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**DEVELOPMENT AND CHARACTERIZATION OF
MODELS OF RESISTANCE TO T-DM1**

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TABLE OF CONTENTS

LIST OF FIGURES	5
LIST OF TABLES.....	6
LIST OF ABBREVIATIONS	7
I. INTRODUCTION.....	8
I.1. ANTIBODY-DRUG CONJUGATES IN CANCER THERAPY	8
I.1.1. THE DEVELOPMENT OF TARGETED THERAPY.....	8
I.1.2. ANTIBODY-DRUG CONJUGATES AS NOVEL ANTI-CANCER THERAPEUTICS	12
I.1.2.1. Mechanism of action and design of ADCs.....	12
I.1.2.2. Clinical development of ADCs	13
I.2. HER2 TARGETED THERAPIES IN BREAST AND ESOPHAGEAL CANCER	14
I.2.1. HER2-POSITIVE CANCER	14
I.2.1.1. ErbB family of receptors	14
I.2.1.2. ErbB receptors and cancer.....	15
I.2.1.3. HER2 positive breast and esophageal cancers	16
I.2.2. HER2 TARGETED THERAPIES FOR BREAST CANCER.....	16
I.2.2.1. HER2 as target in cancer therapy	16
I.2.2.2. Antibody-based therapies.....	17
I.2.2.3. A novel antibody-drug conjugate: ado-trastuzumab emtansine	18
I.2.2.4. Tyrosine kinase inhibitors (TKI).....	19
I.2.3. HER2 TARGETED THERAPIES IN GEJ CANCER.....	20
I.3. RESISTANCE TO ADCS.....	20
I.3.1. RESISTANCE TO T-DM1	20
I.3.1.1. Mechanisms of resistance to trastuzumab.....	20
I.3.1.2. Proposed mechanisms of resistance to T-DM1.....	21
I.3.1.3. Resistance to T-DM1 in preclinical models and clinical trials	22
I.3.2. MECHANISMS OF RESISTANCE TO ADCS	22

II.	RESULTS	23
II.1.	MECHANISMS OF RESISTANCE TO T-DM1 IN AN ESOPHAGEAL CANCER MODEL	24
II.2.	MECHANISMS OF RESISTANCE TO T-DM1 IN A BREAST CANCER MODEL	60
III.	DISCUSSION	80
III.1.	DEVELOPMENT OF T-DM1 RESISTANCE MODELS	80
III.2.	MICROTUBULES AND RESISTANCE TO T-DM1	80
III.3.	CELL ADHESION AND RESISTANCE TO T-DM1	81
III.4.	THE PROSTAGLANDIN PATHWAY AND RESISTANCE TO T-DM1.....	83
IV.	CONCLUSION AND PERSPECTIVES.....	86
V.	FRENCH SUMMARY.....	93
VI.	REFERENCES	96

LIST OF FIGURES

FIGURE 1 STRUCTURE OF ERBB RECEPTORS _____	15
FIGURE 2 PROPOSED MECHANISM OF ACTION OF T-DM1 _____	19

LIST OF TABLES

TABLE 1 MONOCLONAL ANTIBODIES AND ANTIBODY CONJUGATES FOR CANCER THERAPY _____ 9

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
ADC(s)	Antibody-drug conjugate(s)
ADCP	Antibody-dependent cellular phagocytosis
ALCL	Anaplastic large cell lymphoma
AML	Acute myelogenous leukemia
BC	Breast cancer
BV	Brentuximab vedotin
CAM-DR	Cell adhesion mediated-drug resistance
CDC	Complement-dependent cellular toxicity
CNS	Central nervous system
COX-2	Cyclooxygenase 2
CRC	Colorectal cancer
CsA	Ciclosporin A
DAR	Drug to antibody ratio
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
FcγR	Fcγ receptor
GEJ	Gastroesophageal junction
GO	Gemtuzumab ozogamicin
HAMA	Human anti murine antibodies
HER2	Human epidermal growth factor receptor
HL	Hodgkin lymphoma
IgG	Immunoglobulin G
mAb(s)	Monoclonal antibody(ies)
MAP(s)	Microtubule-associated protein(s)
NK	Natural killer
NRG	Neuregulins
NSAID(s)	Non-steroidal anti-inflammatory drug(s)
OS	Overall survival
PFS	Progression free survival
PG(s)	Prostaglandin(s)
PGE₂	Prostaglandin E ₂
SNP	Single nucleotide polymorphism
TBA	Tubulin binding agent
T-DM1	Ado-trastuzumab emtansine
TKI(s)	Tyrosine kinase inhibitor(s)

I. INTRODUCTION

I.1. ANTIBODY-DRUG CONJUGATES IN CANCER THERAPY

I.1.1. THE DEVELOPMENT OF TARGETED THERAPY

The advances in cell biology have shifted the treatment of cancer from conventional chemotherapeutic agents to targeted therapies. These novel drugs are designed to target and interfere with specific cancer molecules involved in tumor growth and progression. Targeted therapies include monoclonal antibodies (mAbs) and small molecules inhibitors that attack cancer cells while doing less damage to healthy cells. The presence of specific antigens expressed by tumor cells and the power of the immune system to fight cancer was observed over a 100 years ago (1–3). Based on those observations, mAbs targeting specific antigens of tumor cells were developed against hematological malignancies and solid tumors (Table 1).

I.1.1.1. Antibody structure

Therapeutic monoclonal antibodies are generally of the immunoglobulin G (IgG) class. IgG are proteins composed of two identical class γ heavy (H) chains and two identical κ or λ light (L) chains grouped in a Y shape. Both heavy chains are linked to each other and to one light chain by disulfide bonds. Each heavy chain contains an N-terminal variable region (VH), three constant regions (CH1, CH2, CH3) and a flexible “hinge” region between CH1 and CH2. Similarly, the light chains are composed of an N-terminal variable region (VL) and one constant region (CL). The pair of VH and VL regions form the antigen binding site. The upper part of the antibody composed of the light chain with VH and CH1 of the heavy chain form the Fab arm (fragment antigen binding). The rest of the antibody fragment forms the Fc (crystallizable fragment).

Drug name	Active ingredient	Target	Specific treatments	Approval date
Naked antibodies				
Rituxan	Rituximab	CD20	Non-hodgkin's lymphoma B-cell chronic lymphocytic leukemia	1997
Herceptin	Trastuzumab	HER2	Breast cancer (HER2+) Gastric cancer (HER2+)	1998
Campath	Alemtuzumab	CD52	B-cell chronic lymphocytic leukemia	2001
Erbix	Cetuximab	EGFR	Colorectal cancer Non-small cell lung cancer Head and neck cancer	2004
Avastin	Bevacizumab	VEGF	Colorectal cancer Lung cancer Renal cancer Ovarian cancer Cervical cancer Fallopian tube cancer Peritoneal cancer Glioblastoma Non-small cell lung cancer	2004
Vectibix	Panitumumab	EGFR	Colorectal cancer (KRAS wild type)	2006
Arzerra	Ofatumumab	CD20	B-cell chronic lymphocytic leukemia	2009
Yervoy	Ipilimumab	CTLA-4	Melanoma	2011
Perjeta	Pertuzumab	HER2	Breast cancer (HER2+)	2012
Gazyva	Obinutuzumab	CD20	B-cell chronic lymphocytic leukemia Follicular lymphoma	2013
Cyramza	Ramucirumab	VEGFR2	Gastric cancer Gastroesophageal junction adenocarcinoma Non-small cell lung cancer	2014
Sylvant	Siltuximab	IL-6	Multicentric Castelman's disease	2014
Blinicyto	Blinatumomab	CD19/CD3	Philadelphia chromosome-negative relapsed B cell precursor acute lymphoblastic leukemia	2014
Keytruda	Pembrolizumab	PD-1	Melanoma Non-small cell lung cancer	2014
Opdivo	Nivolumab	PD-1	Squamous non-small cell lung cancer Melanoma Renal cancer	2015
Unituxin	Dinutuximab	GD2	Pediatric neuroblastoma	2015
Darzalex	Daratumumab	CD38	Myeloma	2015
Portrazza	Necitumumab	EGFR	Squamous non-small cell lung cancer	2015
Empliciti	Elotuzumab	SLAMF-7	Myeloma	2015
Radio-immunotherapy				
Zevalin	Ibritumomab tiuxetan	CD20	Non-Hodgkin's lymphoma	2002
Bexxar*	Tositumomab-I-131	CD20	Non-Hodgkin's Lymphoma	2003
Antibody-drug conjugates				
Mylotarg*	Gemtuzumab ozogamicin	CD33	Acute Myeloid Leukemia	2000
Adcetris	Brentuximab Vedotin	CD30	Hodgkin lymphoma Anaplastic large cell lymphoma	2011
Kadcyla	Ado-trastuzumab emtansine	Her2	Breast cancer (HER2+)	2013

Table 1 Monoclonal antibodies and antibody conjugates for cancer therapy. The approval date corresponds to the approval by the FDA in the USA. (*: withdrawn from most markets)

The first clinical trials using mAbs back in 1980s showed limited efficacy and high toxicity, mainly due to the immunogenicity of the antibodies used which were 100% murine (5). The patient's immune system produced antibodies against these murine mAbs ("Human Anti Murine Antibodies" or HAMAs), which limited their benefit by neutralizing the therapeutic antibodies while causing severe adverse reactions. The advances in cellular and molecular biology have allowed the production of chimeric and humanized antibodies that are less immunogenic and more active. In spite of these advances, many mAbs display insufficient cytotoxicity per se (7) and their selectivity is now being exploited in combination with cytotoxic molecules or radioactive isotopes. The aims are to overcome the toxicity of chemotherapy and the lack of efficacy of mAbs, respectively, by targeting the tumor using antibody-drug conjugates (ADCs) (8).

I.1.1.2. Monoclonal antibody-based therapy

Antibodies can be directed against soluble factors such as cytokines to impair their ability to bind receptors and trigger signaling. But with many mAbs, the therapeutic effect relies on binding tumor-specific antigens (9). The direct action of the antibody is to block signaling, resulting in apoptosis and/or inhibition of growth. Indirectly, mAbs can also activate the immune system through the Fc fraction, resulting in antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) or antibody-dependent cellular phagocytosis (ADCP) (Fig.3, (10)). Other families of mAbs can exert antitumor effects by targeting the tumor vasculature or acting on the immune microenvironment (11,12). Anti VEGF-R mAbs (such as bevacizumab, Table 1) and related molecules have been approved in a number of solid tumor indications (13–15), most often in combination with conventional agents. A more recent and promising family of mAbs target immune checkpoint inhibitors expressed by tumor cells or immune suppressor cells in the tumor microenvironment. MAbs directed against CTLA4 and the PD1/PDL1 complex have recently been approved and a growing number of other targets are currently being explored (16–19). MAbs can also be used as targeting agents when linked to radioactive isotopes or cytotoxic agents. Finally, mAbs can be used in combination with conventional chemotherapy.

The first antibody approved for cancer therapy (rituximab) is directed against CD20 which is overexpressed in 95% of B-cell lymphomas. Although CD20 is also expressed in normal B-cells, this antigen does not circulate and is not internalized or modulated upon antibody binding (11). Since the approval of rituximab in 1997, more than 20 mAbs have been approved for the treatment of different hematological and solid cancers (Table 1).

I.1.1.3. Mechanisms of action of mAbs

Many molecules targeted by mAbs are growth factor receptors that promote proliferation and/or survival. MAbs can inhibit ligand binding and changes in conformation required for dimerization, like cetuximab (20), or block dimerization sites, like pertuzumab (21). Some mAbs like rituximab efficiently induce apoptosis of the targeted cancer cells (22). By inhibiting signaling through growth factor receptors, antibodies can diminish growth rates, induce apoptosis, and in some cases receptor internalization (10).

In addition, the Fc fraction of the antibody can interact with Fcγ receptors (FcγR) on effector cells such as natural killer (NK) cells and macrophages. The subsequent activation of effector cells results in lysis of the targeted tumor cell through ADCC. Lysis can also be achieved through the CDC mechanism that begins with the binding of the C1 complex to the antibody-antigen complex and is followed by activation of complement proteins, the formation of a membrane attack complex, and lysis, opsonization and immune complex clearance (10).

Besides ADCC and CDC, other FcγR-mediated responses are important to induce tumor-directed T cell immunity. The antigen-presenting cells such as dendritic cells, macrophages and B cells use FcγR-mediated endocytosis or phagocytosis of antibody-coated tumor cells. It results in antigen processing and presentation for the activation of the adaptive immune system against the cancer cell (23,24).

The FcγR are hematopoietic cell glycoproteins that bind to the Fc fraction of IgG. They serve as link between humoral and cell mediated immune response and between innate and adaptive immunity (25). There are three classes of FcγR (FcγRI/CD64, FcγRII/CD32 and FcγRIII/CD16) and each is divided into different subclasses (a,b or c). Each class has its own structure, cell distribution and IgG subtype affinity. In addition, functional single nucleotide polymorphism (SNP) has been found on FcγRIIa and FcγRIIIa. A C>T substitution on the *FCGR2A* gene changes the amino acid at position 131 from a histidine to an arginine (FcγRIIa-H131R) and a T>G substitution on the *FCGR3A* gene modifies the amino acid at position 158 from a valine to a phenylalanine (FcγRIIIa-V158F) (23,26). FcγR polymorphisms have been associated with response to mAbs. The FcγRIIIa-V158F polymorphisms have been associated with good response to cetuximab, rituximab and trastuzumab (9).

The most successful antibodies against solid tumors target the ErbB family of growth factor receptors and VEGF. For example, targeting HER2 with trastuzumab has been proven to be very efficient in breast and gastric cancer. Since the approval of trastuzumab in 1998 for breast cancer (BC), other HER2 targeting agents such as the mAb pertuzumab and the ADC T-DM1 have been developed.

I.1.2. ANTIBODY-DRUG CONJUGATES AS NOVEL ANTI-CANCER THERAPEUTICS

I.1.2.1. Mechanism of action and design of ADCs

The lack of specificity of conventional chemotherapeutics makes them highly toxic to healthy tissue and causes severe side effects. The advantages of linking potent cytotoxic molecules to mAbs are the tumor specificity, the potency and reduced non-target toxicity. However, many challenges complicate the design of ADCs (27), that ideally should retain the selectivity of the mAb while being able to release the cytotoxic molecule in quantities sufficient to induce cancer cell death. ADCs are administered intravenously to prevent the degradation of the mAb by gastric acids and enzymes. When circulating in the bloodstream, the stability of the linker is crucial to prevent the release of the conjugated agent.

An important choice when designing ADCs is the chemical nature of the linker connecting the mAb and the toxic compound. There are two types of linkers, cleavable or non-cleavable that have a specific mechanism of release and stability in the bloodstream (28). Non-cleavable linkers depend on endosomal and lysosomal degradation to generate metabolites with or without a portion of the linker (29). Hence, non-cleavable linkers confer the advantage of minimizing the early release of the drug in the plasma. Cleavable linkers depend on the physiological environment in cellular compartments (pH, proteases, glutathione concentrations...) for proteolysis or hydrolysis (30). Therefore, these types of linkers may have some lower stability when circulating in the plasma compared to non-cleavable linkers. The choice of the linker is very important given the high toxicity of the conjugated molecules due to the limited number of ADCs internalized in a given tumor cell (31). In accordance to the chemical nature of linker, the catabolites produced from the ADC will differ. Generally, they are metabolized by cytochrome P450 enzymes and are subject to drug-drug interactions from inhibitors or inducers of P450 (32).

In order to bind the targeted molecule, linking the toxic compound to the mAb must not disrupt its specificity. The conjugation methods have greatly evolved over the past few years and it is currently possible to obtain targeted linking of conjugates on the mAb molecule. This allows a better controlled degree of substitution and the assurance that binding and Fc-related effector functions will not be altered. The "Drug to Antibody Ratio" (or DAR) is typically in the order of three conjugates per molecule but may vary according to the ADC, as the DAR value may impact on the pharmacokinetic of the ADC (33). An ADC traffics through the body in a manner similar to the mAb alone if its DAR is not very high (34).

The antigen should be selected appropriately since it is the most important contributor to the ADCs activity and tolerability (35). Ideally, the antigen should be overexpressed in the cancer cells in comparison to normal cells, undergo minimal shedding so that the target is not bound in circulation and be internalized by receptor-mediated endocytosis (36). Antigens from the tumor vasculature and stroma could also be targeted by ADCs.

The ADC-antigen complex is thought to be internalized via receptor-mediated endocytosis involving clathrin-coated endosomes (37). Endosomes recycling and a low number of surface antigens can be limiting factors by preventing the sufficient accumulation of the cytotoxic compound necessary to induce cell death. The endosome-lysosome degradation and other cellular compartments are crucial to release the active drug in the cytoplasm from non-cleavable and cleavable linkers (38). The compounds linked to mAbs should be very potent drugs given that approximately 1% of the administered dose reaches the intracellular target (39). Thus, the selected drugs are often too toxic to be employed in a non-targeted manner and are able to induce cell death at lower concentrations than conventional chemotherapeutics. The molecules of current use induce cell death by various mechanisms, and are mainly based on two families of conjugates: tubulin binding agents like auristatins and maytansinoids or DNA binding agents such as duocarmycin and calicheamicins.

I.1.2.2. Clinical development of ADCs

Three ADCs have been approved by the FDA to treat hematological malignancies or solid cancers. Gemtuzumab ozogamicin (GO, Mylotarg, Pfizer) was the first ADC approved by the FDA in 2000 for patients over the age of 60 with acute myelogenous leukemia. GO is composed of an anti-CD33, a surface antigen present in 85-90% of AML cases, linked to a calicheamicin derivative. It was withdrawn in 2010 from most major markets after a post-approval phase III trial showed an increased risk of fatal toxicities without any benefits compared to conventional chemotherapy (40). The toxicity of GO was probably due to a lack of selectivity of the antibody and/or stability of the linker, with a significant incidence of severe liver disease (41).

Brentuximab vedotin (BV, Adcentris, Seattle Genetics) was approved in 2012 and is used against CD30-positive hematological malignancies. BV is composed of the chimeric monoclonal antibody brentuximab directed against CD30 linked to monomethyl auristatin E (MMAE). Of interest, brentuximab itself displayed no significant antitumor activity. BV has been approved for patients with relapsed or refractory CD30+ Hodgkin's lymphoma (HL) after autologous stem cell transplant or transplant-ineligible patients who have received at least two other chemotherapeutic regimens, and as second line for patients with anaplastic cell lymphoma.

Ado-trastuzumab emtansine (T-DM1, Kadcyła, Roche, Genentech) was approved in 2013 and is currently the only approved ADC for the treatment of non-hematological malignancies. It combines the monoclonal antibody trastuzumab to the anti-tubulin agent DM1, a derivative of maytansine. T-DM1 was approved as second line therapy for the treatment of HER2+ patients who have previously received trastuzumab and a taxane.

I.2. HER2 TARGETED THERAPIES IN BREAST AND ESOPHAGEAL CANCER

I.2.1. HER2-POSITIVE CANCER

I.2.1.1. ErbB family of receptors

The ErbB superfamily of receptor tyrosine kinase is composed of four members: epidermal growth factor (EGF) receptor (ErbB1/HER1), ErbB2/Neu/HER2, ErbB3/HER3 and ErbB4/HER4. They activate a wide variety of responses involved in key cell functions such as cell growth and survival. ErbB receptors are expressed in tissues derived from epithelial, mesenchymal and neural origin. They play a key role during development, as null mutations in any member of the family are lethal (42,43). ErbB receptors are also important for cell proliferation and differentiation in postnatal and adult organs, such as the mammary gland (42). All members are composed of an extracellular ligand-binding domain, a transmembrane domain and an intracellular domain containing tyrosine kinase activity (Fig. 1). ErbB receptors are activated by the EGF family of ligands, named EGF-related peptides. These growth factors derive from ligand precursors that mature after shedding from the cell membrane by metalloproteases(44,45). Each EGF-related peptide is specific for a type of ErbB receptor: EGF, amphiregulin and transforming growth factor- α bind to ErbB1, while betacellulin, heparin-binding EGF and epiregulin bind ErbB1 and ErbB4. The neuregulins (NRG) NRG-1 and NRG-2 bind ErbB3 and ErbB4, whereas NRG-3 and NRG-4 bind ErbB4. EGF-related peptides bind the extracellular region and induce a conformational change from a closed to an open configuration that exposes the dimerization domain. Interestingly, HER2 is the only receptor to be constantly in an open conformation. The receptor homo/hetero-dimerization followed is by the activation of kinase activity via autophosphorylation of precise tyrosine residues (46). The phosphorylated residues serve as docking sites for adaptor proteins or enzymes involved in signaling cascades. The sites that are phosphorylated are dictated by the ligand and the homo/hetero-dimer. Then, distinct phosphorylated residues engage specific downstream signaling proteins (47). No ligand has been discovered to bind HER2, and HER3 lacks tyrosine kinase activity; however, HER2/HER3 dimers are the most potent (48). HER2 has been proposed to function as a co-receptor since it seems to be the preferred dimerization partner and to play a potentiating role (42,49). The heterogeneity

of ligands and the flexibility of dimer pairs generate different cellular responses. The activation of different signaling pathways by ErbB receptors (such as PI3K/Akt, Ras/Raf/MEK/ERK1/2 and PLC γ) results in proliferation, division, adhesion, migration and/or survival.

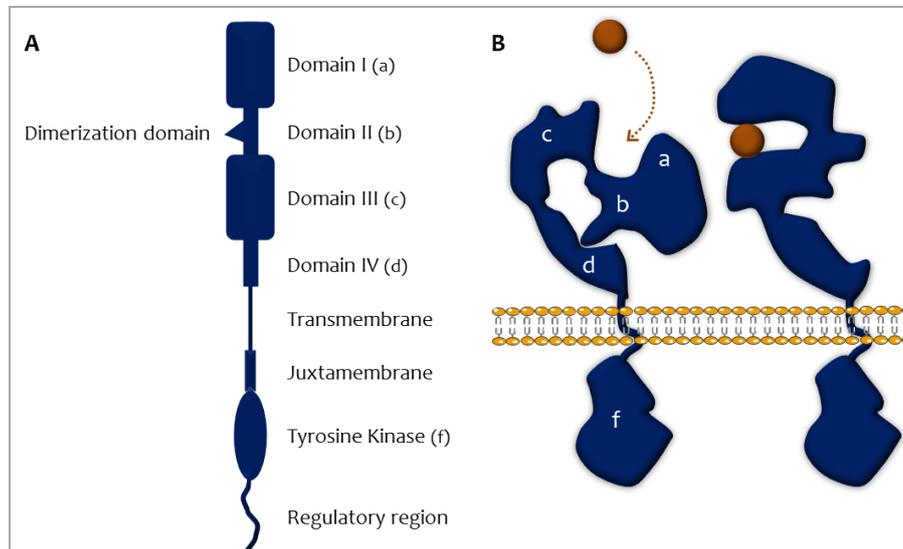


Figure 1 Structure of ERBB receptors. **A.** Schematic representation of the different domains. The extracellular domain is composed of ligand-binding domains (I and III) and cysteine-rich domains (II and IV). The dimerization domain is located in domain II. Following the trans-membrane domain, the intracellular part is composed of a juxta-membrane domain, a tyrosine kinase domain and a C-terminal tail (regulatory domain) containing the main tyrosine residues that are phosphorylated after receptor activation. **B.** Schematic representation of the structural conformation of EGFR. The extracellular domain has two conformations, the closed configuration (inactive) and the open configuration (active). Upon binding with the ligand, the receptor changes its conformation to reveal the dimerization arm. Ligands for HER2 have not been described and the extracellular part exists in an open conformation. Adapted from the Textbook of receptor pharmacology, Third edition (51).

