

**ELUCIDATING THE MECHANISMS OF T-DM1
RESISTANCE IN *IN VITRO* MODELS OF HER2
OVEREXPRESSING BREAST CANCER**

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We certify that we have read this dissertation and that in our opinion it is fully adequate in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

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M.S. in Molecular Biology and Genetics

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Despite the presence of plethora of anti-cancer therapeutics with a variety of different mechanisms of action, it is still not possible to completely eradicate cancer due largely to the occurrence of refractory tumors even years after completion of the treatment. Such “resistant” tumors are formed over time as few cells, which have gained some advantageous genomic alterations eventually populate the entire organ. A lot of *in vitro* and *in vivo* studies are currently being done in order to identify the ways by which cancer cells become resistant to given therapy. This would decipher the weaknesses of the resistant tumors and would provide a means to combat drug resistance. T-DM1 is an anti-HER2 therapeutics, being used in refractory HER2-positive breast cancer patients since 2013. It initially generated a huge excitement owing to the highly favorable clinical findings; however, resistance was developed rapidly after 5-6 months following the initial treatment. Currently; very little is known about the mechanisms of acquired resistance against T-DM1, and therefore, identification of novel targets for the treatment of T-DM1 refractory patients would be highly beneficial.

In this thesis, I have developed and characterized the acquired T-DM1 resistance phenotypically, and demonstrated abrogation of drug induced mitotic arrest and apoptosis as two novel mechanisms of resistance. I have further analyzed the genomic landscape of resistance in terms of the enrichment of cancer related processes. Cell cycle was found to be the most significantly enriched process among genes deregulated in T-DM1 resistance as identified by next-generation RNA sequencing. Cell cycle was also shown to be activated in TCGA patients expressing high levels of the TDM1 resistance signature and further supported the importance of rewiring cell cycle for the acquisition of T-DM1 resistance in patients as well. I have further identified two important mitotic genes; PLK1 and TACC3 as the common mediators of resistance in different HER2-overexpressing models by a targeted siRNA screen. I have showed that their genomic or pharmacological inhibition confers sensitization to T-

DM1 induced growth inhibition, partially through re-induction of apoptotic cell death. I further uncovered a BCL2 dependency in T-DM1 resistant models which was also found to be associated with T-DM1 resistance as inhibition of Bcl2 enhanced T-DM1 induced growth inhibition. Since T-DM1 refractory HER2-positive breast cancer patients are currently not curable, these pre-clinical findings might guide the future clinical test to improve the survival of this patient subgroup *via* the usage of PLK1 or BCL2 inhibitors in combination with T-DM1.

Keyword: T-DM1, drug resistance, HER2-positive breast cancer, mitotic progression, apoptosis, small molecule inhibitors



ÖZET

HER2-POZİTİF MEME KANSERİ HÜCRE MODELLERİNDE T-DM1 DİRENCİNİN MEKANİZMALARININ AÇIĞA ÇIKARILMASI

Özge Saatci

Moleküler Biyoloji ve Genetik, Yüksek Lisans

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Farklı mekanizmalara sahip pek çok kanser ilacının klinikte kullanılıyor olmasına rağmen, günümüzde, halen daha, kanserin tamamen tedavi edilebilmesi çoğu zaman mümkün olmamaktadır. Bunun sebebi, çoğu durumda, hastalığın tedavisinden yıllar sonra bile ortaya çıkabilen, “dirençli” tümörlerin varlığıdır. Bu dirençli tümörler, kanser ilaçlarına maruz kalan kanser hücrelerinin, zaman içerisinde kazandıkları birtakım genetik değişiklikler sebebiyle ortaya çıkmaktadır. Bu değişiklikler, hücrelere ilacın varlığında büyüme avantajı sağlamakta ve zamanla bütün bir organa yayılmalarına sebep olmaktadır. Günümüzde, kanser hücrelerinin bu özellikleri nasıl kazandığına dair pek çok *in vitro* ve *in vivo* çalışma yürütülmektedir. Bu sayede, kanser hücrelerinin zayıf noktaları belirlenebilmekte ve bu da ilaç direncini yenmede yararlı olmaktadır. T-DM1, ilaç dirençli, agresif hastalık seyri gösteren HER2-pozitif meme kanseri hastalarında, 2013 yılından beri kullanılmakta olan bir kanser ilacıdır. T-DM1, ilk olarak test edilemeye başlandığında, elde edilen ümit verici klinik sonuçlar, büyük heyecana yol açsa da, hastaların büyük çoğunluğunda, tedaviyi takiben 5-6 ay içerisinde ortaya çıkan direnç hayal kırıklığı yaşatmıştır. Şu anda, T-DM1’e karşı gelişen direncin mekanizmaları hakkında çok az şey bilinmekte olup, T-DM1 dirençli hastaların tedavisinde kullanılabilecek özgün ilaç hedeflerinin ortaya çıkarılması son derece yararlı olacaktır.

Bu tezde, T-DM1 dirençli hücre modelleri geliştirildi ve bu modellerde ilacın yol açtığı mitozda durdurum (mitotic arrest) ve apoptozun engellenmesi, T-DM1 direncinin özgün mekanizmaları olarak belirlendi. Fenotip karakterizasyonunu takiben, RNA sekanslama teknolojisi ile yapılan genomik karakterizasyon, direnç oluşumunda ekspresyon seviyesi değişimi gösteren genleri açığa çıkardı ve bu genlerde zenginleşen kanser ilintili hücresel olaylar belirlendi. Hücre döngüsü, T-DM1 direncinde rol alan hücresel olaylar arasında istatistiksel anlamlılığı en yüksek olan proses olarak belirlendi. Aynı zamanda, TCGA hastalarında da, T-DM1 direnç puanı yüksek olan hastaların, daha aktif hücre döngüsü

gerçekleştirdikleri gösterildi. Sonrasında yapılan hedefli siRNA taraması sonucu, mitozda önemli göreve sahip iki genin; PLK1 ve TACC3, HER2-pozitif T-DM1 dirençli hücre modellerinde ortak olarak ilaç direncine sebep oldukları gösterildi. Bu iki genin, genomik ve farmakolojik inhibisyonunun, T-DM1'in ilaca duyarlı hücrelerde gerçekleştirdiği proliferasyon azalımını, kısmen apoptozu indükleyerek geri kazandırdığı gösterildi. Son olarak, T-DM1 dirençli modellerin, BCL2 ekspresyonuna daha bağımlı hale geldikleri, ve buna bağlı olarak BCL2 inhibisyonunun T-DM1'e dirençli hücrelerin direncini kırdığı gösterildi. T-DM1 dirençli HER2-pozitif meme kanseri hastaları için şu an herhangi bir tedavi seçeneği bulunmadığı göz önüne alındığında, bu tezde elde edilen klinik öncesi bulgular, ilerde yapılabilecek klinik testlerde PLK1 ve BCL2 inhibitörlerinin T-DM1 ile birleştirilmesinin önünü açacak ve bu hasta grubunun hayatta kalım süresinin arttırılması açısından son derece yararlı olacaktır.

Anahtar Sözcükler: T-DM1, ilaç direnci, HER2-pozitif meme kanseri, mitotik ilerleme

I dedicate this work to my family who showed an endless
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Abbreviations

7-AAD	7-Aminoactinomycin D
ABL1	ABL Proto-Oncogene 1, Non-Receptor Tyrosine Kinase
ADC	Antibody Drug Conjugate
ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute Myeloid Leukemia
APC	Anaphase Promoting Complex
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
BCL2	B-Cell CLL/Lymphoma 2, including
BCR	BCR, RhoGEF and GTPase activating protein
BSA	Bovine Serum Albumin
CDK1	Cyclin-dependent kinase 1
CML	Chronic Myeloid Leukemia
CPC	Chromosome Passenger Complex
DKFZ	The German Cancer Research Center
DMEM	Dulbecco's Modified Eagle Medium
dNTP	desoxynucleotide triphosphate
ER	Estrogen receptor
FBS	Fetal bovine serum
FDA	Food and Drug Administration
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 2
HRP	Horseradish peroxidase
JNK	c-Jun NH ₂ -terminal kinase

kDA	Kilo Dalton
p21	Cyclin-dependent kinase inhibitor 1A (CDKN1A)
p27	Cyclin-dependent kinase inhibitor 1B (CDKN1B)
P/S	Penicillin/Streptomycin
PARP	Poly [ADP-ribose] polymerase 1
PBD	Polo-box Domain
PBS	Phosphate buffered saline
PLK1	Polo-like Kinase 1
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PP2A	Protein phosphatase 2
qRT-PCR	Quantitative real time polymerase chain reaction
RPPA	Reverse phase protein array
SAC	Spindle Assembly Checkpoint
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
SRC	SRC proto-oncogene, non-receptor tyrosine kinase
TACC3	Transforming Acidic Coiled-Coil Containing 3
TBST	Tris buffer saline Tween20
TCGA	The Cancer Genome Atlas
TCPA	The Cancer Proteome Atlas
T-DM1	Trastuzumab Emtansine
TDM1R	T-DM1 Resistant
TNBC	Triple Negative Breast Cancer

CHAPTER 1

Introduction

1.1. Breast cancer

Although better diagnostic and therapeutic strategies implemented into clinics in the last few years have greatly reduced cancer incidence rates, cancer is still one of the leading causes of death worldwide with 1,685,210 estimated new cases in United States (U.S.) in 2016. Breast cancer is the most common cancer and the second most leading cause of death from cancer in women, and is expected to cause death of about 40,450 women in U.S. in 2015 [1]. The disease is the most commonly seen cancer type in women in Turkey as well with 17,531 new cases diagnosed in 2016 [2]. It is clinically classified into three subtypes; Luminal, HER2-positive and Triple Negative Breast Cancer (TNBC) based on overexpression of hormone receptors and specific receptor tyrosine kinases [3]. At the molecular level, there are 5 major subclasses which largely overlap with the clinical subtypes (Figure 1.1) [4]. Luminal breast cancers are further divided into two subclasses; Luminal A, which overexpress estrogen (ER) or progesterone (PR) hormone receptors and Luminal B which overexpress both ER/PR and human epidermal growth factor receptor 2 (HER2). According to the subtype of the disease, there are several treatment regimens recommended to be used in breast cancer patients [5]. Luminal and HER2-positive breast cancers can benefit from targeted therapies due to their overexpression of ER and HER2 receptors unlike TNBC that lacks the amplification of these druggable receptors [6, 7].

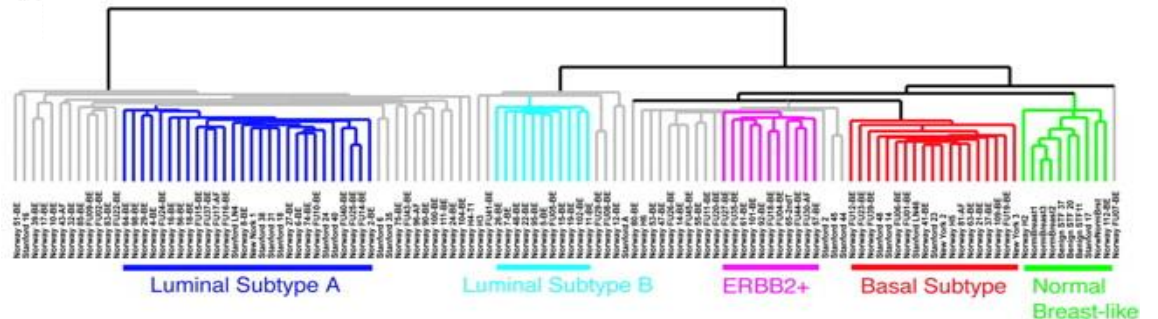


Figure 1.1: Molecular classification of breast cancer according to gene expression profiling [4]. See Appendix for the copyright permission.

1.1.1. HER2-positive breast cancer

HER2-positive breast cancers bear an amplification at the HER2 gene and they comprise of about 25% of all breast cancers. They are associated with higher rates of recurrence as compared to HER2-negative tumors. Young women have more HER2-positive type of breast cancer which usually has higher nuclear grade and shows an aggressive disease progression [8]. Tumor aggressiveness in HER2-positive breast cancer is comparable to TNBC despite the available targeted therapy options in case of HER2 overexpression. In addition, five years survival rates for HER2-positive and Luminal B subtypes of breast cancer were shown to be lower than for TNBC supporting the importance of treating HER2-overexpressing breast cancer [9].

Overexpression of HER2 cell surface receptor makes HER2-positive tumors prone to inhibition of HER2 signaling as they become dependent on HER2 activation for their survival. A variety of agents targeting different elements in the HER2 signaling have been developed so far as depicted in Figure 1.2 [10-19].

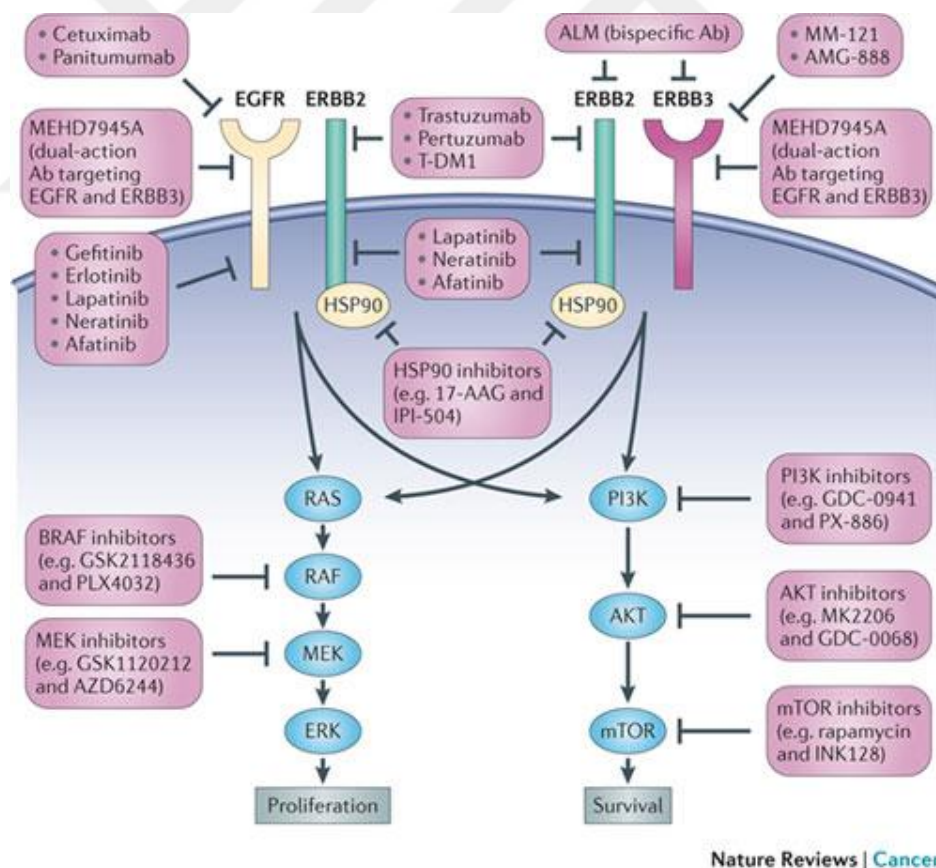


Figure 1.2: Different types of agents used in clinics against HER2 signaling [20]. See Appendix for the copyright permission

1.1.2. Treatment of HER2-positive Breast Cancer

1.1.2.1 Targeted Therapies

Trastuzumab is one of the first targeted therapy agents that received FDA approval in 1998 and since then has been the mainstay therapy option for the treatment of HER2-positive breast cancers. It is a monoclonal antibody targeting the extracellular domain of HER2 which is amplified in nearly 25% of the breast cancers. Trastuzumab binding to HER2 receptor interferes with the downstream signaling by causing the internalization and subsequent degradation of the receptor. At the same time, it inhibits dimerization of the receptor with other ErbB family members which generally tend to dimerize with HER2. Blockage of the ErbB signaling by trastuzumab results in G1 arrest characterized by an increase in G1 arrest markers such as p27 [21]. Besides the intracellular re-shaping of cancer cells, antibody-dependent cell cytotoxicity (ADCC) is another mechanism with which trastuzumab mediates cell killing by recruiting immune cells that recognize the constant region of the trastuzumab antibody [22, 23].

1.1.2.2. Chemotherapy

Trastuzumab is the main therapy option for the treatment of both early and late stage disease; however, if the tumor size is big, it is often given along with chemotherapy as a neoadjuvant treatment. Patients may also be treated with adjuvant chemotherapy in order to reduce the risk of recurrence. Main types of chemotherapy agents used in HER2-positive breast cancer are anthracyclines and taxanes. Anthracyclines are generally not given together with Trastuzumab since they may exacerbate the cardiac toxicity of Trastuzumab [24]. Among taxanes, Paclitaxel (Taxol) and Docetaxol are the two widely used chemotherapy agents that interfere with microtubule dynamics by stabilizing microtubules and simultaneously inhibiting their disassembly [25]. They prevent proper mitotic progression by inhibiting the anaphase promoting complex (APC) and causing arrest at metaphase [26]. Cell fate in response to a mitotic arrest may either be apoptosis following the activation of spindle assembly checkpoint (SAC) or mitotic slippage with incomplete mitosis and formation of a G1-like tetraploid cell state which results in growth arrest or a p53-dependent apoptosis [27].

1.2. Drug Resistance

Despite the presence of plethora of different classes of anti-cancer agents causing shrinkage of tumors during the initial treatment, there are still a significant portion of cancer patients who experience re-growth of their pre-treated tumors or formation of cancerous lesions at a site distant from the primary tumor. Currently the major burden against an effective cancer treatment is the emergence of drug resistance. Long term exposure to anti-cancer agents is, most of the times, followed by occurrence of tumor cells that are no longer affected by the given therapy and results in formation of so-called “recurrent” tumors. How cancer cells manage to survive in the presence of anti-cancer treatments and then to re-dominate the organ they reside in or a distant site are still incompletely understood phenomena. Resistance may either be seen at the very beginning of the treatment which is called as “*de novo* resistance” or it may occur at a later stage following the initial treatment which may sometimes be years after completion of the therapy. This type of resistance is called “acquired resistance” and is mostly related with the genomic or transcriptomic changes in tumors following long-term exposure to drug unlike *de novo* resistance which is mediated by already present molecular signatures that interfere with the anti-cancer mechanisms of the given agent from the beginning.

1.2.1. Novel Strategies to Identify, Delay or Overcome Mechanisms of Drug Resistance in Cancer

Great efforts are being made to regain sensitivity against the given therapy so that survival of the patients shall be augmented. Identifying novel vulnerabilities that has formed as the cells become resistant to the given therapy has been proven to be a highly effective way of tackling with drug-resistant tumors. Significant amount of genomic and functional studies conducted in the last few years have demonstrated the power of “gene hunting” as a means to decipher the weaknesses of aggressive tumors and the recent advancements in genomic and transcriptomic sequencing technologies have greatly eased the detailed characterization of drug resistance. As a result, any change happened during the development of resistance in genomic, transcriptomic or proteomic level can now be detected and tested for its potential to functionally mediate the resistance. Then, it becomes the responsibility of the drug developers to find novel small molecules against the identified therapeutic targets that can be used in clinics with good bioavailability and less toxicity.

