Study of the tumor sensitivity of the marine antitumoral compound elisidepsin and HER3 receptor in breast carcinomas

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Tesis doctoral

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Thesis performed in the Pathology department supervised by **Dr. Javier Hernández** and **Dr. Santiago Ramón y Cajal**.

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Als meus pares
i al meu germà

A la Mireia

Nothing is impossible. Not if you can imagine it.

That's what being a scientist is all about.

Prof. Hubert Farnsworth

Contents

Abbreviations

A	Ampere
Allo-	Allotrope, to denote the more stable of two geometric isomers
ASCO	American Society of Clinical Oncology
ATCC	American Type Culture Collection
b	Base
bp	Base Pair
°C	Grade Centigrade
C-	Carbon
C	Control
CAP	College of American Pathologists
CDDP	Cisplatin
cDNA	Complementary Deoxyribonucleic Acid
CEN	Centromere
CHX	Cycloheximide
CI	Combination Index
cm	Centimeter
CML	Chronic Myelogenous Leukemia
CNS	Central Nervous System
CO_2	Carbon Dioxide
COX-2	Cyclooxygenase-2
D	Dextrarotatory, enantiomers chemically related to a left-handed
Da	Dalton
DAPI	4´, 6-Diamino-2-phenylindole, dihidrochloride
DEP1	Density Enhanced Phosphatase-1
DNA	Deoxyribonucleic Acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
dNTP	Deoxynucleotide Triphosphate
DSS	Disuccinimidyl suberate
DTT	Dithiothreitol
DUB	De-ubiquitylating enzime
DW	Deionized Water
ECD	Extracellular Domain
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EJD	Extracellular Juxtamembrane Domain
ELISA	Enzyme-linked immunosorbent assay
Elisidepsin	Elisidepsin trifluoroacetate
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen Receptor
Exo sap	Exonuclease I and Shrimp Alkaline Phosphatase
F	Forward
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration

FISH	Fluorescence In Situ Hybridization
FGFR	Fibroblasts derived Growth Factor Receptor
5-FU	5-Fluorouracil
g	Gram
GC	Guanine and Cytosine
GPCR	G-protein coupled receptor
GRB2	Growth Factor Receptor Bound-2
GST	Glutathione S-Transferase
h	Hour
H	Hydrogen
H&E	hematoxylin and eosin
HB-EGF	Heparin Binding-EGF
HCL	Hydrochloric acid
HER	Human Epidermal Receptor
IC_{50}	The half maximal inhibitory concentration
ICD	Intracellular Domain
IHC	Immunohistochemistry
Ile	Isoleucine
IP	Immunoprecipitation
k	kilo
KF	Kahalalide F
L	Levorotatory, enantiomers chemically related to a right-handed
l	Liter
LUC	Luciferase
M	Molar
m	Mili
MAPK	Mitogen-Activated Protein Kinase
MDR	Multidrug Resistant
$MgCl_2$	Magnesium Chloride
min	Minute
mRNA	Messenger Ribonucleic Acid
MTD	Maximum Tolerated Dose
mTOR	Mammalian Target Of Rapamycin
n	Nano
N	Nitrogen
Na_3VaO_4	Sodium Orthovanadate
NaCl	Sodium Chloride
NaF	Sodium Fluoride
NaPPi	Sodium Pyrophosphate
NCI	National Cancer Institute
NP40	Nonidet P-40
Nrdp1	Neuregulin Receptor Degradation Protein-1
NRG	Neuregulin
NSCL	Non Small Cell Lung
NSCLC	Non Small Cell Lung Cancer
O	Oxygen

OG	Oregon Green
Orn	Ornithine
Oxa	Indicates that a chemical compound contains oxygen
Oxo	Indicates that a chemical compound contains oxygen link to another atom by a double bond
p	Short arm of a chromosome
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polimerase Chain Reaction
PD	Population Doubling
PDL	Population Doubling Level
pH	Potential Hydrogen
Phe	Phenylalanine
PI3K	Phosphatidilinositol 3-Kinase
PMSF	Phenylmethylsulfonyl Fluoride
PR	Progesterone Receptor
Pro	Proline
PTP1B	protein tyrosine phosphatase-1B
PVDF	polyvinylidene fluoride
R-	Rectus (right)
R	Resistance
R	Reverse
q	Long arm of a chromosome
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RTK	Receptor Tyrosine Kinase
S-	Sinister (left)
SDS	sodium dodecyl sulfate
sec	Second
shRNA	Short Hairpin RNA
SCL	Small Cell Lung
SD	Standard desviation
Shc	Src Homology-2 Containing
STAT	Signal Transducer and Activator of Transcription
TACE	Tumor Necrosis Factor-α Converting Enzyme
TAX	Paclitaxel
TBS	Tris Buffered Saline
TGF-α	Transforming Growth Factor-α.
Thr	Threonine
TKI	Tyrosine Kinase Inhibitors
TMD	Transmembrane Domain
U	Unit
µ	Micro
Val	Valine
WB	Western blot
WT	Wild type

Introduction

The oceans are a unique resource that provides a diverse array of natural products, primarily from invertebrates such as sponges, tunicates, bryozoans, and molluscs, and from marine bacteria and cyanobacteria. As infectious diseases evolve and develop resistance to existing pharmaceuticals, the marine environment provides novel leads against fungal, parasitic, bacterial, and viral diseases.

1. The Kahalalide family

Among opisthobranch marine mollusks (a subclass of Gastropods), members of two orders, the sluglike sea hares and nudibranchs have become frequent targets for chemical research, since most of them are conspicuous and accumulate secondary metabolites from their algal (Scheuer and Lehmann, 1977) or invertebrate animal diets. Another opisthobranch order, the sacoglossans, some of which have shells, are herbivorous with the ability to sequester from their algal diet functioning chloroplasts (Trench et al., 1972), which then may participate in the biosynthesis of secondary metabolites (Ireland and Scheuer, 1979): frequently polypropionates. The sacoglossan genus Elysia, also in the family Plakobranchidae, is represented in Hawaii by several species, among them Elysia degeneri and Elysia rufescens. Elysia degeneri is known to feed on Udotea specie, a green alga from which anti-feedant diterpene aldehydes have been isolated. Instead, Elysia rufescens (figure 1), an orange-fringed blue-green, small (1-4 cm), soft-bodied sacoglossan, which was observed feeding on the green alga Bryopsis specie, contains a series of difficultly separable depsipeptides ranking from a C_{31} tripeptide to a C_{75} tridecapeptide.

The kahalalides are constituents of Elysia rufescens and/or of Bryopsis specie, which are of neither diterpenoid nor polypropionate origin, but are amino and fatty acid-derived depsipeptides.

Figure 1. Herbivorous Marine Molusk Elysia rufescens. Alga Bryopsis species produces Kahalalide F, a major depsipeptide accumulated by Elysia rufescens. This compound protects both Bryopsis species and Elysia rufescens from fish predation, showing a defensive ecologic role and cytotoxicity against several cancer cell lines (Provencio et al., 2009).

1.1 The Kahalalide F family

Kahalalide F (KF) and analogues are the most promising compounds of the Kahalalide family because of their anti-tumoral activities (Hamann, 2004).
Natural KF is a cyclic peptide which occurs in Elysia rufescens and in its diet, Bryopsis specie derived from the marine mollusk Elysia rufescens (Hamann et al., 1996, Becerro et al., 2001).

KF is the largest with one fatty and thirteen amino acid residues of the peptides and it is the only member of the group with significant bioactivity. Among them, kahalalide A shows modest anti-malarial activity against Plasmodium falciparum and kahalalide E exhibits selective activity against Herpes simplex II virus (Hamann et al., 1996).

2. Structure of KF

Members of the KF family, which are head-to-side chain cyclodepsipeptides, have a complex structure, comprising six amino acids, among which are (*Z*)-didehydro-R-aminobutyric acid (formed from dehydratation of a threonine (Thr) residue), as a cyclic part between the carboxylic acid of a L-Valine (Val) and the secondary alcohol of the D-allo-Thr and an exocyclic chain of seven amino acids with a terminal aliphatic/fatty acid group (Bonnard et al., 2003). Compounds in which the fatty acid group is a methylhexanoic acid are of greatest interest (Albericio et al., 2005).

KF contains two positional isomers of similar structure and biological properties: 5-Methyl hexananoic acid KF (C75 H124 N14 O16) and 4S-Methyl hexananoic acid KF (C75 H124 N14 O16) (figure 2). Chemical names are: [1-Oxa-4, 7, 10, 13, 16- pentaazacyclononadecane, cyclic peptide derivative; L-Valine, N-(5- methyl-1-oxohexyl) -D- valyl -L- threonyl -L- valyl -D- valyl -D- prolyl -L-ornithyl -D- alloisoleucyl -D- allothreonyl -D- alloisoleucyl -D- valyl -L-phenylalanyl -(2Z) -2- amino-2-butenoyl-, (13 → 8)-lactone for 5-Methyl hexananoic acid KF; and [1-Oxa-4, 7, 10, 13, 16- pentaazacyclononadecane, cyclic peptide derivative; L-Valine, N-(4S- methyl-1- oxohexyl -D- valyl -L- threonyl -L-valyl -D- valyl -D- prolyl -L-ornithyl- D- alloisoleucyl -D- allothreonyl -D-alloisoleucyl -D- valyl -L- phenylalanyl-(2Z)-2-amino-2-butenoyl-, (13 → 8)-lactone for 4S-Methyl hexananoic acid KF.

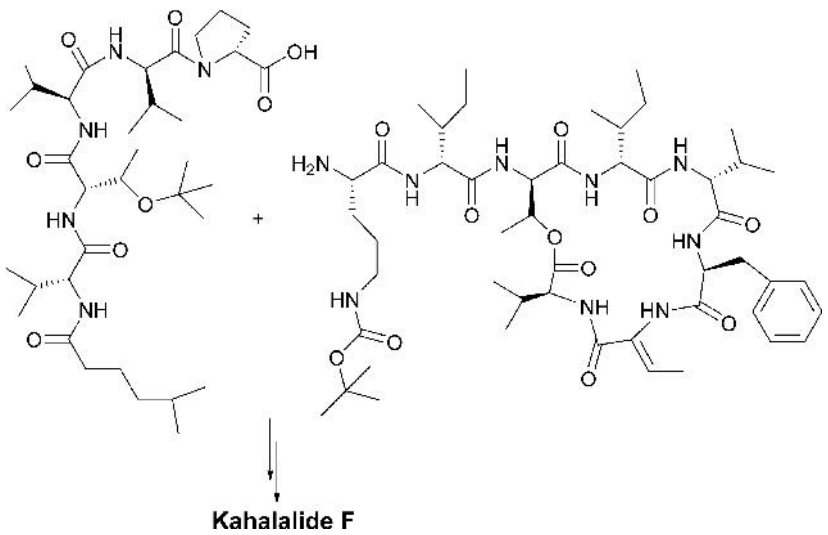


Figure 2. Structural formula of KF. This shows the peptide structure of the compound formed by two isomers: 5-Methyl hexananoic acid KF and 4S-Methyl hexananoic acid KF.

The structure of natural KF was initially elucidated by spectroscopic and physico-chemical methods, which concluded that the N-terminal fatty acid was 5-methylhexanoic acid (Hamann et al., 1996, Lopez-Macia et al., 2001)

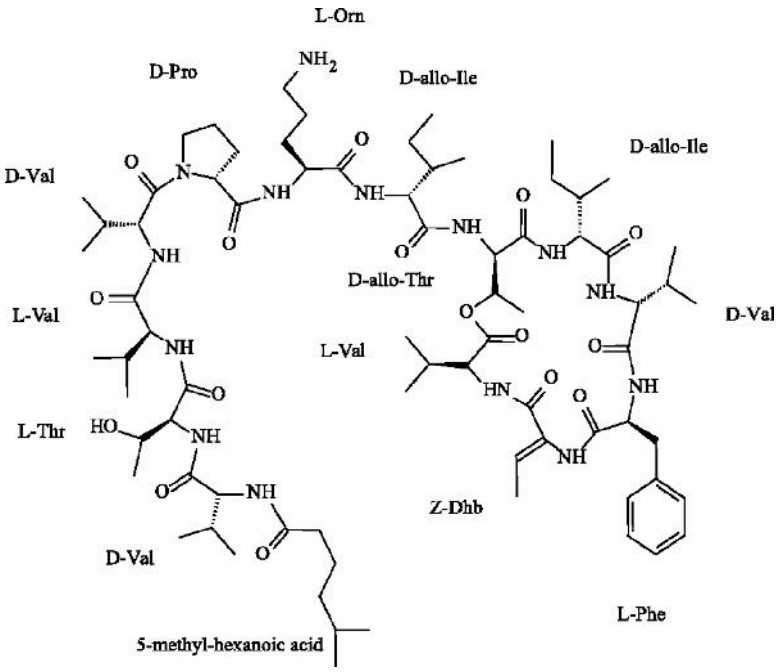


Figure 3. Structure of KF. The fatty acid group is connected to the lateral side. Residues of Thr-Ile-Orn-Pro-Val-Val-Thr-Val and 5-methylbenzoic acid form the lateral chain. The cycled region contains Phe-Val-Ile-Thr-Val and dehydroaminobutyric acid (Rademaker-Lakhai et al., 2005).

KF has the structure indicated in figure 3 involving a lateral chain formed by residues of threonine- isoleucine (Ile)- ornithine (Orn)- proline (Pro)- valine- valine- threonine- valine and 5 methylbenzoic acid and a cycled region containing phenylalanine (Phe)- valine- isoleucine- threonine- valine and dehydroaminobutyric acid. KF molecular formula is C77 H125 F3 N14 O18: (4S)-5-(((1R, 2S)-1-[(((3S, 6Z, 9S, 12R, 15R, 18R, 19R)-9-benzyl-6-ethyldene-3, 12-diisopropyl -19- methyl-15- [(1S)-1 methylpropyl]- 2, 5, 8, 11, 14, 17-hexaoxo- 1oxa- 4, 7, 10, 13, 16, pentaazacyclononadecan-18-yl) amino) carbonyl)] -2- methylbutyl) amino) -4- ([((2R)- 1((2R, 5S, 82, 11R, 16S) -8- [(1R) -1-hydroxyethyl] -2, 5, 11- triisopropyl -16 methyl- 4, 7, 10, 13- tetraoxo- 3, 6, 9, 12-tetraazaoctadec -1- anoyl) pyrrolidinyl) carbonyl] amino) 5-oxo-1-pentanaminum trifluoroacetate.

3. Elisidepsin trifluoroacetate (elisidepsin)

Elisidepsin (PM02734, KF) is a derivative of a natural marine compound extracted from the sacoglossan sea slug, Elysia rufescens.

Elisidepsin is a marine-derived synthetic cyclic depsipeptide with potential antineoplastic activity of the kahalalides family. It is a novel chemical entity resulting from PharmaMar's internal research program for derivatives of marine natural compounds, produced synthetically in-house by PharmaMar.

4. Antitumour activity

4.1 *In vitro*

The early preclinical data identified/characterized elisidepsin as a potent new chemical entity showing significant cytotoxic activity against solid tumor cell lines. Further evaluation demonstrated that this activity is selective for, but not restricted to, prostate tumor cells. Subsequent studies have identified tumor cells that overexpress the HER2 oncogene particularly sensitive target for elisidepsin. More recently, a differential sensitivity is seen among prostate tumor cells in which no activity is apparent against hormone responsive tumor cells. In general, elisidepsin cytotoxicity is not schedule-dependent and it is not a strong multidrug resistant (MDR) substrate as it is effective against many MDR tumor cell lines (Jimeno et al., 2004).

In vitro studies in cell lines of human origin evaluated by the National Cancer Institute (NCI) identified selective activity against breast, colon, central nervous system (CNS), leukemia, non small cell lung (NSCL), small cell lung (SCL), ovary, melanoma, renal and prostate cancer cells with a half maximal inhibitory concentration (IC_{50}) ranging from 200nM (prostate) to 10 μM (leukemia), table 1. The most sensitive cell lines were prostate tumor cells (Garcia-Rocha et al., 1996). Further evaluation demonstrated that elisidepsin shows selectivity for tumor cells compared with non-tumoral cells *in vitro* (Suarez et al., 2003).

Analysis of the pattern of tumor cell line sensitivities to elisidepsin using the NCI 'COMPARE' algorithm produced a weak correlation profile relative to standard chemotherapeutic agents; reinforcing the idea that elisidepsin may be a

novel compound by virtue of its mechanism of action(s).

