Universitat Autònoma de Barcelona

MECHANISMS OF RESISTANCE TO T-DM1 IN HER2-POSITIVE BREAST CANCER

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Doctoral Thesis

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Departament de Bioquímica i Biologia Molecular
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SUMMARY

Summary

English

HER2-positive breast cancer represents around 15-30% of the breast cancer patients. This breast cancer subtype has poor prognosis, followed by the triple negative subtype, which has the worse. Several drugs are currently approved for HER2-positive breast cancer, such as Trastuzumab, Lapatinib and T-DM1. However, often these patients acquire resistance to these therapies in a period of 1 - 2 years. The main goal of this study is defining mechanisms of resistance to T-DM1. First, I have generated T-DM1 resistant cell lines, and I explored *in vitro* different possibilities to explain how these cells escape from HER2-target therapy. I obtained T-DM1 resistant cells using a PDX-derived cell line (PDX118) through continuous treatment of increasing doses of T-DM1. These cells were tested of resistance to T-DM1 in the presence of this HER2 target drugs. I have tested whether the levels of HER2 by mRNA and protein, copy number, downstream signaling effectors, cytotoxic part of T-DM1 or differential lysosome activity might justify the differences between parental and resistant cells. No differences in copy number, neither at transcript or total protein levels of HER2 was observed in parental versus resistant cells. However, in two of three T-DM1 resistant cultures, the protein levels at the surface of the resistant cells were significantly lower, although the downstream signaling activity remained similar. A HER2 rescue experiment resulted in a partial recovery of the sensibility. HER2 protein levels at the surface of the tumor cells is the main mechanism of resistance to T-DM1 therapy, and also, abnormal function of lysosomes maybe a reason to escape this treatment.

Spanish

El cáncer de mama HER2-positivo representa alrededor del 15-30% del total de pacientes con cáncer de mama. Este subtipo tiene un mal pronóstico, seguido del subtipo Triple-negativo (TNBC), que lo tiene aún peor. Actualmente, varios medicamentos están aprobados para las pacientes HER2-positivo, como Trastuzumab, Lapatinib y T-DM1. Sin embargo, a menudo éstas adquieren resistencia a estas terapias en un período de 1 a 2 años. El objetivo principal de este estudio es definir los mecanismos de resistencia a T-DM1. Durante mi tesis, generé líneas celulares resistentes a T-DM1 y exploré diferentes posibilidades *in vitro* que pudieran explicar cómo estas células escapan de la terapia contra HER2. Obtuve células resistentes a T-DM1 usando una línea celular derivada de un tumor primario de una paciente (PDX, Patient-derived xenograft) tratándolas con dosis crecientes de T-DM1. Comprobé los niveles de HER2 por mRNA y proteína, número de copias, efectores de señalización, analicé la región citotóxica de T-DM1 y su actividad lisosómica diferencial comparando la línea parental y las células resistentes. Esta tesis describe punto por punto todos los posibles mecanismos de resistencia que investigamos y cuales resultaron ser los responsables de la resistencia a T-DM1.

ABBREVIATIONS

ABL: Abelson murine leukemia viral oncogene homolog

ADC: Antibody-drug Conjugates

ADCC: Antibody-dependent cell-mediated cytotoxicity

ALK: Anaplastic lymphoma kinase

ATCC: American Type Culture Collection

ATP: Adenosine triphosphate

BMK1: Big mitogen activated protein kinase 1

BTC: Betacellulin

cDNA: Complementary DNA

CSK: C-terminal Src kinase

CT: Carboxy-terminal region

DM1: Emtansine

DMEM: Dulbecco's Modified Eagle's Medium

Dmin: Double minutes

DNA: Deoxyribonucleic acid

ECL: Enhanced chemoluminescence

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

EPR: Epiregulin

ER: Estrogen receptor

ERK: Extracellular-signal-regulated kinase

FAK: Focal adhesion kinase

FDA: Food and Drug Administration (USA)

FGFR: Fibroblast growth factor receptor

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GDF15: Growth differentiation factor 15

HB-EGF: Heparin-binding epidermal growth factor

HER2: Human epidermal growth factor receptor 2

HGFR: Hepatocyte growth factor receptor

HRP: Horseradish peroxidase

HSR: Homogeneously staining region

IC50: Half maximal inhibitory concentration

IGFR: Insulin and insulin-like receptor

IHC: Immunohistochemistry

JAK: Janus kinase

JM: Juxta-membrane region

JNK: c-Jun N-terminal kinase

MAP2K: Mitogen-activated protein kinase kinases

MAP3K: MAP kinase kinase kinase

MAPK: Mitogen activated protein kinase

MCC: (N-maleimi-dimethyl) cyclohexane-1-carboxylate

mTOR: Mammalian target of rapamycin

mTORC1: Mammalian target of rapamycin complex 1

NaCl: Sodium chloride

NaF: Sodium fluoride

NK: Natural killer

NOD-SCID: Non-obese diabetic/severe combined immunodeficiency

NRG: Neuregulin

nRTK: Non-receptor tyrosine kinases

NSCLC: Non-small cell lung cancer

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate-buffered saline

PC-PLC: Phosphatidylcholine-specific phospholipase C

PCR: Polymerase chain reaction

PDGFR: Platelet-derived growth factor receptors

PDX: Patient-derived tumor xenograft

PEI: Polyethylenimine

PES: Polyether sulfone

PFA: Paraformaldehyde

PFS: Progression-free survival

PI3K: Phosphoinositide 3-kinase

PIK3CA: Phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit α

PIP2: Phosphatidylinositol 4,5-triphosphate

PIP3: Phosphatidylinositol 3,4,5-triphosphate

PKB: Protein Kinase B

PR: Progesterone receptor

PTEN: Phosphatase and tensin homolog

qPCR: Quantitative polymerase chain reaction

RCTs: Randomized controlled trials

RNA: Retrotranscription of ribonucleic acid

RSK: Ribosomal S6 kinase

RTK: Receptor tyrosine kinases

SYK: Spleen tyrosine kinase

TAM: Tyro3, Axl, and Mer

T-DM1: Trastuzumab emtansine

TEC: Tec protein tyrosine kinase

TGF: Transform growth factor

TK: Catalytic tyrosine kinase domain

VEGF: Vascular endothelial growth factor

VEGFR: Vascular endothelial growth factor receptor

INTRODUCTION

1. Cancer

1.1 The basis of cancer

Cancer is a disease which involves abnormal cell growth with limitless. Tumors are originated from a single cell, which has acquired certain mutations, favoring their uncontrolled division. During the evolution of this disease, some cells can migrate from their original place to distal organs forming metastases. If the metastases are not effectively controlled the patient can die. There are as many types of cancer as organs; it has been estimated that there are more than 100 types of cancers in humans [1].

The risk factors for cancer might be genetic and/or non-genetic. Several studies showed that 5 to 10% of cancer cases are related to exclusively genetic factors, 25 to 30% to smoking, 30 to 35% to diets (including alcohol), 15 to 20% to infections (Hepatitis B and Human papillomavirus infection, etc.), and 10 to 25% to other environmental factors (ionizing radiation, environmental pollution, etc.) [2].

Every year in Europe there is more than 3.7 million of new cases of cancer that are diagnosed. Moreover, there are 1.9 million deaths per year due to cancer, representing the second most important cause of death [3]. Concretely in Spain, nearly 250,000 new invasive cancer cases were diagnosed in 2015, becoming the second most frequent cause of death [4].

On a global scale, cancer caused about 8.8 million deaths (around 15.7% of the total) in 2015. Lung, breast, stomach, liver, colon and breast cancer cause the most cancer deaths each year [5]. In summary, cancer has become one of the major threats to human health, causing serious physical and psychological deleterious effects in patients and taking an enormous economic burden to our society.

1.2 Definition of a cancer cell

Normal (healthy) and cancer cells have similar biological characteristics, for this reason is so difficult to distinguish tumoral from normal cells. However, cancer cells have some biological functions that are aberrantly active and others inhibited. In 2010, Weinberg and Hanahan proposed 10 hallmarks to define a cancer cell, summarized in **Figure1** [6]:

1. Cancer cells stimulate their own growth. In a normal context, proliferative signals are activated only after growth or injury, whereas in a tumor context proliferating signals are continuously active. Cancer cells acquire these signals already in early stages of the tumorigenesis [7].
2. Cancer cells ignore inhibitory signals to stop their growth. Normal cells stop cell growth through growth inhibitory factors such tumor suppressor genes, while cancer cells inactivate these genes [8].
3. Cancer cells resist their programmed cell death. In normal condition, cells that cannot maintain their correct function will undergo programmed cell death. Cancer cells will suppress and inactivate the genes and pathways related to this process [9, 10].
4. Cancer cells have limitless replication potential. Telomeres are necessary for cell proliferation. In normal cells, after several divisions, telomeres get shorter. Cancer cells activate specific gene pathways that extend the telomeres and thus cells can multiply indefinitely [11].
5. Cancer cells can induce angiogenesis. Cancer cells promote the growth of blood vessels to supply nutrients to tumors [12].
6. Tissue invasion and metastasis. Cancer cells can invade from the primary site to distant tissues or organs, resulting in their spread throughout the body [13].

7. Abnormal metabolic pathways. Most cancer cells use abnormal metabolic pathways to generate energy, which contributes their growth and proliferation [14].
8. Evading the immune system. Cancer cells can avoid interaction with immune system and enhance their ability to escape from the immune responses [15].
9. Cancer cells generally have severe chromosomal abnormalities. The rate of mutations is also enhanced in cancer cells and mutations can be accumulated due to genome instability and DNA repair miss-function [16, 17].
10. Inflammation leads to angiogenesis and more immune response, which can induce and maintain the characteristics of tumors [18, 19].

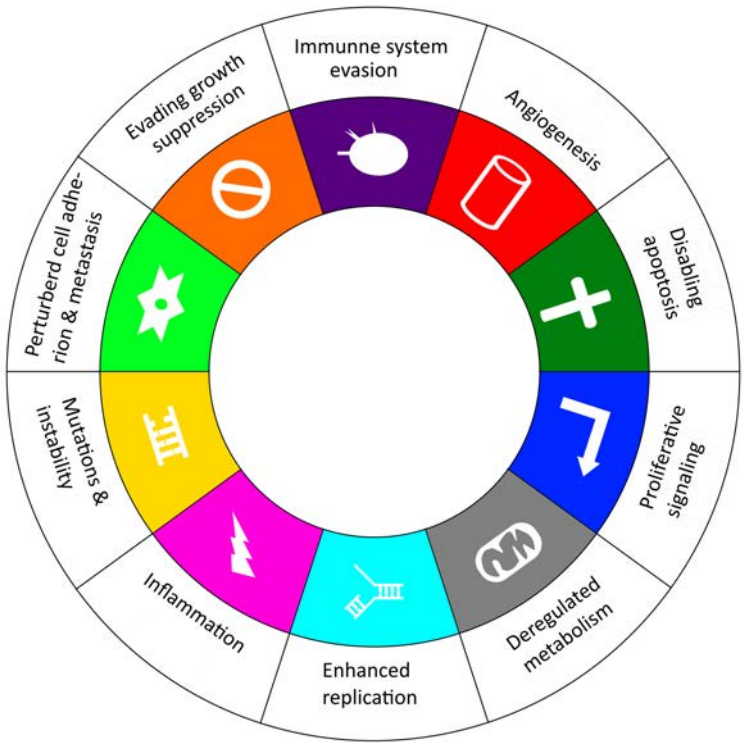

Figure1: Hallmarks of cancer. Schematic model modified from Hanahan and Weinberg, 2011

1.3 Cancer Classification

Cancer can be classified based on the cell of origin of the diagnosed tumor. These types include [20]:

1. Carcinoma: a type of cancer developed from epithelial cells. This group includes many of the most common cancers (i.e. Breast cancer, Lung cancer, etc.).
2. Sarcoma: a type of cancer that arises from transformed mesenchymal cells (i.e. bone, fat, etc.).
3. Myeloma: a type of cancer, which is originated in plasma cells of the bone marrow.
4. Leukemia: a type of blood cancer. They arise from the bone marrow.
5. Lymphoma: this type of cancer arises from the glands or nodes of the lymphatic system, a network of vessels, nodes, and organs that purify bodily fluids and produce infection-fighting white blood cells, or lymphocytes.
6. Mixed type: this type includes all tumor cases that present components of different categories (i.e. adenosquamous carcinoma, adenosquamous carcinoma, etc.).

2. Breast Cancer

Breast cancer is one of the most common malignant tumors in women [21]. In the last years, the incidence of global breast cancer has risen year by year. In 2012, about 1.7 million women in the world were diagnosed with breast cancer, representing 25% out of total women in the world. Breast cancer has the highest mortality by cancer in women in the worldwide [22], which threats women's health and life [23].

In Europe, breast cancer is the most common cancer. It was estimated that 494,100 women were diagnosed and the number of women who died was 142,980 in 2012 [24]. In Spain, the total number of new invasive breast cancer cases was 27,747 in 2015 [4], and it was estimated that in 2012 about 6200 women died by breast cancer [25]. Despite the new therapies and techniques, the prognosis of breast cancer patients improved but some patients still have poor prognosis leading to death.

2.1 Breast cancer subtypes

Breast cancer is a heterogeneous disease. Nowadays, breast cancer is subdivided according to the following molecular markers[26]:

1. **Ki-67**. This protein is a proliferation marker, with 360kD and is present in the nuclei of proliferating cell [27]. Ki-67 was detected in all active phases of the cell cycle (G1, S, G2, M phases), while is absent in resting cells (G0 phase) [28].

2. **Estrogen receptor** (ER). Estrogen is the main steroid hormone in women and is responsible for the development and regulation of the female reproductive system and secondary sexual characteristics. Estrogen can bind to ER. ER belongs to the nuclear receptor family and has two major subtypes, ER-alpha and ER-beta [29, 30]. ER-alpha can

promote the expression of the cell cycle regulatory proteins MYC and Cyclin D1 in cells and plays an important role during mitosis [31].

3. **Progesterone receptor** (PR). Progesterone is an endogenous steroid hormone. It is involved in the menstrual cycle, pregnancy, and embryogenesis of many species of mammals. PR encodes by *PGR* gene. Progesterone can bind to PR. After its binding, the complex enters the nucleus and binds to DNA. The transcription takes place there, resulting in gene transcription such as EGF, TGF-alpha and HB-EGF [32, 33].
4. **HER2**. Also named ERBB2, is a trans-membrane protein. The dimerization of HER2 occurs in the cell surface and leads the auto-phosphorylation of tyrosine residues in the cytoplasmic domain of these receptors, which initiate a variety of signaling pathways involved in cell division and growth.

According to the status of the markers described above, breast cancer can be divided into different subtypes, summarized in **Figure 2** [26, 34, 35]:

1. ER-Positive breast cancer [36]
    a. **Luminal A.** The features of this type of BC are: hormone-receptors ER positive and/or PR positive, HER2 negative, low levels of Ki-67. Tumors of this type are usually low-grade, tend to grow slowly and have the best prognosis [37].
    b. **Luminal B.** The features of this type of BC are: ER positive, HER2 negative, PR less than 20% and Ki-67 higher than 14% [38, 39]. The prognosis of Luminal B is worse than Luminal A.
2. HER2-positive breast cancer [40]
    a. **Luminal HER2.** The features of this type of BC are: hormone-receptors ER and/or PR positive and HER2 positive [41].

b. **HER2-enriched breast cancer.** The features of this type of BC are: hormone-receptors ER and PR negative and HER2 positive. This type of breast cancer presents a higher growth, proliferation and metastasis than luminal cancers. They are highly invasive and have worse prognosis, but they are often successfully responds to HER2 targeted drugs [42, 43].

3. **Triple-negative** or Basal-like breast cancer. The features of this type of BC are: hormone-receptors ER and PR negative and HER2 negative. This type of breast cancer is commonly found in menopausal and *BRCA1* gene mutated women [44, 45]. Although these tumors are sensitive to chemotherapy, this subtype has a poor prognosis [46]. Due to the lack of effective therapeutic targets and drug resistance, the vast majority of these patients eventually will die by metastasis after a relatively short period post-treatment [47, 48].

2.2 Treatments for breast cancer

Breast cancer treatment is a complex issue. Currently, there are a variety of breast cancer treatments. These treatments can be used alone, as single therapy, or in combination following several strategies. These treatments include:

1. **Surgery**. This procedure consists in removing the tumor from the body. In early stages, breast cancer may be cured if surgery succeeds excising entirely the tumor. However, at metastatic stages, the success of the surgery will be limited. In addition, the sample resulted by the surgery can be used for analysis and to predict the prognosis of these patients [49, 50].

2. **Hormonal therapy**. Researchers have found that some types of breast cancers are driven by hormones (i.e. estrogen) and rely on them for

growing [51]. As a result, hormonal therapy can block or reduce hormones levels to stop or slow down tumor growth. For example, Tamoxifen is widely used for ER-positive breast cancer and patients that have received this treatment have been associated to a good prognosis [51, 52].

3. **Chemotherapy**. This treatment uses one or more special chemical drugs. These drugs are cytotoxic agents that can inhibit proliferation in dividing cells within tumors. As chemotherapy has a systemic administration, it can work around the body being able to also target breast cancer metastasis [53, 54].

4. **Radiotherapy**. This is a method is based in the use of high-energy particles or high-energy waves to destroy cancer cells by blocking their ability to proliferate. For a breast cancer patient, radiotherapy can be used alone or combined with other therapies [49, 50]. Radiotherapy has some side effects, including loss of appetite and/or hair loss [55].

5. **Targeted therapy**. This therapy targets specifically proteins that are expressed exclusively or mainly in cancer cells. Targeted therapies can block specific signaling pathways in tumor cells, reducing the vascularization, stimulating the immune response of the body against cancer cells, and thereby inhibiting tumor progression. For HER2-positive breast cancer patient, HER2 targeted drugs, such as Trastuzumab or Lapatinib are widely used and have improved the survival rate of HER2-positive breast cancer patients [56, 57].

In **Figure 2** is shown the incidence, most common alterations, tumor grade, treatment options and prognosis of the different BC subtypes described above.

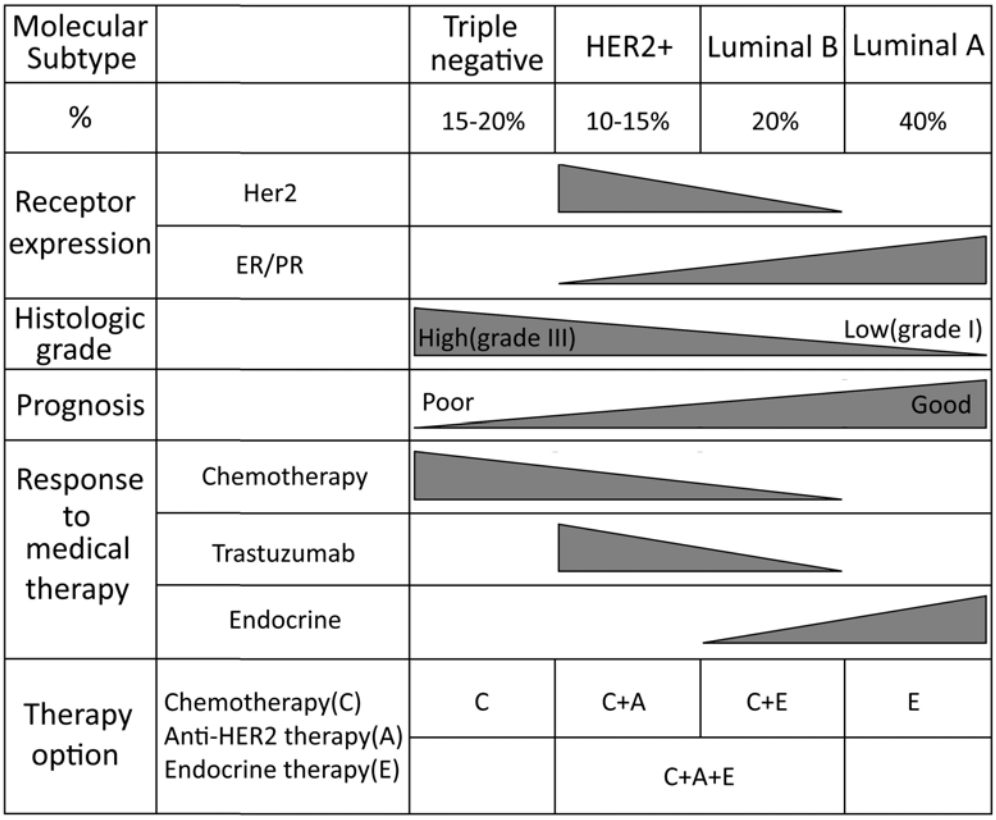

Figure 2: Breast cancer Subtypes and their distinctive features. Figure adapted from Eric Wong, et al., 2012[58]

3 Receptor Tyrosine Kinases

Protein phosphorylation in mammals is mainly divided into serine phosphorylation (86.4%), threonine phosphorylation (11.8%) and tyrosine phosphorylation (1.8%) [59]. Despite tyrosine phosphorylation is less frequent than other types of phosphorylation; it plays a unique role in the cell signal transduction process. These kinases have been closely related to cell proliferation, differentiation, apoptosis and metabolism. There are 90 tyrosine kinases genes in the human genome. According to their structure, tyrosine kinases can be subdivided in two groups: **Non-receptor tyrosine kinases** (nRTK) and **Receptor tyrosine kinases** (RTK) [60, 61].