One of the most well-known examples of overcoming drug resistance is in the case of treatment of chronic myelogenous leukaemia (CML) which is a type of cancer associated with

a chromosomal abnormality. CML patients possess a translocation of the 3' sequence of a gene, ABL1 near to the 5' site of another gene, BCR in chromosome 22. This chimeric BCR-ABL sequence encodes a constitutively active form of a tyrosine kinase which creates the perfect circumstances for the development cancer [28]. Imatinib mesylate, also known as Gleevec was developed by Novartis and approved by FDA in 2001 to be used in CML patients. It is a potent and a highly specific competitive inhibitor of the BCR-ABL tyrosine kinase and it demonstrated a high response rate even in patients that showed resistance to previous therapy [29, 30]. However, drug response was not continuous, and it was followed by rapid development of resistance which was soon associated with point mutations in BCR-ABL that were disrupting imatinib binding and therefore conferring resistance [31, 32]. Soon after the emergence of resistance, drug companies developed second-line agents like nilotinib and dasatinib which bear a higher affinity against the BCR-ABL product and are able to inhibit the activity of 14 out of 15 imatinib resistant BCR-ABL mutants. Five years after the development of dasatinib, FDA gave approval for its clinical usage as a second-line therapy in imatinib-resistant CML patients [33].

Last few years have underpinned the advantages of applying combination therapies composed of two or even more drugs that act in synergy to enhance the anti-cancer effects of the primary therapy and delay the formation of resistance. Although it offers a great promise in terms of the better anti-cancer effect it mediates, it suffers from the accumulated toxicity as more drugs are given in combination [34]. Therefore, it needs more time for many proposed combinations to enter into clinics until the clinical trials with the individual and combination treatments yield favorable results for the combination therapies without much toxicity. Nevertheless, there are also combinations which were proven to synergize and mediate a better anti-cancer effect with reasonable safety profiles. Combination of paclitaxel or docetaxel with trastuzumab which are the mostly used anti-cancer treatment options in HER2-positive breast cancer is one of the few targeted and chemotherapy combinations being used in clinics due to the superior anti-cancer activity they confer over individual treatments [35]. In addition, HER2-positive tumors have been demonstrated to respond better to taxane regimens than HER2-negative tumors which further justifies the usage the combination therapies containing Trastuzumab and taxanes [36].

1.2.2. Tackling Drug Resistance in HER2-positive Breast Cancer

So far, many great efforts like identification of vulnerabilities in drug-resistant tumors or using combination therapies to augment drug response yielded highly promising results which most of the times translated into clinics to be used in patients with refractory disease in order to achieve higher efficacy and increased patient survival. In breast cancer, as well, mechanisms of drug resistance and ways to augment drug response has been immensely studied. Trastuzumab is one of the few drugs that has been characterized in detail in terms of its mechanism of action and the associated *de novo* or acquired resistance.

Plethora of mechanisms responsible for *de novo* or acquired resistance against trastuzumab have so far been identified. Among the many well-studied resistance mechanisms are cleavage of the extracellular domain of HER2, loss of PTEN tumor suppressor, overexpression of ErbB family receptors other than HER2, or activation of oncogenes like SRC which stimulate HER2 signaling pathway despite the presence of trastuzumab [37-40]. Resistance against trastuzumab has also been extensively studied [39-43]. Cleavage of the extracellular domain of the HER2 receptor is one of the mechanisms that trastuzumab treated cancer cells adapt in order to inhibit binding of the antibody to HER2 [44-46]. Cells may also overexpress other members of ErbB family such as HER3 or other tyrosine kinase receptors like IGF1R to promote the continuation of the downstream signaling [47, 48]. Identification of the proteins responsible from resistance has encouraged testing of combination therapies of trastuzumab with inhibitors against the activated proteins [41, 49, 50]. Sequential therapies have also shown to yield better outcome where the treatment was first initiated with trastuzumab and then as tumors started to develop resistance, followed by different types of agents specifically targeting the identified resistance against prior therapy [51].

1.3. Antibody Drug Conjugates (ADCs)

The superior efficacy observed with combination therapies paved the way for the development of antibody drug conjugates (ADCs) which are composed of an antibody molecule targeting a cancer marker and a cytotoxic drug linked through a stable linker. The advantage of ADCs over conventional targeted and chemotherapy agents is their ability to exert a powerful cytotoxic effect targeted specifically towards cancer cells. Their novelty comes from the fact that (1) unlike chemotherapeutic agents causing high toxicities by affecting normal cells along with cancer cells, they target only the tumors through the antibody-receptor interaction; (2) they can exert a more powerful anti-cancer effect than what targeted therapies can ever achieve. Their targeted nature largely depends on the stability of

the linker between the two components. The linker should only be cleaved inside the cellular environment, but not in circulation. Non-specific targeting of the antibody molecule is still an issue that is causing the observed toxicity with this type of agents; nevertheless, ADCs are still good options as anti-cancer agents which have less toxicity than chemotherapy and higher efficacy than targeted therapies [52-54].

The first FDA approved ADC was Mylotarg (gemtuzumab ozogamicin) which is a conjugate of an antibody targeting CD33 expressed in leukemic cells and a cytotoxic agent from the class of calicheamicins [55, 56]. It was approved in 2000 and stayed in market for 10 years until it was withdrawn in June 2010 due to a clinical trial showing increased patient death with no improvement in response [57].

The breakthrough in the field of ADCs was seen in 2011 when FDA approved brentuximab vedotin which is a conjugate of the monoclonal antibody Brentuximab and the anti-mitotic agent monomethyl auristatin E (MMAE). It was approved to be used in refractory Hodgkin's lymphoma and systemic anaplastic large cell lymphoma patients. The antibody, brentuximab targets CD30 protein which is overexpressed in these two types of lymphoma [58].

The next ADC approved by FDA was T-DM1 (Kadcyla) which is currently being used in metastatic breast cancer patients refractory to trastuzumab and taxane therapies. T-DM1 is a conjugate of Trastuzumab and DM1 which is a maytansine derivative that is disrupting microtubule dynamics similar to taxanes. It depolymerizes microtubules whereas taxanes enhance microtubule polymerization. The two molecules are linked with a non-cleavable thioether linker which demonstrates the highest degree of stability among different types of linkers tested. The *in vitro* and *in vivo* efficacy of T-DM1 have been studied in detail by Lewis et al. from Genentech in 2008 [59]. In this study, they showed that even trastuzumab resistant cell lines or tumors showed good response to T-DM1. Supporting these *in vitro* and *in vivo* results, a clinical trial conducted in 2012 demonstrated a superior effect of T-DM1 over lapatinib and capecitabine combination in patients pretreated with trastuzumab and taxane therapies [60]. These promising results led to the FDA approval of T-DM1 in 2013 to be used in metastatic HER2-positive breast cancer patients refractory to trastuzumab and taxane therapies.

1.3.1. Proposed Mechanism of Action of T-DM1

By being a bi-functional compound, T-DM1 exerts both trastuzumab and DM1 mediated anti-cancer effects. It was demonstrated to retain all the mechanisms of action of unconjugated trastuzumab including blockage of PI3K pathway activity and mediating ADCC [61]. In addition to fully exert all the cytostatic effects of trastuzumab, it is also able to mediate an efficient cell killing owing to the DM1 part of the conjugate. DM1 is a maytansine derivative which has a high affinity against the vinca domain of tubulin molecule and upon binding, it stimulates microtubule depolymerization and causes arrest at mitosis similar to vinca alkaloids (Figure 1.3) [62, 63]. T-DM1 has been shown to cause a G2/M arrest and apoptotic cell death in a few HER2-amplified *in vitro* and *in vivo* models [59, 64].

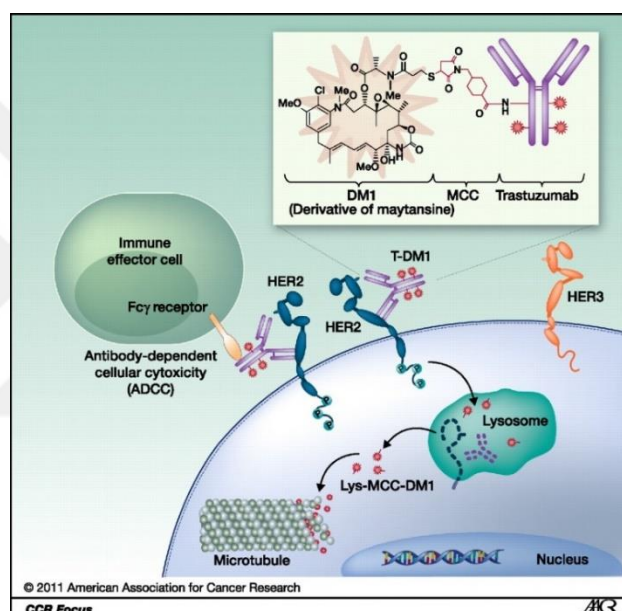


Figure 1.3: The mechanism of action of T-DM1. Trastuzumab and DM1 are linked with a non-cleavable thioether linker, MCC. Upon internalization, the ADC undergoes lysosomal degradation and the linker-containing catabolite, Lys-MCC-DM1 acts on microtubules [65]. See Appendix for the copyright permission.

1.3.2. Proposed Resistance Mechanisms against T-DM1

As for all anti-cancer agents, drug resistance represents a major obstacle against complete cancer eradication with T-DM1 as well. Although the initial clinical trials demonstrated a better outcome with T-DM1, improvement in survival was limited to 5-6 months after which patients experience disease recurrence. Due to the fact that it is a relatively new agent recently

approved by FDA and is being used in clinics for a few years, there are currently no *in vitro* or *in vivo* studies on the identification of resistance mechanisms against T-DM1. This attractive ADC was first marketed in 2013 as a highly promising second-line therapy option for the treatment of trastuzumab refractory HER2-positive breast cancer patients owing to the encouraging preclinical and clinical findings. Observation of acquired resistance against T-DM1 was devastating and somewhat shadowed the initial excitement in the field. For this reason, identification of resistance mechanisms as well as druggable targets that are, in part, responsible for the resistance is highly necessary for the continuation of an effective treatment with this promising ADC in clinics.

Proposing combination therapies containing T-DM1 and a secondary drug targeting the identified resistance mechanism and thereby causing re-gain of response in T-DM1 refractory patients would highly be beneficial in order to prolong patient survival. A combination therapy of T-DM1 and pertuzumab, which is an antibody against HER3 has already been shown to augment the anti-cancer effects of T-DM1 by causing a synergistic cell proliferation inhibition. In this study they also showed that treating HER2-positive breast cancer cells with a HER3 ligand, Heregulin reduces the cytotoxic activity of T-DM1 [66]. Currently, there are a few proposed mechanisms of T-DM1 resistance like low tumor HER2 overexpression, poor internalization of T-DM1-HER2 complex, defects in lysosomal degradation of T-DM1, overexpression of drug efflux pumps or alterations in tubulin which inhibits T-DM1 binding [67]. All these proposed mechanisms have not yet been supported by any *in vitro* or *in vivo* functional study, and they were suggested due to their previous association with resistance to either trastuzumab or anti-mitotic agents like DM1. The exact mechanisms of T-DM1 resistance is currently unknown and there is an urgent need to identify the actual causes of resistance so that the usage of this promising ADC can be prolonged.

Among the proposed mechanisms of T-DM1 resistance, low HER2 expression is unlikely to cause resistance as a clinical study showed that T-DM1 refractory patients are still able to respond to other HER2 targeting agents [68]. PI3K mutation status has also been demonstrated to be unrelated with T-DM1 response unlike what is seen in case of trastuzumab resistance [69]. All these are suggesting that resistance against T-DM1 is more likely to be associated with the activity of the DM1 part of the drug and that cells are trying to evade from the death inducing effects of the more toxic component of the ADC by fine-tuning the levels of mitotic and apoptotic regulators instead of protecting themselves from the relatively less detrimental cytostatic effects of the antibody molecule.

1.4. Enhanced Mitotic Progression as a Resistance Mechanism against Anti-mitotic Agents

Mitotic regulation has been previously associated with resistance against anti-mitotic agents such as paclitaxel or docetaxel. Overexpression of several mitotic genes important for progression through mitosis in the presence of a stress signal has previously been demonstrated to confer a prominent advantage to the cells by causing mitotic slippage or inhibition of apoptosis induced after prolonged mitotic arrest [70-73]. Cells may regulate the expression of several key molecules functioning in different stages of mitosis in order to evade from the mitotic arrest induced by microtubule disrupting agent [74].

1.4.1. Polo-like Kinases in Normal Mitotic Progression and Resistance against Anti-mitotic Agents

Protein phosphorylation has a huge importance in spindle functioning and proper chromosome segregation making protein kinases and phosphatases highly important for the execution of a faithful mitosis. Polo-like kinase family proteins are kinases that have many independent roles in cell cycle regulation [75, 76]. PLK1 is the most ancestral member among the four polo-like kinase family members, and it is the well-studied one. Lots of different functions have been attributed to PLK1 so far, and it was shown to be important in nearly every phase of the cell cycle. During mitosis, it controls both the entry to and exit from mitosis by activating CDK1/Cyclin B1 complex and APC, respectively. During interphase it resides on centrosomes and mediates their maturation and duplication. It can also be recruited to interphase and early mitosis centromeres and control proper segregation during mitosis [77, 78]. It facilitates SAC in the presence of microtubule inhibitory signals; however, it is not strictly required for SAC activation upon microtubule stress. Instead, it is of greater importance in case of the relief of the checkpoint once proper microtubule kinetochore attachments are achieved. This is mediated by the activation of CDC20 by PLK1 and the subsequent degradation of Cyclin B1 which leads to mitotic progression (Figure 1.4) [79].

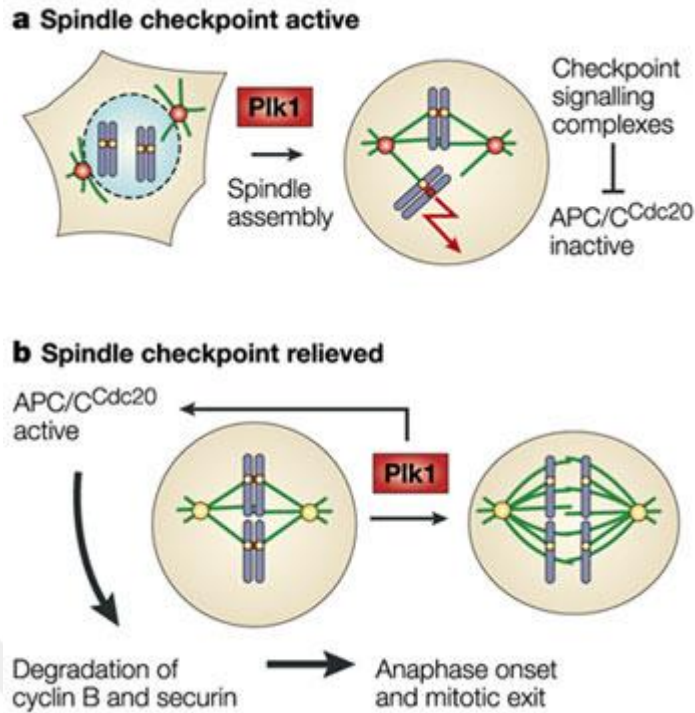


Figure 1.4: The dual-role of PLK1 in SAC activation upon microtubule stress and relief of the checkpoint response after formation of proper microtubule kinetochore attachments [79]. See Appendix for the copyright permission

Inhibition of PLK1 is characterized by large numbers of spindle defects, gain of a “Polo-like” shape and metaphase arrest. The occurrence of the monopolar spindles upon PLK1 inhibition is due to the inhibition of centrosome maturation and separation. Although PLK1 has diverse roles in cell cycle, the phenotype associated with PLK1 knockdown is suggesting the centrosomal protection as the most important function of PLK1. This was also supported by studies showing that the role of PLK1 in mitotic entry during an unperturbed state is quite limited as compared to its functioning as a spindle regulatory protein.

Owing to its active involvement in undertaking a proper mitotic division, it is not surprising that PLK1 is upregulated in different cancer models [80-83]. It is a biomarker of cancer progression and a negative prognostic factor [84, 85]. It has also been associated with resistance against different classes of anti-cancer therapeutics including topoisomerase inhibitors, DNA alkylating agents and targeted therapies [86-88].

1.4.1.1 Targeting Polo-like Kinase 1

Inhibiting PLK1 either genomically or pharmacologically by siRNAs or small molecule inhibitors respectively has proven to be an effective way of combating cancer growth and drug resistance [89, 90]. Several different inhibitors have been designed so far including ATP-competitive inhibitors which bind to the ATP binding pocket of the kinase or novel compounds against the Polo-box domain (PBD) that hinder protein-protein interactions. In addition, there are very recent studies on the development of bifunctional conjugate compounds containing both an ATP-competitive inhibitor and a PBD inhibitor of PLK1. These novel compounds would compete with both ATP and the substrate of PLK1 for binding, and they hold great promise although several challenges need to be overcome in order to eventually find the right conjugate [89, 91].

1.4.1.2. Volasertib

Volasertib (BI6727) is a highly potent ATP-competitive inhibitor of PLK1 belonging to the class of dihydropteridinones. It is effective even at very low concentrations, and it was shown to promote G2/M arrest and apoptosis in *in vitro* and *in vivo* models of different cancers [92, 93]. Several clinical trials with volasertib have so far been conducted both in solid tumors [94-99], and in hematological malignancies [100-102] and lots of promising results were obtained [103]. In a Phase II study with Platinum-resistant ovarian cancer patients, volasertib was compared with chemotherapy in terms of disease control rate as a primary end point and overall and progression-free survival as secondary end points. Volasertib arm demonstrated a better profile with less toxicity than chemotherapy [104]. In a Phase II study with treatment-naïve Acute Myeloid Leukemia (AML) patients who were not eligible for intensive treatment, volasertib combined with the chemotherapy agent LDAC prolonged the event-free survival significantly as compared to LDAC alone [105]. These promising results led to the initiation of a Phase III trial with a similar setup in 2013, the results of which have recently been announced. Although the initial analysis showed a higher percentage of patients with objective response in LDAC+volasertib arm compared to LDAC alone, the results were not statistically significant. The reason of this was the low safety profile seen in LDAC+volasertib treated patients with higher incidence of severe adverse side-effects [106]. Nevertheless, new Phase III trials are underway with a modified dosing and scheduling as to find the best way of administration which does not cause high toxicity without compromising the anti-tumor efficacy. In addition, researchers are now promoting volasertib for testing in different patient groups with unmet needs like higher risk myelodysplastic syndromes (MDS) (ClinicalTrials.gov Identifier: NCT01957644).