Leukemia	NSCL	SCL	Colon	CNS
CCR-CEM	A549	DMS273	COLO 205	U251
10 μM	0,54 μM	30,2 μM	0,71 μM	2,09 μM
Melanoma	Ovary	Renal	Prostate	Breast
SK-MEL-28	SK-OV-3	ACHN	DU-145	T-47D
2,82 μM	1,41 μM	3,09 μM	0,20 μM	0,48 μM

Table 1. In vitro IC_{50} cytotoxicity: most sensitive of NCI tumor panel.

4.2 *In vivo*

The pharmacokinetic behavior of elisidepsin was characterized in mice, confirmed in rats, in conjunction with *in vitro* and *in vivo* antitumor activity studies, to discern the pattern of systemic exposure to the drug associated with efficacious dosing regiments (Brown et al., 2002). Elisidepsin is rapidly eliminated from plasma with limited binding to extravascular tissues.

The maximum tolerated dose (MTD) in tumor-bearing athymic female mice is 280 μg/kg either as an intraperitoneal or intravenous single bolus injection or as a total dose using an intraperitoneal/intravenous fractionated regimen. This compares with an MTD of 490 μg/kg in male athymic mice.

Elisidepsin is moderately active *in vivo* against leukemia and NSCL tumors and it is active against prostate tumors in strong correlation with its *in vitro* profile. Moreover, each of the activities is equally likely against either proximal or distal tumor implants from the intraperitoneal injection site.

Ling et al. observed a synergism of the combination of elisidepsin and erlotinib in *in vitro* and *in vivo* non-small cell lung cancer (NSCLC) models, which provide rational basis for exploring this combination in the clinic (Ling et al., 2009).

4.3 In patients

Elisidepsin has been selected for clinical development based on its *in vivo* activity in xenografted human tumors, as well as an acceptable non-clinical toxicology profile. Elisidepsin appears to be a rapid elimination substance with limited distribution (Stokvis et al., 2002, Stokvis et al., 2004). So far, the clinical phase I program for elisidepsin includes three studies. The dose limiting toxicity has consistently been grade for increases in aminotransferases. These increases occur early after infusion, and are asymptomatic and reversible (Rademaker-Lakhai et al., 2005). Elisidepsin has been tested in two Phase I clinical studies, the primary designed specifically for prostate cancer and a second aimed at solid tumors in general. So far, about 150 patients have been treated with this drug.

Significant doses of circulating compound in plasma have not shown important toxicity, and preliminary results are indicative of clinical benefit. The most important adverse effect was reversible liver toxicity. Asymptomatic elevation of terms was identified and no-other abnormalities of liver function were observed. No signs of renal toxicity, neurotoxicity and myelotoxicity have been detected (Stokvis et al., 2002, Rademaker-Lakhai et al., 2005). This activity together with the lack of myelotoxicity in the preclinical studies warranted the clinical investigation. Lack of myelotoxicity has already been confirmed in the early clinical trials. Some hints of efficacy have been identified in a broad spectrum of tumors tested in clinical trials.

Four exploratory phase II clinical trials have been initiated, including three different malignancies (hepatocellular carcinoma, malignant melanoma and NSCLC) and one in psoriasis (Jimeno et al., 2004).

5. Possible mechanisms of action

The primary mechanism of action of elisidepsin has not been fully identified, although multiple targets have been found and each is a membrane-associated event that may be related to the hydrophobic nature of the compound. Elisidepsin is a COMPARE (NCI) negative compound with in vitro selectivity in the NCI panel against a series of tumor cell lines, where PC-3 and DU-145 prostate

tumor cells are the most sensitive. No specific cell cycle block has been determined in sensitive tumor cell lines.

Elisidepsin has been evaluated in a battery of standard cell-free biochemical assays. It does not inhibit macromolecular synthesis (DNA, RNA and protein), polymerase activities (DNA and RNA) or topoisomerase enzymatic activities (I or II). Moreover, strongly induces p53-independent apoptosis. It is not greatly affected by strong MDR cell lines (PC-3 prostate, CACO-2 colon, UO-31 renal, MCF-7/ breast) or cell lines resistant to topoisomerase II inhibitors (Janmaat et al., 2005).
Furthermore, elisidepsin induces cell death via oncosis preferentially in tumoral cells (Suarez et al., 2003). Preclinical toxicity studies showed CNS and kidney as the main targets, with liver also being affected (Brown et al., 2002).

Normal (COS-1, monkey kidney; Vero, monkey kidney; NRK, rat kidney; BHK, baby hamster kidney) and tumor (HeLa, human epitheloid cervical carcinoma) cells exposed to biologically relevant concentrations of elisidepsin detach from their substrate and become markedly swollen, which is associated with the formation of large intracellular vacuoles (Garcia-Rocha et al., 1996). The effect on cell adhesion has not been explained by changes in organization of cytoskeletal structures, which remain intact. However, the subcellular effect might be explained if elisidepsin is inserted as an ionophore in membranes favoring an increase in cation permeability thus causing a passive water influx and resulting in cisternal dilation. This mechanism would be similar to that of compounds like the carboxylic ionophore, monensin (Tartakoff, 1983).

Other studies found that elisidepsin alters the function of the lysosomal membrane (Garcia-Rocha et al., 1996), a characteristic that distinguishes it from all other known antitumor agents. Moreover, there is an increase in lysosomal pH, but the morphologies of the endoplasmic reticulum and the Golgi apparatus appear to be unaffected by the action of the drug. Cell swelling induced by elisidepsin results in a decrease in cell cytoplasm size, without altering the cytoskeletal (microtubule) network, and in cell detachment (from the culture dish

surface) and death.

This compound also inhibits transforming growth factor-α (TGF-α) expression, blocks intracellular signaling pathways downstream of the epidermal growth factor (EGF) and HER2 (Wosikowski et al., 1997). These growth factors control the tyrosine kinase class I subfamily that normally mediates signal transduction in the "ras" signaling pathway. Many cancer types, especially some breast and ovarian tumors cells overexpress the amount of these components leading to the "activation" of the oncogene.

6. The HER family

The human epidermal growth factor receptor (HER) family of proteins was originally named because of their homology to the erythroblastoma viral oncogene product, v-erbB. It comprises four cell surface receptors, named epidermal growth factor receptor (EGFR, HER1 or c-erbB1), HER2 (neu or c-erbB-2), HER3 (c-erbB-3) and HER4 (c-erbB4) (Witton et al., 2003). These four molecules comprise the type I group of 20 families of receptor tyrosine kinases (RTKs) and regulate several cellular metabolic reactions (van der Geer et al., 1994, Gschwind et al., 2004).

HER1 (Kondo and Shimizu, 1983) was the first receptor of the HER family identified and is expressed by a gene localized on chromosome 7p12.3-p12.1 and its molecular weight is 170 kDa (Boonstra et al., 1995). First identified by Schechter et al. in 1984 (Schechter et al., 1984), HER2 is a transmembrane 185 kDa protein and its gene is localized on chromosome 17q21 (Yamamoto et al., 1986). The third member of the HER proto-oncogene family, HER3, is localized on chromosome 12q13 and expresses a protein with molecular weight of 185 kDa (Kraus et al., 1989). The last member of the HER family classified was HER4 in 1993, and its gene is located on chromosome 2q33 (Plowman et al., 1993), resulting in a protein of 180 kDa.

7. Characteristics of HER family

The receptors are composed of a large extracellular ligand-binding domain, which has four subdomains (I–IV), followed by a transmembrane domain, a small intracellular juxtamembrane domain preceding the kinase domain, and a carboxy terminal tail, on which the docking sites for phosphotyrosine-binding effector molecules are found/localized. Of the four subdomains in the extracellular region of the HER receptors, subdomains I and III are leucine-rich repeats that function in ligand binding, whereas subdomains II and IV are laminin-like, cysteine-rich domains (Citri and Yarden, 2006).

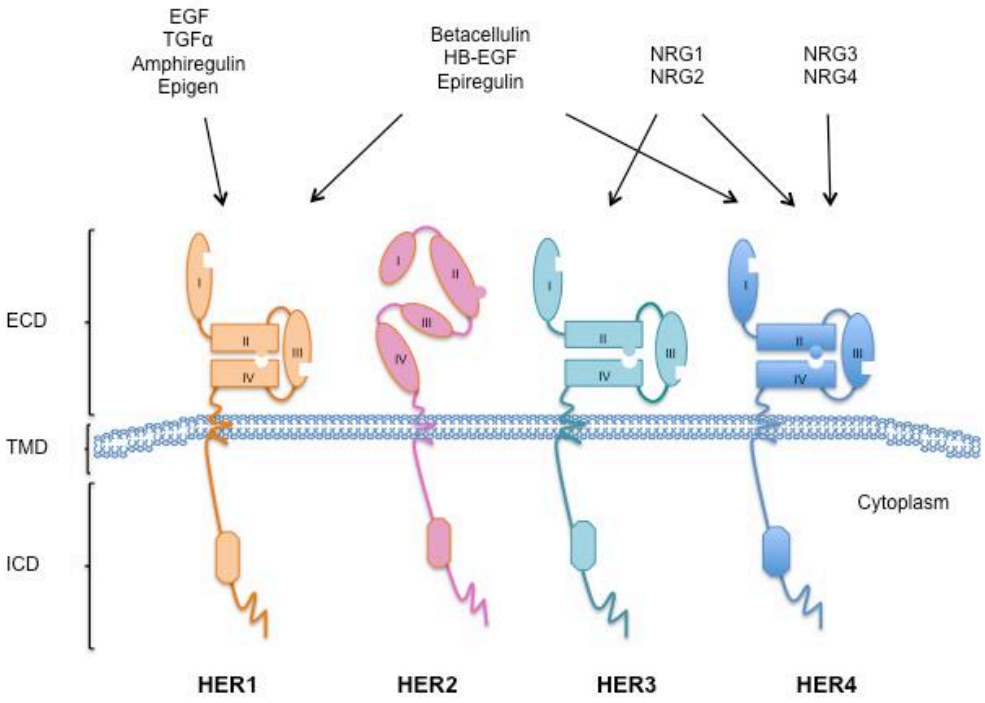


Figure 4. HER and their ligands. HER consist of an extracellular domain (ECD) comprised of two leucine rich ligand binding subdomains (I and III) and two cysteine rich subdomains (II and IV). The transmembrane domain (TMD) connects to the intracellular domain (ICD) containing the bilobed (N and C) protein tyrosine kinase subdomain via a short juxtamembrane sequence. The C-terminal region contains the multiple regulatory tyrosine residues which become phosphorylated on receptor activation.

HB-EGF, heparin-binding EGF-like growth factor; NRG, neuregulin.

The prototypic HER in its monomeric state is autoinhibited through the interaction of domains II and IV, mantaining subdomains I and III at a distance that does not allow simultaneous binding of ligand to both subdomains, and at the same time sequesters the dimerization loop (figure 4). In the dimeric, ligand-bound form, domains I and III are brought together, forcing a separation of domains II and IV and promoting the accessibility of the dimerization loop within domain II for interaction with the docking site of the dimerization receptor (Burgess et al., 2003) forming homo- or hetero-dimers. In contrast, the dimerization loop in HER2 is constitutively extended even in its monomeric state and a strong interaction of domains I and III closes the binding pocket, so that HER2 is always poised for the interactions with ligand-bound receptors of the family (Garrett et al., 2003).

8. Signaling through HERs

HER receptors are cell membrane receptor tyrosine kinases that are activated following ligand binding and receptor dimerization. This dimerization transduces additional conformational changes to the cytoplasmic domains of the receptors, eventually exposing and activating their tyrosine kinase catalytic domains by self- or trans-phosphorylation. Subsequent phosphorylation of tyrosine residues leads to an interaction of the activated receptor with adaptor proteins or enzymes to promote downstream signaling that produces a physiological outcome (figure 5). The HER receptors are well known mediators of cell proliferation, migration, survival, cell cycle, adhesion, motility, apoptosis, and cytoskeletal regulation.

Two of the main pathways activated by these receptors are the phosphatidilinositol 3-kinase (PI3K)-Akt and the mitogen-activated protein kinase (MAPK) pathways (figure 5). Other important HER signaling effectors are the signal transducer and activator of transcription proteins (STATs), which in cancer are often associated with HER1 activation; c-Src tyrosine kinase, the activity of which is increased in response to HER1 and HER2 signaling, and mammalian target of rapamycin (mTOR), a serine-threonine kinase activated downstream of PI3K-Akt and other growth regulators (Hynes and Lane, 2005).

When HER1 when is phosphorylated it recruits signal transducers such as the adaptor protein growth-factor-receptor-bound-2 (GRB2), Src-homology-2-containing (Shc) and STAT5 (Schulze et al., 2005).
The cytoplasmic terminus of HER1 also contains a tyrosine residue whose phosphorylation is recognized by the ubiquitin ligase Cbl, that mediates its proteasome dependent degradation (Levkowitz et al., 1999). No site that can directly recruit the lipid kinase PI3K has been found, so that it cannot directly activate the PI3K-Akt pathway, but it couples to the Ras-MAPK pathway through Shc, as well as to the Ras-PI3K-Akt pathway (Scaltriti and Baselga, 2006).

HER2 is the preferred heterodimeric partner of the other HER receptors; actually, it binds to a much larger subset of phosphotyrosine binding proteins than the other ligand binding receptors of the family (Schulze et al., 2005), and HER2 containing heterodimers have a slower rate of endocytosis (Baulida et al., 1996) and a higher affinity and broader specificity for various ligands than other heterodimers. These particularities can be translated into the strong activation of multiple signaling pathways and potent mitogenic signals (Citri and Yarden, 2006).

In addition to the well understood function of HER2 as a transmembrane receptor, a nuclear function as a transcription factor has also been proposed, based on the observation that a small percentage of cellular HER2 can be found in the nucleus and DNA binding and transcriptional activation of at least one promoter has been reported (Xie and Hung, 1994, Wang et al., 2004). So far, the genes for cyclooxygenase-2 (COX-2) (Wang et al., 2004, Vadlamudi et al., 1999), the chemokine receptor CXCR4 (Li et al., 2004) and the E26 transformation specific transcription factors (Goueli and Janknecht, 2004) have been reported as direct transcriptional targets of HER2.

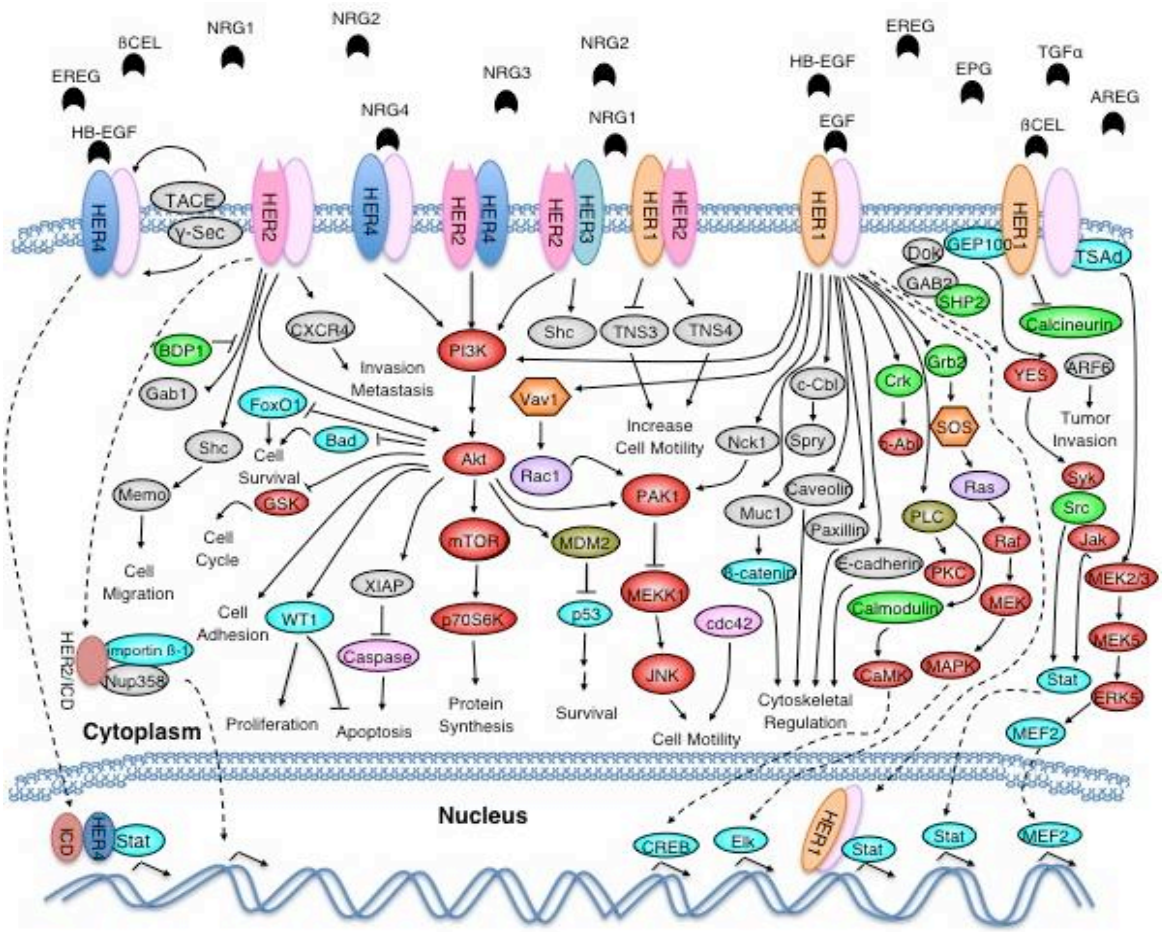


Figure 5. HER pathway activation. General overview of HER driven signaling pathways.