I.2.1.2. ErbB receptors and cancer

Mutations or increased expression of ErbB members have been found in several types of cancer, including lung, breast, ovary, uterus, esophagus, stomach, colorectal, bladder, head and neck, skin, pancreas and brain (52–55). The overexpression of one receptor can bias dimer formation. When HER2 is overexpressed, its intrinsic open conformation can generate homodimers spontaneously. Hence, the amplification of HER2 in cancer causes increased HER-2 containing homo/hetero-dimers. EGFR/HER1 overexpression is frequent in brain tumors, where it is associated with reduced survival (56). The most common mutation of EGFR deletes a part of its extracellular domain, conferring a constitutively active receptor (57). Its mutation has also been found in lung, ovary and breast cancers (58). HER2 overexpression has been mostly studied in breast cancer where it was found to be associated to poor outcome and high chances of recurrence

(59) Most studies have focused on EGFR and HER2 and as a result, multiple targeted therapies have been developed against these receptors. Since HER3 lacks kinase activity it depends on the other ErbB receptors to activate signaling pathways. However, the overexpression of EGFR or HER2 is often accompanied by an increased expression of HER3 (60,61). HER3 overexpression has been associated with poor prognosis, while HER4 could have a favorable effect in the outcome of patients (62). The role of HER4 in cancer is difficult to determine since there are different isoforms with pro or anti-tumoral activities (63). Thus, HER3 and HER4 could serve as prognostic and/or predictive markers (60).

I.2.1.3. HER2 positive breast and esophageal cancers

The amplification of *HER2* gene is observed in different cancer types and often leads to HER2 protein overexpression. In some rare cases, HER2 protein was found to be overexpressed without gene amplification (64). Slamon et al. proposed that in these few cases, the protein overexpression could be due to transcriptional or post-transcriptional deregulations. The amplification/overexpression of HER2 has been observed in a multitude of cancer types, such as breast, bladder, pancreatic, NSCLC, ovarian, gastric, kidney and prostate (65,66). HER2 amplification/overexpression is associated with a shorter disease-free and overall survival with increased chances of recurrence compared to patients whose tumors display normal HER2 expression (67–69).

Female BC is the second most common cancer worldwide after lung cancer (70). Amplification and/or overexpression of HER2 are observed in about 20% cases (71). Gastroesophageal junction (GEJ) cancer is the eight cause of death worldwide with a 5-year survival rate of less than 20% (70,72) and HER2 is overexpressed in approximately 33% of cases.

I.2.2. HER2 TARGETED THERAPIES FOR BREAST CANCER

I.2.2.1. HER2 as target in cancer therapy

HER2 is an oncogenic driver and seems to be sufficient for cell transformation (73). Also, HER2 is of prognostic and predictive value in different types of cancer and its extracellular location renders the receptor an important therapeutic target. Moreover, HER2 overexpression is found in the primary tumor and at metastatic sites and the levels of HER2 are higher in tumors than in healthy tissue. Its critical role in oncogenesis and the large number of cases of overexpression have made HER2 a target for anticancer drugs.

Besides the advances in screening, testing and surgery, the development of HER2-targeted therapy has drastically changed the care and outcome of patients. A large number of HER2-targeted agents have been developed such as mAbs, small tyrosine kinase inhibitors (TKIs) and ADCs (Fig.8 (74)).

I.2.2.2. Antibody-based therapies

I.2.2.2.A. Trastuzumab

Trastuzumab (Herceptin) is a humanized mAb that binds the extracellular domain II of HER2. It represents the first breakthrough in HER2-targeted therapies and was approved by the FDA a decade ago. Trastuzumab has become the standard of care, and the outcome of patients with HER2-positive BC has significantly improved (75). The benefit of trastuzumab has been shown in different clinical trials in combination with standard chemotherapy in early and metastatic BC (71–79).

After binding to HER2, trastuzumab blocks downstream signaling pathways leading to G1 phase arrest and apoptosis (85,86). It also inhibits ligand-independent heterodimerization of HER2/HER3 (87). Whether trastuzumab induces HER2 down-regulation and degradation is currently a subject of debate (88). Some studies demonstrate that trastuzumab increases HER2 endocytosis and degradation rates, inducing its down-regulation (89–92). Other studies did not observe down-regulation of HER2 but instead a rapid recycling of trastuzumab with the co-receptor after endocytosis (93). Clinical studies have also argued against HER2 down-regulation in patients receiving trastuzumab (94,95).

Finally, trastuzumab activates the immune system through its Fc fraction, and is a potent mediator of ADCC (96–98). Clinical data supports that trastuzumab is able to mediate ADCC against HER2-overexpressing cells in patients (94,99).

I.2.2.2.B. Pertuzumab

Pertuzumab (Perjeta) is a humanized mAb targeting the extracellular domain IV of HER2 and thereby inhibiting ligand-dependent dimerization of the co-receptor, a process which cannot be inhibited by trastuzumab. Pertuzumab is very effective in blocking HER2/HER3 signaling pathways and is able to inhibit growth of several types of cancer *in vitro* and *in vivo* (100). The extracellular epitope recognized by pertuzumab is different from the one recognized by trastuzumab (101,102) and both antibodies can bind HER2 at the same time *in vivo* (103).

In the preclinical setting, the combination of pertuzumab and trastuzumab was superior to each mAb alone (103). Clinical studies confirmed these observations since dual inhibition of HER2 showed a survival benefit when pertuzumab was added to trastuzumab and docetaxel (104). Combination of both mAbs was active even in patients who progressed after prior trastuzumab therapy (105). Pertuzumab in combination with trastuzumab has become a standard of care in first-line settings.

I.2.2.3. A novel antibody-drug conjugate: ado-trastuzumab emtansine

T-DM1 was approved for HER2-positive metastatic BC as a second-line therapy. It is composed of the mAb trastuzumab conjugated to the maytansinoid DM1 via an SMCC non-cleavable thioether linker. The proposed mechanism of action is that after binding HER2, T-DM1 triggers receptor-mediated endocytosis of the HER2/T-DM1 complex (Fig. 2). The release of the active DM1-containing metabolite depends on lysosomal degradation. Lysine-N ϵ -SMCC-DM1 is the only metabolite present in quantifiable amounts after T-DM1 internalization (38). Lysine-N ϵ -SMCC-DM1 was shown to be metabolically stable since its chemical properties prevented the interaction with some major hepatic P450 isozymes. These studies showed the absence of depletion of Lysine-N ϵ -SMCC-DM1 by P450 enzymes, of inhibition of P450 and oxidative metabolism in the plasma (32). *In vivo* radiolabeling studies and clinical studies have determined the metabolic fate of T-DM1. Most T-DM1 remains conjugated in the systemic circulation and very low levels of catabolites are found in the plasma (106). In rats, the catabolites appear to be eliminated through the fecal/biliary route with minor elimination in urine (107).

T-DM1 conserves the mechanisms of action of trastuzumab (108), combined to the antimitotic activity of DM1. Even though DM1 is a more potent cytotoxic molecule than trastuzumab, the anti-tumor activity of the mAb should not be neglected. For example, trastuzumab is still beneficial to some patients who have progressed on trastuzumab-containing therapy (109). Maytansinoids bind to microtubules at the same site as *vinca* alkaloids and are 100-fold more cytotoxic than these agents (110,111). As most microtubule disrupting agents, DM1 can cause arrest in G2/M phase, mitotic catastrophe, disruption of the intracellular trafficking network and cell death in a concentration-dependent manner (112–114).

T-DM1 was shown to be efficient in mice bearing trastuzumab-resistant tumors (115,116) and lapatinib-resistant tumors (117). The efficacy and safety of T-DM1 was investigated in the phase III EMILIA trial. The cohort of 991 patients with locally advanced or metastatic BC that were previously treated with trastuzumab and a taxane was randomly assigned to receive T-DM1 or a combination of lapatinib and capecitabine (118). The increase in the progression free survival (PFS) and overall survival (OS) lead

to the approval of T-DM1 by the FDA in 2013. The benefit of T-DM1 in first-line over the standard care therapy trastuzumab plus docetaxel was also proven in a phase II trial (119). T-DM1 was superior to the physician's choice in the third-line setting for pretreated HER2-positive advanced BC in phase III TH3RESA trial (120). However, the phase III trial MARIANNE resulted in non-inferior, but not superior efficacy of T-DM1 or T-DM1 plus pertuzumab to the old standard of care in patients with locally advanced or metastatic HER2-positive BC (121). Ongoing phase III clinical trials KAITLIN and KATHERINE will determine the benefit of T-DM1 in early stage HER2-positive BC (122).

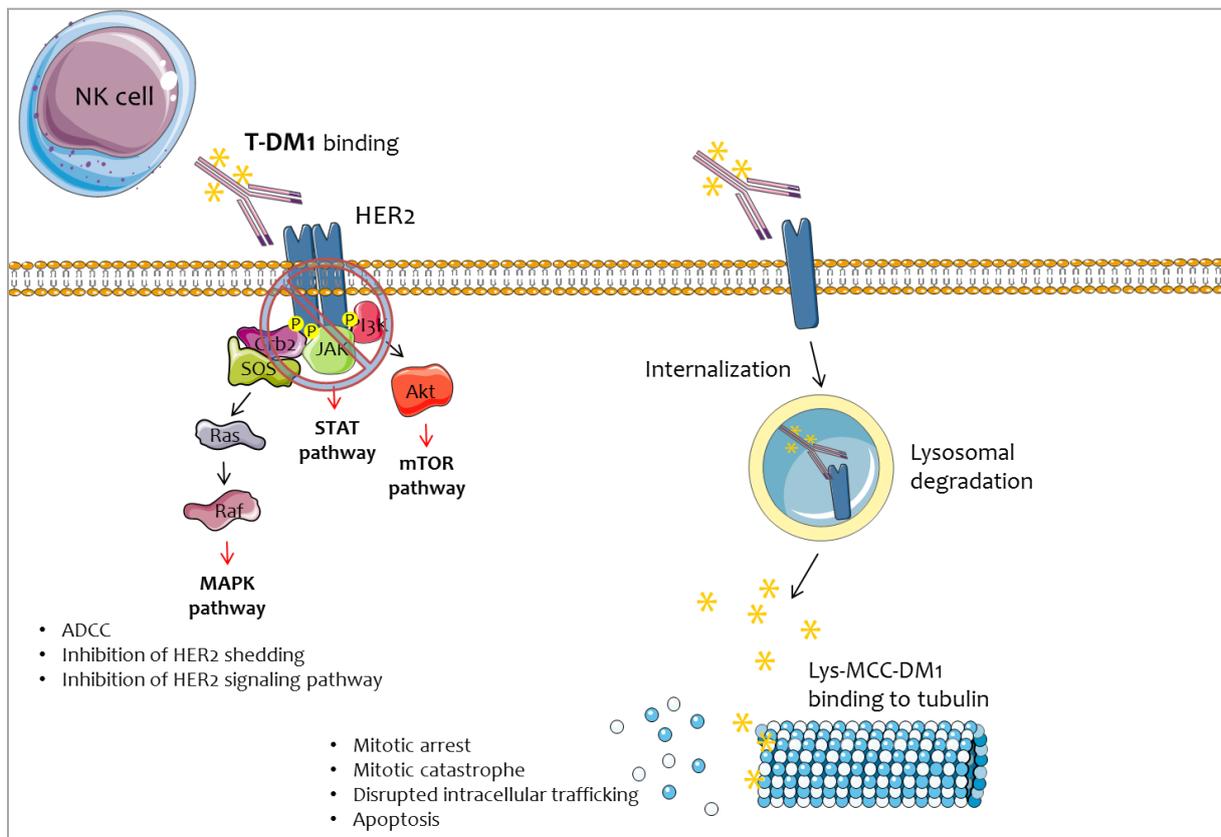


Figure 2 Proposed mechanism of action of T-DM1. T-DM1 maintains a trastuzumab-like activity by binding to HER2. T-DM1 can inhibit HER2 ectodomain shedding, inhibition of HER2-activated signaling pathways and ADCC. Following internalization and lysosomal degradation of HER2/T-DM1 complex, the active metabolite Lys-MCC-DM1 is released in the cytoplasm. The DM1-based metabolite binds to microtubules and induces mitotic arrest, mitotic catastrophe, disruption of intracellular trafficking and apoptosis. (This figure was made using servier medical art)

I.2.2.4. Tyrosine kinase inhibitors (TKI)

Many cellular processes are regulated by tyrosine kinase-mediated phosphorylation and the possibility to modulate their activation is of clinical interest. TKIs are small molecules

that compete with ATP to bind the intracellular catalytic kinase domain, preventing phosphorylation and activation of signaling pathways.

Since TKIs bind to the intracellular domain, they are useful when mAbs cannot bind the extracellular target. For example, trastuzumab or pertuzumab are not beneficial in tumors presenting high expression of cleaved HER2 (p95^{HER2}) or expressing epitope masking molecules (such as MUC-4). Also, mAbs are unable to cross the blood-brain barrier while TKIs have small molecular weight and have been reported to be able to cross into the central nervous system (CNS) (123). Thus, TKIs could be useful in cases of CNS metastasis.

Lapatinib, a dual EGFR/HER2 reversible inhibitor, is the only TKI approved by the FDA for use in combination with chemotherapy in HER2-positive advanced BC that has progressed after previous treatment (124). Besides inhibiting kinase activation, lapatinib induces accumulation of HER2/HER2 and HER2/HER1 dimers at the cell surface, potentiating ADCC of cancer cells (125). Before the approval of T-DM1, lapatinib plus capecitabine was the recommended second-line therapy for metastatic BC. Combining lapatinib and trastuzumab was shown to be beneficial for patients with metastatic BC who progressed on previous trastuzumab-containing regimens (126).

I.2.3. HER2 TARGETED THERAPIES IN GEJ CANCER

Trastuzumab was approved for patients with HER2 overexpressing metastatic cancer or GEJ adenocarcinomas who have not received prior treatment for metastatic disease in 2010. Since, trastuzumab has been incorporated into standard practice in gastric cancer. Ongoing trials are testing the benefit of pertuzumab and lapatinib on gastric cancer. As for T-DM1, preclinical *in vitro* and *in vivo* studies have shown the efficacy of T-DM1 in HER2-overexpressing gastric cancer (116). The open label trial GATSBY was designed to evaluate efficacy and safety of T-DM1 compared to a taxane in patients with HER+ advanced or metastatic gastric or GEJ cancer who progressed during or after first-line treatment with or without HER2-targeted therapy. The trial ended in 2015 without showing superior efficacy of T-DM1 over taxane treatment (127,128).

I.3. RESISTANCE TO ADCs

I.3.1. RESISTANCE TO T-DM1

I.3.1.1. Mechanisms of resistance to trastuzumab

Trastuzumab activity depends on a well-functioning host immune system to activate ADCC against the tumor (129). Trastuzumab-mediated HER2 signaling inhibition may induce tumor cell apoptosis. However, this effect can be overcome by constitutive activation of target proteins or by activation of parallel pathways leading to cell survival. The PI3K/AKT/mTOR signaling pathway triggered by HER2 and inhibited by trastuzumab can be constitutively activated by PIK3CA mutations or PTEN loss. The inability to bind the epitope, due to increased HER2 shedding resulting in high p95^{HER2} expression or masking by overexpression of molecules such as MUC-4, causes resistance to trastuzumab (130,131). The inhibition of HER2 signaling can be by-passed by overexpression of tyrosine kinase receptors such as the insulin-like growth factor-1 receptor (IGF-1R), c-Met or ErbB family members (132).

I.3.1.2. Proposed mechanisms of resistance to T-DM1

The data obtained from T-DM1 clinical trials has not yet led to an understanding of resistance mechanisms. Resistance to T-DM1 could be due to the same factors mediating trastuzumab resistance and/or to the impairment of Lysine-N ϵ -SMCC-DM1 release and cytotoxic activity. In the latter case, mechanisms are expected to be similar to those previously described for tubulin binding agents (133).

Apart from the presence of HER2 at the cell surface, the activity of T-DM1 depends greatly on the rate of its internalization, its lysosomal degradation and the subsequent release of the active metabolite (134). The resulting intracellular concentrations of lysine-N ϵ -SMCC-DM1 need to exceed a threshold to induce cell death (135). Impaired internalization, lysosomal degradation, increased recycling or high expression of efflux pumps could lead to low intracellular concentrations of the active metabolite, resulting in poor T-DM1 efficacy.

The overexpression of membrane ABC transporters, responsible for the efflux of cytotoxic agents outside of the cell, is a commonly described mechanism of resistance to a variety of anti-cancer therapeutics. Maytansinoids are effluxed mainly by MDR1 and overexpression of this ABC transporter has been reported in *in vitro* models resistant to maytansinoids (136,137). Thus, MDR1 or other members of the ABC transporter family could be implicated in resistance to T-DM1.

Impaired activity of T-DM1 could also be due to mutations of tubulin or differential expression of tubulin isoforms. Additionally, modified expression of microtubule associated proteins (MAPs) or modified post-translational modifications of tubulin could result in altered microtubule dynamics and inefficacy of T-DM1 (138–141).

I.3.1.3. Resistance to T-DM1 in preclinical models and clinical trials

Acquired resistance to T-DM1 was observed in mice bearing N-87 gastric cancer tumors. Although the tumors had regressed, half of the mice showed residual tumor cells with high HER2 expression and a very small fraction of proliferating cells (116).

Clinical trials included patients that had received HER2-targeted therapies prior to T-DM1, including those who had progressed after trastuzumab. Primary resistance to T-DM1 was uncommon, but most patients that initially responded ceased to respond despite continued treatment (142).

I.3.2. MECHANISMS OF RESISTANCE TO ADCS

ADCs are promising agents which have been used relatively recently in the clinic, but clinical trials have reported emerging resistance. The identification of mechanisms of resistance still requires in-depth studies.

Only two ADCs are currently used in the clinics, brentuximab vedotin (BV) and T-DM1, against hematological and breast malignancies, respectively. Resistant cell lines to each ADC have been developed so as to study the mechanisms of resistance. Chen et al. selected two *in vitro* resistance models using a HL and an anaplastic large cell lymphoma (ALCL) cell lines by pulsatile and constant exposure to BV respectively (143). Loganzo et al. generated *in vitro* resistance models to a trastuzumab-maytansinoid ADC using the two HER2-positive breast cancer cell lines MDA-MB-361 and JIMT-1 (144).

Both studies concluded that the overexpression of ABC transporters could mediate resistance to ADCs. MDR1 was overexpressed in HL cell line resistant to BV, and MRP1 was overexpressed in MDA-MB-361 cell line resistant to a trastuzumab-maytansinoid. Down-regulation of the extracellular target was also found in ALCL and JIMT-1 resistance models. Immunohistochemistry studies of tumors resistant to BV did not indicate a modification of CD30 but one patient showed overexpression of MDR1 (143). The down-regulation of targeted molecules following targeted therapies has not been described in clinical reports to the best of our knowledge.

Interestingly, the MDA-MB-361 cell line overexpressing MRP1 was resistant to trastuzumab-maytansinoid, but remained sensitive to DM1. Although it has been demonstrated that maytansine binds to MDR1 instead of MRP1 or BCRP (136), the authors hypothesize that the active metabolite released by the ADC may be effluxed by MRP1.

Resistant models to the trastuzumab-maytansinoid ADC showed an upregulation of proteins involved in the regulation of the cell cytoskeleton and the endosomal/lysosomal pathway. More thorough studies need to be performed to confirm the implication of these pathways in resistance to T-DM1.

Resistance to T-DM1 has also been reported to be associated with the expression of the HER3 ligand neuregulin β 3, promoting the formation of HER2/HER3 dimers and activation of the PI3K pathway (145). In this model, the combination of T-DM1 and pertuzumab enhanced antitumor activity *in vitro* and *in vivo*.

II. RESULTS

II.1. MECHANISMS OF RESISTANCE TO T-DM1 IN AN ESOPHAGEAL CANCER MODEL

The human epidermal growth factor receptor 2 (HER2) is a transmembrane tyrosine kinase receptor. HER2 belongs to the epidermal growth factor receptor family that plays critical roles during development and cancer. The amplification and overexpression of HER2 can occur in many types of cancer including breast, ovarian, pancreatic, gastric and non-small cell lung cancer (52,65,66). HER2 is implicated in disease initiation and progression, and serves as an oncogenic driver. Consequently, HER2 is an ideal therapeutic target.

T-DM1 is a novel antibody-drug conjugate for the treatment of HER2-positive breast cancer. It is composed of the mAb trastuzumab linked to DM1 which is a derivative of maytansine, a potent tubulin binding agent (146). Despite the efficacy proven by T-DM1 during pre-clinical and clinical studies, acquired resistance to treatment remains a major obstacle in complete remission of patients.

Resistance to anti-cancer therapy is often mediated by increased expression and/or activity of ABC transporters. They are responsible for the efflux a different cell poisons and therefore for rendering cancer cells multidrug resistant (147). Moreover, the masking or shedding of the epitope recognized by trastuzumab, the hyper activation of HER2 downstream pathway by PI3KCA and PTEN modulation or bypass of HER2 blockade by HER3 or IGF1R activation lead to resistance to trastuzumab, and could mediate resistance to T-DM1 (130–132). Furthermore, alterations in the expression of tubulin isoforms or microtubule-associated factors or tubulin mutations could drive resistance to the maytansinoid component of T-DM1 (140).

The mAb trastuzumab has become a standard of care in HER2-positive breast and gastric cancers. The phase II/III trial GATSBY failed to show superior efficacy of T-DM1 versus standard taxane treatment in patients with HER2-positive advanced gastric cancer. However, other trials are in progress to evaluate the efficacy and safety of T-DM1 for the treatment of gastric cancers. Therefore, we chose an esophageal cancer cell line to develop models of resistance to T-DM1.

The characterization of our esophageal cancer cell lines resistant to T-DM1 showed an increased expression of genes involved in adhesion and the prostaglandin pathway. We found modulations in the role of focal adhesions and production of prostaglandin E₂. Targeting these pathways induced cell death more efficiently in resistant cells compared to parental cells. Our results propose alternative pathways than can be targeted in resistant cells to T-DM1.

Esophageal cancer cells resistant to T-DM1 display alterations in cell adhesion and the prostaglandin pathway

Submitted for publication

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Abstract

Trastuzumab-emtansine (T-DM1) is an antibody-drug conjugate that specifically targets HER2 thanks to its antibody component trastuzumab. In spite of responses to this novel agent, acquired resistance to treatment remains a major obstacle. Prolonged *in vitro* exposure of the gastroesophageal junction cancer cell line OE-19 to T-DM1, in the absence or presence of ciclosporin A resulted in the selection of two resistant cell lines to T-DM1. T-DM1-resistant cells presented an increased expression of adhesion genes, altered spreading and higher sensitivity to anoikis than parental cells. A resistant cell line showed decreased adhesion strength, increased migration speed and increased sensitivity to RhoA inhibition. Genes involved in the prostaglandin pathway were deregulated in resistant models. Addition of prostaglandin E₂ to T-DM1 partially restored its cytotoxic activity in resistant models. This work demonstrates that T-DM1-resistance may be associated with alterations of cell adhesion and the prostaglandin pathway, which might constitute novel therapeutic targets.

Statement of significance

A better understanding of resistance mechanisms to T-DM1 is necessary to improve treatment regimens for HER2-overexpressing cancer patients. Understanding the pathway alterations related to resistance, such as cell adhesion and prostaglandin synthesis, will contribute to propose new therapeutic strategies in combination with T-DM1 or in patients resistant to T-DM1.

Introduction

The human epidermal growth factor receptor 2 (HER2) belongs to the ErbB/HER receptor tyrosine kinase family that is necessary during normal development and plays a role in the oncogenesis of different cancers. HER2 is overexpressed in approximately 20% of breast cancers and is associated with poor outcome and high risk of recurrence (1,2). Gastric and esophageal cancer have a 5-year survival rate of less than 20% (3,4). HER2 is overexpressed in approximately 20% of gastric cancer and 33% of gastroesophageal junction (GEJ) cancers (5). Trastuzumab was approved in 2010 for the treatment of patients with HER2-overexpressing metastatic gastric or GEJ adenocarcinomas who have not received prior treatment for metastatic disease.