1.4.2. Roles of the Mitotic Spindle Protein, TACC3 in Cell Cycle

Transforming Acidic Coiled-Coil Containing 3 (TACC3) is another important mitotic gene which is frequently mutated or amplified in cancer [107, 108]. It is one of the three members of the TACC family proteins which are characterized by having a unique coiled-coil domain at their C-terminal [109]. TACC3 functions in mitotic spindle assembly and ensures a proper chromosomal segregation. The protein is activated upon phosphorylation by Aurora A kinase in early mitosis and recruited to microtubules in order to stabilize microtubule kinetochore attachments [110, 111]. Like PLK1, TACC3 has also been associated with resistance against anti-mitotic agents. Its overexpression was found to attenuate the G2/M arrest mediated by paclitaxel treatment in cervical cancer cells [112]. Although it is suggested as an important mitotic protein, the cellular phenotypes associated with TACC3 inhibition seem to vary with the cancer model being used. In a study conducted with breast cancer cells, siRNA depletion of TACC3 failed to induce cell death, but instead it led to G1 growth arrest and senescence by upregulation of p21 and downregulation of retinoblastoma protein. When TACC3 inhibition was combined with non-toxic doses of paclitaxel, the senescence phenotype increased even more suggesting a synergistic anti-cancer activity of microtubule disruption and TACC3 inhibition in the context of not mitotic arrest, but premature senescence [113]. In hepatocellular carcinoma, downregulation of TACC3 was shown to decrease proliferation, clonogenicity and cancer stemness; however, researchers provided no data related to a possible apoptotic induction in these cells [114]. On the other hand, in human lymphoma cells, TACC3 depletion resulted in aberrant spindle formation and massive apoptosis [115]. In HeLa cells and a non-small cell lung cancer cell line, TACC3 inhibition was shown to greatly enhance the G2/M arrest and apoptosis induced by paclitaxel [112]. The observed synergism between paclitaxel and TACC3 inhibition was explained by the presence of a regulatory effect of paclitaxel on TACC3 expression. It was shown that paclitaxel treatment reduces TACC3 expression at transcriptional level [112]. This finding suggests that paclitaxel may exert its inhibitory effects on mitosis, in part, by downregulating TACC3.

These seemingly contradictory findings on the cellular effects of TACC3 inhibition could be due to the fact that TACC3 protein is also interacting with a hypoxic factor, Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT), and it was actually first identified by its ability to bind to the ARNT protein in a yeast two-hybrid screen [116]. Nevertheless, the high centromeric localization of all three members of the mammalian TACC family suggests an important contribution of these proteins to proper microtubule-spindle assembly [117].

1.4.2.1. Inhibition of TACC3 by KHS101

Despite the presence of large amounts of *in vitro*, *in vivo* and patient data on the oncogenic functions of TACC3, there is currently no inhibitor against TACC3 that is being tested in clinics. KHS101 is a small molecule against TACC3 protein which was first identified as a regulator of neuronal differentiation in rats [118]. An affinity based target identification followed by mass spectrometry revealed TACC3 as the interacting protein of this compound. Later, in another study, TACC3 protein was shown to undergo proteosomal degradation upon KHS101 treatment [119]. Although this inhibitor is unlikely to be translated into clinics due to its low systemic stability [118], more compounds targeting TACC3 that are suitable for *in vivo* usage will surely be developed in the near future [120].

1.5. BCL2 Family Proteins as Important Regulators of Apoptosis

Since a lot of cytotoxic agents used in cancer treatment induce cell death through apoptosis, mechanisms adopted by cancer cells in order to prevent apoptotic death are frequently observed in refractory tumors. BCL2 family proteins are key regulators of both the extrinsic and intrinsic apoptosis, and their expression is frequently deregulated during cancer progression and acquisition of drug resistance [121-125]. There are three subfamilies in BCL2 family. The Bax and BH3-only family proteins promote cell death either by activation of Caspases and formation of apoptosome or by the mitochondrial pathway characterized by the release of cytochromes c into cytosol from the mitochondria. The pro-apoptotic proteins may reside in cytosol or at the outer mitochondrial membrane, and when a cell receives a death signal, some of them work in cytosol while some form oligodimers with each other at the mitochondrial membrane and promote cytochrome c release. Once it is distributed into cytosol, cytochrome c activates Caspases which then mediate the proteolytic degradation of proteins to execute the programmed cell death [125-127]. On the other hand, BCL2 subfamily suppresses apoptotic death and promotes survival *via* antagonizing Bax and BH3-only family proteins by inhibiting their oligomerization on the mitochondrial membrane. BCL2 subfamily can also prevent Caspase activation and formation of the apoptosome [121]. Overexpression of this protein was shown to be a negative prognostic factor and a predictor of poor response to chemotherapy in various cancers [128]. High levels of BCL2 renders cancer cells resistant to apoptosis induced by anti-cancer agents [129] by antagonizing the pro-apoptotic family members.

1.5.1. Regulation of BCL2 in Mitotic Arrest

Besides the relatively well characterized functions of BCL2 in suppressing apoptosis, the mechanisms of the cell cycle dependent regulation of BCL2 protein are largely unknown. It was shown be phosphorylated in response to various stimuli including anti-mitotic agents causing prolonged mitosis; however, whether phosphorylation enhances or weakens its anti-apoptotic activity is highly context dependent. The response of cells to BCL2 phosphorylation largely depends on the cellular model as well as the phosphorylation site. A possible role of BCL2 in mitosis, besides its phosphorylation and regulation in terms of the anti-apoptotic functioning, may also be related to its chromosomal localization and control over mitotic events although such a hypothesis is not yet studied in detail and proposed only by limited number of studies [130-132]. Whether BCL2 follows a cyclic expression pattern with a dominating role in mitotic progression or it solely controls the apoptosis execution has yet to be determined; however, it is for sure that this oncogene is associated with cancer progression and drug resistance by rewiring the apoptosis machinery.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Buffers

1x Anode Buffer I	300mM Tris, 20% (v/v) methanol
1x Anode Buffer II	25mM Tris, 20% (v/v) methanol
1x Cathode Buffer	40mM 6-aminocaproic acid, 20% (v/v) methanol
1x SDS-PAGE Running Buffer	25mM Tris, 14.41g/l glycine, 1% (v/v) SDS
1x TAE	40mM Tris, 20mM acetic acid, 1mM EDTA
1x TBST	20mM Tris, 8g/l NaCl 0.2% (v/v) Tween20

2.1.2. Chemicals and Reagents

4x Protein Loading Dye	250mM Tris HCl (pH:6.8), 10% (w/v) SDS, 0.1% (w/v) Bromophenol blue, 50% Glycerol (v/v), 25% (v/v) mercaptoethanol
6-aminocaproic acid	Sigma Aldrich, St Louis, MO, USA
7-AAD	BD Biosciences, San Diego, CA, USA
Acrylamide/bisacrylamide	Applichem, Darmstadt, Germany
Ammonium peroxisulfate	Carlo Erba, Cornaredo, Italy
Bovine Serum Albumin	Santa Cruz, Dallas, TX, USA

Protease inhibitor cocktail	Roche Applied Science, Mannheim, Germany
ECL	Amersham Pharmacia Biotech, Amersham, UK
Ethanol	Sigma Aldrich, St Louis, MO, USA
Isopronapol	Sigma Aldrich, St Louis, MO, USA
LightCycler 480 SYBR Green I Master	Roche Applied Science, Mannheim, Germany
Lipofectamine 2000	Invitrogen, Carlsbad, CA, USA
Methanol	Sigma Aldrich, St Louis, MO, USA
Milk powder	Sigma Aldrich, St Louis, MO, USA
Nuclease free water	Applied Biosystems/Ambion, Austin, TX, USA
Page Ruler Protein Ladder	Thermo Fisher Scientific, Waltham, MA, USA
Phosstop	Roche Applied Science, Mannheim, Germany
Ponceu S	Sigma Aldrich, St Louis, MO, USA
Sodium Chloride	Merck, Darmstadt, Germany
Sodium Dodecyl Sulfate (SDS)	Merck, Darmstadt, Germany
TEMED	Serva, Heidelberg, Germany
TRIsure	Bioline, Luckenwalde, Germany
Triton X-100	Sigma Aldrich, St Louis, MO, USA
Trizma base	Sigma Aldrich, St Louis, MO, USA
Tween-20	VWR, Radnor, PA, USA

2.1.3. Media and Supplements

DMEM	Lonza, Basel, Switzerland
Fetal bovine serum (FBS)	Biowest, Nuaille, France
Insulin	Sigma Aldrich, St Louis, MO, USA
Non-essential aminoacids (NEAA)	Lonza, Basel, Switzerland
optiMEM	Invitrogen, Carlsbad, CA, USA
Penicillin/streptomycin (P/S)	Lonza, Basel, Switzerland

2.1.4. Kits

BCA Protein Assay Kit	Pierce, Rockford, IL, USA
Cell Titer-Glo cell viability assay kit	Promega, Madison, WI, USA
Caspase-Glo® 3/7 Assay kit	Promega, Madison, WI, USA
MycAlert detection kit	Lonza, Basel, Switzerland
First strand cDNA synthesis kit	Fermentas, St. Leon-Roth, Germany

2.1.5. Equipments

Accuri FACS	BD, Franklin Lakes, NJ, USA
Centrifuges	Thermo Fisher Scientific, Waltham, MA, USA Beckman, Pasadena, CA, USA
Cell culture hood	Nüve, Ankara, Turkey
Cell culture incubator	Nüve, Ankara, Turkey

Counting chamber	Marienfeld, Königshofen, Germany
Freezer (-80)	Hettich, Geldermansen, Holland
Freezer (-20)	Bosch, Stuttgart, Germany
Fridge	Bosch, Stuttgart, Germany
Horizontal Shakers	FinePCR, Seoul, South Korea
	Bellco, Vineland, NJ, USA
LightCycler 96	Roche Applied Science, Mannheim, Germany
Mini-PROTEAN Gel casting module	Biorad, Hercules, CA, USA
Mini-PROTEAN Tetra Cell	Biorad, Hercules, CA, USA
Multichannel Pipette	Thermo Fisher Scientific, Waltham, MA, USA
Nanodrop 1000	Thermo Fisher Scientific, Waltham, MA, USA
Nikon TS300 Inverted microscope	Nikon, Tokyo, Japan
Power supplies for electrophoresis	Biorad, Hercules, CA, USA
qPCR Machine	Roche Applied Science, Mannheim, Germany
Semidry western blot transfer unit	Biorad, Hercules, CA, USA
Synergy HT Multireader	Biotek, Winooski, VT, USA
Vortex	Isolab, Wertheim, Germany
Water bath	Nüve, Ankara, Turkey
X-ray cassette	Amersham Pharmacia Biotech, Amersham, UK
X-ray hyper processor	Amersham Pharmacia Biotech, Amersham, UK

2.1.6. Consumables

100mm dishes	Greiner bio-one, Frickenhausen, Germany
145mm dishes	Greiner bio-one, Frickenhausen, Germany
96-well plates	Greiner bio-one, Frickenhausen, Germany
6-well plates	Greiner bio-one, Frickenhausen, Germany
Filtered pipette tips (10ul, 20ul, 200ul, 1000ul)	Greiner bio-one, Frickenhausen, Germany
Cell scrapers	Greiner bio-one, Frickenhausen, Germany
Coverslips	Marienfeld, Königshofen, Germany
Cryovials	Greiner bio-one, Frickenhausen, Germany
Parafilm	VWR, Radnor, PA, USA
PCR tubes	Axygen, Corning, NY, USA
Plastic pipettes (10ml, 25ml)	Corning Incorporated, Corning, NY, USA
PVDF Membrane	Biorad, Hercules, CA, USA
Reaction tubes (500ul, 1.5ml, 2ml)	Axygen, Corning, NY, USA
Storage bottles (250ml, 500ml, 1L)	Corning Incorporated, Corning, NY, USA
Whatmann paper	GE Healthcare, Little Chalfont, UK
X-ray films	Kodak, Rochester, NY, USA
Cuvettes	VWR, Radnor, PA, USA
LightCycler® 480 Multiwell Plate	
96, white	Roche Applied Science, Mannheim, Germany
White plates	Costar, Corning, NY, USA

2.2.Methods

2.2.1. Cell culturing

2.2.1.1. Culturing Human Breast Cancer Cell lines and Development of Drug Resistant Models

BT474 and SK-BR-3 human breast cancer cell lines were grown in 100mm petri dishes in a 37°C incubator with 5% CO₂ and passaged once in every five days and two times a week, respectively. Growth media (DMEM) of both cell lines contained 10% FBS, 1% NEAA and 1% P/S. Growth medium of BT474 has also supplemented with 0.01 mg/ml of insulin. During passaging, cells were washed with 4 ml of PBS after culture media has been aspirated, and then trypsinized with 1.5 ml of trypsin in 37°C for 5 minutes and resuspended in appropriate culture media. T-DM1 resistant cells were generated by culturing the cells in the presence of drug containing media for three days followed by incubation with fresh media until cells were recovered. All cell lines were tested for mycoplasma contamination periodically.

2.2.1.2. siRNA Transfections

siRNA transfections were performed by using Lipofectamine 2000TM and OptiMEM reagents. Cells were seeded in 96-well plates with 5,000 cells/well and 8,000 cells/well of cell densities for SK-BR-3 and BT474 cells, respectively in culture media without P/S. For 6-well plates; 200,000 cells/well and 300,000 cells/well of SK-BR-3 and BT474 cells were seeded, respectively. The amount of lipofectamine used per well was 0.2 µl lipofectamine diluted in 20 µl of optimem and 2 µl diluted in 250 µl of optimem in 96-well plate and 6-well plate formats, respectively. After siRNAs and lipofectamine were mixed with optimem, they were vortexed for 10 seconds and incubated at room temperature for 5 minutes. Then, lipofectamine and siRNA solutions were mixed in a 1:1 ratio, vortexed for 1 minute and incubated at room temperature for 20 minutes. The final siRNA solutions were mixed with the culture media of the cells without P/S. The final volume in 96-well and 6-well plate formats were 120 µl and 1.5 ml, respectively. The targeted siRNA screen was done with the siRNAs listed in Table 2.1 with a concentration of 20 nM for all 21 genes. Later, the sensitization experiments with PLK1 and TACC3 inhibition was done with 20 nM and 40 nm, respectively.

Table 2.1. List of siRNAs.

siRNAs	Catalog number	Firm
Individual: siGENOME ABCC4 siRNA	D-007313-02-0002	Dharmacon
Individual: siGENOME ADORA1 siRNA	D-005415-05-0002	Dharmacon
Individual: siGENOME AURKB siRNA	D-003326-07-0002	Dharmacon
Individual: siGENOME CCNB2 siRNA	D-003207-05-0002	Dharmacon
Individual: siGENOME GDF15 siRNA	D-019875-01-0002	Dharmacon
Individual: siGENOME HEY1 siRNA	D-008709-06-0002	Dharmacon
Individual: siGENOME ID1 siRNA	D-005051-01-0002	Dharmacon
Individual: siGENOME IKBKE siRNA	D-003723-03-0002	Dharmacon
Individual: siGENOME KCNC4 siRNA	D-006223-02-0002	Dharmacon
Individual: siGENOME LAP3 siRNA	D-005923-01-0002	Dharmacon
Individual: siGENOME MAOA siRNA	D-009369-01-0002	Dharmacon
Individual: siGENOME MLKL siRNA	D-005326-01-0002	Dharmacon
Individual: siGENOME PTGES siRNA	D-008462-01-0002	Dharmacon
Individual: siGENOME PTPRM siRNA	D-006326-01-0002	Dharmacon
Individual: siGENOME S100P siRNA	D-004295-01-0002	Dharmacon
Individual: siGENOME SEC14L2 siRNA	D-012478-01-0002	Dharmacon
Individual: siGENOME SLC16A1 siRNA	D-007402-01-0002	Dharmacon
Individual: siGENOME STK10 siRNA	D-004168-05-0002	Dharmacon
Individual: siGENOME TACC3 siRNA	D-004155-03-0002	Dharmacon

Individual: siGENOME PLK1 siRNA	D-003290-05-0002	Dharmacon
Individual: siGENOME CYP4F8 siRNA	D-009131-01-0002	Dharmacon

2.2.2. Cell-based assays

2.2.2.1. In vitro Sensitization

Treatments with T-DM1 or inhibitors against PLK1 and TACC3 were done on the next day after cell seeding or one day after siRNA transfection in case of sensitization with genomic inhibition. T-DM1 was provided by Genentech under a material transfer agreement. ABT-263 was a kind gift of Asst. Prof. Özgür Kütük (Baskent University, Ankara, TURKEY). Volasertib was purchased from Selleckchem with a catalog number of S2235 and dissolved in DMSO with a stock concentration of 10 mM. KHS101 was purchased from Sigma, with a catalog number of K4019 and dissolved in DMSO with a stock concentration of 50 mM.

The drugs were given in 40 µl and 500 µl volumes per well of 96-well and 6-well plates, respectively. For T-DM1 sensitization with Volasertib and KHS101, the drugs were combined in culture media prior to addition onto the cells. End point viability was measured 3 days after drug or siRNA treatments for all, except for the siRNA sensitization with PLK1 inhibition in which the incubation period in the presence of T-DM1 lasted 2 days. Cell Titer-Glo cell viability assay kit was used to measure cell viability in 96-well plate format. 30 µl of the Cell Titer-Glo reagent per well was mixed with the culture media and incubated for 10 minutes on shaker. Later, the solution was transferred to opaque white flat bottom 96-well plates and the luminescence was recorded with Biotek Multireader. The analysis was done by calculating the percentage cell viability as the ratio of the signal in treated wells to the signal in vehicle treated wells multiplied by 100.

2.2.2.2. Apoptosis Assay

Cells were seeded with the previously indicated cell densities in 96-well plates (2.2.1.2) and treated with 4 µg/ml and 0.02 µg/ml of T-DM1 for BT474 and SK-BR-3, respectively on the next day. After 3 days, 25 µl of the Caspase-Glo® 3/7 reagent per well was added, and the plates were incubated at room temperature for 30 minutes after which the solution was transferred to opaque white flat bottom 96-well plates and, the luminescence was recorded

with Biotek Multireader. The analysis was done by calculating the relative apoptosis as the ratio of the signal in treated wells to the signal in vehicle treated wells.

2.2.2.3. Cell Cycle Analysis

For cell cycle analysis, cells were seeded in 6-well plates in triplicates with the cell densities indicated in Section 2.2.1.2. T-DM1 treatment was done on the next day with 4 µg/ml and 0.06 µg/ml of drug for BT474 and SK-BR-3, respectively and incubated for 48 hours. After 48 hours, cells were trypsinized, counted and transferred into FACS tubes after equalizing the cell number for each group in order to perform an equal staining. Cells were centrifuged and washed once with 1 ml PBS at 1500 rpm for 5 minutes. For fixing the cells, 1 ml of ice-cold 100% methanol was added drop-wise on top of the chilled cell pellet while constantly vortexing, and pellets were incubated for 30 minutes on ice, centrifuged, and washed once with 1 ml PBS. The pellets were first resuspended in 100 µl of PBS and then mixed with the staining buffer containing 5 µl of 7-AAD stain in 100 µl of PBS and incubated at +4 °C for 1 hour. Then, the pellets were centrifuged at 1500 rpm for 5 minutes and washed once with 1 ml PBS. At the end, they were resuspended in 300 µl PBS, and the staining was measured on Accuri FACS.

2.2.3. Molecular Biology

2.2.3.1. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

2.2.3.1.1. RNA isolation

Cells were seeded in 6-well plates with previously indicated cell densities (2.2.1.2), and the siRNA transfections or drug treatments were done as previously described in Sections 2.2.1.2 and 2.2.2.1. After 48 hours of siRNA transfection or drug treatment, cells were collected, and RNA isolation was done by using TRIsure reagent according to manufacturer's instructions.

2.2.3.1.2. cDNA synthesis

RevertAid RT Reverse Transcription kit was used to synthesize cDNA from RNA by following manufacturer's instructions. 1 µg of total RNA was mixed with the reaction components according to the protocol given in Table 2.2, and the reaction was performed in thermocycler using the program given in Table 2.3.

Table 2.2. Components of reverse transcription reaction.