Although HER3 has no site for GRB2 (Schulze et al., 2005), it can recruit Shc to one site and p85, the regulatory subunit of PI3K, to six distinct sites. Despite this, in 1994 Guy et al. found that HER3 has an active ligand binding domain but it lacks intrinsic tyrosine kinase activity. HER3 has a kinase domain that lacks several key conserved (and catalytically important) amino acid residues but has recently been shown to retain sufficient kinase activity to robustly trans-autophosphorylate its intracellular region, although it is substantially less active than HER1 (Shi et al., 2010). Unlike HER1, endocytosed ligand-bound HER3 is more likely to follow the recycling pathway rather than Cbl-mediated degradation (Wallasch et al., 1995, Baulida et al., 1996, Waterman et al., 1999).

HER4 shares recognition and signaling features with HER1 and is associated with differentiation (Srinivasan et al., 1998). Upon ligand binding, the cytoplasmic domain of HER4 also recruits GRB2, Shc and STAT5. Alternative splicing generates four functionally unique isoforms of HER4. Structurally, the HER4 isoforms differ at the extracellular juxtamembrane domain (EJD-1 and EJD-2 isoforms) or at the intracellular domain (ICD-1 and ICD-2 isoforms). The EJD-1 isoform is susceptible to two-step proteolysis by tumor necrosis factor-α converting enzyme (TACE) and γ-secretase activity, generating soluble intracellular domains that can translocate into nucleus and regulate transcription (Ni et al., 2001, Citri and Yarden, 2006). The ICD-1 isoforms, but not the ICD-2 isoforms, contain a direct binding site for PI3K that is necessary for NRG1 induced chemotaxis and survival mediated by non-cleavable HER4 ICD-1 in NIH 3T3 cells (Elenius et al., 1999, Kainulainen et al., 2000). On the other hand, the cleavable EJD-1 and ICD-2 isoform seems to be unique among the isoforms in being capable to induce of ligand-independent signaling by a mechanism involving proteolytic generation of a stable phosphorylated ICD (Maatta et al., 2006, Sundvall et al., 2007, Sundvall et al., 2008).

9. The HER family ligands

The natural mechanism of activation of the HER family is by ligand binding (Schlessinger, 2000). All members of the HER family, apart from HER2, are activated by a group of ligands that are expressed as transmembrane precursor protein molecules, which possess a conserved EGF-like domain (Heldin, 1996). These ligands are expressed by 10 different genes and include EGF (Heimberg et al., 1965), the first ligand to be identified, betacellulin, TGF-α, HB-EGF, epiregulin, amphiregulin and four NRGs (Barros et al., 2010). Alternative splicing of the mRNA products of NRGs genes generates at least 20 different NRG isoforms (Falls, 2003, Breuleux, 2007, Hayes et al., 2007). Most of these isoforms are synthesized as transmembrane molecules that expose the biologically active EGF-like molecule to the extracellular space (Wen et al., 1994).

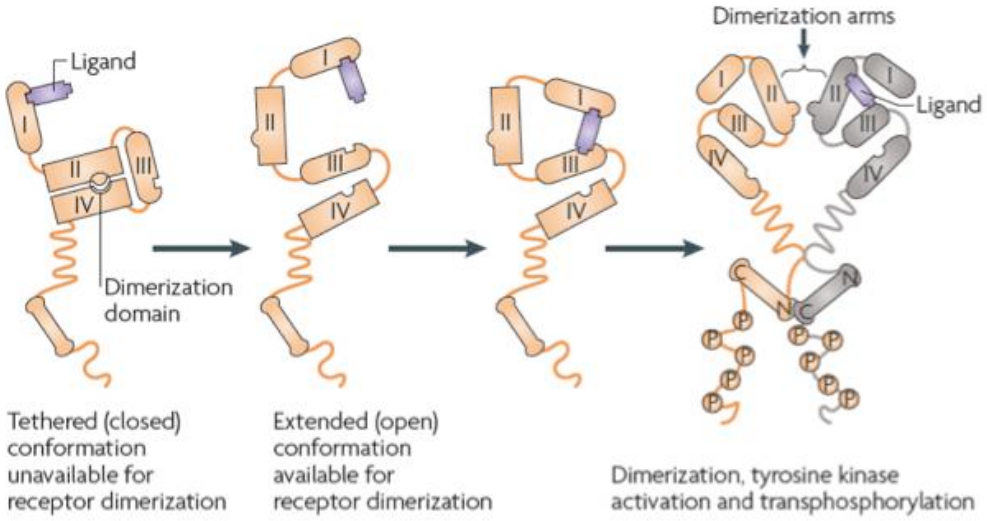


Figure 8. Conceptualization of the receptor conformational change on ligand binding. Ligand binding to HER seems to induce a conformational change in the folded structure of the molecule that exposes the dimerization domain; this step is required for dimer formation and functional activation of HER1, HER3 and HER4 (Baselga and Swain, 2009) .

Ligands can either display receptor specificity (for example, EGF, amphiregulin and epigen bind HER1) or bind to one or more related receptors; NRGs 1-4 bind HER3 and HER4 while HB-EGF, epiregulin and betacellulin activate HER1 and HER4 (figure 4). HER2 has no known ligand (Pinkas-Kramarski et al., 1996). Despite this, MUC4, a member of the mucine family, acts as an intramembrane modulator of HER2 activity (Komatsu et al., 2001). Each HER member has specific ligands, which they interact to encourage dimerization (figure 8).

10. Regulation of HER signaling

The HER network integrates not only its own inputs, but also heterologous signals, including hormones, neurotransmitters, lymphokines and stress inducers. Many of these interactions are mediated by protein kinases that directly phosphorylate HERs, affecting their kinase activity or endocytic transport. There are also autocrine and paracrine loops, in which EGF-like ligands, as well as angiogenic factors are produced following receptor activation (Schulze et al.,

2001). Besides functioning as receptors on the cell surface, HER family proteins are also present in the nucleus to act as transcriptional regulators. For example, membrane-bound HER2 interacts with importin β1 and Nup358 and migrates to the nucleus via endocytic vesicles. Inside the nucleus, HER2 modulates the transcription of multiple downstream genes including the COX-2.
Only a few studies of nuclear localization of HER3 have been reported. In immortalized human breast cells and breast cancer cell lines, full-length HER3 showed prominent nuclear localization with several antibodies and techniques (Offterdinger et al., 2002). HER3 has also been detected in nuclei of Schwann cells (Raabe et al., 2004), lung cancer (Kawano et al., 2008) and prostate cancer, where in the last one, an association with disease progression was found (Koumakpayi et al., 2006).

It is becoming apparent that a large set of inhibitory proteins act to attenuate the signal emanating from activated receptors (Rubin et al., 2005). These negative regulators can either exist prior to receptor activation or they are newly synthesized following signaling initiation. Indeed, receptor activation not only instigates multiple positively acting pathways, such as the Ras-MAPK and the PI3K-Akt cascades, but also sets in motion mechanisms that will ultimately terminate signaling. In mammalian RTK attenuators such as the pan-HER inhibitor RALT/Mig-6 and the fibroblasts derived growth factor receptor (FGFR) inhibitor, Sef, are newly synthesized in response to growth factors. On the other hand, pre-existing attenuators control receptor dephosphorylation and degradation; known are the targeting for internalization and degradation in lysosomes or tyrosine phosphatases as density enhanced phosphatase-1 (DEP1) and protein tyrosine phosphatase-1B (PTP1B) (Schulze et al., 2001).

In many cells, HERs are found in caveolae. HER1 is unique among the HER family, as it undergoes endocytic degradation after ligand mediated activation and homodimerization, in contrast to the other members of the family, which undergo endocytic recycling (Baulida et al., 1996). Internalization into endosomes is followed by recycling back to the membrane in a kinase independent manner (Wang et al., 2005). In the endosomes, autophosphorylated receptors might

recruit the E3 ubiquitin ligase Cbl and undergo ubiquitylation. Ubiquitins then recruit adaptors that contain an ubiquitin interacting motif, such as GRB2, and target internalized receptors to lysosomes for degradation (Levkowitz et al., 1999). De-ubiquitylating enzymes (DUBs) might negatively regulate this process and target HER proteins to the default recycling pathway.

HER2 endocytosis differs significantly from that observed for HER1, as it avoids efficient endocytic down-regulation. Although no consensus model has been achieved, it is proposed that HER2 could suffer an inefficient internalization or an efficient recycling back to the plasma membrane (Roepstorff et al., 2008). Compared with other members of the family, HER2 is less likely to suffer inactivating mechanisms and its recruitment into heterodimeric signaling complexes leads to prolonged signaling (Karunagaran et al., 1996).

Although little is known about endocytosis of HER3 and HER4, both of the receptors can be ubiquitylated. In the case of HER3, the RING finger E3 ubiquitin ligase neuregulin receptor degradation protein-1 (Nrdp1) associates with HER3 in an activation independent manner (Diamonti et al., 2002) and is involved in its trafficking. Nrdp1 mediates the redistribution of HER3 from cell surface to intracellular compartments and induces the suppression of HER3 and HER4 receptor levels but not HER1 or HER2 levels.
HER4 is inefficiently targeted to the endocytic pathway but its known that HER4 is ubiquitylated by the ubiquitin ligase Itch (Roepstorff et al., 2008).

11. HERs and cancer: therapies based on HERs targeting

HER family members are often overexpressed, amplified or mutated in many forms of cancer, making them important therapeutic targets (table 2). HER1 is found to be overexpressed in more than 80% of head and neck cancers, activated by amplification and/or mutation in approximately 50% of gliomas (Kang et al., 2006, Tsugu et al., 1997), and activated by mutation in about 10–15% of NSCLC (Gorgoulis et al., 1992). Overall, deletions in exon 19 and the point mutation of L858R in lung cancer constitute about 90% of all HER1 activating

mutations (Kumar et al., 2008). The most frequent mutant is called EGFRvIII and lacks the dimerization arm and a part of the ligand-binding domain; it is constitutively active at the plasma membrane and evades down-regulation mechanisms (Ge et al., 2002, Okamoto et al., 2003, Sok et al., 2006).

HER2 amplification and/or overexpression are seen in about 20-25% of human breast carcinomas (Slamon et al., 1987, Slamon et al., 1989, Alimandi et al., 1995) and cancers such as stomach, colon, endometrium and ovary. The mutational activation of HER2 is also seen in lung cancer (Soung et al., 2006).

HER3 is expressed at abnormally high levels in several tumor types, including cancers of the breast, ovary, prostate, lung or head and neck cancer (Koumakpayi et al., 2006, Ciardiello et al., 1991, Friess et al., 1995, Kountourakis et al., 2006, Lemoine et al., 1992a, Lozano et al., 2005, Naidu et al., 1998, Tanner et al., 2006), but activating mutations have not been reported. The HER2/HER3 heterodimer is the crucial mediator of HER2 signaling in tumors with HER2 amplification. This dimer is the strongest known transforming and mitogenic receptor complex, with additional functions, such as increased cell motility upon ligand stimulation (Citri and Yarden, 2006, Holbro et al., 2003a, Wallasch et al., 1995).

Although mutations in the kinase domain of HER4 have been reported in some cancers (Soung et al., 2006), in contrast to the other family members, the current evidence suggests that HER4 mediates antiproliferative effects and so, HER4 overactivity does not appear to play a major role in cancer pathogenesis (Sartor et al., 2001, Hsieh and Moasser, 2007). In addition to cancer, HER4 polymorphisms have been associated with schizophrenia (Law et al., 2007).

Overexpression and co-expression of a HER receptor and one or more of its ligands can establish an autocrine loop that drives uncontrolled cell growth. In cancer patients, the autocrine production of TGF-α or EGF is associated with reduced survival (Tateishi et al., 1990). This mechanism was also proposed for NRG (Hansen et al., 2006).

HER1	HER2	HER3	HER4
Bladder*	Bladder		
Breast*	Breast*	Breast	Breast
Cervical*			
			Chronic myelogenous leukemia
Colorectal	Colorectal*	Colorectal*	
Endometrial*	Endometrial*	Endometrial*	
Esophagus*	Esophagus		
Head and neck*	Head and neck*	Head and neck*	Head and neck*
Glioma*			
Lung*	Lung*	Lung*	Lung
Melanoma	Melanoma	Melanoma	Melanoma
Ovary*	Ovary*	Ovary*	Ovary*
Pancreas*		Pancreas*	Pancreas
Prostate	Prosate*	Prostate*	Prostate
Renal	Renal	Renal	
Sarcomas			
Skin			Skin
Stomach*	Stomach*		
Thyroid		Thyroid	Thyroid

* Clinical studies have linked overexpression and /or mutation of this HER receptor to a worse prognosis

Table 2. Malignancies overexpressing wild type and mutated forms of HER receptors. CML, Chronic myelogenous leukemia (Rowinsky, 2004).

The abundance of evidence that implicates overactive HER signaling in the genesis and progression of a number of human cancers has led to the development of drugs that selectively target these proteins (table 3). Two classes of drugs have thus so far shown clinical efficacy and many of the ongoing pharmaceutical efforts continue to be in the realm of these two classes. One is monoclonal antibodies that can target extracellular epitopes and the other is small molecule cell permeable inhibitors of catalytic kinase function, which bind

to the ATP binding site, preventing signal transduction of both the Ras-MAPK and PI3K-Akt pathways.

Antibodies currently used in cancer therapy include trastuzumab (Herceptin, which targets HER2) for treatment of breast tumors, cetuximab (Erbitux, which targets HER1) for treatment of colon and head and neck cancers, and panitumumab (Vectibix, which targets HER1) for treatment of colon cancer. Novel experimental therapeutic approaches target the heterodimerization of HER2 (pertuzumab, Omnitarg), or use antibody combinations to promote receptor degradation (Hynes and Lane, 2005, Citri and Yarden, 2006). Clinical testing of pertuzumab is currently ongoing, and preliminary evidence shows promising activity against HER2 overexpressing breast cancers (Baselga et al., 2010).

Drug	Class	Molecular target	Diseases
Cetuximab (Erbitux)	IgG1 mAb	HER1 ECD	Colon, head & neck cancer
Panitumumab (Vectibix)	IgG2 mAb	HER1 ECD	Colon cancer
Trastuzumab (Herceptin)	IgG1 mAb	HER2 ECD	Breast cancer
Gefitinib (Iressa)	Quinazoline	HER kinase (HER1>HER2)	Lung cancer
Erlotinib (Tarceva)	Quinazoline	HER kinase (HER1>HER2)	Lung & pancreatic cancer
Lapatinib (Tykerb)	Quinazoline	HER kinase (HER1 & HER2)	Breast cancer

Table 3. Therapies targeting HER receptors in clinical use (Sergina et al., 2007).

Improvements in potency and selectivity of tyrosine kinase inhibitors (TKIs) led to the development of clinically useful agents in the late 1990s based

on the quinazoline structure. Three HER-targeting TKIs have thus far been approved for clinical use. These include gefitinib (Iressa, which targets HER1), erlotinib (Tarceva, which targets HER1), and lapatinib (Tykerb), a dual inhibitor that target both HER1 and HER2 (Baselga, 2010).

12. The HER3 gene and its expression

HER3 was discovered in 1989 (Kraus et al., 1989) and it maps to chromosome 12q13.2, which is 23.2 kb in size and consists of 28 exons. The gene and protein sequences for the extracellular ligand-binding domain of HER3 have 43–45% homology with HER1 and HER2, and 56–67% with HER4; the cytoplasmic tyrosine kinase domain sequences have 60–63% homology with those of each of the other HERs (Sithanandam and Anderson, 2008).