1. **nRTKs** have no extracellular domain. They are usually located in the cell membrane or cytoplasm. They performed signaling transductions through cytokine receptors, T-cell receptors and other signaling pathways. There are 32 nRTKs in total, which can be divided in 10 subfamilies. They are SRC, CSK, ABL, TEC, FAK, SYK, JAK, ACK, FRK and FES [60, 62].

2. **RTKs** are a class of cell-surface trans-membrane protein receptors that have endogenous tyrosine kinase activity. By transducing signals from the extracellular environment into the cytoplasm and nucleus, RTKs can play important roles in many cellular functions, such apoptosis, cytoskeleton rearrangement, immune response and DNA transcription [61, 63]. Three domains compose RTKs: extracellular ligand-binding region, a trans-membrane region and intracellular kinase region [64]. The function of the intracellular kinase domain is catalyzing the gamma-phosphate groups of ATPs transferring to tyrosine residues. Phosphorylation activates the protein kinases (active form), then the signal transduction get started [65]. RTKs also play a critical role in many types of cancer and at different stages of the tumor progression

[66]. So far, 58 RTKs have been found, which can be divided in 20 subfamilies based on their amino acid sequence homologies and their similar extracellular domains [61].

3.1 RTK families

The RTKs includes several families of receptors shown in **Figure 3**.

1. Epidermal growth factor receptor (**EGFR**): This receptor family plays important roles in process of the signal transduction, cell proliferation and several regulatory processes, such differentiation [67].
2. Insulin and insulin-like receptor (**IGFR**): This receptor family has two members: Insulin-like growth factor 1 receptor (IGF-1R) and Insulin-like growth factor 2 receptor (IGF-2R). IGF1R signal transduction causes the activation of several intracellular signaling pathways, including the MAPK and the PI3K pathways [68, 69]. IGF2R can induce the activation of small G protein and its downstream signaling pathway [70].
3. Anaplastic lymphoma kinase (**ALK**): This is a receptor tyrosine kinase of the insulin receptor superfamily. The full length ALK can be found in many types of cancer, such as neuroblastomas [71], glioblastomas [72] and melanoma [73]. ALK fusion protein, which formed by chromosomal rearrangements, can also be found in many types of cancers, such as Esophageal squamous cell carcinoma [74] and Non-small-cell lung carcinoma [75].
4. Platelet-derived growth factor receptors (**PDGFR**): These receptors belong to class III receptor tyrosine kinases. The activation of these receptors is associated with many human diseases, such as vascular stenosis [76], atherosclerosis [77, 78] and some types of tumors [79, 80].

5. Vascular endothelial growth factor receptor (**VEGFR**): These receptors are mitogenic and can regulate angiogenesis. They play important roles in mitosis [81] and chemotaxis [82] in vascular endothelial cells.
6. Hepatocyte growth factor receptor (**HGFR**): also called tyrosine-protein kinase Met or c-Met, is a protein encoded by the *MET* gene. It is overexpressed in some cases of human leukemia and lymphoma [83]. HGFR has been implicated in cellular proliferation, cell survival, invasion, cell motility, metastasis and angiogenesis [84].
7. Fibroblast growth factor receptor (**FGFR**): FGFR family comprises of four family members: FGFR1, FGFR2, FGFR3 and FGFR4. FGFR signaling is associated with the cell growth, proliferation, differentiation, and survival [85, 86].
8. **TAM** family: This receptor family consists in three members: Tyro-3, Axl, and Mer. They have a conserved sequence within the kinase domain and adhesion molecule-like extracellular domains. This RTK family can regulate a lot of processes, including cell proliferation/survival, cell adhesion and migration, blood clot stabilization, and regulation of inflammatory cytokine release [87, 88].

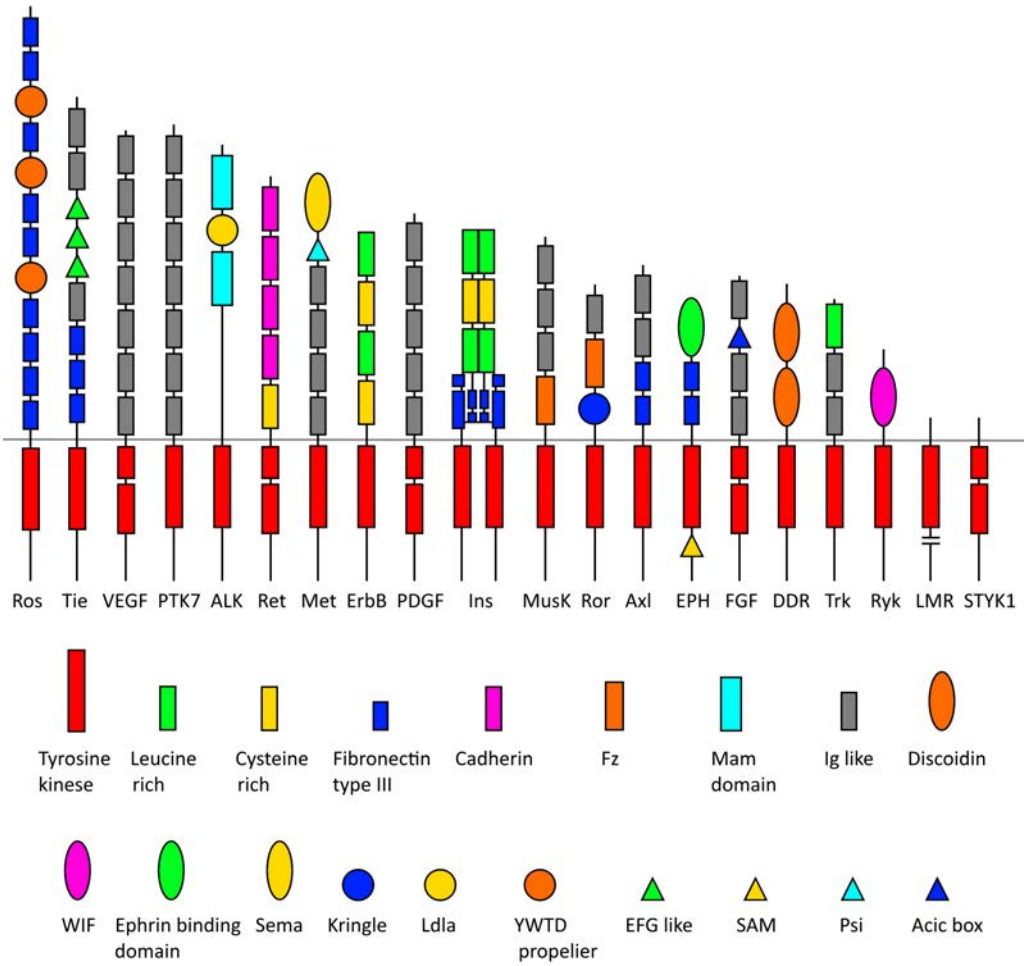

Figure 3: Receptor Tyrosine Kinase Families. Figure modified from the publication of Mark A. Lemmon & Joseph Schlessinger, 2010) Human receptor tyrosine kinases (RTKs) contain 20 subfamilies. The intracellular domains are shown as red rectangles. The rectangle, circle, oval and triangle above are the extracellular regions.

4 ERBB/EGFR Family

ERBB receptor family is also named *EGFR* (Epidermal Growth Factor Receptor) gene family [89].

4.1 Protein structure

ERBB family members share similar structure; all of them contain three parts: an extracellular, trans-membrane and intracellular domain [90]. The structure of ERBB family is shown in **Figure 4**. The extracellular region consists of 621 amino acids, which can bind to the corresponding ligand; the trans-membrane region consists of 23 amino acid residues, which form the alpha helix hydrophobic domain [91], this region anchors the receptor to the cell membrane. The intracellular region consists in 542 Amino acid residues and contains three sub-regions: Juxta-membrane region (JM), Catalytic tyrosine kinase domain (TK) and Carboxyl-terminal region (CT) [92]. The tyrosine kinase activity of this region plays a crucial role in the regulation of cell proliferation and differentiation.

4.2 Family members

ERBB family includes four members: ERBB1 (EGFR), ERBB2 (HER2), ERBB3 (HER3) and ERBB4 (HER4), shown in **Figure 3** [93].

1. Epidermal Growth Factor Receptor (**EGFR**). The human *EGFR* gene is located in Chromosome 7. The mRNA of *EGFR* contains 28 exons and encodes 1186 amino acids [94]. The molecular weight of the EGFR protein is 170kD. EGFR is expressed cellular differentiation [95], and alterations in EGFR signaling pathway are associated to tumor formation. EGFR first needs to homodimerize, this occurs after the binding with a ligand [96, 97]. Alterations in EGFR pathway can be due to:

a) Mutations in *EGFR* gene, for example EGFR VIII, which can be activated independently of the presence of its ligand. This mutation was observed in Glioblastomas and Non-small cell lung cancer (NSCLC) [98, 99].
b) Overexpression of their ligands, for example some tumor cells can overexpress Epidermal growth factor (EGF) or Transform growth factor alpha (TGF alpha), which can activate the downstream of the EGFR signaling pathway [100].
c) EGFR overexpression. This has been observed in head and neck cancers, lung cancer, skin cancer and esophageal cancer [101, 102]. This overexpression can promote tumor cell proliferation, angiogenesis, invasion and metastasis. They can also inhibit apoptosis leading to poor prognosis [103].

2. **HER2**. ERBB2 was originally found in rodent glioblastoma, which is a neurological tumor; as a result, it was named "NEU" [104]. The human *HER2* gene is located in Chromosome 17. The molecular weight of the HER2 protein is 185kD [105]. HER2 receptor does not need the interaction with a ligand to be activated, although homodimers or heterodimers must be formed to generate an activation signal. The most common heterodimer is the formed by EGFR/HER2 [106]. This activation leads to the signal transduction that results in proliferation, differentiation, migration, invasion and anti-apoptotic mechanisms. HER2 is expressed at low levels in the adult human epithelium [107]. Under physiological conditions, HER2 can promote cell growth and differentiation. The overexpression of HER2 is related to tumor progression and metastasis [108]. It is widely known that the overexpression of HER2 is closely related to the occurrence of breast cancer [109]. In addition, HER2 overexpression is significantly associated with shorter survival of breast and gastric cancers [110, 111].

3. **HER3**. The human *HER3* gene is located in Chromosome 12 and it is translated into 1342 amino acids. The gene product of HER3 is a trans-membrane glycoprotein [112]. Its structure is very similar to EGFR and HER2. Unlike other members of the ERBB family, the extracellular domain of HER3 can bind to the ligand Neuregulin, and its intracellular domain does not have a kinase domain [101]. After its binding with the ligand, the intracellular part of HER3 cannot be phosphorylated, neither activated. The extracellular signalization cannot be transmitted to the intracellular domain [113]. Thus, HER3 could only have kinase activity when heterodimerize with other ERBB receptors [114]. HER3 is not only expressed in both adult and fetal tissues [115], but also in some tumors [116]. Some studies have shown that HER3 overexpression is a marker for poor prognosis in some types of cancer, such as gastric cancer and pancreatic cancer [117, 118]. Similarly to HER2, HER3 is generally highly expressed in HER2-positive breast cancer. The presence of heterodimers HER2/HER3 or HER3/EGFR/HER4 is the features for the highly malignant breast cancer with poor prognosis. However, the heterodimer HER3/HER4 is a feature for good prognosis [119].
4. **HER4**. The human *HER4* gene is located in Chromosome 2. The molecular weight of the HER4 protein is 180kD [120]. This receptor can be regulated by Neuregulin, beta-cellulin and heparin-binding EGF ligands. Similar to HER2, HER4 needs to bind to other ERBB receptors to have kinase activity and exert their biological activity. HER4 is commonly expressed in most of the normal tissues at embryonic stages and in adults [121, 122]. It has been reported that HER4 is overexpressed in papillary thyroid and ovarian carcinomas [120, 123]. However, HER4 expression has been correlated with favorable prognostic factors a more positive outcome in patients with breast cancer

[124, 125]. These results indicated that HER4 may have different function in cancer cells from other ERBB family members.

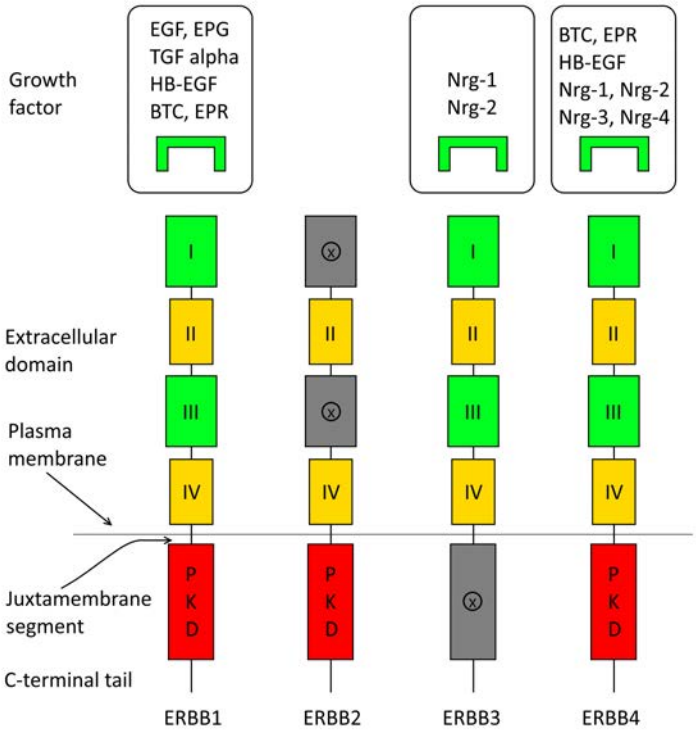

Figure 4: Structure and members of ERBB family. The extracellular domain can bind to specific ligands except the rectangular in grey color, which means it cannot bind any known ligand. The intracellular domain in red rectangular has tyrosine kinase activity (PDK). The one in grey lacks kinase activity.

4.3 ERBB Ligands

EGFRs can be activated by many different molecules, which include EGF, TGF-alpha, Neuregulin (NRG), heparin-binding epidermal growth factor (HB-EGF), Amphiregulin, Epiregulin, betacellulin (BTC) and epiregulin (EPR) [126, 127], shown in **Figure 4**.

Different ligands can recognize different receptors. EGF, AR and TGF-alpha can specifically bind to EGFR; BTC, HB-EGF and EPR can bind both EGFR and HER4.

Regarding NRG ligand family, the NRG 1 and NRG2 can both bind to HER3 and HER4, meanwhile, NRG3 and NRG4 can only bind to HER4 [128, 129].

Up to now, no specific ligands for HER2 have been found.

5 HER2 Signaling Pathway

The dimers formation is essential for the activation of ERBB receptors. What causes the dimerization is the binding of ERBB ligands to the domain I and III of these receptors, and then there is a conformational change opening the domain II. The domain IV is also required for the dimerization process. A variety of external signals then will be transduced into the cell, mediating cell proliferation, differentiation, migration, apoptosis and other biological processes [130]. After the dimerization, the tyrosine residues in the C-terminal tail segments are phosphorylated, which works as adaptor proteins that triggers the activation of other downstream signaling pathways, as shown in **Figure 5**. The most important downstream pathway includes PI3K/AKT/mTOR pathway [131] and MAPK/ERK pathway [132].

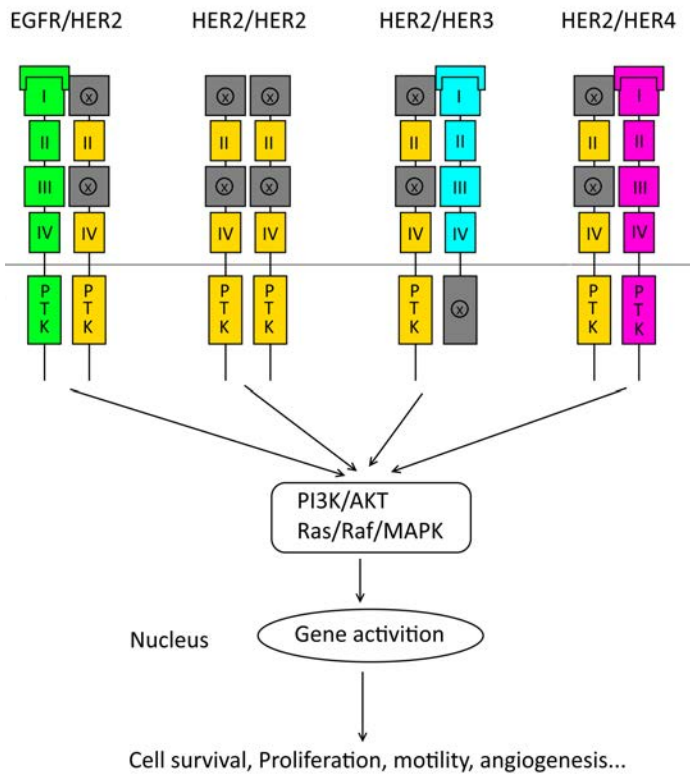

Figure 5: HER2 signaling pathway. The figure shows possible homodimers and heterodimers for HER2.

5.1 PI3K/AKT/mTOR pathway

PI3K/AKT/mTOR pathway is a widely existing intracellular signal transduction pathway that plays an important role in a lot of biological activities such as cell proliferation and apoptosis inhibition [133, 134] **Figure 6**. The central components of this pathway are PI3K (Phosphatidylinositol 3-kinase), PKB (Protein Kinase B) and mTOR (mammalian target of rapamycin).

