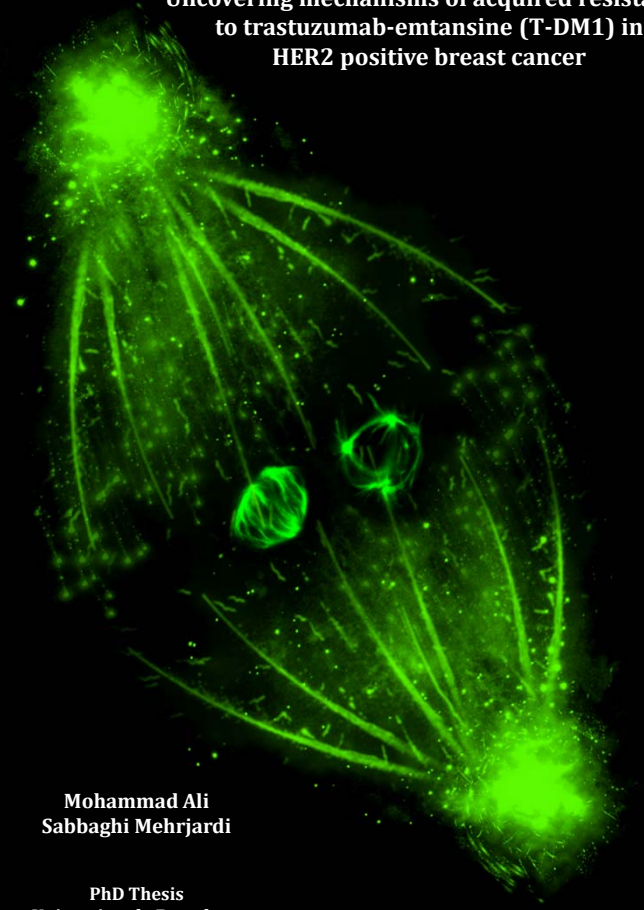
Uncivering mechanisms of acquired resistance to trastuzumab-emtansine (T-DM1) in HER2 positive breast cancer

Mohammad Ali Sabbaghi Mehrjardi

Uncovering mechanisms of acquired resistance to trastuzumab-emtansine (T-DM1) in HER2 positive breast cancer

Uncovering mechanisms of acquired resistance to trastuzumab-emtansine (T-DM1) in HER2 positive breast cancer



Mohammad Ali Sabbaghi Mehrjardi

PhD Thesis
Universitat de Barcelona
2017

Uncovering mechanisms of acquired resistance to trastuzumab-emtansine (T-DM1) in HER2 positive breast cancer

Mohammad Ali Sabbaghi Mehrjardi

PhD Thesis, Universitat de Barcelona
2017

Thesis Directors
Dr. Joan Albanell and **Dr. Ana Rovira**

Molecular Therapeutics in Cancer Laboratory

Cancer Research Program

Hospital Del Mar Medical Research Institute

PhD thesis

Doctoral Program in Biotechnology

Uncovering mechanisms of acquired resistance to trastuzumab-emtansine (T-DM1) in HER2 positive breast cancer

Mohammad Ali Sabbaghi Mehrjardi

This PhD thesis has been performed under the direction of Dr. Joan Albanell and Dr. Ana Rovira in Molecular Therapeutics in Cancer Laboratory, Hospital del Mar Medical Research Institute

Dr. Joan Albanell Dr. Ana Rovira

Mohammad Ali Sabbaghi Mehrjardi

Cover Photo Designed By:
Mahdi Mirahmadi

TABLE OF CONTENT

TO WHOM LOVES JOURNEYS INTO UNKNOWNS

ACKNOWLEDGMENT

I would like to thank important people, without whom this dissertation would not have been possible: my thesis supervisors, my lab-mates, clinical study staffs and my family.

I am indebted to *Dr. Joan Albanell* for his nice supervision and for the important lessons he taught me during my PhD thesis. The challenges area in translational biology may not have ever pursued without his inspiration to only think for patients cure and comforts.

To *Dr. Ana Rovira*, I thank for being a supportive, strong guiding force as supervisor of my thesis. Without her kind recommendations and independence she gave me, I could not reach my story and important achievements during the thesis. Her vision on molecular cancer biology impressed me to work as hard as I can to find answer for many questions.

To *Dr. Gabriel Gil*, he offered a welcome, balancing perspective as a rigorous experimental biologist in cell cycle biology. He opened a new horizon in the project that needs a new vision. My experience working on many techniques related to cell cycle analysis were not achieved without his comprehensive kind comments. The lessons he taught me, I am sure, will help me in future.

To *Dr. Federico Rojo*, for kindly sharing his wisdom in the field of molecular pathology with us. I really enjoyed long

discussing meetings we had and the impressive comments he gave us to progress the project.

To *Dr. Ignasi Tusquets*, *Dr. Sonia Servitja*, *Dr. Clara Montagut* and *Dr. Edurne Arriola* without their kind helps and comments, the clinical part of this study was impossible.

To *Dr. Marta Salido*, *Dr. Aura Montasell, Dr. Jose Yelamos, Dr. Alexandre Calon* and *Dr. Atanasio Pandiella*, whom I am most appreciative for their supportive collaborations. Without them, we did not have these interesting findings. The puzzle never filled up without their help.

To *Dr. Antoni Zorzano*, *Dr. Anna Guma*, *Dr. Marta Camps* and *Dr. Katrin Niisuke*, you gave me the first impressions on working in the field of HER family biology. The short, but beneficial, stay by you gave me many experiences working in the lab.

To *Dr. Montserrat Arumi* and *Dr. Laia Serrano*, pathologists from Hospital del Mar kindly helped me in patients' explants preparation and analysis.

To *Dr. Jose Lloreta* and *Dr. Tarek Salman*, who helped me recover my health and took care of me being again ready for life and science.

I am thankful all the people of lab, formers and lately for the fresh, friendly and warm new atmosphere they provide in the lab. *Oriol Arpí*, *Anna Hernández*, *Israel Cañadas*, *Alba Dalmases*, *Silvia Menéndez*, *Cristina Guardia*, *Aïda Sampera*, *Francisco Javier Sánchez Martín*, *Jenniffer*

Linares, *Max Hardy-Werbin* and *David Casadevall*, I am proud to say my experience in the molecular therapeutics in cancer laboratory was very exciting and fun, and you all, has energized me to continue. We had memorable moments in last five years and I'm sure that life in Barcelona with you was one of my best experiences in my life. I sincerely hope I continue to have opportunities to interact with you all for the rest of my research career.

To *Sílvia Geeraerd*, who kindly helped me to solve the official issues during the PhD and for her generosity and patience to answer the questions.

To *Raúl Peña* and *Marc Bataller*, for their kind technical assistance and kind helps in handling problems.

We thank all the patients participated in the study for their cooperation.

And to my family,

Sepand, you are an angel. Surely, there is no word to describe an angel. Without you, I was not happy in life and carrier. You showed me love is endless.

To *My parents*, always encouraged me to do my best, no matter what I'm doing. Of course, thank you both for your constant support through the ups and downs of my academic career. Your confidence in me has enhanced my ability to get through it all and succeed in the end. Thanks for everything that helped me get to this day.

To my sister, *Fatemeh* and my brothers, *Mehdi* and *Mojtaba*, you were always support me in trouble. Happiness is the gift you are always giving me.

To *my parents in-law*, I found them as kind as my parents. Thank you trusts me in what I'm doing. You taught me to have infinitive love. I sometimes consider our lives a life-long experiment, in which chance and the interesting people we interact with split us on different paths and for sure, you are one of them.

THIS WORK WAS PARTIALLY SUPPORTED BY:

- Instituto de Salud Carlos III (ISCiii): PIE15/00008, PI15/00146, PI12/00680 and RD12/0036/0051

- Agència de Gestió d'Ajuts Universitaris i de Recerca (Generalitat de Catalunya): 2014 SGR 740

- EU through the regional funding development program (FEDER).

We thank the tumor bank of the Pathology Department of Hospital del Mar (MarBiobank) supported by a grant from the Xarxa de Bancs de tumors sponsored by Pla Director d'Oncologia de Catalunya (XBTC).

We thank Fundació Cellex (Barcelona) for a generous donation to the Hospital del Mar Medical Oncology Service.

Printing & Reprographics of A Specific Number of Thesis
Copies was Covered By The Funding Received From IMIM

ABSTRACT

Trastuzumab-emtansine (T-DM1) is an antibody-cytotoxic agent (DM1) conjugated drug. DM1 delivery by trastuzumab inside the HER2 positive cells affects microtubule polymerization, causing cell cycle arrest and finally cell death. Although T-DM1 is approved for the treatment of HER2 positive metastatic breast cancer patients, primary and acquired resistance towards this drug is still a main challenge. Looking for the mechanisms of resistance is necessary to improve patient selection and to develop novel treatment strategies.

Here, we focused on finding mechanisms of acquired resistance to T-DM1 in a panel of HER2 positive breast cancer cell lines (HCC1954, HCC1419 and SKBR3 parental *vs*. resistant cells). Resistant cells were generated by an established protocol of T-DM1 exposure, increasing the concentration of T-DM1 [1-4μg/mL], 3days on/3days off, for 54 days overall.

The binding of T-DM1 to HER2 and its internalization were similar in parental and resistant cell lines. Analysis of T-DM1 effects on cell cycle showed a significant induction of G2-M arrest in the parental cells, while this effect was not observed in the resistant cells. Expression/activity analysis of cyclin B1-CDK1 complex, the main apparatus involved in G2-M cell cycle arrest, showed a cyclin B1 accumulation induced by T-DM1 in the parental but not in the resistant cells. CDK1 activity was also correlated with cyclin B1 expression, increased following T-DM1 treatment in the parental but not in the resistant cells.

Functional analysis revealed that cyclin B1 knock down in the parental cells induced a significant T-DM1 resistance. Furthermore, the silencing of cdc20, a protein mainly involved in APC complex related cyclin B1 degradation, could sensitize the resistant cells to T-DM1. Finally, cyclin B1 induction by T-DM1 was confirmed in *in vivo* xenograft animal model and *ex vivo* fresh HER2 positive human breast cancer explants, respectively. By cyclin B1 induction pattern, we could categorize T-DM1 responsive/non-responsive in fresh breast cancer explants from HER2 positive breast cancer patients.

Our results showed that T-DM1 induced G2-M cell cycle arrest in a cyclin B1 dependent manner. Lack of these effects appeared in acquired T-DM1 resistant cells. Besides, similar pattern in G2-M and cyclin B1 was verified *in vivo* and in patients explants. These data strongly suggest that induction of cyclin B1 is necessary for T-DM1 anti-tumor effects and emerges as a potential pharmacodynamic marker. Our finding raises the question on what are the mechanisms leading to cyclin B1 dysregulation in resistant cells.

PREFACE

The work presented in this PhD thesis has been conducted in the *Molecular Therapeutics in Cancer Laboratory, IMIM Cancer Research Program*. "Uncovering mechanisms of acquired resistance to trastuzumab-emtansine (T-DM1) in HER2 positive breast cancer" was part of FIS project led by Dr. Joan Albanell. The results presented in this thesis are derived from this project and are under second review at Clinical Cancer Research Journal. Part of my work has also contributed to other papers published by the group.

The pre-clinical laboratory, coordinated by Dr. Ana Rovira, and the biomarker research laboratory, coordinated by Dr. Federico Rojo was involved in this project. A multidisciplinary team by oncologists, pathologists and biologists joint the knowledge of the different disciplines to work in a translational project with clinical relevant interest. The work was done in handy collaboration with the *Oncology* and *Pathology Departments* of *Hospital del Mar.* We have also counted on the valuable collaboration of Dr. Gabriel Gil, from *IMIM Cancer Research Program*.

Our finding in this study paved a way to open an Exploratory Clinical Trial (KATTIA GEICAM Study) to perform a comprehensive and integrative characterization of mechanisms of primary and acquired resistance to T-DM1 in a prospective cohort of progressive/recurrent HER2-positive breast cancer patients.

The research in the field of resistance to therapies seemed very easy-going in the beginning, but at the end of story, I can say findings in this field are very difficult and needs patience, hard work, collaborations and luck.

ABBREVIATIONS AND ACRONYMS

T-DM1: Trastuzumab-emtansine

MAPK: Mitogen-activated protein kinases

PI3K: Phosphoinositide 3-kinase

ATP: Adenosine triphosphate

EGFR: Epidermal growth factor receptor

HER2: Receptor tyrosine-protein kinase erbB-2

HER3: Receptor tyrosine-protein kinase erbB-3

HER4: Receptor tyrosine-protein kinase erbB-4

AREG: Amphiregulin

EFG: Epidermal growth factor

HB-EGF: Heparin binding EGF like growth factor

FDA: Food and drug administration

ELISA: Enzyme-Linked ImmunoSorbent assay

FISH: Fluorescence *in situ* hybridization assay

miRNA: microRNA

TP53: Tumor protein p53

Tyr: Tyrosine

NIH: National institute of health

IHC: Immunohistochemistry

ADCC: Antibody-dependent cellular cytotoxicity

ER: Estrogen receptor

PR: Progesterone receptor

IGF-1R: Insulin-like growth factor-1 receptor

c-Met: Tyrosine-protein kinase Met

EpoR: Erythropoietin receptor

c-CBL: Casitas B-lineage lymphoma

SRC: Proto-oncogene, non-receptor tyrosine kinase

ADC: Antibody-drug conjugated

CDKs: Cyclin dependent kinases

c-casp3: Cleaved-caspase3

***i.v.*:** Intravenous injection

Min: Minutes

%: Percentage

INTRODUCTION

INTRODUCTION

I.1 Breast cancer

Breast cancer is the most common cancer in women worldwide, with closely 1.7 million new cases identified in 2012 (second most common cancer overall). This means about 12% of all new cancer cases and 25% of all cancers in women (1). The incidence and mortality of breast cancer are different geographically (**Figure I.1**). In recent decades, the widespread use of mammographic screening has increased the rate of early disease detection, and the development of more effective adjuvant chemotherapeutic regimens, extended use of endocrine therapies, and standard application of targeted anti-HER2 agents have all contributed to improve outcomes of women with primary breast cancer.

I.1.1 Different types of breast cancer

There are different types of breast cancer. The type of breast cancer depends on which cells in the breast turn into cancer. Breast cancer can initiate in different parts of the breast, like the ducts or the lobes.

Ductal carcinoma is the most common type of breast cancer (2). It begins in the cells that line the milk ducts in the breast, also called the lining of the breast ducts. Ductal carcinoma is categorized to (**Figure I.2**):

- Ductal carcinoma *in situ* (DCIS)
- Invasive ductal carcinoma

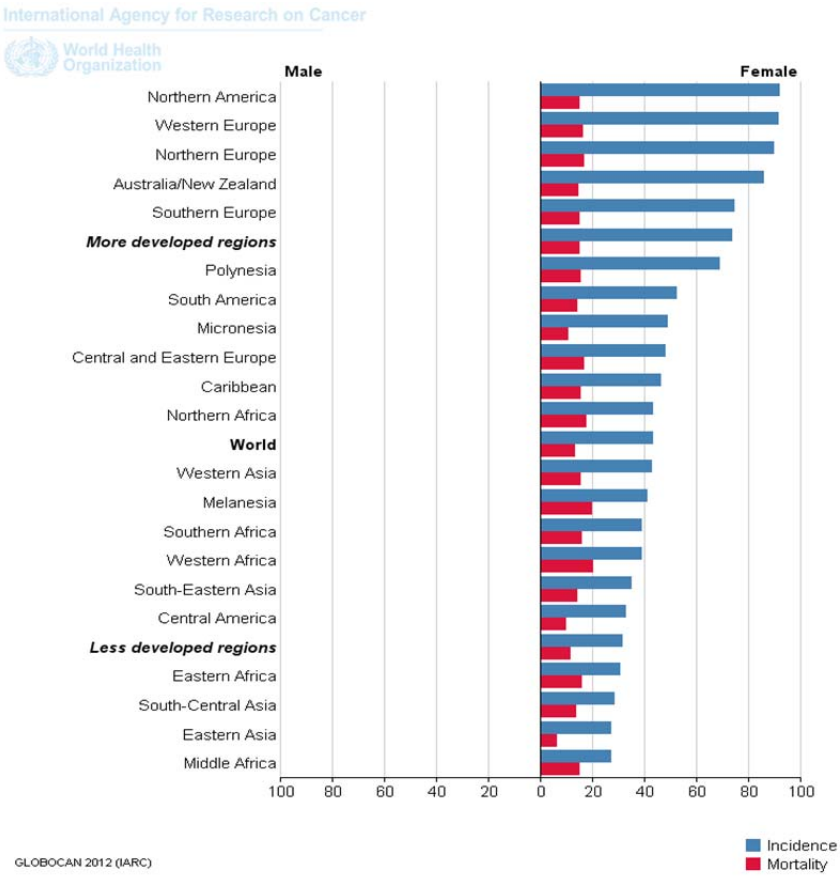


Figure I.1 Breast cancer incidence and mortality worldwide

Breast cancer ranks as the fifth cause of death from cancer overall (522,000 deaths). While breast cancer is the most frequent cause of cancer death in women in less developed regions (324,000 deaths, 14.3% of total), it is now the second cause of cancer death in more developed regions (198,000 deaths, 15.4%) after lung cancer

Another type of breast cancer is called Lobular carcinoma (3). In this kind of breast cancer, the cancer cells begin in the lobes, or lobules, of the breast. Lobules are the glands that make milk. Lobular carcinoma also is divided to two categories (**Figure I.2**):

- Lobular carcinoma *in situ* (LCIS)
- Invasive lobular carcinoma

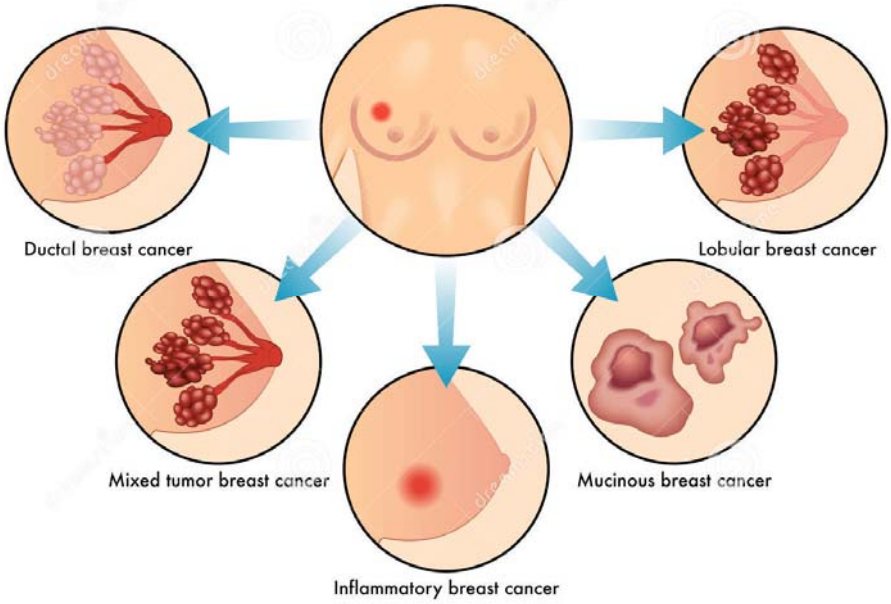


Figure I.2 Different types of breast cancer

Based on a variety of clinical and pathological criterion, patients with breast cancer categorize to several groups in order to decide for suitable treatment

A variety of clinical and pathological factors are normally used to categorize patients with breast cancer in order to evaluate prognosis and to decide for the suitable treatment. These include patient age, axillary lymph node status, tumor size, histological characters, hormone receptor status, and HER2 amplification/expression status (4). Considering these factors in combination are much more clinically valuable than viewing each in separation, and the combined approach forms the basis to group patients into different risk categories such as the St Gallen criteria (5, 6), the NIH consensus criteria (7) and the Nottingham Prognostic Index (8).

Although these risk categories seems valuable assessing prognosis and risk in groups of patients, individual patients with similar features might have a very different clinical consequences. Advance in methods are needed to have better prognosis and determine the most appropriate treatment for patients, the term mainly focused in personalized medicine (9, 10).

I.1.2 Molecular characterization of breast cancer

Various molecular techniques, such as gene expression profiling, have used increasingly to improve the assessment of prognosis and response to therapy in breast cancer (11).

These breast cancer molecular subtypes differ with respect to their patterns of gene expression, clinical features, response to treatment, and prognosis, as summarized in **Table I.1.**

	Molecular subtype		
	Luminal	*HER2*	*Basal*
Gene expression pattern	High expression of hormone receptors and associated genes (luminal A > luminal B)	High expression of HER2 and other genes in amplicon Low expression of ER and associated genes	High expression of basal epithelial genes, basal cytokeratins Low expression of ER and associated genes Low expression of HER2
Clinical features	~70% of invasive breast cancers ER/PR positive Luminal B tend to be higher histological grade than luminal A Some overexpress HER2 (luminal B)	~15% of invasive breast cancers ER/PR negative More likely to be high grade and node positive	~15% of invasive breast cancers Most ER/PR/HER2 negative ('triple negative') *BRCA1* dysfunction (germline, sporadic) Particularly common in African-American women
Treatment response and outcome	Respond to endocrine therapy (but response to tamoxifen and aromatase inhibitors may be different for luminal A and luminal B) Response to chemotherapy variable (greater in luminal B than in luminal A) Prognosis better for luminal A than luminal B	Respond to trastuzumab (Herceptin) Respond to anthracycline-based chemotherapy Generally poor prognosis	No response to endocrine therapy or trastuzumab (Herceptin) Appear to be sensitive to platinum-based chemotherapy and PARP inhibitors Generally poor prognosis (but not uniformly poor)

Table I.1 Major molecular subtypes of breast cancer based on gene expression profiling, clinical features and treatment response

Gene expression profiling in breast cancer have identified a number of major breast cancer subtypes beyond the traditional hormone receptor positive and hormone receptor-negative types. The molecular subtypes among the hormone receptor-positive cancers are the luminal A and luminal B groups. Furthermore, HER2 and basal-like groups are the major molecular subtypes identified among hormone receptor-negative breast cancers (12-19) (**Figure I.3** and **Table I.1**). Luminal breast cancer subtype shows high expression of hormone receptors and related genes. HER2-enriched subtype has highly HER2 expression and other genes located in HER2 amplicon. A high expression level of basal epithelial genes and cytokeratins and

low expression of hormone and HER2 receptors appear in the basal subgroup (4).

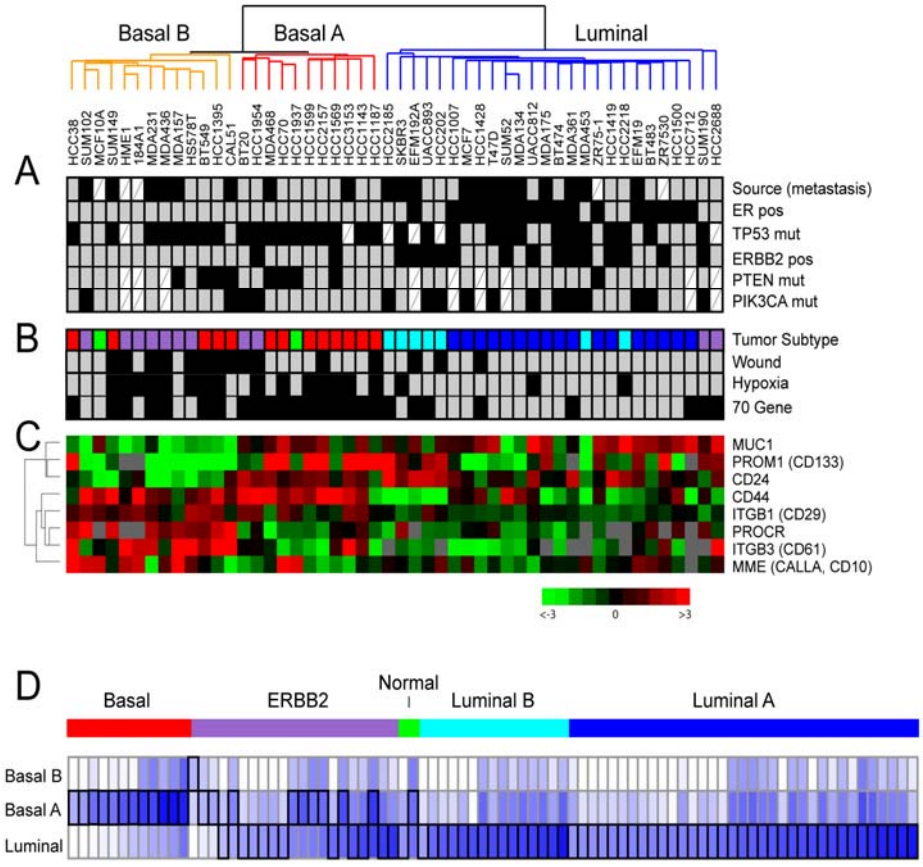


Figure I.3 Molecular characterization of breast cancer based on gene expression and mutation profiling (20)

(A) Clinical, pathological and molecular characteristics of cell line expression subtypes

(B) Classification of cell lines by nearest resemblance to tumor gene-expression subtype

(C) Expression levels of selected stem/progenitor cell relevant markers

(D) Relation of tumor subtypes to cell line subtypes

In recent years, five novel gene expression prognostic tests for breast cancer have been developed: MammaPrint, MapQuantDx, Oncotype DX, PAM50, and Theros Breast Cancer Index. The rationale for developing multi-gene based prognostic tests is not only to add prognostic and predictive information to conventional biomarkers but to provide more reliable and reproducible techniques than the immunohistochemistry based assays, reducing technical errors and subjective interpretation (21).

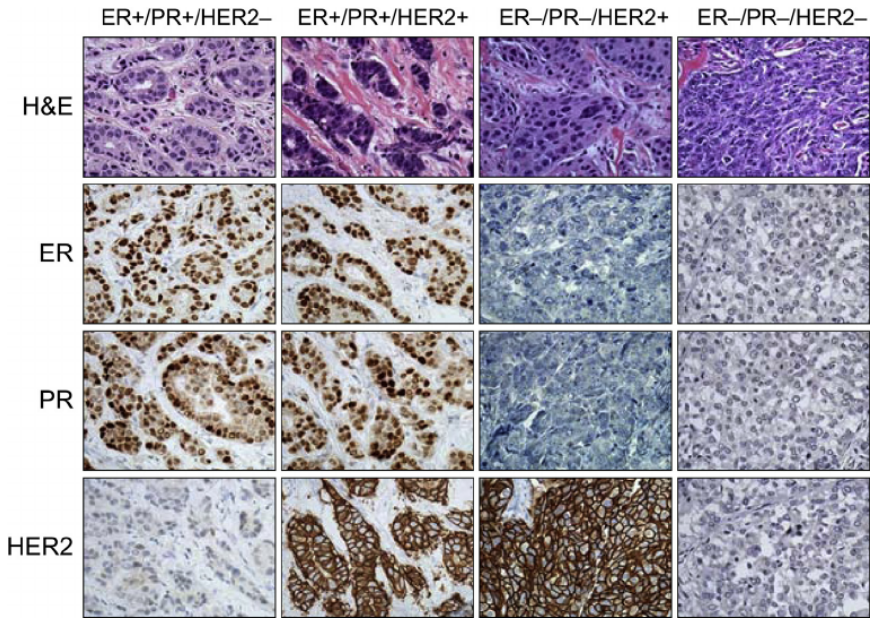


Figure I.4 Breast cancer different subtypes based on receptor expression

Breast tumors are highly heterogeneous and classified based on the expression of estrogen (ER), progesterone (PR) and HER2 receptors into ER (positive), HER2 (positive), and ER (negative) PR (negative) HER2 (negative) (triple-negative breast cancer, TNBC) (22)

Through multi-gene profiling tools, more information can be obtained from tumor tissues (23). Genetic mutations and major structural defects in the DNA strands may permanently compromise gene function. In contrast, epigenetic aberrations can keep the gene structure intact and be partially or completely reverted, renovating the original gene conformation. These transient modifications are dynamically established by enzymes which respond to intrinsic and extrinsic stimuli, and include methylation, histone acetylation, phosphorylation, ubiquitination, citrullination, sumoylation, and ADP-ribosylation (24, 25).