HER3 is transcribed as a 6.2 kb message of 4080 nucleotides and 1342 codons specifying the full-length protein, although there are several HER3 truncated transcripts. A 1.4 kb transcript codes for the first 140 amino acids of extracellular domain I followed by 43 unique amino acids. This transcript is widely expressed in normal and neoplastic cells. It codes for a 24 kDa protein which in mammalian cells forms an intracellular 58 kDa glycosylated dimer that does not appear to bind ligand; the potential functions of this intracellular HER3 form remain to be determined (Sithanandam and Anderson, 2008).

There are four additional alternate transcripts of 1.6, 1.7, 2.1 and 2.3 kb, generated by intron read-through. At least two of this code for truncated, secreted sHER3. A p45 sHER3 consists of extracellular domains I and II and part of domain III, plus 2 unique C-terminal amino acids. A p85-sHER3 is formed by domains I, II and III and part of IV, with addition of 24 unique C-terminal amino acids. Both forms, and especially the p85 sHER3, bound NRG1 and reduced NRG1 activity as a ligand on breast carcinoma cells. Thus, these sHER3 forms may be potential negative regulators of NRG1 (Sithanandam and Anderson, 2008).

Relatively little is known about the regulation of HER3 transcription. The

HER3 promoter region is GC rich (65%) and, like HER1, does not contain a TATA box; there are several transcriptional start sites. AP2-1 (OB2-1) is a potential nuclear factor-binding site, which positively regulates the high expression of HER3 protein in human breast carcinoma cells.
HER3 expression has recently been found to be regulated by α6β4 integrin in breast carcinoma cells, evidently by effects on translation (Folgiero et al., 2007), by miR-205, a new oncosuppressor gene in breast cancer, able to interfere with the proliferative pathway mediated by the HER receptor family (Iorio et al., 2009) and by ZNF217 which contributes to the transcripcional dysregulation of HER3 in breast cancer (Krig et al., 2010).

Attempts to determine the role of HER3 in development have been impeded by the embryonic lethality caused by HER3 gene disruption, which leads to cardiac and neurodevelopmental defects (Erickson et al., 1997). In human fetuses, HER3 transcripts are detected in liver, kidney and brain but not in heart or lung fibroblasts. HER3 is widely expressed in human adult tissues, being consistently detected in brain, spinal cord, liver, prostate, kidney and lung.

13. HER3 in cancer

HER3 is unique in its ability to channel HER signaling to the PI3K-Akt signaling pathway, which undoubtedly favors tumor growth and progression. HER3 is overexpressed in breast, endometrial, head and neck, lung, melanoma, ovary, prostate, colon, renal and thyroid cancer (Rowinsky, 2004).
In primary breast and colon cancers, overexpression of HER3 is associated with overexpression of HER2 (Naidu et al., 1998), suggesting that in these tumors the HER2/HER3 heterodimer functions as an oncogenic unit. Another important observation pertaining to HER heterodimer collaboration during tumor development is that expression of HER3 is seen in many of the same tumor types that overexpress HER2, including breast, bladder and melanomas (Lemoine et al., 1992b) (Rajkumar et al., 1996b) (Rajkumar et al., 1996a) (Bodey et al., 1997). Furthermore, many HER2 overexpressing breast tumors display elevated levels of phosphotyrosine on HER3 (Alimandi et al., 1995), probably as a result of spontaneous dimerization with HER2. Moreover, mammary tumors of transgenic

mice expressing transforming Neu mutants exhibit selective upregulation of HER3 expression and activity (Siegel et al., 1999), suggesting that there might be a selective advantage/pressure leading to co-expression of both receptors.
In 2006, nuclear HER3 was reported in immunohistochemical analysis of prostate cancer samples and association with disease progression was found (Koumakpayi et al., 2006), while HER3 expression in tumor tissue was associated with survival of patients with primary ovarian cancer (Tanner et al., 2006). Also, HER3 overexpression was strongly associated with tumor progression and poor prognosis of patients with gastric cancer (Hayashi et al., 2008) and more recently, HER3 protein levels at the cancer cell plasma membrane were directly correlated with reduced survival in colorectal cancer patients (Gespach, 2011).

A study on melanoma cells has shown that stimulation with NRG induced phosphorylation of HER3 and the acquisition of metastatic properties, including MMP-9 expression, invasion, adhesion and experimental lung metastasis *in vivo*. In addition, phosphorylation of HER1 was rapidly induced by NRG, suggesting that HER1 is a possible heterodimeric counterpart of HER3. Also, HER3 phosphorylation may occur without NRG1, via the activation of HER1 by its specific ligands. RNA interference experiments demonstrated that subcutaneous tumor growth and angiogenesis was attenuated by inactivation of HER3 in cancer cells (Ueno et al., 2008). Complexation of HER3 with HER1 is a prominent phenomenon in some models (Fernandes et al., 1999, Li et al., 2006). In several types of transfected cells, expression of HER1 is sufficient to allow activation of HER3 in response to EGF (Kim et al., 1998, Soltoff et al., 1994, Kim et al., 1994). HER3 is a highly receptive substrate for HER1 tyrosine kinase activity (Sierke et al., 1997), although Tyr1289 in HER3, important as a PI3K binding site, shows a relatively low activity in contrast to other tyrosines (Fan et al., 2005). HER3 also has the potential to activate HER1: in several models involving high expression of transduced HERs, NRG1-stimulated HER3 activated HER1 (Soltoff et al., 1994, Zhang et al., 1996).

The HER network might integrate not only its own inputs, but also heterologous signals, including hormones, neurotransmitters, lymphokines and

stress inducers. Many of these interactions are mediated by protein kinases that directly phosphorylate HERs, affecting their kinase activity or endocytic transport. The best studied mechanism involves activation by G-protein coupled receptors (GPCRs), induced by agonists such as lysophosphatidic acid, carbachol (which specifically activates muscarinic acetylcholine receptors) or thrombin (Citri and Yarden, 2006, Daub et al., 1996, Shah et al., 2006). GPCR stimulate a batimastat sensitive metalloproteinase that induces cleavage and release of HB-EGF, leading to rapid phosphorylation of HER1 (Hynes and Lane, 2005). In addition, the binding of Wnt to its seven-pass transmembrane receptor Frizzled also transactivates HER1 (Hynes and Lane, 2005).
In lung cancer cells with HER1 mutations but with resistance to gefitinib therapy were found to have amplification of the gene for the MET receptor, a transmembrane tyrosine kinase that is activated by hepatocyte growth factor (Engelman et al., 2007, Arteaga, 2007). Physical complexes of MET with HER3 and PI3K were demonstrated. The downstream activation of PI3K and AKT via the MET/HER3 interaction accounted for the acquired gefitinib resistance.

Signaling in *trans* is a key feature of this multimember family and the critically important PI3-Akt pathway is driven predominantly through transphosphorylation of the kinase HER3. For that reason, more potent TKIs or combination strategies would be required to silence oncogenic HER2 signaling effectively (Sergina et al., 2007). HER3 limits the impact of exclusively targeting HER1 or HER2, especially in the presence of HER3 ligands, and provides a route for acquired resistance to anti-cancer drugs that inhibit HERs or other receptors (Stern, 2008). The reasons for this are not entirely clear but recent evidence discussed below suggests that this may be due to ineffective suppression of HER2/HER3 transphosphorylation. Because HER3 is a focal point for both the initial effectiveness of HER1 and HER2 therapies as well as the development of drug resistance, there is considerable effort to develop methods to directly target HER3 with therapeutics. Thus, antibodies directed against HER3 may be the most effective method to disrupt its function. SGP1 is a monoclonal antibody of the ligand-bindig site of the HER3 receptor that can inhibit completely NRG stimulated growth of cultured breast cancer cells (Blackburn et al., 2011), whilst

MM-121, another one, was found to reduce basal HER3 phosphorylation most effectively in cancers possessing ligand-dependent activation of HER3 induced by either HER1, HER2, or MET (Schoeberl et al., 2010).
Extensive crosstalk among HER receptors suggests that blocking signaling from more than one family member may be essential to effectively treat cancer and limit drug resistance. MEHD7945A is a dual HER1/HER3 inhibitor that exhibited dual action by inhibiting HER1- and HER3-mediated signaling *in vitro* and *in vivo* and the ability to engage immune effector functions. Compared with monospecific anti-HER antibodies, MEHD7945A was more broadly efficacious in multiple tumor models, showing that combined inhibition of HER1 and HER3 with a single antibody is beneficial (Kamath et al., 2011).

14. HER3 in breast cancer

HER family and its ligands are critically involved in the carcinogenesis of the mammary gland (Gullick and Srinivasan, 1998, Holbro et al., 2003a). Abnormal function of the members of HER family resulting in receptor hyperactivation (due to gene amplification, protein overexpression or abnormal transcriptional regulation) has been linked with breast cancer prognosis.

HER1 overexpression for the first time has been associated with poor prognosis in breast cancer by Sainsbury et al. (Sainsbury et al., 1987) and it has been confirmed by others (Jardines et al., 1993, Torregrosa et al., 1997). Several studies showed a positive correlation of increased amounts of the receptor not only with shortened survival but failure of endocrine therapy in breast cancer as well (Bolufer et al., 1990, Nicholson et al., 1994).

In 1987, it was revealed that HER2 amplification, independently of the other prognostic factors, predicted time to disease relapse and overall survival in node-positive breast cancer patients (Slamon et al., 1987). Further studies confirmed these results and demonstrated that gene amplification correlates with HER2 receptor overexpression (Slamon et al., 1989). In breast cancers 90-95% of cases of HER2 overexpression result from HER2 gene amplification (Pauletti et al.,

1996). During the next years numerous studies on clinical significance of HER2 have been carried out. Thus, it is generally accepted that there is a significant correlation between HER2 overexpression/amplification and poor prognosis in node-positive patients. The relationship between HER2 status and prognosis in node-negative patients is more controversial.

Although HER1 and HER2 contribution to breast cancer has been extensively studied, the contribution of HER3 and its role in breast cancer progression remains unclear. HER3 overexpression and activation was found to be common in human breast cancer. Some studies have identified an association of HER3 overexpression with HER2 overexpression in breast cancer (Tovey et al., 2004, Witton et al., 2003). The literature data concerning clinical significance of HER3 in breast cancer bring confusing information. Along with the reports that failed to detect association of HER3 and clinical outcome, there are studies that associated HER3 with pathological parameters. The group of Lemoine (Lemoine et al., 1992a) did not find that high HER3 expression was positively associated with the presence of lymph node metastases, whereas the group of Quinn failed to confirm this observation (Quinn et al., 1994) nor did Travis and associates (Travis et al., 1996). No group has so far correlated HER3 expression and patient survival. The work of Naidu suggested that the overexpression of HER3 could play an important role in tumor progression from non-invasive to invasive form. It was also revealed that strong HER3 immunoreactivity occurred in a high percentage of estrogen receptor (ER) negative and lymph node positive tumors (Naidu et al., 1998). On the contrary, Knowlden demonstrated that increased HER3 expression was associated with the prognostically favorable ER positive phenotype (Knowlden et al., 1998).

The majority of published data associate HER4 overexpression with good prognosis and longer survival (Suo et al., 2002, Witton et al., 2003). Furthermore, HER4 contributes to lactation and epithelial differentiation.

Along with the biological impact of HER3 signaling on HER2 amplified breast cancer, increasing evidence links active HER3 to resistance to breast

cancer therapies targeted at HER2 and ER (Menendez and Lupu, 2007). This may be relevant for predicting responses to these agents, and also suggests the usefulness of applying new therapeutic strategies, such as the use of pertuzumab or lapatinib and/or Akt and mTOR inhibitors.

Instead, it appears that HER3 promotes drug resistance by enabling autocrine or paracrine ligands (NRG1 and NRG2) to activate catalytically competent RTKs, and through its capacity to channel signaling to PI3K-Akt signaling pathways. The former has been well demonstrated in cell culture systems, in which, for example, a Herceptin-sensitive cell line becomes resistant in the presence of NRG, provided that HER3 is still present (Holbro et al., 2003a) and in studies of gefitinib-resistance mediated by NRG (Hutcheson et al., 2007).

Recent findings from cell-based studies provide new insight into the mechanisms underlying resistance to TKI in HER2-driven breast cancers. TKIs effectively prevent autophosphorylation of HER1 and HER2 in these tumor cells. However, the transphosphorylation of HER3 is only transiently suppressed and HER3 ultimately escapes inhibition by TKIs in HER2-overexpressing tumor cells (Sergina et al., 2007). The consequence of HER3 mediated resistance is PI3K-Akt pathway mediated resistance, tumor survival, and escape from the proapoptotic consequences of the loss of oncogenic HER2 signaling. The mechanism that underlies HER3 resistance in these tumors appears to be a forward shift in the phosphorylation/dephosphorylation equilibrium steady state of HER3 signaling, in effect buffering HER3 against an incomplete inhibition of HER2 kinase. The resiliency of HER3 is driven by Akt-mediated negative feedback signaling. The failure to stably suppress HER3 significantly attenuates the anti-tumor activities of TKIs and is entirely consistent with the limited clinical activities of these agents in the treatment of HER2-amplified breast cancers (Hsieh and Moasser, 2007).

This study also showed that HER2-driven tumors ultimately cannot escape the total inactivation of HER2 (Sergina et al., 2007), suggesting that more potent TKIs or higher doses of TKIs may yet show significant activity in this disease. The HER2/HER3 complex presents a considerable challenge to drug therapy. In this

complex, HER3 is the principle mediator of resistance, yet HER3 is not a direct target of TKIs. From these data it is clear that the contribution of other HER receptors should be taken into account for future evaluations of HER3 as a target for tumor therapy. Through a clearer understanding of HER interactions, therefore, it is conceivable that future HER-directed approaches may prove to be even more beneficial for cancer treatment.

Hypothesis

PharmaMar Company (a biopharmaceutical company determined to advance cancer treatment through discovering, developing, producing, and marketing innovative drugs of marine origin) identified elisidepsin (obtained from a marine mollusk), and the initial screenings have demonstrated it has antitumor effect in a broad panel of cancer cell lines. These data led it to participate in preliminary clinical studies of phase I and phase II, and a therapeutic effect was found in some patients and tumor types.

Based on the experience of the Molecular Pathology Group studying the chemosensitivity and radiosensitivity of different compounds in the last years, PharmaMar Company collaborated with us to clarify the mechanism of action of this drug, to delineate tumor subtypes that may be more sensitive to the drug treatment and identify possible targets that could have clinical relevance.

In preliminary studies, which have been conducted in our laboratory and by other groups, it was found that elisidepsin exerted a greater cytotoxic effect in carcinoma lines and the possibility that it could have an association or a relationship with the expression of certain epidermal receptors and factors related to epithelial-mesenchymal transition was suggested.

This study was done with an "in vitro" model in a broad panel of cell lines, for the characterization of factors that may be predictive of elisidepsin response. Given that it showed a clear correlation between the expression levels of HER3 receptor and elisidepsin sensitivity, it was decided to focus on the identification of a possible mechanism of action through this receptor. To attain that**,** we generated different constructs for HER3 gene, with expression of mutant and wild type constructs, with RNA interference and tried to correlate the cytotoxic effect of elisidepsin with the functional status of HER3 receptor.

The study of HER3 receptor in human tumors is still not fully characterized, with very small number of publications. Since we saw a higher response of breast tumor cell lines expressing HER3, we decided to continue the project analyzing in more detail the levels of HER3 in a wide range of breast

carcinoma samples from our database of Hospital Vall d'Hebron. For this, we were lucky to collaborate with Dr. Pandiella, an international expert in this family of receptors, who also has one of the best polyclonal antibodies against HER3 receptor, which we set up for studies in paraffin embedded tissues. The evaluation of HER3 in the clinical series (also sent for publication) led to interesting results, correlating the expression of HER3 with ER levels, opening at the same time a new window for understanding the pathophysiological mechanisms of drug sensitivity and resistance to antiestrogen treatments.

Hypothesis:

The present research work focuses on the study and characterization of mechanisms of sensitivity and drug resistance for elisidepsin compound in a broad panel of human cancer cell lines. The study postulated that the cellular sensitivity of elisidepsin could be correlated with the expression of certain members of the HER family. After confirming the association of the cellular response to the expression of HER3, it also raised a possible association of HER3 levels with other important parameters in breast cancer carcinogenesis, such as hormone receptors and the molecular study of the possible interrelationship of the biochemical pathways related to the expression of HER3 epidermal factor and ER.

Objectives

1. To evaluate possible synergistic effects of elisidepsin with other chemotherapeutics (CDDP, TAX and gemcitabine) in a broad panel of cell lines.

