development of the Orbitrap mass analyser in 2005[110], marked another transformative phase in mass spectrometry. They allowed high-speed data acquisition with unprecedented mass accuracy and resolution, opening up new possibilities in proteomics research. These high-resolution MS technological advances have become instrumental in various areas of life sciences research and contributed significant advances in the field. Nowadays, mass spectrometers often employ multiple mass analysers in tandem e.g. quadrupole-Orbitrap and triple quadrupole, representing a strategic approach aimed at enhancing analytical capabilities and resolution.

The field of MS is continuously evolving, with improved hardware, data acquisition strategies, and software for data analysis continuously being developed and introduced. One recent and most remarkable development is the Orbitrap-Astral mass spectrometer introduced in 2023[111], which incorporates a novel mass analyser. The instrument promises unprecedented analysis speed, depth, sensitivity, and accuracy among other capabilities. Its introduction may represent yet another revolution in the field of MS.

MS-based proteomics

MS is one of the most utilized proteomics methods today for protein identification, monitoring the proteome-wide changes between different cellular states, characterizing post translational modifications, among other applications[112–114].

Data acquisition strategies have evolved alongside technological advancements when the hardware developments were still insufficient in solving the limitations encountered when identifying proteins in complex samples. Data dependent acquisition (DDA) is the most commonly used strategy for MS data acquisition in proteomics. In DDA, the mass spectrometer dynamically selects which precursor ions (MS1) to be sent to subsequent fragmentation (MS2) based on their abundance in a previous MS scan[113]. DDA can be performed for quantitative studies in either labelled, label-free, or targeted fashion. With different protein labelling techniques available, the multiplexing of MS samples is possible. For

example, protein labelling using isobaric tandem mass tags (TMTs)[115] nowadays offers the possibility of multiplexing up to 18 samples to be run on the MS simultaneously. This approach helps minimize the issues that may arise by comparing samples ran separately such as differential identification of peptides between runs resulting in missing values or instrument dependent batch effects.

Furthermore, another important consideration are the strategies employed to obtaining the protein identifications. Bottom-up (also known as shotgun) approaches are widely used for analysing complex samples due to their high sensitivity and speed. They involve the digestion of the complex protein mixture into peptides before being further fractionated often using LC and analysed with MS. Peptide identification is done using search engines e.g. Sequest[116] which perform peptide spectral matching to the reference peptide library generated from protein sequence databases. The identified peptides are then assembled into a list of proteins by protein inference[117].

Quantitative proteomics

Quantitative proteomics (QP) refers to the comprehensive study of the expression levels of an as large part of the cellular proteome as methodologically possible. By evaluating protein abundances, attempts are made to draw conclusions regarding the cellular state be it in a normal or disease context or following exposure to different drugs or other means to alter cellular states.

QP approaches have been extensively utilized in cancer research and yielded valuable information in many areas. They have contributed to the characterization of cancer cells and the classification of tumour subtypes[66,118–121], investigating protein synthesis and turnover[122], exploring the MoA of cancer drugs[123], and identifying potential candidate biomarkers for predicting drug efficacy or suggesting therapeutic approaches[121].

CETSA-based functional proteomics approaches

Most of the work presented in this thesis was performed using the proteome-wide implementation of CETSA (MS-CETSA). Therefore, a more in-depth presentation of this method will follow.

Up until 2014 all implementations of CETSA were employing some form of antibody-based technology for protein detection, which presented limited

options when it comes to multiplexing as well as suffered from other shortcomings associated with these types of quantification methods e.g. questionable antibody specificity, limited sensitivity, high background, and variations in signal intensity (especially in the case of WB). One game-changing development occurred when a workflow for shotgun proteomics with TMT-based quantitation of peptide mass spectra acquired in a DDA manner was introduced as a readout method for CETSA. Instead of one or a limited number of proteins at a time as in the case of many antibody-based assays, MS readout allowed the simultaneous measurement of CETSA signals for thousands of proteins in the cell.

The first implementation of MS-CETSA also known as thermal proteome profiling (TPP) explored the proteome-wide effects of kinase inhibitors and highlighted the applicability of the method for identifying drug targets at the proteome level[124]. Subsequent development and application of the MS-CETSA approach demonstrated new ways for studying functional/biochemical aspects of the proteome and for mapping the changes in PRotein INTeraction States (PRINTS) that occur during the transitions between different cellular states e.g. cell cycle transitions or changes upon drug treatment[125,126]. Proteomics studies with CETSA can therefore allow the generation of comprehensive data on an array of cellular responses involving proteins. This in turn can reveal important horizontal biology aspects in the cell that are complementary to the vertical biology information provided by e.g. quantitative proteomics or transcriptomic approaches. MS-CETSA has thus evolved into a relevant proteomics method and several proteome-wide studies performed in either MC- or ITDR-CETSA format have contributed valuable insights regarding drug action and target identification[127–129], protein-metabolite interactions[129–131], protein redox states[132], or monitoring protein complex modulations and dynamics[133–135].

Several MS-CETSA formats have been successfully implemented so far as illustrated in Figure 1.4[125]. Amongst them, the Integrated Modulation of PRINTS (IMPRINTS)-CETSA format was introduced as a more stringent and sensitive alternative to the already existing MC- or ITDR-CETSA MS approaches. IMPRINTS-CETSA owes its improved stringency to an optimized experimental setup, aimed at minimizing sources of measurement error. This is achieved by including three biological replicates for controls and each studied condition within the same TMT set, thus limiting the effect of detection and quantification interference by e.g. variable background signals that are typically present in cases when sample conditions are compared between different TMT sets. This way, the quantified

proteins share the same peptide population as they are simultaneously analysed in the mass spectrometer. Moreover, the samples are processed in parallel throughout the MS sample preparation, further minimizing sources of error related to technical aspects. IMPRINTS-CETSA includes several sets of samples, each containing the same experimental conditions but heated at different temperatures (Fig 1.4)

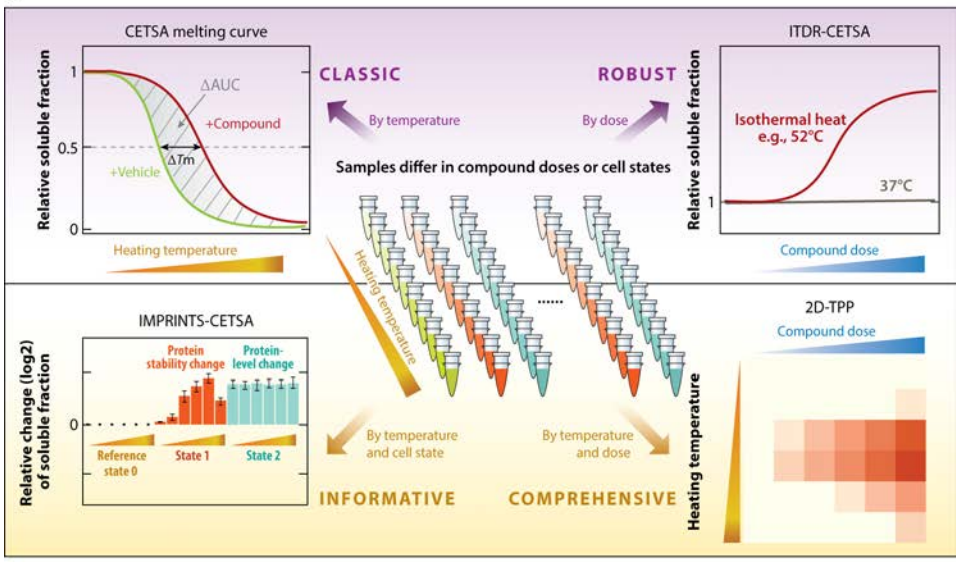

Figure 1.4. Different MS-CETSA formats. CETSA melt curves address the protein responses to treatment with one compound dose (compared to vehicle control) in a range of heating temperatures. The ITDR-CETSA format monitors protein responses to several compound doses at one heating temperature. IMPRINTS-CETSA reports on protein stability changes between different cell states e.g. treated vs. control, within biological replicates, and across a range of temperatures. The 2D-TPP format combines several heating temperatures and drug doses. *Image from Dai et al. Horizontal Cell Biology: Monitoring Global Changes of Protein Interaction States with the Proteome-Wide Cellular Thermal Shift Assay (CETSA), Annu Rev Biochem, 2019, 88:383-408. Reprinted with permission from CCC.*

To also integrate possible changes in protein levels or protein reorganization events, a set of samples heated at 37°C is included. The CETSA data obtained at 37°C differs from data obtained with standard QP approaches in the respect that membranes, organelles, or bulky cellular components still present after lysis would be removed from the soluble fraction along with all associated proteins. The differences seen between the 37°C CETSA and QP data are likely to reflect the relocalisation of proteins between cellular compartments or membranes in the studied samples. Recently we have begun to refer to this aspect as the differential extraction accessibility by relocalisation (DEAR) effect. The IMPRINTS-CETSA

format yields characteristic profiles (which we refer to as IMPRINTS profiles) for protein abundance/relocalisation and thermal stability changes that can provide valuable insights into biochemical effects at the proteome level as cells transition from one state to another.