Regardless of the advances in molecular studies discussed above, the molecular categorization of breast cancer patients in routine clinical practice relies on the expression of the main receptors, HER2, estrogen and progesterone receptors. Based on these categories, several targeted therapies have been designed (**Figure I.4**).

I.1.3 Breast cancer treatment

Women with breast cancer are often treated by more than one treatment, requiring a multidisciplinary team approach. In early stage breast cancer, patients typically undergo surgical treatment, followed by radiation if needed, and adjuvant systemic treatments to reduce the risk of relapse in selected patients. Some patients with early breast cancer, as well as patients with locally advanced disease, receive preoperative systemic treatment, also known as neoadjuvant, to facilitate a more breast cancer surgical approach as well as to assess response to the treatment.

Another group of patients is composed by those with metastatic disease, which can occur *de novo* or due to recurrence months, years or decades following primary breast cancer treatment, different therapy would apply depend on the characteristics of the tumor and the previous treatments.

Among the systemic treatment options, one that has resulted in major advances in the last two decades is in the field of targeted therapy against HER2. Anti-HER2 drugs, particularly antibodies, prolong survival in HER2 positive metastatic disease, reduce recurrence in women with early HER2 positive breast cancer, and significantly increase the pathological complete response rate in the neoadjuvant setting. The biology, advances and challenges in the HER2 field are discussed below.

I.2 Human epidermal growth factor receptors (EGFRs)

I.2.1 Human EGFRs and their functions

HER1, HER2, HER3, and HER4 (also called epidermal growth factor receptors ErbB1, ErbB2, ErbB3, and ErbB4) are transmembrane tyrosine kinase receptors with partial homology that normally regulate cell growth and survival, as well as adhesion, migration, differentiation, and other cellular responses (26, 27) (**Figure I.5**).

All members have an extracellular ligand-binding region (except HER2), a single membrane-spanning region and a cytoplasmic tyrosine-kinase-containing domain. The tyrosine kinase domains are activated by both homo-and hetero-dimerization, generally induced by ligand binding. In contrast to the extracellular domains of the three other HER receptors, the extracellular domain of HER2 can take on a fixed conformation resembling a ligand-activated state, permitting it to dimerize in the absence of a ligand (28). When activated, the signaling pathway of these receptors promote cellular proliferation and survival (29). The ErbB receptors are expressed in various tissues of epithelial, mesenchymal and neuronal origin (30).

Under normal physiological conditions, activation of the ErbB receptors is controlled by the spatial and temporal expression of their ligands (31, 32) (**Figure I.5**).

I.2.2 HER2 signaling and overexpression in breast cancer

HER2 receptor tyrosine kinases have crucial roles in human cancer (31). In particular, the expression or activation of EGFR and HER2 are altered in many epithelial tumors, and clinical studies are consistent

with the fact they have important roles in tumor progression (33). Hence, these receptors have been deeply studied to understand their significance in cancer biology and as therapeutic targets, and many ErbB targeting agents are now used in the clinic (34).

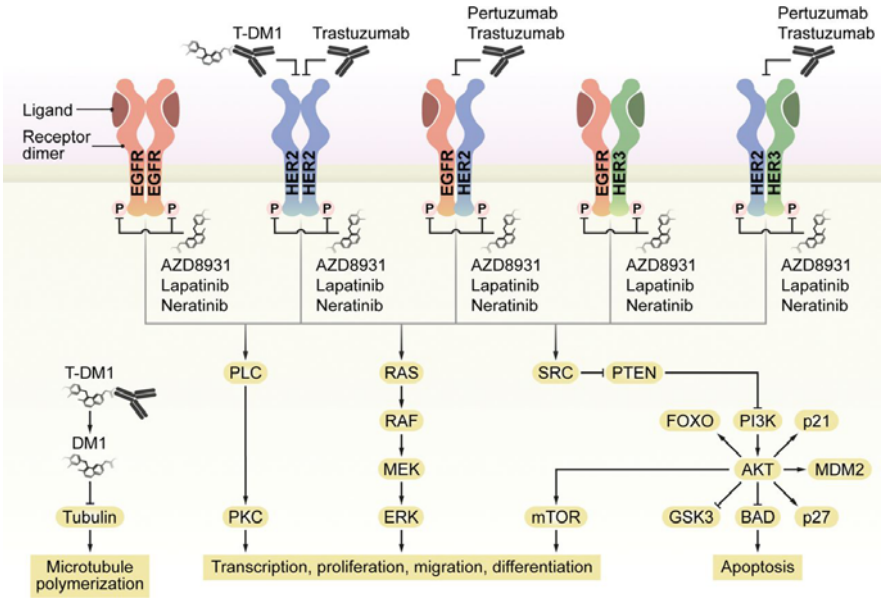


Figure I.5 Members of Human epidermal growth factor receptors, HER1, HER2, HER3 and HER4

Homo- and Hetero-dimerization of different members of this family induces several pathways related to cell proliferation, migration, differentiation in normal and malignant cellular status. Considering the important role of this family in cancer leading pathways, several targeted therapies has been designed (35)

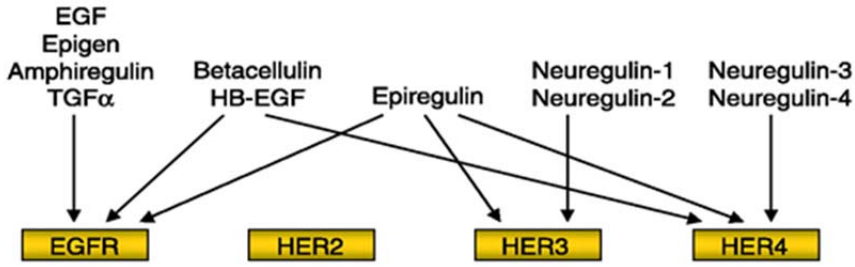


Figure I.6 Epidermal growth factor receptor family members and their ligands

Upon ligand binding, receptors form homo- or hetero-dimers. HER2 is notably different from the other receptors of the family as has no cognate ligand but still is the preferred co-receptor of the family to form dimers (28, 36)

HER2 receptor tyrosine kinase is amplified/overexpressed in 15-20% of invasive breast carcinomas and it is related to poor clinical prognosis (37, 38).

A typical HER2 positive breast cancer cells has 10 to 10,000 times the number of HER2 receptors on the cell surface compared to a HER2 negative (normal) cell (**Figure I.7**).

Overexpression of HER2 in tumors leads to constitutive activation of HER2, presumably because of increased receptor concentrations at the plasma membrane. Many of these tumors contain phosphorylated HER3, which couples HER2 to the phosphatidylinositol3-kinase (PI3K)-AKT pathway.

HER2 signaling promotes cell proliferation through the RAS–MAPK pathway and inhibits cell death through the PI3K–AKT–mammalian target of rapamycin (mTOR) pathway (29).

HER2-dependent cell proliferation was first reported in a rat model of chemically induced neuroblastoma (39). While HER2 overexpression has been described in a variety of human malignant conditions, the gene amplification is rare except in breast cancer. The analysis of HER2 Amplification by fluorescence *in situ* hybridization (FISH) and its overexpression by HercepTest™ are normally used in breast cancer patients.

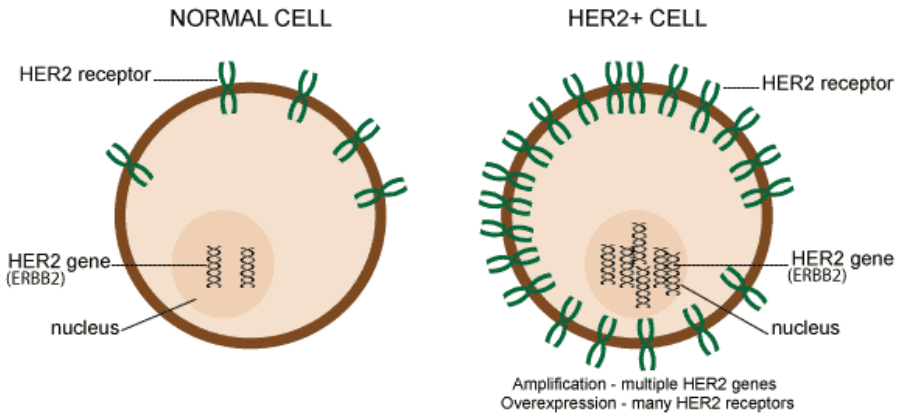


Figure I.7 HER2 gene copy number/expression in normal and HER2 positive breast cancer cells

Normal human cells have around 20,000 HER2 receptors. However, in HER2 positive breast cancer, cancer cells can have up to 2 million HER2 receptors on a cell. This increased, dense concentration of HER2 receptors can promote un-controlled cell growth, over-active cell signaling and malignant (cancerous) tumor formation (40, 41)

I.3 Anti-HER2 targeted therapies

I.3.1 Trastuzumab (Herceptin)

Trastuzumab, also known as Herceptin, consists of two antigen-specific sites that bind to the juxta-membrane portion of the extracellular domain of the HER2 receptor and that prevent the activation of its intracellular tyrosine kinase (42) (**Figure I.8**).

Positive results from clinical studies led to the approval of trastuzumab in the U.S in October 1998 for the treatment of metastatic breast cancer in patients with tumors overexpressing HER2.

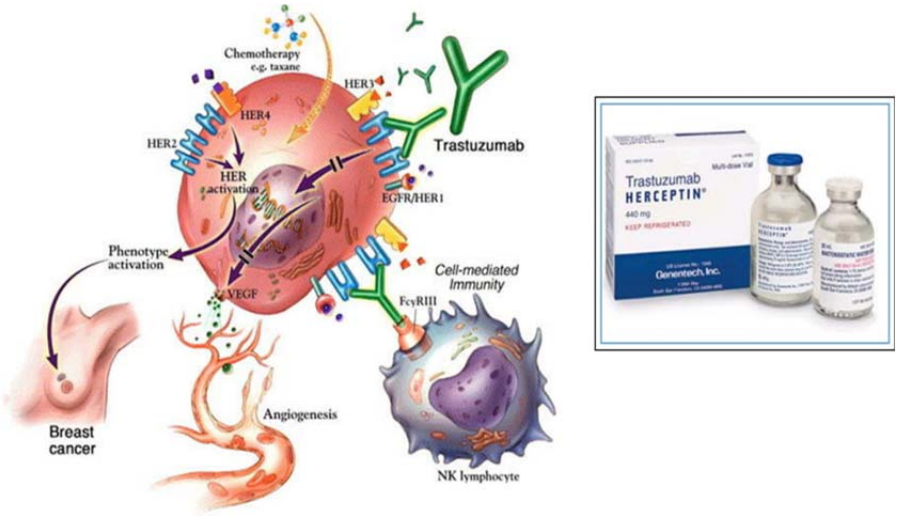


Figure I.8 Trastuzumab treatment in the HER2 positive breast cancer

Trastuzumab is the first humanized monoclonal antibody that used as targeted therapy in cancer. HER2 positive patients are given trastuzumab often in combination with chemotherapy

Results of a multicenter, phase III clinical trial of chemotherapy (doxorubicin- or paclitaxel-based) plus trastuzumab as compared to chemotherapy alone in patients with advanced breast cancers overexpressing HER2 showed a significant enhancement in the effects of chemotherapy on time to disease progression, response rates and survival with co-administration of trastuzumab, without increases in overall severe adverse events (43). Later studies confirmed an important role for trastuzumab in early HER2 positive breast cancer.

I.3.1.1 Trastuzumab mechanisms of action

The molecular mechanisms of action of trastuzumab can divided into three major categories:

I.3.1.1.1 HER2 degradation

Trastuzumab triggers HER2 internalization and degradation through promoting the activity of tyrosine kinase-ubiquitin ligase c-Cbl (44).

I.3.1.1.2 Antibody dependent cellular cytotoxicity (ADCC)

As an antibody, one of the main mechanisms of trastuzumab action is to attract immune cells to tumor sites that overexpress HER2, by a mechanism called antibody dependent cellular cytotoxicity (ADCC) (45, 46) (**Figure I.8**).

I.3.1.1.3 MAPK and PI3K/Akt interference

Trastuzumab may inhibit the MAPK and PI3K/Akt pathways, which leads to an increase in cell cycle arrest, and the suppression of cell growth and proliferation (47).

Trastuzumab, by binding to extracellular domain of HER2, can potently suppress cancer cells growth, proliferation, and survival in both direct and indirect manners (48, 49).

I.3.1.2 Trastuzumab mechanisms of resistance

Several mechanisms of resistance (primary and/or acquired), have been reported for trastuzumab, so far. Less than 35% of patients with HER2-positive breast cancer initially respond to trastuzumab (50, 51), which implies primary resistance. On the other hand, about 70% of patients, who initially responded, experience progression to metastatic disease within a year, reflecting secondary or acquired resistance (52).

Trastuzumab resistance mechanisms which have reported so far can categorize as:

I.3.1.2.1 Steric effects

Some breast cancers have a truncated p95HER2 isoform with constitutive kinase activity that prevents trastuzumab binding (53, 54). In addition, masking of the HER2 trastuzumab binding site due to elevated expression of Mucin4 may cause resistance to trastuzumab (55) (**Figure I.9**).

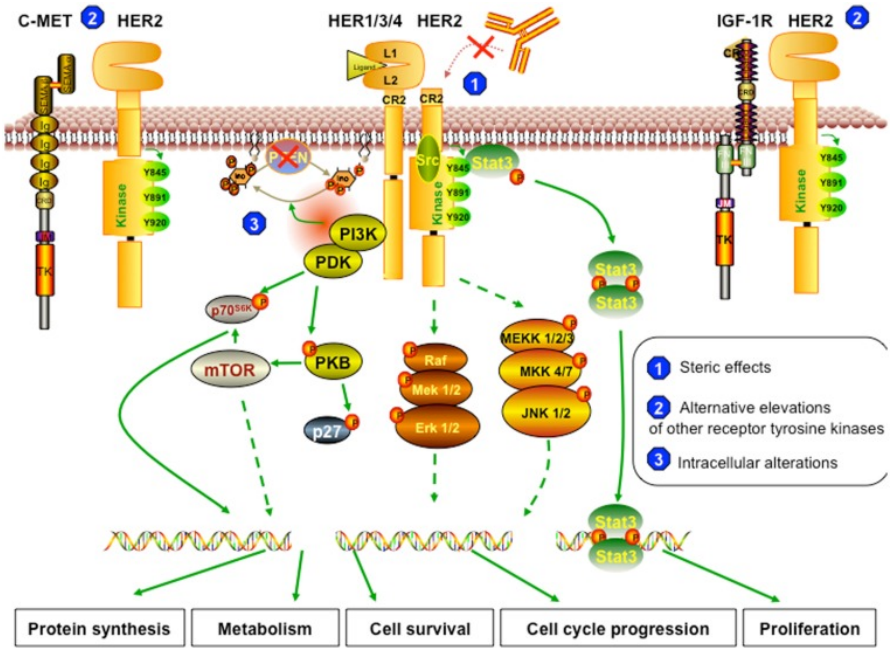


Figure I.9 Mechanisms of trastuzumab resistance

Steric effects, overexpression of other tyrosine kinase receptors and intracellular alterations are the main causes of trastuzumab resistance have been reported. Several pre-clinical efforts performed to find the mechanism of primary and/or acquired resistance to trastuzumab, but a few could apply in the patients (47)

I.3.1.2.2 Overexpression of other tyrosine kinase receptors

Since trastuzumab does not prevent HER3 dimerization, overexpression of HER3 overcome trastuzumab mediated HER2 signaling inhibition (56). The overexpression of insulin-like growth factor-1 receptor (IGF-1R) (57), tyrosine kinase c-Met (58) and also erythropoietin receptor (EpoR)

(59) have been reported as potential mechanisms of trastuzumab resistance (**Figure I.9**).

I.3.1.2.3 Intracellular alterations

The alterations in HER2 signaling downstream molecules such as loss of PTEN activity (48), constitutive active PI3K/Akt pathway (60) and Src activity (61) are potential mechanisms of trastuzumab resistance (**Figure I.9**).

I.3.2 Pertuzumab (Perjeta)

Pertuzumab is approved for the use in combination with trastuzumab and docetaxel in metastatic HER2 positive breast cancer patients (62). Pertuzumab inhibits HER2-HER3 dimerization allowing for a more comprehensive HER2 blockade when combined with trastuzumab (47) (**Figure I.10**).

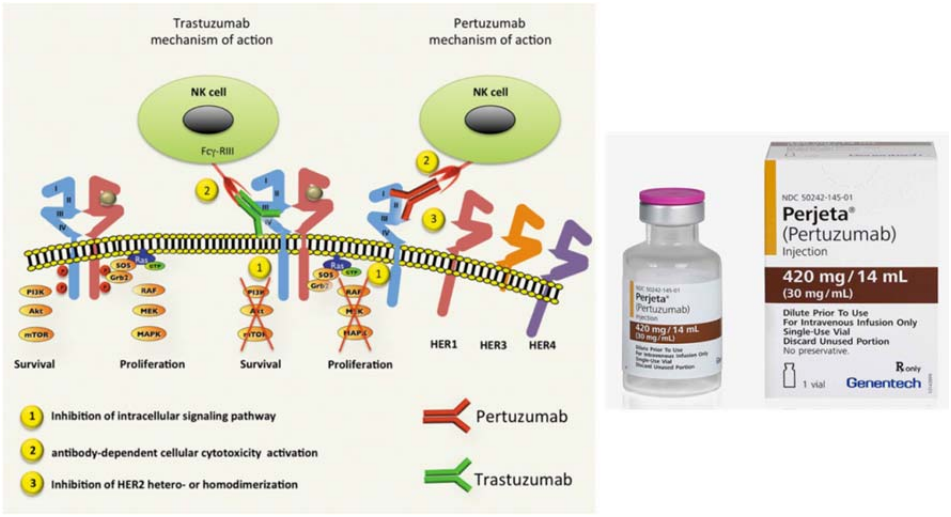


Figure I.10 Pertuzumab treatment for HER2 positive breast cancer patients

Inhibition of survival and proliferation signaling pathways such as PI3K-AKT and MAPK by HER2-HER3 dimerization inhibition and antibody dependent cell mediated cytotoxicity (ADCC) are the most import actions of pertuzumab in HER2 positive breast cancer

I.3.2.1 Pertuzumab mechanisms of action

Although HER2 dimerization with other tyrosine kinase receptors has been reported, HER2-HER3 dimerization is believed to create strong mitogenic signaling and activates two main pathways that regulate cell survival and growth (63):

1- Mitogen activated protein kinase (MAPK) pathway (**Figure I.10**)

2- Phosphoinositide 3-kinase (PI3K) pathway (**Figure I.10**)

Pertuzumab binds to HER2 subdomain II and blocks ligand-dependent HER2 hetero-dimerization with HER1, HER3, and HER4. Furthermore, pertuzumab inhibits HER2-HER3 dimer formation and downstream signaling, while it is also mediating antibody-dependent cell-mediated cytotoxicity (ADCC) (27, 64).

I.3.2.2 Pertuzumab mechanisms of resistance

The mechanisms of pertuzumab resistance, either alone, or in combination therapy has not studied well, yet. Since pertuzumab is mainly used in combination therapy with trastuzumab, down regulation of either HER2 and HER3 alone, or its dimers might consider as possible mechanism of resistance to pertuzumab. The activation of other members of HER2 family by different ligands, such as amphiregulin (AREG), EFG, HB-EGF and Epiregulin might activates several pathways a part of HER2-HER3 leading ones. Neuregulin is the main activator of HER3 that it can induce stemness in the cells and the treatment by pertuzumab may sensitize the neuregulin stem cells

like induced cells to therapy. Further studies needed to uncover the resistance mechanisms to pertuzumab and/or trastuzumab plus pertuzumab combination therapy.

I.3.3 Trastuzumab-emtansine (T-DM1)

I.3.3.1 T-DM1: an antibody-drug conjugated agent (ADCs)

Trastuzumab emtansine (T-DM1), also commercially known as Kadcyla, is a novel drug developed for the treatment of HER2 positive metastatic breast cancer. T-DM1 is a human epidermal growth factor receptor (HER2) targeted antibody drug conjugate, composed of trastuzumab, a stable thioether linker, and the potent cytotoxic agent DM1 (derivative of maytansine) (65-68) (**Figure I.11**). T-DM1 belongs to recently discovered types of drugs called antibody-drug conjugated agents (ADCs).

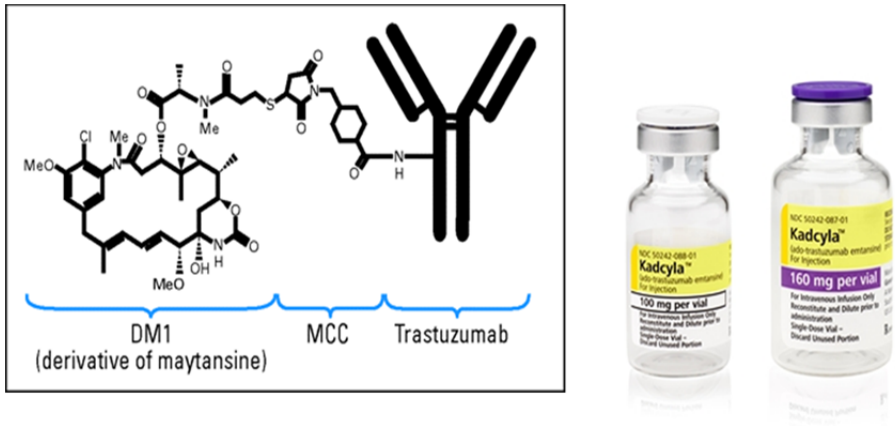


Figure I-11 Trastuzumab-emtansine (T-DM1) chemical structure

Schematic of trastuzumab-DM1 (T-DM1) including the [*N*-maleimidomethyl] cyclohexane-1-carboxylate (MCC) linker. Averages of 3.5 DM1 molecules are conjugated to the Fc region of trastuzumab (69)

The national comprehensive cancer network guideline recommended using T-DM1 as a preferred treatment for patients with trastuzumab-exposed HER2-positive metastatic breast cancer. T-DM1 is generally used after a patient's metastatic disease has progressed following treatment with a combination of a taxane-based chemotherapy and trastuzumab, with or without pertuzumab (Perjeta) (69-72).

The fact that these patients lived longer with less toxicity suggests that T-DM1 is a good option even for patients who have received two or more HER2-targeted treatment regimens before (73, 74).

I.3.3.2 Chemotherapeutic agents classification

To put T-DM1 into context, a general review in chemotherapeutic drug follows. Different types of chemotherapeutic drugs work on different biological processes. Based on this, they can be divided into different groups based on the origins and also their functions.

I.3.3.2.1 Alkylating agents

Alkylating agents are most active in the resting phase of the cell. These types of drugs are cell cycle non-specific. There are several types of alkylating agents used in chemotherapy treatments such as:

- **Mustard gas derivatives**: Mechlorethamine, Cyclophosphamide, Chlorambucil, Melphalan, and Ifosfamide.
- **Ethylenimines**: Thiotepa and Hexamethylmelamine.

- **Alkylsulfonates**: Busulfan.
- **HydrazinesandTriazines**: Altretamine, Procarbazine, Dacarbazine and Temozolomide.
- **Nitrosureas**: Carmustine, Lomustine and Streptozocin. Nitrosureas are unique because, unlike most types of chemo treatments, they can cross the blood-brain barrier. They can be useful in treating brain tumors.
- **Metalsalts**: Carboplatin, Cisplatin, and Oxaliplatin.

I.3.3.2.2 Plant Alkaloids

Plant alkaloids are chemotherapy treatments derived made from certain types of plants. The vinca alkaloids and taxanes are also known as anti-microtubule agents. The plant alkaloids are cell cycle specific. This means they attack the cells during various phases of division.

- **Vincaalkaloids**: Vincristine, Vinblastine, Vinorelbine
- **Taxanes**: Paclitaxel and Docetaxel
- **Podophyllotoxins**: Etoposide and Tenisopide
- **Camptothecananalogs**: Irinotecan and Topotecan

I.3.3.2.3 Anti-tumor antibiotics

Anti-tumor antibiotics are chemo treatments made from natural products produced by species of the soil fungus Streptomyces. These kind of drugs act during multiple phases

of the cell cycle and are considered cell cycle specific. There are several types of anti-tumor antibiotics:

- **Anthracyclines**: Doxorubicin, Idarubicin, Epirubicin, Mitoxantrone, and Daunorubicin
- **Chromomycins**: Dactinomycin and Plicamycin
- **Miscellaneous**: Mitomycin and Bleomycin

I.3.3.2.4 Anti-metabolites

Antimetabolites are types of chemotherapy treatments that are very similar to normal substances within the cell. When the cells incorporate these substances into the cellular metabolism, they are unable to divide. Anti-metabolites are cell cycle specific. They attack cells at very specific phases in the cycle.

Anti-metabolites are classified according to the substances with which they interfere.

- **Folic acid antagonist**: Methotrexate
- **Pyrimidine antagonist**: 5-Fluorouracil, Foxuridine, Cytarabine, Capecitabine, and Gemcitabine
- **Purineantagonist**: 6-Mercaptopurine, 6-Thioguanine
- **Adenosinedeaminaseinhibitor**: Cladribine, Fludarabine, Nelarabine and Pentostatin

I.3.3.2.5 Topoisomerase inhibitors

Topoisomerase inhibitors are types of chemotherapy that interfere with the action of topoisomerase enzymes (topoisomerase I and II). During the process of chemo treatments, these enzymes control the manipulation of the structure of DNA necessary for replication.

- **Topoisomerase I inhibitors**: Ironotecan, topotecan
- **Topoisomerase II inhibitors**: Amsacrine, etoposide, etoposide phosphate, teniposide

I.3.3.2.6 Miscellaneous Anti-neoplastics

Several useful types of chemotherapy drugs are unique.

- **Ribonucleotidereductaseinhibitor**: Hydroxyurea.
- **Adrenocorticalsteroidinhibitor**: Mitotane
- **Enzymes**: Asparaginase and Pegaspargase.
- **Anti-microtubule agent**: Estramustine
- **Retinoids**: Bexarotene, Isotretinoin, Tretinoin (ATRA)

I.3.3.3 T-DM1 mechanisms of action

Once T-DM1 binds to HER2, a receptor-mediated endocytosis triggers for the entry of the HER2 receptor-T-DM1 complex into the cells (75). The active DM1 release in cytoplasm only as a result of proteolytic degradation of the antibody part of T-DM1 in the lysosome (76).