2. To identify new molecular markers associated with primary and acquired resistance to elisidepsin treatment *in vitro.*

3. To study the involvement of HER3 expression and its activation in response to elisidepsin treatment.

4. To evaluate the importance of HER3 expression in breast cancer patients.

5. To analyze the possible value of HER3 as a predictive marker to anti-hormone therapy.

Materials and methods

1. Procurement and processing of tissue samples

Breast tissue specimens with carcinoma diagnosed between 2005 and 2009 were obtained from the tumor bank at the Pathology Department of Vall d'Hebron University Hospital (Barcelona, Spain). All tumors were histologically examined to confirm the diagnosis of carcinoma, using light microscopy and conventional hematoxylin and eosin (H&E) staining. A portion of the biopsied samples was quickly frozen and stored at –80°C immediately after surgery. The remainder of the tumor was fixed in neutral formalin and embedded in paraffin. The Ethics Committee of Vall d'Hebron University Hospital approved all the procedures used in the study.

2. Immunohistochemistry

Immunohistochemical staining using the avidin-biotin-peroxidase technique was performed for each antibody. Five micrometer-thick sections were cut from the tissue specimens and placed on poly-L-lysine–coated glass slides. Sections were deparaffinized by xylene and rehydrated in graded alcohol. Endogenous peroxidase was blocked by immersing the sections in 0.1% hydrogen peroxidase in absolute methanol for 20 min. For antigen retrieval, the tissue sections were heated in a pressure cooker in citric acid monohydrate 10 mM, pH 6.0, for 5 min, and then incubated with the primary antibody at room temperature. Immunohistochemistry (IHC) was performed with the EnVision system (Dako, Glostrup, Denmark) except anti-HER3, E-cadherin, β-catenin and vimentin IHC that were with Benchmark XT (Ventana Medical Systems, Inc, Tucson, AZ).

The primary antibodies and dilutions used were: anti-HER3 (generated by Dr. Pandiella (IBMCC, Salamanca, Spain), 1:75), anti-Ki-67 (Dako, pre-diluted), anti-HER2 (Dako, pre-diluted), anti-ER (Novocastra, Bannockburn, IL, 1:50), anti-progesterone-receptor (PR) (Novocastra, pre-diluted), anti-p53 (clone DO-7, Ventana Medical Systems, pre-diluted), anti-E-cadherin (Dako, pre-diluted), anti-vimentin (Ventana, pre-diluted) and anti-β-catenin (Leica NCL# 2012/10, 1:50). The incubation time for all antibodies was 60 min with the exception of p53, which was incubated for 16 min. All slides were hematoxylin counterstained,

dehydrated, and mounted. Omitting the primary antibody performed negative controls.

2.1. IHC evaluation

All cases were evaluated by two pathologists and me. Hormone receptors (ER and PR) were evaluated taking into account the percentage of positive cells and intensity of the staining, which was assessed semi-quantitatively according to the ASCO (American Society of Clinical Oncology) and CAP (College of American Pathologists) guidelines for hormone receptor evaluation in breast cancer (Hammond et al., 2010). Cases were considered as ER and/or PR positive if ≥1% was seen. For Ki-67, only strong nuclear staining was considered as positive, and then the percentage of positive neoplastic cells was calculated. HER2 was evaluated according to the ASCO and CAP guidelines (Wolff et al., 2007).

All the IHC was performed in whole sections of formalin fixed paraffin embedded blocks. HER3, E-cadherin, β-catenin and vimentin IHC cases were evaluated as follows: 0 (no expression), 1 (weak expression or moderate staining in <10% of neoplastic cells), 2 (moderate staining in >10% neoplastic cells) and 3 (strong staining). HER3 membrane and cytoplasmatic staining was evaluated separately. Cases were then considered as positive (if score 3) or negative (if score 0, 1 or 2). Discordant cases were discussed using a multiheaded microscope.

3. FISH

When an inconclusive HER2 result was found (2+), and when a HER3 positive result was found (3+), fluorescence in situ hybridization (FISH) was carried out (Pathvysion, Vysis Inc., Downers Grove, IL and ZytoVision GmbH, respectively) according to the manufacturer's instructions to confirm HER2 amplification or if its positivity was due to amplification in case of HER3.

In brief, sections were deparaffinized, heat-pretreated in citrate buffer 1 h 80°C, digested with pepsin at room temperature for few minutes and dehydrated in graded ethanol. Then the probe mix was applied to the dry slides. The slides were then incubated in hybridizer (Hybridizer Instrument for *in situ*

hybridization, DAKO, S2450) for denaturation 5 min 80°C and hybridization 18 h 37°C. The slides were re-dehydrated in graded ethanol. FISH analyses were performed according to the HER2 FISH PharmDx (Dako) criteria.

3.1. FISH evaluation

In each case, 100 non-overlapped, intact interphase tumor nuclei identified by DAPI (4´, 6-Diamino-2-phenylindole, dihidrochloride) staining were evaluated, and gene (red signal) and CEN17 or CEN12 (green signal) copy numbers in each nucleus were assessed. The cases were considered to be amplified when the average copy number ratio, HER2/CEN17 or HER3/CEN12 were ≥ 2.0 in all nuclei evaluated or when the HER2 or HER3 signals formed a tight gene cluster. Among the cases in which the gene was not amplified, samples showing more than four copies of the HER2 or HER3 gene and more than four CEN17 or CEN12 in more than 10% of the tumor cells were considered to be polysomic for chromosome 17 or chromosome 12 respectively (Hofmann et al., 2008).

4. RNA extraction

Total RNA was isolated from tumor tissue or cell lines with the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Human samples were manipulated at Vall d'Hebron University Hospital.

Adherent cells were washed twice with PBS 1X, then were lysed directly in the cell culture vessel by addition of a highly denaturing guanidine isothiocyanate-based buffer supplemented with β-mercaptoethanol, so that RNases were inactivated, and using a scraper to collect cell lysates. Homogenization of the samples was performed by passing the lysates through a blunt 20-gauge needle. A volume of 70% ethanol was added and the mixture was transferred to a RNeasy spin column, based in a silica gel membrane that binds total RNA (longer than 200 bp). Columns with bound RNA were washed three times, treated with DNase I to prevent DNA carryout and eluted in RNase free water. RNA was controlled for quantity and quality on a 2100 BioAnalyzer instrument with the *RNA nano Lab Chip Kit Bioanalyzer* (Agilent, Palo Alto, CA).

5. cDNA synthesis

Random primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) were used to carry out cDNA synthesis from 1 µg of total RNA for each sample (200 ng Random Primers, dNTPs 0.5 mM and deionized water (DW) with 12 µl as final volume), and heated for 5 min 70°C. Then, buffer 1x, 10 mM DTT and 40 U RNaseOUT were added and incubated for 2 min 25°C. Then, 200 U SuperScriptII reverse transcriptase were added and incubated for 10 min 25°C and 1h 42°C. Finally the reaction was inactivated by heating for 15 min 70°C. The aliquots were stored at –20°C.

6. PCR

6.1. Conventional PCR

PCR was performed using 50 mM $MgCl_2$, 25 mM dNTPs, 0.2 µM primers forward and reverse, 0.2 U of Taq polymerase and the desired input of cDNA. Amplifications were done in an eppendorf thermocycler, and after a previous step of 94°C for 10 min, cycles were 15 sec 94°C, 15 sec 55°C and 30 sec and a final extension step of 10 min 72°C. Amplified DNA was analyzed by denaturing agarose gel electrophoresis and ethidium bromide staining. The primers used were:

HER3 1*F*: *CTGGGCTTGCTTTTCAGC* *522bp*
HER3 1*R*: *ACAGCTTCTGCCATTGTCCT*

HER3 2*F*: *GACTGGAGGGACATCGTGAG* *559 bp*
HER3 2*R*: *TGTTGCTCGAGTCCACAGTC*

HER3 3*F*: *AGCCTTGTGGGGGACTATGT* *604 bp*
HER3 3*R: TTTCGACAGGACAAGCACTG*

HER3 4*F*: *GTGTGTGACCCACTGTGCTC* *685 bp*
HER3 4*R*: *CTCCTTTGTGCACAGTTCCA*

6.2. Real-time PCR analysis

We used real-time PCR with TaqMan probes incorporation to determine the expression levels of selected genes. An ABI PRISM 7000 instrument (Applied Biosystems, Foster City, CA) was used to do the relative quantification analysis, and data were analyzed with the 7000 Sequence Detection Software, v.1.2.3 (Applied Biosystems).

TaqMan probes and their corresponding primer sets were obtained from Applied Biosystems. HER3 and ZNF217 expression was detected using the Taqman Gene Expression Assay (Hs00951444_m1 and Hs00916723_m1, respectively). 15 ng cDNA of each sample with 1µl TaqMan probe and 10 µl TaqMan Universal PCR Master Mix (20xTaqMan Gene Expression Assays, Applied Biosystems) were used for the PCR analysis. Thermal cycler conditions were 95°C for 10 min and 40 cycles of 95°C for 15 sec and annealing and elongation at 60°C for 1 min for TaqMan assays. All determinations were performed in triplicate and in at least two independent experiments.

Initially, a Taqman Human Endogenous Control Plate (Applied Biosystems) was done to determine which endogenous controls showed less variation between normal and tumoral tissue. With this system we analyzed the expression of 16 reference genes (18S, ACTB, B2M, GAPDH, GUSB, HMBS, HPRT1, IPO8, PGK1, POLR2A, PPIA, RPLP0, TBP, TFRC, UBC and YWHAZ). POLR2A (Hs00172187_m1) was chosen and the FirstChoice® Human Breast Total (AM6952, Applied Biosystems) as an internal control (normal tissue). Target and reference genes showed similar, nearly 100% amplification efficiencies (data not shown).

Since the relative amplification efficiencies of target and reference primers were found to be approximately equal, the ΔΔCt method was applied to estimate relative transcript levels. The final results, expressed as n-fold differences in target gene expression were calculated as follows:

nTARGET=2–[(Ct target – Ct reference) TUMORAL – (Ct target – Ct reference) NORMAL]

6.3. Exo sap and sequentiation

For the removal of unwanted deoxynucleotides and primers with no interference in downstream applications and for an efficient purification of PCR products the Exonuclease I and Shrimp Alkaline Phosphatase (Exo sap, Affymetrix, Wooburn Green, United Kingdom) was used. 2 µl of Exo sap and 5 µl of each sample that we wanted to purify were used for the reaction. Conditions were 37°C for 15 min for treatment and 80°C for 15 min to inactivate it.

PCR sequentiation was performed using 2 µl DNA purified, 1 µl Big Dye, 1 µl of 3.2 µM primer forward or reverse and 1 µl sequencing buffer (Applied Biosystems). Amplifications were done in an eppendorf thermocycler and the thermal cycler conditions were 96°C for 2 min and 30 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min.

7. Reagents

Elisidepsin and its fluorescent derivatives were manufactured at PharmaMar, SA. (Madrid, Spain) and were obtained as a dry powder to be reconstituted with dimethyl sulfoxide (DMSO, Sigma-Aldrich, Taufkirchen, Germany)/ ethanol (1:1) as a 1mM stock solution, and kept in aliquots at -20ºC. Cisplatin (CDDP), paclitaxel (TAX) or gemcitabine were obtained from the Vall d'Hebron University Hospital. Drug dilutions were freshly prepared before each experiment in order to avoid degradation.
NRG-1β was obtained from Sigma-Aldrich, the MG132 proteosome inhibitor from Tocris Bioscience (Ellisville, MO, USA), the cycloheximide (CHX) inhibitor of protein biosynthesis from Sigma-Aldrich and U3-1287 (AMG 888) was provided by U3 Pharma (Munich, Germany).

8. Cell culture

Original cell lines were obtained from the American Type Culture Collection (ATCC), except from DV90 cell line, that was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, and maintained at 37°C in a 5% CO_2 atmosphere and 90% humidity (table 4).

Cell line	Organism	Origin	Culture media
A549	Human	Lung carcinoma	HAM's F-12
AsPC-1	Human	Pancreas adenocarcinoma	RPMI
BT474	Human	Breast ductal carcinoma	DMEM F12
BT549	Human	Breast ductal carcinoma	RPMI
BxPC-3	Human	Pancreas adenocarcinoma	RPMI
CFPAC	Human	Pancreas ductal adenocarcinma derived from liver metastasis	RPMI
DLD1	Human	Colorectal adenocarcinoma	DMEM
DV90	Human	Lung adenocarcinoma	RPMI
HCT 116	Human	Colorectal carcinoma	DMEM
HEK 293T	Human	Embryonic kidney	DMEM
HeLa	Human	Cervix adenocarcinoma	DMEM
HN19	Human	Head and neck carcinoma	RPMI
HN30	Human	Head and neck carcinoma	RPMI
HOP62	Human	Lung carcinoma	RPMI
HPAC	Human	Pancreas adenocarcinoma	DMEM
HT29	Human	Colorectal adenocarcinoma	DMEM
MCF-7	Human	Breast adenocarcinoma derived from pleural effusion metastasis	RPMI
MDA-MB-231	Human	Breast adenocarcinoma derived from pleural effusion metastasis	RPMI
MDA-MB-435	Human	Breast adenocarcinoma derived from pleural effusion metastasis *	RPMI
MDA-MB-468	Human	Breast adenocarcinoma	DMEM
MiaPaCa-2	Human	Pancreas carcinoma	DMEM
NIH 3T3	Mus musculus	Embryonic fibroblast	DMEM
OVCAR-3	Human	Ovary adenocarcinoma	RPMI
PANC-1	Human	Pancreas duct epithelioid carcinoma	DMEM
PC3	Human	Prostate adenocarcinoma	RPMI
SKBR3	Human	Breast adenocarcinoma derived from pleural effusion metastasis	RPMI
T47D	Human	Breast ductal carcinoma	RPMI

Table 4. Cell lines used in this study.

* MDA-MB-435 was found that have a cross-contamination with the M14 melanoma cell line.

RPMI (Roswell Park Memorial Institute medium) or DMEM (Dulbecco's modified Eagle's medium), DMEM F-12 (Gibco-Invitrogen, Barcelona, Spain) and HAM F-12 medium were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Labclinics, Barcelona, Spain), 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 0.1 mM non-essential aminoacids (all from PAA Laboratories, Linz, Austria).

Once per month, a mycoplasma test was performed with cell supernatants with EZ-PCR Mycoplasma Test Kit (Biological Industries, Haemek, Israel) following the manufacturer's instructions.

9. Plasmids and cell transfection

pIRES-Hyg-HER3, pIRES-Hyg-HER2 and the pIRES-Luciferase (LUC) were kindly donated by Dr. Scaltriti (Vall d'Hebron University Hospital Research Institute, Barcelona, Spain) (Scaltriti et al., 2007). The pIRES-LUC was used as a control for transfection. pIRES vectors contain hygromycin resistance.

pCEFL-ss-flag-A1 was provided by Dr. Gutkind (National Institutes of Health, Bethesda, MD, USA). pCEFL-ss-flag-A1-HER3 was constructed by inserting a 6.2 kb human HER3 cDNA into the pCEFL-ssflag-A1 vector. In these vectors, the expression of the clones gene is directed by translation elongation factor 1a promoter and carries the G418 resistance gene and the SV40 replication origin (Teramoto et al., 1996). The pCEFL-ssflag-A1 HER3 truncated was constructed by isolating by digestion of the full-length HER3 cDNA with NdeI and subcloned into the vector.

Cells were transfected for 12 h with Jet Pei (Polyplus-Transfection, Illkirch, France). After transfection, cells were grown in complete medium for 24 h, and processed for different experimental assays.

10. Lentivirus shRNA production and transduction

Short hairpin RNAs (shRNAs) were used for inhibited HER2 and HER3 expression in different cancer cell lines. The following sequences were used:

- shHER2_1F:
GATCCAAGTACACGATGCGGAGACTGTTCAAGAGACAGTCTCCGCATCGTGTACTTTT
TTTTACGCGTG

- shHER2_1R:
AATTCACGCGTAAAAAAAAGTACACGATGCGGAGACTGTCTCTTGAACAGTCTCCGCA
TCGTGTACTTG

- shHER2_2F:
CTAGAAAGTACACGATGCGGAGACTGTTCAAGAGACAGTCTCCGCATCGTGTACTTTT
TTTTAAGCTTG

- shHER2_2R:
AATTCAAGCTTAAAAAAAAGTACACGATGCGGAGACTGTCTCTTGAACAGTCTCCGCA
TCGTGTACTTT

- shHER3_3.1F:
GATCCAAGAGCGACTAGACATCAAGCTTCAAGAGAGCTTGATGTCTAGTCGCTCTTTT
TTTTACGCGTG

- shHER3_3.1R:
AATTCACGCGTAAAAAAAAGAGCGACTAGACATCAAGCTCTCTTGAAGCTTGATGTCT
AGTCGCTCTTG

- SHHER3_3.3F:
GATCCGCCAATACCAGACTGTACTTCAAGAGAAGTACAGTGTCTGGTATTGGTTTTTT
ACGCGTG

- SHHER3_3.3R:
AATTCACGCGTAAAAAACCAATACCAGACACTGTACTCTCTTGAAGTACAGTGTCTGG
TATTGGCG

The different sequences were cloned into the lentiviral vector pLKO.1 (Sigma-Aldrich). The pLKO.1-shRNA LUC was used as a control for transfection. All vectors contain puromycin resistance. Plasmids pVSVG and pCMVΔR8.91 for

the expression of packaging and envelope proteins were kindly provided by Dr. Peeper (VU University Medical Center, Amsterdam, The Netherlands).