Reagent	Concentration/Volume
RNA	1 µg (X µl)
oligoDT	1 µl
5x Revert Aid reaction buffer	4 µl
Ribolock Ribonuclease Inhibitor	1 µl
dNTPs(10mM)	2µl
Revert Aid H Minus M-MulV RT	1µl
H2O	(20-X) µl

Table 2.3. Thermocycler program for cDNA synthesis

Temperature	Time (minutes)
37°C	5
42°C	60
70°C	5
4°C	∞

After the reaction was completed, all samples were diluted with a 1:5 ratio in DEPC-treated water for the following qRT-PCR experiments.

2.2.3.1.3. qRT-PCR for mRNA expression

For qRT-PCR experiments, one reaction mixture per each gene was prepared by mixing the SYBR Green Master mix with the forward and reverse primers of the each gene whose

sequences are given in Table 2.4. Volumes of the reaction components were provided in Table 2.5.

Table 2.4. List of qRT-PCR primers.

Name	Gene ID	Forward Primer	Reverse Primer
PLK1	5347	5'-CGACTTCGTGTTTCGTGGTG-3'	5'-AATAACTCGGTTTCGGTGC-3'
TACC3	10460	5'-GACAGTGGAGCAGAAGACTAAA-3'	5'-GAATCAGACAGGACAGACAGAC-3'
ACTB	60	5'-CCAACCGCGAGAAGATGA-3'	5'-CCAGAGGCGTACAGGGATAG-3'
HPRT	3251	5'-TGACCTTGATTATTTTGCATACC-3'	5'-CGAGCAAGACGTTTCAGTCCT-3'

Table 2.5. Amounts of the qRT-PCR reaction components.

Reagent	Volume
SYBR Green	5 µl
Forward Primer (20uM)	0.25 µl
Reverse Primer (20uM)	0.25 µl
H2O	2.5 µl

After distributing 8 µl per well of the reaction mixture into LightCycler® 480 96-well plate, 20 ng of cDNA in 2 µl volume was added and carefully mixed by pipetting. The plate was tightly sealed, centrifuged at 1000 rpm for 1 minute at 4 °C and placed in LightCycler® 96 qRT-PCR thermocycler (Roche Applied Science, Mannheim, Germany). The reaction was carried out with the parameters shown in Table 2.6.

Table 2.6. qRT-PCR program.

Pre-incubation							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:05:00	4.4	5	0	0	0
Amplification							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:00:10	4.4	5	0	0	0
58	Single	00:00:20	2.2	5	0	0	0
72	None	00:00:20	4.4	5	0	0	0
Melting Curve							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:00:05	4.4	5	0	0	0
55	None	00:01:00	2.2	5	0	0	0
95	Continuous	00:00:00	0.11	5	0	0	0
Cooling							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
40	None	00:00:30	2.2	5	0	0	0

2.2.3.1.4. qRT-PCR Data analysis

Analysis of the qRT-PCR was done by $\Delta\Delta C_t$ method [133] using two housekeeping genes; ACTB and HPRT. Statistical significance between two groups was tested using Student's two-tailed t-test. Three technical replicates were done for each condition.

2.2.4. Protein Biochemistry

2.2.4.1. Protein Isolation

Cells were seeded in 6-well plates with the previously indicated cell densities (Section 2.2.2) and were transfected with siRNAs or treated with inhibitors on the next day. For T-DM1 sensitization with siRNAs, T-DM1 treatment was done one day after siRNA transfections. 24 or 48 hours after incubation with siRNAs or drugs, culture media was collected and centrifuged at 5000 rpm for 5 minutes in order to collect the apoptotic bodies. Meantime, cells were trypsinized and collected. They were added on top of the apoptotic bodies and precipitated with centrifugation at 1500 rpm for 5 minutes. The pellets were carefully washed once with 1 ml PBS. For the isolation of proteins from whole cell, pellets were lysed completely by mixing with 50 μ l RIPA buffer per one 6-well. The RIPA buffer was prepared fresh with the ingredients listed in Table 2.7. For complete lysis, they were incubated on ice for 30 minutes and vortexed vigorously at every 5 minutes. Then, the mixture was centrifuged at 14,000 g for 30 minutes, and the supernatant containing the proteins was collected in a fresh tube and the protein was kept at -20 °C for further experiments.

Table 2.7. Components of the RIPA buffer.

Ingredients	Stock Conc.	Final Conc.	For 1ml
NaCl	2M	150mM	75 μ l
NP-40	10%	1%	100 μ l
Sodium DOC	10%	0.5%	50 μ l
SDS	10%	0.1%	10 μ l
Tris HCl (pH 8.0)	2M	50mM	25 μ l
NAF	1M	50mM	50 μ l
NaVO ₄	100mM	1mM	10 μ l
Protease Inhibitor	25X	4%	40 μ l
Phosphatase Inhibitor	20X	4%	40 μ l
ddH ₂ O			600 μ l

2.2.4.2. Protein Quantification

Protein quantification was done by using the BCA Assay Kit according to manufacturer's instructions. BSA standard solutions were prepared at 9 different concentration ranging from 0 to 2 $\mu\text{g}/\mu\text{l}$, and 25 μl from each standard was placed in 96-well plate in duplicates. 5 μl from each sample was also placed in duplicates, and the dilution factor was taken as 5 for subsequent analysis. Working BCA solution was prepared by mixing reagents A and B in a 50:1 ratio and 200 μl of the working solution was distributed to the wells containing 25 μl and 5 μl of standards and samples, respectively. After 30 minutes of incubation at 37°C, absorbance was measured with the SynergyHT microplate reader (Biotek, VT, USA) at 562nm. Using the absorbance values obtained from BSA standards, a calibration curve was drawn, and sample concentrations were quantified by using the standard curve equation.

2.2.4.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Concentrations of protein samples were equalized by mixing appropriate amounts of protein solution with water and 4X protein loading dye. Immediately after mixing with the loading dye, the samples were boiled at 95°C for 5 minutes. Polyacrylamide stacking and resolving gels were prepared by using the ingredients listed in Table 2.8.

Table 2.8. Components of the stacking and resolving gels at different concentrations.

	8%	10%	12%	5%
Reagent	Resolving gel	Resolving gel	Resolving gel	Stacking gel
H ₂ O	2.3ml	1.9ml	1.6ml	1.36ml
30% acrylamide mix	1.3ml	1.7ml	2ml	340 μl
1 M Tris	(pH 8.8) 1.3ml	(pH 8.8) 1.3ml	(pH 8.8) 1.3ml	(pH 6.8) 260 μl
10% SDS	50 μl	50 μl	50 μl	20 μl
10% APS	50 μl	50 μl	50 μl	20 μl
TEMED	5 μl	5 μl	5 μl	2 μl
Total volume	5ml	5ml	5ml	2ml

Resolving gel was prepared first, and poured between the thin and thick glasses. The top of the gel was covered with isopropanol until it was fully polymerized. After polymerization, isopropanol was removed, stacking gel was casted on top of the resolving gel, and 10 well-comb was placed inside the stacking gel. 3 μ l protein ladder was loaded to the first well and the other wells were filled with 15 μ g of sample in 16 μ l. Electrophoresis was performed at 110V for 90 minutes.

2.2.4.4. Western blotting

For semi-dry transfer, 3 mm whatmann papers were cut in dimensions of 7 to 9 cm. 4 of them were moistened in anode buffer I, 2 of them in anode buffer II and 6 of them in cathode buffer. PVDF membrane was first placed in 100% methanol for 3 minutes and then placed in anode buffer II for 1 minute. The transfer layers were made by putting whatmann papers, membrane and the gel with the following order: 4 anode I paper, 2 anode II paper, PVDF membrane, stacking gel and 6 cathode papers. The stacked layers were placed in Semi-dry Turbo Blot machine and transfer was done for 30 minutes at 25V.

After transfer, PVDF membrane was stained with Ponceu S solution for 3 minutes in order to check if the transfer occurred properly or not. The membrane was washed with ddH₂O until Ponceu S stain was completely removed. Then, membrane was cut according to the kDa of the protein of interest and placed in 5% (w/v) milk:TBST blocking or 5% (w/v) BSA:TBST blocking solution for 1 hour at room temperature with slow agitation. After removal of the blocking solution, membrane was incubated with the primary antibody solution either for 1 hour at room temperature or overnight at 4°C. List of primary and secondary antibodies were given in Table 2.9.

Table 2.9. List of primary and secondary antibodies used in western blot experiments.

Gene Name	Firm	Catalog Number	Dilution
Cyclin B1	Epitomics	1495-1	1:10000
Phospho-Histone H3	Cell Signaling Technology	CST4056	1:1000
BCL2	Cell Signaling Technology	CST15071S	1:1000
Phospho-BCL2 (Ser70)	Cell Signaling Technology	CST2871	1:1000

Cleaved Caspase 3	Cell Signaling Technology	CST9664S	1:1000
Cleaved PARP	Cell Signaling Technology	CST5625S	1:1000
Beta-actin	MP Biomedicals	69100	1:20000
HRP-coupled anti-rabbit IgG	Cell Signaling Technology	CST7074	1:10000
HRP-coupled anti-mouse IgG	Cell Signaling Technology	CST7076	1:10000

After the primary antibody incubation, membranes were washed with TBST for 10 minutes, three times on shaker. Membranes were then incubated in secondary antibody solution for either 1 hour at room temperature or overnight at 4°C with slow agitation. After secondary antibody incubation, membranes were again washed with TBST for 10 minutes, three times on shaker. ECL reagents were mixed in 1:1 ratio and applied onto the membranes which were placed on the X-ray cassette. X-ray films were exposed to membranes for different time periods ranging from 3 seconds to 30 minutes.

2.2.5. Bioinformatic analysis

2.2.5.1. Whole transcriptome sequencing (RNA-Seq)

Sequencing was done by using Illumina Hiseq 2000 technology at German Cancer Research Center (DKFZ). A paired end library of 2×125bp reads were created for each condition (BT474 WT and TDM1R; SK-BR-3 WT and TDM1R) in duplicates. These eight libraries are then given different barcodes, pooled and distributed in two lanes. Around 300 million reads sequenced per lane with 80 million reads for each condition distributed in two lanes.

2.2.5.2. RNA-Seq Analysis

Raw sequence data were aligned with UCSC Homo sapiens reference genome (hg19, GRCh37) using Tophat v2.1.0 with default parameters by allowing up to two mismatches and using up to 20 alignments for reads mapping at multiple positions. Cufflinks was used to assemble transcripts from the sequence alignment/map files generated by Tophat and then to

quantify the expression level of each transfrag in the samples. By utilizing a reference genome annotation (UCSC hg19, GRCh37), Cuffmerge merged reference transcripts with sample transfrags and generated a single gtf annotation file for the differential expression. For the identification of the differentially expressed genes, Cuffdiff, which provides differential expression and regulation at the gene and transcript level, was used. Finally, data produced by Cuffdiff analysis transformed into R statistical computing environment by CummeRbund program in order to check the quality of the generated data.

2.2.5.3. TCGA Data Analysis

mRNA expression and z scores of the 331 genes, found to be commonly differentially expressed genes between the WT and TDM1R models, as obtained by whole genome RNA sequencing, in TCGA breast cancer patients were retrieved from cBio Portal [134, 135]. The scores of the cellular process in TCGA patients were downloaded from TCPA database [136]. For the correlation analysis, the mRNA expression of 331 genes were uploaded to GENE-E software and the correlation matrix was created by setting Pearson correlation as the metric and average linkage as the linkage method. For generating the distance matrix, the Pearson correlation coefficients of the similarity between the z scores of 21 genes targeted in the siRNA screen and the process scores in each patient was again uploaded in GENE-E and the same parameters were applied to create the distance matrix. T-DM1 resistance (TDM1R) signature score for each patient was calculated by subtracting the sum of the z-scores of all the genes upregulated in T-DM1 resistance from the sum of z-scores of the downregulated ones.

2.2.5.4. Pathway Enrichment

The genes found in the most prominent cluster obtained from the clustering analysis were uploaded in the online Reactome Pathway Analysis database [137, 138] and the significant pathways were obtained from the results tab. The Metacore pathway enrichment analysis was performed by uploading 331 commonly differentially expressed genes in Metacore database together with their fold changes in the corresponding cell line models. The pathways that were commonly enriched in both cell line models for selected as the enriched pathways in T-DM1 resistance.

Statistical significance for the each data provided in this thesis was shown as * for $p < 0.05$ and as ** for $p < 0.01$.

CHAPTER 3

RESULTS

3.1. Development and Characterization of T-DM1 Resistant Breast Cancer Cell Line Models

T-DM1 is the newest HER2 targeting agent in clinics and has demonstrated a superior efficacy over current anti-HER2 therapies in metastatic trastuzumab refractory breast cancer patients. However, as an inevitable consequence of the long-term usage of any anti-cancer agents, patients experience disease recurrence within 6 months of T-DM1 therapy. Although several mechanisms that could mediate T-DM1 resistance has been proposed so far in a limited number of studies, the exact mechanisms governing resistance against T-DM1 is currently unknown. Our lab has previously investigated resistance mechanisms against several anti-cancer therapies in the context of different types of breast cancer including ER and HER2 targeting agents; tamoxifen and trastuzumab in ER- and HER2-positive breast cancers, respectively. In these studies, drug resistant cell lines were generated by long-term exposure to drug following molecular characterization by whole genome RNA profiling and identification of the most promising hits as targets to reverse the resistance [43, 139, 140].

3.1.1. Developing T-DM1 resistant cell line models

In order to study the resistance mechanisms against T-DM1, I have started with developing two different *in vitro* models of resistance by continuously treating two HER-2 positive breast cancer cell lines; BT474 and SK-BR-3 with T-DM1 over 8 months. Both of the cell lines are clinically classified as HER2-positive. Molecularly, BT474 cells are of Luminal B and SK-BR-3 cells are of HER2-positive subtype. Therefore, these two cell lines are highly suitable models to study the mechanisms of drug resistance in clinical HER2-positive tumors which may molecularly be either luminal B or HER2-positive. During the development of T-DM1 resistant BT474 cell line (BT474 TDM1R), I have gradually increased the dose of T-DM1 as the cells had started to resist the given dose in terms of their viability. I have measured cell viability regularly by using Cell Titer Glo Reagent and doubled the dose of T-DM1 when I observed a significant difference in cell viability between the WT and TDM1R cells at the given dose. Such a method is one of the established ways of generating drug resistant cell line models which results in formation of highly drug resistant cells [141]. On the other hand, T-

DM1 dose that SK-BR-3 cells were exposed to was kept lower and more time was given in order for the cells to recover in drug-free media. As a result, I obtained one high degree resistant and one clinically relevant resistant model of T-DM1 (Figures 3.1A and 3.1B).

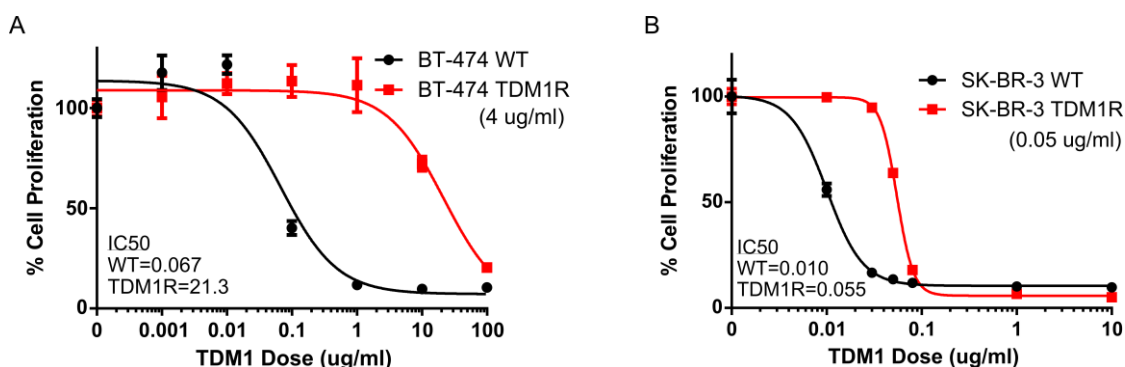


Figure 3.1: Dose response curves of T-DM1 in WT and T-DM1 resistant (TDM1R) models. WT and TDM1R BT474 (A) and SK-BR-3 (B) cells were treated with increasing doses of T-DM1 in quadruplicates for 5 days and cell viability was measured with Cell Titer Glo Reagent. The concentration that causes growth inhibition of 50% of the WT and TDM1R cells (IC_{50} dose) in both models were given at the bottom left side of the graphs.

3.1.2. Phenotypic Characterization of T-DM1 Resistance

After development of two different T-DM1 resistant models, I have set to perform the phenotypic characterization of the resistance. One of the two components of this ADC; DM1 is a maytansine derivative that is disrupting microtubule dynamics by depolymerization of microtubules. Microtubule targeting agents have previously been shown to induce prolonged mitotic arrest followed by activation of the intrinsic apoptosis pathway [142]. Therefore, I have performed a cell cycle and an apoptosis assay with both wild-type (WT) and TDM1R cells treated with T-DM1. 7-AAD staining demonstrated a prominent G2/M arrest in T-DM1 treated WT BT474 and SK-BR-3 cells after 48 hours of drug treatment (Figure 3.2). On the contrary, such an arrest was absent in TDM1R models at the same dose.

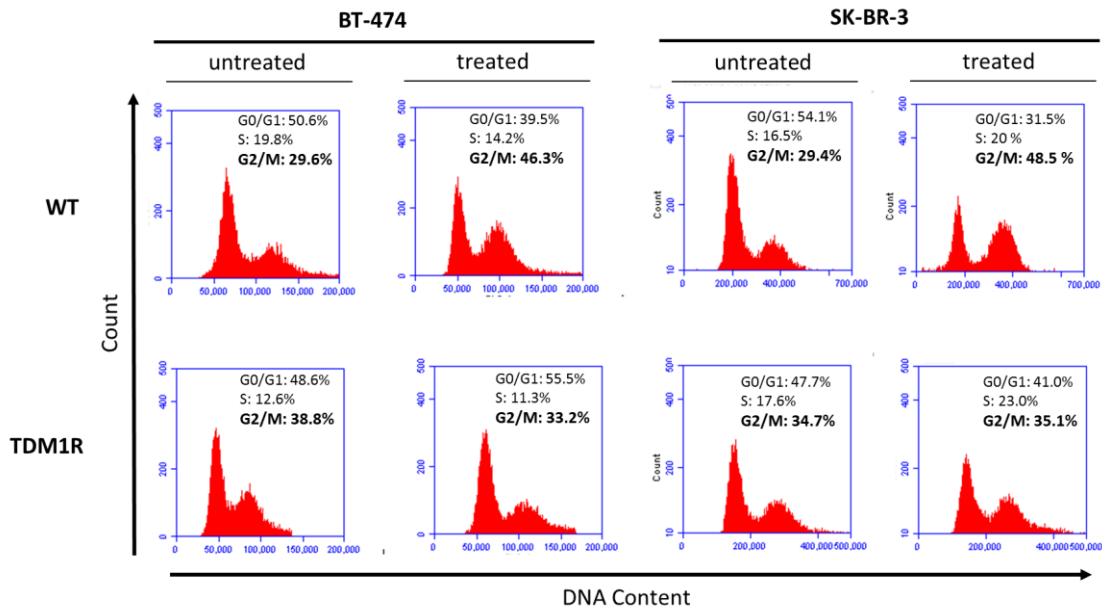


Figure 3.2: 7-AAD staining of the WT and TDM1R models of BT474 and SK-BR-3 cells identified a differential regulation of G2/M transition in T-DM1 resistance. Both WT and TDM1R cells were treated with 4 ug/ml (for BT-474) and 0.02 ug/ml (for SK-BR-3) of T-DM1 for 48 hours and stained with 7-AAD as a measure of DNA content. Cell counts obtained by Flow Cytometry analysis revealed the percentage of cells occupying different phases of the cell cycle. A prominent G2/M arrest was observed in WT cells of both models upon T-DM1 treatment (upper graphs), whereas there was no such accumulation at G2/M phase in TDM1R models with T-DM1 treatment (lower graphs).