Trastuzumab-emtansine (T-DM1) is an antibody-drug conjugate (ADC) that targets HER2 thanks to its antibody component trastuzumab, linked to DM1 via a thioether non-cleavable linker. T-DM1 conserves the mechanisms of action of trastuzumab (6), combined to the antimitotic activity of DM1. DM1 is a derivative of maytansine, which is a

potent antimitotic agent that binds to tubulin at the same site as *Vinca* alkaloids (7). Once the ADC binds to HER2, internalization and processing are necessary for the release of the active metabolites. The lysine-*N*-SMCC-DM1 is the only metabolite present in quantifiable amounts after lysosomal degradation of T-DM1 (8). In patients with HER2-positive metastatic breast cancer patients T-DM1 was approved as a second line therapy in 2013. T-DM1 has also demonstrated efficacy against HER2 overexpressing uterine, bladder, lung and gastric cancers, both *in vitro* and *in vivo* (9–12).

The efficacy of anti-cancer agents is often limited by acquired resistance to treatment. The increased expression and activity of the ABC transporters is responsible for decreasing the intracellular concentration of cytotoxic agents by enhancing drug efflux (13). Resistance to maytansinoids and antibody-maytansinoid conjugates has been reported to be mediated by MDR1 (14,15). Resistance to tubulin binding agents can be due to alterations in tubulin isoforms or mutations and alterations in microtubule-associated factors (16). In patients receiving trastuzumab, resistance can be associated with HER2 shedding leading to a cleaved active form of HER2 (17). Moreover, the epitope recognized by trastuzumab can be masked by molecules such as MUC4 (18). Additionally, HER2 inhibition can be overcome by an intrinsic activation of HER2 downstream pathways, for example by PI3KCA mutation or loss of PTEN activity, or a bypass of HER2 blockage by activation of HER1/3 or IGF1R (19).

Resistance mechanisms to ADC have not yet been extensively studied as they are relatively novel agents, although resistance to T-DM1 has been observed in pre-clinical and clinical reports (20,12,21). *In vitro*, resistance to ADCs may involve alterations of the surface or intracellular targets or to an abnormal endosomal/lysosomal pathway activity, leading to low intracellular concentrations of the cytotoxic agent. Decreases in the expression of the surface targets CD30 and HER2 were observed in lymphoma cell lines resistant to brentuximab vedotin (BV) (22) and breast cancer cell lines resistant to an analogue of T-DM1, respectively (23).

To investigate resistance mechanisms, we selected T-DM1 *in vitro* resistant models using a GEJ cancer cell line continuously exposed to incrementally increased concentrations. The characterization of the resistant cell lines revealed increased various alterations including modified expression of genes involved in adhesion and the prostaglandin pathways.

Results

Selection of in vitro T-DM1 resistant models

OE-19 resistant cells to T-DM1 were selected by continuous exposure to the antibody-drug conjugate (ADC) in the absence or presence of the MDR1 modulator ciclosporin A (CsA). CsA was added simultaneously with T-DM1 at a non-toxic dose of 1 µg/ml. The initial concentration of T-DM1 was 20% of the IC50 for the OE-19 cell line and was gradually increased when stable cell survival was obtained. The final T-DM1 concentration reached was 0.3 nM, which corresponds to 6 times the IC50 of the parental cell line in a 6 days cytotoxicity assay. We obtained two *in vitro* OE-19 resistant models to T-DM1: OE-19 TR in the absence of CsA and OE-19 TCR in the presence of CsA.

Sensitivity phenotype of resistant cell lines

We compared the sensitivity to T-DM1 of the selected resistant cells to that of sensitive parental cells (S cells) using MTT cytotoxicity, xCELLigence and apoptosis assays. The IC50 of T-DM1 determined by the MTT assay was approximately 16-fold higher in TR cells (0.73 nM) and 21-fold higher in TCR cells (0.98 nM) than in S cells (Fig. 1A, Fig. 1D). Real time monitoring by xCELLigence indicated that TR and TCR cells were capable of surviving under prolonged exposure to 0.1 nM T-DM1 contrary to S cells (Fig. 1B). Furthermore, apoptosis was quantified by annexin V staining after a 72h exposure to T-DM1 and we found that TR and TCR cell lines were less sensitive to T-DM1-induced apoptosis compared to S cells (Fig. 1C). We verified that the changes observed were due to cell death and not to reduce proliferation by CFSE staining (Fig. S1).

The sensitivity to HER2 targeted-therapy and standard chemotherapy of resistant cells was assessed by the MTT cytotoxicity assay and xCELLigence (Fig. 1D). Cross-resistance to trastuzumab was observed in both T-DM1 resistant models, with an IC50 approximately 5-fold higher in TR and 10-fold higher in TCR compared to OE-19 S cells. Both resistant models remained sensitive to DNA and tubulin targeting agents. These results suggest that T-DM1 resistant cells did not develop pleiotropic resistance mechanisms influencing cell death pathways and that the prolonged exposure to T-DM1 did not affect the sensitivity to other tubulin targeting or HER2 targeting agents.

T-DM1 resistance is independent of drug efflux

The overexpression of ABC transporters is a well described mechanism conferring multidrug resistance. To examine whether resistance to T-DM1 was due to an increase in the expression and activity of efflux proteins, we studied two main ABC transporters Multidrug Resistance protein 1 (ABCB1, MDR1) and Breast Cancer Resistance Protein (ABCG2, BCRP). Using flow cytometry to detect the expression at the cell membrane, we

found that MDR1 but not BCRP was expressed in parental and resistant cell lines (Fig. 2A). Interestingly, we observed two distinct populations of cells which were MDR1^{low} and MDR1^{high} in OE-19 S. The amount of MDR1^{high} cells in TR and TCR cells was somewhat superior to that in S cells, suggesting that this population was slightly increased during selection of resistance models. To evaluate the activity of ABC transporters in parental and resistant cells, we performed a rhodamine 123 (rho 123) efflux assay (Fig. 2B). Even though a subpopulation expressing MDR1^{high} was selected in TR and TCR cells, the efflux activity was not significantly increased in these resistant models. While these results do not suggest an increased drug-efflux activity in the resistant variants, we observed decreased accumulation of Lys-MCC-DM1 in the TCR cells line compared to the parental cell line (Fig. S3).

Chronic exposure to T-DM1 does not affect HER2 expression or ability to bind antibody

Since the antitumor activity of T-DM1 depends on its ability to bind to HER2, we studied the expression and accessibility of this target. The expression at the mRNA (Fig. 3A) and protein (Fig. 3B) levels, studied by immunoblotting and RT-qPCR respectively, was unchanged between parental and resistant cells. HER2 expression at the cell surface was studied by flow cytometry. No significant difference of HER2 surface levels was found between parental and resistant cell lines (Fig. 3C), suggesting that resistance to T-DM1 was not due to decreased expression of HER2. However, the presence of HER2 at the cell surface does not infer that T-DM1 is able to bind to its target. We therefore studied T-DM1 binding by flow cytometry using an anti-kappa antibody and found that T-DM1 binds similarly to parental and resistant cells (Fig. 3D). These results suggest that resistance to T-DM1 did not arise from downregulation or masking of HER2.

T-DM1-induced cell cycle arrest is reduced in resistant models

We evaluated the effect of T-DM1 on cell cycle distribution by flow cytometry after propidium iodide staining. Parental cells were arrested in G2/M phase after 24h exposure to T-DM1, S-methyl DM1 and vincristine (Fig. S2). Cell cycle arrest was decreased in resistant cells compared to parental cells after exposure to increasing concentrations of T-DM1 for 24h (Fig. 4A). Interestingly, resistant cells were sensitive to G2/M arrest induced by both S-methyl DM1 and vincristine, but the G2/M fraction in TCR was slightly inferior to that of the parental and TR cell lines (Fig.4B). The absence of cell cycle arrest in the presence of T-DM1 may be due to a decreased concentration of the active metabolite or a difference in the microtubule dynamics of resistant cells compared to parental cells.

Expression of β II and β III isoforms, tubulin pools and post-translational modifications of tubulin are altered in T-DM1 resistant models

Tubulin is the major intracellular target of T-DM1. Although prolonged exposure to T-DM1 did not affect total α and β tubulin protein content, isoforms β II and β III were overexpressed in both resistant models compared to parental cells (Fig. 5A). Downregulation of β III tubulin by siRNA did not impact sensitivity to T-DM1 or T-DM1 induced cell cycle arrest in TR cells (Fig. S4). Hence, although β III tubulin is increased in resistant models, its downregulation does not seem to be sufficient to restore T-DM1-cytotoxicity.

To study tubulin pools, fractionation of polymerized tubulin ("microtubule") and non-polymerized tubulin ("free tubulin") was performed (Fig. 5B). We found that α and β tubulins were increased in the microtubule fraction in TR cells but remained unchanged in TCR cells, compared to S cells. Around 80% of tubulin was polymerized in TR cells versus 65% in parental and TCR cell lines. This was associated with a decrease in the free tubulin fraction, confirming that a larger proportion of tubulin was present under polymerized form in the TR resistant cells. Isoforms β II and β III were predominantly present in microtubules in both parental and resistant cell lines. As post-translational modifications (PTMs) of tubulin may affect microtubule dynamics, we studied the acetylation and tyrosination status of α - tubulin by Western Blot. We found a decreased amount of acetylated tubulin in TCR cells and detyrosinated tubulin in both resistant models (Fig.5C). Hence, the modifications of PTMs in resistant cells are likely to be related to altered microtubule dynamics in these cell lines.

Deregulation of adhesion genes is associated with alterations in cell morphology and migration, and shape and strength of focal adhesions.

To gain insight into the resistance mechanisms in TR and TCR cell lines, we performed a pangenomic transcriptomic analysis of OE-19 S, TR and TCR. Bioinformatic analysis OE-19 TR and TCR versus OE-19 S allowed the identification of numerous genes involved in adherens junctions, ECM-receptor interaction, cell adhesion molecules and focal adhesion (Table S1A). To validate these results, we studied the differential expression in resistant cells by RT-qPCR of tyrosine kinase receptors (*EGFR* and *MET*), actin-interacting molecules (*ACTN1* and *VCL*), and regulators of actin cytoskeleton (*ROCK1*, *RAC2* and *DIAPH1*). We found that *EGFR*, *MET*, *ROCK1*, *DIAPH1*, *ACTN1* and *VCL* were upregulated while *RAC2* is downregulated in resistant cells (Table S1B). We examined cell morphology by immunofluorescent staining of α - tubulin and found that parental cells are spread in a round shape while both resistant cell lines spread in a polygonal shape (Fig. 6A, S7A). In order to confirm that the differences in morphology were due to a difference in adhesion, we verified that cell size remained unchanged by mean diameter of suspension cells (Fig.

S5). Next, we assessed the migration capacity by wound-healing assay and we found that although parental cells were capable of migrating, TR and TCR cells closed the wound faster (Fig. 6B). We verified that the changes observed in the wound healing experiment were not due to an increased proliferation of resistant cell lines by CFSE staining (Fig. S1). We studied the shape of focal adhesions by immunostaining of talin (Fig. 6C, S7B) and found that the size of focal adhesions seems to be reduced in resistant cell lines, mostly in TCR cells, compared to the parental cell line. Also, it appeared that the amount of focal adhesions was increased in TR and TCR cells. Finally, we measured the adhesion strength using a centrifugal-force based adhesion assay (Fig. 6D). Following overnight adhesion, the detached fraction was increased in TCR cells and unchanged in TR cells compared to parental. Although spreading, migration speed and focal adhesions were modified in both resistant cell lines, only TCR cells showed decreased adhesion strength.

To evaluate the implications of focal adhesion changes in resistant cells, we assessed their viability under suspension conditions. Cell death was studied after plating cells in low-adherent condition plates for 24h and 48h. We found a decreased number of living cells in both resistant cell lines compared to parental cells (Fig. 6E). This result suggests that resistant cells to T-DM1 are more dependent upon adhesion for survival. Then, in order to determine the relationship between adhesion and resistance we studied the sensitivity of cells to different inhibitors of focal adhesion points. We inhibited RhoA, a major regulator of the actin cytoskeleton, using rhosin and two of its targets, ROCK1 by fasudil and FAK by bortezomib. We found that TCR showed slightly increased sensitivity to rhosin compared to parental cells (Fig. 6F). However, the sensitivity to ROCK1 or FAK inhibitors was unchanged in resistant cells compared to parental cells.

Prostaglandin E₂ increases sensitivity to T-DM1 of OE-19 resistant cell models

The transcriptomic analysis revealed that the prostaglandin pathway was deregulated in cells resistant to T-DM1. The overexpression of *COX2*, *EP2*, *LEF1* and *PGT* was confirmed by RTqPCR (Fig. 7A). Prostaglandins (PGs) are synthesized from arachidonic acid by cyclooxygenase enzymes (COX1 and COX2), and we found that *COX2* gene expression was increased by 22-fold change in TR (P value=0.0005) cells and 3.3-fold change in TCR cells (P value=0.0134). *COX2* expression is inducible and the promoter region contains a TCF/LEF response element. Since *LEF1* is overexpressed in resistant models, it could be involved in the increased expression of *COX2*. The *PGT* or *SLC02A1* gene coding for an SLC transporter that mediates the energy-dependent export of prostaglandins (24), was transcriptionally increased 15-fold in TR (P value=0.0002) and 6.5-fold in TCR cells (P value<0.0001). The *EP2* gene coding for prostaglandin E₂

receptor, was transcriptionally increased by 25-fold change in TR cells (P value=0.0002) and 20-fold change in TCR cells (P value<0.0001). Since *COX2* was overexpressed in cells resistant to T-DM1, we studied their sensitivity to aspirin, a selective inhibitor of COX1 and COX2 that blocks the synthesis of all PGs. We found a small yet significant decrease in the IC50 values of resistant cell lines compared to parental (Fig. 7B). In view of these results, we quantified the extracellular PGE₂ for each cell line and found that the amount of PGE₂ was increased in the TR cell supernatant compared to S cells (Fig. 7C). Interestingly, the increased amount of PGE₂ in TR cells is associated with high *COX2* and *PGT* expression compared to TCR and S cells. Since PGs are involved in cell adhesion, spreading and migration, we exposed parental and resistant cell lines to PGE₂ to study their migration by wound healing assay (Fig. 7D) and found that the addition of PGE₂ delayed migration only of the parental cell line. In order to assess whether PGE₂ was involved in the resistance to T-DM1, we studied the sensitivity to T-DM1 in parental and resistant cells in the presence of PGE₂ by Annexin/PI staining (Fig. 7E) and xCELLigence assays (Fig. 7F). We found that the presence of pharmacological concentrations of PGE₂ increased the sensitivity to T-DM1 in resistant cells but not in parental cells. Altogether these results indicate that the prostaglandin pathway may be an alternative target in T-DM1 resistant cells, in particular the inhibition of the cyclooxygenases.

Discussion

The efficacy of cancer therapies is very often limited by acquired resistance. Although the exact mechanisms of resistance to T-DM1 have not been described, T-DM1 has often been found to lose benefit despite continued treatment in some patients (25,26). To better understand possible mechanisms of resistance to T-DM1, we performed *in vitro* selection of OE-19 cell line resistant to T-DM1 by prolonged exposure at low concentrations over several months in the absence or presence of CsA. CsA was added during selection of resistant cells to prevent MDR1-mediated resistance. However neither TR nor TCR cell lines showed increased MDR1 expression or efflux activity, independently of the presence of CsA.

The downregulation of the ADC target has been found to occur in *in vitro* resistant models (22,27). Also, HER2 shedding leads to the expression of a cleaved transmembrane p95HER2 protein and a circulating extracellular domain and is responsible for the inefficacy of trastuzumab and possibly T-DM1 in patients (17,18). In our resistant models, HER2 expression remained unchanged as well as its ability to bind T-DM1. Our results show that resistance to T-DM1 in TR and TCR cells did not arise from downregulation or masking of HER2 at the cell surface. Additionally resistant cells

retained sensitivity to lapatinib, a small molecule inhibitor of Her2, suggesting that the Her2 pathway remained functionally active.

Resistance to T-DM1 could be due to the inability of DM1 to bind to microtubules or reduced inhibition of microtubule dynamics by DM1. Total α and β tubulin content remained unchanged in resistance models, but the percentage of polymerized tubulin was increased in TR cells. As maytansine binds exclusively to soluble tubulin dimers it is possible that the reduction of the free tubulin pool in TR cells contributes to their resistance to T-DM1. Moreover, we found decreased acetylation of α -tubulin in both resistant cell lines, in particular in TCR cells. Tyrosination was slightly increased and detyrosination was decreased in TCR cells compared to parental cells. Acetylation is associated with stable, long lived microtubules while newly assembled microtubules are highly tyrosinated (28,29). Hence, even though tubulin pools remained unchanged in TCR cells compared to S cells, PTMs indicate that microtubules in these cells seem to be less stable than in parental and TR cells. The contents of tubulin isoforms β II and β III were increased in both resistant cell types. β III has been reported to possess specific characteristics in terms of microtubule dynamics and has also been reported to be associated with drug resistance both in vitro and in vivo (30–35). However, the downregulation of β III tubulin by siRNA in the TR cell line did not reverse the resistance to T-DM1 nor restored T-DM1-induced cell cycle arrest. This suggested that overexpression was not sufficient for resistance to T-DM1. Microtubules are constantly undergoing cycles of polymerization and depolymerization, called “dynamic instability” which are crucial to many of their functions, in particular chromosomal segregation during anaphase. Specific tubulin isoforms and tubulin PTMs have been reported to be associated with tubulin dynamics and possibly drug binding (36–38). Overall, resistant cells displayed a number of microtubule-associated alterations but whether these play a role in the resistance phenotype or are consequences of exposure to T-DM1 remains to be determined.

Transcriptomic analysis of TR and TCR cells showed a deregulation of genes coding for adhesion molecules such as integrins and several regulators of the actin cytoskeleton (Table S1A, Fig. S6). Also, we found that T-DM1 resistant cells have a different shape and increased migration speed compared to parental cells. McGrail et al. found that taxol resistance was associated with decreased adhesion strength and that cells presented small nascent adhesions, characterized by strong traction forces (39). We found that TCR cells have decreased adhesion strength that coincided with small focal adhesions. These results propose that TCR cells present nascent adhesions with strong traction forces, which matches with their increased migration speed. Besides cell motility, adhesion to the extracellular matrix (ECM) is necessary for survival. Cells that detach from the ECM

rapidly undergo apoptosis (40), a phenomenon designated by Frisch and Francis as *anoikis* (41). We found that resistant cells were more sensitive to *anoikis* than parental, which suggests that their survival may be dependent on signaling pathways triggered by adhesion molecules. We studied the response of TR and TCR cells to inhibitors of cell adhesion such as rhosin, an inhibitor of RhoA that blocks actin stress fiber formation and focal adhesion assembly (42) and we observed an increased sensitivity in TCR cells compared to parental cells. In order to identify RhoA downstream pathways responsible for TCR cell death, we inhibited two targets of RhoA. However, the inhibition of ROCK1 or FAK did not modify TCR cell death compared to parental cells, suggesting that the altered pathway signals via other RhoA interactors. In depth studies need to be performed to validate the implication of cell adhesion genes in resistance to T-DM1.

Genes involved in the prostaglandins (PGs) pathway were upregulated in models resistant to T-DM1. PGs are bioactive lipids implicated in normal development and pathological processes such as inflammation and cancer (43). The expression of COX2 is upregulated in many types of cancer and has been associated with decreased survival (43–45). The upregulation of *COX2* in both resistant cell lines coincided with increased sensitivity to inhibition of cyclooxygenases by aspirin. Moreover, PGE₂ has been described to be the most abundant PG in tumors (43), and its amount was increased in the supernatant of TR cells compared to parental cells. In view of this result and that the gene coding for its receptor (*EP2*) was upregulated in both resistant cell types, we studied its effect on T-DM1 resistant cells. We found that the addition of pharmacological concentrations of PGE₂ increased the cytotoxic effect of T-DM1 while PGE₂ alone had no impact on cell survival. PGE₂ has been extensively studied in rodent experiments and in the clinic and shown to be involved in tumor growth and associated with poor prognosis (46,47). However, a dual role of PGE₂ has been observed in some cases and reviewed by Greenhought et al (48). Thus, PGE₂ could have pro or anti-tumoral activities depending on the cell type and the experimental settings. PGE₂ activates many downstream targets such as EGFR, MAPK, angiogenic and antiapoptotic factors, and chemokines (46). These pathways could be implicated in resistance to T-DM1 and their modulation could restore sensitivity to T-DM1. In depth studies are needed to describe the pathways involved in the role of PGE₂ in this setting.

In summary our results show for the first time that resistance to an antibody-drug conjugate may be associated with modifications in cell adhesion and morphology as well as with alterations of the prostaglandin pathways. Additional studies are required to determine the clinical relevance of these observations and whether it is possible these alterations could constitute potential novel therapeutic targets.

Material and methods

Cell culture

OE-19 cell line was purchased from ECACC, tested for *Mycoplasma* once a month and cultured in complete RPMI 1640 medium supplemented with 10% fetal calf serum and 100 µg/ml streptomycin. Counting was performed using Cellometer Auto T4 (Nexcelom Bioscience LLC). Cells were maintained at 37°C and 5% CO₂ at all times. OE-19 cell line was exposed to increasing concentrations of T-DM1 for 6 months in the absence or presence of 1 µg/ml ciclosporin A (C3662; Sigma-Aldrich) to obtain resistance models.

Chemotherapy and targeted agents

T-DM1 was kindly provided by Genentech and S-methyl DM1 by ImmunoGen.

Cytotoxicity Assay

Cell suspensions (100 µL) were inoculated in 96-well plates at a density of 2,500 cells per well and incubated overnight before exposure to therapeutic agents. After 6 days, cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, 20 µL of MTT solution were added to each well and cells were incubated at 37°C for 4h. Then, media/MTT mixture was removed and 100 µL of 4% HCl 1N/isopropanol were added to dissolve the purple formazan crystals. The absorbance was measured at 570 nm with 690 nm as a reference readout using a Thermo MultiSkan EX microplate reader. Percentage of living cells was calculated using the absorbance in drug-exposed cells over control cells. IC₅₀ values were calculated using CompuSyn software.

Real-Time Cell Analysis (RTCA)

The xCELLigence RTCA DP instrument (ACEA Bioscience) was used to monitor cell impedance in real time. Cells were seeded (10,000 cells/well) in E-plate 16 and allowed to adhere overnight before adding the cytotoxic agents or PGE₂. Cells were monitored for one week.

Efflux assay

Cell suspension was prepared at 4^{e6} cells/ml in RPMI media containing 0.5 µg/ml Rhodamine 123 (Santa Cruz, sc-208306) in the absence or presence of 3 µg/mL of CsA and incubated for 30 min at 37°C, 5% CO₂. Cells were washed with cold RPMI media and 2^{e5} cells were suspended in RPMI media with or without 3 µg/mL of CsA and incubated

for 90 min at 37°C, 5% CO₂. Remaining cells were kept at 4°C. All conditions were washed twice with cold DPBS and analyzed by flow cytometry.

Flow cytometry

Cells were incubated for 30 min at room temperature with the corresponding antibodies: HER2 (4225666), BCRP1 (561180) and anti-kappa (214561) from BD Bioscience, MDR1 (348608) from Biologend and mouse IgG1 κ control isotypes from BD Pharmingen. CFSE staining was performed using Cell trace CFSE proliferation kit according to the manufacturer protocol (Invitrogen C34554). For apoptosis measurements, cells (2×10^5) were seeded in 6 well plates and incubated overnight. Then, T-DM1 was added to each well at increasing concentrations up to 100 nM for 72h. After incubation, cells were harvested, washed with cold DPBS + 10% SVF and stained using Annexin-V-FLUOS Staining Kit (Roche) according to the prescribed protocol and analyzed by flow cytometry. Annexin V positive cell percentages in conditions of exposure to T-DM1 were normalized to that of control for each cell line. For cell cycle distribution, cells (2×10^5) were seeded in 6 well plates and incubated overnight. Then, they were exposed to increasing concentrations up to 100 nM of T-DM1, 1 μ M vincristine and 10 nM S-methyl DM1 for 24h. Cells were collected and incubated for 30 min at 4°C with propidium iodide (0.05 mg/mL) containing Nonidet-P40 (0.05%) and 4 μ M of trisodium citrate. Cells were filtered using Falcon tubes with cell-strainer cap (352235) and analyzed by flow cytometry. Analyses were performed using a BD LSRII flow cytometer with BD FACSDiva software (BD Biosciences, San Diego, CA, USA) and FlowJo software (Tree Star, Ashland, OR, USA).