Two plates seeded with 1.5 x 10^6 HEK 293T cells were co-transfected in DMEM 10% FBS with 2 µg of pLKO.1, 2 µg of pCMVΔR8.91 and 2 µg of pVSVG were incubated overnight. Cells were washed and incubated in 10% CO_2 with medium containing 5% FBS and supernatants containing viruses were recovered at 24 h and 48 h and filtered with 0.45 nM-pore filters (Sarstedt, Nümbrecht, Germany).

10.1. Testing shHER2 and shHER3

Titration was performed by infecting cells with the recovered viral particles in the presence of 4 µg/mL polybrene (Sigma-Aldrich). Next day, this process was repeated after the first infection. Equal amounts of viruses were infected into the cells, in parallel with the appropriate shControl. Western blot was performed to assess the silencing levels of the two shHER2 and shHER3 sequences, and the most silencing one, shHER2.1 and shHER3.3, were chosen.

11. Generation of cell lines stably expressing HER3 or depleted of HER2 or HER3

To generate cell lines that stably express HER3, 24 h after transfection, medium supplemented with hygromycin (Sigma-Aldrich) was added (table 5), and cells underwent selection for 10 days to eliminate untransfected cells.

To obtain cell lines with stable depletion of HER2 or HER3, infected cells were selected with puromycin for three days and maintained thereafter in medium with puromycin in half of the selection doses. After 30 h, infected cells were selected by adding the correct puromycin (Sigma-Aldrich) concentration (table 5) for three days.

In order to generate stable cell lines expressing the HER3 or the shRNA of interest we determined the minimum amount of puromycin required to kill non-infected cells by generating a puromycin kill curve.

Cell line	Hygromycin 100 mg/mL	Puromycin 1mg/mL
NIH 3T3	150 µg/ml	x
MCF-7	80 µg/ml	1.5 µg/ml
HEK 293T	300 µg/ml	x
MDA-MB-231	290 µg/ml	2.5 µg/ml
BT474	90 µg/ml	1.5 µg/ml
MDA-MB-435	120 µg/ml	3.8 µg/ml
MDA-MB-468	110 µg/ml	0.7 µg/ml
BxPC-3	x	1-1.25 µg/ml
SKBR3	x	1.4 µg/ml
HPAC	230 µg/ml	0.5 µg/ml
MiaPaCa-2	180 µg/ml	0.5 µg/ml
PANC-1	120 µg/ml	1.5 µg/ml
CFPAC	120 µg/ml	0.75 µg/ml
AsPC-1	120 µg/ml	1.5 µg/ml

Table 5. Antibiotics dose used to select infected cells.

12. Cell growth assay

Cells were plated overnight at a density of 50.000 cells/well in 24-well plates in 1ml of medium. We used at least 3 wells for each condition. Cell lines were treated to various concentrations of elisidepsin, CDDP, TAX or gemcitabine for 72 h as single agents. Cell viability was measured by a crystal violet assay.

Briefly, after each treatment cells were fixed in 1% glutaraldehyde for 20 min, washed twice in PBS 1x, stained with 0.1% crystal violet for 30 min and then washed with abundant DW. Colorant was recovered by 5% acetic acid and optical density was measured at 590 nM using an ELISA plate reader.

13. Analysis of combined drug effects

Cells were plated in 24-well plates as described above. After overnight incubation at 37°C, attached cells were treated for 72 h at a fixed ratio of doses that corresponded to 0.125, 0.25, 0.5, 1 and 2 times the individual IC_{50} values of elisidepsin with combination of CDDP, TAX and gemcitabine. Cell survival fractions were determined by crystal violet assay and the combinational effects

were analyzed by the median effect method of Chou and Talalay by using CalcuSyn software (version 2.1, Biosoft, Cambridge, UK) (Chou and Talalay, 1984). The study was repeated three independent times and representative data are shown.

14. Growth curves (PDL)

Cells were counted and seeded in duplicate every 3 days. Neubauer chamber (Blaubrand®, Germany) was used to count cells, and cell viability was measured with a cell suspension by adding trypan blue (Sigma-Aldrich) at 0.04% in PBS. Living cells exclude trypan blue, and appear white. Thus, the percentage of viable cells can be calculated. To determine the cell concentration (cells/ml), the percentage of living and dead cells, and the total number of cells in a cell suspension was calculated. Population doubling (PD) was determined by the following formula:

PD=Log (Nf/Ni)/Log2, where Nf is the number of cells counted and Ni is the number of cells seeded. Cumulative population doubling level numbers (PDL) represent the sum of PDs from previous passages. The number of cells seeded from the MiaPaCa-2 and MCF-7 lines were $1.25x10^5$ and $1.4x10^5$ cells, respectively. Each curve was performed at least twice with similar results, and each time point was determined in duplicate.

15. Immunocytofluorescence

Cells seeded on sterile coverslips (Afora, Barcelona, Spain) were fixed in cold 4% paraformaldehyde/PBS, permeabilized in 100% methanol (Sigma-Aldrich) for 30 min at -20°C and blocked with PBS/1% BSA for 1 h. Coverslips were incubated with primary antibodies (table 6) diluted in PBS/1% BSA overnight at 4°C, washed twice with PBS and incubated with Alexa 546-conjugated rabbit anti-mouse IgG (Molecular Probes), diluted 1:800 in blocking buffer for 1 h or with AlexaFluor 647 mouse anti-human (Invitrogen, diluted 1:200). Cells were washed twice with PBS and once with distilled water for 10 min each time. Finally, cells were mounted in Citifluor (Citifluor Ltd AF1) before observation and analyzed by fluorescence microscopy. Images were visualized

and captured under a Zeiss Axiophot microscope (Oberkochen, Germany) coupled to an Olympus digital camera.

Antibody	Origin	Company	Reference	Dilution
β-catenin	Mouse	Novocastra	NCL #2012/10	1/50
E-cadherin (NCH-38)	Mouse	Dako	IS059	1/50
Flag	Mouse	Sigma- Aldrich	F3165	1/50
Slug (H-140)	Rabbit	Santa Cruz	SC-15391	1/50
Snail (L70G2)	Mouse	Cell Signaling Technologies	#3895	1/50
Twist (H-81)	Rabbit	Santa Cruz	SC-15393	1/50
Vimentin (V9)	Mouse	Dako	IS630	1/40

Table 6. Antibodies for immunofluorescence used in this study.

16. Protein extractions and western blot

Lysates were obtained from cell lines and tissues. Tissues were ground and sonicated in lysis buffer (20 mM Tris HCL (pH 8), 137 mM NaCl, 2 mM EDTA, 10% Glycerol, 1% NP-40), in the presence of protease and phosphatase inhibitors (1 µg/ml aprotinin, 1 mM DTT, 1 µg/ml leupeptin, 20 mM β-glycerophosphate, 1 mM PMSF, 0.1 M NaPPi , 20 mM NaF, 2 mM Na_3VaO_4) at 4 °C for 20 min. Subconfluent cells were lysed in the same buffer. After clearing the lysates by centrifugation, protein concentrations were determined using the Bradford assay (Bio-Rad Protein Assay, Munich, Germany). Then laemmli buffer (50 mM Tris-HCl pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (w/v) glycerol, 0.05% bromophenol blue and DTT 0.1 M) was added, samples were boiled for 5 min, and analyzed by western blot. Equal amounts of protein were separated (Running buffer containing 25 mM Tris, 190 mM glycine and 1% SDS, usually by 8% SDS polyacrylamide gel electrophoresis (PAGE) and transferred (Transfer buffer containing 25 mM Tris, 190 mM glycine and 20% methanol) to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) for 3 h at constant 300 mA. Blots were blocked with 3% defatted dry milk in TBS (50 mM Tris-HCL pH 7.5, 150 mM NaCl) with 1% Tween-20) for 1 h with Tween-20, and the primary antibody at appropriate dilution was incubated overnight at 4°C with shaking. Antibodies used in this study are summarized in (table 7). Anti-β-actin was used as the

loading control. After washing, blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody, washed again and excited with a chemiluminiscent substrate ECL (Amersham Pharma-Biotech, Dreieich, Germany) for varying times.

ANTIBODY	ORIGIN	DILUTION	COMPANY	REFERENCE
AKT	Rabbit	1/1000	Cell Signaling Technologies	#9272
β-actin	Mouse	1/1000	Sigma-Aldrich	A5060
β-catenin	Mouse	1/500	Novocastra	NCL-β-CAT
USP8	Rabbit	1/500	Abcam	ab38865
E-cadherin	Mouse	1/500	Novocastra	NCL-E-cad
ER	Mouse	1/1000	Novocastra	NCL-L-ER-6F11
HER1 (F4)	Rabbit	1/1000	Sigma-Aldrich	E 3138
HER2 (CB11)	Mouse	1/1000	BioGenex	MU134-UCE
HER3 (2F12)	Mouse	1/1000	NeoMarkers	MS-201-P1
HER4 (111B2)	Rabbit	1/1000	Cell Signaling Technologies	#4795
MAPK (C-14)	Rabbit	1/1000	Santa Cruz	SC-154
Nrdp1	Rabbit	1/10.000	Bethyl	A300-048A
Oregon Green	Rabbit	1/1000	Invitrogen	A889
pAKT (Ser473,587F11)	Mouse	1/1000	Cell Signaling Technologies	#4051
pERα (Ser118, 16J4)	Mouse	1/1000	Cell Signaling Technologies	#2511
pHER2 (Tyr1221/1222, 6B12)	Rabbit	1/1000	Cell Signaling Technologies	#2243
pHER3 (Tyr1289,21D3)	Rabbit	1/1000	Cell Signaling Technologies	#4791
pMAPK (Thr202/Tyr204)	Rabbit	1/1000	Cell Signaling Technologies	#9101
pS6 (Ser240/244)	Rabbit	1/1000	Cell Signaling Technologies	#2215
Snail (L70G2)	Mouse	1/1000	Cell Signaling Technologies	#3895
Slug	Rabbit	1/750	Santa Cruz	SC-15391
Twist (H-81)	Rabbit	1/750	Santa Cruz	SC-15383
Vimentin (V9)	Mouse	1/500	Dako	M0725

Table 7. Antibodies and dilutions used for western blot method in this study.

The secondary antibodies used were: donkey anti-rabbit IgG-HRP (NA9340, Amersham Pharma-Biotech, Uppsala, Sweden; diluted 1:2000) and donkey anti-mouse IgG-HRP (NA9340, Amersham Pharma-Biotech; diluted 1:2000). Bound antibodies were visualized with an enhanced chemiluminescence detection kit (Amersham Pharma-Biotech). Relative protein mass was estimated by addition of a pre-stained marker (BenchMark 10748-010, Invitrogen) that was electrophoresed and transferred simultaneously with samples. Visualization was performed by exposure to autoradiographic film (Agfa Material Corporation, Goose Creek, USA).

Quantification of autoradiograms was performed by using Image J software (version 1.41o, National Institutes of Health), normalized to the intensity of β-actin in each sample, and expressed in arbitrary densitometric units.

17. Cross-linking

Twenty-four hours post-transfection, subconfluent cells were rinsed once with PBS. DSS (disuccinimidyl suberate, Thermo Fisher Scientific, Rockford, IL, USA) was added to cells at a final concentration of 2.5 mM and incubated for 30 min at room temperature. After cross-linking the cells were rinsed with Tris saline and lysed.

18. Immunoprecipitation

Cells were lysed in lysis buffer (see above) and were incubated with Oregon Green (A889, Invitrogen); HER1 (528, Genentech, San Francisco, CA); HER2 (trastuzumab, Roche), HER3 R114, HER4 R113 (generated in Dr. Pandiella´s lab) and AMG 888, 1 μg, overnight at 4°C. The next day, protein G Sepharose 4 Fast Flow (17-0618-01, Amersham Pharma-Biotech) was added, and the solution was gently mixed for 1 h at 4°C. Samples were centrifuged at 12.000 x g for 20 sec. Beads with immune complexes were washed 5 times in lysis buffer, then laemmli buffer was added, samples boiled for 5 min, and analyzed by western blot.

19. Statistical analysis

Statistical studies were performed with the Statistical Package for the Social Sciences (SPSS 15.0; SPSS, Inc, Chicago, IL). Categorical variables were analyzed by cross-tabulation and differences were evaluated by the χ^2 test. The Wilcoxon, Kruskal-Wallis or Mann Whitney U tests were used to seek associations between the parameters analyzed. Statistical significance was set at a two-tailed *P* value of ≤0.05.

Results and Discussion

1. Elisidepsin sensitivity and combination with other chemotherapeutics

The cytotoxic effect of elisidepsin was determined in a large panel of 24 cancer cell lines derived from different tumor types (pancreas, breast, head and neck, lung, colon, ovary and prostate) and an embryonic fibroblast cell line (NIH 3T3) (figure 9). The range of IC_{50} values was narrow, ranging from 0.075 μM to 14 μM after 72 h exposure (median = 2.5 μM). The cells that have an IC_{50} value under or equal to 1 μM were considered sensitive, while the rest were considered less sensitive to the drug. Among the cell lines, CFPAC was the most sensitive and MiaPaCa-2 the most resistant, both pancreatic adenocarcinoma cell lines.

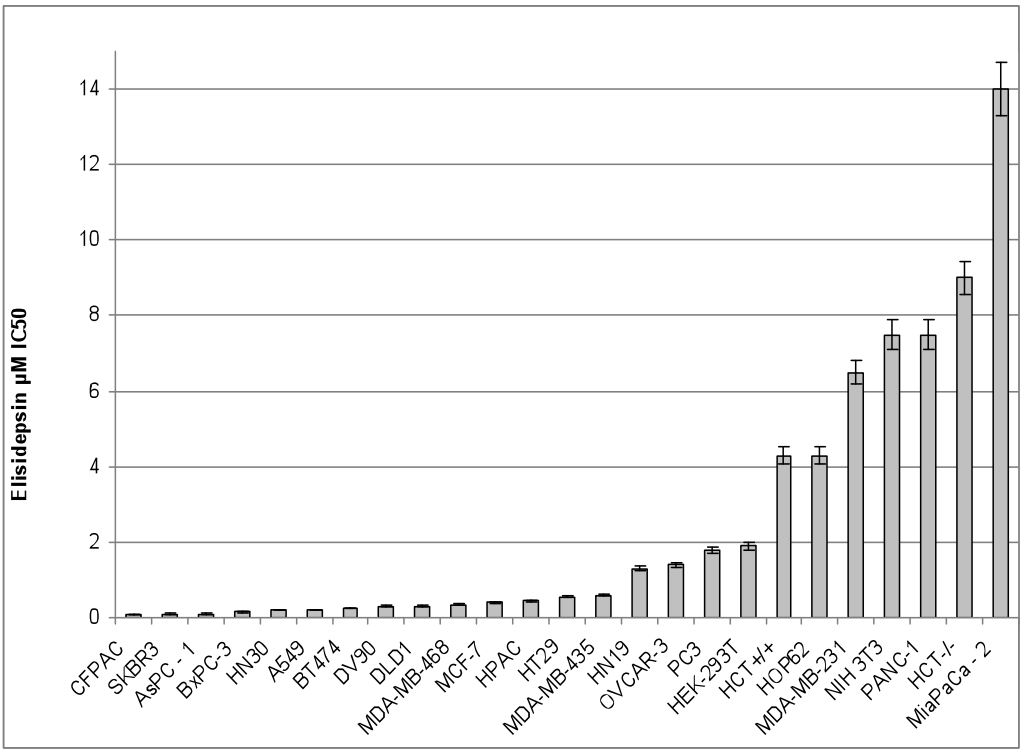


Figure 9. Concentrations of elisidepsin 50% of cell growth on a panel of cell lines from breast, pancreas, head and neck, lung, colon, ovary and prostate as determined by the crystal violet assay. Elisidepsin was exposed to cells for 72 h. Results are mean ± SD of at least three independent experiments.