Since it is not possible to distinguish between a G2 or M phase arrest with individual 7-AAD staining, I have checked the expression of a mitotic marker; phospho-Histone H3, in drug treated WT and TDM1R cells. Histone H3 is phosphorylated at the onset of mitosis predominantly at Ser10 residue, and elevated levels of the phosphorylated form of this protein indicates mitotic arrest [143].

As can be seen in Figures 3.3A and 3.3B, phospho-Histone H3 levels were significantly higher in WT BT474 and SKBR3 as compared to TDM1R cells treated with two different doses of T-DM1 which indicated that the G2/M arrest that I observed with 7-AAD staining, was actually an arrest at the mitotic, but not at the G2 phase of the cell cycle. I further confirmed the T-DM1 mediated mitotic arrest by showing a dose dependent accumulation of Cyclin B1 which is another hallmark of prolonged mitosis [144, 145] (Figures 3.3A and 3.3B).

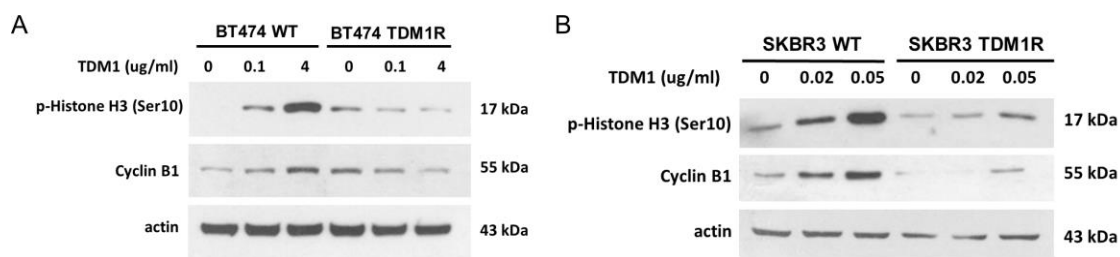


Figure 3.3: T-DM1 induces a dose dependent mitotic arrest in T-DM1 treated WT cells which is completely absent in case of TDM1R models. Western Blot analysis of two mitotic marker proteins; phosphorylated Histone H3 at Ser10 and Cyclin B1 in WT and TDM1R models of BT474 (A) and SK-BR-3 (B) cells treated with two different doses of T-DM1. Actin was used as the loading control.

Mitotic arrest has been previously shown to cause apoptosis though the exact mechanism is not fully known [26, 146, 147]. In line with this, I have observed a prominent increase in the apoptotic index based on intracellular Caspase 3/7 activity in WT cells which was completely absent in TDM1R models (Figures 3.4A and 3.4B). I have further confirmed abrogation of apoptosis in resistant cells by checking the protein levels of cleaved Caspase 3 and cleaved PARP which are the active forms of the corresponding proteins and are mediating the apoptosis process (Figures 3.5A and 3.5B).

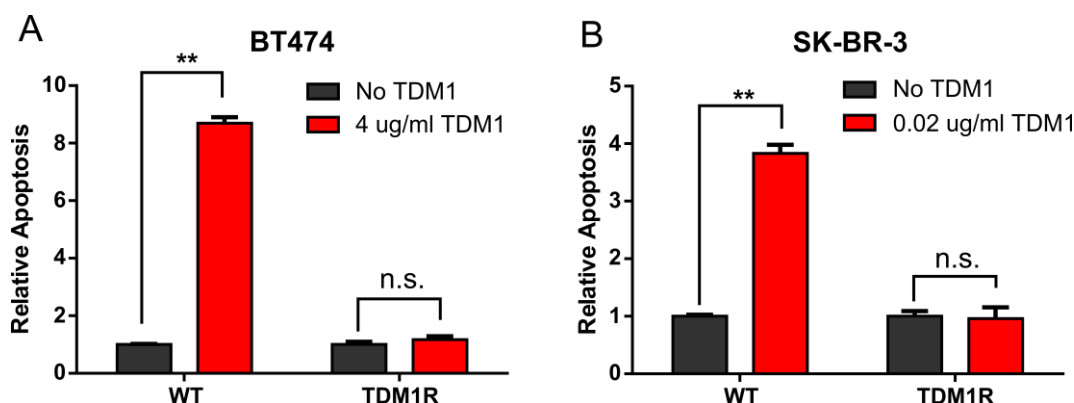


Figure 3.4: Apoptosis activity upon T-DM1 treatment is lost in TDM1R models. WT and TDM1R cells were treated with 4 µg/ml (for BT474) and 0.02 µg/ml (for SK-BR-3) for 72 hours in quadruplicates after which the Caspase 3/7 activity was measured by Caspase Glo 3/7 assay.

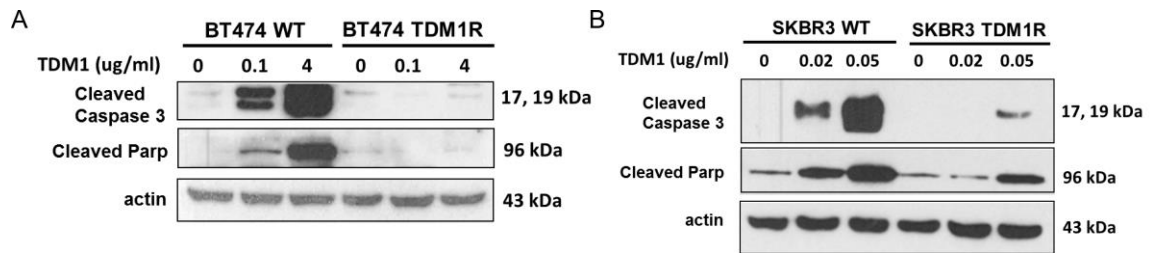


Figure 3.5: Confirmation of loss of apoptotic response in TDM1R models upon T-DM1 treatment by Western Blot analysis of apoptotic markers. Western Blot analysis of cleaved Caspase 3 and cleaved PARP in WT and TDM1R models of BT474 (A) and SK-BR-3 (B) cells, treated with two different doses of T-DM1 for 48 hours. Actin was used as the loading control.

3.1.3. Genomic Characterization of T-DM1 Resistance by Next-Generation Sequencing

Whole genome RNA sequencing has demonstrated to be useful in identifying mRNAs or non-coding RNAs differentially expressed or gene mutations differentially present among groups. In order to uncover the molecular mediators of T-DM1 resistance, I have sent RNA samples isolated from WT and TDM1R BT474 and SK-BR-3 cells to the German Cancer Research Center, DKFZ. There, a paired-end RNA sequencing was performed on the WT and TDM1R samples with two biological replicates.

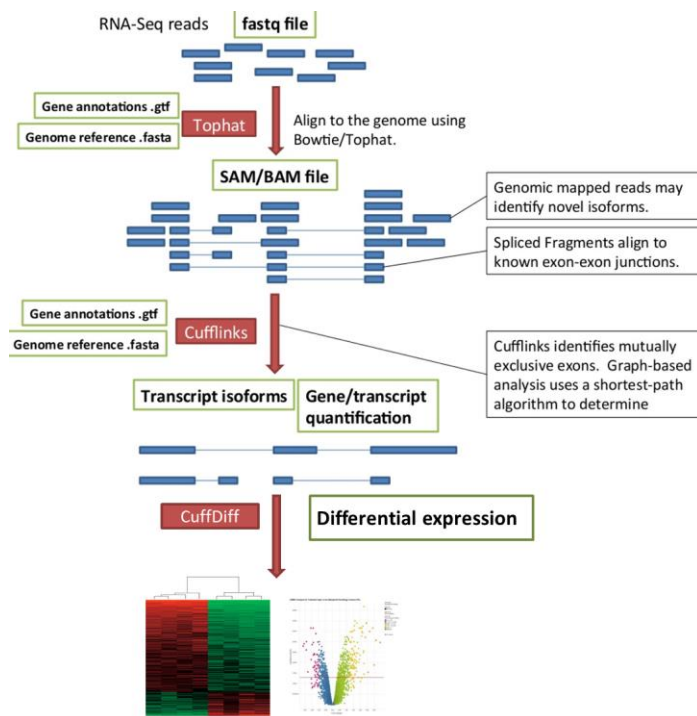


Figure 3.6: RNA sequencing analysis pipeline. CuffDiff algorithm was utilized to obtain the differentially expressed genes between the WT and TDM1R models. First, sequencing reads were mapped by TopHat and aligned to human genome by Cufflinks. Finally, the differential expression between WT and TDM1R samples were assessed by CuffDiff algorithm.

Around 80 million reads for each condition were sequenced and the subsequent analysis was done in our lab by using the CuffDiff methodology depicted in Figure 3.6 [148]. Mapping of the sequencing reads to human genome was done by TopHats. Mapped transcripts were assembled to genes and merged by Cufflinks and Cuffmerge, respectively. Finally, differential expression between the WT and T-DM1 resistant samples was done by Cuffdiff algorithm (Figure 3.6). As a result, I obtained 2000 and 3728 significantly differentially expressed genes between the wild-type and TDM1R models of BT474 and SK-BR-3 cells, respectively. 331 genes were common between the two models. Since I am interested in mechanisms of T-DM1 resistance in HER2-positive breast cancer in general, I have continued the analysis with these 331 commonly differentially expressed genes.

3.2. A Targeted siRNA Screen Identifies Important Mediators of T-DM1 Resistance

In order to identify mediators of T-DM1 resistance that could be targeted in clinic to restore sensitivity against T-DM1 in T-DM1 refractory breast cancer patients, I have focused on genes upregulated in T-DM1 resistant models and that can be inhibited with small molecule inhibitors. After a detailed literature search, I have identified 21 upregulated genes who has druggable protein products (Figure 3.7).

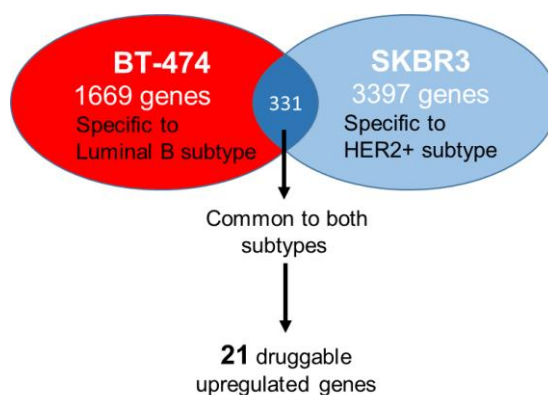


Figure 3.7: The schematic representation of the selection of genes to be silenced in the targeted siRNA screen. The commonly differentially expressed genes in both BT474 and SK-BR-3 models with a significance cutoff of adjusted p-value < 0.05, were further analyzed

in terms of the availability of drugs targeting their protein products which yielded 21 druggable upregulated genes in TDM1R models.

In order to test T-DM1 sensitization with inhibition of these 21 druggable upregulated genes, I have performed a small scale siRNA screen in which the mRNA molecules of these 21 genes were targeted by individual siRNAs and cell viability was assessed in the presence and absence of T-DM1 (Figures 3.8A and 3.8B).

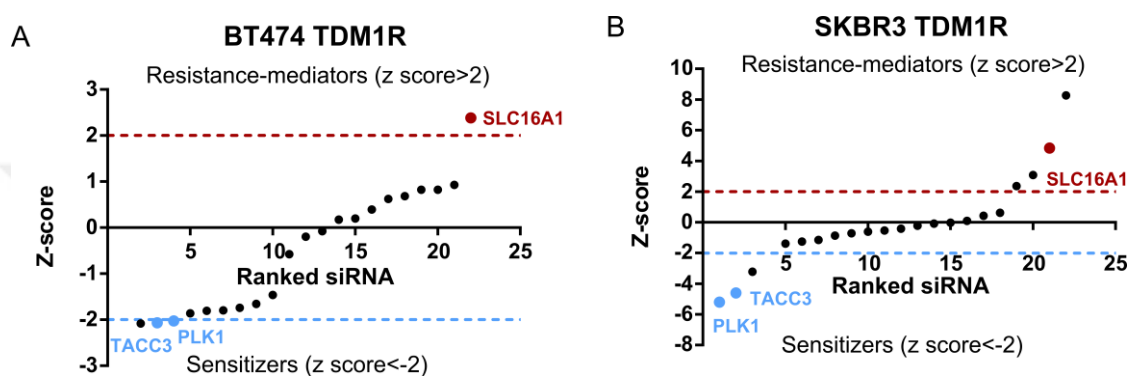


Figure 3.8: z-score plots of the siRNA mediated inhibition of 21 druggable genes in BT474 TDM1R and SK-BR-3 TDM1R models. The targeted siRNA screen was done by transfecting TDM1R cells with 40 nM of each siRNA targeting the 21 upregulated genes in resistance followed by T-DM1 treatment on the next day with doses of 20 ug/ml and 0.06 ug/ml for BT474 (A) and SK-BR-3 (B), respectively. z-scores of the inhibition of each gene was calculated by comparing the growth inhibition upon gene knockdown in the presence of T-DM1 with the growth inhibition upon T-DM1 treatment alone. A cutoff of -2 and +2 was selected to determine the sensitizer and resistance mediator inhibitions, respectively. Inhibition of PLK1 and TACC3 were identified as the common sensitizers to T-DM1 treatment in both models.

I have calculated the z-score of each siRNA inhibition as the change in cell viability with the siRNA transfection in the presence of T-DM1. If the genomic inhibition of an upregulated gene is found to cause a greater growth inhibition than T-DM1 alone, i.e. if the z-score is less than -2, then the gene was called as a sensitizer of T-DM1 resistance. On the contrary, if an siRNA inhibition has a z-score bigger than +2, i.e. if it is enhancing proliferation in the presence of T-DM1, then, that genomic inhibition was called as a T-DM1 resistance mediator. Since I am interested in genes whose inhibition enhances T-DM1

cytotoxicity, I have focused on genes with an siRNA inhibition z-score of less than -2. Among the few genes satisfying this criteria, two of them were common for both cell line models; PLK1 and TACC3. I have validated upregulation of these genes in T-DM1R models with qRT-PCR and showed a good correlation with the RNA sequencing results (Figures 3.9A and 3.9B).

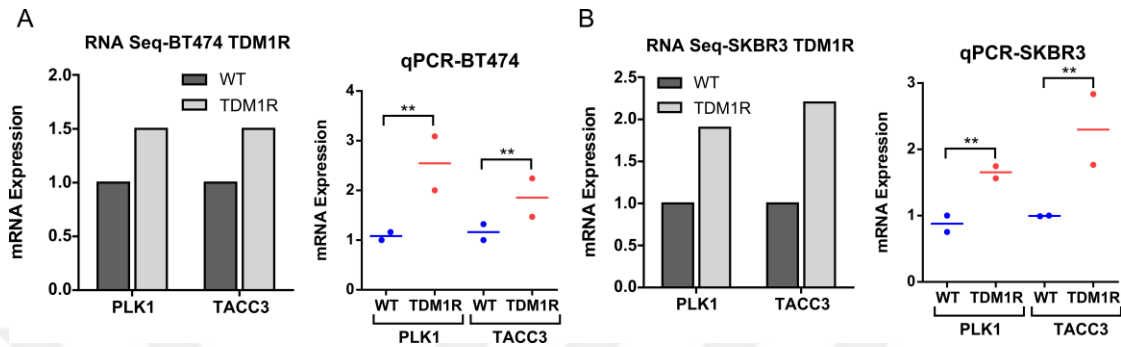


Figure 3.9: Validation of RNA sequencing by qRT-PCR. Upregulation of PLK1 and TACC3 expression in T-DM1 resistance as determined by RNA sequencing was confirmed by qRT-PCR analysis of the two genes in BT474 (A) and SK-BR-3 (B) models. ACTB and HPRT were used as housekeeping genes.

PLK1 is one of the four members of the polo-like kinase family which have diverse and most of the times non-overlapping functions. PLK1 is the most prominent member among the four, and it has ample roles in nearly every stage of cell cycle with mitosis being the well-studied phase in terms of its dependence on PLK1. Without PLK1, animal cells cannot generate proper bipolar spindles at the very beginning of mitosis, and they fail to execute a proper cytokinesis at the end [149]. Inhibition of PLK1 has been shown to induce a prolonged pro-metaphase arrest, and activation of spindle assembly checkpoint followed by apoptotic cell death [150-152]. However, the exact mechanisms of apoptotic induction upon PLK1 inhibition is currently unknown, and activation of spindle assembly checkpoint alone may not be enough to explain the apoptotic cell death with PLK1 inhibition since drugs like Nocadazole or Taxol which were shown to activate SAC may fail to induce apoptosis, but rather causes a prolonged mitotic arrest without affecting cell viability much in some cell line models [153, 154]. This demonstrates that the genetic background of the cells is a key determinant for the anti-cancer effects of PLK1 inhibition. TACC3 is a microtubule binding protein which functions mainly in controlling microtubule dynamics [155]. It has been mostly studied as a biomarker in several different types of cancers including breast and lung [156]. It

may also confer resistance against the anti-mitotic agent, paclitaxel, by inhibiting the G2/M arrest and apoptosis followed by paclitaxel treatment [112].

Before further proceeding into mechanisms of T-DM1 sensitization with PLK1 or TACC3 inhibition, I have verified the siRNA knockdown at mRNA level (Figures 3.10A and 3.10B) and then validated the siRNA screen results within a separate experimental setup and showed that genomic inhibition of PLK1 and TACC3 in the presence of T-DM1 significantly decreases cell viability as compared to the reduction that is seen for T-DM1 alone (Figures 3.11A and 3.11B).

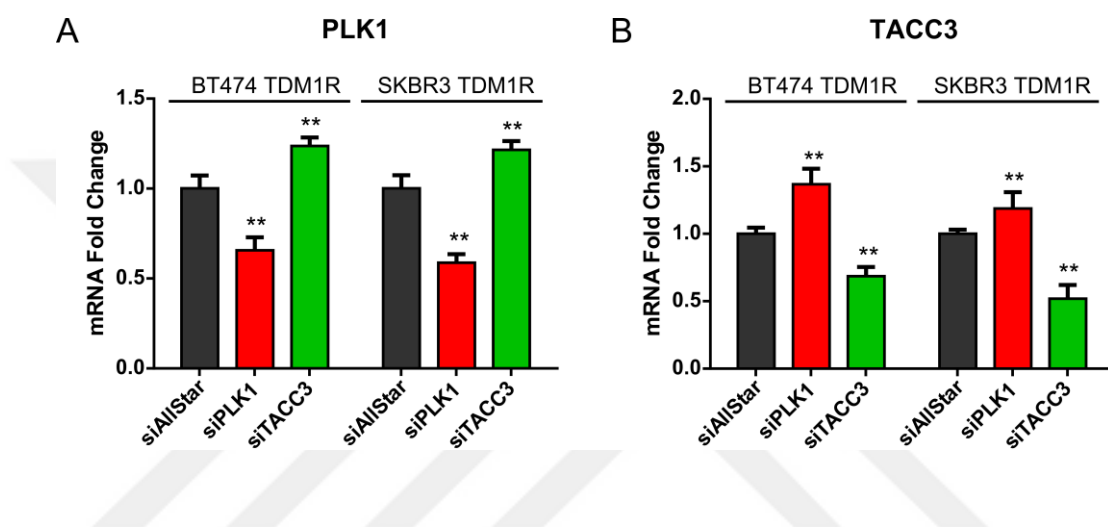


Figure 3.10: Validation of siRNA knockdown of PLK1 and TACC3 in the TDM1R models. Both BT474 and SK-BR-3 TDM1R models were transfected with 40 nM of siRNA against PLK1 (A) and TACC3 (B) and mRNA fold change of these genes were measured by qRT-PCR analysis. ACTB and HPRT were used as housekeeping genes.