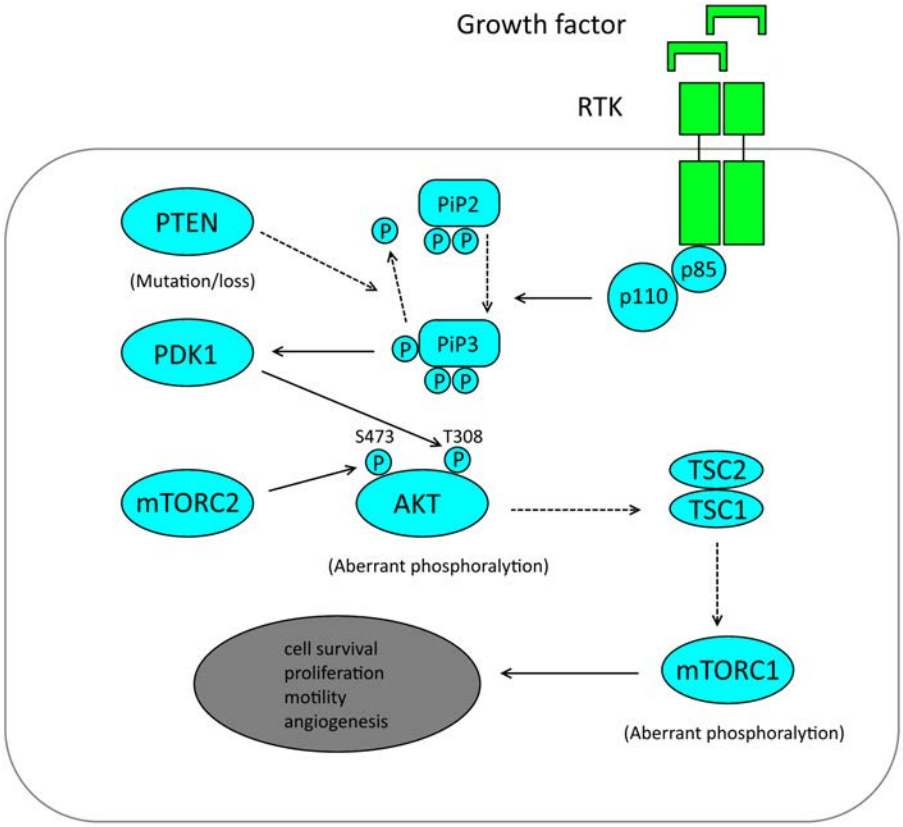

Figure 6: PI3K/AKT/mTOR pathway. RTK, Receptor tyrosine kinase. PiP2, Phosphatidylinositol 4,5-bisphosphate. PiP3, Phosphatidylinositol (3,4,5)-triphosphate. P, Phosphate moiety.

PI3K is a member of the protein tyrosine kinase family and contains eight members. According to its structure and substrate specificity, PI3Ks can be divided in three categories: PI3K I, PI3K II and PI3K III [135]. PI3K I was most clearly studied and closely related to tumor development [136, 137]. This type can further subdivide in two subtypes: PI3K IA and PI3K IB. PI3K IA is a

heterodimer composed by the regulatory subunit P85 and the catalytic subunit P110 [138].

In the absence of an active signaling pathway, the regulatory subunit p85 interacts with the catalytic subunit p110 inhibiting the catalytic activity of p110 [139]. Activated RTKs and GPCRs can recruit PI3K to the cell membrane. The regulatory subunit p85 can interact with them, and then the inhibitory effect on the catalytic subunit p110 activity is released. Activated P110 catalyzes the conversion of PIP2 (phosphatidylinositol 4,5-triphosphate) into PIP3 (phosphatidylinositol 3,4,5-triphosphate). As a second messenger, PIP3 can recruit PH domain-containing proteins, such as Akt (PKB) to the membrane [140].

Helped by PDK1 and mTORC2, AKT can be phosphorylated and then remove the repression over mTOR1. Active forms of AKT and mTORC1 can activate a variety of protein kinases such as ribosomal protein S6 kinases [141-143]. AKT has three subtypes (AKT1, AKT2, and AKT3). Among them, AKT1 has been the most investigated and has been involved in many types of cancer development. AKT can directly phosphorylate several transcription factors such as Foxo [144]. Through the regulation of these transcription factors, AKT can inhibit the expression of pro-apoptotic genes and enhance the expression of anti-apoptotic genes, thereby promoting cell survival [145].

Besides, many studies have shown that *PTEN* gene mutations or deletion are related to many types of tumors [137]. PTEN is a phosphatase of PIP3 protein. In contrast to PI3K, PTEN can change the PIP3 to PIP2, thereby inactivates the PI3K signaling pathway. PTEN can reduce the activation of AKT and block the downstream signaling pathway. As a result, PTEN is an important negative regulator of the PI3K/AKT/mTOR pathway [146].

5.2 MAPK/ERK pathway

Mitogen-activated protein kinase (MAPK) pathway is an important intracellular signal transduction pathway that can transmit an extracellular signal to the nucleus of the cell and participate in many biological processes such as cell growth, development and differentiation [147]. This pathway plays an important role in the initiation and development of tumors [148]. MAPK includes the following four categories: ERK, JNK, p38 kinase and BMK1/ERKS.

The MAPK/ERK signal pathway, shown in **Figure 7**, is one of the important and most well investigated MAPK signaling pathways. The Extracellular signal-regulated kinase (ERK) is the key kinase of this pathway. ERK is represented by two closely related proteins, ERK1 and ERK2. The signal starts with the growth factor binding to the receptor on the cell surface and ends with DNA expression in the nucleus, thereby forming protein and producing cellular changes, such as cell division [147, 149].

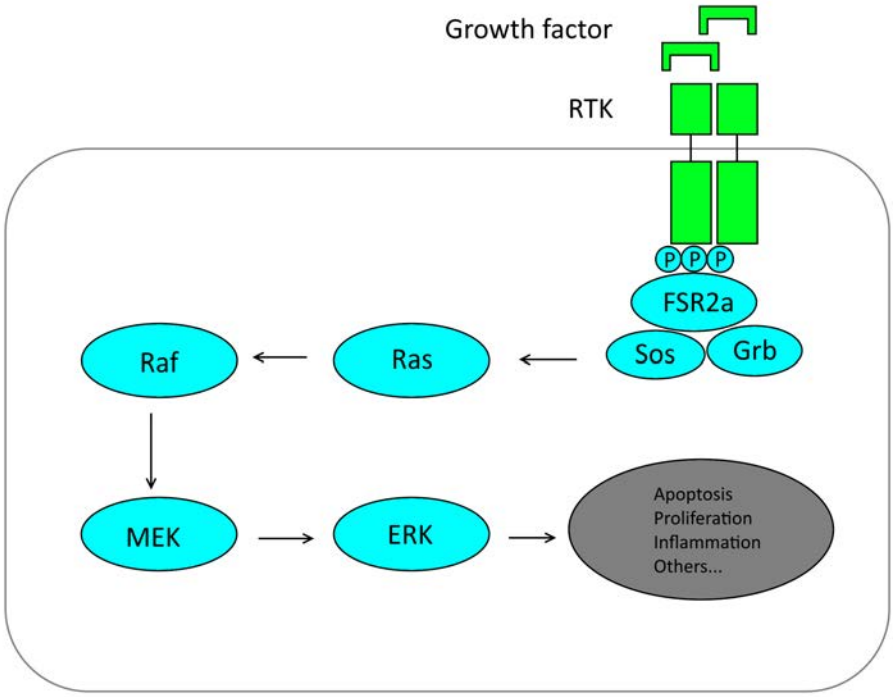

Figure 7: MAPK/ERK pathway. RTK, Receptor tyrosine kinase. P, Phosphate moiety.

The MAPK/ERK pathway can be activated by extracellular signals, such as hormones and growth factors, which can be recognized by receptor tyrosine kinases or receptors that are conjugated to G-proteins [150]. Near the cytoplasmic part of these receptors, a signal complex is assembled, which eventually activates the GTPase Ras. Ras binds and activates MAP3K (MEKK). MAP3K phosphorylates and activates the MAP2K, represented by the two components MEK1 and MEK2. Then MEK1/2 activates ERK1/2 (MAPK) [151, 152].

After the phosphorylation of ERK1/2, the enzyme diffuses into the cytoplasm. This process can phosphorylate signal proteins, such as ribosomal S6 kinase (RSK) [153]. Then the phosphorylate RSK can go to the nucleus and regulate the transcription. ERK1/2 can start the transcription of transcript factors, such as c-Fos and c-Myc [154], which are responsible for proliferation, survival and cell mobility.

6 Targeted therapies for HER2

About 25-30% of breast cancer patients have *HER2* gene overexpression. HER2 is a driver for breast cancer development. As HER2 is overexpressed in the HER2-positive breast cancer subtype, it has become a biological marker for the effective treatment of breast cancer. Now, HER2 targeted therapy for this specific type of BC has widely used in the clinics and has a good outcome [155].

There are three different categories to target HER2:

1. Monoclonal antibodies, such as Trastuzumab and Pertuzumab.
2. Small molecule tyrosine kinase inhibitors, such as Lapatinib.
3. Antibody-drug Conjugates (ADC), such as T-DM1.

6.1 Trastuzumab

Trastuzumab (trade name: **Herceptin**) was approved by FDA in the United States in 1998. As this was the first HER2-targeted drug approved, it dramatically changed the treatment of HER2-positive breast cancer patients. In 2014, a study showed that the combination of trastuzumab and chemotherapy led to a 37% relative improvement in overall survival, increasing 10-year survival from 75.2% to 84%, compare to the chemotherapy alone [156, 157]. Trastuzumab is a humanized IgG1 monoclonal antibody that recognizes the domain IV of HER2 (**Figure 8**) [158].

The anti-tumor mechanism of Trastuzumab may be related to the following aspects:

1. Trastuzumab can recognize the extracellular domain of HER2, preventing the dimerization of HER2 and inhibiting its activation [159].
2. Trastuzumab can reduce pro-angiogenic factors such as Vascular endothelial growth (VEGF) and inhibit tumor angiogenesis [160].

3. Trastuzumab can inhibit non-programmed DNA repair of tumor cells [161].
4. Trastuzumab can activate the NK cells and enhance the Antibody Dependent Cell Cytotoxicity (ADCC) effect. This effect leads to the breakdown of tumor cells [162, 163].
5. Trastuzumab can arrest the G1 phase of the cell cycle, induct the $p27^{kip1}$-CDK2 complex, increase the $p27^{kip1}$ level and decrease the cell proliferation [164, 165].
6. By inhibiting the activity of metalloproteinase, Trastuzumab can block the shedding of the extracellular domain of HER2 [166].
7. Trastuzumab can mediate HER2 endocytosis. After that, degradation of HER2 will occur in the lysosomes [167].
8. Trastuzumab can activate PTEN and block the PI3K signaling pathway [168].

Although Trastuzumab works successfully in HER2-positive breast cancers, its efficacy administered as a single agent is only 12% -34% [158]. Therefore, Trastuzumab is currently used clinically in combination with other chemotherapies, such as Taxanes [169], Capecitabine [170], Cisplatin [171], Doxorubicin [172] or Etoposide [173]. The combination of these drugs can significantly reduce the recurrence rate and prolong the survival of patients [174, 175].

Despite Trastuzumab has many advantages, 70% of HER2-positive patients will undergo resistant to this treatment after one year [176]. In addition, using Trastuzumab for a long time will have some side effects, such as cardiotoxicity and nephrotoxicity [177, 178].

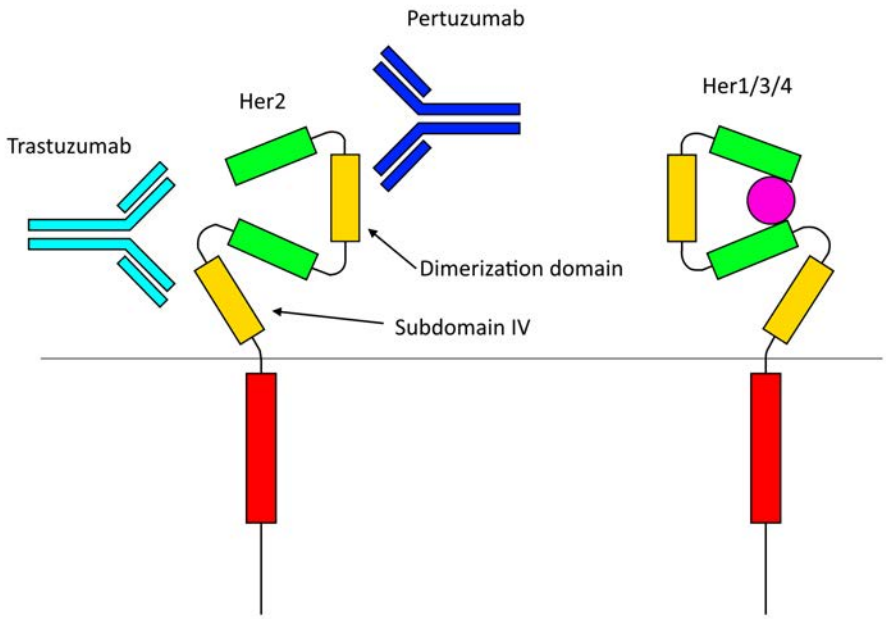

Figure 8: Binding of Trastuzumab and Pertuzumab to HER2. Trastuzumab binds to the Subdomain IV of HER2 while Pertuzumab binds to Subdomain II. The combination of Pertuzumab and HER2 can block the HER2 dimerization.

6.2 Pertuzumab

Pertuzumab (Trade name: Perjeta) was approved in 2012 by FDA and is a type of humanized monoclonal antibody. Pertuzumab can bind to the subdomain II of HER2 and prevent its binding to other ERBB family members (EGFR, HER3, and HER4) to form dimers [179]. This process can inhibit the downstream signaling pathway of PI3K and MAPK, thereby slowing tumor cell proliferation **Figure 8**. As same as Trastuzumab, Pertuzumab can also induce ADCC effect [180].

Pertuzumab has a different binding site to the extracellular domain of HER2 than Trastuzumab. Therefore, the combination of these two drugs can be used to treat HER2-positive breast cancer [181, 182]. After a period of Trastuzumab treatment, some patients develop resistance. HER3

overexpression is one of the mechanisms of Trastuzumab resistance, the HER2/HER3 dimer can activate the downstream signal transduction pathway [183]. As Pertuzumab and Trastuzumab have different sites, the combination of these two drugs can overcome or reduce drug resistance [184]. Clinical studies have shown that Trastuzumab and Pertuzumab combination treatment is more effective than this drug as monotherapy [185].

6.3 Lapatinib

Lapatinib (trade name: Tykerb) is a reversible small molecule tyrosine kinase inhibitor. It was approved in United States in 2007. Lapatinib is mainly used in the clinics for advanced breast cancer patients and can effectively inhibit EGFR and HER2 tyrosine kinase activities [186], as shown in **Figure 9**.

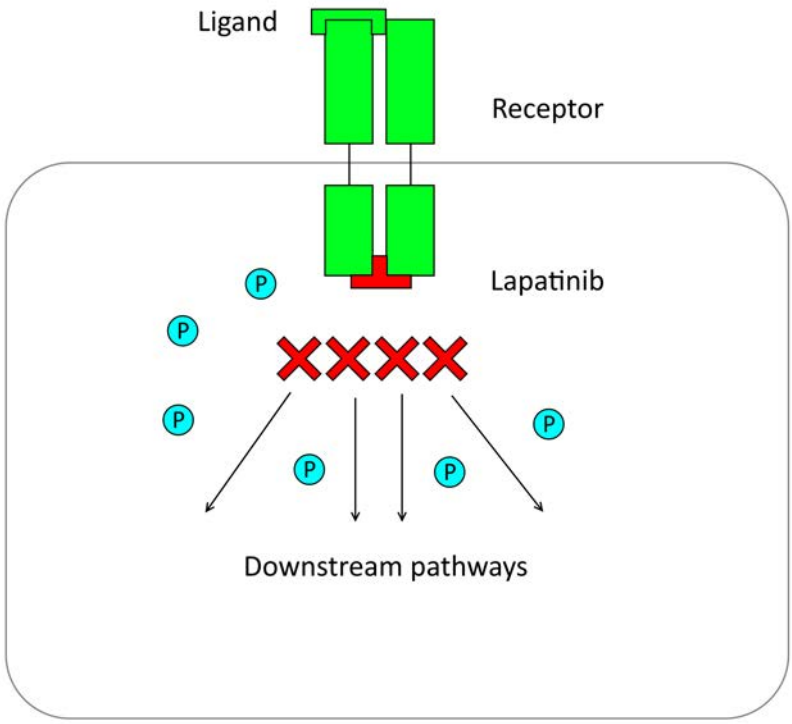

Figure 9: Mechanism of action of Lapatinib. P, Phosphate moiety. Lapatinib can bind to the kinase domain of the HER2 and inhibit the downstream pathway

In HER2-positive breast cancer cells, Lapatinib can inhibit the ATP binding site of EGFR/HER2 and block the ATP binding to the tyrosine kinase domain [187, 188]. By inhibiting tyrosine kinase auto-phosphorylation and activation, Lapatinib can reduce the downstream activity of HER2 receptors: PI3K-AKT and MAPK signaling pathways [189]. Inhibition of these signaling pathways leads to the inhibition of cell proliferation. In addition, Lapatinib induced AKT pathway inhibition promoting apoptosis [190].

The efficacy of Lapatinib as monotherapy is 28% as first-line therapy. After Trastuzumab resistance, the efficiency ratio of Lapatinib becomes 8% as a second-line drug [191]. Due to the high molecular size of Trastuzumab, cannot pass through the hemato-encephalic barrier, therefore, cannot be used to treat brain metastases. Lapatinib is a small molecular drug and can pass through the blood-brain barrier resulting in the perfect candidate for brain metastases [192]. Several studies have showed that the combination of Trastuzumab and Lapatinib has a better outcome than Lapatinib as monotherapy for HER2-positive metastatic breast cancer [193].

Although Lapatinib is an effective drug, its toxicity is a big issue. It has been published that using Lapatinib plus taxanes as first-line therapy for HER2-positive metastasis is associated with shorter progression-free survival rate and higher toxicity compared with Trastuzumab plus taxanes [194]. Similarly to other HER2 therapies, it is quite common to get resistance to Lapatinib [195].

6.4 T-DM1

Currently, there are three drugs widely used to treat HER2-positive breast cancer: the antibody drug Trastuzumab, Pertuzumab and the small molecule inhibitor Lapatinib. Although these drugs have good effects, the resistance and side effect are the two main shortages of these drugs.

Antibody-drug conjugates (ADCs) are a series of drugs that contain a combination of cytotoxic drugs and targeting antibodies fused by a linker [196]. The cytotoxic domain of the ADC is released only when the drug enters into the cancer cell [197, 198]. By this method, the ADC can distinguish between healthy and cancerous cells, presenting fewer side effects [199]. T-DM1 (Trade name: Kadcyla) is an ADC drug developed by the Genentech, which targets HER2 positive breast cancer. It was approved in United States in 2013 [200].

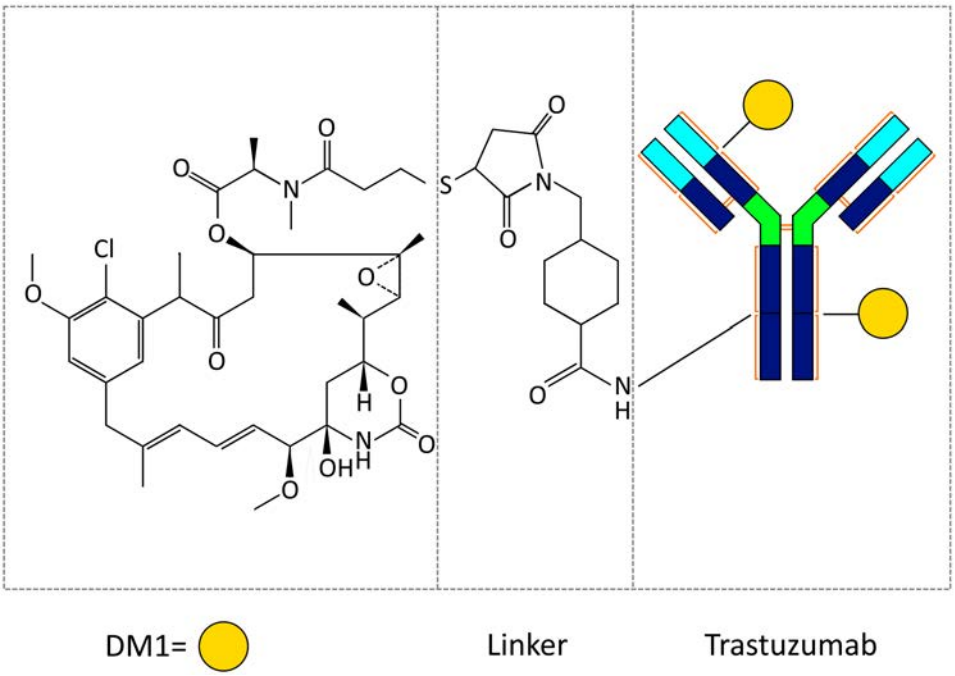

Figure 10: Structure of T-DM1. Left, chemical formula of DM1 (cytotoxic domain) Middle, the linker that binds the cytotoxic domain with the antibody against HER2. Right, a cartoon to illustrate this ADC formed by trastuzumab and DM1.