The effect of elisidepsin it is not time-dependent since the ratio of IC_{50} values observed following 1 h exposure and continuous exposure was not significantly different in most cases by Sewell et al (Sewell et al., 2005). However

when we treated the cells with continuous exposure of subtoxic dose (lower than the IC_{50}) the cells grew more slowly than the parental ones (figure 10).

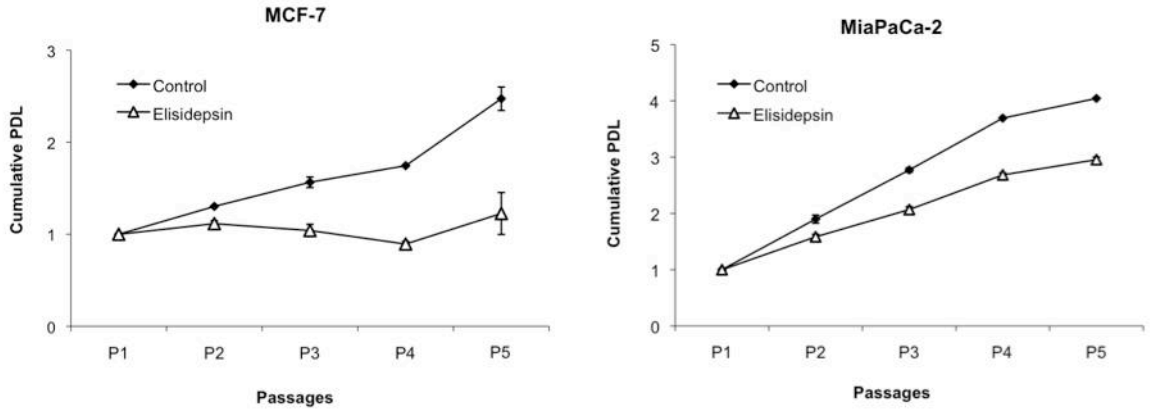


Figure 10. Effect of parental and elisidepsin subtoxic treated cells on cell proliferation. Cumulative numbers of cell divisions (shown as PDL) are shown for MCF-7 and MiaPaCa-2 cells until passage 5. Proliferation of MCF-7 (IC_{50} 0.4) and MiaPaCa-2 (IC_{50} 14) cells was suppressed when elisidepsin was added on them at subtoxic doses, 0.2 and 1 μM, respectively. The number of cells seeded from the MiaPaCa-2 and MCF-7 lines were $1.25x10^5$ and $1.4x10^5$ cells, respectively. Each curve was performed at least twice with similar results, SD are shown, and each time point was determined in duplicate. P, passage.

In vitro and *in vivo* synergism has been described when combining elisidepsin with erlotinib in NSCLC (Ling et al., 2009). Because human carcinomas harbor multiple genetic alterations, the combinations of different antitumor agents are essential in most cases. Classical antitumor agents such CDDP, TAX and gemcitabine are frequently used in carcinomas with unpredictable results. The combination with synergistic drugs could be a novel and relevant approach to increase the rate of tumor responses.

CDDP is a typical DNA-damaging agent with the ability to induce cross-linking between inter and intra DNA strands (Trimmer and Essigmann, 1999). CDDP is also known for its ability to induce apoptosis, although other mechanisms have been reported to induce cell death (Gonzalez et al., 2001). CDDP therapy is

used in several types of tumors such as NSCLC, testicular and ovarian cancer. TAX belongs to a family of drugs that, through the blocking of microtubule formation, interfere with the normal progression of the cell cycle. The drug has therefore been successfully used in the treatment of melanomas, breast, lung and ovary tumors (Blagosklonny and Fojo, 1999, Hortobagyi, 1999). Finally gemcitabine is an anticancer nucleoside that is an analog of deoxycytidine. It is a pro-drug and, once transported into the cell, must be phosphorylated by deoxycytidine kinase to an active form. Both gemcitabine diphosphate and triphosphate inhibit processes required for DNA synthesis. This compound has been widely used in pancreatic and metastatic breast cancer. All this chemotherapeutic agents have shown a limited effect, and in recent years, a huge number of combined studies with other compounds has been proposed (Schwartz, 2009, Raez et al., Squadroni and Fazio).

After characterizing the sensitivity of elisidepsin into 24 cell lines we wanted to focus in a panel of 14 cell lines from different types of human carcinoma (lung, breast, colon and pancreas) and defined their sensitivity by doing cell viability assays with different compounds used routinely in conventional chemotherapeutic treatments, namely CDDP, TAX, and gemcitabine. The conditions of these experiments were the same as the cell viability assays for elisidepsin. The results of the IC_{50} values of the different drugs are represented in table 8.

The values of the different compounds ranged from 0.5 to 24.75 μM of CDDP, from 0.5 to 40 mM of TAX and from 0.004 to 2 μM of gemcitabine. DLD1 cells were the most resistant to CDDP and AsPC-1 cell line the most sensitive. The DLD1 cell line together with HOP62 cell line were the more resistant to TAX treatment and the BxPC-3 cells the most sensitive. For the gemcitabine treatment, DLD1 cell line was again the most resistant and the A549 cells the most sensitive. For all three compounds the DLD1 cell line was the most resistant, in contrast to the elisidepsin treatment, which was one of the most sensitive (0.3 μM).

	Cell lines	CDDP (μM)	TAX (μM)	Gemcitabine (μM)
Lung	A549	8.25	7×10^3	0.004
	DV90	16.5	30×10^3	0.4
	HOP62	8.25	40×10^3	0.75
Breast	MDA-MB-231	8.25	25×10^3	1
	MCF-7	16.5	7.5×10^3	0.9
	MDA-MB-435	2.64	25×10^3	0.04
Colon	DLD1	24.75	40×10^3	2
	HT29	6.6	10×10^3	0.55
Pancreas	AsPC - 1	0.5	1×10^3	0.9
	CFPAC	0.75	0.7×10^3	0.045
	BxPC-3	0.75	0.5×10^3	0.025
	HPAC	2.5	4.5×10^3	0.01
	PANC-1	3	10×10^3	0.75
	MiaPaCa - 2	2	7×10^3	0.075

Table 8. Summary of the IC_{50} of the panel of 14 cell lines chosen in response to 72 h treatment with CDDP, TAX and gemcitabine with a final μM concentration. These experiments were performed in triplicate by crystal violet assay and IC_{50} value resulting from 50% inhibition of cell growth was calculated.

Next we wanted to determine the potential synergism of the combination of elisidepsin with CDDP, TAX, and gemcitabine, performing different combinational drug assays into the lung, breast and colon cancer cell lines, table 9. Only a combination of elisidepsin with gemcitabine was studied on the pancreatic cancer cell lines, table 10 because gemcitabine is indicated for the treatment of adults with locally advanced or metastatic adenocarcinoma of the pancreas and for patients with 5-fluorouracil (5-FU) refractory pancreatic cancer. Furthermore, gemcitabine was the first Food and Drug Administration (FDA) approval of a chemotherapy drug primarily for a non-survival clinical trial endpoint (King, 1996, Rothenberg et al., 1996).

The combination of elisidepsin and CDDP was synergistic in all cell lines except in the HT29 colon adenocarcinoma cell line (table 9A). Several cell lines

present synergistic effect with high doses such as DV90, HOP62, MCF-7, whereas others cell lines have the same effect a low doses of each drug such as MDA-MB-231. However in the breast cancer cell line MDA-MB-435 we observed synergism at 0.25x and 2x times the individual IC_{50} values of the compounds. Interestingly DLD1 cell line presented synergism with elisidepsin and CDDP at a broad range of doses for both drugs.

A

In vitro combination elisidepsin + cisplatin

Cell Line	Origin	CI (at 0.125 x IC50)	CI (at 0.25 x IC50)	CI (at 0.5 x IC50)	CI (at 1 x IC50)	CI (at 2 x IC50)
A549	Lung	4.89	1.14	1.06	***0.75***	1.21
DV-90	"	1.32	1.03	1.40	***0.48***	***0.88***
HOP-62	"	38.88	2.12	3.64	***0.85***	***0.75***
MDA-MB-435	Breast	1.42	***0.72***	1.28	2.28	***0.66***
MDA-MB-231	"	***0.72***	***0.60***	***0.66***	***0.91***	1.60
MCF-7	"	1.82	1.39	1.32	3.45	***0.72***
DLD1	Colon	***0.63***	***0.79***	1.10	***0.60***	***0.82***
HT29	"	1.76	1.22	1.28	1.97	1.10

B

In vitro combination elisidepsin + paclitaxel

Cell Line	Origin	CI (at 0.125 x IC50)	CI (at 0.25 x IC50)	CI (at 0.5 x IC50)	CI (at 1 x IC50)	CI (at 2 x IC50)
A549	Lung	***0.78***	***0.81***	***0.93***	1.35	1.65
DV-90	"	***0.73***	***0.85***	***0.92***	1.13	1.73
HOP-62	"	***0.41***	***0.39***	***0.63***	***0.86***	1.03
MDA-MB-435	Breast	***0.73***	***0.72***	1.35	2.36	***0.78***
MDA-MB-231	"	15.14	***0.92***	1.17	2.27	2.81
MCF-7	"	***0.81***	1.28	1.39	1.05	1.14
DLD1	Colon	***0.55***	***0.75***	1.42	2.54	***0.89***
HT29	"	***0.94***	1.11	1.33	***0.93***	1.18

C

In vitro combination elisidepsin+ gemcitabine

Cell Line	Origin	CI (at 0.125 x IC50)	CI (at 0.25 x IC50)	CI (at 0.5 x IC50)	CI (at 1 x IC50)	CI (at 2 x IC50)
A549	Lung	***0.42***	***0.63***	***0.85***	1.20	1.15
DV-90	"	2.46	2.81	***0.62***	***0.81***	***0.92***
HOP-62	"	1.17	***0.80***	***0.68***	1.29	1.58
MDA-MB-435	Breast	***0.17***	***0.24***	***0.43***	***0.78***	1.17
MDA-MB-231	"	1.69	***0.60***	***0.64***	***0.94***	1.19
MCF-7	"	9.27	1.71	1.46	***0.89***	***0.76***
DLD1	Colon	1.59	***0.57***	***0.62***	***0.90***	1.17
HT29	"	5.06	1.60	1.32	1.12	***0.91***

Table 9. Combination of elisidepsin with CDDP, TAX and gemcitabine**.** Cells were plated overnight and then treated for 72 h at a fixed ratio of doses that corresponded to 0.125, 0.25, 0.5, 1 and 2 times the individual IC_{50} values for each drug alone. Cell viability was measured by a crystal violet assay for optical density using a spectrophotometer. Tables show the combination index (CI) of each combination of elisidepsin with CDDP (A), TAX (B) and gemcitabine (C); CI < 1, CI = 1, CI > 1 indicate synergism, additive effect, and antagonism, respectively. All the experiments were performed in triplicate in different days. The synergistic effects (CI) were analysed by software Calcusyn.

On the contrary, the combination of elisidepsin and TAX showed synergism in all cell lines (table 9B). Several cell lines, exhibit the same tendency of synergism with TAX and CDDP such as MDA-MB-435 and DLD1. Whereas other cell lines such as A549 and DV90 had synergism at low doses, and also in the HOP62 cell line at a broad range of doses of the combinatorial drugs.

The combination results of elisidepsin and gemcitabine are presented in tables 9C and 10. In contrast to the previous combinational assays this is the only one with a

synergistic effect in all cell lines in at least one of the fixed ratio of doses. All lung carcinoma cell lines have a synergism with gemcitabine at low doses. Moreover, all breast carcinoma cell lines and colon cancer cell line DLD1 have a synergism in a broad range of concentrations, whereas the other colon carcinoma cell line, HT29 only has synergism in the highest doses tested. All pancreatic cancer cell lines have synergism at least in the highest doses (table 10). The most synergistic pancreatic cancer cell line was BxPC-3, while the most resistant to this combination was MiaPaCa-2.

Drug combination using each of these three drugs with elisidepsin has shown more efficacy than the monotherapy alone. Interestingly, the above combination was found to be synergistic even in cell lines less sensitive to elisidepsin, such as HOP62, MDA-MB-231, PANC-1 and MiaPaCa-2 cell lines.

In vitro combination elisidepsin+ gemcitabine

Cell Line	Origin	CI (at 0.125 x IC50)	CI (at 0.25 x IC50)	CI (at 0.5 x IC50)	CI (at 1 x IC50)	CI (at 2 x IC50)
AsPC - 1	Pancreas	7.62	2.52	1.40	***0.85***	***0.42***
CFPAC	"	2.71	2.15	1.89	***0.81***	***0.78***
BxPC-3	"	1.72	1.15	***0.94***	***0.79***	***0.66***
HPAC	"	1.39	1.36	1.34	1.33	***0.32***
PANC-1	"	3.60	2.28	1.71	1.29	***0.82***
MiaPaCa - 2	"	3.04	1.78	1.35	1.09	***0.87***

Table 10. Combination elisidepsin with gemcitabine in pancreatic cancer cell lines. Table show the CI of each combination; CI < 1, CI = 1, CI > 1 indicate synergism, additive effect, and antagonism, respectively. All the experiments were performed by triplicate in different days. The synergistic effects (CI) were analysed by software Calcusyn.

In this section we have examined the action of elisidepsin, a novel antitumor agent of marine origin under clinical investigation, in different cancer cell types. To determine whether the effect of elisidepsin in cancer cell lines was cytostatic or cytotoxic, a proliferation assay after incubation with several concentrations of elisidepsin was done. Viability declined with increasing concentrations of the compound, indicating that the drug was cytotoxic to human cancer cell lines. Elisidepsin showed potent cytotoxic activity against a broad spectrum of tumor types (pancreas, breast, head and neck, lung, colon, ovary and prostate) and to embryonic fibroblasts, over a relatively narrow range of IC_{50} values.
The effect of elisidepsin it is not time-dependent, however we found that cells treated with continuous subtoxic doses grow more slowly than the parental ones.

In the second place, and in order to enhance the cytotoxic effect of elisidepsin and other anticancer drugs, we studied if the combination of elisidepsin with other current chemotherapeutic agents could be effective in elisidepsin less sensitive cell lines. In this regard, we have tested the cellular effect from elisidepsin combined with CDDP, TAX and gemcitabine. The combination of elisidepsin with CDDP showed synergism in all tested cell lines except for the colorectal cell line HT29. In contrast, the combination of elisidepsin with TAX and gemcitabine showed synergism in all cell lines studied. We did not find any specific pattern that predicts drug synergy. Synergism was found independently its sensitivity to elisidepsin treatment or the other drugs.

Important membrane alterations were observed after exposure to elisidepsin (Suarez et al., 2003, Varadi et al., 2011). Although elisidepsin can interact with the lipid bilayer, it is unlikely to form pores by itself since at least 20 amino acids are required to span the lipid bilayer (Sewell et al., 2005). The composition of phospholipid bilayer greatly influences the formation of channels by well-known channel-forming cytotoxic peptides and may therefore explain the difference between cell lines' sensitivities (Kourie and Shorthouse, 2000). The differential pattern of cell permeability observed in Sewell et al. between cell lines suggests that specific proteins or membrane interactions are involved (Sewell et al., 2005). Therefore, indentifying these cellular factors may guide the targeted use

of elisidepsin and the development of analogues demonstrating enhanced selectivity.

Taken together all these data, we conclude that the combination of elisidepsin with CDDP, TAX or gemcitabine could be an effective and viable therapeutic option to be evaluated in several *in vivo* studies and provide a rationale for further development of these different combinational treatment in future clinical trials. The combination of elisidepsin with any chemotherapeutic agents shows a synergistic effect in different cell lines. The most probably rationale is that elisidepsin treatment could hit cells on the lipidic bilayer membrane, and this action could enhance the activity of the different tested drugs (CDDP, TAX or gemcitabine).

2. Predictive markers with elisidepsin cell sensitivity

As mentioned before, the primary mechanism(s) of action of elisidepsin has not been identified, although multiple targets have been described. Some of these targets, due to the hydrophobic nature of the compound, are associated with the membrane (Varadi et al., Sewell et al., 2005, Molina-Guijarro et al., 2011, Varadi et al., 2011).

Until the above targets have been validated, robust models that can identify novel/new predictive biomarkers were essential. In order to find new markers of prediction to the drug we studied the epithelial to mesenchymal transition (EMT), because increasing evidence support a role for EMT in the progression of many types of cancer , with critical roles in invasion and metastatic dissemination (Polyak and Weinberg, 2009, Thiery and Sleeman, 2006, Thiery et al., 2009), and the implication of HER3 because different data identify HER3 and Akt as major determinants of cytotoxic activity of elisidepsin *in vitro* (Janmaat et al., 2005).