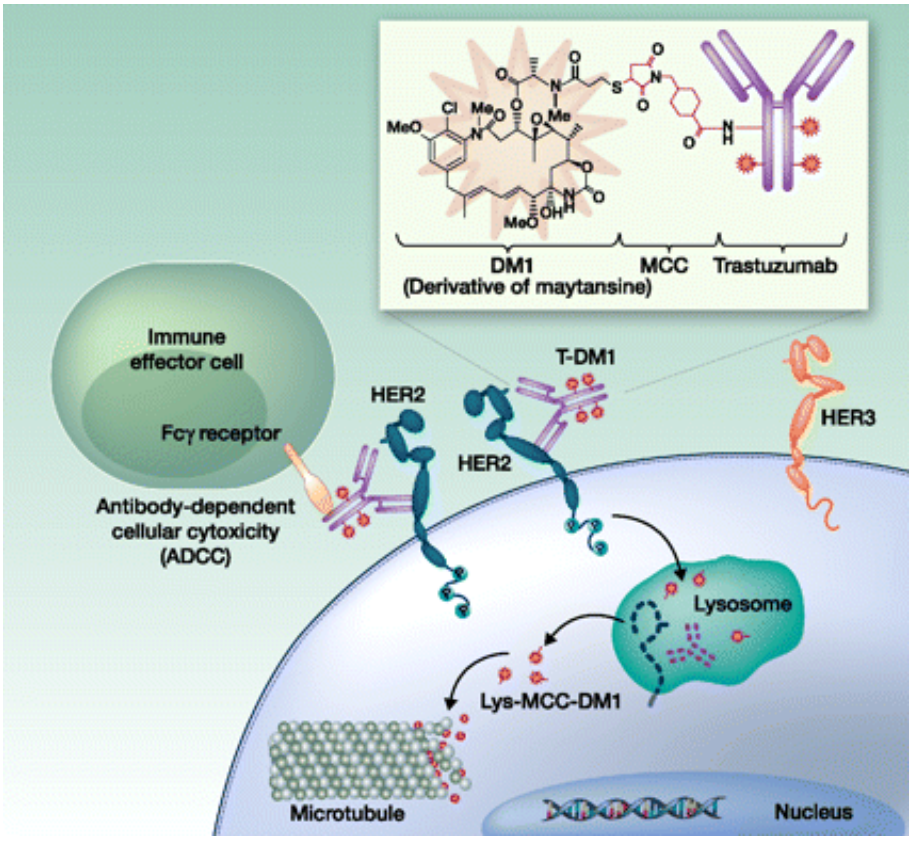


Figure I-12 T-DM1 mechanisms of action

Following release from the lysosome, DM1-containing metabolites inhibit microtubule assembly, eventually causing cell death (77). Trastuzumab-HER2 binding delivers T-DM1 inside the cells, where DM1 release to the cytoplasm and affects microtubule assembly by proteolytic degradation in lysosome and causes mitotic arrest and finally cell death (78, 79)

The inhibition of HER2 ectodomain shedding (67) and PI3K/AKT signaling pathway (67), ADCC (68), mitotic catastrophe (66), disruption of intracellular trafficking and apoptosis are the main

actions of T-DM1 that are caused by trastuzumab and DM1 parts alone and/or together (80).

I.3.3.4 T-DM1 mechanisms of resistance

The studies on mechanisms of resistance to T-DM1, either primary or acquired, are undergoing. Basically, as T-DM1 assembled by the backbone of trastuzumab, the mechanisms of resistance to trastuzumab may contribute to T-DM1 resistance. Overall, the main action of the drug is reported as DM1 effects. Therefore, in the study of T-DM1 mechanisms of resistance both parts should be considered.

The mechanism of resistance to T-DM1 might categorize to three different phases of drug efficacy:

I.3.3.4.1 Receptor-drug binding and internalization

Low HER2 expression, shedding of HER2 ectodomain and masking of the trastuzumab binding epitope on p95HER2 expression are the main alterations may disrupt T-DM1 proper effects (80-82).

I.3.3.4.2 Intracellular trafficking and lysosomal degradation

HER2-drug complex recycling to plasma membrane, inefficient lysosomal degradation of T-DM1 and multi-drug resistance (MDRs) genes overexpression might affect the

processing of T-DM1 to release the active DM1 inside the cytoplasm (81, 83).

I.3.3.4.3 Inefficient DM1 effects

Mutation in tubulin different compartments such as β1, the overexpression of a β3-tubulin isoform and microtubule-associated proteins can affects DM1 effective disruption of microtubules. The activation of cell survival mechanisms such as mitotic slippage and the activation of AMPK pathway also can consider as the mechanism of resistant which are reported similarly in the other microtubule disrupting drugs (67, 81).

Further studies needed to elucidate the mechanisms of resistance to T-DM1 and the best strategy to concur resistance to this drug. The lack of trustable biomarkers for T-DM1 activity in the patients and also for the screening of the patients treated by T-DM1 is the main concern in the future of T-DM1 therapy.

I.4 Cell cycle and cancer

I.4.1 Cell cycle

Cell cycle is a set of different highly controlled stages that guaranty the cell division in a very complex biological process. Cell division is the main component of the cell cycle which is followed by interphase proceeds in three sub phases: the first gap (G1), the synthesis phase (S), and the second gap (G2) (**Figure I.13**).

About 10 percent of the cell cycle consists of Mitosis (M). M phase is divided into two processes: Mitosis and Cytokinesis. The nucleus division occurs in mitosis and is followed by cytokinesis. The cell growth stops in M phase and the cellular energy spends for the division of one cell to two daughter cells.

I.4.2 Cell cycle regulation

Cell cycle is controlled by numerous mechanisms ensuring correct cell division. The cell cycle control is included a set of molecules that work in each phase of cell cycle to ensure that the amount of genetic information goes to each daughter cells are appropriate and equally. In case of any damage happened in any phase, checkpoints stop the cell cycle to provide a time to repair.

Cell cycle checkpoints control appropriate process in different steps:

1) The end of G1 before entry to S phase: It makes sure that the cells are enough big in size and assembles enough material to go to the S phase

2- The end of G2 before entry to M phase: It makes sure that the process of DNA synthesis is correctly done in S phase, the errors are fixed and the cells are ready to go to M phase

3- The end of M phase: It makes sure that the chromosomes are correctly attached to the spindles

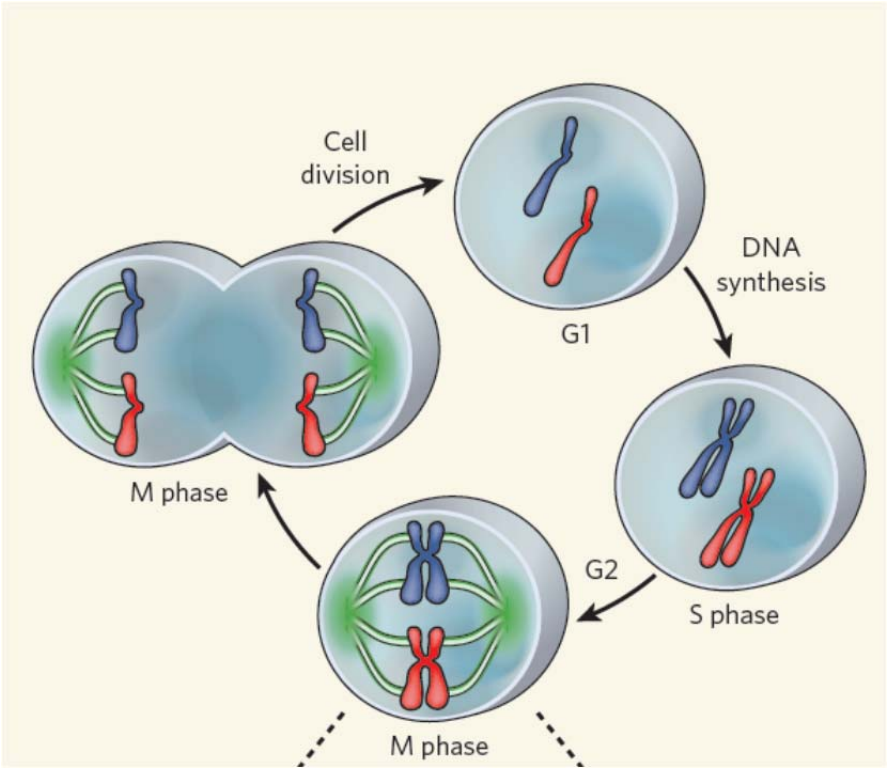


Figure I.13 Cell cycle different phases

G1 is the starting phase of the cell cycle which cell grows and increases in size. DNA replication occurs in S phase. In the end of S phase, when the chromosomes are doubled, cell goes to G2 where it is more grows and prepare for division (adapted from (84))

A complex network of protein interactions are needed for transition between different stages of cell cycle. Therefore, understanding the dynamics of these proteins and their interactions are important for the dynamics of cell cycle.

The main regulatory proteins are protein kinases and cyclins. The kinases are inactive most of the time in cell cycle and they are activated when it binds to a cyclin. The concentration of cyclins, then, regulates the activity of the kinases. For this reason, the kinases are called cyclin dependent kinases (CDKs) (85, 86). The activation of kinase through cyclins triggers biochemical reactions in their targets such as phosphorylation. These modifications are considering as signals for different phases transition (87-89).

I.4.3 Cell cycle deregulation in cancer

A well-known characteristic of the transformed state is the lack of cell cycle checkpoints control. The complexity of checkpoints control as described before makes cell cycle susceptible for alterations in many types of cancer (**Figure I.14**).

However, targeting the cell cycle components in general and CDKs in particular presents unique chance for drug discovery and cancer treatment (88). Both genetics and epigenetics alterations affect the cell cycle apparatus, causing cyclin deregulation as well as loss of the expression of CDKs inhibitors (90). Deregulation of CDKs activity mainly affect cell growth modification and finally transform the cells to malignant tumors. For this reason, CDKs activity has been focused for the development of specific kinase inhibitors to stop the cell cycle and induce growth arrest (91).

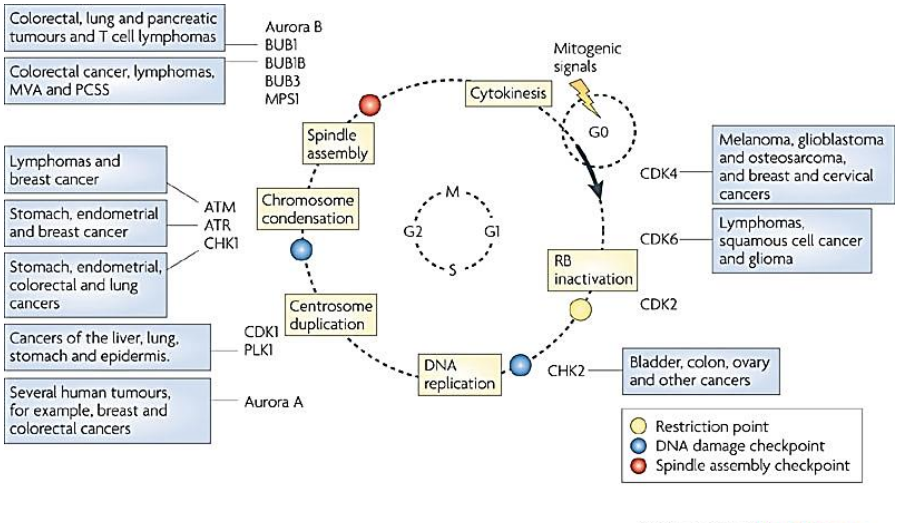


Figure I.14 Cell cycle alterations in different types of cancer

Uncontrolled cell proliferation is the hallmark of cancer, and tumor cells have typically acquired damage to genes that directly regulate their cell cycles (92)

There are many compounds under study as anti-tumor agents, act at different stages of cell cycle, with cytostatic or cytotoxic effects, that is depend on the cell cycle status of the target cells. In fact, the checkpoint control disruption in the cells makes them more sensitive to genotoxic or microtubule damage (93). On the other hand, using DNA damaging treatments makes the checkpoints activation in order to stop the cell cycle to edit the errors and in worse situation make programmed cell death (94). Since the finding of this study focused on the effects of trastuzumab-emtansine (T-DM1) on G2-M phase in cell cycle, its regulation and abrogation in cancer is focused.

I.4.3.1 G2 and mitosis phases in cell cycle

The fundamental studies on G2-M transition in diverse organisms showed that the same protein could control entry into mitosis in yeast, mammals, frogs, starfish and many other organisms. A complex called mitosis promoting factor (MPF) found as the main component which regulates G2 to mitosis entry. MPF contains two main members: CDK1 and cyclin B1 (95).

CDK1 activity peaks at the G2-M border and turned off when the cells enter the anaphase in mitosis. The activity of CDK1 is regulated by the special cyclin, cyclin B1, that accumulated when cells progress through G2 and it is degraded when the cells pass mitosis metaphase to anaphase (96). Cyclin B1 is a member of the highly conserved cyclin family and cyclin A/B subfamily. Cyclin B1 begins to increase during G2, peaks in mitosis, and is rapidly degraded before the cell cycle is completed (**FigureI.15**).

At the entry into mitosis, cyclin B1-CDK1 promotes chromosome condensation, nuclear lamina resolution, and mitotic spindle assembly (96-98). When chromosomes are properly aligned during anaphase, rapid degradation of cyclin B1 by anaphase promoting complex/cyclosome (APC/C) is required for mitotic exit and completion of the cell cycle (97, 99-101).

I.4.3.2 MPF complex deregulation in cancer

Cyclin B1 overexpression and/or mislocalization has been depicted in several primary cancers such as breast (102), gastric (103), non-small cell lung cancer (104), etc. In most primary tumors, the expression of cyclin B1 is unrestricted to particular phases of the cycle, while in normal tissues, the expression of cyclin B1 is limited to very late S and G2-M phases of the cell cycle (97).

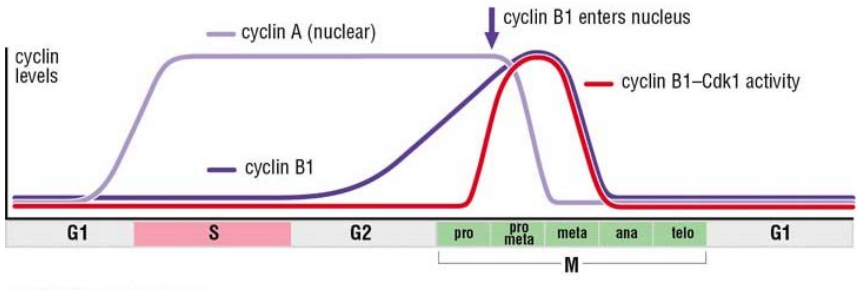


Figure I-15 Schematic cyclin B1 expression and CDK1 activity pattern during cell cycle different phases

The peak of cyclin B1 expression at the end of G2 causes the elevation in the activity of CDK1 in MPF complex and the cells proceed to mitosis. In the end of mitosis, the degradation of cyclin B1 and consequently, the drop down in the activity of CDK1 let the cells to exit mitosis

In addition, the abnormal activation of CDK1 has been reported in a number of primary tumors (breast, colon, prostate, oral, lung and esophageal carcinomas), most commonly owing to overexpression of cyclin B1, and in some cases correlates with poor prognosis (97).

Cyclin B1-CDK1 complex deficiency could lead to a type of non-apoptotic cell death which is known as mitotic catastrophe (105, 106). During mitotic death, a mitotic imbalance results in the activation of the cell death machinery in the presence of elevated cyclin B1 levels, that is, when cells have not yet exited mitosis (107, 108). Mitotic catastrophe can trigger a lethal pathway that is not carry out until cells reach interphase of the next cell cycle. In this regards, cell death can occur quickly, within hours after mitotic exit, or in a delayed fashion (109, 110).

As G2 and mitosis are two phases of the cell cycle should control tightly by the regulators, any alterations in the main players or regulators may causes cellular transformation and cancer.

I.4.3.3 G2-M checkpoints and therapeutic strategies

Drugs that interfere with the normal progression of mitosis belong to the most successful chemotherapeutic compounds currently used for anti-cancer treatment. Classically, they are represented by microtubule binding drugs that inhibit the function of the mitotic spindle in order to stop the cell cycle in mitosis and to induce apoptosis in tumor cells.

However, these compounds act not only on proliferating tumor cells, but exhibit significant side effects on non-proliferating cells including neurons that are highly dependent on intracellular transport processes mediated by microtubules (111).

Therefore, there is a particular interest in developing novel anti-mitotic drugs that target non-microtubule structures. In addition, approaches of cell cycle checkpoint abrogation during mitosis and at the G2-M transition inducing mitosis associated tumor cell death are promising new strategies for anti-cancer therapy. It is expected that this next generation of anti-mitotic drugs will be as successful as the classical anti-microtubule drugs, while avoiding some of the adverse side effects (112).

In general, G2-M arresting drugs targets different components in G2 and mitosis phases of the cell cycle typically (**Figure I.16**):

- The mitotic spindle inhibitors (113)
- Microtubules stabilizer/destabilizer (113)
- Mitosis components and regulators
- Mitotic kinesin inhibitors: KSP/Eg5, MKLP1, Kif4, Kid, MCAK and CENP-E (114)
- Polo-like kinases inhibitors (115)
- Aurora kinases inhibitors (116)
- CDK1 inhibitors (117)

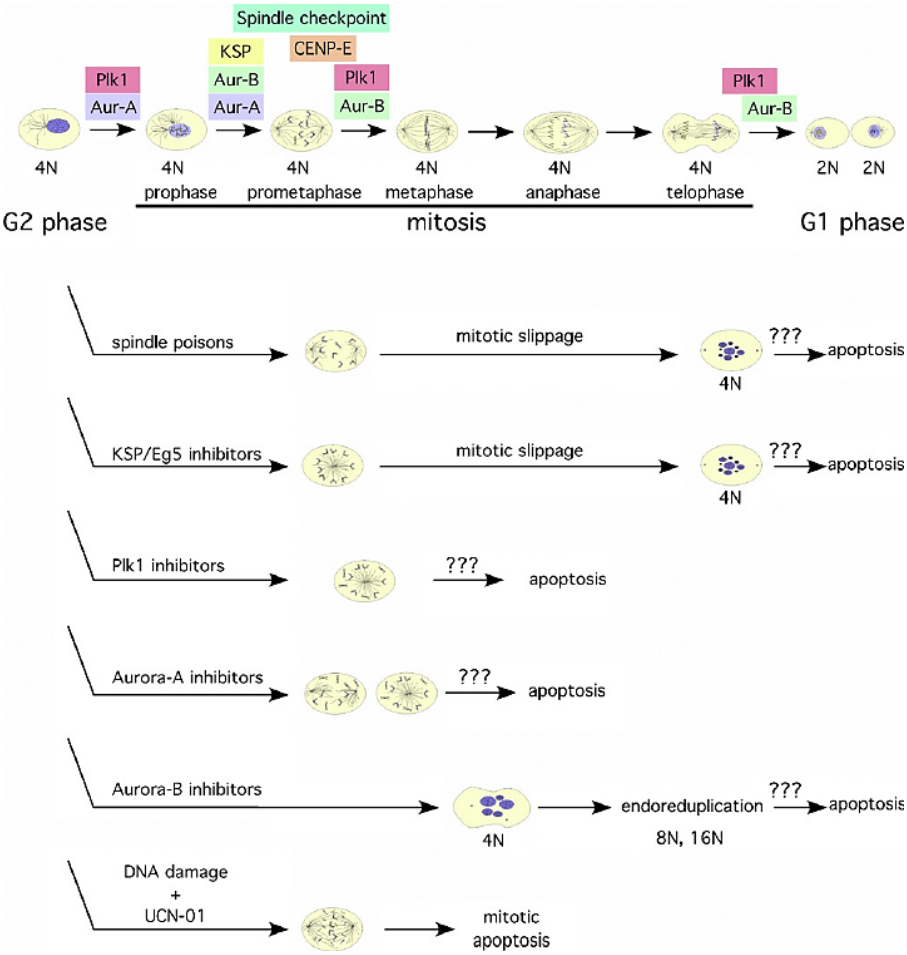


Figure I.16 Mitosis stages and the phenotypes of chemotherapeutic drug treatment

The approaches of cell cycle checkpoint abrogation during mitosis and at the G2-M transition inducing mitosis associated tumor cell death are promising new strategies for anti-cancer therapy. Even though, the G2 and mitosis inhibiting drugs have different effects on cells and finally might have different consequences in cell death and/or drug resistance (112)

I.4.3.4 Mitotic catastrophe and apoptosis

Mitotic catastrophe is an event in which a cell is destroyed during mitosis. This is believed to be caused through apoptosis as a result of an attempt at aberrant chromosome segregation early in mitosis or as a result of DNA damage later, during the metaphase/anaphase transition (106, 118).

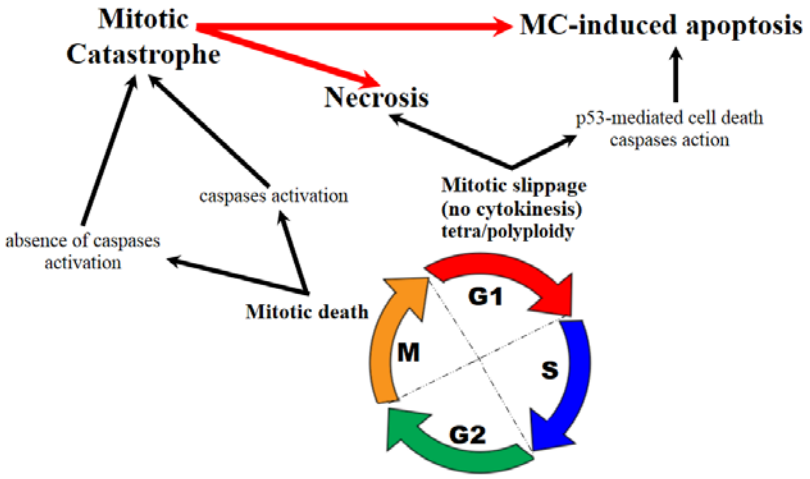


Figure I.17 Schematic representation of main cell death pathways related to mitotic catastrophe

Mitotic arrest might lead mitotic cell death through mitotic catastrophe which consequently caused cell death, senesce and/or necrosis. Mitotic slippage let some cells scape from mitotic arrest and re-enter normal cell cycle which might consider as a possible mechanism of resistance towards the drugs leading mitotic catastrophe (110)

Defective mitotic cells can engage the cell death machinery and undergo death in mitosis, when cyclin B1 levels remain high.

Alternatively, defective cells can exit mitosis, known as slippage, and undergo cell death execution during G1 in the subsequent cell cycle (**Figure I.17**).

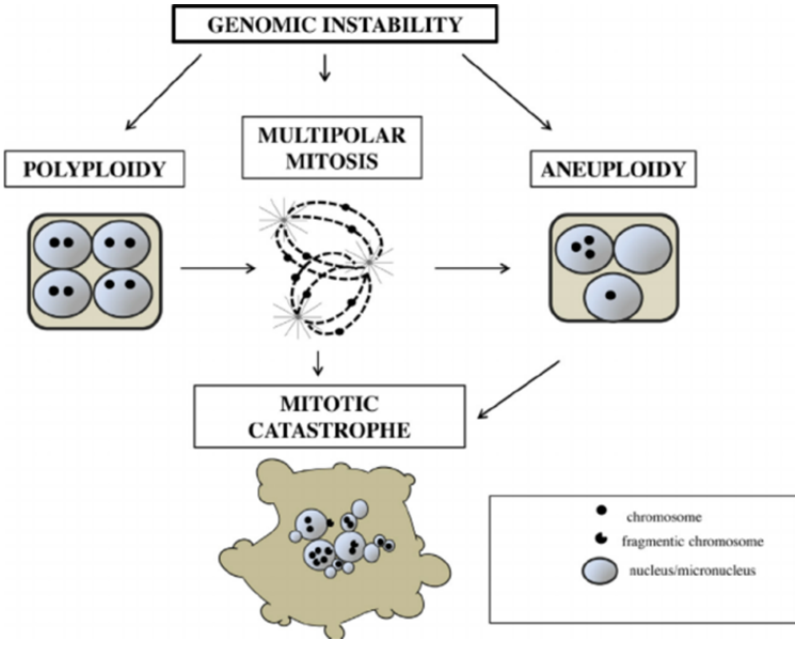


Figure I.18 Genomic instability may lead to development of mitotic catastrophe

Genomic instability causes different types of abnormalities in cell division (polyploidy, multipolar mitosis and aneuploidy). All these abnormalities are interconnected to each other and, subsequently, can lead to development of mitotic catastrophe and cell death (119)

Defective cells can exit mitosis and undergo senescence. Thus, mitotic catastrophe senses mitotic damage and directs the defective cell to

one of the three possible anti-proliferative fates such as death in M phase, death in G1 and senescence (120, 121).

Mitotic catastrophe thus may be conceived as a molecular device that prevents aneuploidy, which may participate in oncogenesis (**Figure I.18**). Mitotic catastrophe is controlled by numerous molecular players, in particular, cell cycle specific kinases such as the cyclin B1-dependent kinase CDK1, polo-like kinases, Aurora kinases and cell cycle checkpoint proteins including survivin, p53, caspases and members of the Bcl2 family (105).

Clearly, the features of mitotic catastrophe are heterogeneous (109). In fact, although these events are usually viewed as changes from the normal cell cycle, they involve phenomena that are not necessarily associated with mitosis, such as micro-nucleation, restitution, and polyploidy (122).

The molecular environment of G2-M arrest is much unknown and consequently what happen after G2-M arrest is unclear. It has a potential to return to the mitotic cycle and may therefore provide a survival advantage. However, the *Achilles heel* of G2-M arresting drugs might be hidden in this part, where cells re-enter the cell cycle and become drug non-responsive.

HYPOTHESIS

HYPOTHESIS

Trastuzumab-emtansine (T-DM1) is an antibody-drug conjugate that uses trastuzumab to specifically deliver the maytansinoid anti-microtubule agent DM1 to HER2 positive cells. Based on the previous reports (80, 81, 83, 123, 124), mainly emphasized T-DM1 mitotic catastrophe induction, we hypothesized that mechanisms leading to resistance to the DM1 component might play a key role in T-DM1 resistance.

OBJECTIVES

OBJECTIVES

The general objective of this PhD thesis was to elucidate mechanisms of acquired resistance to trastuzumab-emtansine (T-DM1).

The specific objectives were:

1. To define T-DM1 response in a panel of trastuzumab primary sensitive and resistant HER2 positive breast cancer cell lines

2. To generate models of acquired resistance to T-DM1 in order to find the mechanisms of acquired resistance
    a. Whether HER2 amplification (copy number)/expression pharmacodynamics related characters alteration, including drug binding and internalization, in the resistant cells are involved in T-DM1 resistance
    b. Whether cell cycle alteration might affect T-DM1 response and resistance
    c. To define the predictive biomarkers for T-DM1 acquired resistance

3. To validate the *in vitro* and *in vivo* results in fresh breast cancer explants obtained from HER2 positive breast cancer patients

METHODS AND MATERIALS

METHODS AND MATERIALS

M.1 Cell lines

SKBR3 and BT474 (DMEM-HAM´S-F12, supplemented with 10% FBS, L-glutamine and Penicillin-Streptomycin), AU565, HCC1954 and HCC1419 (RPMI1640, supplemented with 10% FBS, L-glutamine and Penicillin-Streptomycin), EFM192A (RPMI1640, supplemented with 20% FBS, L-glutamine and Penicillin-Streptomycin) and MCF7 and JIMT1 (DMEM, supplemented with 10% FBS, L-glutamine and Penicillin-Streptomycin) purchased from ATCC.

M.2 Drugs

T-DM1 (Kadcyla, ado-trastuzumab emtansine) was provided by Dr. Mark Sliwkowski and Dr. Gail Lewis Phillips (Genentech, South San Francisco) under MTA agreement and trastuzumab by Hospital del Mar pharmacy. The stock concentration of both drugs was 20mg/mL.