T-DM1 is composed by a humanized monoclonal antibody, Trastuzumab; a linker, MCC ((N-maleimi-dimethyl) cyclohexane-1-carboxylate) and a cytotoxic agent, Emtansine (DM1) [201] as shown in **Figure 10**. DM1 is a thiol-containing maytansinoid, a microtubule polarization inhibitor [202]. Maytansine is an antineoplastic agent isolated from plants, which shows strong cytotoxicity and has anti-mitotic effects as vincristine and colchicine [203, 204].

When T-DM1 binds to HER2, both proteins are internalized and degraded in the lysosome, releasing the DM1 and promoting the cell lysis. *In vitro* studies have shown that DM1 has a very strong anti-tumor effect (**Figure 11**) [205, 206].

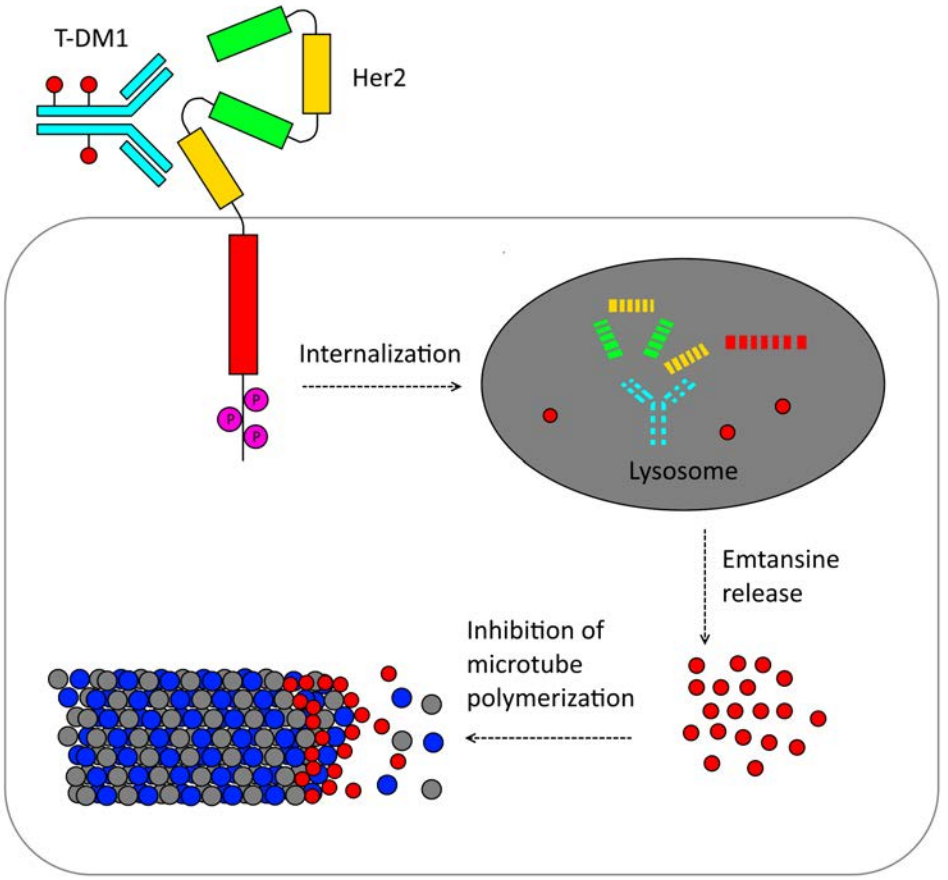

Figure 11: The mechanism of action of T-DM1. P, Phosphate moiety. The red circles are Emtansine, the tube blue and grey represents the microtubules.

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After the binding of T-DM1 to HER2, this antibody gets into the cell and is degraded in the lysosome [206]. After that, the cytotoxic region (DM1) is released, bound to the tubulin and cause the destruction of the cell microtubule formation. This process leads to inhibition the division and proliferation of the cells [207].

In addition, studies have demonstrated that T-DM1 also has the pharmacological effects of trastuzumab monotherapy. T-DM1 can inhibit the HER2 downstream signaling pathway and mediate the ADCC effect in HER2 positive breast cancer cells [206]. However, the main effect of T-DM1 does not depend on the HER2 downstream signaling pathway; it mainly utilizes the targeting effect of Trastuzumab to transport DM1 into target cells. As a result, T-DM1 can overcome the Trastuzumab resistance due to the abnormal signaling pathway [208]. Preclinical studies found that T-DM1 still has the antitumor effect of the trastuzumab and lapatinib-resistant cells [208, 209].

In 2017, a meta-analysis study which included 5 studies with randomized controlled trials (RCTs) for T-DM1, showed that T-DM1 significantly prolonged Progression-free survival (PFS) (HR=0.73, 95% CI: 0.61, 0.86; $P < 0.05$) [210]. Besides, HER2-positive patients are initially sensitive to this treatment; some of them will eventually acquire resistance. Unfortunately, this acquired resistance to T-DM1 is a common problem. Thus, it is very important understanding mechanisms of resistance and exploring strategies for overcoming T-DM1 resistance.

My thesis has as **main objective** to investigate all possible mechanism of resistance to T-DM1 in HER2-positive breast cancer, more specifically in primary cells derived from a human HER2-positive tumor.

MATERIALS AND METHODS

1. Cell culture

All the cells used in this work were cultured at 37ºC with 5% CO_2 in the humidified incubator. The condition medium was DMEM/F12 supplemented with 10% FBS and 2 mM glutamine.

Here is the list of all cells used in this experiment:

- PDX118 cells, which were obtained from a patient-derived tumor xenograft (PDX). This tumor comes from a HER2-positive breast cancer patient.
- Human stable cell lines:
    - MCF-7 cell line. This is a HER2 negative breast cancer cell line which was originally isolated in 1970 from a patient.
    - BT474. This is a HER2 positive breast cancer cell line, which was established from a solid invasive ductal carcinoma of patient.

  Both cell lines were obtained from ATCC.

For the generation of stable cellular clones, I selected used:

- Zeocin™ Selection Reagent (Thermo Fisher Scientific, 1.25mL)

2. Virus production and Infection protocols

Viral vectors are tools commonly used in molecular biology. This can be used to introduce exogenous DNA into the nucleus of a cell. In this experiment, PRZ-CMV-740/nt (ERBB2) was used to introduce the *HER2* gene into the PDX188 cells (both the parental cells and the T-DM1 resistant cells). The experiment was performed as followed:

2.1 Before transfection

- Plate the 293T packaging cells at 3.8×10^6 cells per plate in complete medium in 10 cm Ø tissue culture plates.

- Incubate the cells at 37 ºC in a humidified incubator in an atmosphere of 5% CO_2 for ~20 hours.
- Gently remove media from plate, add 10 mL fresh complete medium containing 25 µM (10 µL) chloroquine di-phosphate and incubate ~5 hours.

2.2 Transfection

- Prepare a mixture of the 3 transfection plasmids for 10cm Ø dish:

psPAX2	1.3 pmol
pMD2.G	0.72 pmol
PRZ-CMV-740/nt(ERBB2)	2 pmol
OptiPro SFM to total volume	500 µL

- Dilute the above 500 µL mixture into 500 µL PEI-OptiPro SFM with PEI (the ratio of µg DNA:µg PEI is 1:3, 1000 µL total per 10 cm Ø dish).
- Gently add the diluted PEI to the diluted DNA. Add the diluted PEI dropwise while gently flicking the diluted DNA tube. Incubate the mixture 15-20 min at room temperature.
- Carefully transfer the transfection mix to the Lenti-X 293T packaging cells. Add the transfection mix dropwise being careful not to dislodge the cells.
- Incubate the cells for 18 hours.
- The following morning, carefully aspirate the media. Replace the media with 15 mL of DMEM complete.
- Incubate the cells.

2.3 Collect the virus

- Virus can be harvested at 48, 72, and 96 hours post transfection in individual harvests or a combined harvest where all the individual harvests are pooled. If pooling harvests, transfer the harvested media to a polypropylene storage tube and store at 4 ºC between harvests.

- Centrifuge the viral supernatant at ~500 g for 5 minutes to pellet any packaging cells that were collected during harvesting.
- Filter supernatant through a 0.45 µm PES filter.
- The viral supernatant can be stored at 4 ºC for several hours but should be aliquoted and snap frozen in liquid nitrogen and stored at -80 ºC as soon as possible to avoid loss of titer.

2.4 Infection and selection

- Infect the PDX118, PDX118-TD44R and PDX118-TD55R with the lentivirus with *HER2* gene.
- Use Zeocin™ Selection Reagent (1:5000) to select the cells infected with the virus.

3. Analysis of protein expression by Western Blot

The western blot is an analytical technique used in molecular biology to detect and identify specific protein in a biological sample. The process of the Western Blot is as follows:

3.1 Protein extraction:

- Wash the cells with PBS twice.
- Lyse cells with Lysis Buffer (150mM NaCl, 50mM Tris pH=7.4, 1% NP-40, 0.25% Sodium deoxycholate, 5mM β-glycerophosphate, 5mM NaF, 1mM Na3VO4, 0.02 tablet/ml of cocktail inhibitors in water).
- Incubate on ice for 30 min.
- Centrifuge the cell lysates at 13000rpm during 30 min.
- Use 60-120pg of the supernatant for Western Blot analysis.

3.2 Electrophoresis and gel transfer:

Separate the samples by 10-12% polyacrylamide gel electrophoresis (PAGE). After, transference to a nitrocellulose membrane

3.3 Western blot

Before incubating with the primary antibodies, block the nitrocellulose membranes with 5% non-fat milk in TBS-T (50mM Tris, 150mM NaCl, 0.05% Tween; adjust pH to pH7.6) for 1 hour at room temperature. Then, incubate the membranes overnight at 4ºC with the primary antibody in blocking solution. After three washes with TBS-T, incubate the membranes with secondary antibodies for 1 hour. Next, remove the excess of secondary antibody washing three times with TBS-T and then, incubate the membranes for 1 minute with ECL solution, containing a HRP substrate. Develop the chemoluminescence signal in a radiography film or by “Amersham Imager 600” system (GE Corp.).

4. RNA extraction and quantitative qRT-PCR analysis

The quantitative polymerase chain reaction (qPCR) or real time PCR, is a technique widely used in molecular biology. Based on the PCR, qPCR is used to amplify and meanwhile quantify a targeted DNA fragment. It enables both detection and quantification of one or more specific sequences in a DNA sample. In many cases the template used for quantitative PCR is not from the beginning DNA, but may be single stranded complementary DNA (cDNA) obtained by retro-transcription of ribonucleic acid (RNA); in this case, the technique is called quantitative RT-PCR (qRT-PCR).

TaqMan Gene Expression Assays is developed by Applied Biosystems Corp. This product contains of a pair of unlabeled PCR primers and a TaqMan probe. Combine to the qRT-PCR, this product can detect the level of RNA of samples very efficient and with high repeatability. The process is followed:

- Cell culture: Seed cells in 10cm Ø dishes and growth for 48h. Then lyse the plates for mRNA extraction.

- RNA extraction: Extract mRNA by using the RNA extraction kit (Qiagen). The process of extraction followed the instructions from the manufacturer. Then, use NanoDrop spectrophotometer to measure the amount of the RNA of samples.
- cDNA synthesis: Using RT-First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech) was used to synthesize the cDNA, the process followed manufacturer's instructions.
- qRT-PCR amplification (Taqman): TaqMan Gene Expression Assays was used to detect the presence of gene expression in the obtained cDNA. Performed qRT-PCR in Applied Biosystems 7500 Real-Time PCR system. Calculate the fold expression of the different genes relative to a housekeeping gene, the 18s or GAPDH expression. The probes used for TaqMan are listed in **Table 1**.

Gene	Assay ID	Amplicon Length	Dye
GAPDH	Hs03929097_g1	58	FAM-MGB
RNA18S5	Hs03928990_g1	61	FAM-MGB
ERBB2	HS01001580_m1	60	FAM-MGB

Table 1. Probes used for TaqMan Gene Expression Assays

5. Copy number variation

TaqMan Copy Number Assays is a product of Applied Biosystems Crop. It was used to evaluate the copy number of genomic DNA targets. Combine to the Applied Biosystems real-time PCR instruments and software; this system can be used to obtain copy number results of a specific gene in a very short time.

- Seed cells in 10 cm Ø dishes and growth for 48h. Then lyse the plates for DNA extraction.
- DNA extraction: DNeasy Blood & Tissue Kits(Qiagen) was used to extract DNA. The process of extraction followed the instructions from the manufacturer. Then, use NanoDrop spectrophotometer to measure the amount of the DNA of samples.

- qRT-PCR amplification (Taqman): TaqMan™ Copy Number Assays was used to detect the copy numbers of ERBB2. Performed qRT-PCR in Applied Biosystems 7500 Real-Time PCR system. Use a cell line without ERBB2 amplification as a control, such as MCF7, then we calculate the copy numbers of each sample.
- The probes used for TaqMan Copy Number Assays are listed in **Table 2**.

Gene	Assay ID
RNase P	4403326
ERBB2	Hs00641606_cn

Table 2. Probes used for TaqMan Copy Number Assays

6. *In vivo* experiment

The NOD-SCID was first reported in 1995 which cannot produce the mature functional T cells and B cells, and has low level of NK cells and macrophage. It is widely used and as a type of immunodeficient mice.

Protocol:

- Three days before injection, seed each type of cells (parental and resistant cells lines) in five-15cm Ø dish. The day of injection, cells were trypsinized and counted. 1×10^7 PDX118-parental, PDX118-TD44R, PDX118-TD55R cells were resuspended in 1000 ul of PBS for each injection.
- 1.5×10^6cells were injected into one side of the mammary fat pads of each mouse.
- Measure the size of the tumor by Vernier scale twice a week and then calculate the volume of the tumor.
- When the volume of the tumors in one groups (PDX118-parental, PDX118-TD44R or PDX118-TD55R) all reached 200 mm2, we started

the treatment of the T-DM1. When the tumor regrows and the volume of it reach to 500 mm^2, another dose of the T-DM1 was given.

- When the size of the tumor reaches 1000mm^3, the mouse was sacrificed and the tumor was passed to a new mouse.
- Six months later, all mice were sacrificed and the in vivo experiment stopped here.
- After the mice were sacrificed, tumors were removed and use for two purposes: re-growth in vitro and Immunohistochemistry.

7. Immunohistochemistry

Immunohistochemistry (IHC) is a method of localizing protein in the cells of a tissue section by detecting antigens using antibodies. In general, the antibody is conjugated to an enzyme, which can catalyze a color-producing reaction. This technique allows detecting characteristic antigenic markers of a cell line, such as secreted protein, or membrane receptors.

- Paraffin sections rehydration: The section was re-hydrated by 2x Xylene for 5 min and then treat with 2x Absolute Alcohol for 3 min. After that treat with 95% Alcohol for 3 min and then 70% Alcohol for 3 min. then in water.
- Antigen retrieval: Treat the section by Citrate Buffer PH6 (ready to use 1X) in microwave for 20 min. After that treatment, put it in Citrate Buffer PH6 for 20 min.
- Blocking and permeabilization: Wash the section with Tris Saline Wash Buffer 1X PH7.6 or PBS 1X for 5 min. After that the section was treated in 3% Hydrogen Peroxide Blocking Solution 5 min. (stock: 30%; ready to use: 3% in Absolut Methanol), then blocked with 3% BSA in PBS 10 min.

Finally, the section was washed with Tris Saline Wash Buffer 1X PH7.6 or PBS 1X 5 min.

- Primary antibody: Incubate with primary Antibody Ab c-erbB2; concentration: 1/500, 30 min. (in Envision Flex antibody diluent Dako.) Then, washed with Tris Saline Wash Buffer 1X PH7.6 or PBS 1X 5 min.
- Secondary antibody: Incubated with Envision System- HRP Labeled Polymer Anti-rabbit 30 min. After that, Wash with Tris Saline Washing Buffer 1X PH7.6 or PBS 1X 5 min twice.
- Development: **DAB** Substrate Chromogen treatment (1ml diluent + 1 drop of DAB). 1-5 min. Washing with Tris Saline Wash Buffer 1X PH7.6 or PBS 1X 5 min.
- Counter-staining: Harris Haematoxylin (1/4 in water) 2 min. Washing with distilled water.
- Dehydration and mounting: 70% Alcohol 3 min. 95% Alcohol 3 min. 2x Absolute Alcohol 3 min. 2x Xylene 5 min. Cover the sections with coverslips and seal with DPX (BDH Chemicals)

8. Cell cycle analysis by flow cytometry

Cell cycle analysis is a flow cytometry method. It allows the cell population to be grouped by the cell cycle stage. The method is based on quantification of cellular DNA, which varies during the individual stages. Before replication (stage G1; G0), the amount of DNA is equal to 2n. During the replication (stage S), the DNA is duplicated and the amount of DNA is larger than 2n. The next step(G2) and as well as in the stage M, the amount of DNA is 4n. The measured data is presented in the form of a histogram where the number of cells is plotted against the amount of DNA.

- Seeds 1.5x10^5 cells in a 6cm Ø dish (in triplicates in each condition), 3 days before the cell cycle analysis.
- Trypsinized adherent cells and add into the cytometer tube.
- Centrifuge at 3500 rpm for 2 minutes at 4 ºC. Discard supernatant. Wash with 4ml 1x PBS and centrifuge again.
- Add 1 ml Ethanol 70% at 4 ºC by drops while vortexing. Put the FACON tube on the ice to incubate at least 30 minutes. (protocol can be stopped at that point)
- Centrifuge at 3500 rpm for 2 minutes at 4 ºC. Wash with 1x PBS and centrifuge again. (resuspend in 1xPBS and the protocol can be stopped at this point and store at 4 ºC)
- Mix by vortexing. Add 300ul DNA extraction solution. Vortex and incubate at 37 ºC for 10 minutes.
- Centrifuge at 3500 rpm for 2 minutes at 4 ºC. Discard supernatant.
- Prepare PI (propidium iodide)/ RNase solution. Add 300µl PI/RNase solution to each tube. Vortex. Incubate 30 min 37ºC in dark (cover tubes).
- Analyze by flow cytometry (FL-2)

Reagents:

- DNA extraction solution: 190ml Na2PO4 0,2M; 8ml Cítric ac. 0,1M pH= 7.8. (store at 4ºC, take out of the fridge 1h before starting and put on the magnet stir)
- PI/RNase Solution: RNase (10µg/ml); PI (40µg/ml); PBS (filtered).