Western Blot

Proteins were extracted with RIPA buffer (RIPA buffer, 1 mM DTT, 1M NaF, 100 mM sodium orthovanadate and protease and phosphatase inhibitors). After SDS PAGE separation, and transfer onto a PVDF membrane by iBlot dry blotting system (Invitrogen), membranes were incubated overnight at 4°C with primary antibodies: HER2 (GTX50425; Genetex), β III-tubulin (clone TUJ1), β II-tubulin (clone 7B9) α -tubulin (T6199), β -tubulin (T4026) and β -actin (A5441) from Sigma-Aldrich, and 1h at room temperature with secondary antibodies (IRDye Infrared Dyes from LI-COR Biosciences). Membranes were scanned using Odyssey infrared imaging system (LI-COR Biosciences) and densitometric quantification was performed with Odyssey software. Expression levels of proteins were normalized against β -actin.

Separation of soluble tubulin and microtubules

Cells (20^{e6}) were lysed in 300 μ L of PEM 50DP Buffer (50mM Pipes, 1mM EGTA, 1mM MgSO₄, 0.05% sodium azide, 1mM DTT and proteinase inhibitors at pH 6.7) by three freeze-thaw cycles. Cells were ultracentrifuged (100000 g for 1h at 20°C) and the supernatant was separated from the pellet. The pellet fraction was resuspended in 100 μ L of PEM 50DP buffer, incubated on ice for 30 min for depolymerization and ultracentrifuged at 50000g for 45 min at 4°C to recover the supernatant containing "soluble tubulin". The supernatant was incubated at 35°C with 1mM GTP for 30 min for polymerization and ultracentrifuged at 50000g for 45 min at 35°C. The supernatant was discarded and the pellet containing the "microtubules" was resuspended in 50 μ L of PEM 50DP buffer. Alpha and beta tubulin isotypes were then analysed by Western Blot.

Microarray

The microarray was performed by ProfileXpert-LCMT platform using OE-19 S, TR and TCR cell lines as previously described by Dumontet et al (49). Data was analyzed with GeneSpring and Ingenuity softwares (Agilent Technologies, Santa Clara, CA, USA).

RT-qPCR

RNA was extracted using the QIAamp RNeasy Mini Kit (QIAGEN). Random primers (Life Technologies) were used for reverse transcription. Primer sequences were based on Roche database and quantitative PCR was performed using the LightCycler 480 Real-Time PCR system (Roche Life Science, Indianapolis, IN, USA).

Immunofluorescence

Immunostaining was performed as previously described (50). The primary antibodies used were obtained from Sigma: the anti- α -tubulin (T6199 clone DM1A) diluted at 1/50 and anti-talin (T3287, clone 8d4) diluted at 1/100.

Adhesion strength assay

The centrifugal force-based adhesion assay was based on previous published methods (39). Briefly, cells were seeded at 20,000 cells/well in 96-well plate and left to adhere overnight in media without red phenol. Cells were stained with 2 μ M Calcein AM (Sigma-Aldrich, C1359) in PBS-dextrose 2mM for 20 min at 37°C and rinsed with PBS. Cells were covered with PBS-dextrose before an initial fluorescence reading at 485nm excitation, 535nm emission on a Plate Chameleon multilabel detection platform. Next, the supernatant was discarded and the inverted plates were centrifuged at 60g for 5 min and

rinse with PBS. Then, PBS-dextrose was added before a final reading. The detached fraction was calculated as $1 - \text{final fluorescence} / \text{initial fluorescence}$.

Wound healing assay

Wound healing assays were conducted using Culture-Inserts form ibidi (80209). To each well were added 100 μL of cell suspension 1.5×10^6 cells/ml and allowed to adhere overnight. The Culture-Insert was removed and wells were washed before filling with growth media with or without 10 μM PGE₂. Pictures were taken at 24h intervals using a Leica DMI3000B microscope equipped with a Leica DFC425C camera.

PGE₂ quantification

Cells were washed and suspended in SVF free media to plate 2×10^5 cells per well in a 12-well plate. Supernatants were recovered 24h after plating and PGE₂ was quantified with ELISA High sensitivity (ENZO Life Sciences).

Quantification of Lys-MCC-DM1

Analysis was performed with a Q-Exactive-Plus (hybrid quadrupole-Orbitrap mass spectrometer) coupled with liquid chromatography (Ultimate 3000) from Thermo ScientificTM. Lys-MCC-DM1 was separated on a Hypercarb (5.0 μm , 150 mm x 2.1 mm i.d.) Thermo ScientificTM column. Gradient elution with water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) was applied. The mobile phase was delivered through the column (temperature at + 30° C) at a flow rate of 250 $\mu\text{L}/\text{min}$. At start, (B) was maintained for 1.5 min at 30 % (v/v). After, (B) increased linearly until 3 min to 80 % (v/v) and this composition is fixed for 4 min. Then (B) was reset to 30 % (v/v) for 5 min. Analysis of Lys-MCC-DM1 was carried out in positive ion mode using a heated electrospray ion source. The signal of Lys-MCC-DM1 (C₅₃H₇₅N₆O₁₅ClS) was collected using target-sim mode with a resolution of 70,000 and following the [M+H]⁺ ion at m/z 1103.4765. Lys-MCC-DM1 eluted as sharp peak at 7.2 min and cell components did not interfere with the analysis. Cells were exposed to 5nM T-DM1 for one hour and washed twice with phosphate buffered saline and extracted with 300 μL of a mixture containing acetonitrile and water (80/20; v/v). The extract was transferred in Eppendorf tube, then shaken for 5 min, and conserved at -80°C until analysis. The day of analysis, samples were vigorously vortexed and centrifuged for 10 minutes at 13000 g. The supernatant was evaporated to dryness under nitrogen at 37°C. Finally, the residue was resuspended in 150 μL of water and 20 μL were injected into liquid chromatography-mass spectrometry device. For calibration curves, blank cell samples were spiked with the appropriately diluted standard solutions to final concentrations of

1.25, 2.5, 5, 12.5 and 25 ng/ml. Calibration curves were constructed by plotting the ion abundance peak area as function of cell Lys-MCC-DM1 concentration. Data were fitted by weighted (1/concentration) for least-squares regression, and standard curves were determined using linear regression analysis.

Statistical analysis

Experiments were performed at least three times and shown in graphs as the mean \pm SD. Graphs and statistics were done using GraphPad Prism software. Statistics on cell survival experiments such as AnnexinV/PI staining or MTT assay were calculated by Two Way Anova followed by Bonferroni post-test. Statistics on gene expression by RT-qPCR were performed by Student t test.

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Figure 1

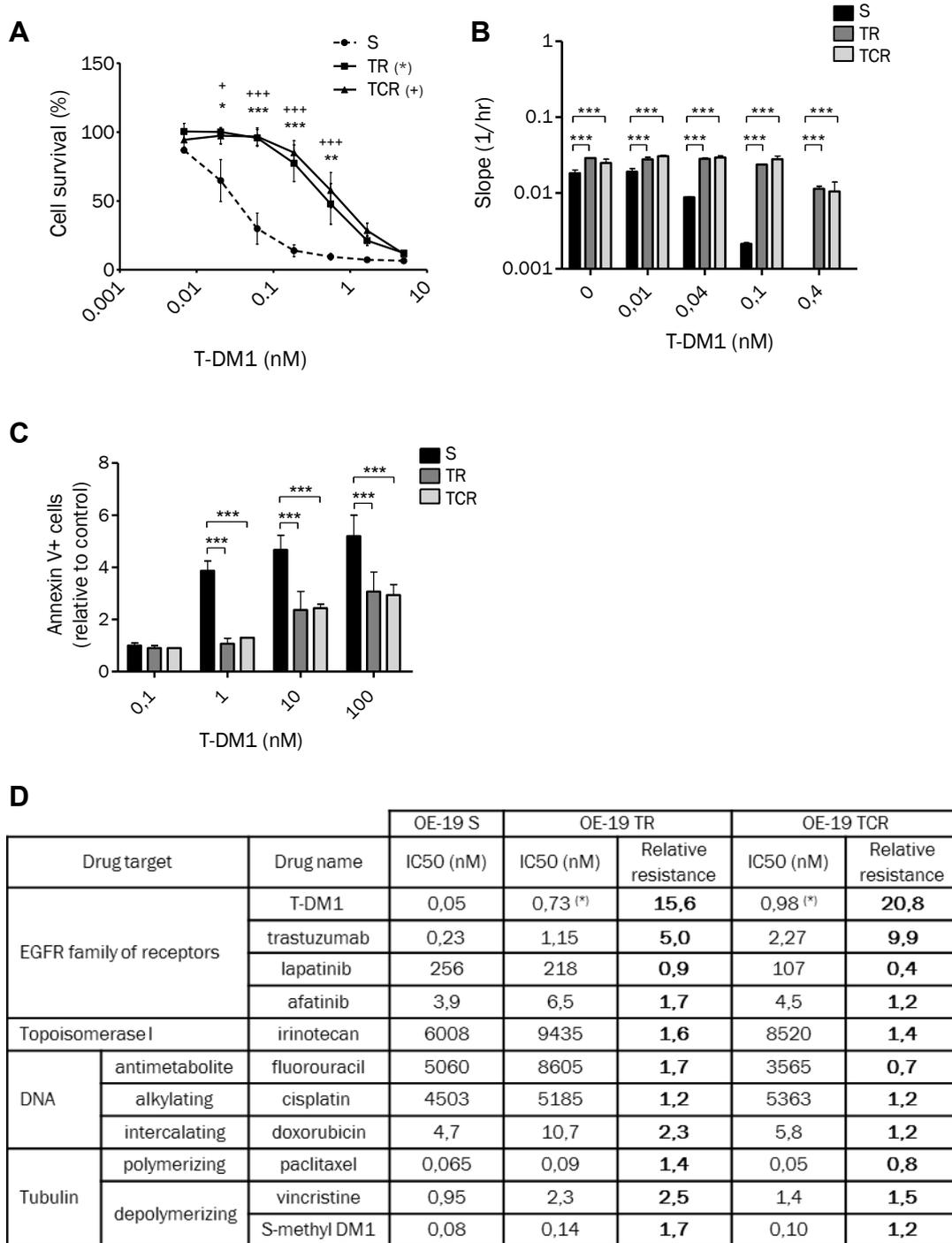


Fig. 1. Chronic exposure to T-DM1 of OE-19 cell line results in resistance to this immunoconjugate. **A.** Cytotoxicity of T-DM1 on OE-19 S, TR and TCR cells determined by MTT cytotoxic assays revealed an increase in the IC50 of TR and TCR cells compared to parental cells. **B.** Cytotoxicity of T-DM1 was studied using xCELLigence. The cell index slope was calculated using RTCA software and plotted. A single experiment is shown, representative of 3 experiments. **C.** Cell death after 72h exposure to T-DM1 was assessed by annexin V staining using flow cytometry. The fold change in cell death relative to control was plotted for each cell line. The amount of cell death was decreased in TR and TCR compared to parental cells. Statistically significant differences were found for TR (***: P<0,001; **: P<0,01; *: P<0,05) and TCR (+) compared to S cells. **D.** Parental and resistant OE-19 cells were exposed to the indicated anti-cancer agents and their sensitivity was assessed by MTT assay (or xCELLigence for trastuzumab). Data are shown as the mean IC50 calculated from 3-4 independent experiments and the relative resistance is the ratio of the IC50 for OE-19 TR or TCR over the IC50 for OE-19 S cell line (*: p<0,05).

Figure 2

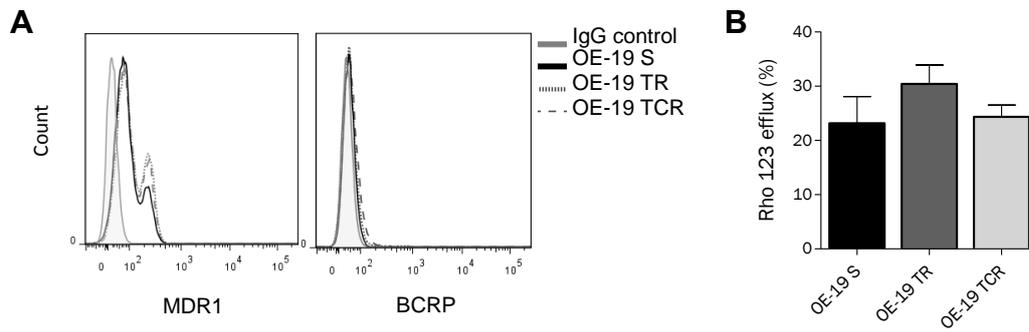


Fig. 2. ABC transporters MDR1 and BCRP expression and activity are not significantly modified in resistance models. **A.** Surface expression of MDR1 and BCRP, studied by flow cytometry, does not show an increased expression of these ABC transporters in resistant cells. **B.** Efflux activity was determined by Rhodamine 123 (Rho123) accumulation using flow cytometry. Rho123 efflux percent was not significantly different in resistant cells and in parental cells. The percentage of Rho123 efflux was calculated by comparing the mean fluorescence intensity (MFI) after uptake and the MFI after efflux ((Uptake-Efflux)/Uptake*100).

Figure 3

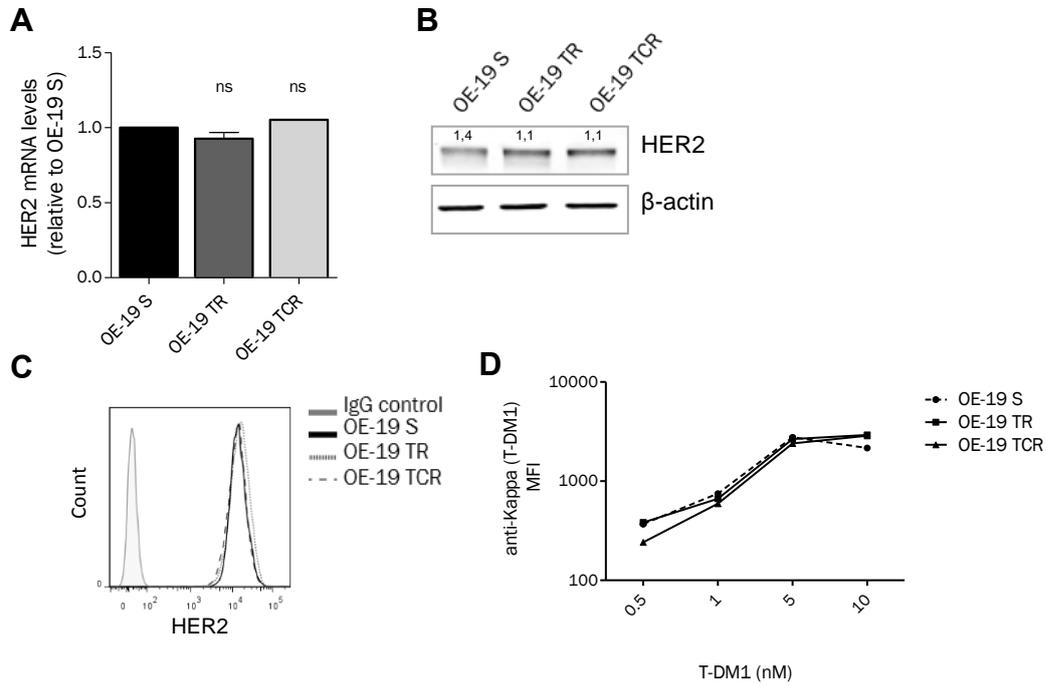


Fig. 3. HER2 expression remains unchanged after chronic exposure to T-DM1. **A.** mRNA and **B.** protein expression from total cell lysates show that HER2 levels are unaffected in resistant cells. **C.** HER2 expression at the cell surface determined by flow cytometry shows that parental and resistant cells express the same amount of HER2. **D.** After exposure to T-DM1 for 1h at 4°C, cells were stained with anti-Kappa antibody and the mean fluorescence intensity (MFI) was studied using flow cytometry. T-DM1 was found to bind similarly parental and resistant cells. A single experiment is shown, representative of 3 experiments.

Figure 4

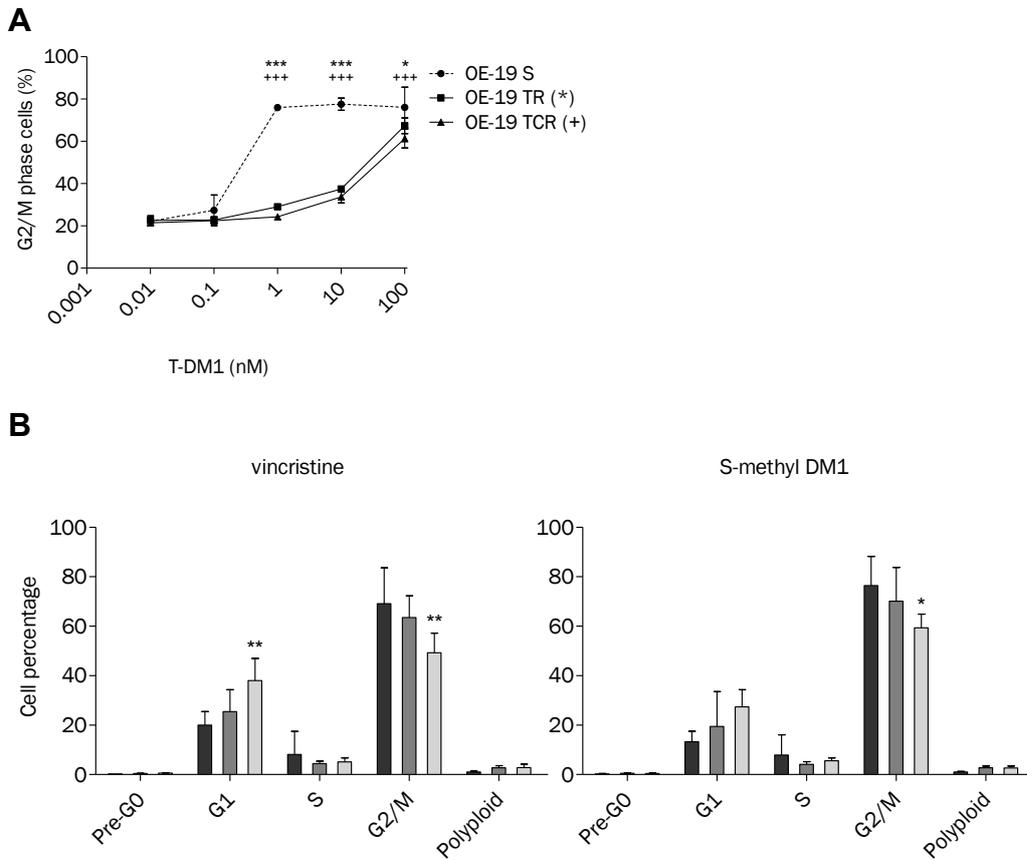


Fig 4. T-DM1-induced cell cycle arrest is impaired in OE-19 TR and TCR compared to OE-19 S. **A.** Exposure to increasing concentrations of T-DM1 for 24h and analysis of cell cycle distribution shows that the G2/M population was decreased in resistant cells compared to parental. Statistically differences are shown for TR (***: $P < 0,001$; *: $P < 0,05$) and TCR (***: $P < 0,001$) compared to S. **B.** Cell cycle distribution was studied by propidium iodide staining using flow cytometry after 24h exposure to 1 μ M vincristine or 10 nM S-methyl DM1. Control conditions are not shown; only OE-19 S, TR and TCR exposed to drugs are plotted. Vincristine and S-methyl DM1 induced G2/M phase arrest in parental and resistant cells. The TCR cell line showed decreased sensitivity to cell cycle arrest in comparison to the parental cell line (*: $P < 0,05$; **: $P < 0,01$).

Figure 5

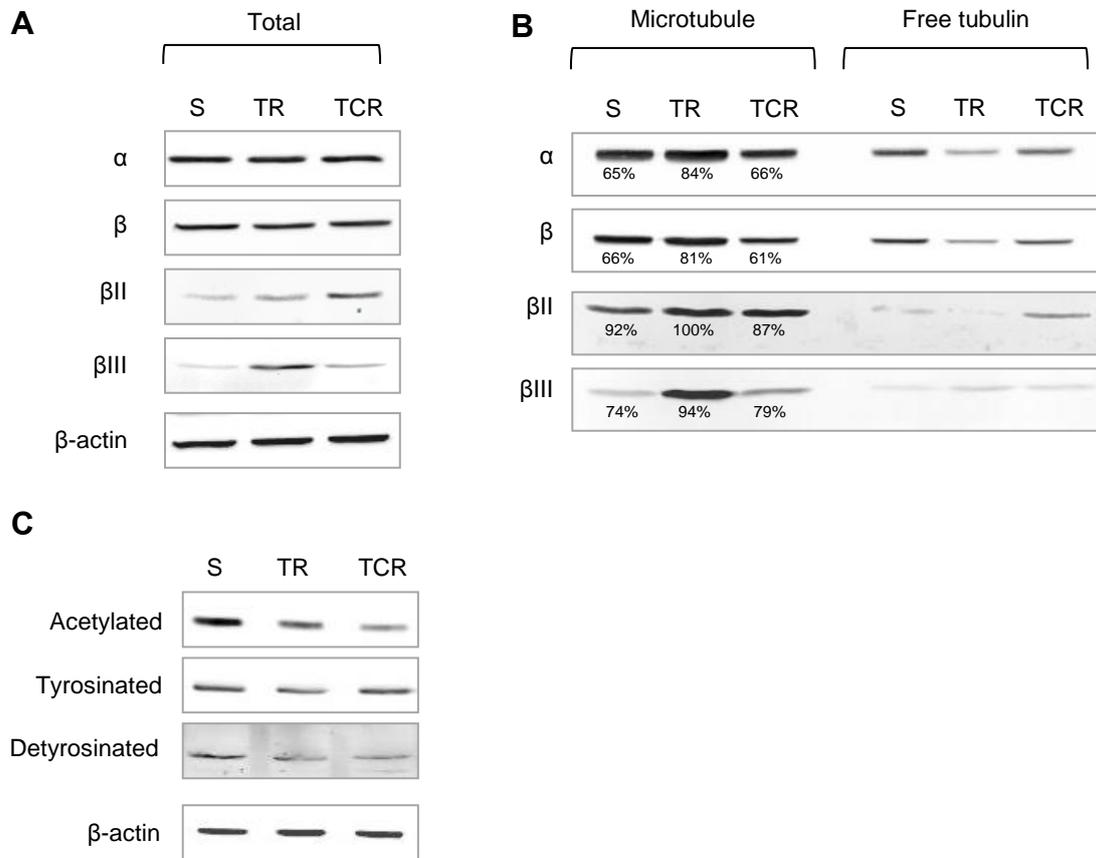


Fig. 5. Tubulin expression and polymerized/soluble tubulin fractions in resistant models. Expression of total α and β tubulin and isoforms β II and β III was examined from total cell lysates or from purified fractions of tubulin. A single experiment is shown, representative of 3 experiments. **A.** Protein levels from total cell lysate were studied by Western Blot and the density of the bands was normalized with actin to determine the expression fold-change. Cells resistant to T-DM1 express higher levels of β II and β III tubulin than parental cells. **B.** Protein expression after purification of polymerized (microtubule fraction) and soluble (free tubulin fraction) tubulin. The percentage of polymerized tubulin indicated on the figure shows that TR cells have an increased amount of polymerized tubulin compared to parental. **C.** Acetylation, tyrosination and detyrosination state of α tubulin was studied by Western Blot.