The first IMPRINTS-CETSA implementation extracted extensive information on the modulation of proteins along the cell cycle[135] and together with a 2D-TPP approach[134] (also on the cell cycle), constituted the beginning of multidimensional proteome-wide CETSA exploration. In the past years, such multidimensional CETSA approaches have contributed insights in different areas of cancer research such as monitoring drug TE and target deconvolution for clinically administered drugs[100,136] or bioactive natural products[137], comparing different cell states e.g. cell cycle phases[134,135], investigating the effects of point mutations[138] or gene knock-out [139].

TPP in 2D format (2D-TPP), also addresses the CETSA-based changes in PRINTS in the cell but utilizes a slightly different sample arrangement for MS analysis. Instead of biological replicates and different experimental conditions at one heating temperature being packaged and analysed within one TMT set and several different temperatures analysed in different sets as is the case with IMPRINTS-CETSA, the 2D-TPP format analyses different experimental conditions at different temperatures packaged in one TMT set. Several such sets are then analysed in separate MS runs, while biological replicates are also analysed in separate MS runs.

The protein integral solubility alteration (PISA) approach[140] also utilizes the CETSA principles for investigating changes in PRINTS but employs a slightly different workflow. Instead of analysing the soluble protein fraction from differentially heated samples as different measurements, PISA integrates the melting profile of the proteins over a temperature gradient into one sample by combining differentially heated subsets of the same starting material into one, followed by MS analysis. The relative quantification of protein abundances obtained in this manner yields information regarding the differences between the cellular states analysed without the need of protein melting curves being fitted or T_m values needing to be determined as it is required in the case of e.g. TPP.

One limitation of CETSA-based proteomic studies with MS is that changes in PRINTS are not currently trackable over the entire proteome. This is partly due to sample complexity and limitations in the dynamic range of MS

instruments. In addition, some proteins e.g. membrane proteins generally can't be reliably accessed in CETSA studies without the need of extensive optimization of protein extraction protocols specifically for this purpose. Extraction protocols for CETSA are typically designed to access proteins without perturbing the aggregates formed in the heating step and therefore are focused on employing lysis methods e.g. freeze-thawing, without including any additives that may interfere with protein precipitates e.g. strong ionic detergents. However, in a few cases, thermal shifts have been reported for membrane proteins when a non-ionic detergent was included in the cell lysis step[128,141].

2 RESEARCH AIMS

Aims of Paper I

CETSA interaction proteomics define specific RNA-modification pathways as key components of fluorouracil-based cancer drug cytotoxicity.

- Obtain a better understanding of the mechanism of action of 5-FU and its metabolites FUDR and FUR
- Investigate the cellular processes that contribute to acquiring resistance to 5-FU
- Identify candidate biomarkers for 5-FU efficacy

Aims of Paper II

The mechanism of action of trifluridine and TAS-102 in a colon cancer model: A proteomics study using IMPRINTS-CETSA.

- Investigate the mechanism of action of trifluridine and TAS-102
- Determine if and how could 5-FU resistance be overcome by TFT/TAS-102 or by 5-FU metabolites FUDR or FUR

Aims of Paper III

CETSA-based target engagement of taxanes as biomarkers for efficacy and resistance.

- Determine the applicability of β-tubulin CETSA for monitoring the efficacy of taxanes in clinically relevant cancer models
- Evaluate taxane target engagement for β-tubulin using CETSA in breast cancer patient samples

Aims of Paper IV

Proteome-wide CETSA reveals diverse apoptosis inducing mechanisms converging on an initial apoptosis effector stage focused at the nuclear matrix proximal region.

- Obtain a better understanding of apoptosis-related cellular processes
- Investigate the mechanism of action of several cytotoxic cancer drugs utilised in the clinic

3 RESULTS AND DISCUSSION

3.1 PAPER I

CETSA interaction proteomics define specific RNA-modification pathways as key components of fluorouracil-based cancer drug cytotoxicity.

In this study we implemented IMPRINTS-CETSA with the aim of mapping the proteome-wide cellular responses to 5-FU and its metabolites, 5-fluorodeoxyuridine (FUDR) and 5-fluorouridine (FUR) so that a more complete understanding of the 5-FU MoA could be obtained. We also aimed to identify key proteins in the development of 5-FU resistance that could become novel candidate biomarkers for 5-FU efficacy.

3.1.1 CETSA responses to FUDR and FUR

Deciphering the MoA of 5-FU, a prodrug that is activated in several subsequent steps producing multiple metabolites, each affecting cellular processes in different ways (as discussed in section 1.3), was expected to be a rather complicated endeavor. Therefore, we first attempted to simplify the task by studying 5-FU metabolites FUDR and FUR separately, as each represent metabolic pathways that ultimately lead to DNA-related and RNA-related toxicities respectively. IMPRINTS-CETSA experiments were performed with the drugs in MCF-7 breast cancer cells at two time points, 2 h and 12 h. As we hoped, this approach provided a detailed understanding of the early cellular effects taking place and revealed quite separate protein responses to each of the compounds (Fig. 3.1.1).

FUDR produced a considerable effect on the (deoxy)nucleotide metabolism with CETSA shifts observed for proteins that engage with different thymidine metabolites. TYMS, considered the main target of 5-FU, had one of the strongest shifts observed. Several other key enzymes (TK1, DTYMK, RRM1, and SAMHD1) were thermally destabilized, which is likely to reflect a decrease in thymidine nucleotide levels, consistent with previous CETSA studies[131,135]. DUT, the enzyme that catalyses the conversion of dUTP to dUMP was stabilized, and follow-up experiments revealed a potential direct binding of FdUTP, in agreement with previous proposals for this metabolite functioning as a DUT substrate[142,143].

FUDR also led to early responses in several proteins involved in DNA repair out of which the DNA abasic site shielding protein HMCES[144], not previously linked to the MoA of 5-FU, was most noteworthy. Proteins with roles in the repair of DNA double strand breaks FANCD2, FANCI, and XRCC6 also showed changes in thermal stability following FUDR treatment indicating the presence of this type of DNA lesions already at this early time point.

On the other hand, FUR predominantly affected proteins involved in RNA modification and processing. Prominent CETSA stabilizations were observed for several types of 5xU modifying proteins: pseudouridine synthases (e.g. PUS1, TRUB1, and RPUSD2), the tRNA uracil-5-methyltransferase TRMT2A, and dihydrouridine synthases (DUS1L and DUS3L). The responses seen in the abovementioned proteins likely reflect on an increased and potentially covalent protein-RNA interaction due to the misincorporated 5-FU bases into different types of RNA.

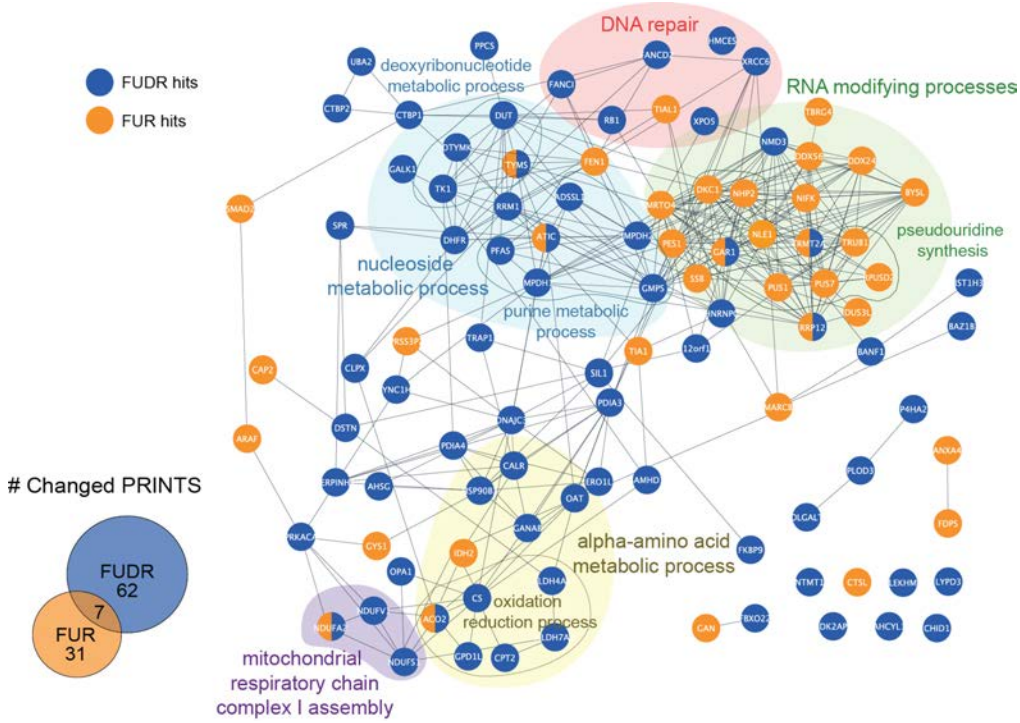

Figure 3.1.1. Protein association network generated with STRING for FUDR and FUR hits following 2 h treatment in MCF-7 cells.