9. Analysis of the HER2 protein on the cell surface

Immunophenotyping is a technique used to study the protein expressed by cells. This method uses the antibody that can recognize the specific proteins on the cells. Combine to the flow cytometer; this technic can measure the level of the protein on the surface of the cells. Here is the protocol:

- Cells and types to be analyzed: PDX118-Parental, PDX118-TD44R, PDX118-TD55R
- Staining: Human IgG + anti-human-Alexa-488; Trastuzumab + anti-human-Alexa-488
- Before starting: Prepare and setting all the reagents and equipment for the experiment: First, Label 15mL Falcon tubes, 1.5ml Eppendorf tubes and 96-well (V-bottom) plate and store them on ice. Label cytometer tubes and store them at 4ºC (store them in the fridge). After that, Set centrifuges for Falcon Tubes and Eppendorf tubes at 4ºC. Then warm the following reagents at 37ºC: 50m PBS, 50mL medium, and 10mL accutase. Then, prepare Blocking Solution: 5% Horse Serum (750µL Stock); 1% BSA (1.5mL 10% Stock in PBS); 2.5mM-EDTA (750µL Stock in PBS) in PBS 1X (12mL)
- Detach the cells (accutase): Take all the cell plates from the incubator and move to the bench. Then, aspirate de cell medium from all the plates and wash once with 5mL/plate of PBS, add 1mL/plate of accutase or PBS-EDTA (5mM). After that, incubate at 37ºC in the laboratory incubator until the cells are visually detached. Then add 5mL/plate of medium and transfer the cell suspension to the 15mL Falcon tubes. After that, Centrifuge 5 seconds 1000rpm at RT and discard supernatant by aspiration. Then add 1mL/tube of PBS to the cell pellet and resuspend and count the cells (store samples at 4ºC during cell count). After that

transfer 3X10^5 cells for each cell type to the Eppendorf tubes, centrifuge 15 min 8000rpm at 4ºC and discard supernatant by aspiration.

- Blocking: Add 300µL of blocking solution the cell pellet (=100µL/1xl0^5 cells). Resuspend cell pellet. Pippete 2 samples of 100pL/each to the 96-well plate (1x10s cells/staining). Incubate 20' on ice. Centrifuge the plate 5' 1000rpm at 4ºC. Check visually for a cell pellet in each of the wells. Discard supernatant
- Prepare the Primary antibodies during the blocking: Human IgG: 1mL blocking solution + 2.5µL Human IgG 1µg/µL; Herceptin: 1mL blocking solution +2.5µL Trastuzumab 1µg/µL
- Incubation with Primary Antibodies: Add 100µL of primary antibodies solution the cell pellet, accordingly (=2.5µg/mL) (A column on the plate for Human IgG and B column for Trastuzumab). Resuspend with the multichannel pipette. Incubate 20' on ice. Centrifuge the plate 5' 1000rpm at 4ºC. Check visually for a cell pellet in each of the wells. Discard supernatant. Wash: Add cold-PBS 200µL/well. Resuspend with the multichannel pipette. Centrifuge the plate 5' 1000rpm at 4ºC. Check visually for a cell pellet in each of the wells. Discard supernatant
- Prepare the Secondary antibody during 1ry Antibody Incubations: Anti-human Alexa-488: 1mL blocking solution + 2µL anti-human-Alexa 488, final concentration: 4µg/mL (1:500 dilution) and store on ice until used
- Incubation with Secondary Antibodies and preparation of PBS-PI. Add 50µL of secondary antibody solution (=4µg/mL) in all the wells. Resuspend with the multichannel pipette. Incubate 20' on ice and covered with foil paper. Prepare PBS+propidium iodine (2µg/mL) 8ml,
  cold PBS + 16µL propidium iodine (1mg/mL). Centrifuge the plate 5' 1000rpm at 4ºC. Check visually for a cell pellet in each of the wells. Discard supernatant. Wash: Add cold-PBS 200µL/well. Resuspend with

the multichannel pipette. Centrifuge the plate 5' 1000rpm at 4ºC. Check visually for a cell pellet in each of the wells. Discard supernatant

- Fixation and Resuspension of the samples. Add 200µL/well PBS-PI solution. Resuspend with the multichannel pipette. Incubate for 5' (covered with foil paper). Centrifuge the plate 5' 1000rpm at 4ºC. Check visually for a cell pellet in each of the wells. Discard supernatant. Add 100µL/well of PFA 4% solution. Resuspend with the multichannel pipette. Incubate for 10' on ice (covered with foil paper). Centrifuge the plate 5' 1000rpm at 4ºC. Check visually for a cell pellet in each of the wells. Discard supernatant. Add 200µL/well of ice-cold PBS. Resuspend with the multichannel pipette. Transfer the stained cells to the cytometer tubes according to the numeration. Cover the rack of tubes with foil paper.
- Analysis of the samples. Evaluate fluorescence at FL1-FL3 channels using a cytometer (FACScalibur/Navios). Acquire 10000 events of FL3- cells.

10. Crystal Violet Cell Staining

Crystal Violet Staining Solution can bind to DNA. The nucleus of the cell can be stained in a violet color. As a result, this protocol can be used for determining viability of cultured adherent cells in the plate.

- Reagents: Glutaraldehyde solution (10%), Crystal Violet solution (0.1%), Acetic Acid solution (10%).
- Equipment: VICTOR3 Multilabel Readers, Container for washing the plate (box or tank), Orbital Shaker.

Procedure:

- Fix the cells. First, Remove the medium from the plate without washing and add 10% glutaraldehyde, the volume depends on the type of plate, which listed below. Then, incubate the plate for 10 minutes at room temperature and empty the glutaraldehyde inside a bag with paper. After that, wash by immersion 3 times in a container with tap water and empty the plate.
- Staining. First, add 0.1% Crystal Violet to cover dish, the volume depends on the type of plate, which listed below. Then, Incubate the plate for 20 minutes at room temperature and empty the Crystal Violet inside a bag with paper. After that wash the plate by immersion for 3 times in a container with tap water. Then, let the plate dry on bench overnight.
- Sample solubilization. First, add 10% Acetic Acid in to the plate, the volume depends on the type of plate, which listed below. After that, shake the plate for 10 minutes at room temperature on orbital Shaker. Then read the plate with the TECAN Infinite M200 PRO Multimode Microplate Reader at 560 nm.

Volume to add into the plate

- 96 well plate 50µL
- 48 well plate 150µL
- 24 well plate 250µL
- 12 well plate 500µL
- 6 well plate 1mL

11. Cellular sub-cloning from the cell culture

In order to study the heterogeneity of a cell line, a sub-clonal population from the original culture was done. This is a method to generate a sub-clonal population by limiting dilution.

Reagents and equipment required:

- Cell culture media
- Phosphate buffered saline (PBS)
- Trypsin-EDTA (0.05%)
- Vi-CELL XR, Cell Counter

Procedure:

- Remove the media from plate. Then wash cells with PBS. After that, add Trypsin-EDTA (0.05%) to cells.
- Incubate the cells for 10 minutes at 37ºC and 5% CO2 until the cells detach. After that add 5mL cell culture media into the plate. Then, mix well the cells by pipetting with 5mL stripette.
- Check the plate under the microscope to make sure most cells have been detached. Then count the number of live cells with the Cell Counter. After that, calculate and dilute cells to 1 x 10^4 cells/mL in 1 mL of fresh media.
- Transfer the diluted cells to 96 well plates by multichannel pipette, every well with 100ml. The day after checking the 96 well plates under microscope, mark the well only has one cell.
- Change the media every week until the cells grow fully in the marked well. Then transfer the cells to 24 well plate.
- Change the media every week until the cells grow fully in the 24 well plate. After that transfer the cells to 6 well plate.

- Change the media every 3 days until the cells grow fully in the 6 well plate. Then, transfer the cells to 10 cm dish.
- Change the media every 3 days until the cells are enough to expend to two plates.
- Now sub-clone cells already obtained. The cells can be frozen or done the experiment.

12. Cell Lysosome Lysis Extraction

Lysosomes have a very important role in the protein degradation. This protocol is used for isolate and lysate the lysosomes.

Reagents and equipment required:

- IKA T 10 Basic Ultra Turrax Homogenizer
- Lysosome Extraction Kit (BestBio, China)

Procedures:

- Take 1-2×10^7 cells and centrifuge at 4°C for 5 min at the speed of 500g. Carefully aspirate the medium as much as possible and collect the cells.
- Wash twice with cold PBS. Aspirate the supernatant as much as possible after each wash.
- Add 400μl of cold reagent A and keep on ice for 10 minutes.
- Homogenize in the homogenizer until most cells were disrupted. Centrifuge for 5 minutes at 4°C at the speed of 1000g.
- Quickly transfer the supernatant into another pre-cooled clean centrifuge tube. Then Centrifuge for 10 minutes at 4°C at the speed of 3000 g.
- Quickly transfer the supernatant into another pre-cooled clean centrifuge tube. Then Centrifuge for 10 minutes at 4°C at the speed of 5000 g.

- Quickly transfer the supernatant into another pre-cooled clean centrifuge tube. Then Centrifuge for 20 minutes at 4°C at the speed of 20000-30000 g.
- Discard the supernatant. Then add 400µl of cold reagent B to the pellet and mix.
- Centrifuge for 20 minutes at 4°C at the speed of 20000-30000g.
- Discard the supernatant and add the lysis buffer of the protein.
- Obtain the lysis of the lysosomal sample, measure the concentration and use it for downstream experiments.

RESULTS

1. *In vitro* characterization of T-DM1 resistant cells

1.1 Generation of the T-DM1 resistance cells

In order to study the mechanism of resistance to T-DM1 *in vitro*, first I decided to generate a cell line unable to respond to T-DM1 treatment.

With this purpose, I used **PDX118 cells**. These cells are a primary culture, which came from a Patient-Derived Xenograft of a woman who was diagnosed with a HER2-positive breast cancer. In the lab, we established a 2D-culture from this PDX118, and we tested by Western Blot their levels of HER2 and their sensitivity to T-DM1.

MCF7, BT474 and PDX118 cells were seeded in a 10 cm dish for 3 days. The expression of HER2 was analyzed by Western blot. Their HER2 levels of PDX118 cells were similar to BT474 cells, a HER2-positive breast cancer cell line, and both presented higher levels than the control MCF7cell line (**Figure 12**).

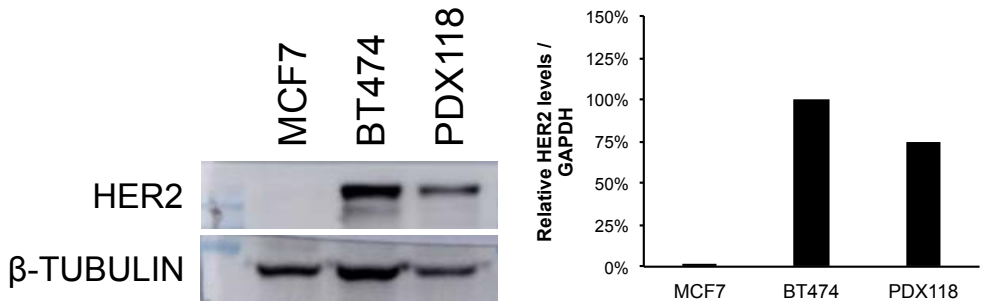


Figure 12: HER2 levels in different cell lines. Left, WB which measures HER2 protein levels of PDX118 cells compared to known breast cancer cell lines. β-TUBULIN was used as a loading control. Right, quantification of WB by ImageJ Software

PDX118, MCF7 and BT474 cells were treated at different concentrations of T-DM1 for 6 days. Then their cell viability was determined by Crystal Violet method and analyzed with Magelian Software (**Figure 13**). Only the HER2-positive cells lines (BT474 and PDX118 cells) were sensitive to T-DM1 treatment.

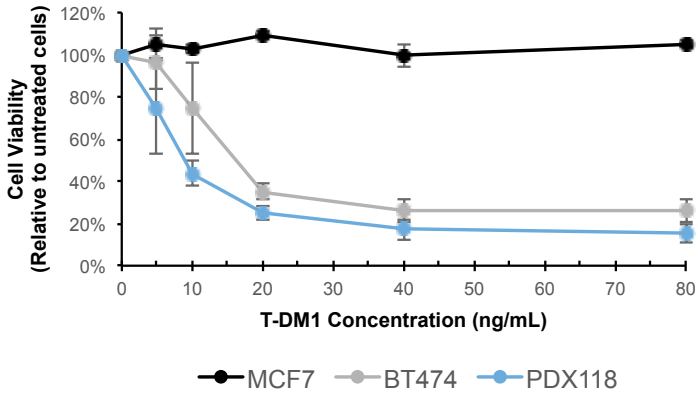


Figure 13: Effect of T-DM1 in different cell lines. Sensibility to T-DM1 measured by crystal violet. Data are expressed as Mean ± SD. N = 2 independent experiments, each including technical replicates.

In order to get an *in vitro* T-DM1 resistant model, I treated PDX118 cells with a low concentration of T-DM1. Then, I increased the concentration every week until reaching a concentration that let resistant cells growing but not the parental cells. PDX118 cells were seeded in 10 cm dish, 3 days after were treated at the indicated concentration at the indicated time points described in **Table 3**.

TD44R	Day	1	10	17	24					
	Concentration(ng/mL)	11	22	33	44					
TD55R	Day	1	10	17	24					
	Concentration(ng/mL)	22	33	44	55					
TD200R	Day	0	17	34	69	142	177	188	206	212
	Concentration(ng/mL)	20	40	80	100	120	140	160	180	200

Table 3: T-DM1 concentrations used to generate resistant clones. Left, name of the resistant cellular clones. Right, the T-DM1 concentrations used at different indicated time points.

I named the clones with the highest T-DM1 concentration used during its generation: 44ng/mL (TD44R cells), 55ng/mL (TD55R cells) and 200ng/mL (TD200R). After reaching the final concentration, the cells were culture with T-DM1 for 1 month and then analyzed.

1.2 Testing T-DM1 resistance

First, I tested whether these clones were resistant to T-DM1. In order to verify their sensitivity, I performed cell viability experiments using increasing doses of T-DM1 and measuring the cellular growth at *day 6* after treatment by

crystal violet (**Figure 14**). The results indicated that, whereas parental cells (PDX118 cells) showed an IC_{50} = 20 ng/mL, the resistant TD44R, TD55R, TD200R cells had a very strong ability to resist to T-DM1 (IC_{50} >80 ng/mL; >80 ng/mL and >200 ng/mL, respectively).

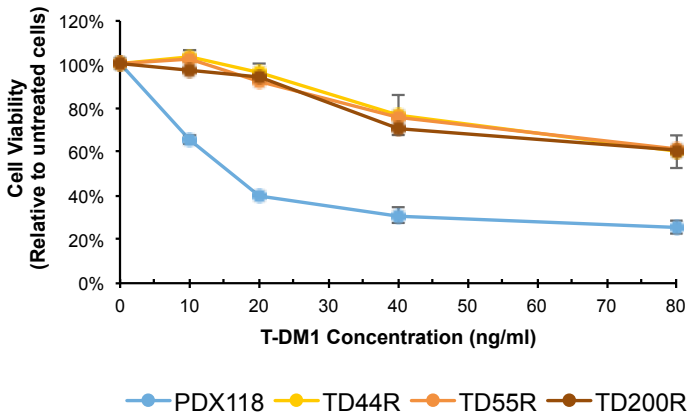


Figure 14: Sensitivity to the T-DM1 in TD44R, TD55R and TD200R cells. TD44R cells (yellow), TD55R cells (orange) and TD200R (brown) cells were grown in increasing doses of T-DM1 for 6 days. Cell viability was determined using Crystal violet assay. Data are expressed as Mean ± SD. N = 2 independent experiments, each including technical replicates.

Then, I decided to evaluate whether this resistance was stable. With this purpose, I removed the T-DM1 drug from the media of the resistant cells for more than 3 months. Thus, I re-checked the T-DM1 resistance of these cells (**Figure 15**) and I observed that they maintained their low sensibility to this treatment, comparing parental cells with TD44R, TD55R and TD200R. These results prove that all these cellular clones have an established resistance to T-DM1.

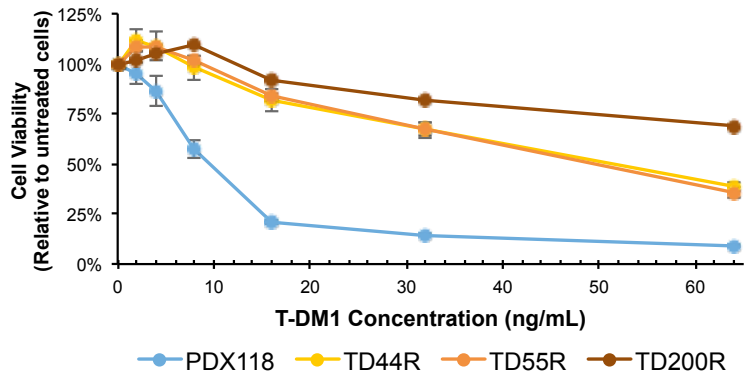


Figure 15: Stable resistance to T-DM1 in TD44R, TD55R and TD200R. PDX118 (blue) and. TD44R cells (yellow), TD55R cells (orange) and TD200R (brown) cells were grown after removing T-DM1. Then their sensibility to T-DM1 was tested using increasing doses of T-DM1 for 6 days. Cell viability was determined using Crystal violet assay. Data are expressed as Mean ± SD. N = 2 independent experiments, each including technical replicates.

1.3 T-DM1 causes cell cycle arrest

After T-DM1 binds to HER2 in the surface of the cells, the DM1 (cytotoxic part) can interact to the Tubulin, and then the microtubules cannot be formed, resulting in a cell cycle arrest, blocking the cells in S phase. First I checked how T-DM1 was affecting the cell cycle of PDX118 parental cells and resistant clones (**Figure 16**). After treating the cells with 200 ng/mL of T-DM1 for 72h, I measured the percentage of cells at the different cell cycle phases by Flow Cytometry and I observed that all resistant clones showed a decreased percentage of cells in G_0/G_1. Indeed, TD44R, TD55R and TD200R confirmed an increased percentage of cells in S phase due to the DM1 action.

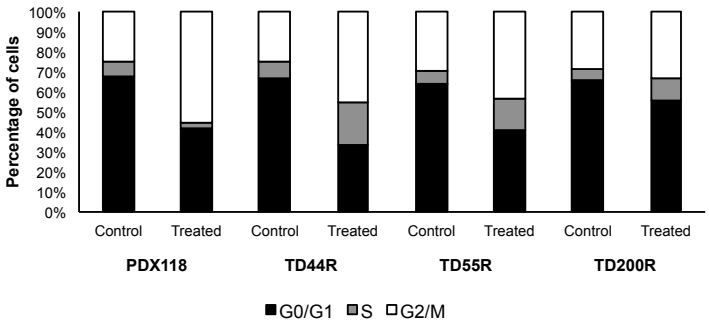
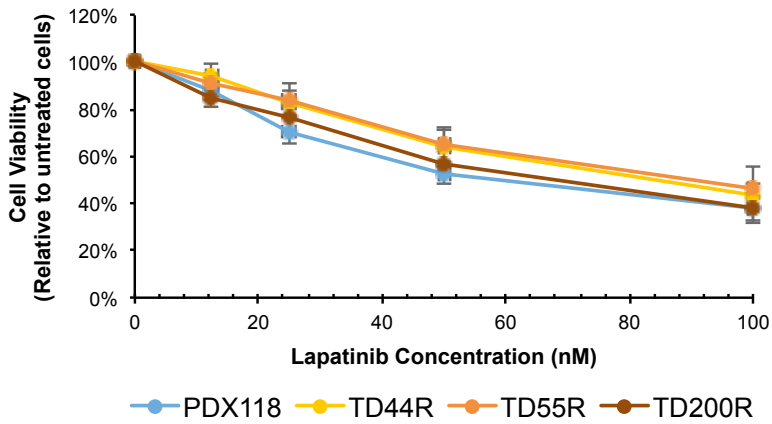


Figure 16: Cell cycle analysis after T-DM1 treatment. Cells were treated with T-DM1 at 200 ng/mL concentration for 72h and then analyzed by Flow Cytometry. Bar graphs show the percentage of PDX118, TD44R, TD55R and TD200R cells in G_0/G_1 (black), S (grey), and G_2/M (white) phases of the cell cycle.

These results, in agreement to previous data, suggested that these clones, although they are resistant to T-DM1, at high concentrations of this drug they are able to partially block their cell cycle, slowing down their proliferation (**Figure 14**).