M.3 Cell proliferation and viability assay

M3.1 Luminescence assay

To assess short term effects of T-DM1 on different cell lines, CellTiter-Glo® Assay (Promega) was used. HER2 breast cancer cell lines seeded as the density of 1000 cells per well in a 96-well plates. After 24 hours, a range of T-DM1 concentrations [0-100μg/mL] added to the medium. Wells containing medium without cells prepared as the control. To read the wells, 72 hours post treatment, 96 well plates

equilibrated at room temperature for 30 min. Then after, CellTiter-Glo® reagent added in equal volume of cell culture medium to each well and the control. After 2 min of orbital shaking to induce the cell lysis and 10 min room temperature incubations to stabilize luminescence signals, wells read by Centro LB 960, Berthold Technologies.

M3.2 Automated cell counting

Long term T-DM1 effects measured by 3, 7 and 10 days proliferation assay in different cell lines (according to (125) with small modification). Briefly, cells seeded in a density of 8000-12000 cells per well in a 12-well plates and 24 hours later, treated with T-DM1 [0.1µg/mL]. Cell numbers were counted (by automated cell counter, scepter, Millipore) after 3, 7 and 10 days post T-DM1 treatment. The effects of T-DM1 on MCF7, a non-HER2 amplified cell line, evaluated to determine the specific concentration of the drug with the same methods of short and long exposure.

M.4 The generation of T-DM1 acquired resistant cells

We established a protocol of T-DM1 acquired resistant generation in HER2 positive breast cancer cell lines. T-DM1 resistant cell lines were derived from original parental cell lines by exposure to stepwise increasing concentrations of T-DM1 in a pulse-fashion (126). SKBR3, HCC1954 and HCC1419 were exposed to increasing concentrations of T-DM1 [1-4µg/mL]. The protocol was included 3 days T-DM1 treatment and 3 days without drug (started from T-DM1 [1µg/mL]

(18 days) and continued with T-DM1 [2μg/mL] (18 days) and T-DM1 [4μg/mL] (18 days) (**Figure R.4**). Previous studies reported T-DM1 [3μg/mL] as clinically relevant concentrations of T-DM1 (127, 128). However, T-DM1 [4μg/mL] considered as a top concentration of the drug in this protocol. It is worthy to note that the same protocol applied for BT474 cell line to generate T-DM1 resistance, but several attempts failed.

M.5 *HER2* fluorescence *in situ* hybridization (FISH)

To assess the genetic status of *HER2* (*ERBB2*) gene in parental and the resistant HCC1954, HCC1419 and SKBR3 cells, we applied FISH technique by using the PathVysion commercial probe (Abbott Molecular Inc, Des Plaines, IL, USA). This probe consists of two different probes, one with the centromeric alfa-satellite region, specific for chromosome 17 (Spectrum green), and a locus specific probe from the *HER2* gene (Spectrum orange).

FISH was performed in Carnoy fixed cells (suspension of nuclei and metaphases) obtained from the cell lines after application of cytogenetic technique. Slides prepared and the probe was co-denaturated at 75°C for 5 min and hybridised overnight at 37°C in a hot plate (Hybrite chamber, Abbot Molecular Inc.).

Post-hybridization washes were performed with 0.4xSSC 0.3% NP-40 solutions for 2 min at 72°C and 2xSSC 0.1% NP-40 at room-temperature for 1 minute. Samples were counterstained with 4,6-diamino-2-phenilindole (DAPI)(Vysis, Inc.). Results were analysed in a fluorescent microscope (Olympus, BX51) using the Cytovision software (Applied Imaging, Santa Clara, CA). A minimum of 300 nuclei per cell line were scored at low magnification (100X). Normal nuclei

showed two copies of *HER2* gene and CEP17 region. We classified nuclei as (1) amplified: nucleus had one HER2 cluster (50 copies of HER2 gene); (2) high amplified: nucleus had two clusters (100 copies of HER2) (3) low amplified nucleus (between 6 to 10 HER2 copies). When the average number of chromosome 17 signal numbers exceeded 2.5 per cell, the case was considered polysomic.

M.6 T-DM1/HER2 receptor binding assay

HCC1954, HCC1419 and SKBR3 prenatal and resistant cells (0.2-0.5×10^6) were incubated with T-DM1 and Trastuzumab [50nM] for 30 min on ice. Then cells were washed and incubated with phycoerythrin labeled goat anti-human Fc secondary antibody (1:3000 dilution) for 30 min. The samples, then, washed and stained by DAPI. Finally, samples were acquired on LSR Fortessa flow cytometer (BD Biosciences), and data analyzed with DIVA software (BD Biosciences).

M.7 T-DM1 internalization assay

T-DM1 internalization was evaluated by immunofluorescence staining. Cells (1.5× 10^5) were seeded on coverslips and treated with T-DM1 [10nM] for 15 min. After washing out the drug, cells were cultured for 24 hours with or without chloroquine [50 μM] (a drug that change lysosomal pH) to accumulate intracellularly T-DM1. Cy3-conjugated anti-human was used to detect T-DM1, phalloidin-FITC (P5282, Sigma) was used for actin staining and nuclei were counterstained with DAPI. Processing of the samples was performed as previously reported (129).

M.8 Cell cycle assay

The cells seeded in the concentration of 0.6-0.7×10^6 cells in p60 plates. 24 hours later, cells treated with T-DM1 [0.1µg/mL]. Finally, After 24 hours, culture medium removed from cells, replaced with BrdU labeling fresh medium and incubated for 1 hour at 37°C. After that, the labeling solution removed and cells washed two times with PBS. Then, the cells harvested and the pellet prepared. Then after, cells fixed by adding ice cold 70% ethanol. Continue with the protocol of BrdU staining, pellet resuspended in denaturing solution (2M HCL) and incubated for 20 min at room temperature. The cells washed by adding wash buffer (PBS containing 0.5% BSA) twice and then, 0.1 M sodium borate (Na2B4O7, pH 8.5) added to neutralize any residual acid for 2 min. In the next step, the pellet incubated 30 min with purified mouse anti-BrdU monoclonal antibody (555627, BD Pharmingen™) diluted in dilution buffer (PBS containing 0.5% Tween®-20, 0.5% BSA). Pellet then washed, incubated with FITC-conjugated goat anti-mouse Ig secondary antibody for 30 min and finally resuspended in 0.5 ml propidium iodide (10 µg/ml in PBS). The cellular staining analyzed by flow cytometry, exciting at 488 nm and measuring the BrdU-linked green fluorescence (FITC) through a 514 nm bandpass filter and the DNS linked red fluorescence (PI) through a 600 nm wave-length filter by using a Becton Dickinson FACScan operated by the CELLQuest software.

M.9 Cdc2/CDK1 activity assay

To evaluate the activity of CDK1 in parental and T-DM1 acquired resistant cells, we used MESACUP® Cdc2/CDK1 Kinase Assay Kit (MBL, International Corporation). Principally, CDK1 activity assay kit

is based on an ELISA that uses a synthetic peptide as a substrate for CDK1 kinases and the phosphorylation form of the peptide is recognized by a monoclonal antibody.

Following the protocol, the lysates for different conditions were prepared by lysis buffer recommended in the kit. After protein quantification, 0.2mg/mL protein is used for each assay. In the first step, phosphorylation reaction was performed. This reaction mixture was included the sample buffer, 10Xcdc2 reaction buffer, Biotinylated MV peptide, distilled water and 1Mm ATP that incubated 30 min in 30^{oC}. In the second step, the phosphorylated MV peptide is detected by ELISA. In a brief, 100µL phosphorylated reaction mixture in incubated 1h in monoclonal antibody 4A4 coated micro-wells strip. After several washes, wells incubated 30 min with 100µL POD conjugated streptavidin. Then after, 100µL of substrate solution added each wells, incubated 3-5 min and stop solution poured into each wells. Finally, the absorbance read at 492nm by Infinite® M200, Tecan.

M.10. Apoptosis and cell death analysis

For measurement of apoptosis, the Annexin V and Dead Cell Assay Kit (Millipore) was used according to the manufacturer's instructions. Briefly, after treatment, the cells were incubated with Annexin V and Dead Cell Reagent (7-AAD) for 20 min at room temperature in the dark, and the events for dead, late apoptotic, early apoptotic, and live cells were counted with the Muse Cell Analyzer (Millipore, Hayward, CA, USA) and analyzed with MuseSoft 1.4.0.0 (Millipore).

M.11 Protein detection

M.11.1 Total protein extraction

For whole cell protein extracts, we followed a common protocol mostly used in many molecular biology labs. Briefly, cells were cultured in plates and lysed in ice-cold Nonidet P-40 buffer (Tris-HCL (pH = 7.4) 50mM, NaCl 150mM, 1 % NP40, EDTA 5mM, NaF 5mM, Na3VO4 2mM, PMSF 1mM, Leupeptin 5µg/mL and Aprotinin 5µg/mL) mechanically with the help of an scrapper. After shaking during 30 min at 4°C, the samples were centrifuged 10 min at 13200rpm and the supernatant was aliquoted and stored at -20°C.

M.11.2 Western blot analysis

Whole cell extracts were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with 5% non-fat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 min, the membrane incubated with primary antibodies at 4°C for 12 hours. Membranes were washed three times for 10 min and incubated with a 1:3000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies for 1 hour. Blots were washed with TBST three times and developed with the ECL system (Amersham Biosciences) according to the manufacturer's protocols. The antibodies used were: HER2 (Biogenex), cyclin B1 (sc-245) and Cdc2 p34 (CDK1) (sc-54) purchased from SantaCruz and β-actin (A-5316) was purchased from Sigma.

M.12 Cyclin B1 and cdc20 silencing

For transient transfection, we performed electroporation with Amaxa 4D-Nucleofector™ device, according to manufacturer's instruction. Briefly, after trypsinization, 0.5-1×10^6 cells were separated per tube, centrifuged and the cell pellet, then, resuspended carefully in 100 µL of complete Nucleofector® solution combined with 300 nM siRNA against cyclin B1, cdc20 and scrambled siRNA as a control (duplex were purchase from GE Darmacon). The cell suspension transferred to the certified cuvette and the specific Nucleofector® Program (SKBR3 E-009) was applied for transfection. Once the program finished 500 µL of complete growth media added and ultimately, the cells seeded by enough intensity for proliferation assay and lysate preparation in defined time lines.

M.13 *In vivo* studies

M.13.1 animals

Six week old male BALB/c Beige mice were purchased from Charles River Laboratories (Wilmington, MA) and hosted in the pathogen free animal facility at the Barcelona Biomedical Research Park (PRBB). Animal treatments were done according to institution approved protocols.

M.13.2 Subcutaneous xenograft model

In order to generate a tumor xenograft model, 10-15×10^4 HCC1954 cells mixed with matrigel and then injected subcutaneously (n=12)

for tumor generation. Tumors were clearly visible in all mice inoculated with a mean volume of 100-150mm^3 before starting the drug treatment. Drug treatment with control antibody (n=6) or T-DM1 (5mg/kg) (n=6) (*i.v.*) was initiated 7 days after HCC1954 inoculation in each treatment group. The animals sacrificed in two time lines: 1) day 12: after the first round of 5 days treatment (n=3, control and n=3, T-DM1 [5mg/kg] and 2) 33 days: after the first round of 5 days treatment (n=3, control and n=3, T-DM1 [5mg/kg].

M.14 *Ex vivo* studies

M.14.1 T-DM1 *ex vivo* treatment protocol for patient samples

The biopsies of the HER2 positive breast cancer patients verified by the clinical criteria sliced and cultured in the RPMI medium supplemented by (10%FBS, L-glutamine and Penicillin-Streptomycin for 120 hours treated by T-DM1 [0.1µg/mL] and trastuzumab (Herceptin) [15 µg/mL].

M.14.2 Immunohistochemistry (IHC)

After the preparation of paraffin-embedded blocks for the samples, 3 µm tumor tissue sections were stained for HER2 (Herceptest P980018/S010, Dako), cyclin B1 (sc-245, Santacruz), Ki-67 (GA62661-2, MIB1 clone, Dako), phosphorylated (Ser10) Histone 3 (9701, cell signaling) and c-casp3 (9664, cell signaling) followed by incubation with an anti-rabbit Ig dextran polymer (Flex+, Dako) and

3,3′-diaminobenzidine as chromogen in a Dako Link platform. HER2 staining was scored following ASCP/CAP guidelines (130). For the other markers, the percentage of positive tumor cells was scored.

M.15 Statistical analysis

Data presented as mean±SEM and mean±SD. $P<0.05$ considered as significance. To find the significant difference between two groups, *t*-student test and for more than two groups, one way ANOVA applied by GraphPad (version 6.0). Tukey's and Post Hoc were used to perform pairwise comparison.

RESULTS

RESULTS

R.1 HER2 amplification/expression analysis

To verify the amplification/overexpression of HER2 in a panel of HER2 positive breast cancer cell lines, similar methods and criterion using in clinical practice were applied in the panel of breast cancer cell lines.

R.1.2 HER2 expression analysis

HercepTest™ (DAKO), a semi-quantitative immunohistochemical assay for determination of HER2 protein overexpression in breast cancer was used to determine the expression of HER2 in a panel of cells (131, 132).

Using this method, we found that except JIMT1 HER2 (1+) and MDA-MB361 HER2 (2+), the remaining cell lines tested were HER2 (3+) (**Figure R.1** and **Table R.1**). It is important to note that anti-HER2 targeted therapies are applied only in HER2 3+ patients.

R.1.3 HER2 amplification analysis

Besides HER2 expression assessment in the panel of cell lines, HER2 amplification was analyzed in all the cell lines by fluorescence *in situ* hybridization (FISH). Based on the latest ASCO guideline (133), HER2 amplification in breast cancer patients considered as positive, negative and equivocal.

FISH analysis in the cell lines showed all of them are HER2 amplified even in those cell lines showed HER2 (1+) and HER2 (2+) in IHC analysis (**Figure R.1**).

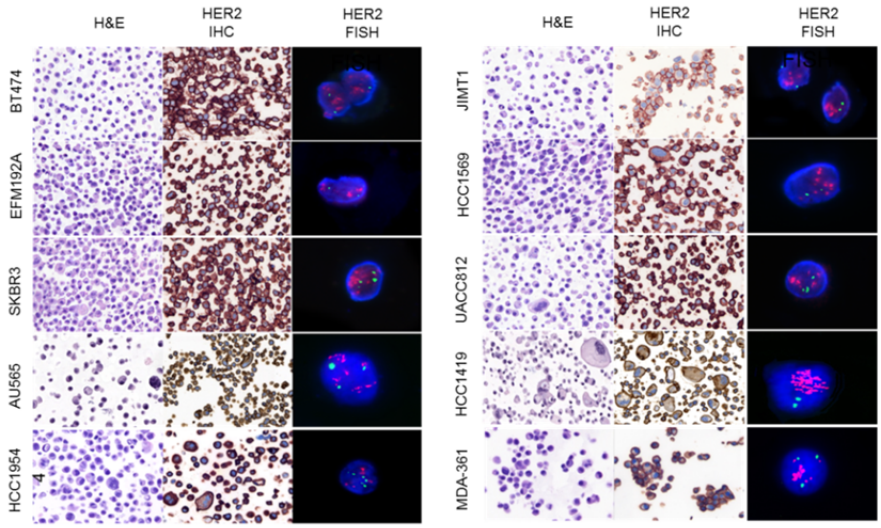


Figure R.1 HER2 amplification/expression evaluation in a panel of breast cancer cell lines

HER2 amplification/expression analysis by FISH and HercepTest™ (DAKO), respectively, in the panel of HER2 amplified/overexpressed breast cancer cell lines (134, 135). (Red: HER2 probe) (Green: Chr17 centromere probe) (H&E: Hematoxylin-Eosin staining)

In addition to the analysis of HER2 expression/amplification, we assessed the expression of estrogen and progesterone receptors in HER2 positive cell lines. Results showed that BT474 and MDA-MB361 express both receptors (**Table R.1**).

The estrogen receptor (ER) and epidermal growth factor receptor (EGFR) pathways play pivotal roles in breast cancer progression. In

addition, both EGFR and ER receptors use the mitogen activated protein kinase extracellular signal regulated kinase pathway as a major route of cellular activation (136).

Cell line	HER2_IHC	HER2_FISH (Mean)	ER	PR
BT474	3+	24 HER2 : 3.5 CEP17	+	+
EFM192A	3+	30 HER2 : 1.8 CEP17		
SKBR3	3+	35 HER2 : 2.5 CEP17		
JIMT1	1+	14 HER2 : 2.4 CEP17		
HCC1569	3+	18 HER2 : 1.5 CEP17		
UACC182	3+	22 HER2 : 2.5 CEP17		
AU565	3+	34 HER2 : 2.5 CEP17		
HCC1419	3+	32 HER2 : 2.2 CEP17		
MDA-MB361	2+	18 HER2 : 2.5 CEP17	++	+

Table R.1 A summary of HER2 amplification/expression status besides the expression of estrogen (ER) and progesterone (PR) receptors in a panel of HER2 amplified breast cancer cell lines

JIMT1 showed low HER2 amplification as well as HER2 1+ pattern. MDA-MB361 cells were HER2 (2+) with the expression of estrogen (ER) and progesterone (PR) receptors. ER and PR expression observed also in BT474

R.2 T-DM1 effects on cellular proliferation and viability

T-DM1 response was analyzed in our panel of HER2 positive breast cancer cell lines. MCF7 cells, which do not overexpress HER2 and do not have HER2 gene amplification, were used as a control in our experiments (**Figure R.2**).

By defining the EC50 of different cell lines using Sigmoidal non-linear regression analysis in GraphPad Prism software, we divided the cell lines to three different groups:

1) Highly sensitive to T-DM1

[0.001μg/mL]<EC50<[0.01μg/mL]: SKBR3, AU565, EFM192A,

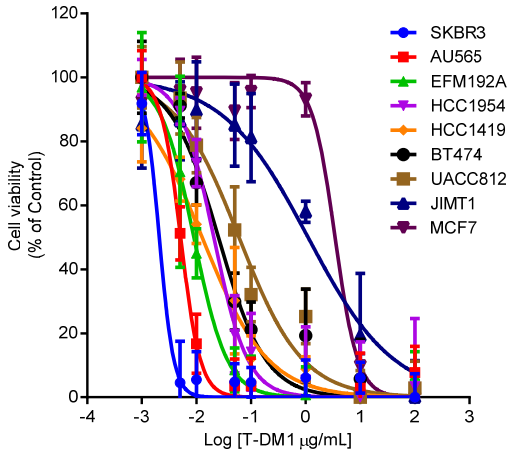
2) Intermediate sensitivity to T-DM1

[0.01μg/mL]<EC50<[1μg/mL]: HCC1954, HCC1419, BT474, UACC812, MDA-361

3) Less sensitive to T-DM1

[1μg/mL]<EC50: JIMT1

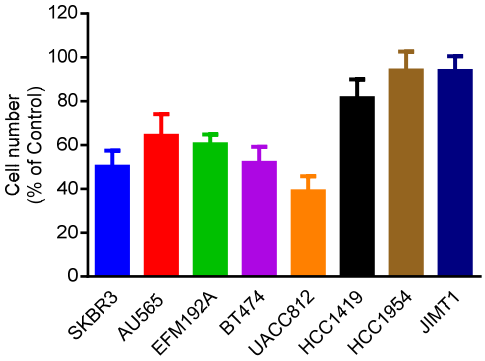
The EC50 for MCF7 was calculated as 4.984 μg/mL, which suggest that T-DM1 at this concentration or above exerts non-specific antitumor effects. The EC50 for T-DM1 in both trastuzumab sensitive and trastuzumab resistant cell lines was similar (**Figure R.2** and **Figure R.3**).



Cell lines	EC50 [μg/mL]
SKBR3	0.002
BT474	0.025
UACC812	0.058
AU565	0.005
EFM192A	0.009
HCC1419	0.018
HCC1954	0.020
JIMT1	1.980
MCF7	4.984

Figure R.2 T-DM1 response in HER2 positive cell lines

72 hours of drug treatment discriminated 3 groups for sensitivity to T-DM1 (highly, intermediate and less sensitive). SKBR3 and JIMT1 categorized as the most sensitive and resistant cell lines to T-DM1, respectively



Cell lines	Trastuzumab Response
SKBR3	S
BT474	S
UACC812	S
AU565	S
EFM192A	S
HCC1419	R
HCC1954	R
JIMT1	R

Figure R.3 Trastuzumab response in HER2 positive cell lines

By 7 days proliferation assay treating the cells by trastuzumab [15μg/mL], three cell lines, HCC1419, HCC1954 and JIMT1 were confirmed as trastuzumab primary resistant cell lines (according to (125)). Less than 20% cell number decrease considered as trastuzumab resistance

R.3 T-DM1 acquired resistant models

R.3.1 T-DM1 acquired resistance generation

We established a protocol of T-DM1 acquired resistant generation in HER2 positive breast cancer cell lines. SKBR3 (the most T-DM1 sensitive cell line) and HCC1954 and HCC1419 (as two intermediate responsive cell lines to T-DM1) were exposed to increasing concentrations of T-DM1 [1-4μg/mL]. The protocol included 3 days of T-DM1 treatment and 3 days without drug (started from T-DM1 [1μg/mL] (18 days) and continued with T-DM1 [2μg/mL] (18 days) and T-DM1 [4μg/mL] (18 days) (**Figure R.4**).

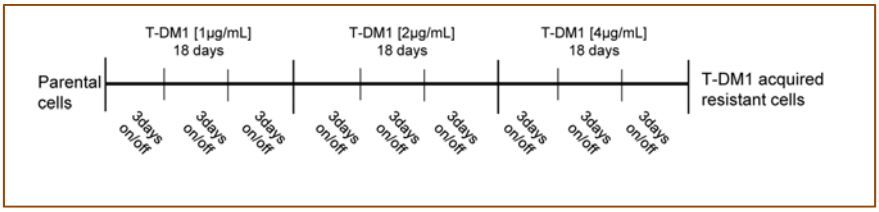


Figure R.4 The protocol of T-DM1 acquired resistant generation

The T-DM1 acquired resistant cells generated by original parental cell lines exposure to stepwise increasing concentrations of T-DM1 in a pulse-fashion (126) as mentioned in the protocol

The protocol set up based on similar cellular exposure to stepwise increasing concentrations of chemotherapeutics agents in a pulse-fashion (126). Previous studies reported T-DM1 [3μg/mL] as clinically relevant concentrations of T-DM1 (127, 128). However, in

this study, T-DM1 [4µg/mL] was considered as a top concentration of the drug.

R.3.2 T-DM1 acquired resistance verification

Once the protocol completed, we tested different concentrations of T-DM1 [0.1, 1, 2 and 4µg/mL] for short (3 days) and long (7 and 10 days) exposure time to assess the stability of T-DM1 resistance (/TDR) cells (**Figure R.5**).

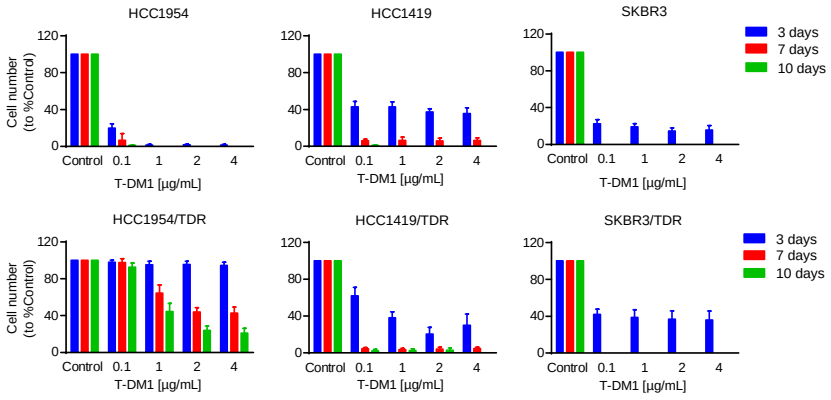


Figure R.5 The analysis of T-DM1 acquired resistance stability

Short (3 days) and long term (7 and 10 days) T-DM1 treatment in parental and resistant HCC1954, HCC1419 and SKBR3 cell lines. HCC1954/TDR considered as stable and SKBR3/TDR and HCC1419/TDR seemed that were partially resistant to T-DM1

The results showed that HCC1954/TDR cells were completely resistant even after 10 days at the concentration of 0.1µg/mL. Based on this, we termed HCC1954/TDR as stable acquired resistant to T-DM1. On the other hand, SKBR3 and HCC1419, at the concentration of 0.1µg/mL demonstrated 20-30% of resistance to the drug at 3 days. In a long time exposure, we could not find the difference between the parental and the resistant cells treated with T-DM1. However, HCC1419/TDR and SKBR3/TDR were considered as partial T-DM1 resistant cells (**Figure R.5**).

Although the level of resistance to T-DM1 was varied between different cell lines, later, the results showed that these levels of resistance was enough to find a similar behavior of resistant cells against those parental counterparts.

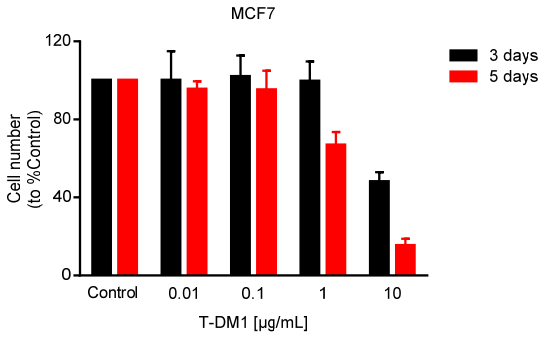


Figure R.6 MCF7 cell line was used as a model of non-HER2 amplified cell line to define the specific concentration of T-DM1

The results showed that the concentrations of 10µg/mL and 1µg/mL are non-specific for 3 and 5 days drug treatment, respectively. 0.1µg/mL considered as specific concentration of the drug used in later experiments

To establish the criterion for resistance discrimination amongst different cell lines, we determined the specific concentration of T-DM1 by testing a range of T-DM1 concentrations [0.01-10μg/mL] in MCF7, a model of non-overexpressing HER2 cell line. Results showed that the concentrations of 10μg/mL and 1μg/mL were non-specific 3 and 5 days post T-DM1 treatment, respectively (**Figure R.6**).

To analyze the stability of resistance, cells expanded and then freeze/thawed and the resistance checked again by proliferation assay. Freeze/thawed following proliferation assay showed similar results to cells before freezing. It is worthy to note that the process of T-DM1 acquired resistance generation did not change the cell proliferation rates (**Figure R.7**).

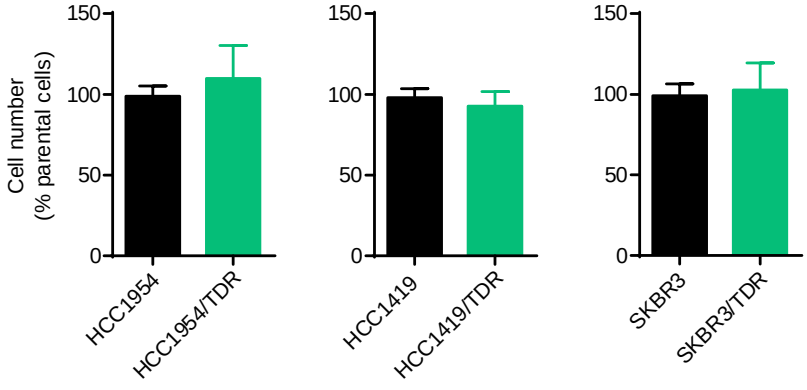


Figure R.7 The analysis of cellular proliferation rates in parental and the resistant cells

The results did not show differences in proliferation rates of parental and the resistant cells, showing that the protocol of acquired resistance generation did not affect the cellular growth rate

The established protocol of T-DM1 acquired resistance generation did not affect the sensitivity of the resistant cells to trastuzumab. HCC1954 and HCC1419 cell lines, both parental and resistant cells were trastuzumab primary resistant. SKBR3 and SKBR3/TDR cells showed the same sensitivity to trastuzumab (around 50% cell viability after 7 days) (**Figure R.8**).