Figure 6

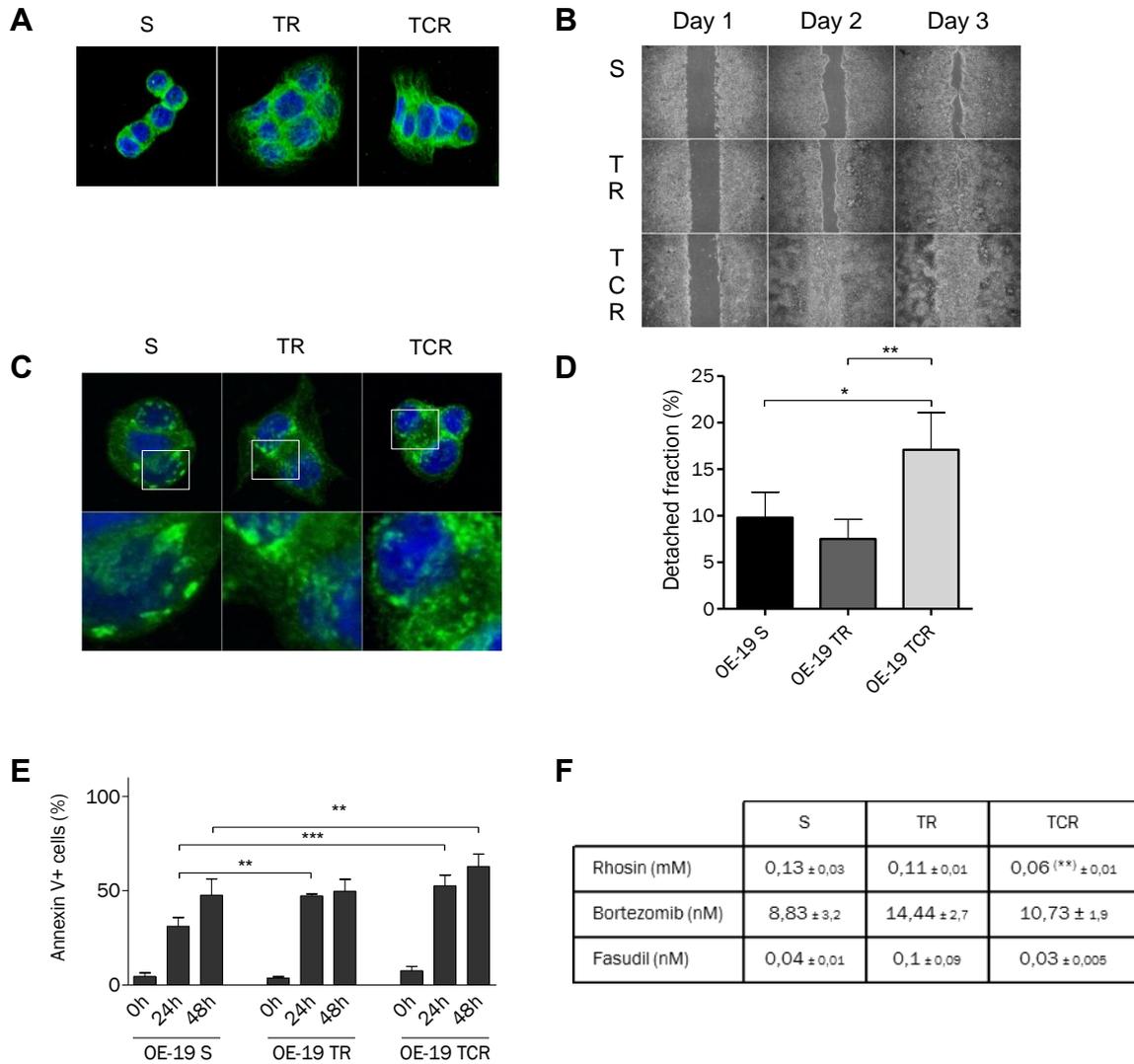


Fig. 6. Morphology is modified in both resistant cell lines but only TCR cells have increased migration speed and decreased adhesion strength. **A.** Immunofluorescence staining of α -tubulin (green) and DAPI (blue) observed by confocal microscopy shows morphological differences between parental and T-DM1-resistant cell lines. **B.** Migration speed determined by wound healing assay shows an increase in TR and TCR cells compared to parental **C.** Immunofluorescence staining of talin (green) and DAPI (blue) observed using a confocal microscope. Focal adhesions in parental cells appear bigger and in least amount that those in resistant cells. **D.** Detached fraction of parental and resistant cells following detachment by centrifugal force shows a decreased adhesion strength in TCR cells (*: $P < 0,05$; $P < 0,01$). **E.** Cell death was quantified by Annexin/PI staining following 24h and 48h of incubation in suspension conditions. The percent of living cells was decreased in TR cells at 24h ($P < 0,01$) and in TCR cells after 24h ($P < 0,001$) and 48h ($P < 0,01$) compared to parental. **F.** Sensitivity to rhosisin, bortezomib and fasudil was studied using MTT cytotoxic assays after 6 days exposure to the corresponding cytotoxic agents. Sensitivity to rhosisin was increased in TCR cells compared to parental (**: $P < 0,01$).

Figure 7

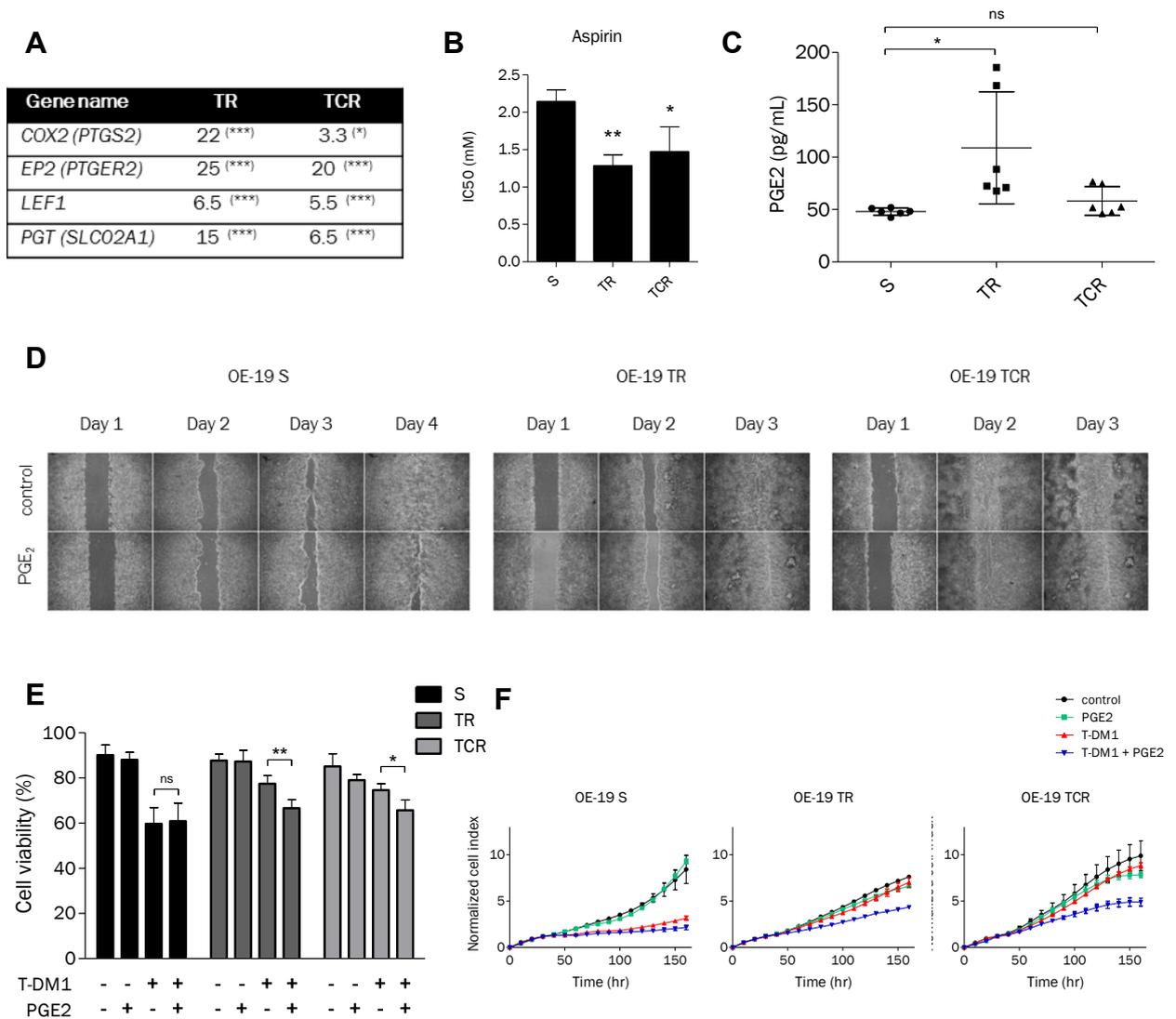


Fig 7. Prostaglandin E₂ mediates sensitivity to T-DM1 in resistant cells. **A.** The expression of genes involved in the prostaglandin pathway is highly modified in resistant cells to T-DM1. The results shown are the expression fold changes of TR or TCR over parental cells for the indicated genes determined by RT-qPCR. **B.** Sensitivity to aspirin studied by MTT assay was found to be increased in TR and TCR cells compared to S cells (*: P<0,05; **: P<0,01). **C.** Quantification by ELISA of PGE₂ in the supernatant of each cell line shows an increased amount in TR cells compared to parental cells. **D.** Wound healing assay performed on OE-19 S, TR and TCR cell lines in the absence or presence of 10 μ M PGE₂. The addition of PGE₂ decreased the migration speed of the parental cell line but had no effect on the migration speed of both resistant cell lines. **E.** Cell survival after exposure to 1 nM T-DM1 and 10 μ M PGE₂ was studied by Annexin V/PI staining after 72h exposure. The presence of PGE₂ increases the sensitivity to T-DM1 of resistant models. **F.** The increased sensitivity to T-DM1 in the presence of PGE₂ was confirmed by xCELLigence. After overnight incubation cells were exposed to 1 μ M PGE₂ and/or 0,1 nM T-DM1. The normalized cell index of TR and TCR cells exposed to T-DM1 and PGE₂ is inferior to control, PGE₂ and T-DM1. A single experiment is shown, representative of 3 experiments.

Table S1:

A

	Pathway	P-value	Genes
OE-19 TR	ECM-receptor interaction	0,032	<i>ITGA2, ITGA3, ITGA6, ITGAV, ITGB1, ITGB4, ITGB5, LAMA1, LAMA3, LAMA5, LAMB1, LAMB2, LAMB3, LAMC2, SDC1, THBS1</i>
	Adherens junction	0,033	<i>SMAD3, SMAD4, WASF3, ACP1, ACTN1, CDH1, CTNNA1, EGFR, LEF1, MET, PVRL3, PTPRF, PAC2, TJP1, VCL</i>
	Cell adhesion molecules	0,041	<i>CD276, CD58, CDH1, CLDN18, CLDN2, CLDN3, CNTNPA2, ESAM, GLG1, ALCAM, ITGA6, ITGB1, HLA-A, HLA-E, HLA-G, HLA-DRA, NRCAM, LOC647859, PVRL3, PTPRF, SDC1</i>
	Focal adhesion	0,058	<i>SHC1, ACTN1, BIRC3, EGFR, FLNA, FLNB, GSK3B, ITGA2, ITGA3, ITGA6, ITGAV, ITGB1, ITGB4, ITGB5, LAMA1, LAMA3, LAMA5, LAMB1, LAMB2, LAMB3, LAMC2, MET, MAPK9, PAK4, PARVA, PDGFB, RAC2, THBS1, VCL, ZYX</i>
OE-19 TCR	Adherens junction	0,002	<i>BAIAP2, LMO7, SMAD3, SMAD4, WASF3, ACP1, ACTN1, CDH1, CTNNA1, EGFR, LEF1, MET, PVRL3, PTPRM, RAC2, TJP1</i>

B

		Fold change			
		Microarray		qPCR	
		TR	TCR	TR	TCR
Tyrosine kinase receptors	<i>EGFR</i>	1,4	1,3	1,8 (**)	1,2
	<i>MET</i>	1,5	1,4	1,8 (**)	1,2
Actin-interacting proteins	<i>ACTN1</i>	1,6	1,7	2,1 (**)	1,7 (**)
	<i>VCL</i>	1,7		1,9 (**)	1,3
Regulators of actin cytoskeleton	<i>ROCK1</i>		1,4	2,0 (**)	1,6 (**)
	<i>RAC2</i>	0,5	0,7	0,3 (***)	0,5 (***)
	<i>DIAPH1</i>			1,9 (***)	1,5

Table S1. Deregulated genes in OE-19 TR and OE-19 TCR cell lines compared to parental. A. Transcriptomic analysis of OE-19 S, TR and TCR cell lines shows several genes involved in cell adhesion that are deregulated in resistant cells compared to parental. **B.** Expression fold change values of the genes of interest from the microarray and from RT-qPCR assays. The fold change was calculated as the level of expression in each resistant cell line over the one of the parental cell line (**: P<0,01; ***: P>0,001).

Figure S1:

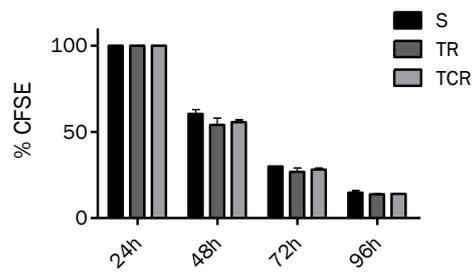


Fig. S1. Cell proliferation remained unchanged in resistant cells. Cells were stained with CFSE and the staining intensity was measured by flow cytometry. The percent of CFSE was normalized to the values observed at 24h after staining for each cell line. T-DM1 resistant cell lines proliferate at the same rate as the parental cell line.

Figure S2 :

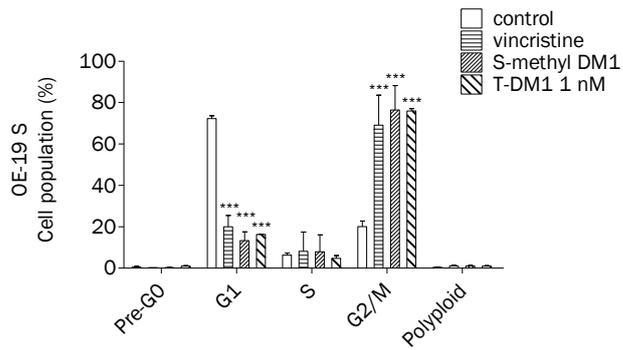


Fig. S2. Cell cycle arrest is induced by T-DM1, vincristine and S-methyl DM1 in OE-19 parental cell line. Cell cycle distribution determined by propidium iodide using flow cytometry after 24h exposure to 1 nM T-DM1, 1 μ M vincristine and 10 nM S-methyl DM1 shows a G2/M phase arrest in parental cells. Statistics analysis comparing cells exposed to the cytotoxic agents to control condition was performed by Two way ANOVA followed by bonferroni posttest (***: P<0,001).

Figure S3 :

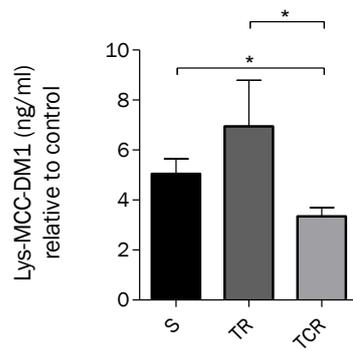


Fig. S3. Lys-MCC-DM1 accumulation after exposure to T-DM1 is decreased in TCR cells compared to parental cells. Lys-MCC-DM1 quantification by LC-MS/MS after 1 hour exposure to T-DM1 shows a decreased amount of the metabolite in TCR cells compared to TR and S cells (*: $P < 0,05$).

Figure S4:

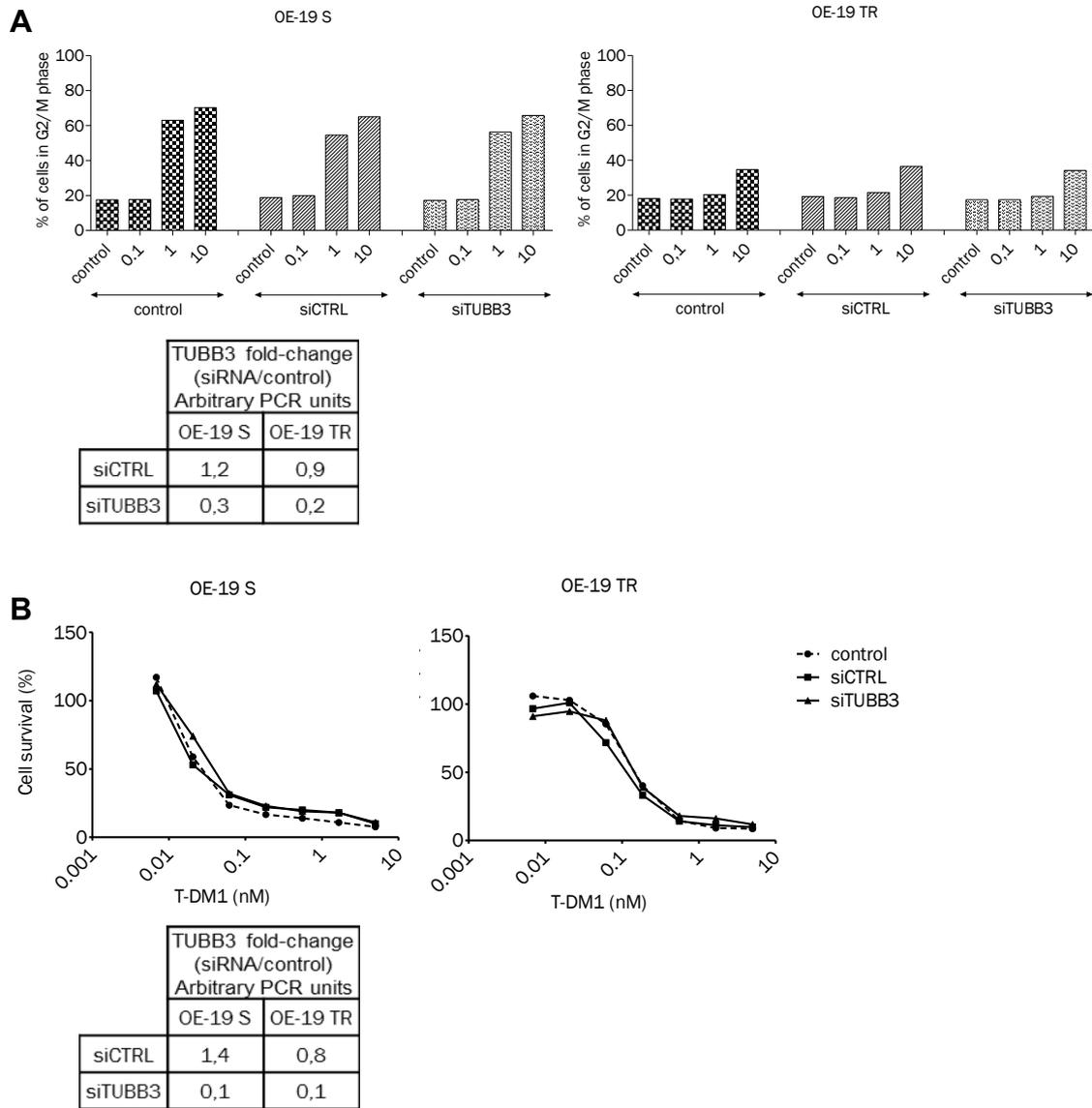


Fig. S4. Downregulation of β III tubulin by siRNA did not affect T-DM1-induced cell cycle arrest or T-DM1-cytotoxicity in OE-19 S and OE-19 TR cell lines. Cells were transfected with siRNA control (siCTRL) or siRNA targeting β III tubulin (siTUBB3) 24h prior exposure to T-DM1. The fold-change expression of TUBB3 was evaluated by RT-qPCR and normalized using non-transfected cells (control) and is indicated for each experiment at 24h post-transfection. **A.** Cell cycle distribution was studied by propidium iodide using flow cytometry after 24h exposure to increasing concentrations of T-DM1. Downregulation of β III tubulin did not affect cell cycle distribution in OE-19 S or OE-19 TR cells. **B.** Cell survival was determined by and MTT assay after 6 days exposure to T-DM1. The sensitivity to T-DM1 was not modified in OE-19 S or OE-19 TR cells transfected with siTUBB3.

Figure S5:

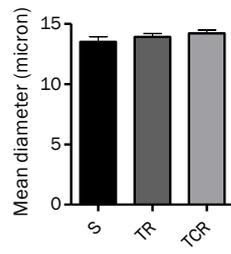


Fig. S5. Cell size was not modified in resistant cells. The mean diameter of cells in suspension measured by a Cellometer counter shows no modification of size in resistant cells compared to parental.

Figure S6:

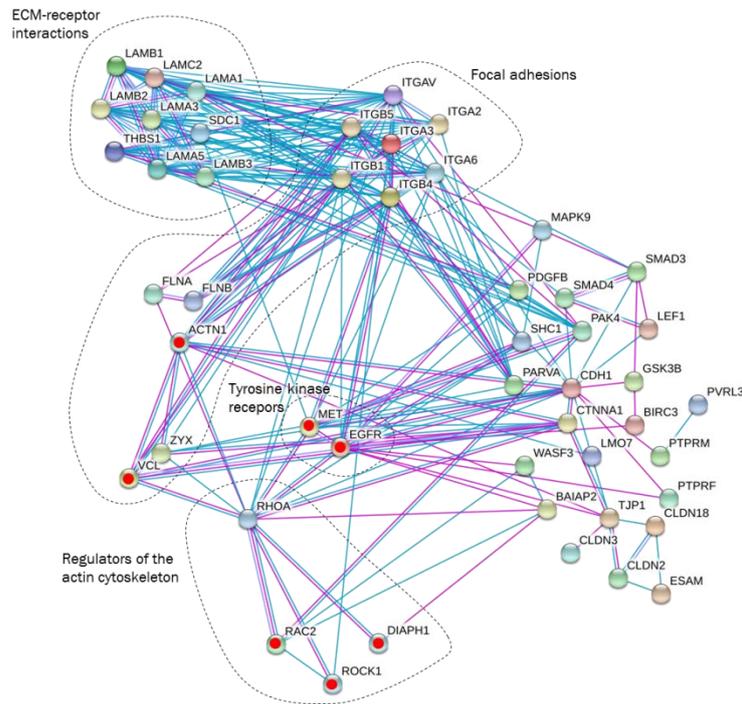


Fig S6. Network of proteins encoded by the genes deregulated in resistant cell lines. The interactions between the genes listed in **Table S1** were represented using STRING. Pink interactions are predicted from databases and blue interactions have been experimentally determined. The interactions were represented using a medium confidence score (0,400). The red dots indicate the molecules for which the gene expression was verified by RT-qPCR after the transcriptomic analysis, showed in **Table S2**. RhoA did not appear to be deregulated but was represented since it is an important interactor of our molecules of interest.

Figure S7:

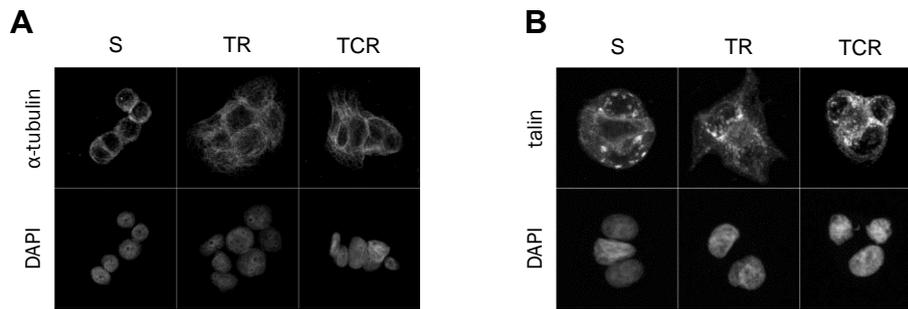


Fig S7. Single-channel images for tubulin and talin immunostaining. A. Immunostaining of α -tubulin and DAPI shows morphological changes in resistant cell lines compared to the parental cell line. **B.** Immunostaining of talin and DAPI shows differences in size and amount of focal adhesions between parental and resistant cells.

The efficacy of T-DM1 is limited by acquired resistance to treatment (118,119). In order to understand the underlying resistance mechanisms and to propose therapeutic alternatives, we selected OE-19 cancer cell lines resistant to T-DM1.