1.4 Testing sensitivity to other HER2-target therapies

In the last years, other HER2-target drugs such as Trastuzumab [211] or Lapatinib [186] have been approved in EU and US. All these drugs have the same target as T-DM1, HER2. Since I was characterizing these T-DM1 resistant clones, I found interesting to know whether they could also be resistant to Trastuzumab and/or Lapatinib. Therefore, I compared the sensibility to these drugs in parental and resistant cells measuring cell growth by Crystal Violet (**Figure 17**).



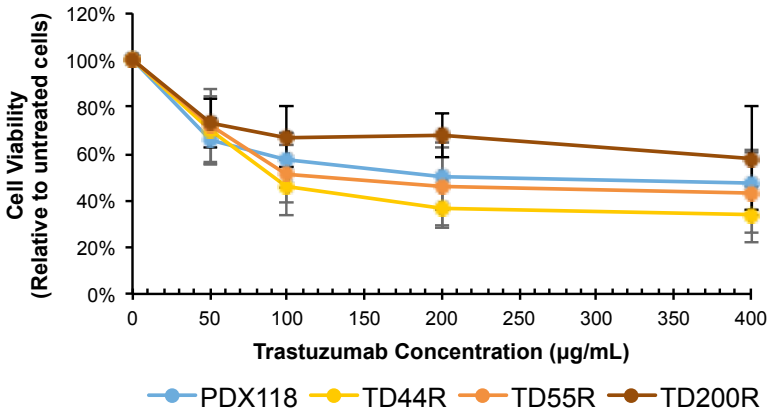


Figure 17: Cell viability of TD44R, TD55R and TD200R cells after treatment of Trastuzumab and Lapatinib. PDX118, TD44R, TD55R and TD200R cells were grown in increasing doses of Trastuzumab (bottom) or Lapatinib (upper) for 6 days. Cell viability was determined using Crystal Violet assay. Data are expressed as means ± SD. N = 2 independent experiments, each including technical replicates.

These analyses showed non-significant difference between TD44R, TD55R and TD200R cell growth compared to parental PDX118 cells after been treated with Trastuzumab or Lapatinib.

The mechanism of action of Lapatinib depends on the kinase activity of HER2 receptor suggesting that the mechanism of resistance of these clones to T-DM1 is independent of the downstream HER2 signaling pathway. Interestingly, Trastuzumab, which is the same antibody forming part of T-DM1, showed similar sensibility in the resistant clones compared to PDX118 cells suggesting that the T-DM1 resistance might be independent of the antibody binding to HER2.

2. Mechanisms of resistance to T-DM1

The drug-resistant to targeted therapy may have many origins; it may be caused by mutations at DNA level, which then can be translated in genes expression changes [212], and also non-DNA related, which could imply post-translational changes involving different signalling pathways [213].

2.1 Evaluating HER2 protein levels

Since T-DM1 is an antibody-drug conjugate, which consists in the presence of Trastuzumab (HER2 antibody) linked to a cytotoxic agent DM1. The mechanism of action of this drug depends on the presence of HER2 expression at the cell membrane of the cells, for this reason is important to know if the levels of HER2 protein are comparable to the parental PDS118 cells. Thus, I measured the total levels of HER2 in PDX118, TD44R, TD55R and TD200R cells by Western Blot (**Figure 18**).

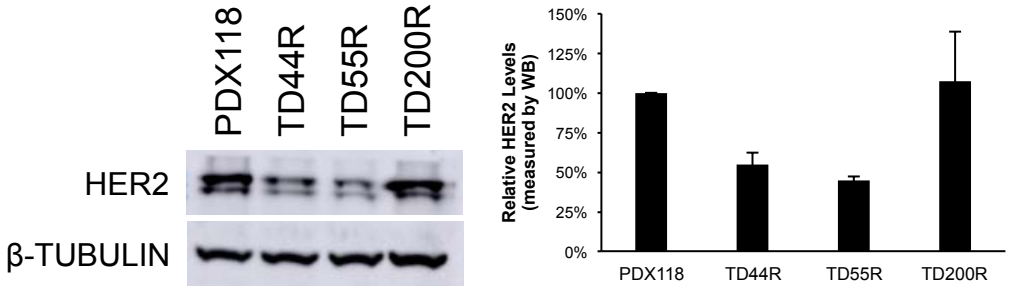


Figure 18: HER2 level in PDX118 parental cells and T-DM1 resistant clones by Western blot. Left, PDX118, TD44R, TD55R and TD200R cells were seeded in 10 cm dish, 3 days later the cell lysates were collected and analyzed by Western Blot. It is shown one representative experiment of n= 3 independent experiments, quantified in right shown as Mean ± SD.

These results showed that TD44R and TD55R resistant cells had lower protein levels of HER2 compared to parental cells (54.8% and 44.1%, respectively). Interesting, TD200R cells showed similar levels of HER2 as parental PDX118 cells.

Similarly, I quantified by Flow Cytometry the levels HER2 at the cell surface (**Figure 19**).

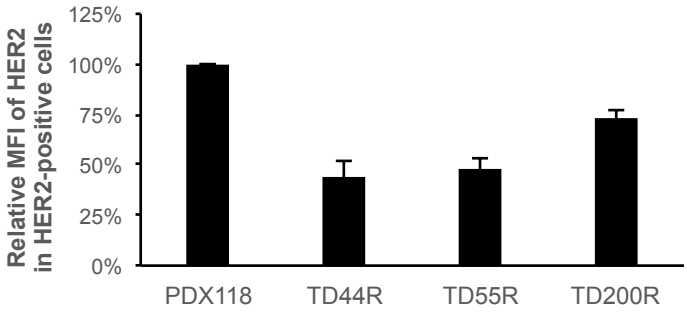


Figure 19: HER2 expression of PDX118 and T-DM1 resistant cells by Flow Cytometry. PDX118, TD44R, TD55R and TD200R cells were seeded in 10 cm dish and analyzed 3 days later. Bars represent Mean Fluorescence Intensity (MFI) in HER2-positive cells measured by Flow Cytometry, after normalizing the parental cells to 100%. These results are shown in Mean ± SD. N = 3 independent experiments, each including technical replicates.

These experiments showed similar results than obtained in **Figure 18**, confirming that the levels of HER2 expressed in the membrane were different in some resistant clones (TD44R and TD55R), compared to the parental.

In order to know whether this HER2 reduction in TD44R and TD55R was permanent or transient, I checked the protein levels of HER2 of these cell lines after removing T-DM1 for more than three months. Then, I analyzed by Western blot (**Figure 20**) and Flow Cytometry (**Figure 21**), and I found that HER2 levels in these long time-T-DM1 removed resistant cells were maintained stably lower than in parental cells.

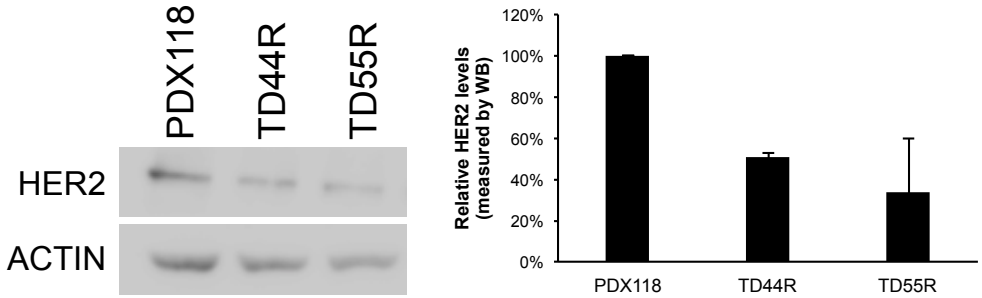


Figure 20: Total HER2 levels of TD44R and TD55R after 3 months without treatment. Left, Western blot shows HER2 levels of PDX118, TD44R-R and TD55R-R cells, ACTIN was used as a loading control. Right, bars represent the quantification of 3 independent experiments in Mean ± SD.

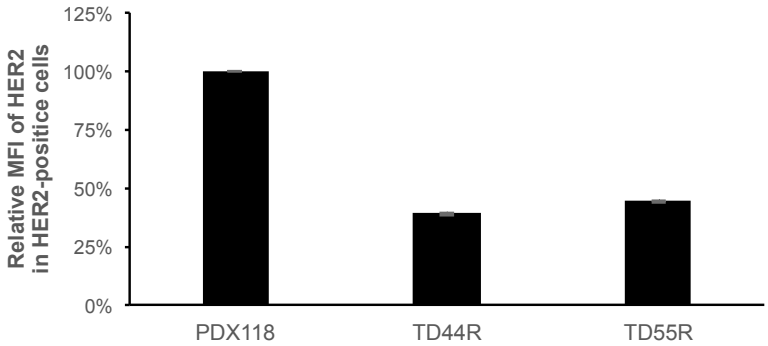


Figure 21: Levels of HER2 in the surface of resistant clones after 3 months without treatment. HER2 levels of PDX118 cells and indicated resistant clones detected by Flow Cytometry. Bars represent MFI of HER2 in HER2-positive cells ± SD, after normalizing the parental cells to 100%. N = 3 independent experiments, each including technical replicates.

With the intention of understanding why TD44R and TD55R cells had lower protein levels of HER2 than parental cells, I performed qRT-PCR based on TaqMan Gene Expression Assays to measure the amount of *HER2* mRNA levels in parental and T-DM1 resistant cells (**Figure 22**).

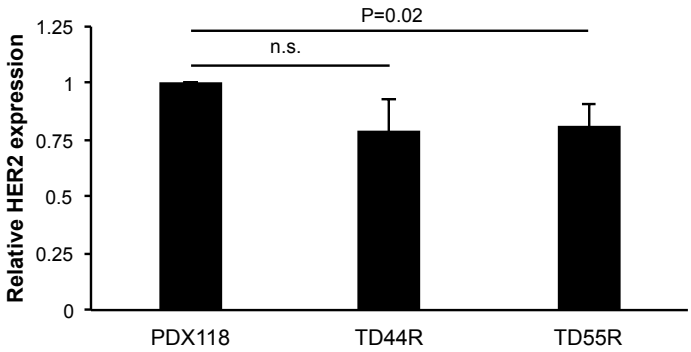


Figure 22: HER2 expression at mRNA level of PDX118, TD44R and TD55R cells. Cells were seeded for 3 days, harvested, extracted mRNA, performed reverse transcription and qPCR analysis. Results are shown in mean ± SD. P-values calculated with *t.test*. N = 3 independent experiments, each including technical replicates.

Surprisingly, the differences in *HER2* expression were minimal or not significant between TD44R and TD55R compared to parental cells. Then, I decided to perform several experiments in order to find an explanation for the reduced HER2 protein levels of these clones. Therefore, I analyzed the

degradation of HER2 in PDX118, TD44R and TD55R cells using Cycloheximide, which is a eukaryote protein synthesis inhibitor (**Figure 23**).

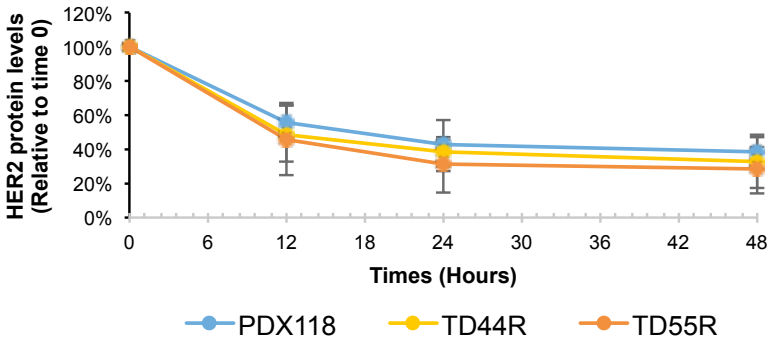


Figure 23: Lifespan of HER2 protein in different clones. The degradation process of HER2 protein was determined by Western blot at 0h, 12h, 24h and 48h in the presence of Cycloheximide. N= 5 independent experiments were performed. The standard deviation was calculated. T-test between each group was shown

From these results, I concluded that HER2 degradation did not show any significant difference (P>0.5) between TD44R and TD55R compared to PDX118. Then, I explored the possibility that the resistant clones had different genomic levels of HER2 in comparison to PDX118 cells. To do this, I measured their copy number of *HER2*.

The amplification of a cancer gene usually has two forms: Homogeneously Staining Region (HSR) and Double Minutes (Dmin). Dmin are circular, extra-chromosomal amplifications of specific acentric DNA fragments. It has been reported that Dmin can be decreased after using some drugs, such as Gemcitabine [214]. From previous data generated in our laboratory, we already showed that HER2 in PDX118 cells is mainly found in Dmin form [215]. I also know that the protein levels of HER2 are decreased in TD44R and TD55R cells compared to the parental cells, suggesting that maybe the copy number of HER2 may change during T-DM1 treatment.

In order to validate this hypothesis, I used the TaqMan Copy Number Assay to detect the copy numbers of HER2 in TD44R, TD55R and parental cells (**Figure 24**).

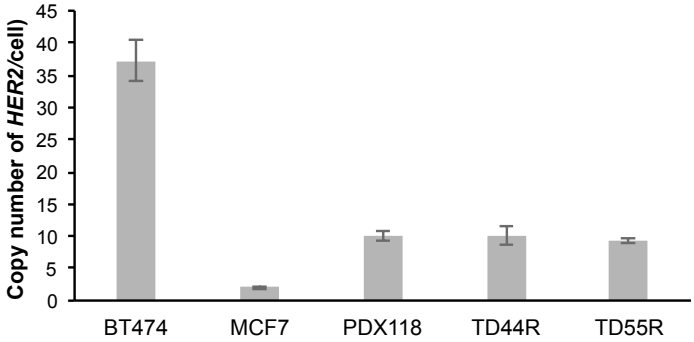


Figure 24: HER2 copy number in PDX118, TD44R and TD55R cells. Cells were seeded for 3 days, harvested and extracted DNA. Then TaqMan Copy Number Assays were performed. Bars show mean ± SD.

My data showed that there is no significant difference between the PDX118, TD44R and TD55R cells, demonstrating that the reduced levels of HER2 protein is not caused by changes in their copy number.

2.2 Assessing HER2 protein levels as a mechanism of resistance

My results have illustrated that the difference in the levels of HER2 levels in TD44R and TD55R cells, could explain that the T-DM1 resistance of these clones. To prove this hypothesis, I performed a HER2 rescue experiment. I transfected *HER2* containing vector and selected with Zeocin (the antibiotic resistance included in this vector). After two weeks of selection, I got three HER2-rescued cell clones: PDX118-HER2, TD44R-HER2 and TD55R-HER2. Then, I checked HER2 levels by Western Blot and Flow Cytometry (**Figure 25**).

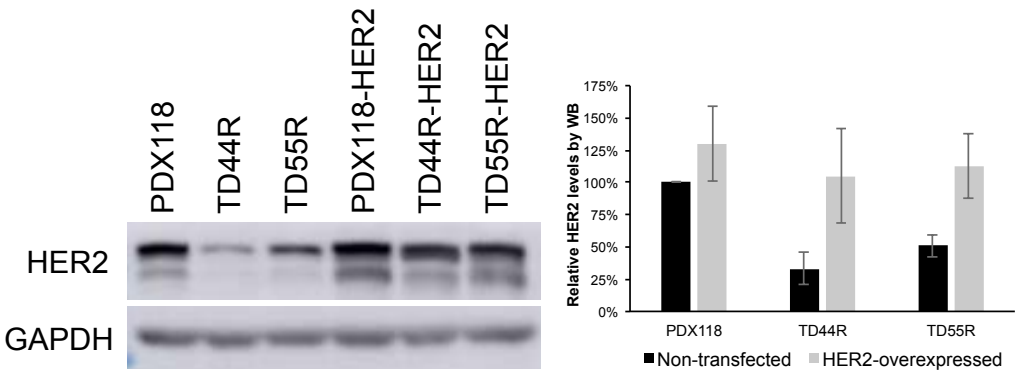


Figure 25: HER2 expression in HER2-rescued clones. PDX118, TD44R, TD55R, PDX118-HER2, TD44R-HER2 and TD55R-HER2 cells were seeded in 2*10 cm dish and 3 days later, cells were lysated and performed WB (upper); or analyzed by Flow Cytometry (bottom). In the top right, bars represent the quantification of 3 independent experiments in Mean ± SD. Non-transfected cells (black) and clones overexpressing HER2 (grey).

These control experiments showed that all the HER2-rescued clones expressed higher levels of HER2, compared to their parental**.** Then, I examined the sensitivity of these HER2-rescued clones to T-DM1 compared to the non-transfected clones. I quantified their viability after T-DM1 treatment by crystal violet (**Figure 26**).

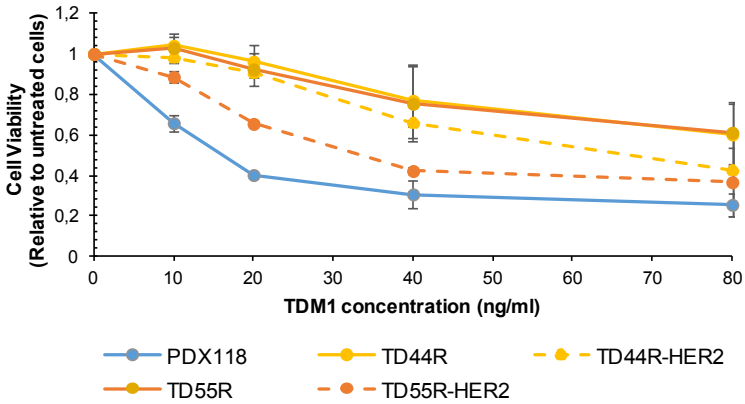


Figure 26: Cell viability of HER2-restored cells under T-DM1 treatment. The sensitivity to T-DM1 was measured by Crystal Violet. T-DM1 resistant cells and HER2-rescued cells were grown in increasing doses of T-DM1 for 6 days in 96 well plates. Data is expressed as means ± SD; n= 2 independent experiments.

These results showed that restoring the levels of HER2 in the TD44R and TD55R cells is sufficient to increase their sensibility to T-DM1 (IC_{50}= 60ng/mL and IC_{50}= 30ng/mL, respectively). However, they were not able to reach the

same sensibility observed in the PDX118 parental cells (IC_{50}= 15ng/mL), suggesting that although the levels could partially explain their T-DM1 resistance, might not be the only mechanism of resistance.

Overall, this data together with the fact that TD200R had similar levels of HER2 as parental cells (**Figure 18**) suggest that protein levels of HER2 might be a mechanism of resistance to T-DM1, but definitively not the only one.

2.3 Measuring heterogeneity in T-DM1 resistant cells

During the clinic therapy, the resistance always occurs after a period of treatment. There are at least two hypotheses about the source of these resistant cells that have been proposed: on one hand, it has been postulated that during a drug treatment, several somatic mutations can happen resulting in “mutant” cells able to replace the originally sensitive-cells. Another hypothesis is that a tumour is composed by a group of heterogenic cells with different chemo-sensibility, meaning that resistant cells already existed in the tumour. During a treatment, however, under the selecting pressure of a specific drug, the resistant cells could finally expand becoming a resistant tumor.

In order to rule out any of the hypothesis described above, I tested whether different cell sub-populations might form the T-DM1 resistant clones. In order to explore the heterogeneity of these cells, I performed sub-cloning of the PDX118, TD44R and TD55R separately and checked their sensibility to T-DM1 (**Figure 27**).