In another word, the trastuzumab component of the drug might be excluded from T-DM1 resistance related mechanism, suggesting that the resistance mechanisms are related mainly to DM1 component.

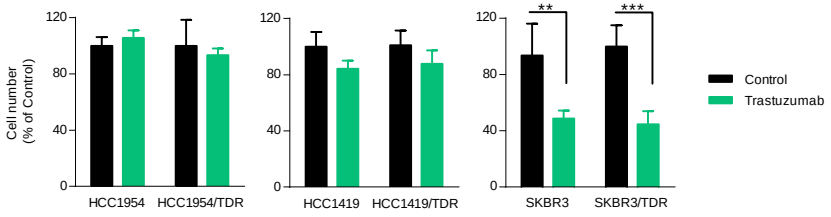


Figure R.8 The effects of trastuzumab, as the backbone of T-DM1, in the parental and the resistant cells

HCC1419 and HCC1954 were two trastuzumab primary resistant cell lines. HCC1419/TDR and HCC1954/TDR cells were still trastuzumab resistant. SKBR3 was sensitive to trastuzumab and similar sensitivity observed in SKBR3/TDR cells (** $p<0.01$, *** $p<0.001$)

We assessed the effects of paclitaxel in both parental and the resistant cells to exclude the mutation in microtubules (tubulin α/β) as a mechanism of resistance. The changes in microtubule stability caused

by tubulin mutation should render resistant cells more sensitive to the addition of paclitaxel (137, 138). The results showed that there were no differences between the parental and the resistant cells regarding paclitaxel response (**Table R.2**).

	Paclitaxel (EC50) (nM)	
	Parental	Resistant
HCC1954	4.5	6.5
HCC1419	6.0	5.6
SKBR3	3.2	3.1

Table R.2 Paclitaxel sensitivity in the parental and T-DM1 acquired resistant cells

Paclitaxel EC50 calculation in parental and resistant cells showed similar sensitivity towards this drug in parental and resistant cells.

Furthermore, these results showed that multi-drug resistance (MDR) mechanism of resistance was not activated in the resistant cells as both parental and the resistant cell showed similar response to paclitaxel. Examining the expression of breast cancer resistance protein (BCRP) gene did not prove differences between the parental and the resistant cells (data not shown).

R4. HER2 pharmacodynamics features in parental and the resistant cells

R4.1. HER2 amplification by FISH

We characterized the parental and T-DM1 acquired resistant cells for HER2 amplification as well as the capacity of the receptor to bind the drug and internalize the cytotoxic agent, DM1, inside the cells.

First of all, we analyzed the amplification of HER2 by FISH. The results showed that HER2 amplification in SKBR3/TDR and HCC1419/TDR was similar to the parental counterparts. In contrast, HCC1954/TDR showed reduced HER2 amplification (**Figure R.9**).

Besides that, HercepTest verified lower level of HER2 expression in HCC1954/TDR than the parental cells, as the resistant cells were considered as 1+ in comparison to 3+ in parental cells (**Figure R.9**).

Further analysis for Estrogen (ER) and progesterone (PR) receptor showed negative expression for both markers either in parental, or in resistant cells (**Figure R.9**).

R4.2. HER2-drug binding efficacy

In the next step, we analyzed T-DM1-HER2 binding capacity in both parental and the resistant cells to find whether less HER2 amplification, at least in one cell line, disturb the drug binding efficacy. The analysis showed similar drug binding capacity in all the resistant cells to parental cell, even in HCC1954/TDR cells that showed less HER2 amplification (**Figure R.10**).

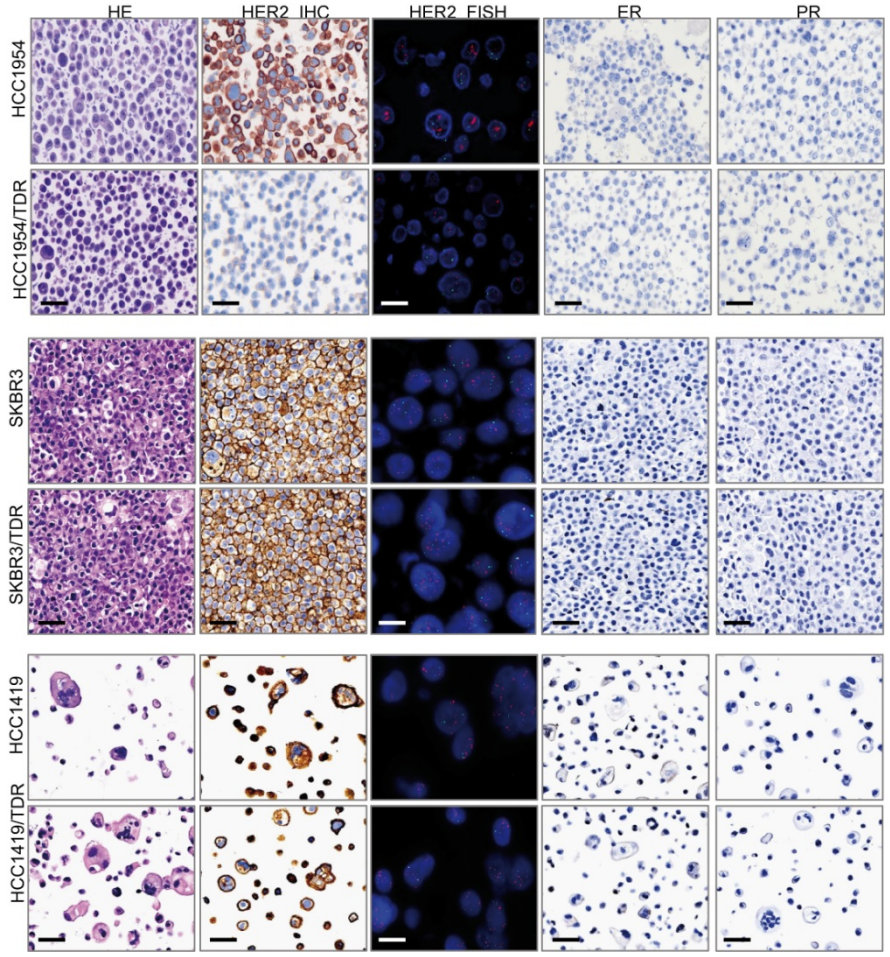


Figure R.9 The evaluation of HER2 expression (HercepTest), amplification (FISH) and the analysis of estrogen (ER) and progesterone (PR) receptors by immunohistochemistry in parental and the resistant cells

In SKBR3 and HCC1419, the expression and amplification of HER2 was not changed between the parental and the resistant cells. For HCC1954, we found less HER2 amplification and reduction in HER2 expression in the resistant cells. In all three pairs of parental and the resistant cells, the expression of estrogen (ER) and progesterone (PR) receptors was negative

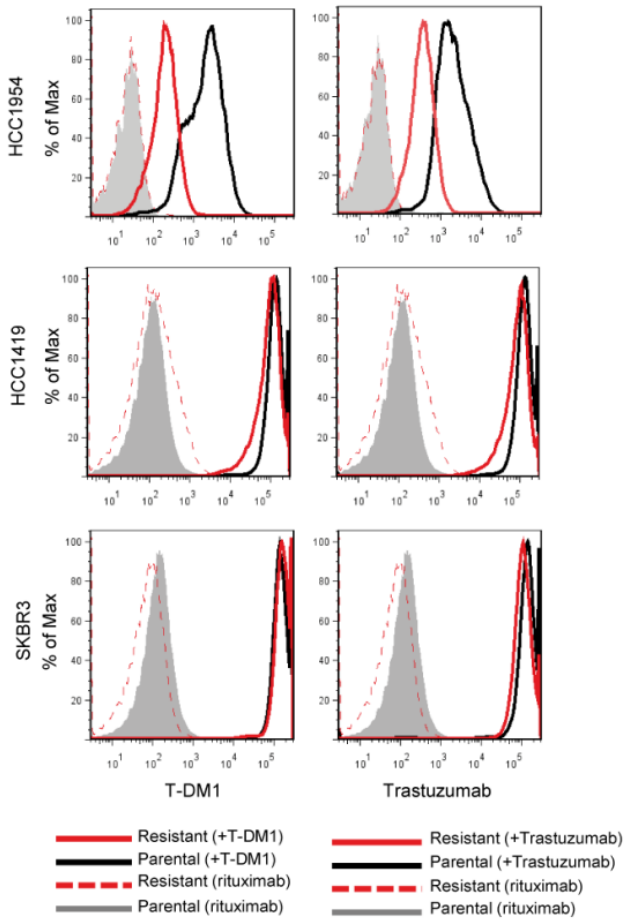


Figure R.10 T-DM1-HER2 receptor binding analysis in the parental and resistant cells

Similar T-DM1 binding capacity was observed in both parental and the resistant cells, while in HCC1954/TDR cells, we previously observed the loss of HER2 amplification and expression. This means that we have still enough number of the receptor in the resistant cells to have enough binding capacity. Trastuzumab used as control

R4.3 HER2-drug internalization efficacy

Antibody-drug conjugates (ADCs) such as T-DM1 represent a rapidly growing class of bio-therapeutics that deliver drugs specifically to target cells by binding of the antibody component to surface receptors. The majority of ADCs require receptor internalization depending on intrinsic features of the specific ADC-antigen interaction.

As we found similar HER2-drug binding capacity in both parental and resistant cells, we next tested if the HER2-T-DM1 internalization was similar in parental and resistant cells (**Figure R.11**).

Internalization assay showed similar T-DM1 internalization for HCC1419 and SKBR3 parental and the resistant cells. In HCC1954/TDR cells, we observed very weak internalization of T-DM1. By using chloroquine (CQ), which changes the lysosomal PH and accumulates T-DM1 in the cytoplasm, we verified enough internalization of the drug, even if there was less HER2 amplification (**Figure R.11**).

Similarly Wang et al reported that the kinetics of binding and internalization of T-DM1 were the same in N87 parental and resistant generated cells (139).

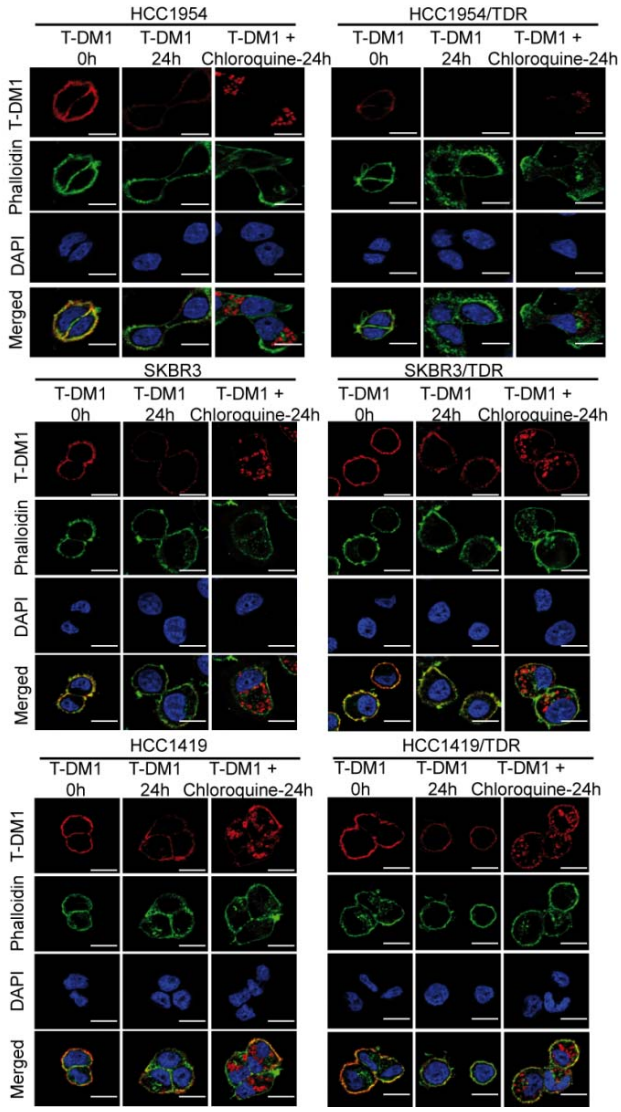


Figure R.11 T-DM1 Internalization assay in parental and resistant cells

T-DM1 internalization was evaluated by immunofluorescence staining. Cells were cultured for 24 hours with/without chloroquine to accumulate intracellularly T-DM1 (129). Similar internalization capacity was observed between the parental and resistant cells. In HCC1954/TDR cells, internalization was observed to a lesser extent than the parental cells (scale bar: 7.5 μm)

R5. T-DM1 treatment and cell cycle profiling

R5.1 Differential cell cycle profile in parental and resistant cells

In the previous analysis (**Section R.4**), we observed HER2-drug pharmacodynamics was similar in parental and T-DM1 resistant cells. In the next step, we checked parental and resistant cell cycle profiles with/without T-DM1 treatment (**Figure R.12**).

Our results showed that T-DM1 induced an obvious G2-M arrest in the parental cells, besides a significant decrease in G1 percentages. In addition, in HCC1954, we found a significant decrease in percentage of S phase (in another two parental cells it was not evident). Surprisingly, T-DM1 effects on G2-M phase of cell cycle were not evident in the resistant cells (**Figure R.12**).

Moreover, the confocal microscopy in the cells with/without T-DM1 treatment staining for α-Tubulin showed a clear microtubule depolymerization in the parental cells, while this effect was not observable in resistant cells (**Figure R.13**). We found also multi-nucleated giant cells that are one of the main characteristics for the anti-mitotic drugs (*i.e.* mitotic catastrophe) (**Figure R.13**).

R5.2 MPF complex alteration in the resistant cells

The differential behavior of T-DM1 on G2-M phase of cell cycle in the parental and resistant cells (123, 124) led us to check the regulator of G2-M phase. The main regulator of G2-M phase of the cell cycle is

mitotic promoting factor (MPF), which includes two members, cyclin B1 and CDK1.

First of all, we checked the activity of CDK1 in parental and resistant cells treated by T-DM1. In the HCC1954 parental cells, we observed the elevation in CDK1 activity 12 hours post T-DM1 treatment. The activity increased further until 24 hours and then dropped down after 48 hours. In contrast, no changes in CDK1 activity were observed in HCC1954/TDR cells (**Figure R.14**).

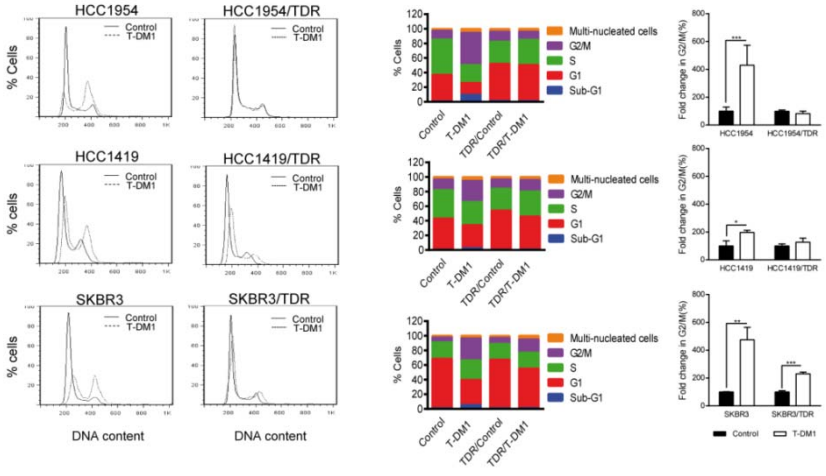


Figure R.12 The effects of T-DM1 on cell cycle profile in the parental and resistant cells

In the parental cells, we observed previously reported effects of T-DM1 in G2-M arrest. G2-M phase percentage of the cells increased post T-DM1 treatment. This effect was not evident in the resistant cells (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

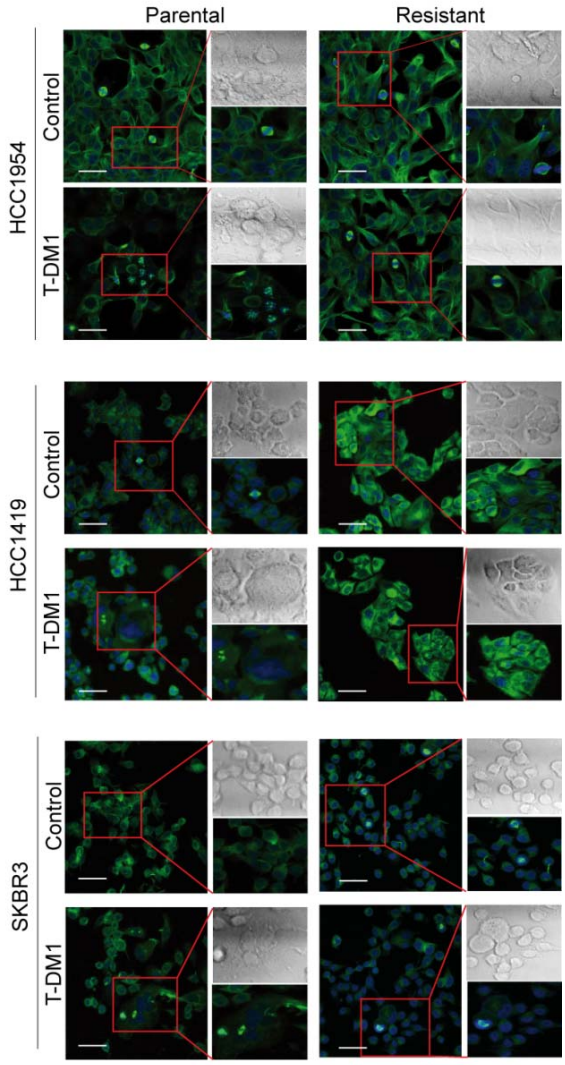


Figure R.13 the effects of T-DM1 on microtubule organization

Microtubule staining by α-tubulin antibody showed that T-DM1 induced structural spindle microtubule abnormalities. Multi-nucleated cells were also observable in the parental cells. Confocal microscopy showed that the final T-DM1 effects on microtubule polymerization after drug-receptor binding, internalization, processing in lysosomes and cytoplasm release was lost in the resistant cells (scale bar: 24μm)

In parallel, we checked the expression of cyclin B1 in a time response T-DM1 treatment in HCC1954 parental cells. In correlation with CDK1 activity analysis, we observed cyclin B1 accumulation after 12 hours T-DM1 exposure, boosts until 24 hours and decreased after 48 hours. Again, no changes in cyclin B1 expression were observed following T-DM1 treatment in the resistant cells (**Figure R.14**). In addition, the basal level of cyclin B1 was lower in resistant than the parental cells.

We checked also SKBR3 and HCC1419 parental and the resistant cells for CDK1 activity and cyclin B1 expression after 24 hours, in the time we observed maximum effects of T-DM1 in time response analysis. Interestingly, in those parental cells, we observed CDK1 activity elevation and cyclin B1 accumulation following T-DM1 treatment, while in the resistant cells, no changes in CDK1 activity and cyclin B1 expression was observed (**Figure R.14**).

Altogether, the cell cycle profiling and the molecular analysis in the parental and resistant cells showed a clear alteration in G2-M regulatory apparatus which might change the sensitivity of the cells to T-DM1. In another word, the main T-DM1 cell cycle effect which is G2-M arrest did not occur in the resistant cells perhaps because of changes in cell cycle regulators, CDK1 and cyclin B1.

R5.3 T-DM1 induced apoptosis in T-DM1 acquired resistant cells

Mitotic catastrophe or mitotic arrest is a type of cell death that occurs during mitosis. Mitotic catastrophe is accompanied by chromatin condensation, mitochondrial release of pro-apoptotic proteins (in particular Cytochrome *c* and AIF), caspase activation and DNA

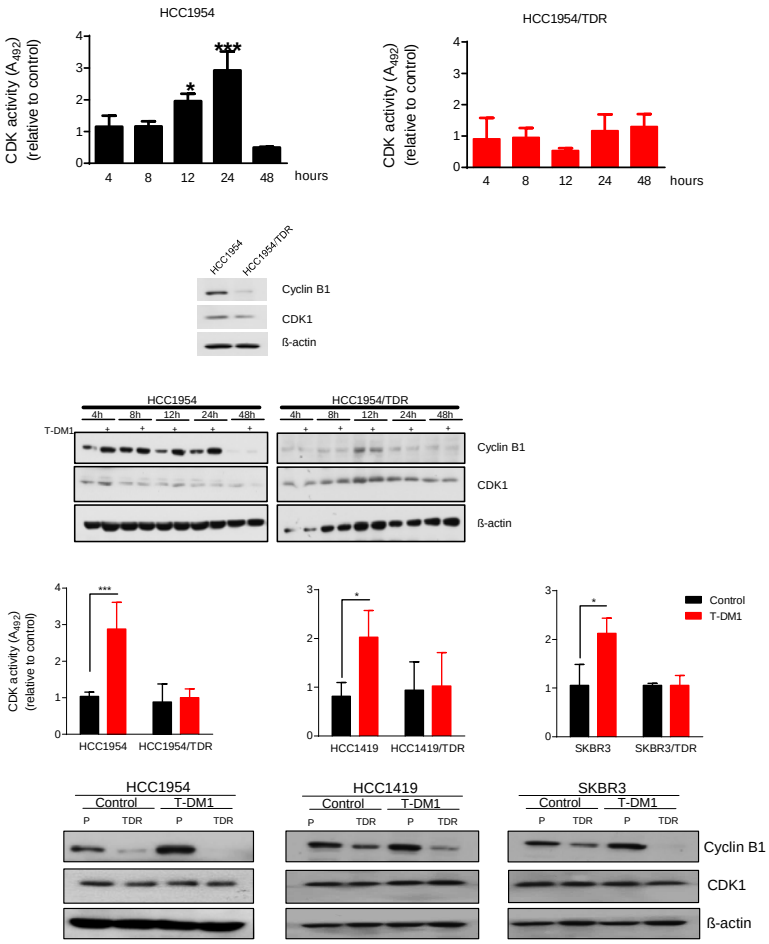


Figure R.14 Differential behavior of parental and T-DM1 acquired resistant cells on MPF complex members

The activity of CDK1 and the accumulation of cyclin B1 observed after 12 hours treatment by T-DM1 and boosted until 24 hours. We found the peak of the activity of CDK1 and accumulation of cyclin B1 was at 24 hours and those drop down by 48 hours. There was less expression of cyclin B1 in resistant cells than the parental cells in the basal level. Similar expression/activity pattern was observed in all three pairs of parental and resistant cells by 24 hours T-DM1 exposure (* $p<0.05$, *** $p<0.001$)

degradation (105, 108). This implies that mitotic catastrophe is accompanied by the key molecular events defining apoptosis, namely, caspase activation and mitochondrial membrane permeabilization (140). Considering above mentioned background, we checked the apoptosis induction in the parental and resistant cells as a consequence of T-DM1 mitotic arrest (**Figure R.15**).

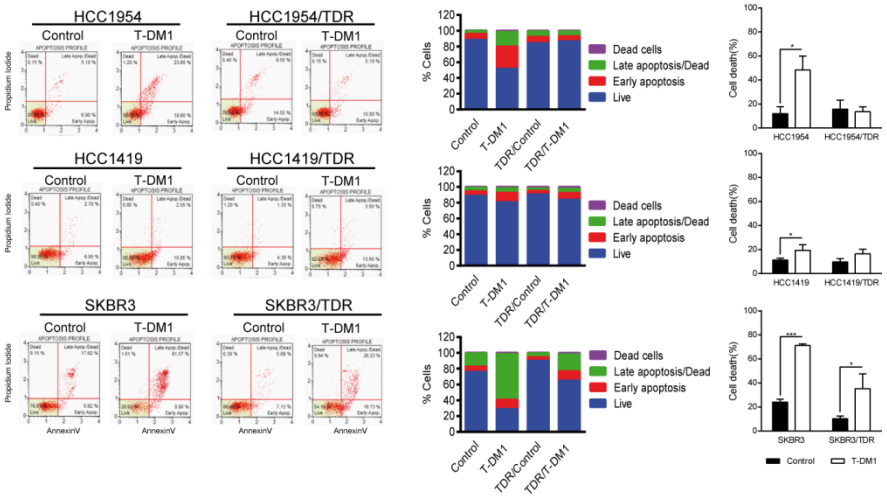


Figure R.15 Mitotic arrest induced apoptosis by T-DM1

Muse Annexin V and Dead Cell analysis showed apoptosis in HCC1954 HCC1419 and SKBR3 parental cells. In contrast, in HCC1954/TDR and HCC1419/TDR, no apoptosis detected. SKBR3/TDR showed less extent apoptosis than parental SKBR3 (* $p<0.05$, *** $p<0.001$)

By using Muse Annexin V and Dead Cell Assay, we detected various levels of apoptosis following T-DM1 treatment in the HCC1954, HCC1419 and SKBR3 parental cells after 48 hours. It is worthy to note

that the apoptosis induction in HCC1419 parental cells were less than the other parental cells. In contrast, no (HCC1954/TDR and HCC1419/TDR) and less apoptosis (in SKBR3/TDR) than SKBR3 parental observed in the resistant cells. Cell cycle arrest in mitosis ultimately led to killing of the cancer cells by apoptosis. In total, these results showed that the alteration in G2-M phase of cell cycle in the resistant cells, finally, prevented the apoptosis in the resistant cells.

R5.4 T-DM1 anti-tumor activity in trastuzumab resistance

R5.4.1 T-DM1 activity in trastuzumab primary resistant cells

Previous results showed T-DM1 had anti-tumor activity in both trastuzumab primary sensitive and resistant cells (**Figure R.2 and Figure R.3**).

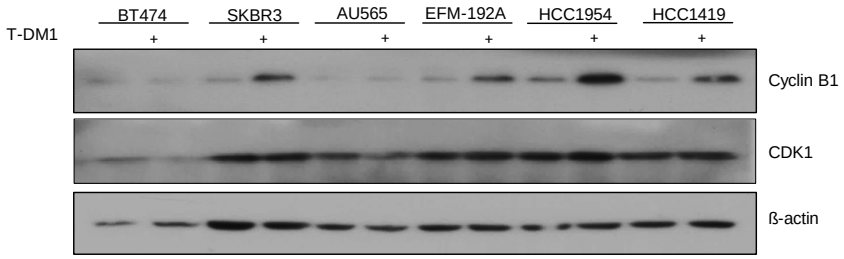


Figure R.16 Cyclin B1 expression in a panel of HER2 positive breast cancer cell lines

A clear accumulation of cyclin B1 observed post T-DM1 treatment in the panel of HER2 positive breast cancer cell lines, except BT474

The analysis of cyclin B1 expression showed similar T-DM1 induced cyclin B1 accumulation in trastuzumab primary resistant and sensitive cells (**Figure R.16**). The only exception was BT474 cell line. In this cell line cyclin B1 expression decreased after T-DM1 treatment.

R5.4.2 T-DM1 activity in trastuzumab acquired resistance

We had available a number of cell lines with acquired trastuzumab resistance (AU565, SKBR3, EFM-192A and BT474 parental and trastuzumab acquired resistance (/TR)).