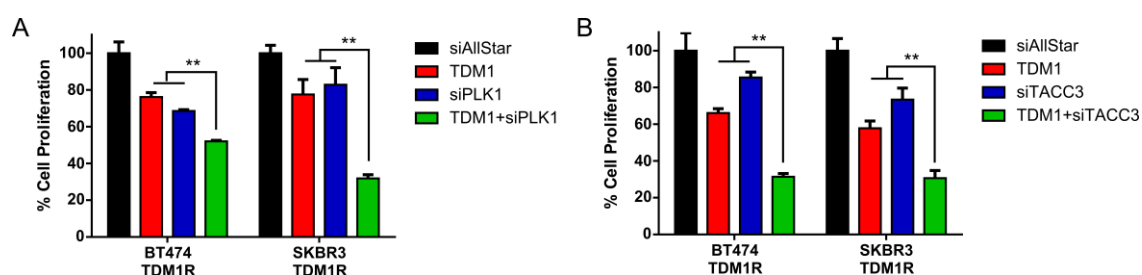


Figure 3.11: Validation of T-DM1 sensitization with PLK1 and TACC3 knockdowns. BT474 and SK-BR-3 TDM1R cells were transfected with 20 nM and 40 nM of siRNAs against PLK1 and TACC3, respectively. On the next day, 20 ug/ml and 0.06 ug/ml of T-DM1 was added, and cells were incubated for 48 and 72 more hours for PLK1 and TACC3 knockdown, respectively. Inhibition of both PLK1 (A) and TACC3 (B) significantly enhanced the growth inhibition mediated by T-DM1 treatment alone.

Protein products of both of these genes have small molecule inhibitors against, and I have utilized these inhibitors as a next step to further confirm T-DM1 sensitization, this time with pharmacological inhibition of the two genes. Volasertib is a kinase inhibitor that is currently in Phase III trials for the treatment of AML patients [106]. It is a small molecule kinase inhibitor of PLK1 and similar to PLK1 genomic depletion, it induces early mitotic arrest and apoptosis [157]. KHS101 is a small molecule against TACC3, and it was shown to induce proteosomal degradation and downregulation of TACC3 protein [119]. Currently, there are no clinical studies done with this inhibitor, and the preclinical testing is also quite unsatisfactory. Nevertheless, the clinical importance of TACC3 inhibition has been increasingly appreciated as reviewed in [120], and it has already been shown that TACC3 inhibition sensitizes cells to microtubule targeting agent; paclitaxel. The observed dependence of cancer cells on TACC3 makes it a highly suitable target in cancer therapy whose inhibition can mediate a targeted cell killing by affecting survival of TACC3 overexpressing cancer cells without affecting the microtubule dynamics in non-dividing cells. Although there are currently no inhibitors against TACC3 being tested in clinics, it is very likely that within a few years, inhibitors against this important mitotic protein will start to emerge as a means to treat TACC3 overexpressing cancers as well as patients refractory to microtubule targeting agents. I have performed sensitization experiments with PLK1-targeting volasertib and TACC3-targeting KHS101 in BT474 and SK-BR-3 TDM1R models. Similar to the sensitization I observed with genomic inhibition of PLK1 and TACC3, pharmacological inhibition of the protein products of these two genes was shown to confer a similar degree of sensitization against T-DM1 (Figures 3.12A and 3.12B).

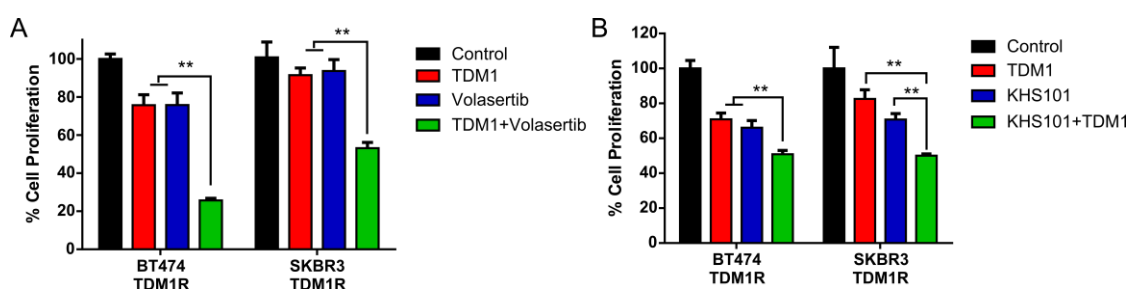


Figure 3.12: Pharmacological inhibition of PLK1 and TACC3 by Volasertib and KHS101, respectively, sensitized cells to T-DM1. 20 ug/ml and 0.06 ug/ml of T-DM1 was combined with 25 nM and 15 nM of Volasertib (A) or 3 uM or 1 uM of KHS101 (B) in BT474 and SK-BR-3 TDM1R cells in quadruplicates, respectively, and cell viability was measured after 72 hours.

3.3. T-DM1 Sensitization with PLK1 and TACC3 Inhibition is Partially Mediated by Re-induction of Apoptotic Cell Death

So far, I have showed prolonged mitotic arrest and activation of the subsequent apoptosis as a mechanism of T-DM1 cytotoxicity which is abrogated in case of T-DM1 resistance. I have identified PLK1 and TACC3 as two important mediators of T-DM1 resistance whose genomic or pharmacological inhibition by siRNAs or small molecule inhibitors respectively, sensitizes resistant cells by conferring a significantly higher levels of growth inhibition (Figures 3.11 and 3.12).

Since inhibition of both of these mitotic genes was previously associated with mitotic arrest and apoptosis, I have hypothesized that the observed T-DM1 sensitization with volasertib and KHS101 might be *via* re-gain of apoptotic induction which was seen in WT cells upon T-DM1 treatment, but that was absent in case of T-DM1 resistance (Figure 3.4). Western Blot analysis of cleaved Caspase 3 which was previously shown to be upregulated in response to T-DM1 (Figure 3.5), demonstrated that combination of volasertib and KHS101 with T-DM1 resulted in an increase in the levels of these proteins suggesting an activation of apoptosis (Figure 3.13). The increase in these apoptotic markers was more prominent in case of volasertib and T-DM1 combination as compared to KHS101 and T-DM1 which indicates that there must be a mechanism other than apoptosis by which TACC3 overexpression confers resistance against T-DM1.

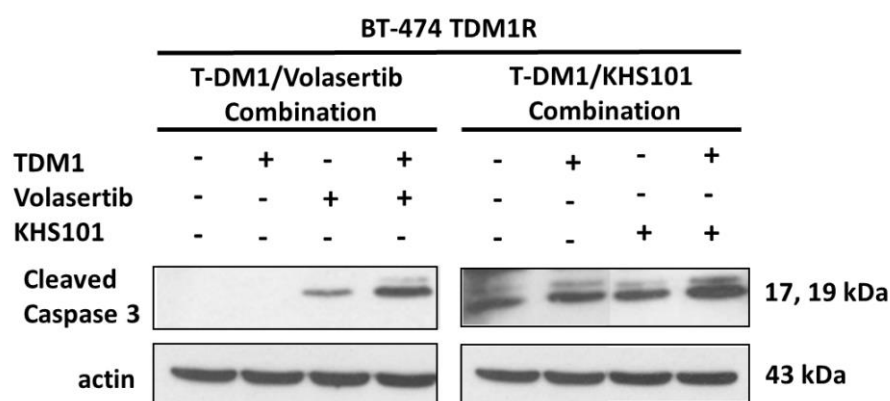


Figure 3.13: Combination of Volasertib and KHS101 with T-DM1 induces apoptosis. Western Blot analysis of cleaved Caspase 3 showed an increase in apoptosis when 20 ug/ml of T-DM1 was combined with 25 nM of Volasertib or 3 um of KHS101 in BT474 TDM1R cells. Actin was used as the loading control.

3.4. Cell Cycle Progression is Associated with T-DM1 Resistance as a Process

Since both PLK1 and TACC3 which were obtained as a result of an unbiased analysis of the differentially expressed genes in T-DM1 resistance, are important for cell cycle progression, especially for the mitotic phase, I have suspected the involvement of an activated cell cycle as a process, in mediating T-DM1 resistance. In order to test this hypothesis, I have re-analyzed the 331 commonly differentially expressed genes and looked for the pathways significantly enriched among these genes. To do this, I have utilized the Metacore Pathway Analysis software and uploaded the 331 differentially expressed genes to obtain pathways significantly enriched among them. Supporting a possible key role of progression through mitotic cell cycle in mediating T-DM1 resistance, pathways related to mitotic phase of the cell cycle were found among the top mostly enriched pathways (Figure 3.14).

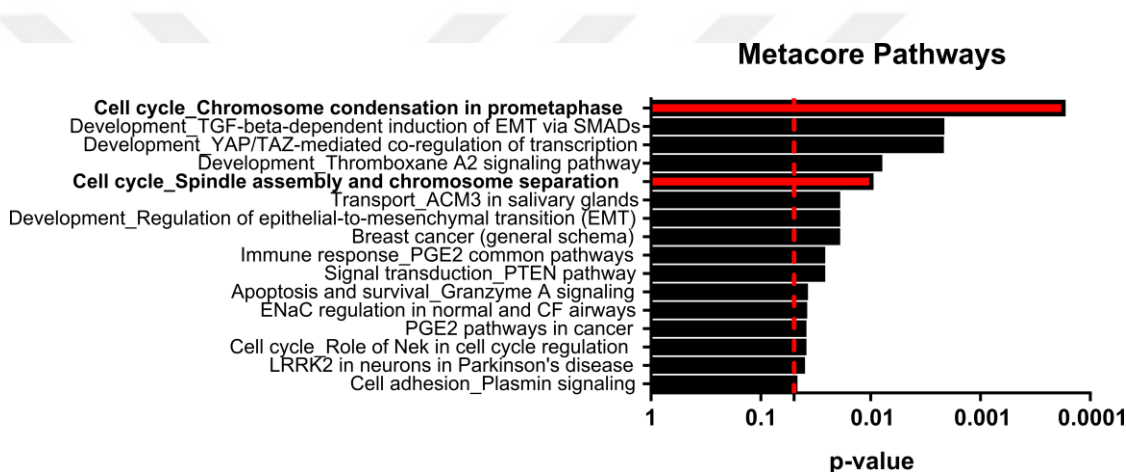


Figure 3.14: Metacore Pathway Enrichment Analysis revealed the cellular processes enriched in T-DM1 resistance. Analysis of the commonly differentially expressed genes in both models in terms of their enrichment with cellular process was done by using the Metacore software. Mitosis related gene sets were significantly enriched in T-DM1 resistance signature with a p-value cutoff of <0.05 .

To support this finding in breast cancer patients as well, I have analyzed the TCGA breast cancer data. First, I have obtained the z scores of each of the 331 differentially expressed genes from the cBioPortal and calculated a T-DM1 Resistance Signature Score for each patient by subtracting the sum of z scores of the upregulated genes from downregulated ones. Meantime, I have downloaded the scores of several processes important for cancer progression like EMT and cell cycle in the same set of patients from TCPA (The Cancer Proteome Atlas) database. Scores for these process were created based on the expression of

proteins involved in these processes assessed by Reverse Phase Protein Array (RPPA) Analysis done on the tumor samples of TCGA patients [136]. In order to link the expression of TDM1R signature with the activity of these processes, I have correlated the TDM1R signature score with the process scores of the patients. Among the ten processes, cell cycle was the top process that is significantly positively correlating with the T-DM1 resistance signature (Figure 3.15) which was further supporting the Metacore Pathway Analysis results that I obtained with the RNA-Seq data of the commonly differentially expressed genes. These analysis showed that the T-DM1 resistance signature found to be enriched with genes related to cell cycle is likely to be functionally related with the cell cycle process and that patients having a high T-DM1R signature has an active cell cycle.

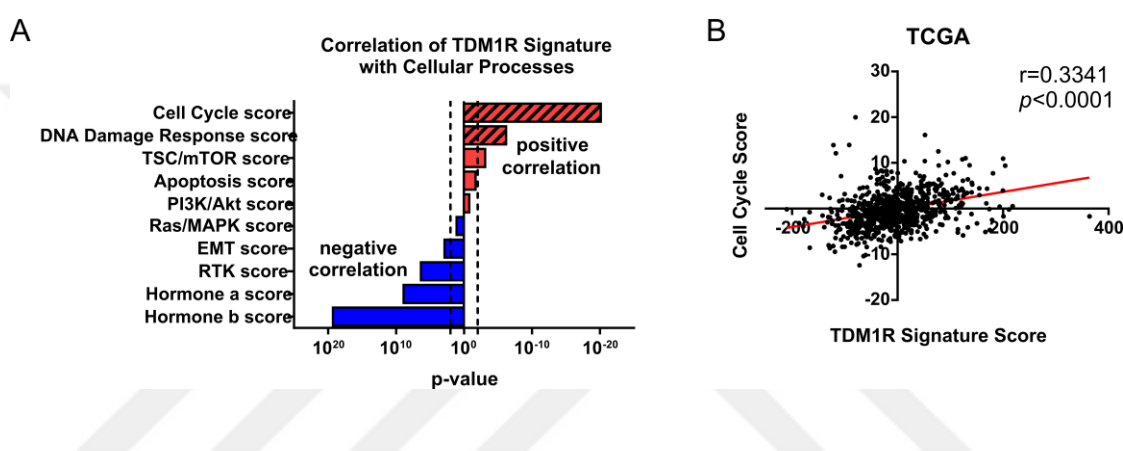


Figure 3.15: Analysis of TCGA data in terms of the correlation between TDM1R signature score and the score of cancer associated cellular process in breast cancer patients. (A) Cell cycle was identified as the top most significantly positively correlating cellular process with T-DM1 signature score in TCGA breast cancer patients. **(B)** The individual correlation plot of T-DM1R signature score with cell cycle score.

Genes functioning in similar cellular processes tend to be regulated in a similar way as to amplify the overall response that could be achieved from activation of individual proteins [158]. This tendency can be exploited in order to identify groups of genes that are working together in a process by looking at their co-expression patterns. The method is called correlation analysis, and it is also the idea behind personalized medicine which aims to suggest individual targeted therapies by looking at the co-expression profiles of drug sensitivity biomarkers in tumors [159]. I have hypothesized that if the cell cycle process is indeed the most significantly enriched process among my differentially expressed gene list, then an unsupervised clustering of those genes would unravel all the cell cycle related genes as a cluster. In order to test this, I have again utilized the TCGA breast cancer data and created

the correlation matrix of all the differentially expressed 331 genes by using their mRNA expression among these patients. Such an unsupervised clustering would strengthen the importance of cell cycle activation in T-DM1 resistance and would show that cell cycle related genes dominate the whole differentially expressed gene list more than any other cellular process that could possibly have a role in T-DM1 resistance. In order to test this, I have created the correlation matrix of all the differentially expressed 331 genes by using their mRNA expression data in TCGA breast cancer patients. As a result, I have obtained 4 main clusters among the upregulated genes that consist of genes positively correlating with each other based on their mRNA expression in patients (Figure 3.16).

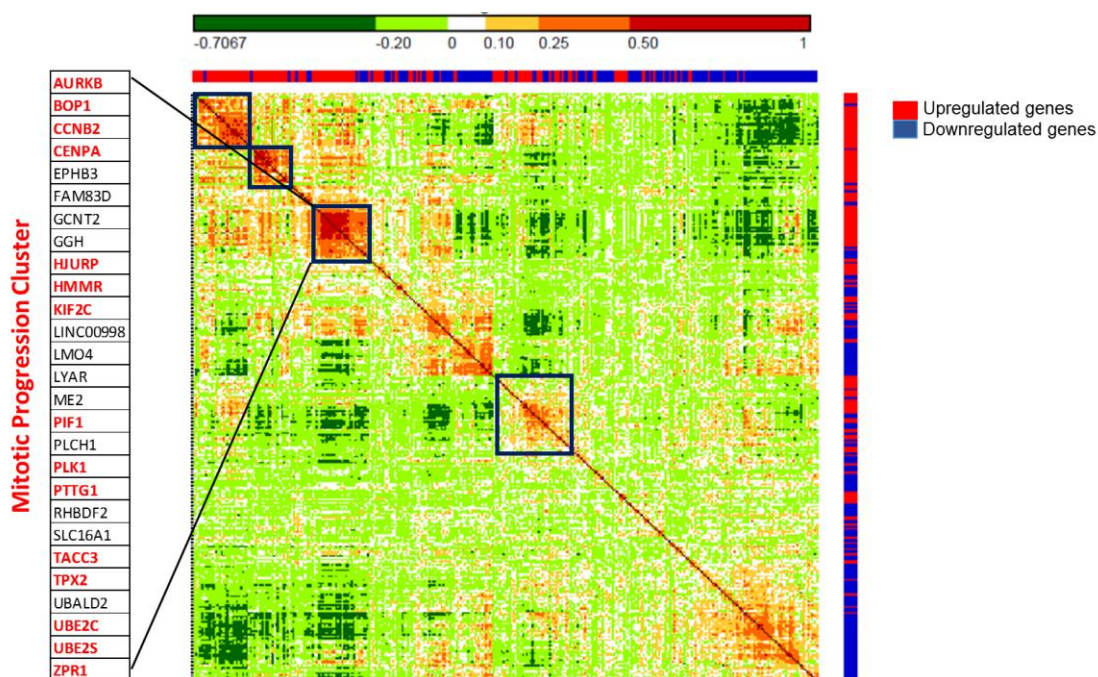


Figure 3.16: The correlation matrix of the 331 commonly differentially expressed genes in T-DM1 resistance obtained by using the mRNA expression data of TCGA breast cancer patients. mRNA expressions of the 331 differentially expressed genes in T-DM1 resistance in TCGA breast cancer patients were downloaded from cBio Portal, and the correlation matrix was generated by using GENE-E software. Pearson correlation was chosen as the metric, and average linkage was used as the linkage method. Red and blue colored genes were down and up-regulated genes in T-DM1 resistant cell line models, respectively. Color coding of the matrix depicts the Pearson correlation coefficients between two genes going from green to red as the correlation increases. The four major clusters formed mostly among the upregulated genes were depicted in squares. Genes in the most prominent cluster were listed.

Interestingly, when I analyzed the genes found in the most prominent cluster of all, I have seen enrichment of a lot of cell cycle related processes by Reactome Pathway Enrichment Analysis (Figure 3.17). This finding further strengthened the importance of cell cycle progression in T-DM1 resistance as a process and corroborated the results of the siRNA screen which yielded two cell cycle related genes; PLK1 and TACC3 as the common mediators of T-DM1 resistance in HER2-positive breast cancer models.