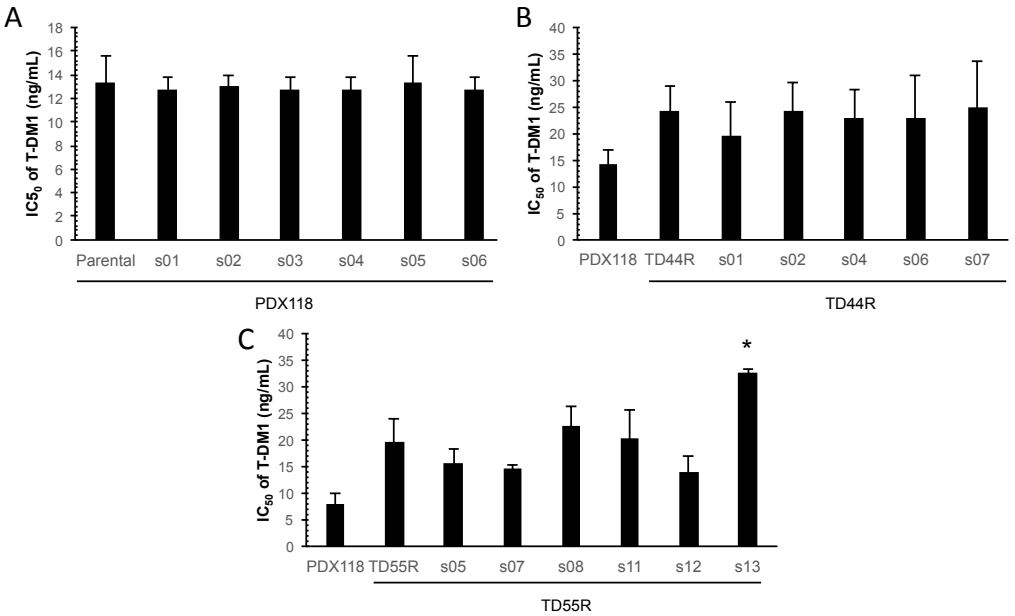
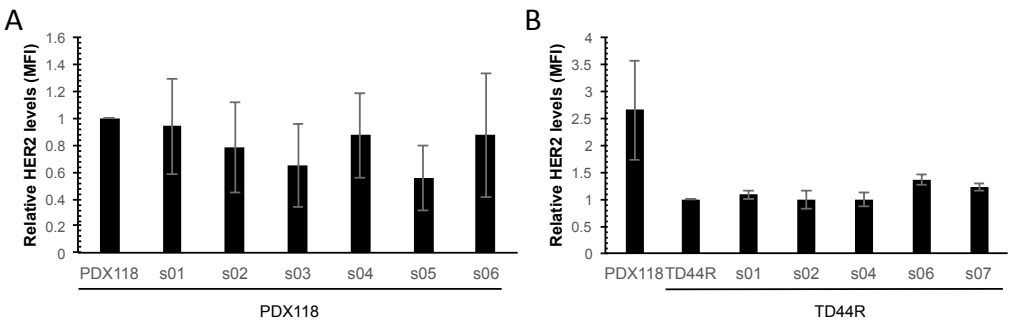


Figure 27: Sensibility to T-DM1 in different cellular sub-cloning. IC_{50} for T-DM1 in PDX118 (A), TD44R (B) and TD55R (C) sub-clones. Standard deviation bars are shown. A t-test between the whole population and the sub-clones were done, asterisk indicates p-value <0.05.

In fact, I could not find any significant difference in the IC_{50} of the sub-clones of PDX118 and the TD44R sub-clones. However, in one sub-clone of TD55R (sub13), the *t-test* showed that the IC_{50} of the parental TD55R (whole population) and TD55R-sub13 was significant (p= 0.015). This indicates that TD55R cells may have a slightly higher heterogeneity than the PDX118 and TD44R. In order to know if their sensibility to T-DM1 is related to their levels of HER2, I checked the HER2 levels of the different sub-clones by Flow Cytometry (**Figure 28**).



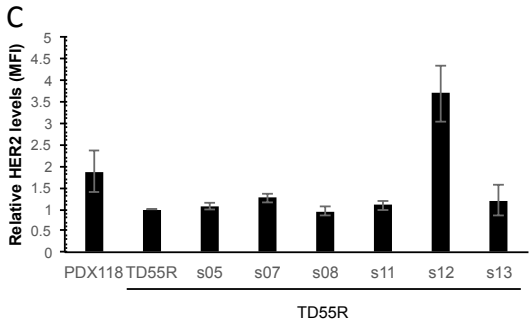


Figure 28: HER2 protein levels by Flow Cytometry of different sub-clones. PDX118 (A), TD44R (B) and TD55R (C) sub-clones were seeded in 10 cm dish and were analyzed by Flow Cytometry 3 days later. Bars represent the quantification of 3 independent experiments in Mean ± SD.

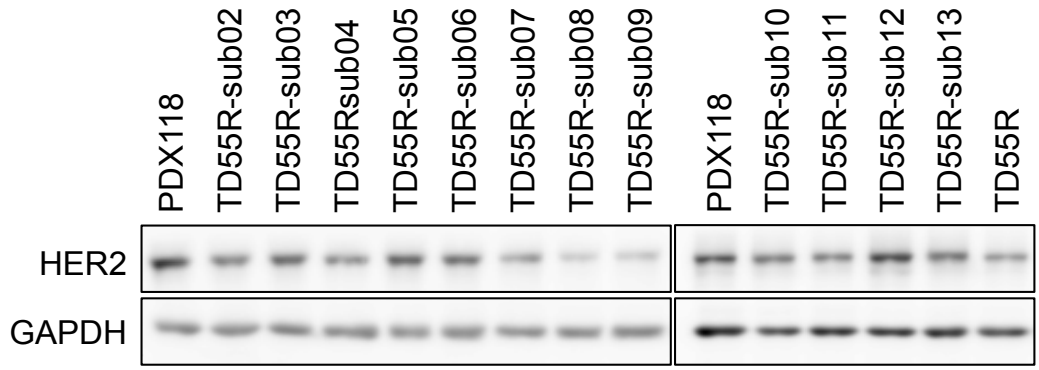


Figure 29: HER2 levels of TD55R sub-clones. TD55R sub-clone cells were seeded in 10 cm dish and 3 days later, cells were lysated and performed WB. GAPDH was used as loading control.

For TD55R sub-clones, I also checked the HER2 level by WB (**Figure 29**).

From these results I concluded that there is neither positive nor negative correlation between HER2 levels and IC_{50} after T-DM1 treatment.

2.4 Exploring the HER2 downstream signaling pathway

In the cell surface, HER2 exists as monomers or dimers. The dimmers can be homodimers or heterodimers. When HER2 binds to another ERBB receptor, there are two ways to activate its downstream pathway: 1) depends on the ligand; 2) independent of the ligand. Then, the activation of the downstream

pathway occurs, and the cells proliferate. Here, in order to find a reason that caused T-DM1 resistance, I performed several experiments:

a) Downstream signal cascade: AKT/PI3K and MAPK/ERK pathways

There are two main signaling pathways downstream of HER2: AKT/PI3K pathway and MAPK/ERK pathway. To know whether an aberrant activation of HER2 signal pathway caused the T-DM1 resistance, I checked these downstream pathways by WB (**Figure 30**). As PTEN loses often occur in the activation of the PI3K pathway, I also checked the level of PTEN in TD44R and TD55R cells.

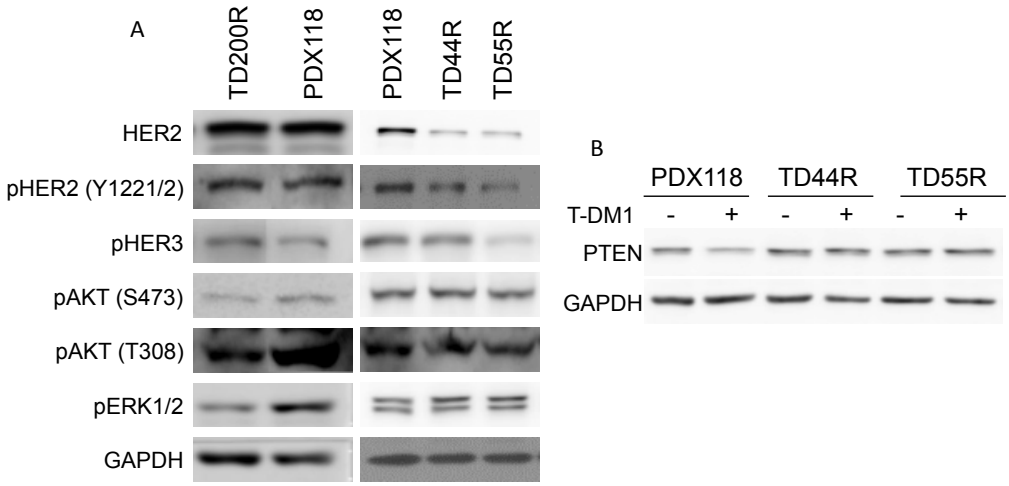


Figure 30: WB of the downstream effectors of HER2 in resistant clones. (A) PDX118, TD44R, TD55R cells were seeded in 10 cm dish, 3 days later the cell lysates were collected and performed WB. (B) WB of PDX118, TD44R and TD55R cells treated with T-DM1 for 3 days and analyzed the levels of PTEN by WB. Data are expressed as means ± SD. N = 2 independent experiments.

TD44R and TD55R cells have lower HER2 levels than parental PDX118, however, only TD55R cells, but not the TD44R, had different levels of phospho-HER2 (both phospho-HER2-1221/2), when compared to the parental cells. I could not find any significant difference between PDX118, TD44R and TD55R in neither AKT nor MAPK signaling pathways (**Figure 30**).

For TD200R cells, although the phospho-ERK1/2 and phospho-AKT (T308) seemed higher than in PDX118, the results were not significant (p=0.34 and 0.12 respectively).

b) Modulator of HER2 dimerization: HSP90

It has been demonstrated that HSP90 can limit the formation of ERBB2-centred receptor complexes [216]. To identify whether the T-DM1 resistance of TD44R and TD55R is related to this phenomenon, I checked the Hsp90 levels of these resistant clones by WB (**Figure 31**).

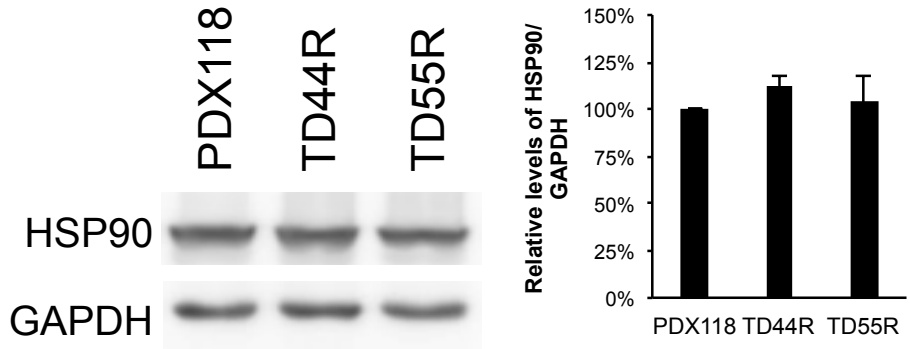


Figure 31: Hsp90 level of PDX parental cells and T-DM1 resistant cells. PDX118, TD44R and TD55R cells were seeded in 10 cm dish, 3 days later the cell lysates were collected and performed Western Blot. Data are expressed as means ± SD. N = 2 independent experiments.

These results showed that there is no significant difference in Hsp90 levels between the PDX118, TD44R and TD55R cells

c) Activators of the HER2 signaling pathway: Neuregulin

Besides HER2, another important ERBB member present in HER2-positive cells is HER3. On the surface of the cell, HER2 binds to HER3 and form heterodimers. If it is a ligand-independent process, T-DM1 could bind to HER2 and block directly cell proliferation. On the other hand, if NRG, which is the ligand of HER3, was required, under high concentrations of this NRG ligand, the downstream dimer HER2-HER3 will be hyperactive. Persistent activation of the anti-apoptotic NRG-HER3-HER2-PI3K signaling axis can block emtansine-

induced apoptosis [217]. This process could help the cells to escape of the T-DM1 therapy.

Here, I checked the mRNA levels of NRG1 and NRG2 in PDX118, TD44R and TD55R cells by the qPCR (**Figure 32**).

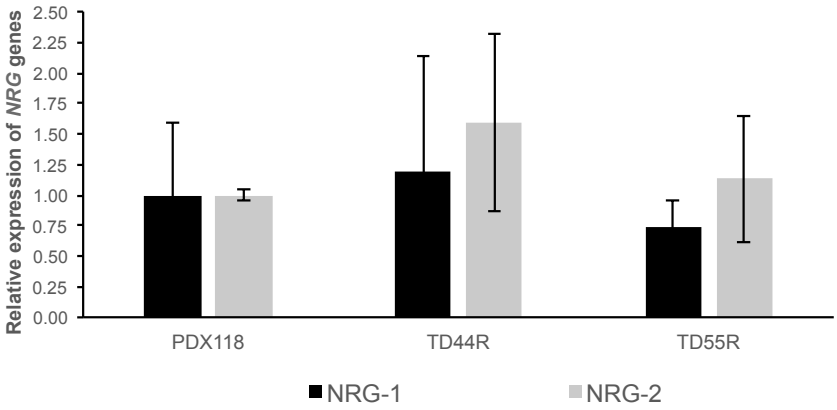


Figure 32. The mRNA level of NRG1 and NRG2 in PDX118 and T-DM1 resistant cells. Bars represent the fold change expression of NRG1 and NRG2 in mRNA level compared to *GAPDH*. The mRNA level was determined by qPCR normalizing the parental cells to 1. Standard deviation bars are shown.

However, the obtained results showed that there was not significant difference in NRG1 neither NRG2 expression in resistant clones compared to PDX118. This indicates that NRG presence is not a mechanism of resistance to T-DM1 in these cells.

2.5 Investigating the role of DM1 (cytotoxic part) in resistant cells

The most common therapy for treating cancer in the clinics is chemotherapy. Some chemotherapeutic drugs can bind to the cytoskeleton and stop proliferation of the cells. Among them, one widely used drug is Paclitaxel, which can bind and stabilize the Tubulin and inhibit the cell proliferation. Besides Paclitaxel, Maytansine is another cytotoxic agent, which can bind to the Tubulin. T-DM1 contains two parts: Trastuzumab (antibody which targets HER2) and DM1 part. DM1 is a thiol-containing Maytansinoid, which is a chemical derivative of Maytansine. It can bind to the microtubules and inhibits cell division. The

different response to this cytotoxic part might cause T-DM1 resistance. For this reason, I decided to check the proliferation of the PDX118 parental cells and the resistant cells under the treatment of the Maytansinol (**Figure 33**).

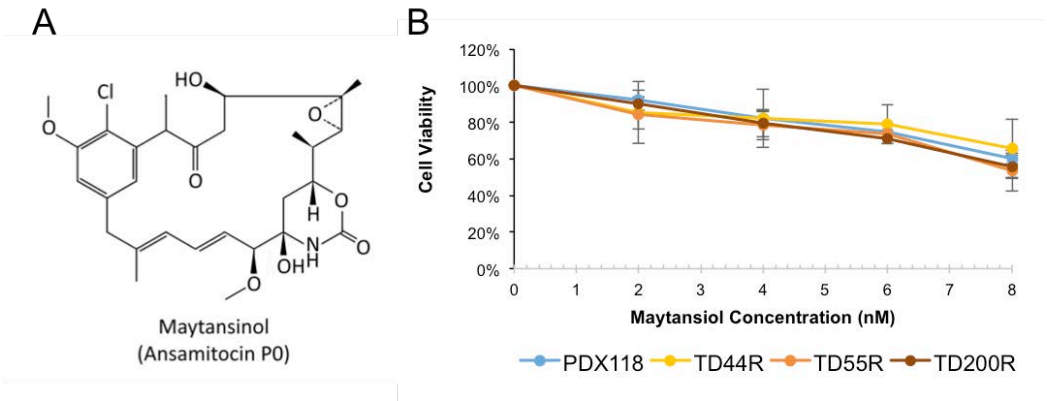


Figure 33. The role of DM1 part for T-DM1 resistant cells. (A) The structure of the Maytansinol. (B) Viabilities of the cells under the treatment of Maytansinol. TD44R, TD55R and TD200R were grown in increasing doses of Maytansinol for 6 days. Cell viability was determined using Crystal Violet violate assay. Data are expressed as means ± SD. (N = 2 independent experiments, each in duplicate).

There was no significant difference in the Maytansinol treatment between the parental and resistant cells. These results indicate that parental and resistant cells have the same response to Maytansinol, suggesting that is not this the mechanism of resistance to T-DM1.

2.6 Searching for distinct degradation rates for T-DM1

After the binding of Trastuzumab (part of **T**-DM1) to HER2 in the cell surface, the HER2/T-DM1 complex is internalized and degraded by the lysosomes. These lysosomes contain hydrolytic enzymes, which can break in many biomolecules T-DM1 drug.

The non-cleavable linker between Trastuzumab and DM1 will be degraded in the lysosomes and the free DM1 part then will bind to the Tubulin in the cell. This binding will inhibit the formation of microtubules and finally cause the death of the targeted cell.

In order to know whether the inhibition of the lysosome activity might affect the T-DM1 resistance, I tested the proliferation of PDX118 cells under T-DM1 treatment in the presence or absence of a lysosome inhibitor (**Figure 34**).

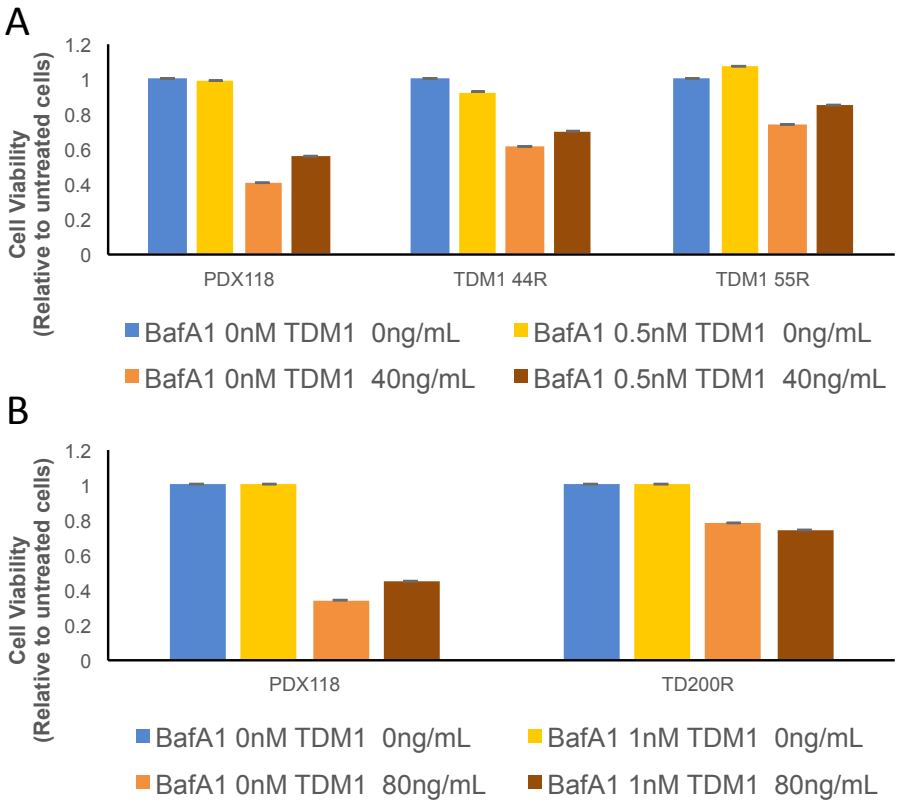


Figure 34: Inhibit the activity of lysosome can change the sensitivity of T-DM1 in resistant cells. (A) PDX118, TD44R and TD445R cells (B) PDX118 and TD200R cells. Cells were seeded in 96 well plates for 6 days. Cell viability was determined by Crystal Violet assay.

BafilomycinA1 (BafA1) is a V-ATPase inhibitor that can block the function of the autophagosome-lysosome complex. In **Figure 34**, we can see that compared to the BafA1 untreated cells, BafA1 treated cells have less sensitivity to T-DM1 in PDX118, TD44R and TD55R cells. However, in TD200R cells, I observed that regardless the presence of this lysosome inhibitor, the sensibility to T-DM1 did not change. A plausible explanation could be that these cells

already have a lysosome miss-function, and this was the causing their T-DM1 resistance.