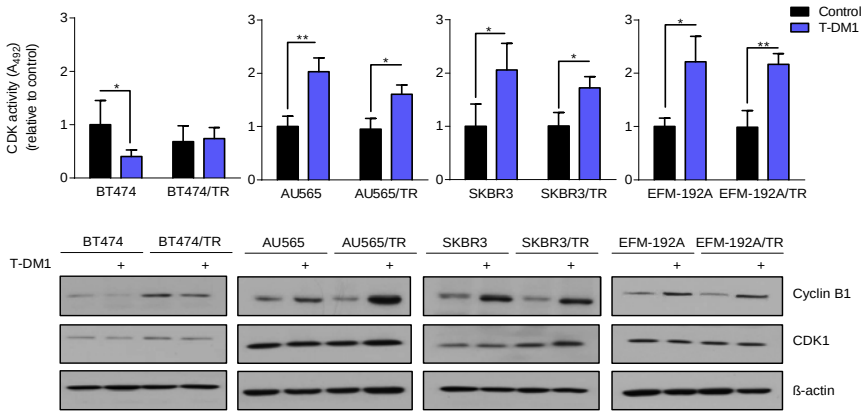


Figure R.17 T-DM1 effects in trastuzumab acquired resistant cells

Similar effects of T-DM1 on MPF complex was observed in EFM-192A, AU565 and SKBR3 parental and resistant cells. We observed different behavior in BT474/TR cells, where the level of cyclin B1 and the activity of CDK1 was not changed by T-DM1 treatment, while in its parental cells we observed the decrease in cyclin B1 expression and CDK1 activity (* $p<0.05$, ** $p<0.01$)

In these cell lines, we tested T-DM1 response regarding its effects on cyclin B1 expression/CDK1 activity (**Figure R.17**). We observed accumulation of cyclin B1 and CDK1 activity elevation in parental and trastuzumab acquired resistance EFM-192A, AU565 and SKBR3 cells. In BT474/TR cells, in contrast to its parental cells, the level of cyclin B1 did not change post T-DM1 treatment (**Figure R.17**).

R5.5 T-DM1 induced apoptosis in trastuzumab acquired resistant cells

We tested apoptosis following T-DM1 treatment in AU565, SKBR3 and EFM-192A parental and trastuzumab acquired resistant cells using Muse Annexin V and Dead Cell Assay after 48 hours (**Figure R.18**).

The results showed clear apoptosis induction following T-DM1 in both parental and trastuzumab acquired resistant AU565, SKBR3 and EFM-192A cells. In contrast, no signs of apoptosis observed in BT474 and BT474/TR cells which had a different T-DM1 behavior in BT474 compared to the rest of HER2 positive breast cancer cell lines (**Figure R.18**).

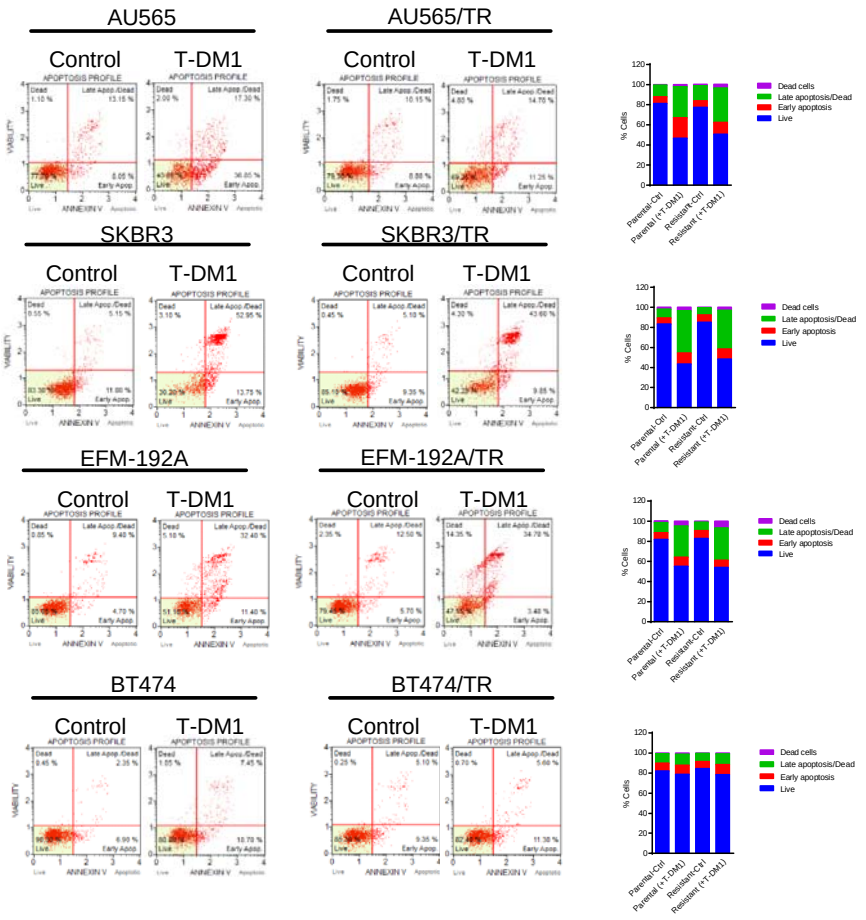


Figure R.18 T-DM1 induced apoptosis in the parental and trastuzumab acquired resistant cells

Muse Annexin V and Dead Cell analysis showed apoptosis in AU565, SKBR3 and EFM-192A parental and trastuzumab resistant cells after 48 hours. In contrast, in BT474 both parental and trastuzumab resistant cells, no signs of apoptosis were detected

R6. Cyclin B1 functional studies

R6.1 Cyclin B1 silencing induced T-DM1 resistance in parental cells

To answer the question whether there was a mechanistic link between cyclin B1 and T-DM1 effects in parental cells, we silenced cyclin B1 in the parental cells. We used nucleofector technology to silent cyclin B1 and then, the expression of cyclin B1 checked by western blot 24 ad 48 hours post transfection (**Figure R.19**).

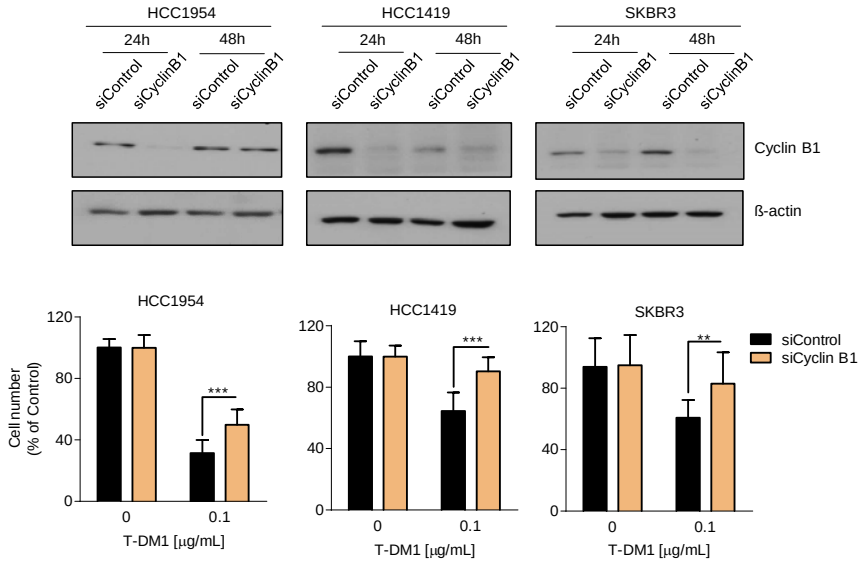


Figure R.19 Cyclin B1 silencing in parental cells induced T-DM1 resistance

Western blot analysis showed an efficient cyclin B1 silencing 24 and 48 hours post-transfection. The effects of cyclin B1 silencing on T-DM1 resistance induction checked by proliferation assay illustrated different level of resistance (** $p<0.01$, *** $p<0.001$)

The blotting analysis verified cyclin B1 silencing in parental HCC1954, HCC1419 and SKBR3 cells. Then, we assessed the proliferation assay in scrambled and silenced cyclin B1 with/without T-DM1 treatment. The results showed the acquisition of resistance (19% in HCC1954, 25% in HCC1419 and 22% in SKBR3) in silenced cyclin B1 parental cells (**Figure R.19**).

R6.2 Cyclin B1 accumulation recovered T-DM1 sensitivity in the resistant cells

Since we found a role of cyclin B1 expression in parental cells T-DM1 response, we hypothesized that the recovery of cyclin B1 in resistant cells might recover the sensitivity to T-DM1. For this reason, we silenced cdc20 in the resistant cells. cdc20 is responsible for cyclin B1 degradation at the end of mitosis.

cdc20 and cyclin B1 expression checked by western blot showed an efficient silencing for cdc20 and cyclin B1 accumulation 48 and 72 hours post transcription (**Figure R.20**). Proliferation assays showed a significant decrease in cell number in HCC1419/TDR and SKBR3/TDR cdc20 silenced cells (**Figure R.20**). In HCC1954/TDR cells even with a potent recovery of cyclin B1 by cdc20 silencing, T-DM1 sensitivity did not recover.

Altogether, the functional studies by cyclin B1 silencing and expression recovery clarified the importance of cyclin B1 in T-DM1 response

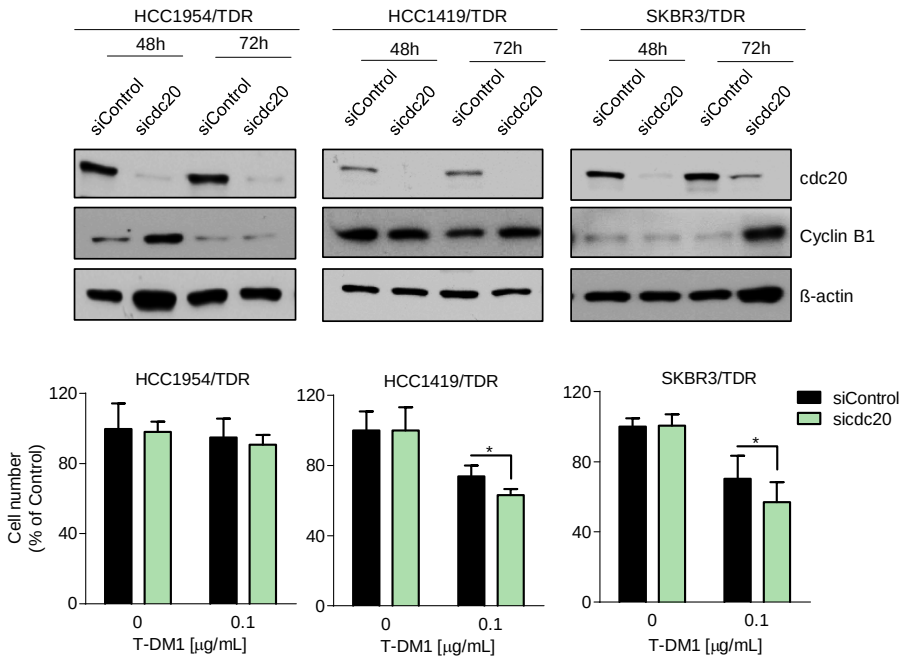


Figure R.20 Cyclin B1 accumulation in the resistant cells recovered T-DM1 sensitivity

cdc20 and cyclin B1 expression checked by western blot showed an efficient silencing for cdc20 and cyclin B1 accumulation 48 and 72 hours post transcription. Proliferation assay showed a significant decrease in cell number in HCC1419/TDR and SKBR3/TDR cdc20 silenced cells in comparison to scrambled cells (* $p<0.05$)

R.7 T-DM1 response *in vivo*

R.7.1 HCC1954 xenograft model

In order to generate a tumor xenograft model, HCC1954 (in a concentration of 10-15×10^4 cells) mixed with matrigel and then injected subcutaneously in the dorsal of each mice (n=12) for tumor generation. Tumors were clearly visible in all mice inoculated with a mean volume of 100-150mm^3 before starting the drug treatment (**Figure R.21**).

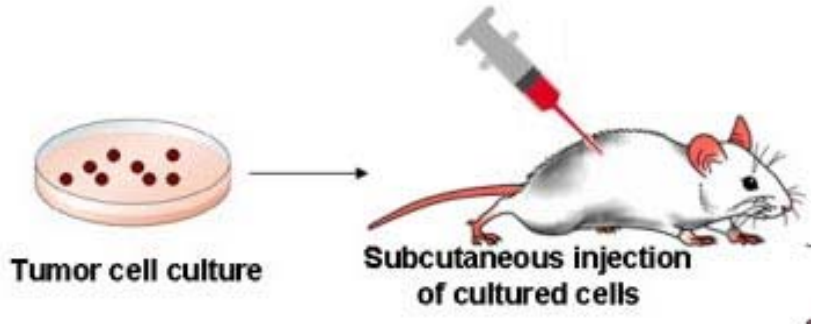


Figure R.21 Schematic of subcutaneous injection

To generate a tumor xenograft model, HCC1954 cells mixed with matrigel and then injected subcutaneously in the dorsal of each mice for tumor generation

Drug treatment with control antibody (n=6) or T-DM1 [5mg/kg] (n=6) (*i.v.*) (141-143) was initiated 7 days after HCC1954 inoculation in animals.

T-DM1 was injected (*i.v.*) every 21 days, based on clinical practice (144). The growth curves showed T-DM1 *in vivo* anti-tumor activity in breast cancer xenografts (**Figure R.21**).

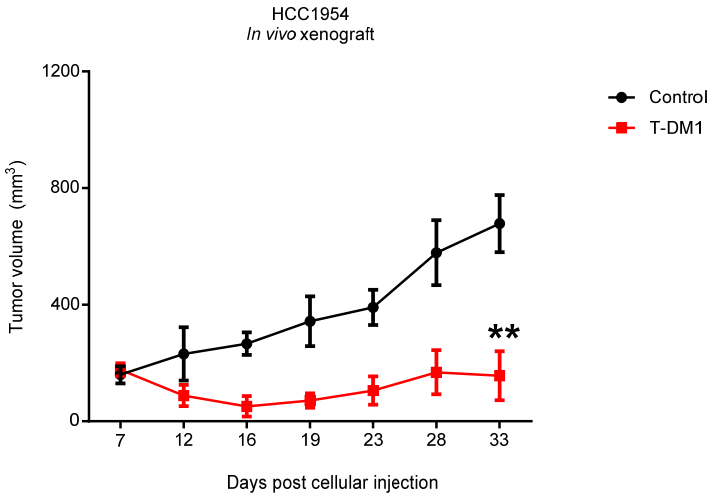


Figure R.22 T-DM1 response *in vivo*

n=12 animals injected by HCC1954 cells mixed with matrigel and then injected subcutaneously in each animal. The first group of tumors collected after 5 days of the treatment, in the day 12, control (n=3) and treated (n=3) animals. Same was done in the day 33 control (n=3) and T-DM1 treated animals (n=3) (** $p<0.01$)

The first group of animals (n=6, 3 controls and 3 treated) were sacrificed after 5 days of T-DM1 treatment and tumor specimens collected were analyzed by immunohistochemistry. At this time point, mice treated with T-DM1 achieved a grossly complete response when compared to vehicle group. The control group had a tumor size

(mean±SEM) of **246.80±31.80 mm³** and the T-DM1 group was **73.26±9.658 mm³**. The difference was statistically significant when both conditions were compared (** p<0.01). The same was true when the weight of the tumors were compared (**Figure R.22** and **Figure R.23**).

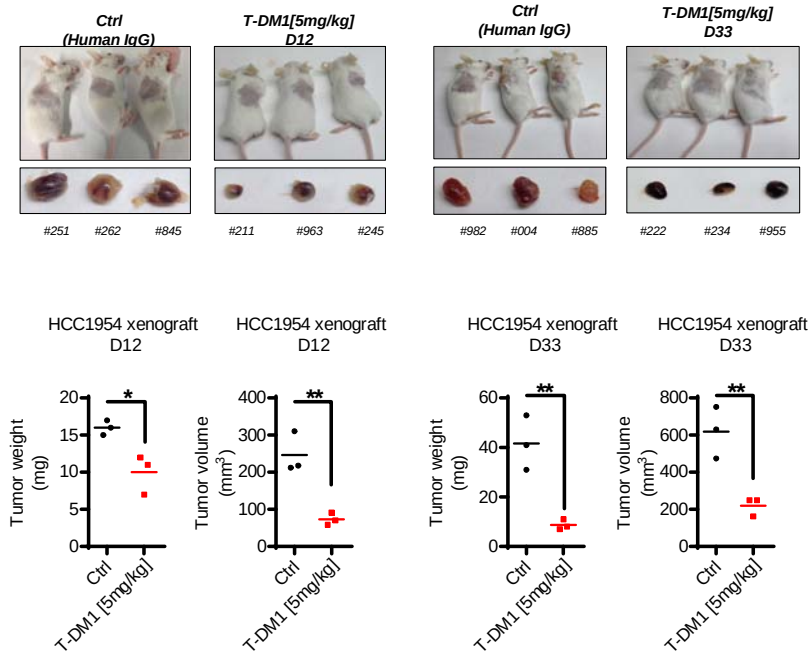


Figure R.23 Tumor volume and weight measurement in control and T-DM1 treated animals after 12 and 33 days

When the tumor reached 100-150mm³, T-DM1 [5mg/kg] treatment started and 5 days later, the specimen collected. Then, after 21 days, second dose of the drug applied and samples collected for further analysis. In each time line, the tumor volume and weight measured (* p<0.05, ** p<0.01)

In the remaining animals (n=6), a second round of treatment with T-DM1 was performed after 21 days of the first drug injection and a second group of animals (3 controls and 3 treated) were sacrificed after 5 days of the treatment and tumor specimens were collected. At this point, the control group had a tumor size (mean±SEM) of **619.10±80.63 mm³** and the T-DM1 treated group was **220.20±29.22 mm³** (** p<0.01) (**Figure R.22** and **Figure R.23**).

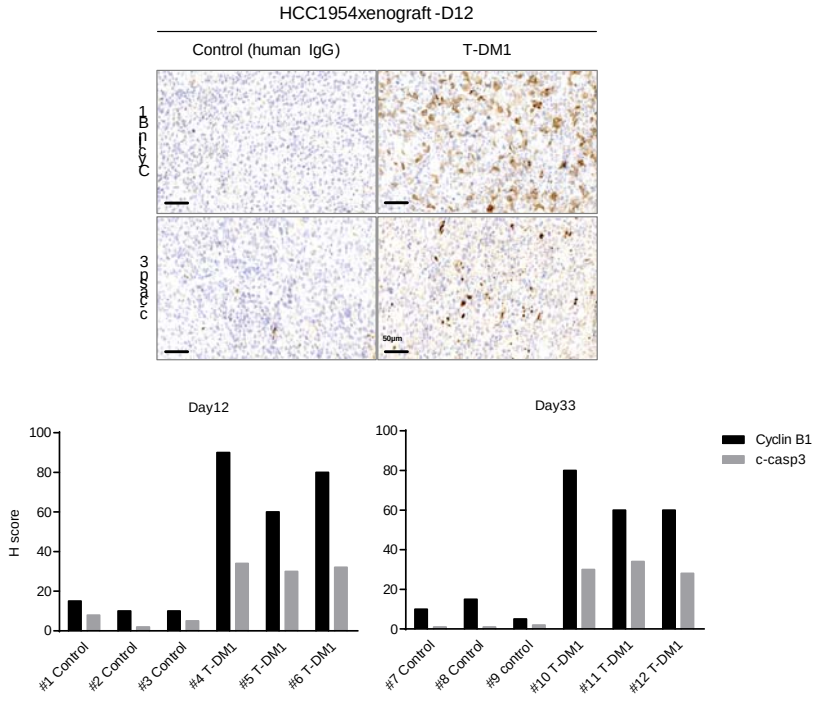


Figure R.24 Cyclin B1 and c-casp3 staining in *in vivo* samples collected in different time lines

In day 12, when we observed a clear effect of T-DM1 in tumor volume and weight, cyclin B1 and c-casp3 expression increased. Similar results observed at day 33

R.7.2 T-DM1 *in vivo* effects on cyclin B1 expression and apoptosis

We aimed to illustrate the effects of T-DM1 *in vivo* on cyclin B1 expression (as the marker of mitotic arrest) as well as c-casp3 (as the marker of apoptosis) (**Figure R.24**).

The staining for these two markers in the samples collected at day 12 (the first round of 5 days treatment) showed a clear elevation in the expression of cyclin B1 and c-casp3 that was correlated with T-DM1 effects on tumor volume and weight measurement. Similar results were observed in the day 33 (**Figure R.24**).

R.8 T-DM1 *ex vivo* response

R.8.1 T-DM1 *ex vivo* effects on cyclin B1 expression and apoptosis

With the background we observed in *in vitro* and *in vivo* models, we investigated the *ex vivo* activity of T-DM1 on tumor samples derived from breast cancer patients using an *ex vivo* model according to our experience (145).

The aims were to investigate differences in drug sensitivity between various individual patient samples and to investigate whether these differences were associated with cyclin B1 modulation within the subsets of patients. Patient samples treated *ex vivo* with T-DM1 for 120 hours and c-casp3 determinations by immunohistochemistry were used to assess T-DM1 sensitivity *ex vivo* (**Figure R.25**).

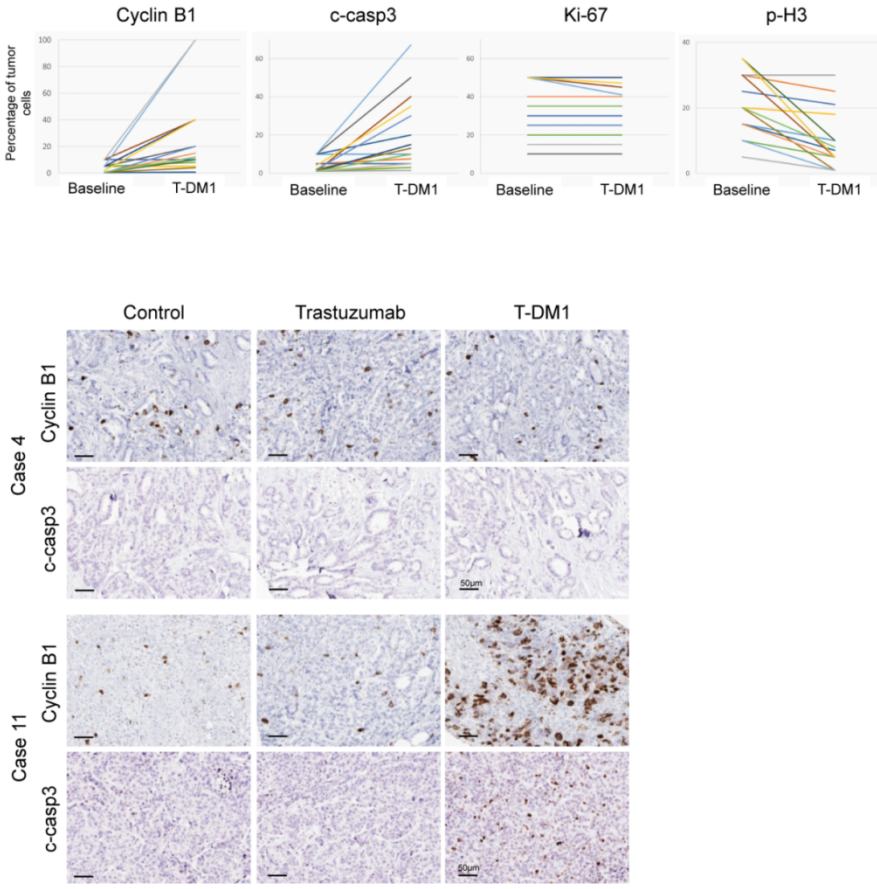


Figure R.25 T-DM1 *ex vivo* response in breast cancer patients' samples

In case 11, the expression of cyclin B1 and c-casp3 was elevated in comparison to trastuzumab used as control. c-casp3 expression besides cyclin B1 expression elevated. In case 4, no signs of cyclin B1 and c-casp3 expression elevation were observed. In summary, based on the modulation in expression of cyclin B1 and c-casp3, we could categorize case 11 as T-DM1 responsive (sensitive) and case 4 as T-DM1 non-responsive (primary resistance). The proliferation markers, Ki67 and pH3 were also analyzed. Ki67 did not showed modulation following T-DM1, but we observed a decrease in the expression of pH3 in the samples

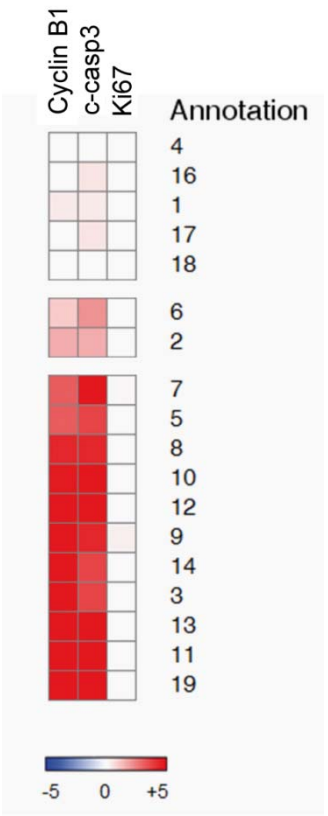


Figure R.26 HER2 positive patients divided into three groups based on the modulation of cyclin B1 and c-casp3

Group 1) No cyclin B1 regulation by T-DM1 and very low up-regulation of apoptosis (c-casp3); Group 2) group with a low cyclin B1 up-regulation by T-DM1 and weak up-regulation of apoptosis (c-casp3); and Group 3) a strong cyclin B1 up-regulation by T-DM1 and high up-regulation of apoptosis (c-casp3). We did not observe Ki67 modulation by T-DM1 in the samples

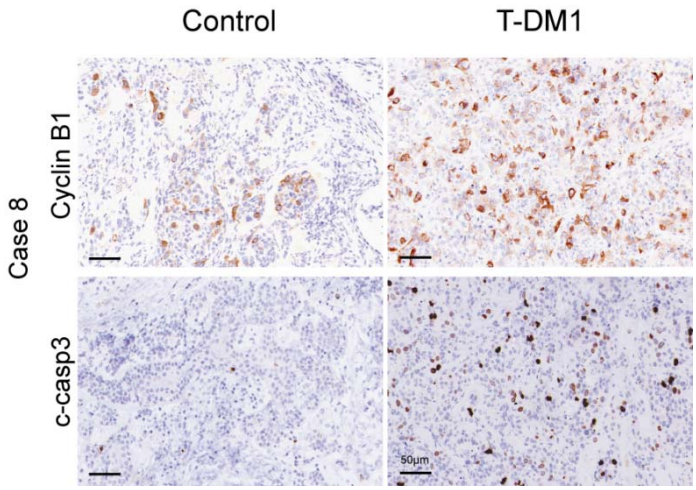


Figure R.27 Representative IHC images of control and T-DM1 treated explant obtained from a HER2 positive patient liver metastasis

Cyclin B1 and c-casp3 (apoptosis) staining performed in explants from metastatic patients (liver) showed a partial induction of cyclin B1 and elevation of c-casp3 following T-DM1 treatment *ex vivo*

Our results showed that cyclin B1 accumulation by T-DM1 varies between individual patients and grouped into three classes: 1) no cyclin B1 regulation by T-DM1 and very low up-regulation of apoptosis (c-casp3); 2) a low cyclin B1 up-regulation by T-DM1 and weak up-regulation of apoptosis (c-casp3); and 3) the third group showing a strong cyclin B1 up-regulation by T-DM1 and high up-regulation of apoptosis (c-casp3) (**Figure R.26**). Although there were few samples, the analysis of correlation between *ex vivo* T-DM1 sensitivity and cyclin B1 was conclusive. Some samples were

essentially unaffected by the concentration of T-DM1 tested, whereas tumor cells from other samples showed increases cyclin B1 at the concentration tested (**Figure R.26**). These latest samples were mostly more sensitive to T-DM1 treatment as demonstrated by c-casp3 staining. On the other hand, there were clearly some individual samples being resistant to T-DM1 in which we found a weak cyclin B1 and cleaved c-casp3 up-regulation post T-DM1 treatment.