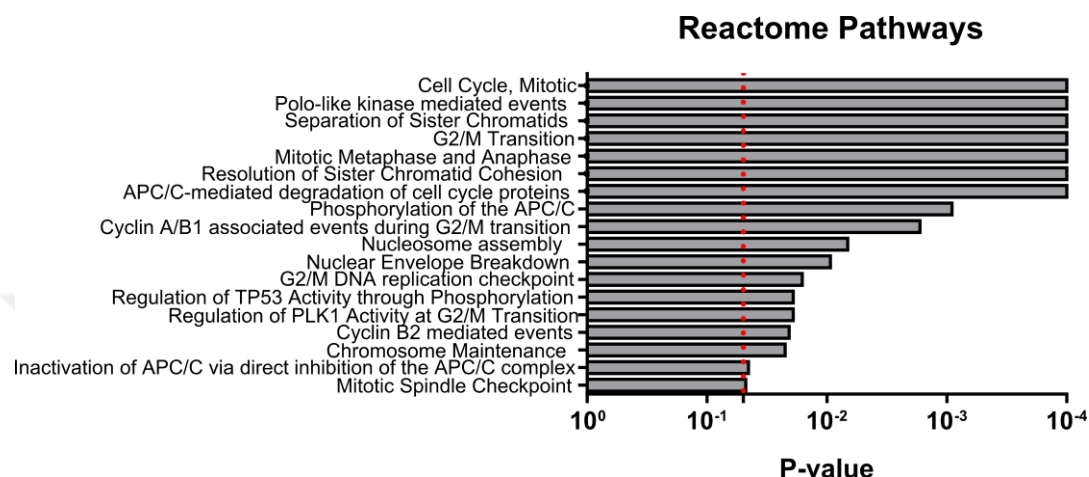


Figure 3.17: The enriched pathways among the most prominent cluster in Figure 3.16. Genes which formed the most prominent cluster among all the differentially expressed genes from Figure 3.16 were tested for their enrichment in cellular processes by using Reactome database which showed enrichment of a lot of cell cycle related gene sets with a significance cutoff of $p < 0.05$.

Interestingly, a similar clustering of the cell cycle related genes was also obtained within the 21 genes that were selected for the siRNA screen. This time, I have utilized the RPPA data of around 200 proteins involved in different processes again in TCGA breast cancer patients obtained from the TCPA database [136]. I have calculated the correlation coefficient of each gene with the individual RPPA proteins and looked for the similarity of these coefficients between genes. The generated distance matrix shows how close the two genes are to each other in terms of the pattern of correlation with the RPPA network. This can further be interpreted as follows; if two genes are connected with a blue color; i.e. if they have a short distance between each other, then they correlate with the RPPA proteins in a similar manner. As a result, I have again seen a similar clustering of the cell cycle related genes among the 21 candidates I chose for the siRNA screen (Figure 3.18)

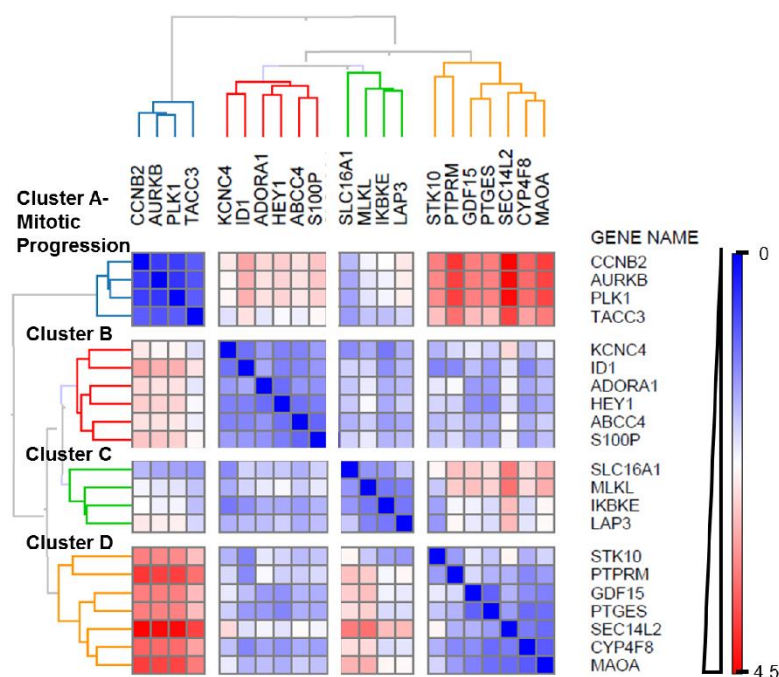


Figure 3.18: Distance matrix of the 21 druggable genes in terms of the correlation of their mRNA expression with the RPPA data of around 200 proteins involved in various cancer associated processes in TCGA breast cancer patients. The color coding depicts how close the two genes are with each other in terms of their correlation with the RPPA network. Distance between two genes are increasing as the color at their intersection of the genes goes from blue to red.

3.5. A BCL2 Dependency Emerges in T-DM1 Resistance for Growth in the Absence and Presence of T-DM1

BCL2 is a well-studied anti-apoptotic protein functioning upstream of Caspase activation mainly in the intrinsic apoptosis pathway [125, 126]. As I have seen a differential regulation of apoptosis in our WT and TDM1R models, I wanted to check the levels of BCL2 mRNA and protein in T-DM1 resistant cell lines. As can be seen in Figures 3.19A and 3.19B, BCL2 was found to be highly upregulated in TDM1R cell lines at both mRNA and protein levels. This might indicate the emergence of a BCL2 dependence for survival in T-DM1 resistance. To check if the resistant cells are more dependent on BCL2 for their growth, I have inhibited BCL2 with a BCL2 family inhibitor, ABT-263 and looked at the changes in cell viability in WT and TDM1R models. As can be seen in Figures 3.20A and 3.20B, both of the TDM1R models had significantly less survival in the presence of BCL2 inhibition than WT cells.

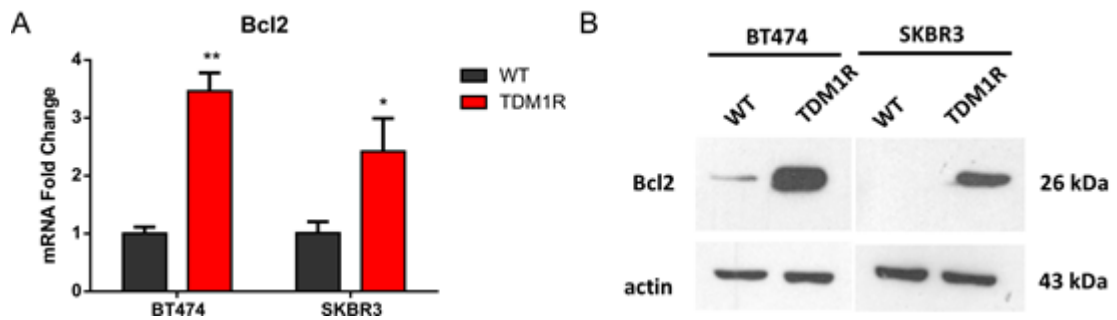


Figure 3.19: BCL2 is overexpressed in T-DM1 resistance. qRT-PCR (A) and Western Blot (B) analysis of BCL2 in WT and TDM1R models. ACTB and HPRT were used as housekeeping genes for qRT-PCR. Actin was used as the loading control for Western Blot analysis.

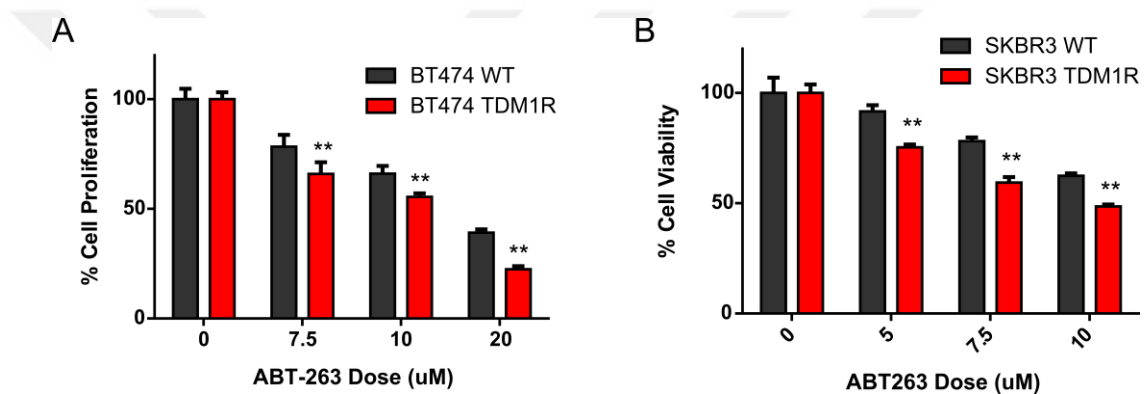


Figure 3.20: TDM1R cells become dependent on BCL2 for their survival. WT and TDM1R models of the BT474 (A) and SK-BR-3 (B) cells were treated with ABT-263 in quadruplicates and cell proliferation was assessed after 72 hours.

BCL2 protein was shown to be phosphorylated during prolonged mitosis, usually from multiple sites which is associated with the inhibition of its anti-apoptotic activity, and subsequently apoptosis [160-163]. Ser70 was shown to be the major site phosphorylated upon treatment with anti-mitotics [164]. Therefore, I wanted to check the phosphorylation status of Ser70 residue of BCL2 in the presence of T-DM1 alone and T-DM1 combined with volasertib to see if there is any regulation on BCL2 phosphorylation during T-DM1 sensitization with PLK1 inhibition. I have seen that although T-DM1 alone did not cause an increase in BCL2 phosphorylation, when combined with volasertib, it prominently increased phosphorylated BCL2 on Ser70 (Figure 3.21).

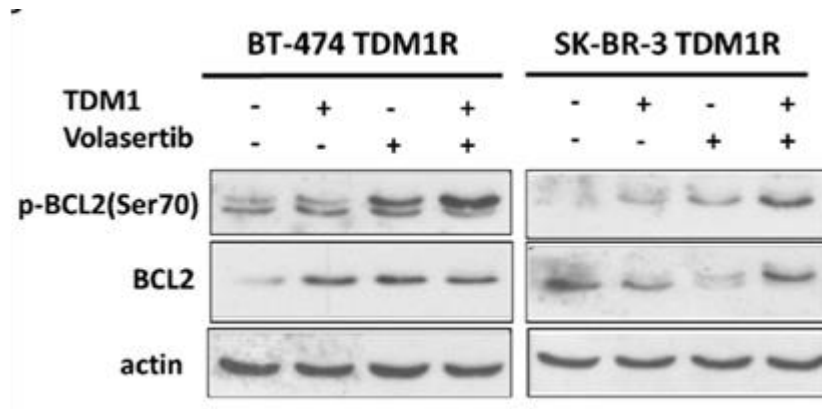


Figure 3.21: Inhibition of PLK1 by Volasertib enhances BCL2 phosphorylation which may partially be responsible from re-induction of apoptosis by T-DM1 upon PLK1 inhibition in both TDM1R models. Western Blot analysis of Ser70 phosphorylated and wild-type BCL2 showed a synergistic increase in BCL2 phosphorylation at Ser70 residue after 24 hours of treatment with 25 nM Volasertib and 20 ug/ml T-DM1.

This indicates that re-gain of apoptosis by PLK1 inhibition in the presence of T-DM1 is partly by inactivation of BCL2 through phosphorylation of Ser70 site. If this is the case, then inhibition of BCL2 by ABT-263 should partially mimic the T-DM1 sensitization obtained by volasertib. As shown in Figure 3.22, ABT-263 combination with T-DM1 significantly reduced cell viability as compared to either T-DM1 or ABT-263 alone.

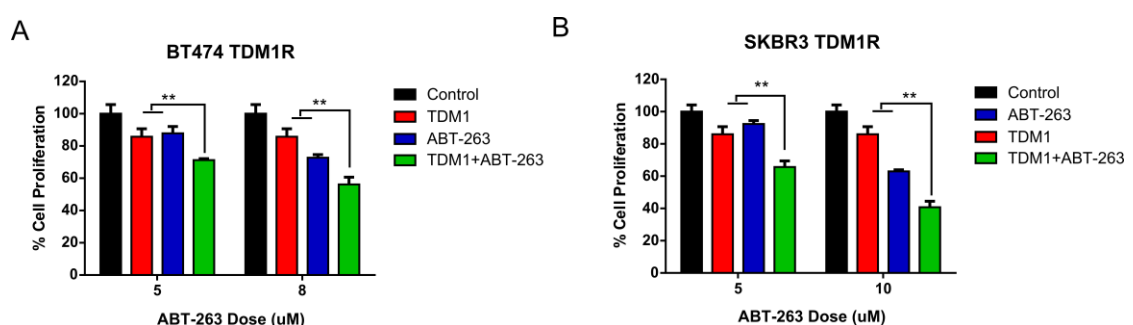


Figure 3.22: Inhibition of BCL2 family proteins by ABT-263 sensitizes resistant cells to T-DM1. Both T-DM1 resistant BT474 (A) and SK-BR-3 (B) cells were treated with combination of T-DM1 and ABT-263 with two different doses of ABT-263 (5 and 8uM for BT474 TDM1R & 5 and 10 uM for SK-BR-3 TDM1R) in quadruplicates for 72 hours and cell proliferation was measured with Cell Titer Glo Reagent.

CHAPTER 4

DISCUSSION

Resistance against anti-cancer therapeutics is a frequently observed phenomenon which severely hinders the efficacy of many initially promising agents. T-DM1 is an ADC of trastuzumab and DM1, and it is currently being used in clinics for the treatment of refractory HER2-positive breast cancer patients since 2013. Resistance against T-DM1 was detected in patients after 5-6 months of treatment, and there is an urgent need for the identification of resistance mechanism so as to augment the response to this promising targeted therapy agent.

Here, I have deciphered two novel mechanisms of resistance against T-DM1, which are abrogation of mitotic arrest and inhibition of apoptosis. I have genomically characterized two different *in vitro* models of acquired resistance and performed a targeted siRNA screen with the druggable genes upregulated in T-DM1 resistance. As a result, I have identified two mitotic genes; PLK1 and TACC3 as the common mediators of resistance against T-DM1 in HER2-overexpressing models and later showed that pharmacological inhibition of these proteins by small molecule inhibitors sensitized cells to T-DM1 induced growth inhibition and apoptosis. I have uncovered cell cycle regulation as the most prominently activated process in T-DM1 resistance and associated the signature of resistance with the cell cycle activation score in breast cancer patients. I have decoded an increased dependency of the resistant cells on the anti-apoptotic protein; BCL2 for cellular growth and identified phosphorylation and inactivation of BCL2 as one of the possible mechanisms of volasertib induced T-DM1 sensitization. Based on these results, I proposed a model for T-DM1 cytotoxicity and the acquired resistance against this HER2-targeted therapy where overexpression of PLK1, TACC3 and BCL2 protects cells from the T-DM1 induced mitotic arrest and apoptosis. Upregulation of PLK1 and TACC3 favors mitotic exit even in the presence of the anti-mitotic stress induced by T-DM1. At the same time, the overexpressed BCL2 confers a growth advantage for the resistant cells and protects them from apoptosis. Upon inhibition of PLK1 and TACC3 by volasertib and KHS101, respectively; T-DM1 makes the cell arrested in mitosis and undergo apoptotic cell death by inactivation of BCL2 through phosphorylation at Ser70 (Figure 4.1).

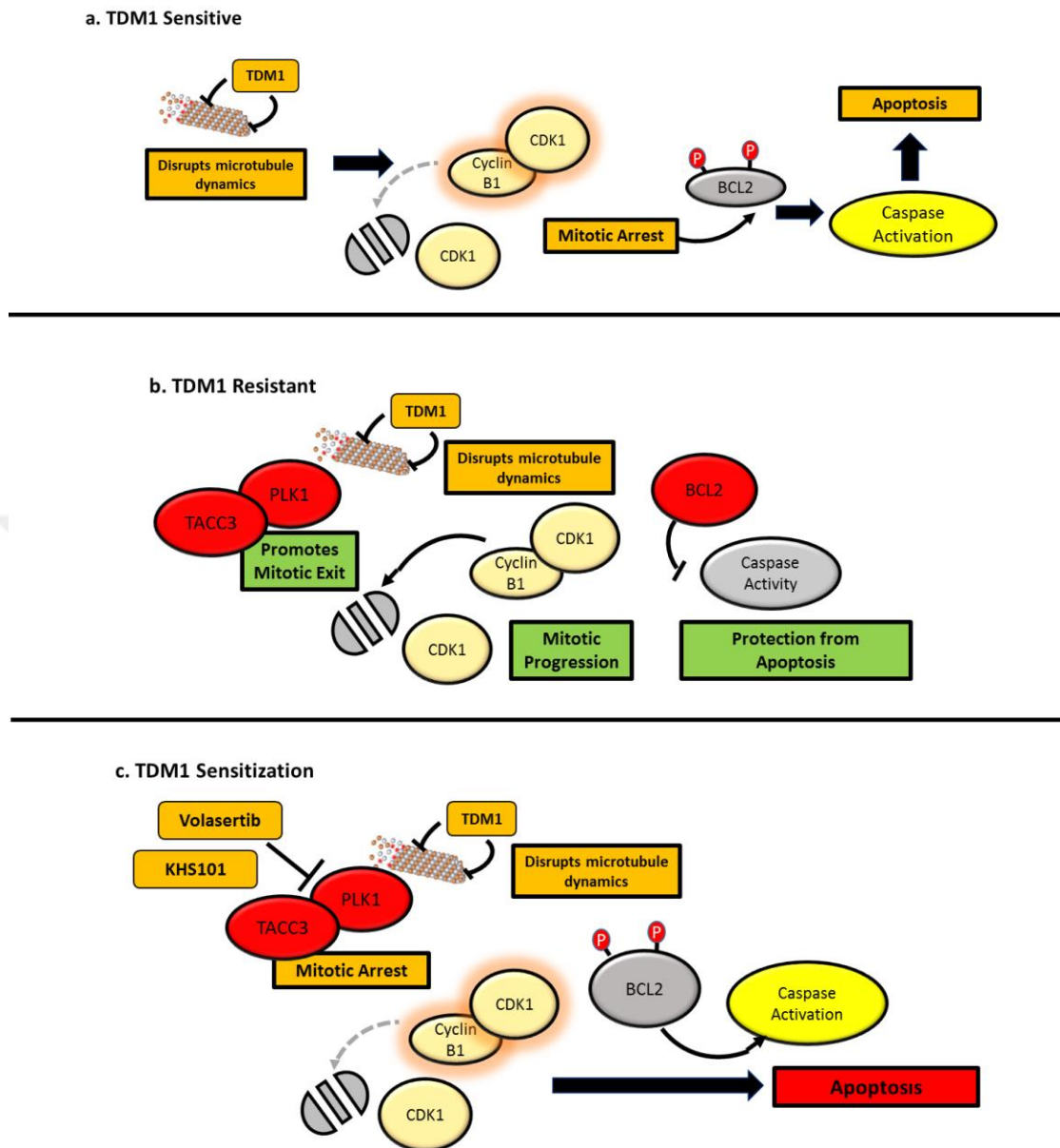


Figure 4.1: The predicted mechanisms of cytotoxicity of T-DM1, resistance against T-DM1 and T-DM1 sensitization with PLK1 or TACC3 inhibition by Volasertib and KHS101, respectively. In T-DM1 sensitive cells, T-DM1 induces mitotic arrest upon disruption of microtubules which is characterized by accumulated Cyclin B1 protein. Prolonged mitotic arrest leads to BCL2 inactivation by phosphorylation which further results in Caspase activation and apoptosis. In case of T-DM1 resistance, upregulated PLK1 and TACC3 proteins promote mitotic progression and at the same time, overexpressed BCL2 confers growth advantage both in the absence and presence of T-DM1 and protect cells from apoptosis. Upon PLK1 or KHS101 inhibition by Volasertib and KHS101, respectively, cells

get arrested at mitosis and BCL2 gets phosphorylated in case of Volasertib-T-DM1 combination which further causes regain of apoptosis by activating Caspases.