2.7 Investigating drug combinations to increase T-DM1 sensitivity

As the NRG-HER3-HER2-PI3K signaling cascade is an important pathway for cell proliferation, some researchers have already focused on the relationship between this signaling pathway and T-DM1 combination. In that study, the authors found that the combination of Pertuzumab with T-DM1 can inhibit the formation of HER2 dimers, resulting in enhanced activity of T-DM1 [216]. In order to restore the sensibility to T-DM1 in our resistant clones, I treated TD44R and TD55R cells with the combination T-DM1 and Pertuzumab (**Figure 35**).

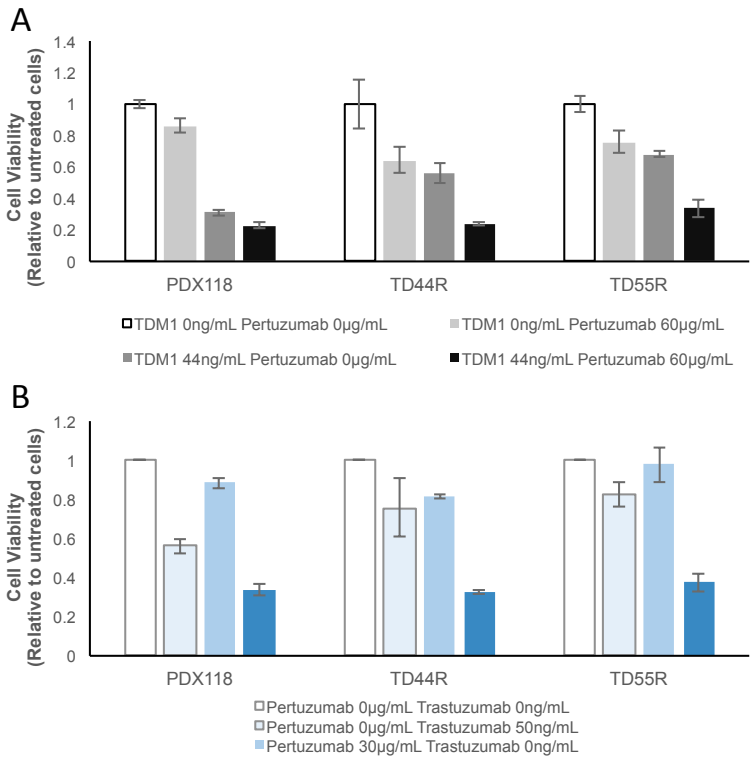


Figure 35: Viabilities of the PDX118 and T-DM1 resistant cells under the combination treatment of the drugs. PDX118, TD44R and TD55R were seeded in 96 well plates (6000 cells/well). The cells were grown in different concentration of the combination of the drugs for 5 days. (A) The combination of the T-DM1 and the

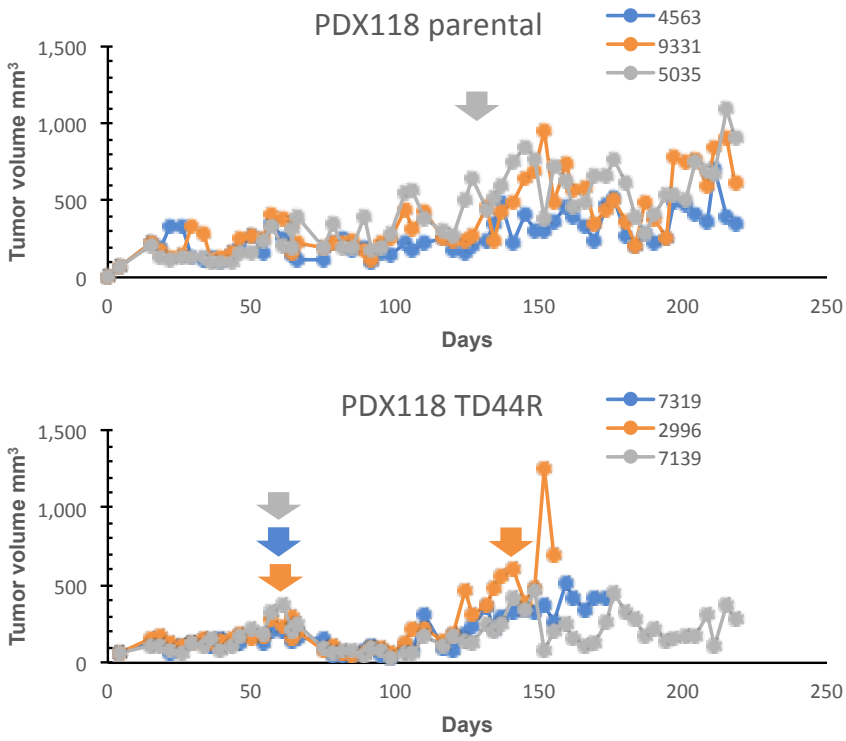
Pertuzumab (B) the combination of the T-DM1 and the Pertuzumab. Cell viability was determined using Crystal Violet assay. Data are expressed as means ± SD.

In T-DM1 resistant cells, Pertuzumab can increase the sensitivity to T-DM1. To know whether the enhancement of T-DM1 is only related the Trastuzumab part of this drug; I checked the cell viability after combining Trastuzumab and Pertuzumab (**Figure 35**). The combination of Trastuzumab and Pertuzumab can significantly reduce the proliferation of the cells, suggesting that, similarly to what it was published, this combination can impair the dimerization of HER2. This means that, despite HER2 levels is lower in the TD44R and TD55R cells than that in the PDX118 cells, the downstream of HER2 is an important pathway for the proliferation of these cells.

3 Establishment of resistance to T-DM1 *in vivo*

In order to know whether the T-DM1 resistant cells selected *in vitro* in this project were also resistant to T-DM1 *in vivo*, I performed orthotopic experiments injecting these cells into immunodificient mice. After the cell injection, I waited until the tumour was palpable (200 mm^3) and then I started the T-DM1 treatment (**Figure 36**). When the tumour size reached a volume of 1000 mm^3, the mouse was euthanized and the tumors analyzed by immunohistochemistry (**Figure 37**).

Only some tumors derived from TD55R cells were resistant to T-DM1 *in vivo* (**Figure 36**). These results showed that definitively not all the *in vitro* resistant cells are able to generate resistant tumors *in vivo*. Several studies has shown that some *in vitro* cell lines resistant to Trastuzumab, for instance, resulted to be sensitive to this drug *in vivo* [218]. This phenomenon could be explained due to the differences in the microenvironment surrounding the tumor *in vitro* and *in vivo*.



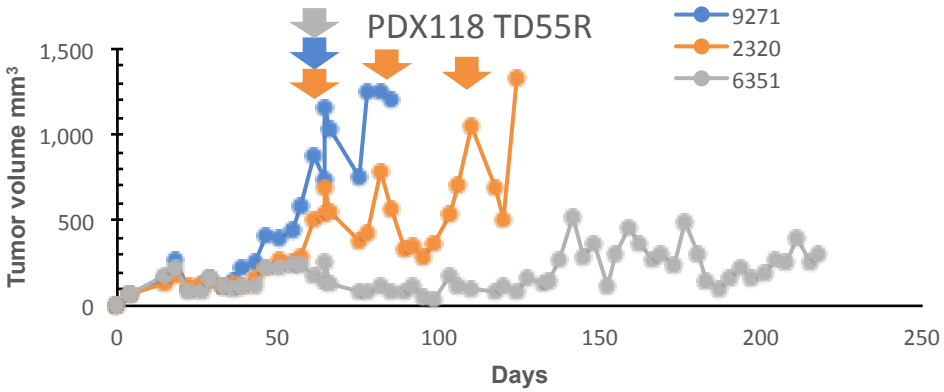


Figure 36: The effect of T-DM1 on tumour growth in vivo for in vitro derived cells. Mean tumour size in each mouse group was measured weekly. The arrows indicated the time of the T-DM1 administration. Each color represents a different mouse.

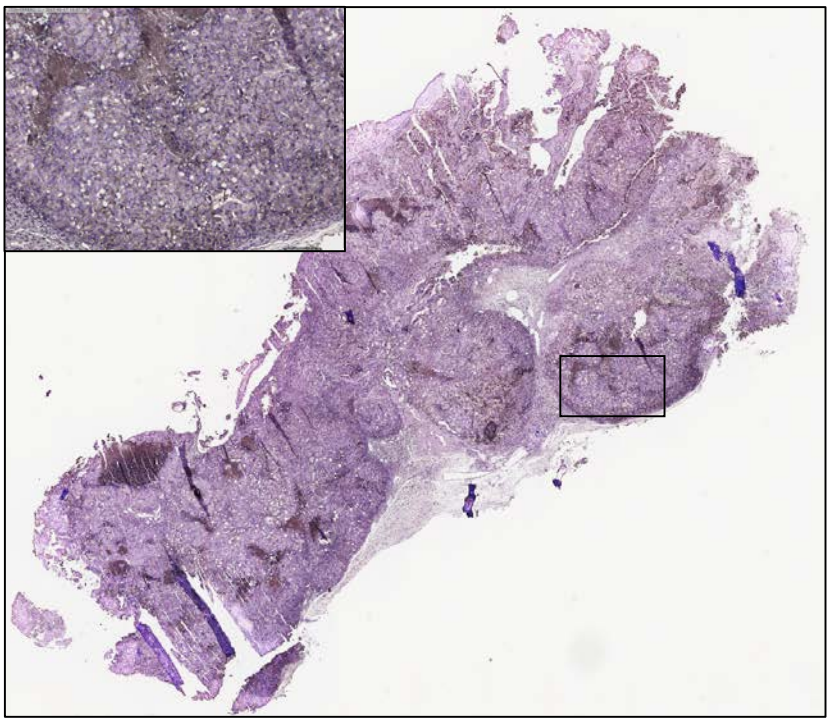


Figure 37: Immunohistochemistry of a T-DM1 resistant tumor generated *in vivo.* Immunostaining anti-HER2 counterstained with Hematoxylin to label nuclei in blue. Magnification in the upper left corner of the figure.

DISCUSSION

1. HER2 levels, Trastuzumab resistance and T-DM1 resistance

Like Trastuzumab, T-DM1 can also recognize HER2 on the surface of the cell and inhibit proliferation. Therefore, there may be some similar mechanisms shared by these two drugs. Here I will discuss the publications related to the Trastuzumab resistance.

In my experiments, I found that in some resistant cells, HER2 levels are involved in the resistance to T-DM1, since TD44R and TD55R presented lower HER2 levels than parental PDX118 cells. There are many reasons that can cause the decrease of the HER2 level: it may be caused by a change at the genomic level, in mRNA levels or post-translational modifications.

In HER2-positive breast cancer cells, the amplification of *HER2* gene is in the form of Double Minutes (DMs) or Homogeneously Staining Regions (HSR) [215, 219]. Theoretically, the copy number of HER2 at genomic level can change the protein level of HER2. However, no publications have ever shown that HER2 copy number decreases after Trastuzumab therapy. From my data, I can conclude that there were no differences in the copy number variation of HER2 between parental and T-DM1 resistant cells. However, it has been reported that after the Trastuzumab treatment, the copy number of HER2 will increase in metastasis cancer cell [220]. These results indicate that the mechanism of resistance of cells to Trastuzumab and T-DM1 is likely to be different.

It is also well established that changes at transcriptional levels of HER2 can also affect their protein levels. Studies have shown that *HER2* at the mRNA level in breast cancer samples positively correlated with HER2 at the protein level [221-223]. However, in my experiments I saw that the mRNA levels of *HER2* in TD44R and TD55R were only slightly lower than in the parental cells, while the TD44R and TD55R have a significant reduced expression of HER2 at protein level when was compared to the parental cells. The reason for this

phenomenon may be caused by either the different protein synthesis rates or the different protein degradation rates.

A study showed that down-modulation of HER2 expression at the protein level in the cell membrane could cause a Trastuzumab resistance [224]. However, another publication showed that the overexpression of HER2 is not enough to explain the Trastuzumab resistance [225]. With my data, I found that in T-DM1 resistant cells (TD44R, TD55R), the HER2 levels were lower than that in parental cells, whereas the TD200R cells had similar HER2 levels as parental cells. These results demonstrated that the reduction of HER2 levels is not the only reason that causes the Trastuzumab or T-DM1 resistance.

2. Reasons that might expained the reduction of HER2 levels

In our study, we have found that the HER2 levels in TD44R and TD55R are lower than in PDX118 cells. This indicates that the reduction of the drug target (HER2) may be the reason causing the T-DM1 resistance. In previous publications, several groups have found different reasons to explain the reduction in HER2 protein levels.

A publication showed that inactivation of phosphatidylcholine-specific phospholipase C (PC-PLC) causes an enhanced HER2 internalization and lysosomal degradation. This procedure induces down-modulation of HER2 levels on the cell membrane [224]. Another reason for the HER2 reduction is the HSP90. A publication showed that inhibit the activity of HSP90 can cause the degradation of HER2 [226]. Moreover, a carboxyl-terminal HER2 fragment named p95HER2, maybe another reason leads to missing the target of the T-DM1. This type of fragment lack of extracellular domain where can bind to Trastuzumab [225].

In summary, the above reasons may cause the loss of T-DM1 target on the cell surface, resulting in a drug resistance.

3. Downstream signaling pathways of HER2

I found that the total and phosphorylated HER2 in drug-resistant cells TD44R and TD55R were relatively lower than that in the parental cells. However, the downstream pathway of HER2, both AKT-PI3K and MAPK pathway retained similar activity in both parental and T-DM1 resistant cells.

I did not find out why the downstream pathway of HER2 was higher than expected. According to the published literature, here I describe possible reasons.

Some publications showed that most Trastuzumab resistance cells maintain their high levels of HER2. A possible mechanism for this is the loss of PTEN or mutation of PIK3CA, which leads the activation of the AKT-PI3K pathway, but not causes the loss of HER2 [227, 228].

In addition to PTEN inactivation and PIK3CA mutations, it has been reported that the crosstalk between HER2 and other RTK family members may also be responsible for the activation of the AKT-PI3K pathway. For example, IGF-IR overexpressed cells can increase the level of IGF-IR/HER2 heterodimer [229, 230]. This heterodimer activates the downstream AKT-PI3K pathway, creating drug resistance.

MAPK is another important signalling pathway. A publication reported that the Growth differentiation factor 15 (GDF15) could stimulate p38 phosphorylation in HER2-positive cells and cause the MAPK pathway activation leading to the Trastuzumab resistance. Besides, the crosstalk between HER2 and other RTK family members, such as IGFIR can also lead to the Trastuzumab resistance, by activating the MAPK pathway.

These publications are all about Trastuzumab resistance, but if I consider that the PI3K/AKT levels observed in our T-DM1 resistant clones are also higher than expected, the reasons above may also explain the T-DM1 resistance [229, 230].

4. T-DM1 degradation and T-DM1 resistance

T-DM1 is an ADC drug. The linker between the Trastuzumab part and the Emtansine part of T-DM1 is not cleavable. After T-DM1 binds to HER2 on the cell surface, the complex of HER2 and T-DM1 will enter the lysosome. Then, T-DM1 will be degraded, releasing free Emtansine. After that, Emtansine can bind to tubulin, thus inhibiting cell division. From my results, I know that the lysosomal inhibitor can reduce the efficacy of T-DM1. I can then speculate that inhibiting the T-DM1 degradation can prevent the free Emtansine release, which may lead to the T-DM1 resistance. This shows that the degradation process of T-DM1 in lysosomes is an important source of T-DM1 resistance.

Several studies have shown that T-DM1 resistant cells are still sensitive to T-ADC, a Trastuzumab-coupled drug that uses a cleavable linker [231]. The ADC drug with this linker can release of cytotoxic part of the drug by a lysosomal independent process. Thus, because T-DM1 uses a non-cleavable linker, once the lysosomal function is abnormal, it may lead to cell resistance to T-DM1.

5. HER3 activation and T-DM1 resistance

As a member of the ERBB family, HER2 can interact with other ERBB members to form a heterodimer. Therefore, when the HER2 levels decrease, overexpression or activation of other ERBB family members may counteract and be responsible for the T-DM1 resistance. Some studies have shown that Neuregulin is associated with the drug resistance for Trastuzumab [232] and Lapatinib [233].

As an important ERBB member, HER3 has been widely studied. Neuregulin is an epidermal growth factor that can be combined with HER3. Although the intracellular part of HER3 does not have kinase activity, Neuregulin can activate the heterodimer, such as HER2/HER3, increase their phosphorylation, and activate downstream signaling pathways. There is

literature showing that Neuregulin levels can also be related to T-DM1 resistance [216]. Moreover, in this bibliography, the authors mentioned that the use of Pertuzumab might revert the T-DM1 resistance caused by Neuregulin. No over-expression of Neuregulin was observed in my experiments. However, I observed that the phosphorylation of HER2 in the drug-resistant group (TD44R and TD55R) was also lower than that in the PDX118 cells. Despite this, when we use Pertuzumab, the effect of T-DM1 significantly increases.

Combining the published literature with my experimental results, I speculate that a possible explanation could be that Pertuzumab can block the non-receptor pathway of the HER2/HER3 heterodimer, thereby enhancing the efficacy of T-DM1.

6. How to overcome the T-DM1 resistance?

This study explored possible causes of *in vivo* T-DM1 resistance. When the HER2 breast cancer cells appear to be resistant to T-DM1, how can we deal with them? Based on the literature and our results, here I discuss about this question.

Reduction of HER2 is one of the reasons for T-DM1 resistance. And the HER2/other ERBB member heterodimer, especially HER2/HER3 heterodimer, shows a sustained activation of signaling pathways downstream of HER2. For this reason, the combination of T-DM1 and Pertuzumab may be used to overcome T-DM1 resistance. Pertuzumab can reduce the formation of heterodimers, weaken the activity of downstream signals, and thus reduce drug resistance.

In addition to ERBB members, HER2 can bind to other RTK members and result in the activation of downstream signals and drug resistance. In this case, the corresponding RTK inhibitors, or inhibitors of downstream signaling pathways, such as PI3K or MAPK cell signaling pathway inhibitors, may also be used to eliminate drug resistance.

Sometimes, the process of T-DM1 degradation is abnormal. For example, the abnormal lysosomal function can slow down the release of the free Emtansine, resulting in drug resistance. In this case, ADC drugs with cleavable linkers can be used to overcome drug resistance.

The causes of T-DM1 resistance are various. As this study continues, more and more drug resistance mechanisms will be revealed, and the accompanying strategies will be established. The survival quality of HER2-positive breast cancer patients will also be improved.

CONCLUSIONS

1. A reduction in HER2 protein levels is the main cause of T-DM1 resistance. Since I found that 2 out of 3 resistant clones acquired spontaneously this type of resistance. However, this is not the only cause.

2. Reduced levels of HER2 are not due to changes in copy number of *HER2* gene.

3. Reduced levels of HER2 are not due to changes of HSP90, a modulator of HER2/HER3 dimerization.

4. The tumor heterogeneity of some resistant clones could partially explain the T-DM1 resistance. In this case, the HER2 levels do not correlate with the sensitivity of T-DM1.

5. The activation of the downstream signalling pathways of HER2, such as AKT/PI3K and MAPK/ERK, are similar between the resistant and parental group.

6. Although Neuregulin is not over-expressed in T-DM1 resistant cells, the use of Pertuzumab can be used to prevent the formation of HER2 heterodimers increasing their sensitivity of T-DM1.

7. There are not significant differences in the sensitivity of the cytotoxic part of T-DM1 between parental and T-DM1 resistant cells.

8. The abnormal Lysosomal function can be a mechanism of resistance to T-DM1.

9. Not all the T-DM1 resistant cells are able to maintain their resistance to T-DM1 *in vivo*.

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ANNEXES