Following 12 h exposure to either FUDR or FUR, while many of the CETSA responses seen at 2 h were maintained, the overall captured changes in PRINTS appeared to be more evenly distributed between the two drugs. This indicated that within the 12 h timeframe, the sugar moieties of the 5-FU metabolites are interconverted (shuffled) between deoxyribose and ribose and so, proteins that only shifted with one metabolite at 2 h, began to show CETSA shifts with the other metabolite at 12 h, albeit oftentimes smaller.

An intriguing effect emerging at the 12 h time point was reflected by changes in the levels of several p53-regulated proteins following FUR treatment indicative of p53 activation, which we confirmed using orthogonal methods. This effect was not observed for FUDR which was surprising considering the wide-spread assumption that DNA damage is the key factor leading to p53 activation[145].

3.1.2 The mechanism of action of 5-FU and resistance

Our next aim was to obtain a better understanding of the 5-FU MoA and resistance in CRC cells. For these purposes we performed IMPRINTS-CETSA with 5-FU in parental HCT15 cells (HCT15-P) as well as in 5-FU resistant HCT15 cells (HCT15-R).

Following 12 h incubation with 5-FU in HCT15-P cells, it was apparent that the overall cellular effect was dominated by RNA-related protein responses alongside other proteins seen following treatment with FUDR or FUR in MCF-7 cells.

We also evaluated the 5-FU CETSA responses in HCT15-R cells in order to uncover some of the cellular mechanisms by which resistance to 5-FU could be acquired, and to identify potential efficacy predictive biomarkers. Most intriguing was the CETSA response of TYMS, which was equally thermally stabilized in both HCT15-P and HCT15-R cells following 5-FU treatment while the resistant cells were able to grow apparently unhampered in this situation.

Another interesting aspect revealed by these data was that the CETSA responses of some protein hits in HCT15-P cells were attenuated or even absent in HCT15-R cells. Many of these were the previously mentioned RNA-modifying proteins responding after FUR treatment in MCF-7 cells e.g. PUS1, TRMT2A, DUS3L (Fig. 3.1.2). We believe these response attenuations are reflective of critical components in the 5-FU MoA that had to be primarily addressed by these cells to establish resistance.

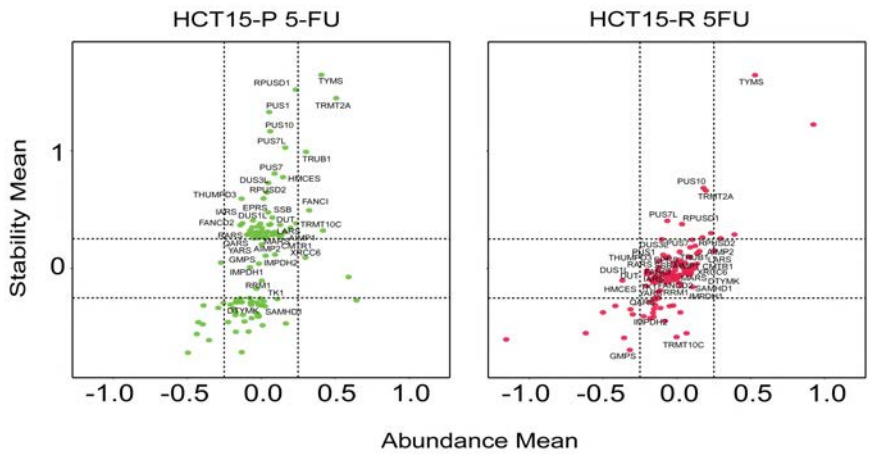

Figure 3.1.2. Scatter plots of mean abundance changes versus mean stability changes for responding proteins in HCT15-P cells treated with 100 µM 5-FU and compared to vehicle. FUDR- and FUR-characteristic responses are also represented (left panel). Many of these CETSA responses are attenuated in HCT15-R cells exposed to the 5-FU concentration used for cell culture (16 µM) when compared to vehicle-treated HCT15-P cells (right panel).

All the proteins mentioned up until now showed only changes in thermal stability i.e. CETSA responses. We also recorded many proteins in HCT15-R cells that showed changes in levels compared to HCT15-P as reflected by the data measured at 37 °C. One notable example was seen for UMPS which displayed a decrease in levels in HCT15-R cells compared to HCT15-P cells, potentially leading to a decreased incorporation of FUTP into different RNAs.

3.1.3 Candidate biomarkers for 5-FU efficacy

As discussed in sections 1.5 and 1.6, no reliable biomarkers for predicting 5-FU efficacy exist today while drug resistance is a common occurrence. Based on our findings described above, we envisioned that the CETSA responses of some of the differentially responding proteins in resistant cells could potentially serve as mechanistic biomarkers for 5-FU efficacy. To test this hypothesis, we monitored the thermal stability changes of TYMS, PUS1 and HMCES following 5-FU treatment in different cancer cell lines (Fig 3.1.3 A). These experiments supported a potential connection between the CETSA response of PUS1 and 5-FU sensitivity and therefore PUS1 qualifies as candidate biomarker for 5-FU efficacy.

We further explored the applicability of CETSA responses as candidate biomarkers in a cancer model that was more relevant from a clinical point of view. A clear stabilization was seen for several of the abovementioned 5xU modifying

proteins (Fig. 3.1.3 B) in mouse MCF-7 xenografts exposed *in vivo* to different doses of 5-FU, confirming that these CETSA responses could be monitored in animal tissues.

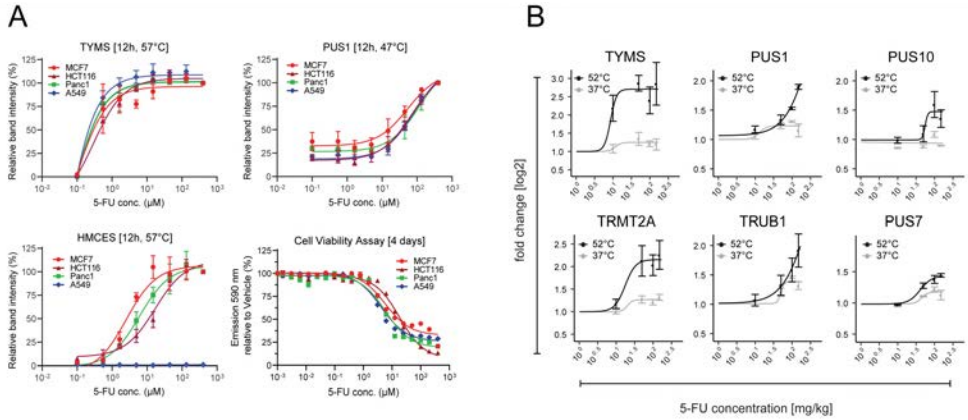

Figure 3.1.3. A. ITDR-CETSA with WB readout for TYMS, PUS1, and HMCES in different cancer cell lines treated with 5-FU for 12 h. Cell viability assays with 5-FU in these cell lines. Data are presented as mean ±SEM from biological replicates (n=3). B. ITDR-CETSA profiles of 5xU modifying proteins in mouse MCF-7 xenografts exposed *in vivo* to 5-FU and analyzed with MS-CETSA. Data are presented as mean fold change ±SEM compared to vehicle from technical replicates.

3.1.4 Discussion

IMPRINTS-CETSA experiments for investigating drug MoA often generate vast amounts of complex data that can prove challenging to interpret and to understand from both a mechanistic and cell biological point of view. Dissecting the 5-FU MoA by using the FUDR and FUR metabolites yielded valuable and at times novel information regarding the initial proteome-wide responses occurring once each of these metabolites would begin to appear in the cell, the time frame in which these responses are to be expected, as well as the way they would progress during drug incubation, information not easily accessible with other methodologies.

An interesting aspect revealed by this study was that 5-FU-containing nucleosides and nucleotides could be quickly interconverted from ribose-containing forms to deoxyribose-containing forms and vice versa, a process we refer to as ribose shuffling. This interconversion is overall reflected by the increase in overlapping responses with FUDR and FUR observed at 12 h and is also highlighted by the stabilization observed for TYMS with FUR (already detectable at 2 h).

When it comes to 5-FU, the overall picture emerging from this work was that its MoA in the utilized 5-FU-sensitive cell model is dominated by effects on proteins performing RNA modifications or involved in other RNA-related processes.

We also identified CETSA responses that we believe are reflective of critical components of the 5-FU MoA. The attenuated or absent CETSA shifts seen in 5-FU resistant cells for e.g. RNA-modifying proteins, report on the presence of a cellular adaptation that results in a decrease in the pools of the different 5-fluorouridine nucleotides available as compared to the situation in 5-FU sensitive cells, which in turn would lead to a decreased misincorporation of 5-FU metabolites into different RNAs. Our data revealed RNA-related toxicities to be a core component of the 5-FU MoA, which needed to be abrogated if the cells were to continue living. Our overall impression from the vast literature on 5-FU was that the role of RNA effects in the MoA of the drug has been acknowledged but so far underestimated.

Following the publication of this paper, another study presented RNA-related cytotoxicities as core components of 5-FU MoA in CRC models[146]. rRNA damage and impaired ribosome biogenesis were reported to be important events leading to cell death. Moreover, the study highlighted CRCs as particularly sensitive to the cytotoxic effects conveyed by the accumulation of 5-FU bases into RNA compared to other cancers, which may explain the utility of 5-FU-based therapies in this cancer type.