The characterization of OE-19 resistant models exposed an overexpression of β III tubulin. However, its downregulation by siRNA did not impact the sensitivity to T-DM1 or cell cycle in OE-19 parental and TR cell lines. Although the expression of β III tubulin has been associated with prognosis and efficacy of treatment (138), our results suggest that β III tubulin does not mediate response or resistance to T-DM1.

One of the variants (TCR) was obtained in the presence of ciclosporin in order to circumvent the expression of Pgp or other ciclosporin A-sensitive efflux transporters. Pgp is frequently found to be over-expressed in lines exposed to tubulin-binding agents, as well as to other ABC substrates. The TR variant, in spite of being selected in the absence of ciclosporin, was not found to overexpress significantly and homogeneously Pgp or BCRP. This suggests that ABC overexpression might not be as frequent a mechanism of resistance as for conventional cytotoxic agents.

A transcriptomic analysis of resistant cell lines revealed a deregulation of several genes implicated in adhesion and regulation of the actin cytoskeleton. Both resistant cell lines were more sensitive to anoikis than the parental cell line. This result indicates that the survival of TR and TCR cells may depend on the signaling pathways activated by adhesion molecules such as integrins. The role of adhesion in drug resistance was first observed in myeloma cells and has been observed in other hematological and solid cancers since (148–152). This mechanism, called cell adhesion-mediated drug resistance (CAM-DR), seems to protect cells from T-DM1 cytotoxicity. Moreover, targeting RhoA by rhosin induced cell death more efficiently in TCR cells than in TR or parental cells. Our results suggest that cell adhesion plays a protective role and that they can be targeted to induce death of cells resistant to T-DM1.

The OE-19 TR cell line expressed *COX-2* 20-fold times higher than the parental cell line and had an increased amount of PGE_2 in the supernatant. Also, the sensitivity to aspirin, a cyclooxygenase 1 and 2 inhibitor, was increased in both resistant cells compared to the parental cells. The overexpression of *COX-2* and the increased production of PGE_2 are found in many types of cancer and are often associated with poor prognosis (153,154). Thus, the hyper activation of the prostaglandin pathway found in the OE-19 TR cell line could contribute to resistance to T-DM1. The PGE_2 is able to activate pro-survival signals that probably by-pass the inhibition of HER2 by T-DM1 (155). Our results show that targeting the prostaglandin pathway could be an alternative to trigger death of T-DM1 resistant cells.

II.2. MECHANISMS OF RESISTANCE TO T-DM1 IN A BREAST CANCER MODEL

HER2 is a transmembrane tyrosine kinase receptor that belongs to the epidermal growth factor receptor family. HER2 plays an important role during development but is also implicated in tumor initiation and progression (156). HER2 is amplified and/or overexpressed in about 20% of breast cancers and was previously associated with poor prognosis (59). The development of targeted therapy has drastically improved the management of HER2-positive breast cancer patients. T-DM1 is an antibody-drug conjugate that combines the anti-cancer activity of trastuzumab to the tubulin targeting agent DM1. The selectivity of trastuzumab permits the delivery of the maytansinoid DM1 within cancer cells. Despite the efficacy of novel targeted treatments, acquired resistance to treatment is observed in a subset of patients.

The study of the mechanisms of resistance to ADCs is of great interest as these agents are of relatively new use in the clinic. The activity of ADCs can be dissected into major steps that are essential for their efficacy. The binding of the antibody to the extracellular epitope can be disrupted by downregulation, masking or shedding. Resistance to trastuzumab can be mediated by shedding of HER2 by metalloproteases, or by masking by glycoproteins or MUC-4 for example (130,157). Then, the proposed mechanism of internalization of ADCs is receptor-mediated endocytosis. Caveolin-1 has been shown to enhance the sensitivity of breast cancer cell lines to T-DM1 (158). Hence, a decreased expression of caveolin-1 might mediate resistance to T-DM1. Finally, lysosomal degradation and release of the active metabolite into the cytoplasm are necessary to reach the second target of the ADC. The lysosomal transporter SLC46A3 was shown to be required for the transport of T-DM1 metabolites from the lysosome to the cytoplasm (159). Hence, the downregulation of SLC46A3 or similar transporters could prevent the maytansinoid metabolite to bind tubulin. Also, tubulin mutations or differential expression of tubulin isotypes or MAPs could drive resistance to T-DM1.

We selected MDA-MB-361 breast cancer cell lines resistant to T-DM1 to investigate resistance mechanisms. We found a decrease of β III tubulin expression that was associated with an increased number of chromosomes.

Breast cancer cells resistant to T-DM1 contain giant aneuploid cells

Manuscript in preparation

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Abstract

The development of targeted therapy has drastically improved the outcome of patients with different types of cancer. T-DM1 is a novel antibody-drug conjugate for the treatment of HER2-positive breast cancer. Despite the clinical success achieved by targeted therapies, a number of patients develop resistance during treatment. To select resistant cells, MDA-MB-361 breast cancer cell line was exposed *in vitro* to T-DM1 in the absence or presence of ciclosporin A. Both resistant cell lines remained sensitive to the tyrosine kinase inhibitor lapatinib and DNA-damaging agents, but the sensitivity to vincristine was increased. The expression of β III tubulin was decreased in resistant cell lines and was associated with a decreased percent of polymerized tubulin. The down-regulation of β III tubulin by siRNA in the parental cell lines did not modify the sensitivity to T-DM1, but did increase S and G2/M phase during cell cycle. Both resistant cell lines to T-DM1 presented giant aneuploid cells. The increased number of chromosomes might be due to cell division problems caused by T-DM1. Taken together, these results demonstrate alterations in expression of β III tubulin, polymerization of tubulin and increased ploidy that correlate with resistance to T-DM1.

Introduction

The human epidermal growth factor receptor 2 (HER2) is amplified / overexpressed in about 20% of breast cancers and is associated with poor outcome and high chances of recurrence (1–3). Direct targeting of HER2 has dramatically improved the management of HER2-positive breast cancer patients (4). The monoclonal antibody (mAb) trastuzumab represents the first breakthrough in HER2-targeted therapies and was followed by pertuzumab. Despite them being a major step forward in cancer therapy, the insufficient toxicity displayed by mAbs has propelled the development of antibody-drug conjugates (ADCs) (5). The selectivity of mAbs combined with potent cytotoxic molecules permits the delivery of the drugs within cancer cells while doing less damage to healthy cells (6).

Ado-trastuzumab emtansine (T-DM1) is the first ADC for the treatment of HER2-positive metastatic breast cancer as second-line therapy. T-DM1 is composed of the monoclonal antibody trastuzumab conjugated to a derivative of maytansine (DM1) via a non-cleavable linker SMCC (7). T-DM1 selectively binds to HER2 and delivers a potent tubulin binding agent within cancer cells. Despite the clinical success of T-DM1, some patients initially responding develop resistance during treatment.

ADCs are of relatively new use in the clinics and even though resistance has been reported in patients, the exact mechanisms still need in-depth studies. Resistance to T-DM1 was reported in pre-clinical and clinical reports (8). Brentuximab vedotin (BV) and T-DM1 are the only ADCs approved for the treatment of CD30-positive hematological malignancies and HER2-positive breast cancer, respectively. Resistant cell lines to each ADC have been developed to undercover resistance mechanisms. The overexpression of ATP-binding cassette (ABC) transporters that are responsible for the efflux of toxic molecules outside of cells was detected in both cases (9,10). The upregulation of proteins involved in the regulation of the actin/tubulin cytoskeleton and the endosomal/lysosomal pathway was observed in resistance models to the trastuzumab-maytansinoid ADC.

We selected resistant cells to T-DM1 using MDA-MB-361 breast cancer cell line to study the resistance mechanisms. Our results showed that the prolonged exposure to T-DM1 induced expression changes of β III tubulin and aneuploidy.

Results

Generation of MDA-MB-361 in vitro resistance models to T-DM1

MDA-MB-361 resistant cells were selected *in vitro* by constant exposure to increasing concentrations of T-DM1. The initial concentration of T-DM1 was 20% of the IC₅₀ and was gradually augmented when cells reached stable survival. The final concentration of T-DM1 was 0.4 nM, which corresponds to x times the initial IC₅₀. The selection of resistant cells to T-DM1 was performed in the absence or presence of a modulator of MDR1, a member of the ABC transporters family. The efflux of DM1 outside the cells is mostly executed by MDR1. Consequently, ciclosporin A (CsA) was used to inhibit MDR1 and avoid increased efflux activity. Two cell lines resistant to T-DM1 were selected in the absence (MDA-MB-361 TR) and in the presence of CsA (MDA-MB-361 TCR), and compared to the parental cell line (MDA-MB-361 S).

Sensitivity to anti-cancer agents

To evaluate the resistance to T-DM1 of TR and TCR cell lines, its cytotoxicity was studied by different techniques. The IC₅₀ determined by MTT assay increased by 5-fold in TR cell line and by 8-fold in TCR cell line compared to the parental cell line (Fig. 1A). The IC₅₀ calculated by xCELLigence was also increased in TR cells by 73-fold and TCR cells by 12-fold compared to S cells (Fig. 1B). Even though the absolute IC₅₀ values obtained by MTT and xCELLigence are different, both techniques indicate a decreased sensitivity to T-DM1 in TR and TCR cells. Apoptosis was analyzed by Annexin V staining

after exposure to T-DM1 for 6 days and we found a decreased sensitivity to T-DM1-induced apoptosis in TR and TCR cells compared to S cells (Fig. 1C). Altogether, these results indicate that the selected TR and TCR cell lines are resistant to T-DM1.

So as to assess the sensitivity to different anti-cancer agents of parental and resistant cell lines to T-DM1, we performed MTT and xCELLigence assays using targeted therapies and chemotherapy agents (Table 1). Both resistant cell lines to T-DM1 remained sensitive to DNA damaging agents, indicating that DNA-repairing machinery may not be involved in resistance to T-DM1. The sensitivity to tubulin binding agents such as paclitaxel, vincristine and S-methyl DM1 was slightly decreased in both resistant cell lines, but only the increased IC50 for vincristine in TCR cells was found to be significant. These results indicate that the resistance to T-DM1 may be due to altered tubulin binding.

The efflux activity is increased in resistant models

The increased activity and/or the overexpression of ABC transporters is a common mechanism of multidrug resistance (11–13). To verify whether resistance to T-DM1 was due to the activity of ABC transporters, we performed a rhodamine 123 (Rho 123) accumulation assay (Fig. 2A). We observed an increased efflux activity in TCR cells compared to parental cells, while it remained unchanged in TR cells. To determine if the augmented activity was due to the overexpression of two main ABC transporters, MDR1 and BCRP, we studied their expression by flow cytometry. We found that both proteins are poorly expressed in the parental cell line and that the prolonged exposure to T-DM1 of TR and TCR cells did not select MDR1 or BCRP overexpressing cells. As MDR1 is the main transporter of maytansinoids, we studied Rho 123 accumulation in the presence of CsA (Fig. 2C). Although CsA induced a significant decrease of Rho123 efflux, the percent of Rho123 efflux remained high in all cell lines, indicating that other transporters are involved in efflux and might be implicated in resistance to T-DM1.

HER2 expression is decreased in resistant cells to T-DM1

The presence of HER2 at the cell surface is essential for T-DM1 cytotoxicity, so we studied its expression by RT-qPCR and Western Blot. We found a downregulation of HER2 at the mRNA and the protein levels of total cell lysates in both resistant cell lines compared to the parental cell line (Fig. 3A, 3B). The amount of extracellular HER2 detected by flow cytometry was decreased in TR and TCR cells compared to S cells (Fig. 3C). We found a heterogeneous population expressing HER2^{high} and HER2^{low} in TR and TCR cell types, indicating that during the prolonged exposure to T-DM1 we selected cells expressing low levels of HER2 and so with reduced sensitivity to the cytotoxic agent.

Tubulin β III expression and the polymerized tubulin fraction were decreased in resistant models

Following HER2, the second major target of T-DM1 are tubulins. We studied the expression of total α and β tubulin by Western Blot and found that it remained unchanged in TR and TCR cells compared to S, while the isotype β III was downregulated in TR and TCR cells (Fig. 4A). To determine the relation between β III tubulin expression and sensitivity to T-DM1, we transfected MDA-MB-361 S cell line with an siTUBB3. The downregulation of β III tubulin did not impact the sensitivity to T-DM1 in parental cells. However, we noticed that the population in S and G2/M phase was increased in S cells transfected with siTUBB3 (Fig. 4C). Thus, the downregulation of β III tubulin found in both resistant cell lines could affect cell division.

The percent of polymerized tubulin was studied after separation of soluble tubulin and microtubules (Fig. 4C). Total α and β tubulin affected to microtubules were decreased in both resistant cell lines, especially in TCR cells. Around 72 to 77% tubulin was found in the microtubule fraction of parental cells against 35%-41% in the TR cells and 18%-20% in TCR cells. The amount of β III tubulin affected to microtubules in the parental cell line was superior to that in both resistant cell lines.

T-DM1-induced cell cycle arrest is maintained in resistant cell lines

We studied cell cycle distribution after 24h exposure to T-DM1 in parental and resistant cells by propidium iodide staining and flow cytometry. MDA-MB-361 S cells were arrested in G2/M phase after exposure to T-DM1 (Fig. 6A). We found that the G1 population in non-exposed MDA-MB-361 TR and TCR cells was decreased compared to S cells while G2/M and polyploid populations were increased (Fig. 6B, 6C), suggesting that resistant cells contain an abnormally increased number of chromosomes. Even though the exposure to T-DM1 did not induce an increased G2/M phase population, we noticed a decrease in G1 phase in TCR cells and an increased amount of polyploid cells in both cell lines, indicating that resistant cells were sensitive to T-DM1-induced cell cycle arrest.

Resistant models to T-DM1 contain giant aneuploid cells

In order to confirm the differences in cell cycle distribution observed in resistant cells, we studied the DNA profile by propidium iodide staining and flow cytometry after cells reached confluence (Fig. 6A). The number of tetraploid (4N) and aneuploidy (>4N) in TR and TCR cells was increased compared to parental cells. These results lead us to evaluate the ploidy in parental and resistant cells by chromosome counting (Fig. 6B). The parental cell line contains 90% near diploid cells and 10% near triploid cells and while the number

of diploid cells was decreased in TR cells, it remained unchanged in TCR cells. The number of 4N and 5N cells was increased in both resistant cells lines, confirming the results obtained by flow cytometry. Also, we noticed that resistant cells have an increased size compared to parental cells. The mean diameter measured by the cellometer cell counter of cells in suspension and the SSC-FSC parameters determined by flow cytometry are increased in TR and TCR cells compared to parental cells (Fig. 6C, 6D). Altogether, these results indicate that resistant cell lines contain giant aneuploidy cells.

Discussion

Clinical studies have demonstrated that T-DM1 can lose its benefit in some patients despite continued treatment (14,15). We selected *in vitro* resistant models to T-DM1 using MDA-MB-361 breast cancer cells to characterize possible mechanisms of resistance.

The downregulation of HER at the cell surface may interfere with T-DM1 cytotoxicity in the selected cell lines. However, TR and TCR populations are heterogeneous for HER2 expression meaning that they contain HER2^{high} expressing cells that were resistant to T-DM1. We did not observe cross-resistance to DNA-damaging agents or TBA in TR cell lines even though the sensitivity to paclitaxel, vincristine and S-methyl DM1 was moderately but non-significantly decreased. Although TCR cells showed increased efflux activity, they did not develop cross-resistance to DNA-damaging agents. These results suggest that the increased efflux is not sufficient to protect cells against the toxicity of DNA-damaging molecules. The sensitivity to vincristine of TCR cells was significantly decreased. Altogether, the decreased sensitivity to TBA in TR and TCR could imply that resistance to T-DM1 is due to minor microtubule alterations. Both resistant cell lines remained sensitive to T-DM1-induced cell cycle arrest. However, we found aneuploid cells in TR and TCR cell lines. Taken together, these results suggest that TR and TCR cells survive in spite of cell cycle arrest which would lead to an increased chromosome number. The expression and interactions of cell cycle checkpoints and the integrity of the mitotic spindle should be explored to verify whether cycle arrest induces apoptosis.

Beside the increased number of chromosomes, we found a downregulation of β III tubulin associated with a decreased percent of polymerized tubulin. The different isoforms of tubulin have been described to possess different characteristics and dynamics (16,17). Hence, the decreased expression of β III tubulin in TR and TCR cell lines could be responsible for the decrease in the percent of polymerized. Interestingly, the downregulation of β III tubulin by siRNA in the parental cell line increased S and G2/M

populations while the sensitivity to T-DM1 remained unchanged. We hypothesize that the downregulation of β III tubulin was implicated in aberrant mitoses that lead to an abnormal chromosome number. Therefore, it is necessary to study whether β III tubulin downregulation is a direct cause of T-DM1 exposure or merely a consequence of other pathways being disrupted.

In summary, the downregulation of β III tubulin in MDA-MB-361 resistant cell lines to T-DM1 could be involved in cell cycle defects leading to an abnormal number of chromosomes. Yet, the downregulation by siRNA of β III tubulin did not modify sensitivity to T-DM1. Consequently, β III tubulin does not drive resistance to T-DM1. The pathways involved in cell cycle arrest and subsequent induction of apoptosis should be explored to identify targets to treat T-DM1 resistant cells.

Material and methods

Cell culture

The human Caucasian breast adenocarcinoma cell line MDA-MB-361 was cultured in DMEM medium supplemented with 10% fetal calf serum and 100 μ g/ml streptomycin at 37°C and 5% CO₂. Cells were counted using a Cellometer Auto T4 (Nexcelom Bioscience LLC).

Selection of TR and TCR cells was performed by exposure to increasing concentrations of T-DM1 for 6 months. CsA (C3662; Sigma-Aldrich) at 1 μ g/ml at the same time than T-DM1 for the selection of TCR cell line.

Anti-cancer agents

T-DM1 and S-methyl DM1 were kindly provided by Genentech and ImmunoGen respectively.

Cytotoxicity Assay

Cells were seeded in 96-well plates at a density of 5000 cells per well and incubated overnight. Increasing concentrations of chemotherapy agents were added to the media and 6 days later viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. 20 μ L of a 5 mg/ml MTT solution were added and plates were incubated at 37°C for 4h. The media/MTT mix was removed and 100 μ L of

4% HCl 1N/isopropanol per well were added to dissolve the purple formazan crystals. The absorbance was measured at 570 nm with 690 nm as a reference readout using a Thermo MultiSkan EX microplate reader. The absorbance of drug-exposed and control cells was compared to determine the percentage of living cells. IC50 values were calculated using CompuSyn software.

Real-Time Cell Analysis (RTCA)

The xCELLigence RTCA DP instrument (ACEA Bioscience) monitors cell impedance in real time. Cells were inoculated in E-plate 16 at a density of 10 000 cells per well and incubated overnight before addition of cytotoxic agents or PGE₂. The cell index was monitored for 1 week.

Efflux assay

Cell suspension was prepared with 4^{e6} cells in 10 ml of DMEM media containing 0.5 µg/ml Rhodamine 123 (Santa Cruz, sc-208306) and incubated for 30 min at 37°C, 5% CO₂. Cells were washed three times in cold DPBS on ice and some cells were taken for flow cytometry analysis ("uptake"). The remaining cells were incubated in DMEM media in the absence or presence of 3 µg/mL of CsA and incubated during 24h. Cells were suspended with trypsin and washed on ice before flow cytometry analysis.

Flow cytometry

Cells were incubated for 30 min at room temperature with the corresponding antibodies: HER2 (4225666) and BCRP1 (561180) from BD Bioscience and MDR1 (348608) from Biolegend or mouse IgG1 κ control isotypes from BD Pharmingen. Analysis was performed using a BD LSRII flow cytometer with BD FACSDiva software (BD Biosciences, San Diego, CA, USA) and FlowJo software (Tree Star, Ashland, OR, USA).

Annexin-V/Propidium Iodide Apoptosis Assay

Cells were seeded in 6 well plates at a density of 2^{e5} cells per well and incubated overnight. Then cells were exposed to T-DM1 for 72h. Cells were harvested, washed with cold DPBS + 10% SVF and stained using Annexin-V-FLUOS Staining Kit (Roche) according to the prescribed protocol and analysed by flow cytometry. Annexin-V positive cells exposed to T-DM1 were normalized to that of control for each cell line.

Cell cycle distribution analysis

Cells were seeded as described for the apoptosis assay, incubated and exposed to 100nM T-DM1 for 24h. Cells were harvested, washed with cold DPBS and incubated for

30 min at 4°C with propidium iodide (0.05 mg/mL) containing Nonidet-P40 (0.05%) and 4 µM of trisodium citrate. Cells were filtered using Falcon tubes with cell-strainer cap (352235) before flow cytometry analysis.

Western Blot

Protein extraction was performed using complete RIPA buffer (RIPA buffer, 1 mM DTT, 1M NaF, 100 mM sodium orthovanadate and protease and phosphatase inhibitors). After SDS PAGE separation, proteins were transferred onto a PVDF membrane by iBlot dry blotting system (Invitrogen). Membranes were incubated overnight at 4°C with primary antibodies and 1h at room temperature with secondary antibodies (IRDye Infrared Dyes from LI-COR Biosciences). Primary antibodies used were: HER2 (GTX50425; Genetex), βIII-tubulin (clone TUJ1), βII-tubulin (clone 7B9) α-tubulin (T6199), β-tubulin (T4026) and β-actin (A5441) from Sigma-Aldrich. Membranes were scanned using Odyssey infrared imaging system (LI-COR Biosciences) and densitometric quantification was performed with Odyssey software. Expression levels of proteins were normalized against β-actin.

Separation of soluble tubulin and microtubules

20 million cells were lysed in 300 µL of PEM 50DP Buffer (50mM Pipes, 1mM EGTA, 1mM MgSO₄, 0.05% sodium azide, 1mM DTT and proteinase inhibitors at pH 6.7) by three freeze-thaw cycles. Cells were ultracentrifuged (100000 g for 1h at 20°C) to separate soluble tubulin (supernatant) and microtubules (pellet). The pellet was suspended in 100 µL of PEM 50DP buffer, incubated on ice for 30 min to depolymerize tubulin and ultracentrifuged at 50000g for 45 min at 4°C to recover the supernatant. The supernatant was incubated with 1mM GTP for polymerization at 35°C for 30 min and ultracentrifuged at 50000g for 45 min at 35°C. After centrifugation, the supernatant was discarded and the pellet containing was suspended in 50 µL of PEM 50DP buffer. Tubulin amount in both fractions was studied by Western Blot.

RT-qPCR

RNA extraction was performed with the QIAamp RNeasy Mini Kit (QIAGEN) and followed by reverse transcription. Primers were design according to Roche sequences and quantitative PCR was performed with LightCycler 480 Real-Time PCR system (Roche Life Science, Indianapolis, IN, USA).

Statistical analysis

Each experiment was performed three times minimum and the results were presented in graphs as the mean \pm SD. Graphs and statistical analysis were performed using GraphPad Prism software. Statistics on cell survival experiments such as AnnexinV/PI staining or MTT assay were done by Two Way Anova followed by Bonferroni post-test. Statistics on gene expression by RT-qPCR were performed by Student t test.