Most of the explants were from diagnostic specimens derived from patients that received neoadjuvant treatment without T-DM1. Besides that, we had some samples from metastatic patients. Two received T-DM1 and had cyclin B1 and apoptosis induction *ex vivo*. One had *de novo* metastatic disease, the explant was from the diagnostic breast cancer biopsy, and received T-DM1 as second line achieving a partial response. The second patient had bone and liver disease and after several lines of treatment received T-DM1. The explant was obtained from a liver metastasis just before T-DM1 (**Figure R.27**) and subsequently had a partial response.

DISCUSSION

DISCUSSION

D.1 Introduction

HER2 amplification/overexpression has been reported in 15-20% of the breast cancer patients (146). This proposes the susceptibility of this proportion of breast cancer patients to anti-HER2 targeted therapy.

So far, several anti-HER2 targeted therapies approved by FDA. This includes trastuzumab, a humanized monoclonal antibody against HER2 (8, 147), pertuzumab, HER2-HER3 dimerization inhibitor (14, 148), and recently trastuzumab-emtansine (T-DM1), a novel antibody-drug conjugated agent (ADC) (24, 149). Antibody-drug conjugates (ADCs) are an evolving novel class of anti-cancer treatment agents that combines the selectivity of targeted therapy with the cytotoxic potency of chemotherapeutic drugs.

T-DM1 is a novel antibody-drug conjugated agent, whereby trastuzumab is linked to a microtubule depolymerizing agent, DM1 (a derivative of maytansine) using a non-reducible thio-ether linker (11, 66, 80, 82, 142). T-DM1 displayed superior activity compared to unconjugated trastuzumab (150). However, T-DM1 has limited activity as a single therapy and many patients treated with T-DM1 fail to respond to therapy *de novo*. On the other hand, in patients in whom T-DM1 works, or after a period of response, they develop resistance to T-DM1 (acquired resistance) (149, 151, 152).

This study sought to address this by developing new cell line models of acquired T-DM1 resistance and characterizing the resistance phenotype, with a view to identifying mechanisms of acquired resistance to this drug.

D.2 *In vitro* T-DM1 acquired resistance

Cancer drug resistance continues to be a major obstacle in medical oncology. Clinically, resistance can arise prior (primary) to or as a result of cancer therapy (acquired resistance).

One of the main theories in cancer drug resistance obeys the *Darwinian law* of evolution, hence, under therapeutic pressure, those populations that are most adaptive or resistant to treatment will be selected (153). *Inter*- and/or *intra*-tumoral heterogeneity often reports as one of the powerful forces in being resistance to anti-cancer therapies (154, 155). The changes in metabolism of cells (156), epigenetic alterations (157), and cancer cells microenvironment modifications (158, 159) have been also suggested as cancer drug resistance inducers.

To our knowledge, there are a few studies focused on T-DM1 mechanism of resistance in breast cancer or in the other types of cancer. Nevertheless, T-DM1 resistance is observed in pre-clinical and clinical models (149, 152, 160).

Loganzo *et al.* generated *in vitro* resistant models to trastuzumab-maytansinoid ADC using two HER2 positive breast cancer cell lines MDA-MB-361 and JIMT-1 (161). The results showed that multi-drug resistance associated protein 1 (MRP1) was overexpressed in MDA-MB361 cell line resistant to T-DM1.

Phillips *et al.* reported that the resistance to T-DM1 was associated with the expression of the HER3 ligand neuregulin (NRG) in selected cellular models. This can promote the formation of HER2-HER3 dimers and PI3K pathway activation (162).

In another study, Mellor *et al.* observed a decrease in the surface expression of HER2 in breast cancer cell lines resistant to analogue of T-DM1 (163). The correlation between the expression of HER2 and T-DM1 response was further reported in the clinical trials (128, 164-168).

Moreover, the modifications in cell adhesion molecules and the alterations in prostaglandin pathways were suggested as the mechanism of resistance to T-DM1 using MDA-MB361 breast cancer cell line model and OE-19, a model of esophageal cancer cells (169).

Besides that, a decrease in β3 tubulin expression that might be associated with an increased number of chromosomes suggested as a T-DM1 mechanism of resistance (169). Tubulin alterations/mutations have been proposed in similar microtubule depolymerizing agents such as paclitaxel (170-173) and vincristine (174-176).

More, Wang *et al.* reported a decrease in T-DM1 metabolites induced by aberrant V-ATPase activity contributes to T-DM1 resistance. They proposed V-ATPase activity in lysosomes as a novel biomarker for predicting T-DM1 resistance (139). Abnormal endosomal/lysosomal activity which can lead low intracellular concentrations of the cytotoxic agent might be considered as a potent mechanism of T-DM1 resistance.

In our study, firstly, T-DM1 response was assessed in a panel of HER2 positive breast cancer cells. The results showed T-DM1 efficacy in all the cells, either with trastuzumab primary resistance (HCC1954, HCC1419), or sensitivity. Similar efficacy of T-DM1 in panel of HER2 positive cells has been also reported by others (67, 159, 177).

In the next step, based on the EC50 of different cell lines, SKBR3, HCC1419 and HCC1954 were selected for the generation of T-DM1 acquired resistant model. With on/off exposure strategy (126) by increasing the concentration of T-DM1, for 54 days, we generated acquired resistant cells, SKBR3/TDR, HCC1419/TDR and HCC1954/TDR. The T-DM1 resistance was verified by viability and proliferation assay. The cells verified with different level of resistance to T-DM1 (2-80 times *vs.* parental).

The generation of resistant cell lines in a short time frame may be caused by several mechanisms and may vary between cell lines. In HCC1954, a specific finding was a marked reduction of HER2 gene amplification after the first round of exposure to T-DM1. In parental HCC1954 cells, there was a predominant subpopulation (~93% of cells) with high HER2 amplification and a minor subpopulation (~7%) with low, but amplified HER2 gene. An early clonal selection of the subpopulation with lower HER2 amplification following T-DM1 exposure appears to contribute to resistance. Regardless of this, the rapid emergence of resistance, also in cell lines that retain the same level of HER2 amplification, suggests a mechanistic link with the cytotoxic DM1 component rather than to trastuzumab, by as yet unknown mechanisms such as epigenetic and/or cellular pathway rewiring.

Several attempts failed to generate T-DM1 acquired resistant in BT474 cell line. Later, we found a different molecular effect of T-DM1 in this cell line. In concordance, Chung *et al.* showed Caveolin-1 dependent endocytosis enhanced the sensitivity of HER2 positive breast cancer cells to T-DM1 (178). BT474 showed lower Caveolin-1

expression than SKBR3 and the Caveolin-1 overexpressing BT474 were more sensitive to T-DM1 treatment.

All in all, these results showed the efficacy of T-DM1 in trastuzumab primary resistant and sensitive cells. This was an important finding, since T-DM1 mostly used in trastuzumab pre-treated patients in clinical practice.

D.3 HER2 receptor related T-DM1 pharmacodynamics

HER2 surface receptor expression seemed important for trastuzumab (52, 179) and T-DM1 response *in vitro* (163) and in clinical models (128, 164-168). Trastuzumab, as the backbone of T-DM1, must bind and internalize correctly inside the cells to have a sufficient delivery of DM1 in the cells. Therefore, the parental and resistant cells analyzed based on HER2 receptor related T-DM1 pharmacodynamics features. The main studies were:

1. **HER2 amplification/overexpression**

   The HER2 amplification analysis in the parental and the resistant cells showed similar level of HER2 amplification in SKBR3 and HCC1419 parental and the resistant cells. We found reduced HER2 amplification in HCC1954 resistant cells (but still in the range considered as amplified, FISH +)

2. **HER2-drug binding**

   Drug-receptor binding assay was performed in both parental and the resistant cells specially in HCC1954/TDR cells that showed less HER2 amplification. The results illustrated

similar binding capacity in all of the cell lines, even the cells with reduced HER2 amplification.

3. **Drug internalization**

   For finding the capacity of receptors to internalize the drug inside the cells, the internalization of T-DM1 was assessed in the parental and the resistant cells. The results showed similarity in internalization capacity in both parental and resistant cells.

Altogether, our results illustrated that HER2 related T-DM1 pharmacodynamics aspects did not change between the parental and resistant cells. Although there are some reports about the role of HER2 expression in trastuzumab response and resistance (52, 179), it was not crucial for T-DM1 efficacy in our models. Indeed, several preclinical studies mentioned the activity of T-DM1 in HER2 low expressed cell lines and low HER2 expressed clinical cases (128, 168)

D.4 Cyclin B1: a predictive biomarker for T-DM1 activity

D.4.1 Cyclin B1 and T-DM1: *in vitro* studies

There are few reports about T-DM1 mechanisms of action and resistance. Among them, mitotic catastrophe has been stressed by several studies (80, 81, 83, 180, 181).

By the definition of mitotic catastrophe, T-DM1 effects through DM1 component seemed probable (105-107). Mitotic catastrophe has been

reported in DNA damaging agents (182, 183) and microtubule modifying agents (184, 185).

However, we analyzed the effects of T-DM1 in both parental and the resistant SKBR3, HCC1419 and HCC1954 cell lines. The results showed that in the parental cells, T-DM1 induced a significant G2-M cell cycle arrest, as others reported (124, 186). In the resistant cells, the effects of T-DM1 on G2-M phase were diminished. These results altogether led us to hypothesize that the apparatus involved in G2-M arrest, cyclin B1-CDK1 complex, might be altered in the resistant cells, and let those cells escape from G2-M arrest.

Our analysis showed that while the activity of CDK1 was elevated and the expressions of cyclin B1 enhanced by T-DM1 in the parental cells, these effects were not evident in the resistant cells. These results clearly verified the difference in cell cycle profile of parental and resistant cells post T-DM1 exposure.

Other CDK-cyclin complexes have been previously reported as implicated in resistance to anti-HER2 therapies (187-190). For example, cyclin E has a role in trastuzumab resistance and treatment with CDK2 inhibitors has been proposed for tumors displaying cyclin E amplification/overexpression (88, 191, 192).

On the other hand, the inhibition of CDK/cyclin complexes has been shown promising combination therapy with T-DM1. Witkiewicz *et al.* reported CDK4/6 inhibition provided a potent adjunct to anti-HER2 targeted therapy in pre-clinical breast cancer models (193). Goel *et al.* showed the effects of CDK4/6 Inhibitors for overcoming resistance to anti-HER2 targeted therapies (194).

The importance of MPF complex further verified where its silencing caused a partial resistance in the parental cells responding to T-DM1. On the other hand, the cdc20 silencing in the resistant cells, which indirectly revived the expression level of cyclin B1 (195, 196), sensitized those cells to T-DM1.

D.4.2 Cyclin B1 and T-DM1: *in vivo* studies

T-DM1 effects *in vivo* have been evaluated in several studies. Barok *et al.* reported T-DM1 caused tumor growth inhibition by mitotic catastrophe in trastuzumab-resistant breast cancer cells using JIMT1 xenograft model (80). By histological analysis, they found the cellular response to T-DM1 consisted of apoptosis and mitotic catastrophe. They observed evidence of an increased number of cells with aberrant mitotic figures and giant multinucleated cells. English *et al.* reported that T-DM1 was highly effective against primary HER2 overexpressing uterine serous carcinoma *in vitro* and *in vivo* (165). In another study, Nicoletti *et al.* found T-DM1 was highly effective uterine and ovarian carcinosarcomas overexpressing HER2 (167). Cretella *et al.* showed that T-DM1 is active on HER2 overexpressing NSCLC cell lines and overcomed gefitinib resistance (123).

In our study, to test further the importance of cyclin B1 as a predictive pharmacodynamic marker for T-DM1, *in vivo* analyses were performed. The histological analysis showed the accumulation of cyclin B1 as an indicator of tumor response to the drug and very interestingly it was correlated with the expression of apoptosis marker, c-casp3.

D.4.3 Cyclin B1 and T-DM1: *ex vivo* studies

To complete this translational study, we tried to define the effects of T-DM1 on patients´ explants. In detail, some explants were from diagnostic specimens derived from patients that received neoadjuvant treatment without T-DM1. Also, we had three samples from metastatic patients. We aimed to analyze the explants treated 120 hours with T-DM1 for cyclin B1 induction and its correlation with apoptosis marker expression.

In a similar study, Witkiewicz *et al.* studied the effects of T-DM1 on primary tumor explants by pH3 and Ki67 staining analysis (193). Multiple aberrant mitotic figures were detected in HER2-positive tumors by histological analysis. Staining for Ki67 and pH3 in our patients´ explants showed a reduction in the level of pH3 and no changes in Ki67. Indeed, it is reported that the cells arrested in G2-M phase stain positive to Ki67 (185).

We could categorize the patients to three groups:

1) Cyclin B1 up-regulation/apoptosis induction by T-DM1 exposure

2) Weak Cyclin B1 expression/apoptosis induction by T-DM1 exposure

3) No sign of Cyclin B1 expression/apoptosis induction by T-DM1 exposure

In summary, our results suggested T-DM1 *in vitro* molecular activity happened similarly *in vivo* in animal tumor models as well as patient samples by *ex vivo* analysis for the markers of mitotic arrest and apoptosis. Our results showed a remarkable alteration in cell cycle especially in G2-M phase apparatus. We showed MPF complex status

was different in the resistant cells and consequently the apoptosis induction by T-DM1 did not occur in the resistant cells.

Altogether, *in vitro*, *in vivo* and *ex vivo* analysis illustrated an important role of cyclin B1 induction in T-DM1 response and resistance. By analyzing different models, we found a clear correlation between the T-DM1 response and cyclin B1 expression. It was also evident that in the time of T-DM1 resistance, the main effects of the drug leading G2-M cell cycle arrest and consequently, the apoptosis was lost.

The question ¨why T-DM1 cannot induce cyclin B1 accumulation and CDK1 activity enhancement¨ needs further investigation.

CONCLUSIONS

CONCLUSIONS

1) T-DM1 showed a dramatic efficacy in HER2 positive breast cancer cell lines, including both trastuzumab sensitive and trastuzumab resistant cells.

2) In cell lines with acquired resistance to T-DM1, drug binding and internalization was similar than in parental T-DM1 sensitive cells.

3) T-DM1 main effect in parental cells was G2-M arrest in a cyclin B1 dependent manner. In the resistant cells, the effect of T-DM1 on cell cycle G2-M phase was significantly reduced.

4) The reduced effect of T-DM1 on G2-M was linked to a defective induction of cyclin B1, consistent with a dysregulation of the mitotic promoting factor (MPF) complex.

5) The genetic modulation of cyclin B1 and its regulator, cdc20, showed a mechanistic link between cyclin B1 induction and T-DM1 response.

6) Analysis in *in vivo* xenografts and in patient biopsy samples cultured *ex vivo* validated the effects of T-DM1 on cyclin B1 expression and its link to anti-tumor effects.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Ferlay J, Shin H, Bray F, Forman D, Mathers C, Parkin D. Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 10 [Internet]. International Agency for Research on Cancer. 2010.

2. Erbas B, Provenzano E, Armes J, Gertig D. The natural history of ductal carcinoma in situ of the breast: a review. Breast Cancer Research and Treatment. 2006;97(2):135-44.

3. Cristofanilli M, Gonzalez-Angulo A, Sneige N, Kau S-W, Broglio K, Theriault RL, et al. Invasive lobular carcinoma classic type: response to primary chemotherapy and survival outcomes. Journal of Clinical Oncology. 2005;23(1):41-8.

4. Schnitt SJ. Classification and prognosis of invasive breast cancer: from morphology to molecular taxonomy. Modern Pathology. 2010;23:S60-S4.

5. Goldhirsch A, Wood W, Gelber R, Coates A, Thürlimann B, Senn H-J. Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. Annals of Oncology. 2007;18(7):1133-44.

6. Goldhirsch A, Ingle JN, Gelber R, Coates A, Thürlimann B, Senn H-J. Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2009. Annals of Oncology. 2009;20(8):1319-29.

7. Panel NIoHCD. National Institutes of Health Consensus Development Conference statement: adjuvant therapy for breast cancer, November 1–3, 2000. Journal of the National Cancer Institute. 2001;93(13):979-89.

8. Galea MH, Blamey RW, Elston CE, Ellis IO. The Nottingham Prognostic Index in primary breast cancer. Breast Cancer Research and Treatment. 1992;22(3):207-19.

9. Hamburg MA, Collins FS. The path to personalized medicine. New England Journal of Medicine. 2010;363(4):301-4.

10. Aspinall MG, Hamermesh RG. Realizing the promise of personalized medicine. Harvard Business Review. 2007;85(10):108.

11. Network CGA. Comprehensive molecular portraits of human breast tumours. Nature. 2012;490(7418):61-70.

12. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proceeding of the National Academy of Sciences (PNAS). 2001;98(19):10869-74.

13. Sørlie T, Tibshirani R, Parker J, Hastie T, Marron J, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proceedings of the National Academy of Sciences (PNAS). 2003;100(14):8418-23.

14. Brenton JD, Carey LA, Ahmed AA, Caldas C. Molecular classification and molecular forecasting of breast cancer: ready for clinical application? Journal of Clinical Oncology. 2005;23(29):7350-60.

15. Rakha E, El-Sayed M, Reis-Filho J, Ellis I. Expression profiling technology: its contribution to our understanding of breast cancer. Histopathology. 2008;52(1):67-81.

16. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. Journal of Clinical Oncology. 2009;27(8):1160-7.

17. Sotiriou C, Pusztai L. Gene-expression signatures in breast cancer. New England Journal of Medicine. 2009;360(8):790-800.

18. Van't Veer LJ, Dai H, Van De Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002;415(6871):530-6.

19. Geyer FC, Reis-Filho JS. Microarray-based gene expression profiling as a clinical tool for breast cancer management: are we there yet? International Journal of Surgical Pathology. 2009;17(4):285-302.
20. Kao J, Salari K, Bocanegra M, Choi Y-L, Girard L, Gandhi J, et al. Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. PLOS ONE. 2009;4(7):e6146.
21. Prat A, Ellis MJ, Perou CM. Practical implications of gene-expression-based assays for breast oncologists. Nature Reviews Clinical Oncology. 2012;9(1):48-57.
22. Rivenbark AG, O'Connor SM, Coleman WB. Molecular and cellular heterogeneity in breast cancer: challenges for personalized medicine. The American Journal of Pathology. 2013;183(4):1113-24.
23. Toss A, Cristofanilli M. Molecular characterization and targeted therapeutic approaches in breast cancer. Breast Cancer Research. 2015;17(1):60.
24. Bojang P, Ramos KS. The promise and failures of epigenetic therapies for cancer treatment. Cancer Treatment Reviews. 2014;40(1):153-69.
25. Vesuna F, Lisok A, Kimble B, Domek J, Kato Y, van der Groep P, et al. Twist contributes to hormone resistance in breast cancer by downregulating estrogen receptor-α. Oncogene. 2012;31(27):3223-34.
26. Holbro T, Hynes NE. ErbB receptors: directing key signaling networks throughout life. Annual Review of Pharmacological Toxicology. 2004;44:195-217.
27. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nature Reviews Cancer. 2005;5(5):341-54.

28. Cho H-S, Mason K, Ramyar KX, Stanley AM, Gabelli SB, Denney DW, et al. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. Nature. 2003;421(6924):756-60.
29. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nature Reviews Molecular Cell Biology. 2001;2(2):127-37.
30. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. The EMBO Journal. 2000;19(13):3159-67.
31. Holbro T, Civenni G, Hynes NE. The ErbB receptors and their role in cancer progression. Experimental Cell Research. 2003;284(1):99-110.
32. Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. Experimental Cell Research. 2003;284(1):31-53.
33. Harari P. Epidermal growth factor receptor inhibition strategies in oncology. Endocrine-Related Cancer. 2004;11(4):689-708.
34. Guarneri V, Barbieri E, Dieci M, Piacentini F, Conte P. Anti-HER2 neoadjuvant and adjuvant therapies in HER2 positive breast cancer. Cancer Treatment Reviews. 2010;36:S62-S6.
35. Creedon H, Byron A, Main J, Hayward L, Klinowska T, Brunton VG. Exploring mechanisms of acquired resistance to HER2 (human epidermal growth factor receptor 2)-targeted therapies in breast cancer. Biochemical Society Transactions. 2014;42(4):822-30.
36. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiological Reviews. 2003;83(3):835-70.
37. Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, et al. Efficacy and safety of trastuzumab as a single

agent in first-line treatment of HER2-overexpressing metastatic breast cancer. Journal of Clinical Oncology. 2002;20(3):719-26.

38. Owens MA, Horten BC, Da Silva MM. HER2 amplification ratios by fluorescence in situ hybridization and correlation with immunohistochemistry in a cohort of 6556 breast cancer tissues. Clinical Breast Cancer. 2004;5(1):63-9.

39. Bargmann CI, Weinberg RA. Oncogenic activation of the neu-encoded receptor protein by point mutation and deletion. The EMBO Journal. 1988;7(7):2043.

40. Rosen LS, Ashurst HL, Chap L. Targeting signal transduction pathways in metastatic breast cancer: a comprehensive review. The Oncologist. 2010;15(3):216-35.

41. Curigliano G, Viale G, Bagnardi V, Fumagalli L, Locatelli M, Rotmensz N, et al. Clinical relevance of HER2 overexpression/amplification in patients with small tumor size and node-negative breast cancer. Journal of Clinical Oncology. 2009;27(34):5693-9.

42. Hudis CA. Trastuzumab—mechanism of action and use in clinical practice. New England Journal of Medicine. 2007;357(1):39-51.

43. Albanell J, Baselga J. Trastuzumab, a humanized anti-HER2 monoclonal antibody, for the treatment of breast cancer. Drugs Today (Barc). 1999;35(12):931-46.

44. Klapper LN, Waterman H, Sela M, Yarden Y. Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. Cancer Research. 2000;60(13):3384-8.

45. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. Nature Medicine. 2000;6(4):443-6.

46. Arnould L, Gelly M, Penault-Llorca F, Benoit L, Bonnetain F, Migeon C, et al. Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? British Journal of Cancer. 2006;94(2):259-67.

47. Claret FX, Vu TT. Trastuzumab: updated mechanisms of action and resistance in breast cancer. Frontiers in Oncology. 2012;2:62.

48. Nagata Y, Lan K-H, Zhou X, Tan M, Esteva FJ, Sahin AA, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell. 2004;6(2):117-27.

49. Junttila TT, Akita RW, Parsons K, Fields C, Phillips GDL, Friedman LS, et al. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. Cancer Cell. 2009;15(5):429-40.

50. Wolff AC, Hammond MEH, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. Archives of Pathology & Laboratory Medicine. 2007;131(1):18-43.

51. Narayan M, Wilken JA, Harris LN, Baron AT, Kimbler KD, Maihle NJ. Trastuzumab-induced HER reprogramming in "resistant" breast carcinoma cells. Cancer Research. 2009;69(6):2191-4.

52. Gajria D, Chandarlapaty S. HER2-amplified breast cancer: mechanisms of trastuzumab resistance and novel targeted therapies. Expert Review of Anticancer Therapy. 2011;11(2):263-75.

53. Zagozdzon R, Gallagher WM, Crown J. Truncated HER2: implications for HER2-targeted therapeutics. Drug Discovery Today. 2011;16(17):810-6.

54. Arribas J, Baselga J, Pedersen K, Parra-Palau JL. p95HER2 and breast cancer. Cancer Research. 2011;71(5):1515-9.

55. Nagy P, Friedländer E, Tanner M, Kapanen AI, Carraway KL, Isola J, et al. Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line. Cancer Research. 2005;65(2):473-82.

56. Wehrman TS, Raab W, Casipit C, Doyonnas R, Pomerantz J, Blau H. A system for quantifying dynamic protein interactions defines a role for Herceptin in modulating ErbB2 interactions. Proceedings of the National Academy of Sciences (PNAS). 2006;103(50):19063-8.

57. Lu Y, Zi X, Zhao Y, Mascarenhas D, Pollak M. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). Journal of the National Cancer Institute. 2001;93(24):1852-7.

58. Agarwal S, Zerillo C, Kolmakova J, Christensen J, Harris L, Rimm D, et al. Association of constitutively activated hepatocyte growth factor receptor (Met) with resistance to a dual EGFR/Her2 inhibitor in non-small-cell lung cancer cells. British Journal of Cancer. 2009;100(6):941-9.

59. Liang K, Esteva FJ, Albarracin C, Stemke-Hale K, Lu Y, Bianchini G, et al. Recombinant human erythropoietin antagonizes trastuzumab treatment of breast cancer cells via Jak2-mediated Src activation and PTEN inactivation. Cancer Cell. 2010;18(5):423-35.

60. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, et al. A functional genetic approach identifies the PI3K

pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell. 2007;12(4):395-402.

61. Zhang S, Huang W-C, Li P, Guo H, Poh S-B, Brady SW, et al. Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. Nature Medicine. 2011;17(4):461-9.

62. Swain SM, Baselga J, Kim S-B, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. New England Journal of Medicine. 2015;372(8):724-34.

63. Lee-Hoeflich ST, Crocker L, Yao E, Pham T, Munroe X, Hoeflich KP, et al. A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy. Cancer Research. 2008;68(14):5878-87.

64. Hsieh Aa, Moasser M. Targeting HER proteins in cancer therapy and the role of the non-target HER3. British Journal of Cancer. 2007;97(4):453-7.

65. Boyraz B, Sendur MA, Aksoy S, Babacan T, Roach EC, Kizilarslanoglu MC, et al. Trastuzumab emtansine (T-DM1) for HER2-positive breast cancer. Current Medical Research & Opinion. 2013;29(4):405-14.

66. Phillips GDL, Li G, Dugger DL, Crocker LM, Parsons KL, Mai E, et al. Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody–cytotoxic drug conjugate. Cancer Research. 2008;68(22):9280-90.

67. Junttila TT, Li G, Parsons K, Phillips GL, Sliwkowski MX. Trastuzumab-DM1 (T-DM1) retains all the mechanisms of action of trastuzumab and efficiently inhibits growth of lapatinib insensitive

breast cancer. Breast Cancer Research and Treatment. 2011;128(2):347-56.

68. Burris HA, Tibbitts J, Holden SN, Sliwkowski MX, Phillips GDL. Trastuzumab emtansine (T-DM1): a novel agent for targeting HER2+ breast cancer. Clinical Breast Cancer. 2011;11(5):275-82.

69. Krop IE, Beeram M, Modi S, Jones SF, Holden SN, Yu W, et al. Phase I study of trastuzumab-DM1, an HER2 antibody-drug conjugate, given every 3 weeks to patients with HER2-positive metastatic breast cancer. Journal of Clinical Oncology. 2010;28(16):2698-704.

70. Olson EM, Lin NU, DiPiro PJ, Najita JS, Krop IE, Winer EP, et al. Responses to subsequent anti-HER2 therapy after treatment with trastuzumab-DM1 in women with HER2-positive metastatic breast cancer. Annals of Oncology. 2011; 23(1):93-7.

71. Sadeghi S, Olevsky O, Hurvitz SA. Profiling and targeting HER2-positive breast cancer using trastuzumab emtansine. Pharmgenomics and Personalized Medicine. [Review]. 2014;7:329-38.

72. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. New England Journal of Medicine. 2005;353(16):1659-72.

73. Krop IE, Kim S-B, Martin AG, LoRusso PM, Ferrero J-M, Badovinac-Crnjevic T, et al. Trastuzumab emtansine versus treatment of physician's choice in patients with previously treated HER2-positive metastatic breast cancer (TH3RESA): final overall survival results from a randomised open-label phase 3 trial. The Lancet Oncology. 2017;18(6):743-754.