UMPS downregulation may be one important way through which resistant cells limit the production of FUMP and subsequently FUTP, ultimately reducing the misincorporation of 5-FU bases in RNA and avoiding RNA-related toxicity down to a level that is manageable for survival. Still, resistance is likely more complicated than the expression level attenuation of one single enzyme.

Intriguingly, the CETSA response of TYMS in 5-FU resistant cells suggests that even with this key enzyme apparently fully inhibited, these cells continue to proliferate unhampered. TYMS inhibition has for a long time been believed to be a critical component of the 5-FU MoA, yet our data rather point towards a non-essential role of TYMS inhibition in the efficacy of 5-FU in these cells within the specific conditions used. Studies in yeast reported dTTP level decreases to be well-tolerated which provides additional support to our findings[147]. Another possibility is that the cells can sufficiently supply their thymidine pools from the growth medium.

The CETSA responses for many of the proteins mentioned in this work could function as biomarkers for 5-FU efficacy and we confirm the applicability of CETSA for monitoring drug responses in potential candidate biomarker proteins in multiple cell lines and in mouse xenografts. Still, further evaluation is required concerning the applicability of the method in clinical samples from patients, but this work was outside of the scope of this study and remains to be considered as a future perspective for us and others.

3.2 PAPER II (Manuscript)

The work presented in this manuscript is an expansion of our study on 5-FU (Paper 1). Here, IMPRINTS-CETSA was used for the evaluation of the MoA of trifluorothymidine (TFT) and TAS-102 in the 5-FU-sensitive and -resistant CRC cells, referred to as HCT15-P and HCT15-R respectively (also used in paper I). In addition, FUDR and FUR were included in this study to gain additional information regarding their MoA particularly in these cells as well as to perform a comparison with the responses seen with TFT/TAS-102. The experiments were performed at 2 h and 12 h incubation, to obtain a better picture over the evolution of cellular responses to the drugs over time.

This manuscript is a short overview of the research findings resulting from the performed IMPRINTS-CETSA experiments, the focus being on some of the main aspects uncovered.

3.2.1 The overall CETSA response of TFT and TAS-102 in CRC cells

Firstly, we evaluated the CETSA responses of TFT and TAS-102 in HCT15-P cells and observed that while TAS-102 treatment generally induced more responses than TFT, the two drugs still exhibited a considerable amount of common hits. The overlapping hits included proteins involved in e.g. the deoxyribonucleotide metabolism and tRNA-related processes. Clusters of proteins involved in the DNA damage response, chromatin maintenance, and cell cycle regulation were observed solely at the later time point used in the experiment, with many of the representative protein hits in these processes overlapping between TAS-102 and FUDR (FUR as well at times) (Fig. 3.2.1). This indicates that TAS-102 appears to share a part of its MoA with FUDR. Proteins involved in RNA-related processes also showed changes in thermal stability, most of them being only FUDR and FUR hits, which indicates that the TAS-102 MoA does not rely on this type of cytotoxic effects. One thing to be noted is the interconversion between FUDR and FUR (ribose shuffling) discussed in paper I, which can explain e.g. the presence of DNA-related hits in the case of FUR, a ribose-containing compound (Fig. 3.2.1).

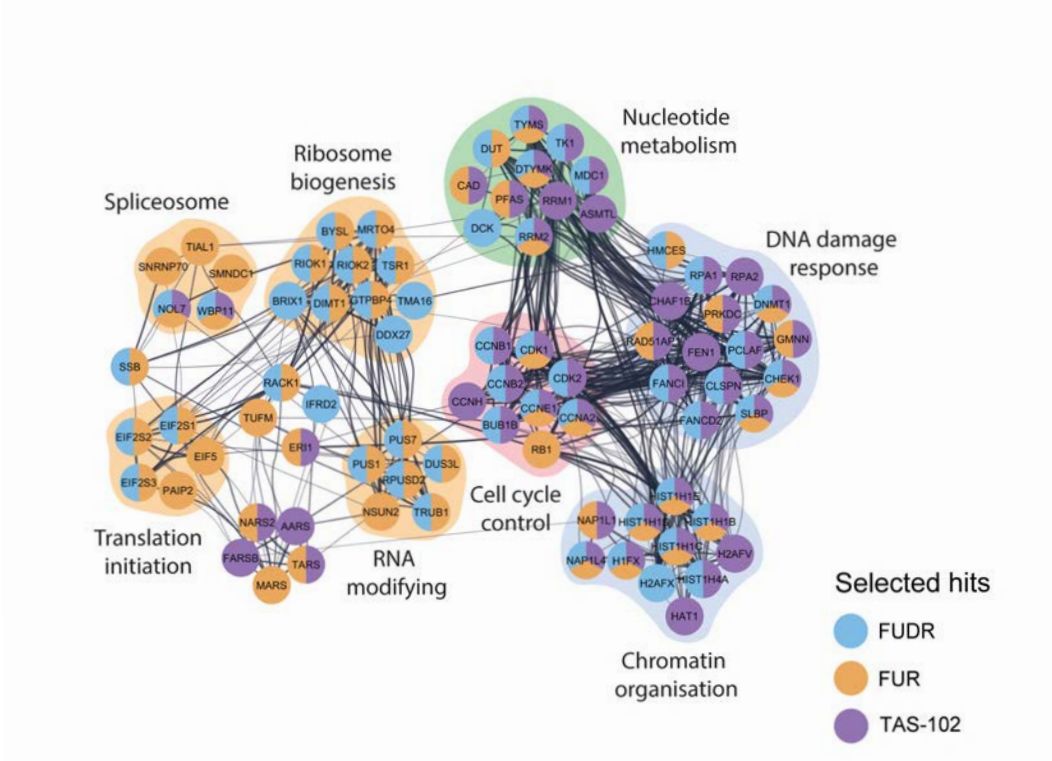

Figure 3.2.1. Protein association network of selected hits at 12 h incubation in HCT15-P cells. The network depicts the main effects present in the data following FUDR, FUR, or TAS-102 treatment.

3.2.2 Notable CETSA responses in HCT15-P cells

The CETSA responses observed for enzymes involved in the nucleotide metabolism in HCT15-P cells following FUDR and FUR treatment, were similar to the responses seen in our previous study in MCF-7 cells (paper I). The shifts observed for TYMS with FUDR/FUR and TFT/TAS-102 reflect on the anticipated inhibiting capacity of the corresponding metabolites FdUMP and trifluorothymidine monophosphate (TFdTMP). As previously discussed in paper I, TYMS inhibition was expected to produce variations in the pools of the different deoxynucleotides that can affect the CETSA responses of DTYMK, TK1, SAMHD1, and RRM1. Interestingly, after 2h incubation we only observed a destabilization in DTYMK following treatment with all four drugs, while TK1 and SAMHD1 appeared destabilized following FUDR treatment but remained unaffected following incubation with TFT/TAS-102. RRM1 on the other hand was stabilized with TFT/TAS-102, indicative of a direct trifluorothymidine triphosphate (TFdTTP) binding, most likely in the substrate specificity site. This was note-worthy as RRM1 has not been previously indicated as a target for TFT/TAS-102.

As mentioned in section 1.3, FUDR and TFT will be further metabolized to FdUTP and TFdTTP respectively, which will then be misincorporated into DNA. Due to the ribose shuffling process (described in paper I), FUR will also eventually be metabolized to the same deoxyribonucleotides as FUDR and hence misincorporation into DNA will also occur in this case. CETSA shifts in proteins involved in the DNA damage response were observed as early as after 2h of treatment with FUDR and TFT/TAS-102. These DNA damage related effects are more pronounced at 12 h with additional proteins exhibiting changes in thermal stability.

A CETSA shift was also observed for HMCES following FUDR treatment in HCT15-P cells, as was seen in MCF-7 cells in paper I. We interpreted the HMCES stabilization also observed in this context as potentially occurring due to its interaction with an increased number of abasic sites within DNA. Interestingly no response was recorded following TFT/TAS-102 treatment (Fig 3.2.2) which likely excludes a role of HMCES in the MoA of TFT/TAS-102 in this model.