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Figure 1

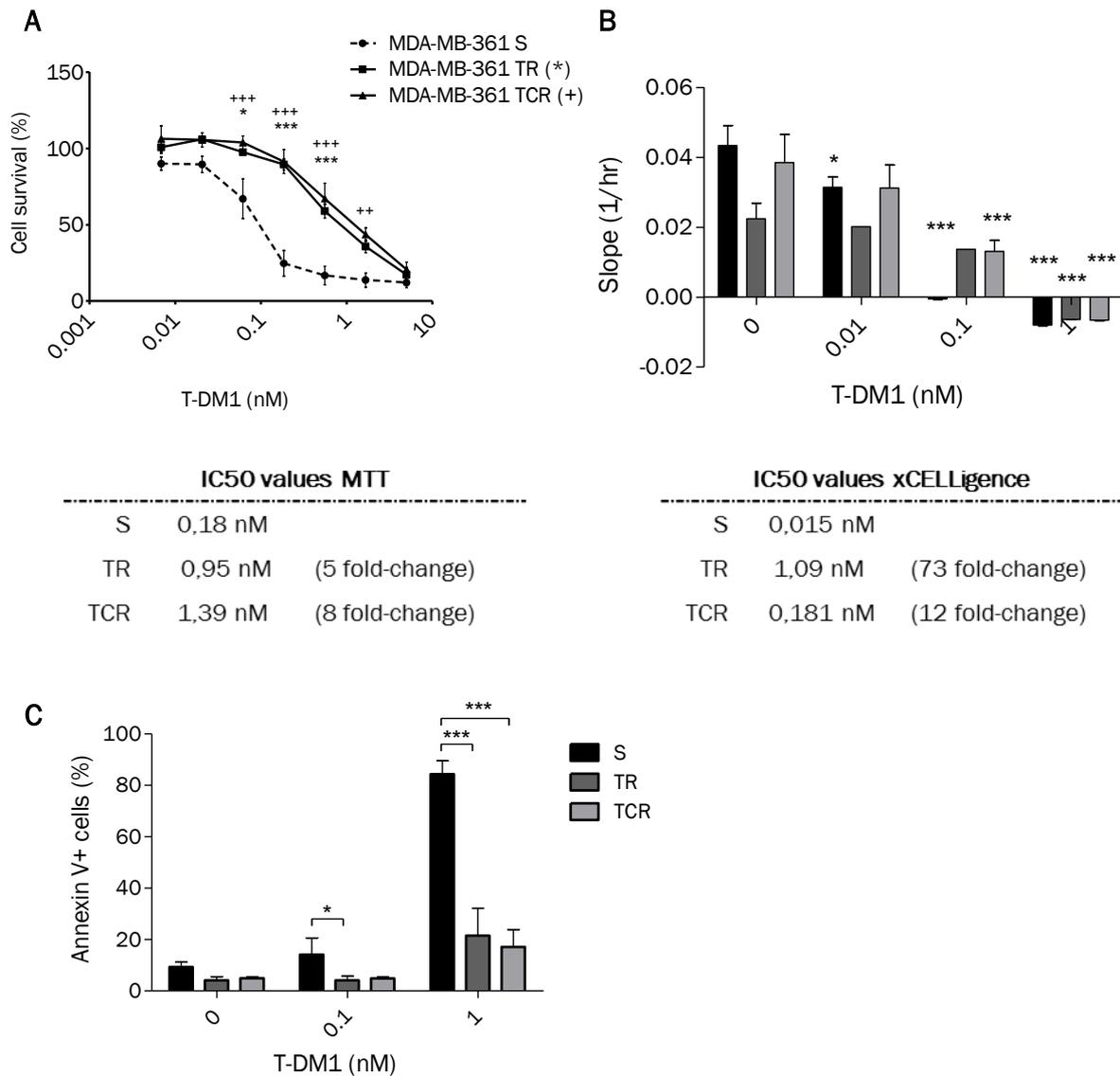


Fig. 1. Chronic exposure to T-DM1 of MDA-MB-361 cell line results in decreased sensitivity to the ADC. **A.** MTT cytotoxic assay of T-DM1 on MDA-MB-361 S, Tr and TCR shows an increase in the IC50 values of both resistant cells compared to parental. Statistics analysis was performed by Two way ANOVA followed by bonferroni posttests and differences are shown for TR (***: $P < 0,001$; **: $P < 0,01$; *: $P < 0,05$) and TCR (+) compared to S. **B.** Parental and resistant cells were exposed to increasing concentrations of T-DM1 and the cell index was followed by xCELLigence. The slopes of the normalized cell index determined the RTCA software were plotted. Statistics analysis was performed by Two way ANOVA followed by bonferroni posttests and differences are shown for each cell line between control and exposed conditions (*: $P < 0,05$; ***: $P < 0,001$). **C.** Annexin positive cells were studied by flow cytometry after 6 days exposure to T-DM1. The percent of Annexin positive cells decreased in TR and TCR compared to parental cells. Statistical analysis was performed by two-way ANOVA followed by bonferroni posttest (*: $P < 0,05$; ***: $P < 0,001$).

Table 1

Drug target		Drug name	MDA-MB-361 S	MDA-MB-361 TR		MDA-MB-361 TCR	
			IC50 (nM)	IC50 (nM)	Relative resistance	IC50 (nM)	Relative resistance
HER2		T-DM1	0,18	0,95	5,2^(*)	1,39	7,5
		lapatinib	4817	4428	0,9	3227	0,7
Topoisomerase I		irinotecan	3443	5203	1,5	3797	1,1
DNA	antimetabolite	fluorouracil	16227	9020	0,6	15323	0,9
	alkylating	cisplatin	3867	7023	1,8	9457	2,4
	intercalating	doxorubicin	6,57	7,31	1,1	8,81	1,3
Tubulin	polymerizing	paclitaxel	0,08	0,17	2,0	0,23	2,7
	depolymerizing	vincristine	0,12	0,40	3,2	0,67	5,3^(*)
		S-methyl DM1	0,07	0,16	2,3	0,13	1,8

Table 1. Sensitivity to HER2-targeted therapies and standard-care chemotherapeutics. The sensitivity after 6 days exposure to the indicated anti-cancer agents was studied by MTT cytotoxic assay. Data are shown as the mean IC50 calculated from 3-4 independent experiments and the relative resistance is the ratio of the IC50 for each resistant cell line over the parental cell line. Statistics were calculated by Student t test (*: $p < 0,05$).

Figure 2

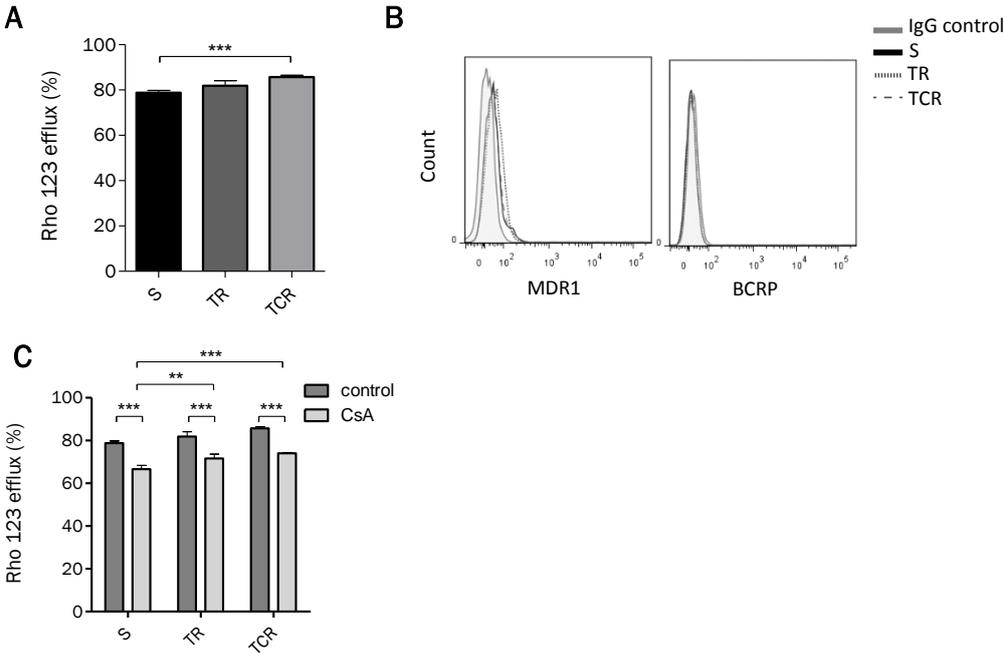


Fig. 2. Efflux activity is increased in TCR cells but MDR1 and BCRP expression remain unchanged.
A. Efflux activity was studied by accumulation of rhodamine 123 (Rho 123) using flow cytometry. The efflux activity is increased only in TCR cells compared to parental cells. **B.** The expression of MDR1 and BCRP detected by flow cytometry indicates that resistant cell lines do not overexpress neither ABC transporter. **C.** Efflux activity was studied in the absence of presence of CsA, an inhibitor of MDR1. The addition of CsA reduces the Rho123 efflux percent in parental and resistant cell lines.

Figure 3

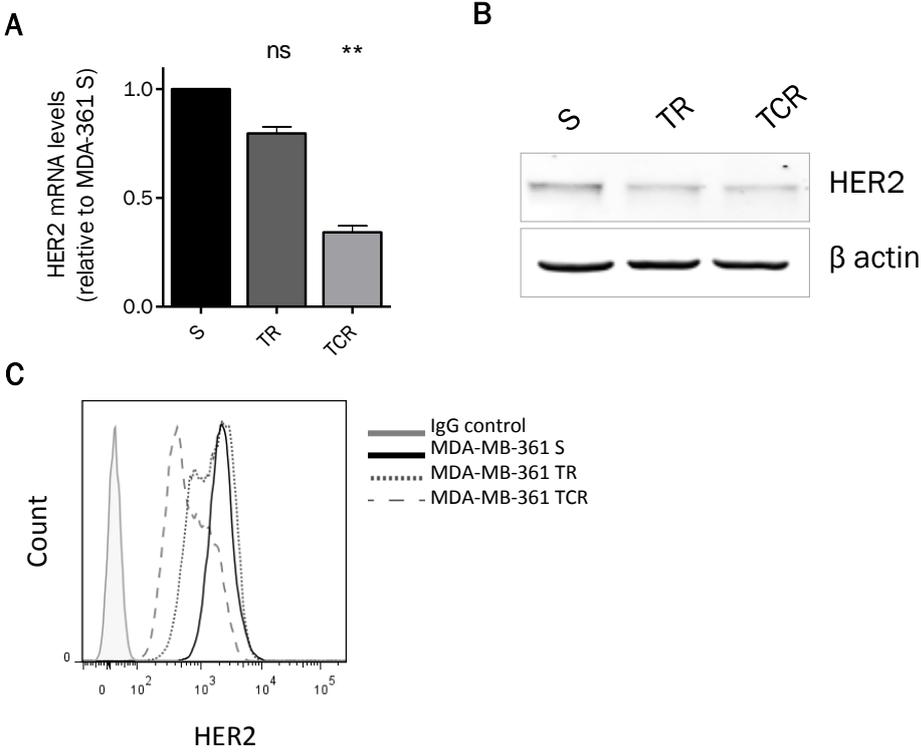


Fig. 3. HER2 expression is decreased in resistant cells to T-DM1. **A.** mRNA and **B.** protein expression from total cell lysates show that HER2 levels are decreased in resistant cells. Statistics were calculated by Student t test (**: $p < 0,01$). **C.** HER2 expression at the cell surface determined by flow cytometry shows distinct populations in resistant cells expression HER2^{high} and HER2^{low}. The total mean fluorescence intensity of HER2 is decreased in resistant cells compared to parental cells.

Figure 4

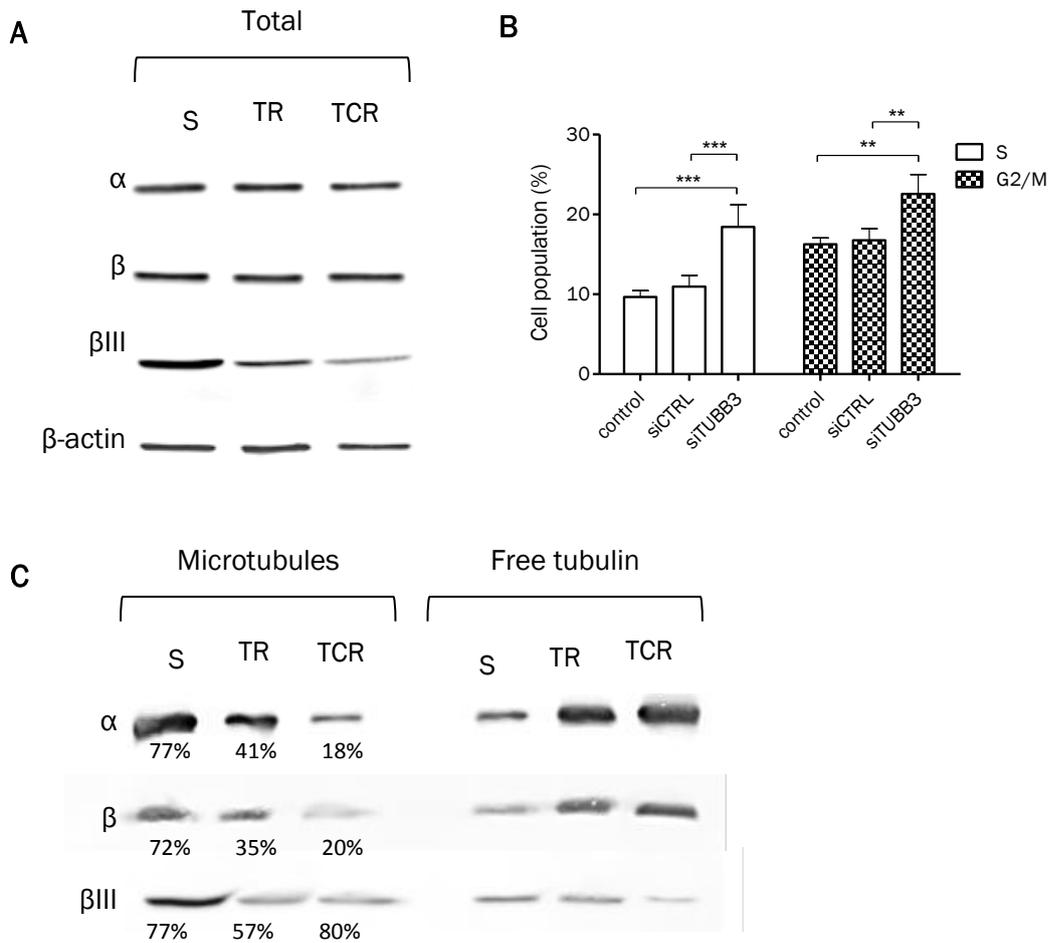


Fig. 4. Tubulin β III decreased expression coincides with decreased polymerized tubulin fraction and increased S and G2/M phase fractions. The protein expression of total α and β tubulin and isoform β III was studied in total cell lysates (**A**) or after purification of tubulin fractions (**C**). **A.** Western Blot of tubulins α , β and β III shows that while total α and β tubulin expression are unchanged, β III protein expression is decreased in resistant cells to T-DM1. The density of each band was normalized to actin and the value indicates the fold-change of expression. **B.** Down-regulation of β III tubulin in the parental cell line leads to increased S and G2/M populations after 48h of transfection by siRNA. **C.** Tubulin purification was performed to separate the polymerized (microtubules) and soluble (free) tubulin fractions. The percent values shown correspond to the percent of polymerized tubulin in each cell line, for each tubulin type. The percent of total α and β tubulin in microtubules is decreased in resistant cell lines compared to parental cells. The percent of β III tubulin in microtubules is decreased in TR cell line. Even though the percent of β III tubulin in microtubules is unchanged in TCR cells compared to parental cells, the density of the bands indicate a higher amount of β III tubulin in parental than resistant cells in the microtubules.

Figure 5

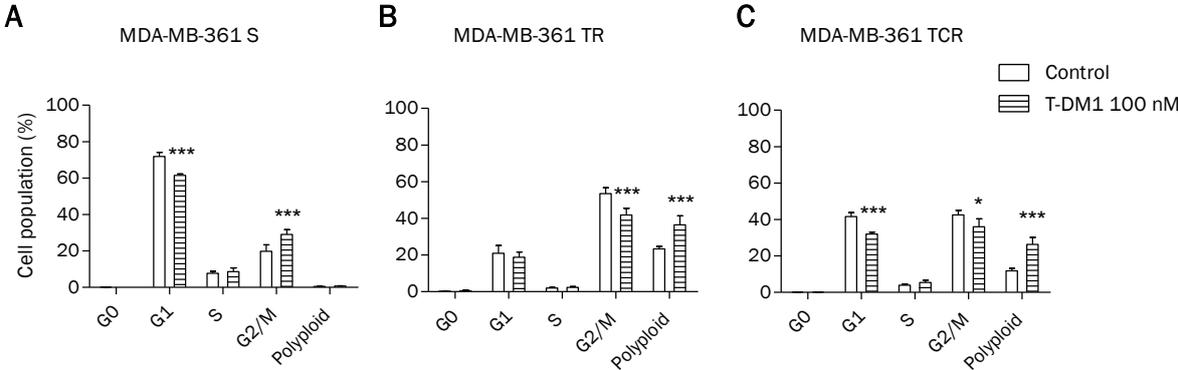


Fig. 5. T-DM1-induced cell cycle arrest is maintained in resistant cells. Cells were exposed to T-DM1 for 48h and cell cycle distribution was analyzed by propidium iodide staining using flow cytometry. T-DM1 induces G2/M phase arrest in the parental cell line. In both resistant cell lines, the DNA content is increased by $n > 2$ in control conditions and cells are arrested at G2/M phase ($2n$) as well as in a $n > 2$ phase.

Figure 6

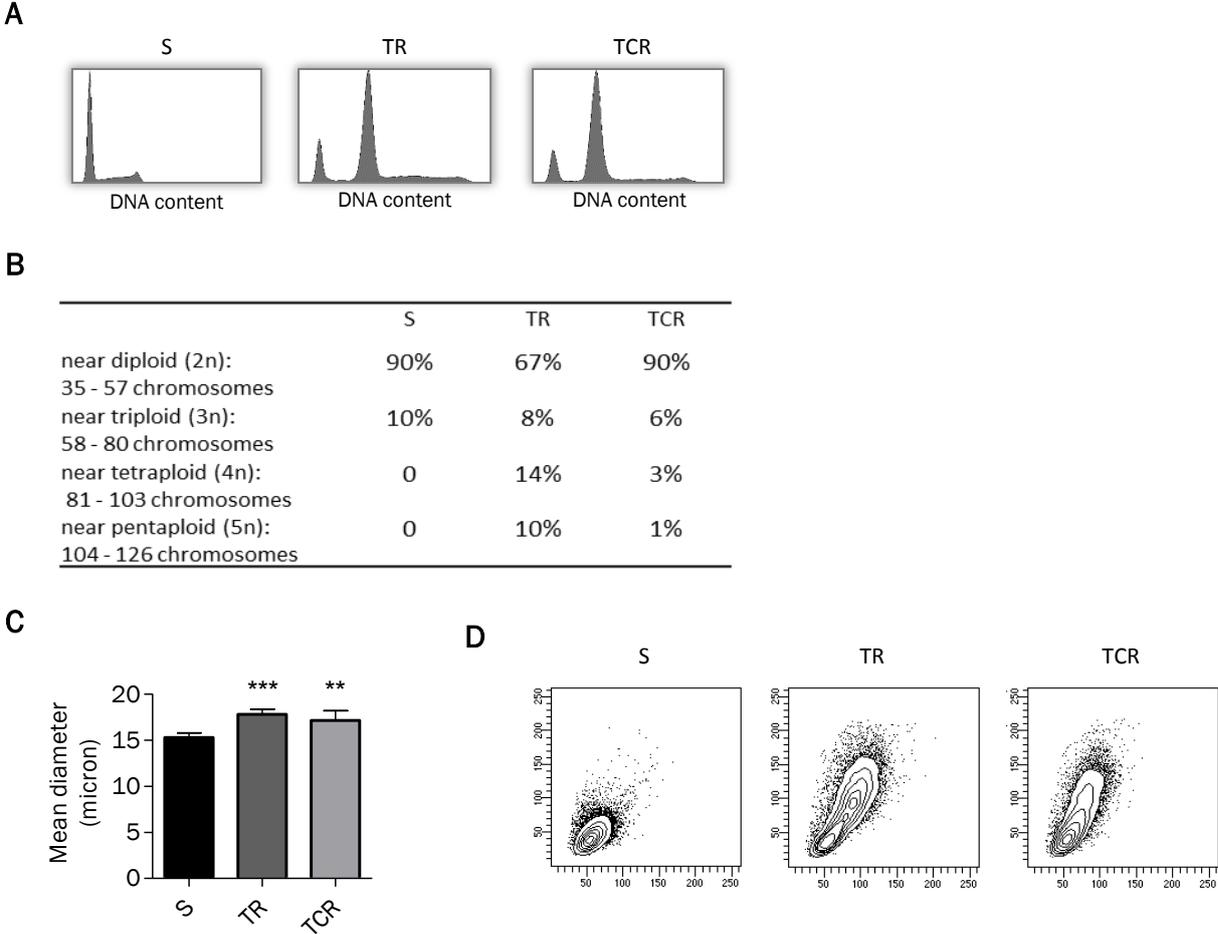


Fig. 6. Resistant cells to T-DM1 are giant aneuploid cells. A. The cell cycle distribution profile was studied by propidium iodide staining in confluent cells without exposure to any agent. The G2/M phase is increased and cells containing $n > 2$ appear in resistant cell lines. **B.** Chromosome count shows an increase of 3n, 4n and 5n cells in resistant cell line, especially in MDA-MB-361 TR. **C.** The mean diameter of cells in suspension was measured using a Cellometer counter. Cell size is increased in MDA-MB-361 TR and TCR cell lines compared to MDA-MB-361 S. **D.** The FSC (relative size) and SSC (Relative granularity) parameters determined by flow cytometry indicate a different size and complexity of cells in the TR and TCR populations compared to the S cells.

Breast cancer cell lines resistant to T-DM1 were selected by prolonged exposure to increasing doses of T-DM1. The efflux activity of both resistant cell lines was increased compared to the parental cell line. However, the expression of MDR1 and BCRP remained unchanged, suggesting that other ABC transporters are responsible for the efflux of the Lys-MCC-DM1 catabolite. In spite of the augmented efflux activity, the resistance models did not develop cross-resistance to DNA-damaging agents or TBA, although the sensitivity to paclitaxel, vincristine and S-methyl DM1 was slightly decreased. This data suggests that modifications of tubulin expression and/or activity could have occurred during the selection of resistant cell lines. The alterations in the microbutule/tubulin pools are complex and may be involved in resistance but the validation of this hypothesis is limited by the difficulty of reproducing the key alterations in model systems. In fact, the expression of β III tubulin was decreased in both resistant cell lines and was associated to a decreased percent of polymerized tubulin. Altogether, the alterations of tubulin could lead to a defective mitotic spindle and aberrant mitosis. The presence of aneuploid cells in the TR and TCR populations suggests that these cells are able to survive after an aberrant cell division. However, it is not clear if aneuploidy is the cause or the consequence of resistance to T-DM1. Interestingly, the parental cell line presented 10% of near triploid cells, proposing that it is prone to chromosomal instability. Combined to the hypothetic defects in the mitotic spindle, possibly caused by the prolonged exposure to T-DM1, the chromosomal instability could ensure survival. It could aloud cells to survive throughout aberrant mitoses in spite of the increasing number of chromosomes.

III. DISCUSSION

III.1. DEVELOPMENT OF T-DM1 RESISTANCE MODELS

The major obstacle for the complete remission of patients remains acquired resistance to treatment. Despite the efficacy shown by T-DM1 during the clinical trial leading towards its approval, it is expected that some patients become refractory to T-DM1 therapy. To propose alternative treatments in combination with or after T-DM1 regimen, the mechanisms leading to resistance need to be studied. We selected breast cancer models since T-DM1 is used for HER2-positive breast cancer patients, but also gastric cancer because trastuzumab is used as a targeted therapy and T-DM1 is currently being evaluated in this indication in clinical trials.

The concentration of drug chosen for the selection of resistance models *in vitro* should be relevant to the clinic. Very often, high concentrations induce resistance mechanisms such as an overexpression of ABC transporters or development of multiple cross-resistance (160,161). To develop our resistance models, we chose low concentrations of T-DM1 that were increased each time cells were able to sustain stable survival. Also, cells were constantly under T-DM1 exposure during the selection period in order to avoid the stochastic selection of non-dividing cells in which DM1 would not be expected to have a cytotoxic effect. The prolonged exposure to T-DM1 was performed in the absence of presence of ciclosporin A (CsA). CsA was first prescribed as an immunosuppressor following organ or marrow transplantation. Subsequently, CsA has been used as a MDR modulator since it binds to MDR1 at a common binding site to *vinca* alkaloids inhibiting its efflux activity (162,163). It was added at the same time as T-DM1 at non-lethal doses to prevent the efflux of Lys-MCC-DM1 mediated by MDR1.