4.1. T-DM1 Resistance is Characterized by Abrogation of Mitotic Arrest and Apoptosis

In order to characterize resistance against T-DM1 and to identify novel targets that can be inhibited for T-DM1 sensitization, I have generated two *in vitro* models of resistance by continuously exposing BT474 and SK-BR-3 breast cancer cell lines to T-DM1. One of the two resistant models (BT474 TDM1R) was designed to be a high-degree resistant model which was developed by following a dose escalation procedure whereas for the other model (SKBR3 TDM1R), the dose was kept constant and more time was provided for the cells to recover. The latter methodology may reflect the clinical application of T-DM1 better. However, high degree resistant models are always more stable and easier to maintain in culture and display a higher degree of genomic and phenotypic separation from their wild-type counterparts. Since I have concentrated on the common changes in both models, my results would represent the most prominent changes in the resistance that are also clinically applicable.

I have seen a lack of mitotic arrest and apoptosis in T-DM1 resistant models, whereas a prominent increase in both of the cellular phenotypes was seen in WT cells upon T-DM1 treatment (Figures 3.2-3.5). Although G2/M arrest and apoptosis have previously been shown to occur upon T-DM1 treatment, they have never been associated with resistance against T-DM1 before. Therefore my results on the loss of mitotic arrest and apoptosis in T-DM1 resistance are unique. In order to identify the molecular mechanisms of this resistance against T-DM1, I have genomically characterized the resistance by whole genome RNA sequencing in collaboration with DKFZ. Next-generation sequencing has proven to be a highly effective way of decoding the mechanisms underlying a cellular phenotype. By tracing the genomic changes associated with acquisition of drug resistance, one can obtain list of genes deregulated in resistance and that may be important for the observed phenotype. Identifying the functional mediators among the differentially expressed genes requires the implantation of a screening strategy where you screen some or all of the deregulated genes in terms of their potential to reverse the resistance when their expression is altered in the opposite way [165]. Here, I have genomically characterized the resistance against T-DM1 by whole genome RNA sequencing and identified the list of deregulated genes among which I have selected the upregulated ones with druggable protein products for the following targeted siRNA screen. As a result of the screen, I have identified genes whose genomic inhibition by siRNAs in the

presence of T-DM1, significantly reduces cellular growth of the resistant cells as compared to T-DM1 alone.

4.2. PLK1 and TACC3 were Identified as the Common Mediators of T-DM1 Resistance

The targeted siRNA screen yielded two mitotically important genes; PLK1 and TACC3 as the common mediators of T-DM1 resistance whose genomic inhibition caused sensitization to T-DM1 (Figure 3.8). PLK1 has previously been intensely studied as a biomarker of disease progression and response to anti-cancer therapy. It has an important role in promoting mitotic exit by activating the APC, and therefore, its overexpression in T-DM1 resistant models could partially explain the lack of mitotic arrest in TDM1R cells upon T-DM1 treatment. It is overexpressed in several different malignancies and was proposed as a highly feasible therapeutic target that only affects cancer cells upon inhibition [76, 80, 84, 89, 166]. Depletion of PLK1 in normal breast cells, MCF10A, resulted in no apparent growth inhibition or cell cycle arrest [166]. Since PLK1 is a serine/threonine kinase mediating the phosphorylation and activation of many cell cycle regulated genes [75, 149, 152], a possible dependency to PLK1 expression may likely be owing to its kinase activity. In the sensitization experiments that I did with the siRNA knockdown of the gene and the pharmacological inhibition of the kinase activity of PLK1, I have seen a better sensitization with volasertib as compared to siRNA against PLK1. This may be due to the lower siRNA concentration that I had to use during the experiments so as not to cause too much toxicity. PLK1 depletion alone has greatly affected the viability of the cells with even 50% reduction in its mRNA level. For this reason I had to decrease the siRNA concentration by half to decrease the cell death due to PLK1 depletion. In this case, the sensitization remained at a minimal level. On the other hand, inhibition of the kinase activity alone, did not result in much toxicity, but it greatly synergized with T-DM1 at the same dose. These results suggest that the T-DM1 resistant cells may become dependent on both the presence of the PLK1 protein as well as its kinase activity. In basal levels, in the absence of T-DM1, kinase activity may not be as important as the physical presence of the protein; however, when the cells are under drug treatment, they may strongly require the protein phosphorylations mediated by PLK1.

In the targeted siRNA screen that I have performed with genomic inhibition of several genes upregulated in T-DM1 resistance, the second gene I obtained as the common mediator of T-DM1 resistance in both models besides PLK1 was TACC3. TACC3 is a spindle protein that is important for proper chromosomal segregation like PLK1. Upregulation of TACC3 in the T-DM1 resistant models may contribute to resistance by preventing the disruptive effects

of T-DM1 on the microtubule dynamics. TACC3 was also shown to be overexpressed in several different cancers and associated with anti-cancer therapy resistance [156, 167-170]. In case of TACC3, I have seen a better sensitization with siRNA knockdown of the gene as compared to pharmacological inhibition with KHS101. Since TACC3 is a spindle protein whose main function is to control proper chromosome segregation, it is plausible to observe a more pronounced anti-proliferative effect in case of protein depletion than pharmacological inhibition. Although one study with KHS101 showed TACC3 protein degradation upon KHS101 treatment [119], downregulation of TACC3 with KHS101 in the T-DM1R models needs to be tested.

Interestingly, although the targeted siRNA screen identified inhibition of PLK1 and TACC3, which are among the four cell cycle related genes in the list of 21 upregulated targets, as sensitizers of T-DM1, inhibition of the other cell cycle related genes; Cyclin B1 and Aurora B failed to mediate sensitization. Cyclin B1 silencing was among the top most powerful sensitizers in SK-BR-3 model; however, it had no effect on BT474 model. On the other hand, Aurora B inhibition was inducing sensitization in none of the models. Since the lack of the phenotype could be due to an absence of knockdown by the siRNAs, I have first checked the knockdown efficiency of these genes. I have confirmed knockdown of both of these genes with the siRNAs being used which indicated that the lack of sensitization is actually related to the biological functions of these genes. The absence of sensitization could be explained by the nature of microtubule disruption with DM1. DM1 inhibits microtubule polymerization and generates unattached microtubules. Aurora B is a CPC component which acts on incorrectly attached microtubules by sensing loss of tension at the microtubule-kinetochore junctions [171]. It may seem that the functioning of CPC is dispensable for the protection against T-DM1 mediated microtubule disruption due to a complete loss of instead of an incorrect attachment at the kinetochores. However, studies with nocodazole which has very similar mechanisms of action with DM1, showed that other components of the CPC may still be important since only genomic depletion, but not the pharmacological inhibition of Aurora B, the latter being unable to completely inactivate CPC interferes with nocodazole mediated mitotic arrest. It may also be that in T-DM1 resistance, at low doses of T-DM1, a certain degree of CPC activation confers a growth advantage by keeping the CPC complex at the kinetochore-microtubule junctions at a basal level so as to repair the damage induced by T-DM1. However, when drug dose is increased to a level where some degree of growth inhibition starts to occur, the overexpressed Aurora B might become a death inducer by activating SAC.

4.3. Pharmacological Inhibition of PLK1 and TACC3 In the Presence of T-DM1 Re-induces Apoptosis in TDM1R Models

Since apoptotic cell death is a highly effective way of cell killing, resistance against this phenomenon induced by many anti-cancer agents provides a great advantage for those cells who manage to evade from the activation of the apoptotic cascade. I have identified the apoptotic cell death mediated by Caspase 3 as the mechanism of T-DM1 induced growth inhibition. Therefore it was highly plausible to see an abrogation of T-DM1 induced apoptosis in my resistant models. As I have shown with an apoptosis assay and a western blot analysis of cleaved Caspase 3 and cleaved PARP, it seems that TDM1R cells found a way to silence the activation of the apoptotic machinery upon drug treatment (Figures 3.4 and 3.5). This could be due to an absence of mitotic arrest in resistant models with T-DM1 treatment (Figure 3.3) and the regain of apoptosis I observed with PLK1 and TACC3 inhibition may therefore be through re-induction of mitotic arrest. Since both of these genes were depicted to exert most of their functions in mitosis, it is highly probable that the re-induction of mitotic arrest with PLK1 and TACC3 inhibition in the presence of T-DM1 is responsible for the apoptotic cell death. However, it should still be tested if apoptosis observed in TDM1R cells treated with the combination of T-DM1 and volasertib or KHS101 is due to a regain of mitotic arrest.

4.4. A BCL2 Dependency Emerges upon Acquisition of T-DM1 resistance

It could be hypothesized that since the T-DM1 resistant cell line models are able to evade from the induction of mitotic arrest upon treatment with T-DM1, they might not necessarily adapt a resistance mechanism related to protection from apoptosis. However, such an assumption would be misleading since there could be many parallel mechanisms formed at similar time points all contributing to the overall resistance achieved as the cells treated with T-DM1 continuously. Studies have shown the contribution of a variety of genetic alterations to the final resistant phenotype [172, 173]. For this reason, it is plausible to expect a change in the expression of pro or anti-apoptotic genes in TDM1R models as a means to attenuate the execution of drug induced apoptosis, parallel to abrogation of mitotic arrest. The most well-known anti-apoptotic BCL2 family protein is BCL2 and its contribution to resistance against many cytotoxic agents has strongly supported by plenty of *in vitro*, *in vivo* and clinical studies [122-124, 174-178]. For this reason I wanted to check the differential expression of BCL2 in the T-DM1 resistant models. I have seen an upregulation in both models although the RNA sequencing showed overexpression only in BT474 cell line. This could be because SK-BR-3 is a BCL2-negative cell line and therefore BCL2 mRNA might not have been detected in

RNA sequencing in the WT cells with Western Blot due to very low levels of expression. Supporting this, I could detect no band for BCL2 in WT SK-BR-3 cells. Importantly, although there was no BCL2 expression in WT cells, the T-DM1 resistant cells were expressing high levels of BCL2 (Figure 3.19). This was also the case in BT474 cells although in this model, WT counterpart also expresses certain level of BCL2 (Figure 3.19B). BCL2 overexpression has previously suggested to increase the dependency of those cells overexpressing BCL2 on the expression of this anti-apoptotic pro-survival gene [179]. Consistent with this, I have observed a significantly more growth inhibition with the BCL2 family mimetics, ABT-263 treatment in TDM1R cells as compared to WT counterparts (Figure 3.20). This inhibitor inhibits the dimerization of the anti-apoptotic BCL2 family proteins with the pro-apoptotic BH3-only and Bax family members. The enhanced dependency on BCL2 that I have observed in TDM1R models could be due to the presence of high levels of pro-apoptotic proteins accumulated in cells during treatment with T-DM1. In such a case, overexpression of BCL2 would be highly necessary in order to sequester the pro-apoptotic members so that they cannot initiate cell death [180].

4.5. Phosphorylation of BCL2 May, In Part, Be Responsible For T-DM1 Sensitization with PLK1 Inhibition by Increasing Apoptosis

BCL2 protein was shown to be phosphorylated in response to several stimuli. Treatment with survival agonists or protein kinase activators was demonstrated to cause phosphorylation of BCL2 at Ser70 residue which followed a highly dynamic pattern controlled by Protein Kinases and the BCL2 phosphatase PP2A. Agonist mediated phosphorylation was found to augment the anti-apoptotic activity of BCL2 and to be important for cellular survival [181]. On the contrary, phosphorylation of BCL2 at the same site was also observed upon treatment with anti-mitotic drugs but in this case, it was identified as an inhibitory phosphorylation which was disrupting the interaction of BCL2 with the pro-apoptotic family members. In line with this, I have observed an increase in BCL2 phosphorylation at Ser70 residue when I combined T-DM1 with volasertib in TDM1R models. Although BCL2 phosphorylation was previously shown to occur upon treatment with anti-mitotics, it has never been associated with PLK1 inhibition before. This finding suggests that the regain of T-DM1 induced apoptosis with PLK1 inhibition in TDM1R models could, in part, be mediated by inhibition of BCL2 through phosphorylation at Ser70 residue.

The kinases mediating BCL2 phosphorylation in response to different stimuli are largely unknown, although several kinases were proposed to be responsible such as CDK1,

JNK and Protein Kinase C (PKC) [181-184]. In case of anti-mitotics induced phosphorylation, CDK1 seems to be the most plausible kinase that links prolonged mitotic arrest with apoptosis. However, it seems that it is not a universal BCL2 kinase functioning in mitosis, since in one study, JNK but not CDK1 was shown to induce BCL2 phosphorylation in G2/M phase of the cell cycle [183]. From these findings, it is obvious that the phosphorylation status of BCL2 is controlled by a lot of different protein kinases, most of which have not been identified yet. The Ser70 phosphorylation of BCL2 that I have observed in case of T-DM1 sensitization with volasertib suggests the presence of a possible link between PLK1 inhibition and BCL2 phosphorylation, mechanisms of which should further be studied in detail. Supporting the involvement of BCL2 anti-apoptotic activity in mediating T-DM1 resistance, I have seen a higher T-DM1 induced growth inhibition when BCL2 is inhibited with ABT-263. Overall, BCL2 overexpression seems to confer a growth advantage and a mechanism for evasion from T-DM1 induced apoptosis to T-DM1 resistant cells which make it a highly attractive target to be inhibited in T-DM1 refractory patients.

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, I have uncovered some of the resistance mechanisms against T-DM1 which is the newest anti-HER2 therapy currently used in clinics for the treatment of refractory HER2-positive breast cancer. I have developed two *in vitro* models of T-DM1 resistance and identified cell cycle as the most prominently activated process upon acquisition of resistance based on the expression profiles both in the cell line models as well as in breast cancer patients. PLK1 and TACC3 were among the two significantly upregulated genes in resistance whose inhibition with siRNAs and small molecule inhibitors significantly enhanced the growth inhibition induced by T-DM1 *via* activating apoptosis. The rewiring of the apoptotic machinery has made the resistant cells more dependent on BCL2 expression that could further be exploited as a way of antagonizing the growth of T-DM1 resistant tumors in the combat of reversal of drug resistance in HER2-positive breast cancer. The importance of apoptotic re-activation in T-DM1 sensitization has also been supported by an enhanced T-DM1 sensitivity with inhibition of anti-apoptotic BCL2 family proteins. Phosphorylation of BCL2 at a site previously shown to inactivate the anti-apoptotic activity of the protein was proposed as a possible mechanism of T-DM1 sensitization with PLK1 inhibition.

Volasertib is a drug that is currently in Phase III trials for AML patients ineligible for intensive treatment and so far its clinical usage was supported by several studies showing good anti-cancer efficacy either as a single agent or as a combination therapy with other anti-cancer treatments. Therefore, any result presenting PLK1 as a target in the context of cancer progression or drug resistance can easily be translated into clinics. The *in vitro* T-DM1 sensitization that I have shown with PLK1 and TACC3 inhibition should further be supported with a preclinical study where xenografts of T-DM1 resistant cells or PDX models of T-DM1 refractory patients are treated with volasertib in combination with T-DM1 and tumor progression is to be monitored. Unfortunately, there is currently no inhibitor against TACC3 with a good stability to allow its usage in *in vivo* models. However, owing to the results of recently conducted studies on TACC3 importance as a biomarker of disease progression and drug resistance, it is a matter of time until an effective inhibitor becomes available for *in vivo* and clinical usage for TACC3 as well.

The findings presented in this thesis, propose cell cycle progression which is frequently altered in cancer progression and acquisition of drug resistance as a mechanism of

resistance against T-DM1 and it suggests PLK1 and TACC3 as two important mitotic genes that are partially responsible for the resistance against T-DM1 by promoting the continuity of mitosis even in the presence of microtubule disruption. Inhibition of PLK1 and TACC3 which was shown to induce apoptosis in *in vitro* models of T-DM1 resistance can be exploited as a means to treat T-DM1 refractory HER2-positive breast cancer patients. Although both of these genes were shown to be important in executing a faithful mitosis, whether there is a link between the two, needs to further be tested. Both of these genes function at the centromeric regions of the chromosomes and their genomic inhibition results in highly similar phenotypes. It is very likely that there is a physical association between PLK1 and TACC3 which can be elucidated by checking the co-localization of the two proteins on centromeres.

Although G2/M arrest and apoptosis are the two previously identified mechanisms of T-DM1 cytotoxicity which were also presented in this thesis by using two different HER2-positive breast cancer cell line models, the link between prolonged mitotic arrest and apoptosis has not yet fully established. Phosphorylation of BCL2 protein at different sites has previously been reported to be highly context dependent which may either stimulate or inhibit the anti-apoptotic functions of BCL2 [161, 185-187]. Ser70 phosphorylation was previously shown to occur in response to anti-mitotic agents and cause the loss of BCL2's anti-apoptotic activity. Although there are a few identified BCL2 kinases, the exact mechanisms of the regulation of BCL2 through phosphorylation are largely unknown. PLK1 inhibition has never been associated with BCL2 phosphorylation and whether this phosphorylation is a consequence of the mitotic arrest or there is a link between PLK1 and BCL2 independent of prolonged mitosis need to be studied in detail. Analyzing the time dependent phosphorylation pattern of BCL2 with the treatment of T-DM1 alone or in combination with volasertib in the resistant models would provide a clue on how BCL2 is regulated in cells resistant to microtubule targeting agents.

It should also be studied if there is any relation between the p53 status and the degree of T-DM1 sensitization with PLK1 inhibition since p53 status was previously shown to determine dependency on PLK1. P53 mutant cells were found as being more prone to cell death induced by PLK1 depletion [166]. This could be due to the fact that cells with functional p53 can undertake apoptosis even if the cells that are arrested at mitosis slipped into next G1. For those cells apoptosis during mitotic arrest becomes dispensable. On the other hand, cells with mutant p53 need to be eradicated in mitosis since they may not mediate an apoptotic cell death in G1. However, it should also be noted that p53 mutations are not necessarily interfere with p53 functioning as we have also showed that the pro-apoptotic gene, Noxa can mediate apoptotic cell death following arrest at G2/M in p53-mutant cells [188]. Elucidating a possible

relation between the p53 status and the degree of T-DM1 sensitization with volasertib would be useful in order to identify the patient subgroups that will benefit from volasertib in combination with T-DM1 the most.

The BCL2 overexpression and an observed dependency on BCL2 for survival in T-DM1 resistant models can further be exploited as a means to inhibit the growth of T-DM1 refractory tumors. Identification of protein kinases and phosphatases linking PLK1 inhibition with BCL2 phosphorylation would uncover novel tumor suppressors or oncogenes that could further be regulated to inhibit BCL2 by enhancing its phosphorylation. However, more studies need to be conducted in order to fully decipher the mechanisms of T-DM1 sensitization obtained with volasertib and KHS101 and to verify the reversal of resistance in *in vivo* models of resistance.



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APPENDIX

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