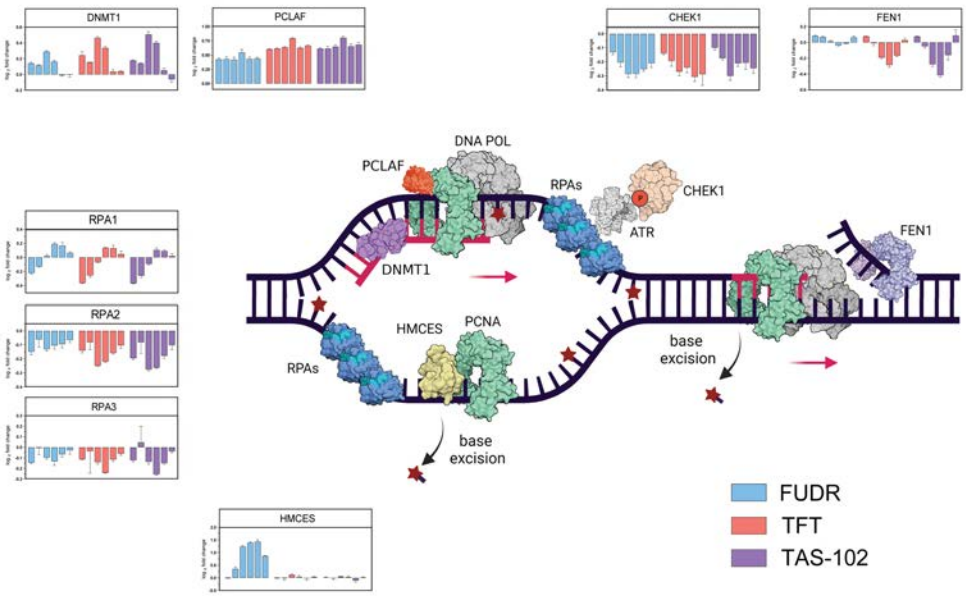

Figure 3.2.2. DNA-related responses to FUDR, TFT, or TAS-102 in HCT15-P cells at 12 h incubation with the compounds. IMPRINTS-CETSA data are presented as $\log_2$ fold change compared to vehicle ± SEM from biological replicates (n=3).

Replication protein A (RPA) plays a role as a shielder and stabilizer of ssDNA sections present at the replication fork during DNA replication or long-patch base excision repair (BER)[148,149]. After 2 h incubation with TFT/TAS-102 we observed a CETSA shift in RPA1, the large subunit of the RPA heterotrimer, a response that extended to RPA2 and RPA3 following 12 h treatment with the drugs (Fig 3.2.2). The CETSA responses point towards a possible increase in interaction between ssDNA and RPAs, possibly as a consequence of delayed/stalled replication forks or increased occurrence of long-patch BER attempts. Interestingly, we also observed a decrease in RPA levels at the 12 h time point, which might reflect either a degradation or reorganization of the RPAs. Another protein involved in long-patch BER for which we observed a CETSA shift was FEN1, tasked with the removal of the DNA flap created while DNA is being repaired.

One consequence of increased ssDNA-RPA interaction is the triggering of the ATR-CHEK1 axis[150], a cellular mechanism employed for e.g. keeping the cell cycle in check while DNA damage repair attempts are made [151]. Intriguingly, the cell cycle related responses we observed in the HCT15-P data were not indicative of cell cycle arrest. CHEK1 exhibited a CETSA shift following 12 h incubation with FUDR or TFT/TAS-102 that likely occurs due to its activation (Fig 3.2.2). However, several cyclins and their associated CDKs produced responses that, according to previous IMPRINTS-CETSA data on the cell cycle[135], indicate that the checkpoints are "open" for continued cell cycle phase transitions. Furthermore, no discernible responses indicating an induction of cell death were recorded in these cells within the time frame of the study.

3.2.3 RNA-related effects and the response in HCT15-R cells

Exposing the HCT15-P cells to FUR, also led to CETSA responses in many of the proteins reported in MCF-7 cells (paper I) e.g. PUS1, TRMT2A, and DUS3L. An important observation discussed in paper I, were the CETSA shifts seen with 5-FU in RNA modifying proteins in HCT15-P cells that were diminished or absent in HCT15-R cells. Interestingly, clear shifts were seen for these proteins in HCT15-R cells treated with FUR already at 2 h, and even with FUDR at 12 h in some cases. The decreased levels of UMPS reported in HCT15-R cells when compared to HCT15-P cells (Paper I) might account for some of the attenuated CETSA stabilizations seen for these proteins with 5-FU, but FUR and to some extent FUDR appear to be able to bypass this. However, some resistance to FUR and FUDR is still present in the HCT15-R cells as indicated by cell viability data, which points towards a more intricate resistance mechanism

where the downregulation of UMPS is only one component.

3.2.4 Discussion

The present study is essentially an extension of the work presented in paper I. Several of the affected processes as judged from the CETSA data for TFT/TAS-102 in 5-FU sensitive cells were expected. In the nucleotide metabolism, one noteworthy observation was that not all previously described thymidine "sensor" proteins were reflecting a thymidine depletion with TFT/TAS-102 in HCT15-P cells. This at first glance was an indication that the thymidine nucleoside and nucleotide pools were not affected to a similar extent with these drugs as it was the case with FUDR/FUR. However, it is very likely that we are observing the combined outcome of multiple thermal-stability-altering events in these proteins. For example, in the case of TK1, a thymidine depletion would typically manifest as a CETSA destabilization, but TFT is a TK1 substrate, which would convey a thermal stabilization effect. These two effects could occur simultaneously, likely to different degrees, therefore the result displayed in the IMPRINTS profile would reflect the superimposed response of these two effects on the enzyme.

In HCT15-P cells, TFT/TAS-102 also elicited cellular responses in proteins involved in tRNA metabolic processes and translation initiation, which was not previously linked to the MoA of these drugs. Still, the vast majority of RNA-related processes reported as an essential component of the 5-FU MoA in paper I, were not affected by TFT/TAS-102. This indicated that these drugs do not manifest their toxicity via the same RNA-related processes as FUDR/FUR. This is also likely why the 5-FU resistant HCT15-R cells did not display any resistance to TFT/TAS-102 while some resistance to FUDR/FUR was present. TFT/TAS-102 more readily appeared to share a part of their MoA with FUDR when it came to affecting processes as the DNA damage response, chromatin organization, and cell cycle regulation.

Our data also pointed towards a DNA damage response leading to the activation of CHEK1 in HCT15-P cells. However, the CETSA responses observed for key cell cycle control proteins did not indicate the expected cell cycle arrest but more towards the opposite, that transitions between cell cycle phases would be free to occur. Moreover, we have not observed any responses indicative of imminent cell death in these cells within the studied time frame. Still, the cell killing effects may occur at a later time point.

Another interesting finding resulting from this study was that FUR and FUDR produced CETSA responses for RNA-modifying proteins in HCT15-R cells, apparently bypassing the downregulation of UMPS previously documented in these cells (paper I). However, cell viability data reflect on the presence of additional mechanisms of resistance when the FUR and FUDR EC_{50} values still differ between the parental and resistant cell lines by ~1 order of magnitude.

Overall, this study yielded valuable insights that enhanced our understanding of the four drugs investigated both in 5-FU-sensitive and -resistant settings. This manuscript presents an initial overview of the main findings resulting from the performed experiments. A more comprehensive presentation of the obtained data remains as a future prospect.

3.3 PAPER III

CETSA-based target engagement of taxanes as biomarkers for efficacy and resistance.

The first phase of my PhD was dedicated to implementing the CETSA methodology to monitor TE for microtubule-binding drugs in different clinically relevant models and ultimately in samples collected from patients. The goal was to evaluate the feasibility of using CETSA responses for tubulins as candidate biomarkers for taxane efficacy. We first aimed at developing a readout method for CETSA that was able to sensitively detect β-tubulin in a straightforward and convenient way even when the available cell material to be analysed came in very low amounts, as it often is the case with patient samples. The next step involved applying our developed readout method in different models to evaluate the kind of information we would be able to obtain and further assess the applicability of the method in these models. Ultimately patient samples were analysed.

3.3.1 Developing a sensitive protein detection method for β-tubulin

The first step towards developing a readout method for monitoring β-tubulin TE for future use in patient samples was to better understand the CETSA behaviour of the said protein. These preliminary steps were performed using WB as protein detection method for CETSA, while MS-CETSA was in the process of being introduced and optimized in our research group at that point in time. During the project, β-tubulin data generated with different CETSA formats revealed that clear thermal stability shifts were observable in living cells upon treatment with different microtubule-binding drugs.

Several considerations arose during the initial establishment and optimization of CETSA for β-tubulin, one being the high melting temperature observed for tubulins that raised the question of potentially losing membrane integrity during the CETSA heating step, which would lead to drug leaking inside the cell as well as disturbing the cellular microtubule organization among other things. Additional experiments revealed that membrane integrity would not be a concern when the majority of the cells maintained membrane integrity up to 63°C within the first minute of heating, when most thermal stability changes are actually manifesting. Furthermore, when CETSA for β-tubulin was performed in cell lysates, no shifts were detectable. This was a good indication that drug binding and thermal

stabilization only occur while microtubules are intact, as microtubules quickly depolymerize when membrane integrity is lost.

We then established a β-tubulin detection assay based on AlphaLISA technology. We selected this methodology due to its ability to specifically detect soluble β-tubulin in a homogenous assay format i.e. without having to remove the protein aggregates following the CETSA heating step, an aspect achieved via the selection of two different antibodies, one monoclonal and one polyclonal, which would only produce a signal when both antibodies bind to the native protein. After testing multiple antibody combinations, we have found two pairs that performed well for β-tubulin and captured its CETSA behaviour in an accurate and robust manner (example in Fig. 3.3.1) while exhibiting enhanced sensitivity compared to WB.