III.2. MICROTUBULES AND RESISTANCE TO T-DM1

The abnormal expression of tubulin isotypes has been shown to be correlated with tumor aggressiveness and response to treatment, as reported in many pre-clinical and clinical studies (140). In clinical trials, the expression of β III tubulin has been correlated to the response and resistance to specific anti-cancer agents (164–169).

We found a modulation of β III tubulin expression in both cell line models resistant to T-DM1. In the esophageal cell lines β III expression was increased, particularly in OE-19 TR, while in the breast cancer cell lines it was decreased, particularly in MDA-MB-361 TCR. Each beta tubulin isotype has unique assembly properties and the isotype composition

can greatly influence microtubule dynamics (170,171). Thus, these expression changes may be responsible for the changes in microtubule assembly observed in the resistant cell line models. The percent of polymerized tubulin was increased in OE-19 TR compared to OE-19 and was associated to increased β III tubulin. Additionally, the fraction of polymerized tubulin in MDA-MB-361 resistant cell lines was decreased as well as β III tubulin expression. In the breast cancer cell lines, MDA-MB-361 TCR expressed lower levels of β III tubulin than TR and had a lower percentage of polymerized tubulin. Altogether, these results suggest that microtubule assembly was correlated with β III tubulin expression. Whether these alterations are a direct cause of resistance to T-DM1 or merely a consequence of other deregulated pathways needs to be investigated. To better understand the influence of β III tubulin on the response to T-DM1, we performed downregulation studies in the OE-19 TR cell line and the MDA-MB-361 parental cell line. In both cases, the downregulation of β III tubulin did not have an influence on the sensitivity to T-DM1. However, in MDA-MB-361 parental cell line, the downregulation increased the S and G2/M phase. Interestingly both breast cancer resistant cell lines showed a decreased sensitivity to vincristine and the presence of aneuploid cells. These results suggest that the prolonged exposure to T-DM1 affected the microtubule spindle and cell division. What remains to be studied is how these breast cancer cell lines adapted to survive after mitotic problems and with an increased number of chromosomes.

III.3. CELL ADHESION AND RESISTANCE TO T-DM1

Expression levels of adhesion molecules and regulators of the actin cytoskeleton were modified in resistant cell lines. Beside these alterations, the morphology of the cells became flat and polygonal compared to the rounded shape of the parental cells. Although both resistant cells lines presented these characteristics, only the OE-19 TCR cells showed an increased migration speed and decreased adhesion strength. Weak adhesions can correlate with high migration speed if we consider that in order to move, cells need a fast adhesion turnover. While new focal adhesions are being created at the migration front, the ones located at the back are being destroyed. New cell adhesions are described as small adhesions with high traction forces and weak adhesion strength (160,172). Besides migration, adhesion to the extracellular matrix (ECM) is necessary for survival since cells that detach from the ECM rapidly undergo apoptosis (173,174), a phenomenon designated by Frisch and Francis as anoikis. Upon contact with the ECM, integrins form clusters and serve as bridges between the extracellular and intracellular compartments. In a second step integrins then recruit a variety of proteins that enable cell motility and regulate cell survival (175,176). Changes in the integrin repertoire of cancer cells leads to anoikis resistance since it allows cells to attach to different ECM

(177). The sensitivity to anoikis was increased in both resistant cell lines compared to the parental cell line. These results indicate that T-DM1 resistant cell lines are probably more dependent upon survival signals triggered by adhesion molecules than the parental cell line. However, even though there was increased sensitivity to anoikis *in vitro*, cells may react differently *in vivo*. Since the integrin repertoire and the expression of other adhesion molecules were changed, resistant cells may attach to distant ECM more easily than the parental cells *in vivo*. The metastatic potential of these cell lines should be investigated in order to validate this hypothesis. Together, these results indicate that the prolonged exposure to T-DM1 altered the repertoire of adhesion molecules, probably granting an increased migration potential to TCR cells but rendering both resistant cell lines more dependent upon adhesion to survive than parental cells.

Tubulin binding agents (TBA) have been shown to induce cell death via a disorganization of the actin cytoskeleton leading to detachment and anoikis (178). In fact, microtubules can regulate focal adhesion turnover (179) and focal adhesions can alter microtubule dynamics (180,181). *In vitro* ovarian cancer cells resistant to taxol showed alterations in cell adhesion as well as altered microtubule dynamics (160). The prolonged exposure to T-DM1 altered the expression of β II and β III isoforms in our OE-19 resistant models, the percent of polymerized tubulin and its post-translational modifications. Accordingly, alongside the alterations in tubulin, T-DM1 appears to have modified cell adhesion in OE-19 TR and OE-19 TCR. Damiano et al. showed for the first time in 1991 that interactions of multiple myeloma cells with the ECM was an important determinant of drug response. The inhibition of drug-induced apoptosis mediated by adhesive molecules such as integrins was named cell adhesion-mediated drug resistance (CAM-DR) (148). Although CAM-DR was described for hematological malignancies at first, it has also been observed in solid tumors (152,182). Interestingly, OE-19 cell lines were cultured in standard flasks without any specific ECM coating. The transcriptomic changes in the integrin repertoire and other adhesion molecules may induce the expression of new adhesion proteins at the cell surface responsible for survival signaling. Most likely, the pro-survival signaling that can be triggered by integrins protects TR and TCR cells from T-DM1-induced cell death.

To validate whether the exposure to T-DM1 could have altered adhesion pathways contributing to resistance, we used targeted inhibitors of regulators of the actin cytoskeleton. The inhibition of RHOA increased the mortality of OE-19 TCR cells compared to OE-19 TR and S cells. This result indicates that TCR are more dependent on RHOA signaling pathway for survival than S and TR. Even though TR cells were sensitive to anoikis, the inhibition RHOA did not modify their survival in comparison to parental cells. Thus, the modifications in cell adhesion in TR cells do not seem to be associated with resistance to T-DM1 at the same degree as in TCR cells. To further investigate the

altered signaling pathway in OE-19 TCR cells, we used inhibitors of two RHOA targets: FAK and ROCK1. The inhibition of these proteins did not impact the sensitivity of either resistant cell line compared to the parental cell line. In depth studies need to be performed in order to identify the pathways downstream of RHOA contributing to resistance to T-DM1 in TCR cells.

III.4. THE PROSTAGLANDIN PATHWAY AND RESISTANCE TO T-DM1

Prostaglandins (PGs) are bioactive lipids synthesized by cyclooxygenases from arachidonic acid. They are necessary for normal development, but are also involved in inflammation and cancer. The expression of cyclooxygenase-2 (COX-2) is increased in premalignant and malignant cells compared to surrounding tissues. The overexpression of COX-2 is found in different types of cancer, including colorectal, stomach, esophagus, liver, pancreas, head and neck, lung, prostate and bladder and is associated with poor prognosis (183). Its overexpression most likely drives uncontrolled production of PGs, since increased COX-2 expression in cancer is often associated with an increased production of the downstream mediator PGE₂. The pro-inflammatory PGE₂ is abundantly produced by different types of cancer cell types and their surrounding cells and modulates proliferation, apoptosis, migration and invasion (184).

Several genes involved in the PGs pathway were upregulated in both resistant cell lines. The COX-2 gene was highly upregulated in OE-19 TR cell line compared to the parental cell line and moderately upregulated in OE-19 TCR. Genes coding for the PGs transporter PTG and the PGE₂ receptor EP2 were also found to be upregulated in both resistant cell lines. Besides an overexpression of *COX2*, the amount of PGE₂ was increased in the supernatant of OE-19 TR cell line compared to the parental cell line; but remained unchanged in the OE-19 TCR cell line. The increased quantity of PGE₂ could be due to the high expression of *COX2* leading to increased production and/or the overexpression of the PTG leading to increased transport of PGE₂. PGE₂ can support tumor growth by binding to its receptor located on cancer cells to promote survival (184). Thus, the increased amount of PGE₂ in OE-19 TR cells may be directly involved in resistance to T-DM1. PGE₂ might be released promoting cell survival and abolishing the anti-tumor effects of T-DM1. Increased COX-2 and PGE₂ induce resistance to EGFR targeted therapy in lung cancer. In non-small cell lung cancer, PGE₂ supports survival by phosphorylation of Erk in a PKC-dependent manner. Exposure to EGFR, Src or PKA inhibitors did not decrease Erk phosphorylation (155). The activation of pro-survival signals such as the MAPK pathway could be a mechanism to bypass the inhibition of HER2 downstream pathways by the antibody component of T-DM1. This hypothesis is plausible given that a

known mechanism of resistance to trastuzumab is the compensation of HER2 inhibition by increased IGF1R signaling, resulting in increased PI3K signaling (157,185).

In view of these observations we explored whether the addition of PGE₂ could protect the cells from T-DM1 cytotoxicity. Interestingly, when T-DM1 was combined to PGE₂ compared to the exposure to T-DM1 alone, the cell viability was decreased in both resistant cell lines with no effect on the parental cell line. However, PGE₂ alone did not modify cell survival. Even though the overexpression of *COX2* was higher in OE-19 TR cells than in OE-19 TCR cells, and that only TR cells showed increased amount of PGE₂, both resistant cell lines expressed high levels of PTG transporter and EP2 receptors. This could explain why the addition of extracellular PGE₂ had an effect on both cell lines independently of the endogenous levels of PGE₂.

This finding was unexpected according to the pro-tumoral role of PGE₂ that has been widely described in the literature. However, a dual role for COX-2/PGE₂ has been described in the literature and reviewed by Greenhough et al (186). For instance, the exogenous administration of an analogue of PGE₂ in *Apc^{Min/+}* mice resulted in reduction of number and size of intestinal tumors (187). Similarly, overexpression of COX-2 and increased levels of PGE₂ in the keratinocytes of transgenic mice protected them against skin tumor development (188). Likewise, PGE₂ was found to stimulate growth at low concentrations and to inhibit growth at high concentrations in human colorectal carcinoma cells; but to stimulate growth at low and high concentrations in colorectal carcinoma cells (189). Altogether, these results indicate that PGE₂ can have both pro and anti-tumoral activities, probably depending on the cell types and the experimental settings. In our experiments, the concentrations of PGE₂ used were in the pharmacological range, or 10⁶ fold higher than the ones observed in the supernatants of parental and resistant cell lines. At high concentrations, PGE₂ might induce a feed-back negative control on the PGs pathway. Hence, the down-regulation of EP receptors and/or increased degradation could abolish the pro-survival effects of the endogenous PGE₂, biasing cell signaling towards T-DM1-induced cell death. Also, PGE₂ at high concentrations may bind to less specific receptors and trigger anti-tumoral signaling pathways. However, cell death was induced only by the combination of T-DM1 with PGE₂ and not by the PG alone exclusively in the resistant cell lines. The pathways that are induced by PGE₂ could re-sensitize the cells to T-DM1 toxicity. Additionally, PGE₂ could modulate a target of T-DM1 that was previously deregulated as a mechanism of resistance. In depth studies are necessary to uncover the signaling pathways responsible for T-DM1/PGE₂-induced cell death. Overall, these results indicate that the prostaglandin pathway could be targeted in T-DM1 resistant cells.

The exposure to the nonsteroidal anti-inflammatory drug (NSAID) aspirin resulted in increased cell death of both resistant OE-19 cell lines compared to the parental cell line. The sensitivity to aspirin of OE-19 TR cells was higher than that of OE-19 TCR cells. Whether the increased sensitivity in OE-19 TR is related to the high expression of COX-2 still needs to be investigated. Nonetheless, aspirin is a non-selective inhibitor of COX-2, so we will explore the sensitivity to the selective COX-2 inhibitor celecoxib (190).

Combining T-DM1 to the inhibition of the COX-2 signaling pathway could be beneficial for HER2+ cancer patients overexpressing COX-2. Aspirin can be administered as chemoprevention for colorectal cancer or after the diagnosis of early stages of colorectal cancer (CRC), especially those overexpressing COX-2 (191). Major obstacles for the prolonged use of NSAIDs as anti-cancer agents are cardiovascular and gastrointestinal side effects. To avoid the toxicity of COX-2 inhibitors, one solution is to target the COX-2 derived signaling. The antagonists of PGE₂ receptors have shown promising inhibitory effects on tumor growth during preclinical studies (192–194).

IV. CONCLUSION AND PERSPECTIVES

The resistant models derived from breast and esophageal cancer appear to have developed different resistance mechanisms. In MDA-MB-361 resistant cell lines, T-DM1 resistance is associated with the presence of aneuploid cells. In these cells, pathways regulating cell cycle check points may have been altered, which would explain how cells can survive with an abnormal number of chromosomes. The cell cycle check point regulators and apoptotic proteins should be carefully studied in order to discover the mechanisms responsible for resistance and target these with specific inhibitors. In order to discover the underlying resistance mechanisms in MDA-MB-361 models, a transcriptomic or proteomic analysis remains to be performed. The deregulation of the adhesion molecules and prostaglandin pathway genes was found in both resistant OE-19 cell lines. However, their implications in the resistance to T-DM1 seem to differ. Even though both resistant cell lines were selected at the same time and same concentrations of T-DM1 they seem to have developed different resistance mechanisms. OE-19 TR cells express higher levels of COX-2 than TCR and have increased amount of PGE₂. Their sensitivity to aspirin is higher than that of TCR cells. Concerning TCR cells, the modulations in adhesion genes expression had an impact on migration, adhesion strength and sensitivity to the RHOA inhibitor Rhosin, contrary to TR. The biological processes leading to the overexpression of COX-2 and adhesion genes by T-DM1 needs to be further explored.

Our models are limited to those of a 2D system and consequently by the absence of 3D interactions as well as as the tumor microenvironment. The development of resistance models *in vivo* is necessary to confirm that the resistance mechanisms that we observed are found in a whole organism and to study their interactions with the tumor microenvironment. For example, increased PGE₂ levels or differential expression of adhesion molecules could modulate the microenvironment to support tumor growth. Moreover, the adhesion molecules could favor the invasion of surrounding tissues or increase the metastatic potential of cancer cells.

Resistant tumors developed *in vivo* might present other resistance mechanisms. We developed an *in vivo* resistance model using BT-474 breast cancer cell line. Our preliminary characterization showed that HER2 expression was unchanged and that *wt* tumors and T-DM1-resistant tumors both responded to vincristine treatment. Further analyses need to be performed to characterize cross-resistance to targeted therapy and chemotherapy, tubulin isotype composition and microtubule assembly. A global analysis

such as transcriptomic or proteomic assay or sequencing should be performed to identify the pathways involved in resistance.

The molecular pathways involved in resistance will need to be decrypted in order to discover the responsible genes and alternative therapeutic targets. Afterwards, these findings need to be explored in samples from patients treated with T-DM1. This comparison can validate which mechanisms of resistance found in the pre-clinical experiments are relevant in patients. Once the genes are validated, they could have a prognostic and hopefully predictive value for the response to T-DM1. Moreover, they can be targeted by different therapeutic strategies to induce death of cancer cells resistant to T-DM1.

V. FRENCH SUMMARY

La toxicité et l'efficacité limitée des thérapies conventionnelles contre le cancer ont poussé au développement de nouvelles stratégies. Des nouveaux médicaments sont conçus pour cibler et interférer avec des molécules spécifiques aux cellules cancéreuses. HER2 (Human Epidermal growth factor receptor 2) est un antigène de surface à activité tyrosine kinase qui est surexprimé dans à peu près 20% des cancers du sein. Il était associé à un mauvais pronostic, mais grâce au développement des thérapies ciblées, le pronostic des patients de cancer du sein HER2 positif est désormais amélioré. Les anticorps monoclonaux trastuzumab et pertuzumab sont utilisés pour cibler HER2 dans le cancer du sein, ainsi que le lapatinib qui est un inhibiteur de tyrosine kinase. A ces thérapies anti-HER2 s'est rajouté en 2013 le T-DM1, un immunoconjugué composé de l'anticorps trastuzumab lié au DM1, un agent anti-tubuline dérivé de la maytansine. Malgré l'efficacité démontrée par le T-DM1 lors des tests cliniques qui ont abouti à son approbation, la résistance acquise aux traitements anti-cancéreux reste un obstacle majeur dans le rétablissement complet des patients. Notre objectif était de développer des modèles de résistance au T-DM1 afin d'étudier les mécanismes qui en sont à l'origine et proposer des nouvelles stratégies thérapeutiques.

HER2 est surexprimé dans plusieurs types de cancers, dont le cancer du sein et le cancer gastrique. Le trastuzumab a été accepté pour le traitement des cancers gastriques en 2010. De ce fait, nous avons choisi de travailler avec ces deux modèles de cancer en utilisant la lignée de cancer de l'œsophage OE-19 et la lignée de cancer du sein MDA-MB-361. Dans le but de sélectionner des lignées résistantes *in vitro*, les cellules ont été exposées au T-DM1 à doses croissantes pendant une durée de 6 mois en absence ou en présence de ciclosporine A (CsA). La CsA est un inhibiteur du transporteur ABC MDR1 (P-gp), qui prend en charge les maytansinoides pour les effluer à l'extérieur des cellules. Nous avons obtenu deux lignées résistantes par type de cancer, que nous avons nommé TR celles qui ont été sélectionnées uniquement avec le T-DM1 et TCR celles qui ont été exposées au T-DM1 et à la CsA.

La première cible du T-DM1 étant la molécule de surface HER2, nous avons étudié son expression. Elle est restée inchangée chez les lignées OE-19 résistantes comparées à la lignée parentale. Au contraire, les lignées MDA-MB-361 TR et TCR étaient composées d'une population hétérogène, avec des cellules exprimant HER2 au même niveau que la lignée parentale et des cellules avec une diminution de l'expression en surface de HER2. Malgré cette diminution qui pourrait entraîner une baisse d'internalisation du T-DM1 et

donc une résistance, des cellules avec une forte expression de HER2 persistent. Ceci indique que d'autres mécanismes de résistance ont été acquis parmi ces lignées de cancer du sein.

Suite à l'internalisation du T-DM1, le métabolite actif est libéré et se lie aux microtubules. De ce fait, nous avons étudié la composition en isotypes de tubuline des cellules résistantes. L'expression de l'isotype β III était augmentée chez les lignées OE-19 TR et TCR alors qu'il était diminué chez les MDA-MB-361 TR et TCR par rapport à leur lignée parentale respective. Cependant, la diminution de l'expression de tubuline β III par siARN chez la lignée OE-19 TR ou MDA-MB-361 parentale n'a pas entraîné d'hypersensibilité au T-DM1. De ce fait, les changements d'expression observés chez les lignées résistantes semblent être la conséquence d'autres dérégulations plutôt que la cause de résistance.

En plus des altérations microtubulaires, les agents anti-tubuline peuvent entraîner des altérations du cytosquelette d'actine. Nous avons trouvé des dérégulations transcriptomiques des molécules d'adhésion chez les lignées OE-19 TR et TCR. La sensibilité à l'anoikis, ou mort cellulaire par détachement, était augmentée chez les deux lignées résistantes par rapport à la parentale. Ceci indique qu'elles seraient plus dépendantes que la lignée parentale aux signaux de survie transmis par les molécules d'adhésion. Cependant, seul la lignée TCR a eu une augmentation de la vitesse de migration et une diminution de la force d'adhésion. Aussi, uniquement la lignée OE-19 TCR a présenté une sensibilité accrue à un inhibiteur de RHOA, une protéine régulatrice du cytosquelette d'actine. Ces résultats mettent en évidence la possibilité de cibler la voie des adhésions focales comme stratégie thérapeutique en cas de résistance au T-DM1.

L'hyperactivité de la voie des prostaglandines est observée dans différents types de cancer et est associée à un mauvais pronostic. L'expression de la cyclooxygénase-2 (COX-2) et la quantité de prostaglandine 2 (PGE_2) extracellulaire étaient augmentées chez la lignée OE-19 TR. L'inhibition des cyclooxygénases 1 et 2 par l'aspirine a entraîné plus de mort cellulaire chez les OE-19 TR et TCR que chez la lignée parentale. Aussi, la combinaison de PGE_2 et de T-DM1 a induit plus de mort cellulaire chez les lignées résistantes que l'exposition au T-DM1 seul. La voie des prostaglandines pourrait donc être ciblée dans le cas de cancers résistants au T-DM1 qui surexpriment COX-2.

En conclusion, le développement des modèles de résistance au T-DM1 et leur caractérisation ont permis de proposer des nouvelles stratégies thérapeutiques. Etant donné l'hétérogénéité observée entre types de cancers et entre lignées cellulaires, des

analyses préalables seraient nécessaires afin de vérifier quelle voie de signalisation est affectée et utiliser des nouveaux traitements en conséquence. Les agents ciblant les régulateurs du cytosquelette d'actine ou la voie des prostaglandines devraient être testés en combinaison avec le T-DM1 afin d'étudier leur efficacité en préclinique.

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DEVELOPMENT AND CHARACTERIZATION OF MODELS OF RESISTANCE TO T-DM1

T-DM1 is an antibody-drug conjugate composed of the monoclonal antibody trastuzumab linked to DM1, a potent tubulin binding agent. Despite its efficacy in the treatment of HER2-positive breast cancer patients, acquired resistance to T-DM1 was observed during clinical trials. In order to study resistance mechanisms to T-DM1, we developed resistance models using OE-19 (esophageal) and MDA-MB-361 (breast) cancer cell lines in the absence or presence of ciclosporin A (CsA), an inhibitor of MDR1 mediated efflux. Resistant cells selected with T-DM1 alone are named "TR" and cells selected in the presence of T-DM1 and CsA are called "TCR". OE-19 TCR cells showed modifications in adhesion gene expression, migration and adhesion strength, combined with an increased sensitivity to a RHOA inhibitor. Also, OE-19 TR cells presented an overexpression of COX-2 associated with an increased amount of PGE₂ in the supernatant. A deregulation of the genes involved in the prostaglandin pathways was found in OE-19 TR and TCR cells, associated with increased sensitivity to aspirin. In conclusion, we found two signaling pathways deregulated in cell lines resistant to T-DM1. These results need to be validated using samples from patients resistant to T-DM1. Targeting the adhesion or the prostaglandin pathway could be of benefit for patients with T-DM1 resistant cancers.

DEVELOPPMENT ET CARACTERISATION DE MODELES DE RESISTANCE AU T-DM1

Le T-DM1 est un immunoconjugué composé de l'anticorps trastuzumab qui cible HER2 lié au DM1, un agent anti-tubuline dérivé de la maytansine. Malgré son efficacité, la résistance acquise au T-DM1 a été démontré lors des tests précliniques et chez certains patients. Nous avons développé des lignées résistantes à partir de la lignée de cancer du sein MDA-MB-361 et de la lignée de cancer de l'œsophage OE-19, que nous avons exposées au T-DM1 à doses croissantes pendant une longue durée en absence ou en présence de ciclosporine A (CsA). A partir de ces conditions nous avons obtenus les lignées "TR" qui ont été exposées uniquement au T-DM1 et "TCR" qui ont été exposées au T-DM1 et CsA. Nous avons observé une augmentation de la vitesse de migration et une diminution de la force d'adhésion chez OE-19 TCR associées à une sensibilité accrue à un inhibiteur de RHOA. Aussi, la voie des prostaglandines était dérégulée chez OE-19 TR et TCR, avec une forte augmentation de l'expression de COX-2 et de prostaglandine E2 dans la lignée OE-19 TR. La sensibilité à l'aspirine, un inhibiteur des cyclooxygénases 1-2, était accrue chez les deux lignées OE-19 résistantes par rapport à la lignée parentale. En conclusion nous avons démontré que différentes voies de signalisation peuvent être impliquées dans la résistance au T-DM1. Nos résultats restent à être validés chez les patients. Nous suggérons que cibler la voie de régulation de la composition du cytosquelette ou la voie des prostaglandines pourrait permettre d'obtenir un effet thérapeutique dans le cas de cancers résistants au T-DM1.

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