74. Krop IE, Kim S-B, González-Martín A, LoRusso PM, Ferrero J-M, Smitt M, et al. Trastuzumab emtansine versus treatment of physician's choice for pretreated HER2-positive advanced breast

cancer (TH3RESA): a randomised, open-label, phase 3 trial. The Lancet Oncology. 2014;15(7):689-99.

75. Kovtun YV, Goldmacher VS. Cell killing by antibody–drug conjugates. Cancer Letters. 2007;255(2):232-40.

76. Erickson HK, Park PU, Widdison WC, Kovtun YV, Garrett LM, Hoffman K, et al. Antibody-maytansinoid conjugates are activated in targeted cancer cells by lysosomal degradation and linker-dependent intracellular processing. Cancer Research. 2006;66(8):4426-33.

77. Chari RV. Targeted cancer therapy: conferring specificity to cytotoxic drugs. Accounts of Chemical Research. 2007;41(1):98-107.

78. Sadeghi S, Olevsky O, Hurvitz SA. Profiling and targeting HER2-positive breast cancer using trastuzumab emtansine. Pharmgenomics and Personalized Medicine. 2014;15(7):329-38.

79. LoRusso PM, Weiss D, Guardino E, Girish S, Sliwkowski MX. Trastuzumab emtansine: a unique antibody-drug conjugate in development for human epidermal growth factor receptor 2–positive cancer. Clinical Cancer Research. 2011;17(20):6437-47.

80. Barok M, Tanner M, Köninki K, Isola J. Trastuzumab-DM1 causes tumour growth inhibition by mitotic catastrophe in trastuzumab-resistant breast cancer cells in vivo. Breast Cancer Research. 2011;13(2):R46.

81. Barok M, Joensuu H, Isola J. Trastuzumab emtansine: mechanisms of action and drug resistance. Breast Cancer Research. 2014;16(2):209.

82. Barok M, Isola J, Pályi-Krekk Z, Nagy P, Juhász I, Vereb G, et al. Trastuzumab causes antibody-dependent cellular cytotoxicity–mediated growth inhibition of submacroscopic JIMT-1 breast cancer xenografts despite intrinsic drug resistance. Molecular Cancer Therapeutics. 2007;6(7):2065-72.

83. Barok M, Tanner M, Köninki K, Isola J. Trastuzumab-DM1 is highly effective in preclinical models of HER2-positive gastric cancer. Cancer Letters. 2011;306(2):171-9.

84. Bosco G. Retinoblastoma, a trip organizer. Nature. 2010;466(7310):1051–1052.

85. Arellano M, Moreno S. Regulation of CDK/cyclin complexes during the cell cycle. The International Journal of Biochemistry & Cell Biology. 1997;29(4):559-73.

86. Morgan DO. Principles of CDK regulation. Nature. 1995;374(6518):131.

87. Hochegger H, Takeda S, Hunt T. Cyclin-dependent kinases and cell-cycle transitions: does one fit all? Nature Reviews Molecular Cell Biology. 2008;9(11):910-6.

88. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. Nature Reviews Cancer. 2009;9(3):153-66.

89. Deshpande A, Sicinski P, Hinds PW. Cyclins and cdks in development and cancer: a perspective. Oncogene. 2005;24(17):2909-15.

90. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes & Development. 1999;13(12):1501-12.

91. Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Proliferation. 2003;36(3):131-49.

92. Lapenna S, Giordano A. Cell cycle kinases as therapeutic targets for cancer. Nature Reviews Drug Discovery. 2009;8(7):547-66.

93. Shackelford RE, Kaufmann WK, Paules RS. Cell cycle control, checkpoint mechanisms, and genotoxic stress. Environmental Health Perspectives. 1999;107(Suppl 1):5.

94. Hartwell LH, Kastan MB. Cell cycle control and cancer. Science. 1994;266(5192):1821.

95. Castedo M, Perfettini J, Roumier T, Kroemer G. Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe. Cell Death and Differentiation. 2002;9(12):1287-93.

96. Takizawa CG, Morgan DO. Control of mitosis by changes in the subcellular location of cyclin-B1–Cdk1 and Cdc25C. Current Opinion in Cell Biology. 2000;12(6):658-65.

97. Jackman M, Lindon C, Nigg EA, Pines J. Active cyclin B1–Cdk1 first appears on centrosomes in prophase. Nature Cell Biology. 2003;5(2):143-8.

98. Lindqvist A, van Zon W, Karlsson Rosenthal C, Wolthuis R. Cyclin B1-Cdk1 activation continues after centrosome separation to control mitotic progression. PLOS Biology. 2007;5(5):e123.

99. Pesin JA, Orr-Weaver TL. Regulation of APC/C activators in mitosis and meiosis. Annual Review of Cell and Developmental Biology. 2008;24:475.

100. Jallepalli PV, Lengauer C. Chromosome segregation and cancer: cutting through the mystery. Nature Reviews Cancer. 2001;1(2):109-17.

101. Kops GJ, Weaver BA, Cleveland DW. On the road to cancer: aneuploidy and the mitotic checkpoint. Nature Reviews Cancer. 2005;5(10):773-85.

102. Aaltonen K, Amini R-M, Heikkilä P, Aittomäki K, Tamminen A, Nevanlinna H, et al. High cyclin B1 expression is associated with poor survival in breast cancer. British Journal of Cancer. 2009;100(7):1055-60.

103. Begnami MD, Fregnani JHT, Nonogaki S, Soares FA. Evaluation of cell cycle protein expression in gastric cancer: cyclin B1 expression and its prognostic implication. Human Pathology. 2010;41(8):1120-7.
104. Soria J-C, Jang SJ, Khuri FR, Hassan K, Liu D, Hong WK, et al. Overexpression of cyclin B1 in early-stage non-small cell lung cancer and its clinical implication. Cancer Research. 2000;60(15):4000-4.
105. Castedo M, Perfettini J-L, Roumier T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: a molecular definition. Oncogene. 2004;23(16):2825-37.
106. Vakifahmetoglu H, Olsson M, Zhivotovsky B. Death through a tragedy: mitotic catastrophe. Cell Death & Differentiation. 2008;15(7):1153-62.
107. Roninson IB, Broude EV, Chang B-D. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. Drug Resistance Updates. 2001;4(5):303-13.
108. Castedo M, Perfettini J, Roumier T, Kroemer G. Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe. Cell Death and Differentiation. 2002;9(12):1287.
109. Erenpreisa J, Ivanov A, Dekena G, Vitina A, Krampe R, Freivalds T, et al. Arrest in metaphase and anatomy of mitotic catastrophe: mild heat shock in two human osteosarcoma cell lines. Cell Biology International. 2000;24(2):61-70.
110. Lombardi D, Lasagni L. Cell-cycle Alterations in Post-mitotic Cells and Cell Death by Mitotic Catastrophe. Cell Biology-New Insights: InTech; 2016.
111. Chan K, Koh C, Li H. Mitosis-targeted anti-cancer therapies: where they stand. Cell Death & Disease. 2012;3(10):e411.

112. Schmidt M, Bastians H. Mitotic drug targets and the development of novel anti-mitotic anticancer drugs. Drug Resistance Updates. 2007;10(4):162-81.

113. Gascoigne KE, Taylor SS. How do anti-mitotic drugs kill cancer cells? Journal of Cell Science. 2009;122(15):2579-85.

114. Sakowicz R, Finer JT, Beraud C, Crompton A, Lewis E, Fritsch A, et al. Antitumor activity of a kinesin inhibitor. Cancer Research. 2004;64(9):3276-80.

115. Garuti L, Roberti M, Bottegoni G. Polo-like kinases inhibitors. Current Medicinal Chemistry. 2012;19(23):3937-48.

116. Kitzen J, de Jonge M, Verweij J. Aurora kinase inhibitors. Critical Reviews in Oncology/Hematology. 2010;73(2):99-110.

117. Goga A, Yang D, Tward AD, Morgan DO, Bishop JM. Inhibition of CDK1 as a potential therapy for tumors over-expressing MYC. Nature Medicine. 2007;13(7):820-7.

118. Nichat P, Mishra N, Bansal R, Kaur H. Mitotic Catastrophe–Role in Programming of Cell Death. International Journal of Oral Craniofac Science 2. 2016; 4634(3):003-005.

119. Denisenko TV, Sorokina IV, Gogvadze V, Zhivotovsky B. Mitotic catastrophe and cancer drug resistance: A link that must to be broken. Drug Resistance Updates. 2016;24:1-12.

120. Molz L, Booher R, Young P, Beach D. cdc2 and the regulation of mitosis: six interacting mcs genes. Genetics. 1989;122(4):773-82.

121. Galluzzi L, Vitale I, Abrams J, Alnemri E, Baehrecke E, Blagosklonny M, et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death & Differentiation. 2012;19(1):107-20.

122. Erenpreisa J, Cragg M. Mitotic death: a mechanism of survival? A review. Cancer Cell International. 2001;1(1):1.

123. Cretella D, Saccani F, Quaini F, Frati C, Lagrasta C, Bonelli M, et al. Trastuzumab emtansine is active on HER-2 overexpressing NSCLC cell lines and overcomes gefitinib resistance. Molecular Cancer. 2014;13(1):143.

124. Montero JC, García-Alonso S, Ocaña A, Pandiella A. Identification of therapeutic targets in ovarian cancer through active tyrosine kinase profiling. Oncotarget. 2015;6(30):30057.

125. O'Brien NA, Browne BC, Chow L, Wang Y, Ginther C, Arboleda J, et al. Activated phosphoinositide 3-kinase/AKT signaling confers resistance to trastuzumab but not lapatinib. Molecular Cancer Therapeutics. 2010;9(6):1489-502.

126. McDermott M, Eustace AJ, Busschots S, Breen L, Crown J, Clynes M, et al. In vitro development of chemotherapy and targeted therapy drug-resistant cancer cell lines: a practical guide with case studies. Frontiers in Oncology. 2014;4.

127. Girish S, Gupta M, Wang B, Lu D, Krop IE, Vogel CL, et al. Clinical pharmacology of trastuzumab emtansine (T-DM1): an antibody–drug conjugate in development for the treatment of HER2-positive cancer. Cancer Chemotherapy and Pharmacology. 2012;69(5):1229-40.

128. Diessner J, Bruttel V, Stein R, Horn E, Häusler S, Dietl J, et al. Targeting of preexisting and induced breast cancer stem cells with trastuzumab and trastuzumab emtansine (T-DM1). Cell Death & Disease. 2014;5(3):e1149.

129. Montero J, Garcia-Alonso S, Ocana A, Pandiella A. Identification of therapeutic targets in ovarian cancer through active tyrosine kinase profiling. Oncotarget. 2015;6(30):30057-71.

130. Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for human epidermal growth

factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. Journal of Clinical Oncology. 2013;31(31):3997-4013.

131. Allison M. The HER2 testing conundrum. Nature Biotechnology. 2010;28(2):117-119.

132. Yaziji H, Taylor CR. Begin at the beginning, with the tissue!: the key message underlying the ASCO/CAP task-force guideline recommendations for HER2 testing. Applied Immunohistochemistry Molecular Morphology. 2007;15(3):239-41.

133. Press MF, Sauter G, Buyse ME, Fourmanoir Hln, Quinaux E, Tsao-Wei DD, et al. HER2 gene amplification testing by fluorescence in situ hybridization (FISH): Comparison of the ASCO-CAP guidelines with FISH scores used for enrollment in breast cancer international research group (BCIRG) clinical trials. American Society of Clinical Oncology; 2016;34(29): 3518-3528.

134. Ragazzi M, Bisagni A, Gasparini E, Kuhn E, Bassano C, Tamagnini I, et al. Impact of 2013 ASCO/CAP guidelines on HER2 determination of invasive breast cancer: A single institution experience using frontline dual-color FISH. The Breast. 2017;34:65-72.

135. Donaldson AR, Shetty S, Wang Z, Rivera CL, Portier BP, Budd GT, et al. Impact of an alternative chromosome 17 probe and the 2013 American Society of Clinical Oncology and College of American Pathologists guidelines on fluorescence in situ hybridization for the determination of HER2 gene amplification in breast cancer. Cancer. 2017;123(12):2230-2239.

136. Wong A, Lamothe B, Lee A, Schlessinger J, Lax I. FRS2α attenuates FGF receptor signaling by Grb2-mediated recruitment of

the ubiquitin ligase Cbl. Proceedings of the National Academy of Sciences (PNAS). 2002;99(10):6684-9.

137. Minotti AM, Barlow S, Cabral F. Resistance to antimitotic drugs in Chinese hamster ovary cells correlates with changes in the level of polymerized tubulin. Journal of Biological Chemistry. 1991;266(6):3987-94.

138. Cheung C, Wu S-Y, Lee T-R, Chang C-Y, Wu J-S, Hsieh H-P, et al. Cancer cells acquire mitotic drug resistance properties through beta I-tubulin mutations and alterations in the expression of beta-tubulin isotypes. PLOS ONE. 2010;5(9):e12564.

139. Wang H, Wang W, Xu Y, Yang Y, Chen X, Quan H, et al. Aberrant intracellular metabolism of T-DM1 confers T-DM1 resistance in HER2-positive gastric cancer cells. Cancer Science. 2017.

140. Vitale I, Galluzzi L, Castedo M, Kroemer G. Mitotic catastrophe: a mechanism for avoiding genomic instability. Nature Reviews Molecular Cell Biology. 2011;12(6):385-92.

141. Barok M, Tanner M, Koninki K, Isola J. Trastuzumab-DM1 causes tumour growth inhibition by mitotic catastrophe in trastuzumab-resistant breast cancer cells in vivo. Breast Cancer Research. 2011;13(2):46-57.

142. Askoxylakis V, Ferraro GB, Kodack DP, Badeaux M, Shankaraiah RC, Seano G, et al. Preclinical Efficacy of Ado-trastuzumab Emtansine in the Brain Microenvironment. Journal of the National Cancer Institute. 2016;108(2):313-23.

143. Yamashita-Kashima Y, Shu S, Harada N, Fujimoto-Ouchi K. Enhanced antitumor activity of trastuzumab emtansine (T-DM1) in combination with pertuzumab in a HER2-positive gastric cancer model. Oncology Reports. 2013;30(3):1087-93.

144. Krop IE, Modi S, LoRusso PM, Pegram M, Guardino E, Althaus B, et al. Phase 1b/2a study of trastuzumab emtansine (T-DM1), paclitaxel, and pertuzumab in HER2-positive metastatic breast cancer. Breast Cancer Research. 2016;18(1):1.

145. Rojo F, Gonzalez-Navarrete I, Bragado R, Dalmases A, Menendez S, Cortes-Sempere M, et al. Mitogen-activated protein kinase phosphatase-1 in human breast cancer independently predicts prognosis and is repressed by doxorubicin. Clinical Cancer Research. 2009;15(10):3530-9.

146. Pauletti G, Dandekar S, Rong H, Ramos L, Peng H, Seshadri R, et al. Assessment of methods for tissue-based detection of the HER-2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. Journal of Clinical Oncology. 2000;18(21):3651-64.

147. Feldman AM, Lorell BH, Reis SE. Trastuzumab in the treatment of metastatic breast cancer. Circulation Journal. 2000;102:272-274.

148. Blumenthal GM, Scher NS, Cortazar P, Chattopadhyay S, Tang S, Song P, et al. First FDA approval of dual anti-HER2 regimen: pertuzumab in combination with trastuzumab and docetaxel for HER2-positive metastatic breast cancer. Clinical Cancer Research. 2013;19(18):4911-6.

149. Amiri-Kordestani L, Blumenthal GM, Xu QC, Zhang L, Tang SW, Ha L, et al. FDA approval: ado-trastuzumab emtansine for the treatment of patients with HER2-positive metastatic breast cancer. Clinical Cancer Research. 2014;20(17):4436-41.

150. Lambert JM, Chari RV. Ado-trastuzumab Emtansine (T-DM1): an antibody–drug conjugate (ADC) for HER2-positive breast cancer. Journal of Medical Chemistry. 2014; 57(16):6949–6964.

151. Higgins MJ, Baselga J. Targeted therapies for breast cancer. The Journal of Clinical Investigation. 2011;121(10):3797-803.

152. Ferguson K. Active and inactive conformations of the epidermal growth factor receptor. Biochemical Society Transactions. 2004;32(5):742-745.

153. Zahreddine H, Borden K. Mechanisms and insights into drug resistance in cancer. Frontiers in pharmacology. 2013;4(28).

154. Bedard PL, Hansen AR, Ratain MJ, Siu LL. Tumour heterogeneity in the clinic. Nature. 2013;501(7467):355-64.

155. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. New England Journal of Medicine. 2012;366(10):883-92.

156. Kroemer G, Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell. 2008;13(6):472-82.

157. Easwaran H, Tsai H-C, Baylin SB. Cancer epigenetics: tumor heterogeneity, plasticity of stem-like states, and drug resistance. Molecular Cell. 2014;54(5):716-27.

158. Mbeunkui F, Johann Jr DJ. Cancer and the tumor microenvironment: a review of an essential relationship. Cancer Chemotherapy and Pharmacology. 2009;63(4):571-82.

159. Liotta LA, Kohn EC. The microenvironment of the tumour–host interface. Nature. 2001;411(6835):375-9.

160. Schrama D, Reisfeld RA, Becker JC. Antibody targeted drugs as cancer therapeutics. Nature Reviews Drug Discovery. 2006;5(2):147-59.

161. Loganzo F, Tan X, Sung M, Jin G, Myers JS, Melamud E, et al. Tumor Cells Chronically Treated with a Trastuzumab–Maytansinoid Antibody–Drug Conjugate Develop Varied Resistance Mechanisms but

Respond to Alternate Treatments. Molecular Cancer Therapeutics. 2015;14(4):952-63.

162. Phillips GDL, Fields CT, Li G, Dowbenko D, Schaefer G, Miller K, et al. Dual targeting of HER2-positive cancer with trastuzumab emtansine and pertuzumab: critical role for neuregulin blockade in antitumor response to combination therapy. Clinical Cancer Research. 2014;20(2):456-68.

163. Mellor JD, Brown MP, Irving HR, Zalcberg JR, Dobrovic A. A critical review of the role of Fc gamma receptor polymorphisms in the response to monoclonal antibodies in cancer. Journal of Hematology & Oncology. 2013;6(1):1.

164. Perez EA, Hurvitz SA, Amler LC, Mundt KE, Ng V, Guardino E, et al. Relationship between HER2 expression and efficacy with first-line trastuzumab emtansine compared with trastuzumab plus docetaxel in TDM4450g: a randomized phase II study of patients with previously untreated HER2-positive metastatic breast cancer. Breast Cancer Research. 2014;16(3):R50.

165. English DP, Bellone S, Schwab CL, Bortolomai I, Bonazzoli E, Cocco E, et al. T-DM1, a novel antibody–drug conjugate, is highly effective against primary HER2 overexpressing uterine serous carcinoma in vitro and in vivo. Cancer Medicine. 2014;3(5):1256-65.

166. Miller KD, Diéras V, Harbeck N, Andre F, Mahtani RL, Gianni L, et al. Phase IIa trial of trastuzumab emtansine with pertuzumab for patients with human epidermal growth factor receptor 2–positive, locally advanced, or metastatic breast cancer. Journal of Clinical Oncology. 2014;32(14):1437-44.

167. Nicoletti R, Lopez S, Bellone S, Cocco E, Schwab CL, Black JD, et al. T-DM1, a novel antibody-drug conjugate, is highly effective against

uterine and ovarian carcinosarcomas overexpressing HER2. Clinical & Experimental Metastasis. 2015;32(1):29-38.

168. Wong AL, Lee S-C. Mechanisms of resistance to trastuzumab and novel therapeutic strategies in HER2-positive breast cancer. International Journal of Breast Cancer. 2012;2012.

169. Sauveur J. Development and characterization od models of resistance to T-DM1: Université de Lyon; 2016 (Dissertation).

170. Martello LA, Verdier-Pinard P, Shen H-J, He L, Torres K, Orr GA, et al. Elevated levels of microtubule destabilizing factors in a Taxol-resistant/dependent A549 cell line with an α-tubulin mutation. Cancer Research. 2003;63(6):1207-13.

171. Monzó M, Rosell R, Sánchez JJ, Lee JS, O'Brate A, González-Larriba JL, et al. Paclitaxel resistance in non–small-cell lung cancer associated with beta-tubulin gene mutations. Journal of Clinical Oncology. 1999;17(6):1786-93.

172. Giannakakou P, Sackett DL, Kang Y-K, Zhan Z, Buters JT, Fojo T, et al. Paclitaxel-resistant human ovarian cancer cells have mutant β-tubulins that exhibit impaired paclitaxel-driven polymerization. Journal of Biological Chemistry. 1997;272(27):17118-25.

173. Mozzetti S, Ferlini C, Concolino P, Filippetti F, Raspaglio G, Prislei S, et al. Class III β-tubulin overexpression is a prominent mechanism of paclitaxel resistance in ovarian cancer patients. Clinical Cancer Research. 2005;11(1):298-305.

174. Wang Y, O'Brate A, Zhou W, Giannakakou P. Resistance to Microtubule-Stabilizing Drugs Involves Two Eventse: β-Tubulin Mutation in One Allele Followed by Loss of the Second Allele. Cell Cycle. 2005;4(12):1847-53.

175. Kavallaris M. Microtubules and resistance to tubulin-binding agents. Nature Reviews Cancer. 2010;10(3):194-204.

176. Hari M, Wang Y, Veeraraghavan S, Cabral F. Mutations in α- and β-Tubulin That Stabilize Microtubules and Confer Resistance to Colcemid and Vinblastine1. Molecular Cancer Therapeutics. 2003;2(7):597-605.

177. Poon KA, Flagella K, Beyer J, Tibbitts J, Kaur S, Saad O, et al. Preclinical safety profile of trastuzumab emtansine (T-DM1): mechanism of action of its cytotoxic component retained with improved tolerability. Toxicology and Applied Pharmacology. 2013;273(2):298-313.

178. Chung Y-C, Kuo J-F, Wei W-C, Chang K-J, Chao W-T. Caveolin-1 dependent endocytosis enhances the chemosensitivity of HER-2 positive breast cancer cells to trastuzumab emtansine (T-DM1). PLOS ONE. 2015;10(7):e0133072.

179. Perez EA, Baehner FL, Butler SM, Thompson EA, Dueck AC, Jamshidian F, et al. The relationship between quantitative human epidermal growth factor receptor 2 gene expression by the 21-gene reverse transcriptase polymerase chain reaction assay and adjuvant trastuzumab benefit in Alliance N9831. Breast Cancer Research. 2015;17(1):133.

180. Teicher BA, Doroshow JH. The promise of antibody-drug conjugates. New England Journal of Medicine. 2012;367(19):1847-8.

181. Carlson JA, Nooruddin Z, Rusthoven C, Elias A, Borges VF, Diamond JR, et al. Trastuzumab emtansine and stereotactic radiosurgery: an unexpected increase in clinically significant brain edema. Neuro-Oncology. 2014;16(7):1006-9.

182. Zhou B-BS, Elledge SJ. The DNA damage response: putting checkpoints in perspective. Nature. 2000;408(6811):433-9.

183. Nitta M, Kobayashi O, Honda S, Hirota T, Kuninaka S, Marumoto T, et al. Spindle checkpoint function is required for mitotic

catastrophe induced by DNA-damaging agents. Oncogene. 2004;23(39):6548-58.

184. Klein LE, Freeze BS, Smith III AB, Horwitz SB. The microtubule stabilizing agent discodermolide is a potent inducer of accelerated cell senescence. Cell Cycle. 2005;4(3):501-7.

185. Jackson JR, Patrick DR, Dar MM, Huang PS. Targeted anti-mitotic therapies: can we improve on tubulin agents? Nature Reviews Cancer. 2007;7(2):107-17.

186. Oroudjev E, Lopus M, Wilson L, Audette C, Provenzano C, Erickson H, et al. Maytansinoid-antibody conjugates induce mitotic arrest by suppressing microtubule dynamic instability. Molecular Cancer Therapeutics. 2010;9(10):2700-13.

187. Le X-F, Claret F-X, Lammayot A, Tian L, Deshpande D, LaPushin R, et al. The role of cyclin-dependent kinase inhibitor p27Kip1 in anti-HER2 antibody-induced G1 cell cycle arrest and tumor growth inhibition. Journal of Biological Chemistry. 2003;278(26):23441-50.

188. Ye D, Mendelsohn J, Fan Z. Augmentation of a humanized anti-HER2 mAb 4D5 induced growth inhibition by a human-mouse chimeric anti-EGF receptor mAb C225. Oncogene. 1999;18(3).

189. Le X-F, Pruefer F, Bast RC. HER2-targeting antibodies modulate the cyclin-dependent kinase inhibitor p27Kip1 via multiple signaling pathways. Cell Cycle. 2005;4(1):87-95.

190. Nahta R, Yu D, Hung M-C, Hortobagyi GN, Esteva FJ. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. Nature Clinical Practice Oncology. 2006;3(5):269-80.

191. Scaltriti M, Eichhorn PJ, Cortes J, Prudkin L, Aura C, Jimenez J, et al. Cyclin E amplification/overexpression is a mechanism of

trastuzumab resistance in HER2+ breast cancer patients. Proceeding of the National Academy of Sciences (PNAS). 2011;108(9):3761-6.

192. Tormo E, Adam-Artigues A, Ballester S, Pineda B, Zazo S, Gonzalez-Alonso P, et al. The role of miR-26a and miR-30b in HER2+ breast cancer trastuzumab resistance and regulation of the CCNE2 gene. Science Reports. 2017;25(7):41309-18.

193. Witkiewicz AK, Cox D, Knudsen ES. CDK4/6 inhibition provides a potent adjunct to Her2-targeted therapies in preclinical breast cancer models. Genes & Cancer. 2014;5(7-8):261-72.

194. Goel S, Wang Q, Watt AC, Tolaney SM, Dillon DA, Li W, et al. Overcoming therapeutic resistance in HER2-positive breast cancers with CDK4/6 inhibitors. Cancer Cell. 2016;29(3):255-69.

195. Clute P, Pines J. Temporal and spatial control of cyclin B1 destruction in metaphase. Nature Cell Biology. 1999;1(2):82-7.

196. Malureanu LA, Jeganathan KB, Hamada M, Wasilewski L, Davenport J, van Deursen JM. BubR1 N terminus acts as a soluble inhibitor of cyclin B degradation by APC/C Cdc20 in interphase. Developmental Cell. 2009;16(1):118-31.

APPENDIX

Article as first author based on this PhD thesis

1) **Sabbaghi M,** Gil-Gómez G, Guardia C, Servitja S, Arpí O, García-Alonso S, et al. Defective cyclin B1 induction in trastuzumab-emtansine (T-DM1) acquired resistance in HER2-positive breast cancer (under review-Clinical Cancer Research Journal-2017).

Articles as co-author as a result of my participation in different studies within the breast cancer research group

1) García-Parra J, Dalmases A, Morancho B, Arpí O, Menendez S, **Sabbaghi M,** et al. Poly (ADP-ribose) polymerase inhibition enhances trastuzumab antitumour activity in HER2 overexpressing breast cancer. European Journal of Cancer. 2014;50(15):2725-34.

2) Rojo F, González-Pérez A, Furriol J, Nicolau MJ, Ferrer J, Burgués O, **Sabbaghi M,** et al. Non-canonical NF-κB pathway activation predicts outcome in borderline oestrogen receptor positive breast carcinoma. British Journal of Cancer. 2016;115(3):322-31.

3) Hernandez A, Guardia C, Casadevall D, Gavilan E, **Sabbaghi M,** Arpí O, et al. Increasing the *in vitro* and *in vivo* efficacy of T-DM1 by modulating the mTOR pathway in HER2-positive breast cancer (in preparation).