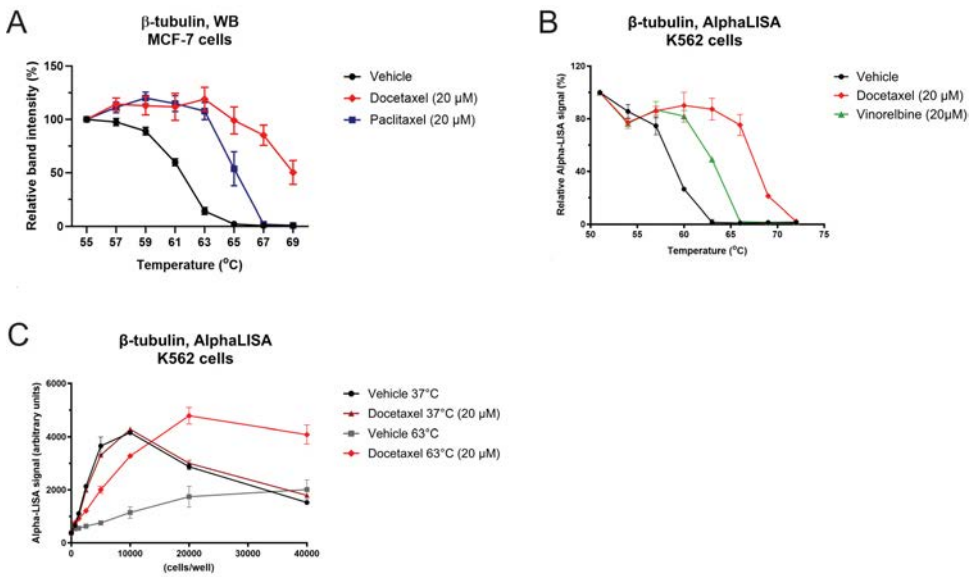

Figure 3.3.1. A. CETSA melt curves in MCF-7 cells treated with docetaxel or paclitaxel. β-tubulin detected with WB. Data are presented as mean ±SEM from independent experiments (n=5-6). B. CETSA melt curves in K562 cells treated with docetaxel or vinorelbine. β-tubulin was detected in total cell lysate using AlphaLISA. The data represent the mean ± S.E.M from technical replicates. C. Cell density titration with AlphaLISA in total cell lysate from K562 cells treated with docetaxel.

In the context of clinical applications, being able to monitor TE at the level of individual cells holds significant importance as it would reveal valuable aspects concerning the heterogeneous cellular makeup present in a tumour. A potential strategy to achieve single-cell resolution with CETSA involves quantifying differences in protein thermal stability using antibody-based cell imaging approaches where the selected antibodies can specifically identify the native

conformation of the protein. We established the Imaging-CETSA format for β-tubulin, and we demonstrated the feasibility of this approach for detecting differences at TE level associated with resistance to docetaxel and paclitaxel.

3.3.2 TE of microtubule-binding drugs in different cancer models

Monitoring TE for taxanes in sensitive and resistant cell lines

An important requirement for tubulin CETSA if it eventually was to be used in patient samples was to assess whether differences in thermal stability could be detected at TE level and linked to variations in drug efficacy i.e. resistance. For this we selected a cell model consisting of sensitive and resistant K562 cells referred to as K562-P and -R respectively, to study with CETSA and AlphaLISA detection for β-tubulin. The K562-R cells, were initially developed to be resistant to the vinca alkaloid vincristine but cross resistance was observed in these cells to several other drugs, taxanes included, due to the overexpression of the P-gp efflux pump (discussed in sections 1.5 and 1.6).

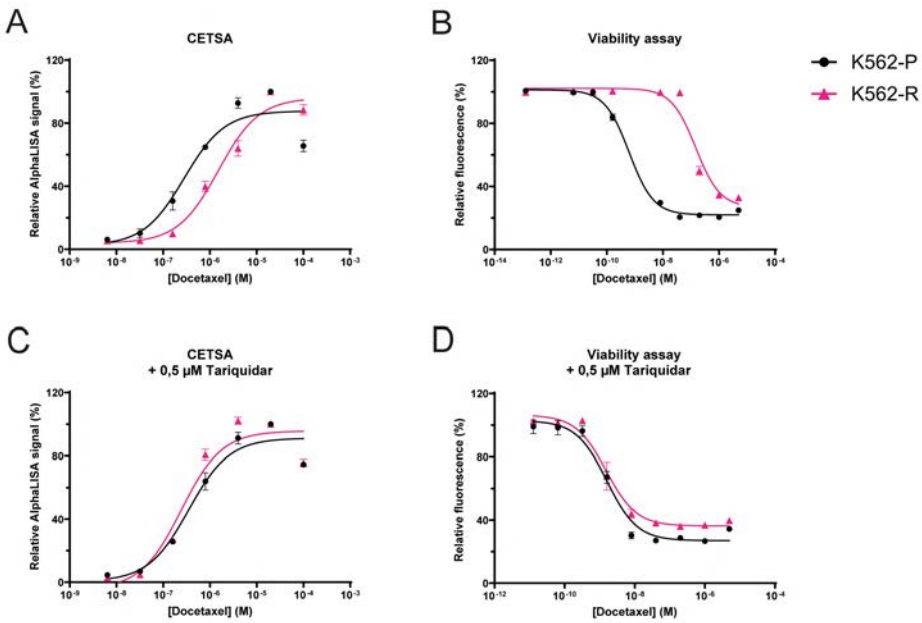

Figure 3.3.2. A. ITDR-CETSA for β-tubulin in K562-P and K562-R treated with docetaxel. B. Viability assays for docetaxel in K562-P and K562-R cells. C. ITDR-CETSA for β-tubulin in K562-P and K562-R cells treated with docetaxel and the P-gp inhibitor tariquidar. D. Viability assays in K562-P and K562-R cells treated with docetaxel and tariquidar. All data represent the mean ± S.E.M from independent experiments (n = 3–4).

Differences in CETSA responses to taxanes were recorded for β-tubulin between K562-P and K562-R indicating a resistance mechanism that affects drug TE (Fig. 3.3.2). Co-incubation with the P-gp inhibitor tariquidar abolished the observed differences which further supported that the resistance mechanism was attributed to drug efflux via P-gp transporters.

This part of the study established CETSA as a successful way to monitor drug resistance when TE is affected, as well as to aid in identifying the resistance mechanism in certain cases.

Taxane TE in ex vivo and in vivo setting

Having established that CETSA studies for tubulin TE are feasible and can yield important information for different drugs in cell lines, the next step was to test the applicability of the method in more relevant cancer models. For this purpose, we performed CETSA experiments in two types of breast cancer xenograft tumours grown in mice where taxane treatment was applied in *ex vivo* or *in vivo* setting. TE on β-tubulin was detected successfully for docetaxel at several doses some of which similar to the ones clinically administered to patients.

Monitoring drug resistance at TE level in PDX models

Patient-derived xenograft (PDX) models represent a valuable clinically relevant approach to anticipating drug efficacy. We wanted to investigate whether monitoring TE with CETSA when treatment with microtubule-binding drugs is performed *ex vivo* could reveal the presence of resistance mechanisms in this setting.

For this purpose, we first studied the effect of several microtubule-binding drugs in cell lines established from prostate cancer PDX models and saw that a difference between the apparent affinities of the drugs could be recorded in these cell lines. Our data also indicated that a resistance mechanism that involves TE could successfully be monitored in this model. Next, we performed comparable experiments on PDX tumour slices treated *ex vivo* with the taxanes docetaxel or cabazitaxel and observed that even in this model differences in TE could be monitored with CETSA between sensitive and resistant setting. Together, these data showed that taxane resistance mechanisms that affect TE could indeed be tracked using CETSA in complex samples originating from clinically relevant cancer models.

3.3.3 The applicability of CETSA for β-tubulin TE in patient samples

We next aimed at evaluating CETSA for β-tubulin and monitoring taxane TE in breast cancer samples obtained from patients. CETSA analyses for β-tubulin were therefore performed on FNAs collected from surgically removed primary breast cancer tumours as well as on FNAs collected directly from the primary tumours of breast cancer patients. A dose-dependent stabilization was observed for β-tubulin as a response to docetaxel in the majority of the samples analysed and clear differences were observable at TE level between patients as the EC_{50} values differed radically (Fig. 3.3.3).

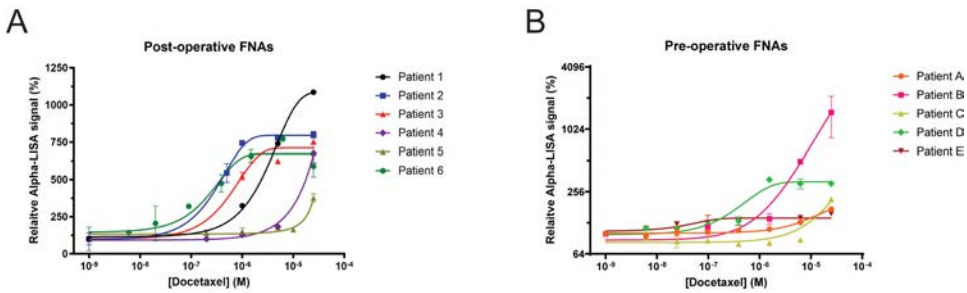

Figure 3.3.3. ITDR-CETSA for β-tubulin in FNAs collected from surgically removed tumours (A) or directly from patients (B) following *ex vivo* treatment with docetaxel. The data represent the mean ± S.E.M from technical replicates.

3.3.4 Discussion

The ultimate goals in this work were to establish a sensitive CETSA assay for β-tubulin that would be applicable for investigating the effects of microtubule-binding drugs at TE level and to evaluate whether the obtained responses can be used as candidate biomarkers for treatment efficacy in patient material.

First, we monitored the melting and stabilization behaviour of β-tubulin with different CETSA formats and confirmed that valuable data can be obtained regarding TE for different microtubule-binding drugs after very short incubation times, when cellular processes e.g. gene expression have likely not yet been altered.

We then established an AlphaLISA-based readout method for detecting β-tubulin in CETSA samples that showed increased sensitivity compared to WB. The applicability of this readout method for CETSA was evaluated for monitoring drug binding at TE level in cancer models where resistance to different classes of microtubule-binding drugs was present.

We demonstrate that resistance at TE level could be tracked with CETSA for β-tubulin and could be correlated to drug efficacy in the studied models. Moreover, we show that CETSA studies could be utilized to study the resistance mechanism present. When several studies report taxane resistance mechanisms to include cellular strategies that affect the effective intracellular concentration of the drug, it was most informative to see how this was reflected in the CETSA data where a P-gp efflux pump inhibitor was utilized in cell lines. Still, for other drugs resistance would not necessarily be evident at TE level. In such situations MS-CETSA can be employed for monitoring the downstream modulations of protein interaction states.

Evaluating CETSA for β-tubulin in patient samples yielded valuable insights in the form of considerable differences observed at TE level between patients. These are indications that could ultimately translate in differences in drug efficacy but correlating CETSA data with clinical treatment outcome was outside of the scope of this work.

While performing CETSA in patient samples it is important to note the challenges encountered when working with this type of material. The most significant obstacle was acquiring a sufficient quantity of viable cells, which posed a limitation for our experimental objectives. Many of the received samples unfortunately displayed low cell viabilities independent of cell amount. Given that performing CETSA analyses requires living cells, our experiments were only executable on a relatively small proportion of the samples and in most cases the limited amount of material hindered us from performing the full panel of planned treatments.

3.4 PAPER IV (Manuscript)

Proteome-wide CETSA reveals diverse apoptosis inducing mechanisms converging on an initial apoptosis effector stage focused at the nuclear matrix proximal region.

The first aim of this work was to obtain a better understanding of apoptosis-related cellular processes by systematically evaluating the proteome-wide CETSA responses arising after treatment with drugs designed to induce apoptosis either via the intrinsic pathway (venetoclax) or extrinsic pathway (AT-IAP in a TNFα background). We also employed IMPRINTS-CETSA to investigate the MoA of the APR-243 metabolite MQ, PI3K inhibitors buparlisib and alpelisib, and taxanes docetaxel and paclitaxel. These are cancer drugs expected to induce more complex cellular responses leading to cell death, where the insights gained in the first part of the study would prove useful.

My role in this project was focused on the taxanes docetaxel and paclitaxel for which I have implemented IMPRINTS-CETSA with the aim of gaining a better understanding of their MoA in breast cancer cells.

3.4.1 CETSA responses as a result of intrinsic apoptosis

In order to better understand the protein-related effects occurring when the intrinsic apoptotic pathway is induced, we performed IMPRINTS-CETSA and quantitative proteomics (QP) for the BCL2 inhibitor venetoclax at two different time points.

The early CETSA responses to venetoclax mainly consisted of an ensemble of proteins involved in nuclear processes out of which several were targets of effector caspases e.g. PARP1, MATR3 and nuclear lamins LMNB1 and LMNB2. At the later time point this effect was more prominent with many additional proteins involved in nuclear processes exhibiting CETSA shifts (Fig. 3.4.1 A). Follow-up experiments supported that the responses seen for these nuclear proteins were indeed dependent on apoptosis being triggered.

The collected QP data was quite devoid of responses compared to the 37°C CETSA data where many proteins showed a change that likely reflected on the relocalisation of proteins between cellular compartments or membranes. As described in section 1.9, we refer to this phenomenon as the differential extraction accessibility by relocalisation (DEAR) effect. Since many of the proteins displaying

DEAR effect were involved in nuclear processes, and out of which some were caspase targets e.g. LMNB1, LMNB2 (Fig. 3.4.1 A), we hypothesized that a protein reorganization event may be occurring in the proximity of the nuclear membrane following the induction of apoptosis, with the potential release of proteins or caspase-generated fragments of these proteins.

We also noted that for some caspase targets, the displayed CETSA shifts were being produced only by portions of the protein, deduced from analysing the abundance of the different detected peptides for each protein (Fig. 3.4.1 B). An example can be given for PARP1, for which the stabilized peptides originate predominantly from the DNA-binding region of the protein located at the N-terminus. We refer to this phenomenon as the regional stabilization after proteolysis (RESP) effect and we noted that these regional effects are consistent with known caspase cleavage sites.

3.4.2 CETSA responses as a result of extrinsic apoptosis

Next, we performed IMPRINTS-CETSA for AT-IAP, an IAP antagonist that was shown to induce apoptosis via the extrinsic pathway.

Similar to what we previously noted with venetoclax, many of the proteins exhibiting responses were localized in the nucleus, nuclear lamina, and played roles in e.g. DNA repair (Fig. 3.4.1 A). Again, several of these proteins were caspase targets and displayed RESP effects in accordance with known caspase cleavage sites. Considering the notable similarity in CETSA response between AT-IAP and venetoclax when it came to proteins involved in processes taking place at or in the proximity of the nuclear matrix, we defined the common protein hits as the core CETSA apoptosis ensemble (CCAE). We propose that the responses in CCAE proteins reflect a considerable overlap existing between the biochemical events of the early phases of the intrinsic and extrinsic apoptosis pathways.

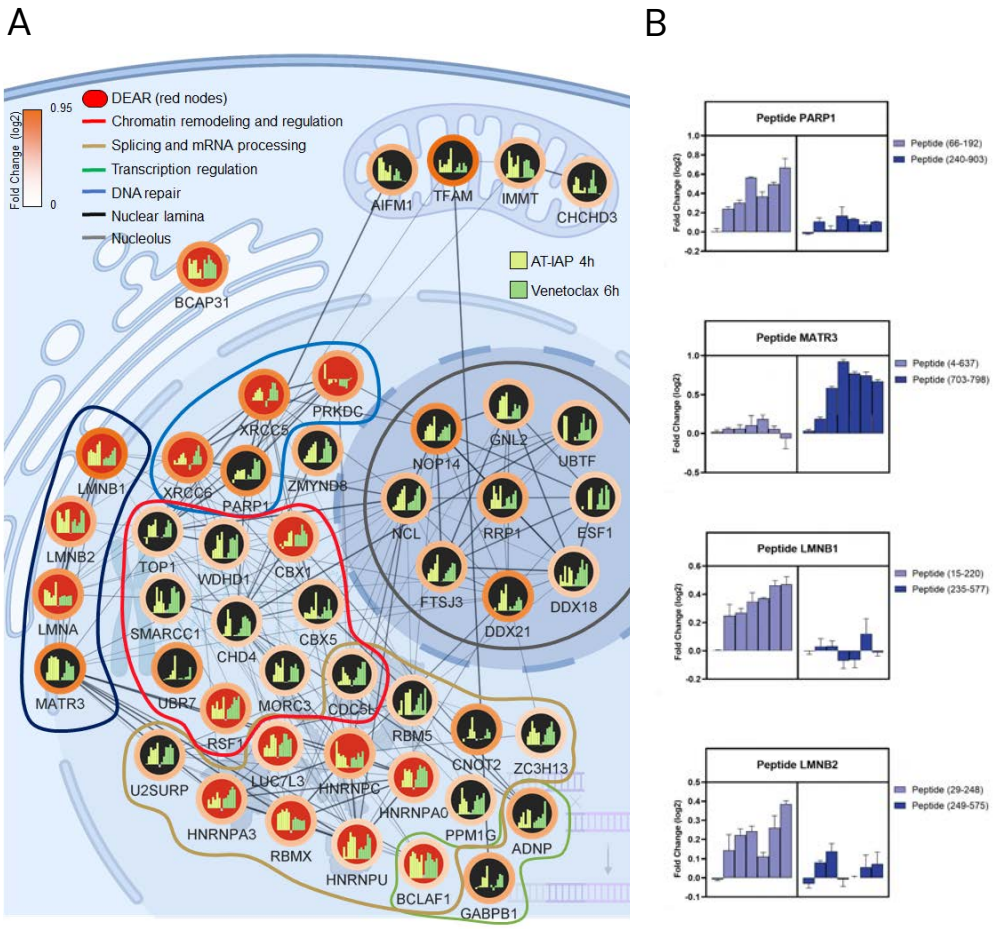

Figure 3.4.1. A. The core CETSA apoptosis ensemble (CCAE). B. Examples of RESP effects in proteins responding to venetoclax. Known caspase cleavage sites are found in between shifting and non-shifting regions. Data are presented as $\log_2$ fold change compared to vehicle ±SEM (n=3).

3.4.3 The CETSA responses to additional apoptosis-inducing drugs

The APR-246 metabolite MQ

Next in the study, we collected IMPRINTS-CETSA data for MQ in ovarian cancer OVCAR-3 cells. MQ is a metabolite of APR-246 that induces cell death via a MoA that includes the targeting of mutant p53 leading to its reactivation and the induction of oxidative stress by binding cellular GSH and inhibiting proteins involved in reactive oxygen species (ROS) control[152].

The CETSA data following MQ treatment revealed a subset of early responses (2h) in pathways that might be involved in inducing apoptosis, including redox and translational modulations. At the later timepoint (6h), response similarities to

venetoclax and AT-IAP were seen, represented by a large number of proteins associated with the nuclear matrix proximal regions, many of these being part of the CCAE. Interestingly, the RESP effects observed for venetoclax and AT-IAP were mostly absent for MQ indicating that different caspase activation profiles may be at work in this case. Several other insights on the MQ MoA were conveyed by responses in proteins involved in modulating GSH pools or translation initiation.

Notably, no effect was observed for p53 nor an induction of proteins under the transcriptional control of p53 is seen up to 6 h, suggesting that the apoptosis-related effects reflected by the CCAE responses are p53 independent during the time window of the experiment. However, effects downstream of p53 activation might instead appear at later time points.

PI3K inhibitors buparlisib and alpelisib

We also collected IMPRINTS-CETSA and QP data for MCF-7 cells treated with PI3K inhibitors alpelisib or buparlisib. CETSA responses were observed for several proteins that are core components of PI3K signalling, while some responding proteins have not previously been reported to play roles in this pathway. Buparlisib showed a bigger overlap with the CCAE than alpelisib, but overall both drugs seemed to lack the extensive CCAE responses seen in the previously discussed drugs, indicating a possible slower induction of apoptosis. Furthermore, apart from PARP1, no other caspase targets exhibited RESP effects. Instead, a cluster of proteins involved in cell cycle control displayed CETSA responses, which was more indicative of cell cycle arrest. Other responses were seen for proteins involved in mitochondrial respiration, out of which some displayed both thermal stability changes and DEAR effects, indicative of a drug-induced reorganization of proteins. Autophagy is expected to be regulated by the PI3K-pathway but only alpelisib affected the stability of proteins involved in autophagy – we show that this is likely due to buparlisib inhibiting PIK3C3 (VPS34), a key kinase in autophagy activation.

Taxanes docetaxel and paclitaxel

IMPRINTS-CETSA was employed for exploring the elusive non-mitotic aspects of taxane MoA (described in section 1.3) in MCF-7 breast cancer cells.

Within the experimental time frame, docetaxel and paclitaxel produced CETSA shifts for several different tubulins as well as for proteins involved in microtubule

assembly and dynamics. A clear indication that cell killing signals have been initiated in the cells were the CCAE proteins responding already at 1 h treatment with paclitaxel, while the RESP analysis of the caspase targets among them intriguingly did not support cleavage, but instead suggested that these proteins remain intact. In conjunction, these observations may reflect on a non-mitotic cell death mechanism being triggered and propagated via microtubules and microtubule-interacting proteins with effects on the CCAE that are not dependent on caspase cleavage within the studied time frame.

Furthermore, both taxanes induced responses in additional cellular processes. Notably, the most prominent responses were observed in protein folding complexes in the ER, out of which many are known to be sensitive to changes in Ca^{2+} levels and in some cases directly bind Ca^{2+} [153]. One example was calreticulin (CALR), for which we showed that the observed CETSA stabilization is associated with Ca^{2+} level increases in the ER while not dependent on caspase activation. Additional follow-up experiments revealed that paclitaxel triggers apoptosis at later time points (15 h), a response that was more pronounced when Ca^{2+} level increases were also present. This observation could signify the induction of the mitotic taxane MoA that occurs at later time points.

3.4.4 Discussion

Several interesting insights resulted from this work. Firstly, the discovery of an ensemble of proteins exhibiting CETSA responses to both venetoclax and AT-IAP, an observation that indicated the presence of a shared initial mechanism or consequence of initializing apoptosis, common to both the intrinsic and extrinsic pathways. We refer to these common CETSA-responding proteins as the core CETSA apoptosis ensemble (CCAE), which constitutes an event trackable with CETSA in the early stages of drug action that becomes more advanced at later exposure times. The CCAE consists largely of proteins involved in nuclear processes out of which several are targets of effector caspases e.g. PARP1, MATR3, and nuclear lamins LMNB1 and LMNB2.

The DEAR effect was another important insight explored in this study. As described before in section 1.9, the differences seen between the 37°C CETSA and QP data could be reflecting the relocalisation of proteins between cellular compartments or membranes. Based on the DEAR effects observed in the venetoclax and AT-IAP data, we hypothesized that an apoptosis-induced protein

reorganization event may occur in the proximity of the nuclear membrane detectable with CETSA already at early phases of apoptosis induction.

Another notable aspect highlighted in this work was the RESP effect observed for known caspase targets at known caspase cleavage sites. Monitoring different regions of the protein at the peptide level, allowed for the direct assignment of caspase cleavage events in intact cells. The number of target proteins cleaved were different between the studied drugs. For venetoclax and AT-IAP, a collection of several caspase targets were cleaved, while in the case of the taxanes, the same caspase targets appeared to be intact. This suggests that the induction of the early CCAE response by taxanes is not brought on by the activation of effector caspases in the nucleus but through alternative mechanisms.

Overall, the RESP effect contributes important information and represents a complementary dimension to the data obtained with CETSA at the protein level, as it allows the tracking over time of additional MoA particularities. The RESP effect constitutes the first method for monitoring protein cleaving events directly in intact cells, where the risk of erroneous results resulting from cell lysis or enrichments steps often employed by other methods are minimized. However, the proportion of caspase targets that exhibit RESP effect upon cleavage remains to be further investigated.

Several additional interesting insights emerged when we expanded our IMPRINTS-CETSA studies to other drugs. For example, in the case of the microtubule-stabilizing taxanes, apart from a collection of CETSA-responses involving tubulins and tubulin-associated proteins, we also observed changes in PRINTS indicative of a Ca^{2+} increase in the ER following paclitaxel treatment, an event not previously reported as a component of taxane MoA.

Collectively, the CETSA responses for all drugs evaluated in our study revealed valuable information regarding their MoA and many of the proteins or ensembles of proteins responding could now be evaluated as candidate biomarkers for monitoring the initiation of apoptosis and consequently predicting drug efficacy in multiple cancers with potential applicability in the clinic.

4 CONCLUSIONS AND PERSPECTIVES

Several formats of CETSA were employed in the work presented in this thesis with the aim of further exploring the MoA of different cancer drugs in both sensitive and resistant contexts in the hope of identifying proteins that could potentially function as biomarkers for predicting drug efficacy.

This strategy allowed different aspects of drug MoA to be tracked simultaneously in living cells, revealing thermal stability changes for multiple proteins, some already linked to the MoA while some either previously uncharted or exhibiting unexpected yet informative responses. Different ensembles of proteins showed potential in functioning as reporters on the occurrence of certain drug-induced cellular processes e.g. the CCAE. Novel approaches to analysing CETSA data were also introduced, best represented by the analysis of the RESP effect, which allows protein cleaving events to be monitored directly in intact cells and providing yet another complementary perspective over cellular states and processes. These considerations and insights will become an integral part of future CETSA studies performed in our research group.

Our work revealed important information regarding drug MoA, best exemplified by the identification of RNA-related toxicities as a core component of 5-FU efficacy. As emerging studies support and expand upon these findings, a potential appears for a paradigm shift in the way we regard and approach the 5-FU MoA. Generating this type of data on MoA and resistance mechanisms for multiple drugs might also assist in developing strategies for how to more rationally combine drugs that differ in their MoAs and mechanisms of resistance in order to optimize efficacy.

A number of proteins that show potential in becoming candidate biomarkers for drug efficacy were also highlighted and could be further explored for clinical application. Additional studies are required to fully assess the applicability of these candidate biomarkers in more complex cancer models as well as clinical samples. In the case of β-tubulin and taxanes, we took a first step into evaluating CETSA responses in clinical samples. The results were compelling and continuing this work would be of great interest and promise for the future. Subsequent investigation could similarly be conducted for other of the highlighted candidate biomarkers either by our research group or others.

CETSA proved to be a valuable method for dissecting the intricacies of drug action and contributed vast amounts of information from a protein-centric point of view that is complementary to other methods. As the MS-CETSA methodology becomes increasingly utilized and evolving alongside the available technologies for protein detection, future studies will likely address the MoA of numerous other drugs in different models and disease types. The first CETSA experiments to be detected with the Orbitrap-Astral mass spectrometer will likely be opening another level of possibilities when it comes to proteomic analysis depth, sensitivity, and throughput. Therefore, it is possible that future CETSA studies with MS readout would be applicable in clinical diagnostics and contribute to therapy selection in patients with the hopes of a more positive outcome in patient care.

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