2.1 Implicated markers into EMT

The EMT is a developmental program in which epithelial cells down-regulate their cell-cell junctions, acquire spindle cell morphology and exhibit cellular motility (Huber et al., 2005, Thiery, 2002). EMT plays an important role in development (Thiery, 2003, Polyak and Weinberg, 2009), particularly in

gastrulation and neural crest migration (Yamashita et al., 2004). A critical component is the loss of type I cadherins that maintain stable cell-cell contacts through adherents junctions and desmosomes (Nagar et al., 1996, Shapiro et al., 1995).

To preserve cellular shape and polarity, the intracellular domains of cadherins connect to the actin cytoskeleton through α-catenin and β-catenin (Nagafuchi et al., 1987, Patel and Gumbiner, 1995, Cavey et al., 2008).

In most cases this is associated with transcriptional repression of E-cadherin (Hazan et al., 2004, Schmalhofer et al., 2009), augmenting cell invasiveness (Thiery, 2002, Nagafuchi et al., 1987, Perl et al., 1998, Schmalhofer et al., 2009). Several specific repressor factors have been identified, such as the zinc-finger domain containing factors snail and slug (Peinado et al., 2007) and the basic helix-loop-helix factor twist, all of which can bind with so called E-boxes within the CDH1 gene promoter (Moreno-Bueno et al., 2006, Peinado et al., 2007). Their function is regulated by oncogenic pathways, particularly by Akt, GSK-3β, NF-κB, RAS and SRC.

2.1.1 Associated EMT factors in pancreatic and breast cancer cell lines

In order to evaluate the EMT protein markers expression levels and correlate them with the sensitivity of the cell lines to elisidepsin, we performed different analysis based on western blot, immunofluorescence and IHC in a panel of 12 cell lines (6 pancreatic and 6 breast cancer cell lines). The protein expression of E-cadherin, β-catenin, vimentin, slug, snail and twist-1 were assessed by immunocytochemical and western blot analysis, while by immunohistochemical analysis were only performed for E-cadherin, β-catenin, and vimentin protein expression.

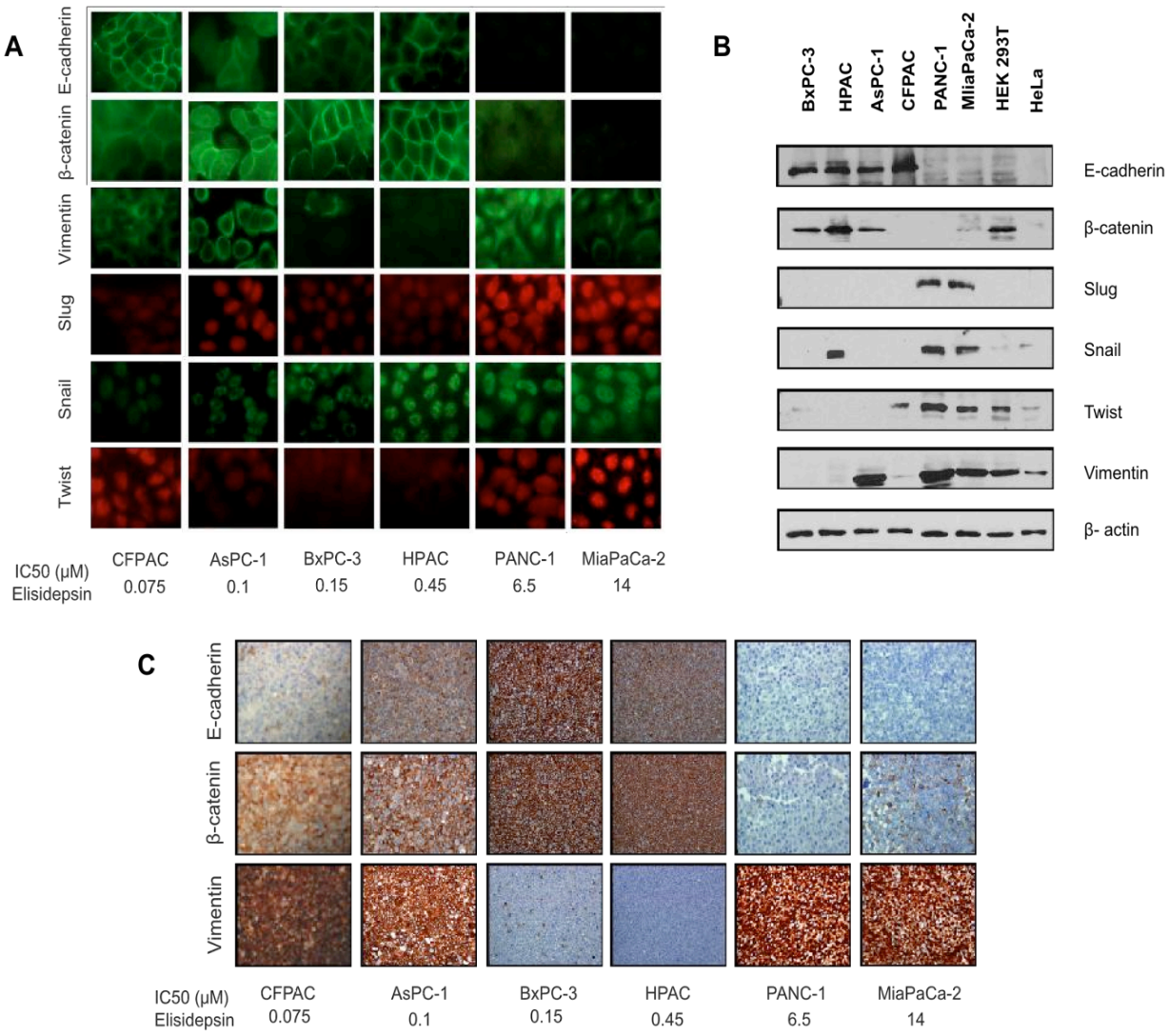


Figure 11. Expression of EMT markers with elisidepsin sensitivity in pancreatic cancer cell lines. Basal E-cadherin, β-catenin, vimentin, slug, snail and twist expression levels were evaluated by immunocytochemistry (A) and western blot (50 μg of protein/lane) (B). Immunocytochemistry photographs were taken in a magnification 100x. Membranes were stripped and reproved with anti-β-actin and these were used as an internal control. C) E-cadherin, β-catenin and vimentin protein expression levels were evaluated by IHC. Magnification 20x. These analyses were performed by duplicate.

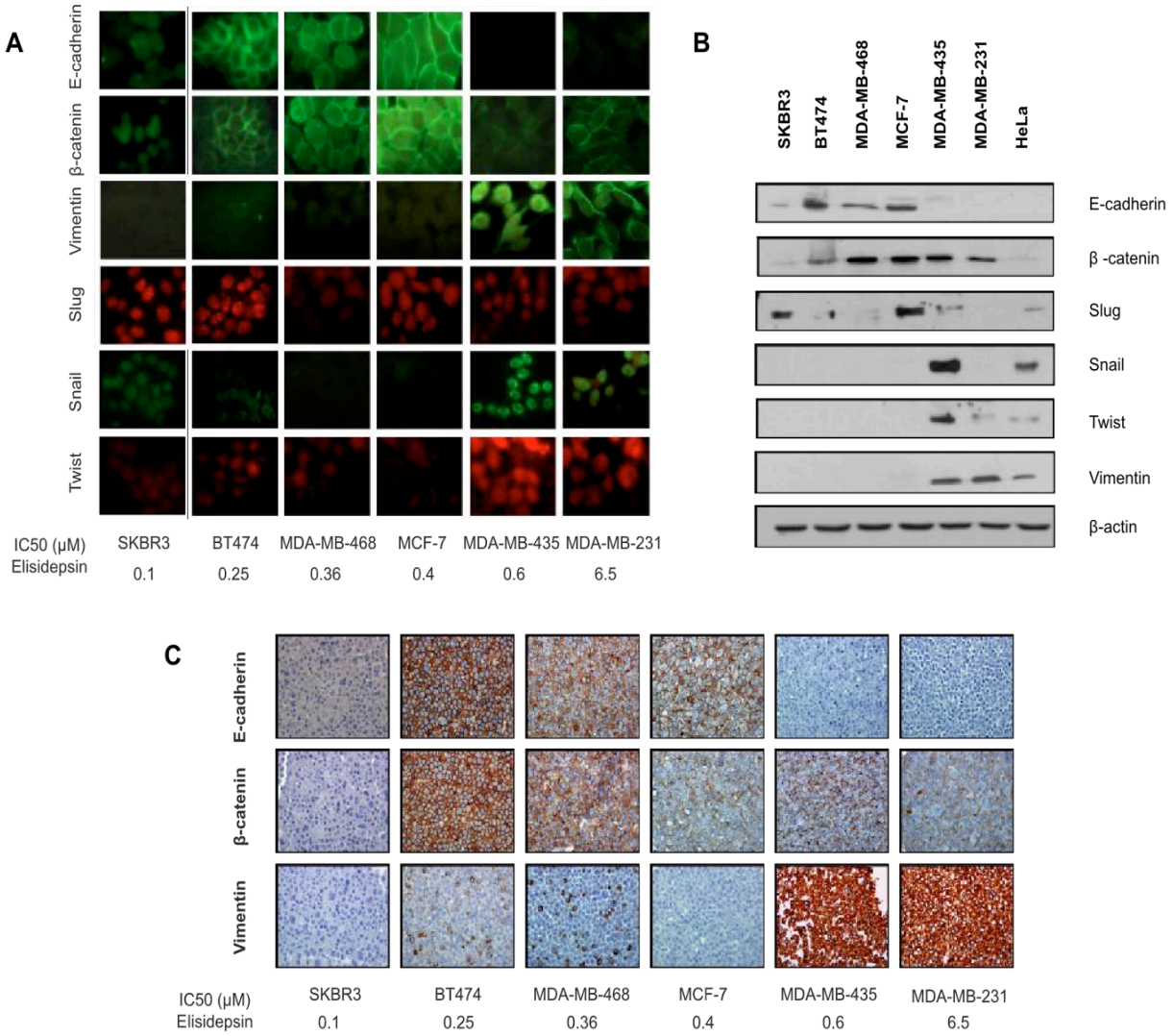


Figure 12. Expression of EMT markers with elisidepsin sensitivity in breast cancer cell lines. Protein expression levels of different EMT markers were evaluated by immunocytochemistry (A), western blot (B) and IHC (C). A) Immunocytochemistry of 2 epithelial (E-cadherin and β-catenin) and 4 mesenchymal markers (vimentin, slug, snail and twist). Magnification 100x. B) E-cadherin, β-catenin, slug, snail, twist, vimentin and β-actin (loading control) were detected by western blot analysis of 50 μg of total protein. C) Basal levels of E-cadherin, β-catenin were analyzed by IHC. Magnification 20x. These experiments were performed at least by duplicate.

We determined whether the different cancer cell lines share common EMT genes in their basal levels when they are sensitive to elisidepsin treatment. The pancreatic carcinoma cell lines presented E-cadherin and β-catenin when they are sensitive to elisidepsin treatment and the less sensitive ones had slug expression . Furthermore, snail, twist-1 and vimentin expression was found in sensitive and less sensitive cell lines (figure 11). In contrast, in the breast cancer cell lines we found a high E-cadherin expression in the sensitive cell lines. All cell lines have detectable expression of β-catenin, whereas slug expression was found independently of their sensitivity to elisidepsin. Lastly, snail expression was only found into MDA-MB-435, and all cell lines that exhibit levels of twist-1 and vimentin where the less sensitive to the drug (figure 12).

E-cadherin protein was significantly expressed in the sensitive cell lines, while the vimentin, twist-1 and snail proteins were found in all cell lines with less sensitivity to the drug with an exception of two sensitive cell lines for vimentin expression (CFPAC and AsPC-1) and with one sensitive cell line for the twist-1 (CFPAC) and snail (SKBR3). With these results we can conclude that sensitivity of pancreatic and breast cancer cell lines to elisidepsin positively correlates with an epithelial phenotype.

2.1.2 Acquired resistance to elisidepsin induces a mesenchymal phenotype

Three elisidepsin resistant cancer cell lines (HCT 116 R, A549 R and the MCF-7 R) were generated by chronically exposing the cancer cell lines to increasing concentrations of the drug. Cancer cell lines were exposed to elisidepsin at a starting concentration of their IC_{50} (HCT 116, 4.3 μM; A549, 0.2 μM; MCF-7, 0.4). Elisidepsin was increased every week until cells became resistant to it for over 12 months. Cell clones resistant to the antiproliferative effects of elisidepsin were identified (HCT 116, 100 μM; A549, 25 μM; MCF-7, 8 μM). Figure 13 shown the proliferation of a representative HCT 116, A549 and MCF-7 elisidepsin-resistant cell lines are undisturbed in the presence of increasing concentrations of elisidepsin. HCT 116 R and A549 R cancer cell lines were generated by PharmaMar, while MCF-7 R cancer cell line was generated in Vall d'Hebron Hospital. As shown

in figure 14 the morphology of the resistant cancer cell lines was modified after the continuous exposure to the drug, compared with the parental cell lines. Cells that became resistant to elisidepsin treatment had a phenotype more mesenchymal, loss of cell-cell adhesions, disorder and larger. Our hypothesis was that loss of epithelial markers observed in our panel of cancer cell lines could be responsible for the resistance of elisidepsin treatment, which would in turn result in the acquisition of mesenchymal markers of these cells.

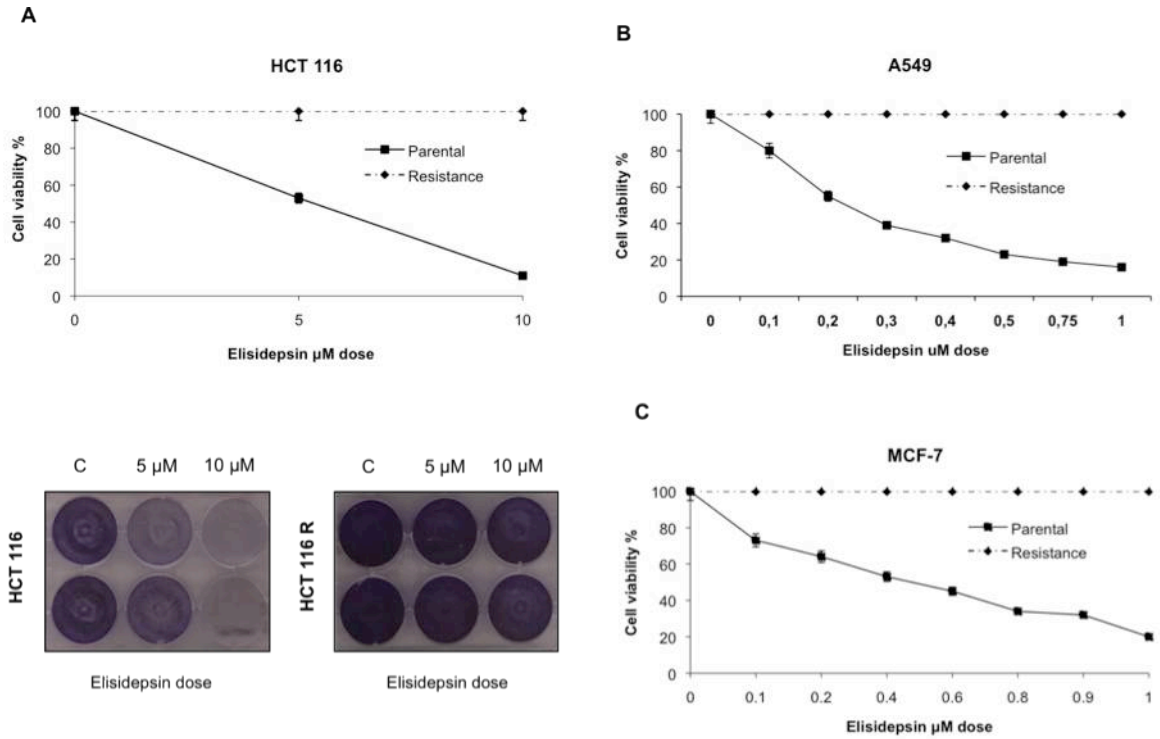


Figure 13. Generation and characterization of elisidepsin resistant cell lines. Elisidepsin sensitive cancer cell lines were made resistant by persistent exposure to increasing concentrations of elisidepsin. Cells were treated with elisidepsin at the indicated concentrations for 72 h and proliferations were measured by crystal violet. (A) HCT 116, (B) A549 and (C) MCF-7. Error bars, SD, of three replicate experiments. C, control.

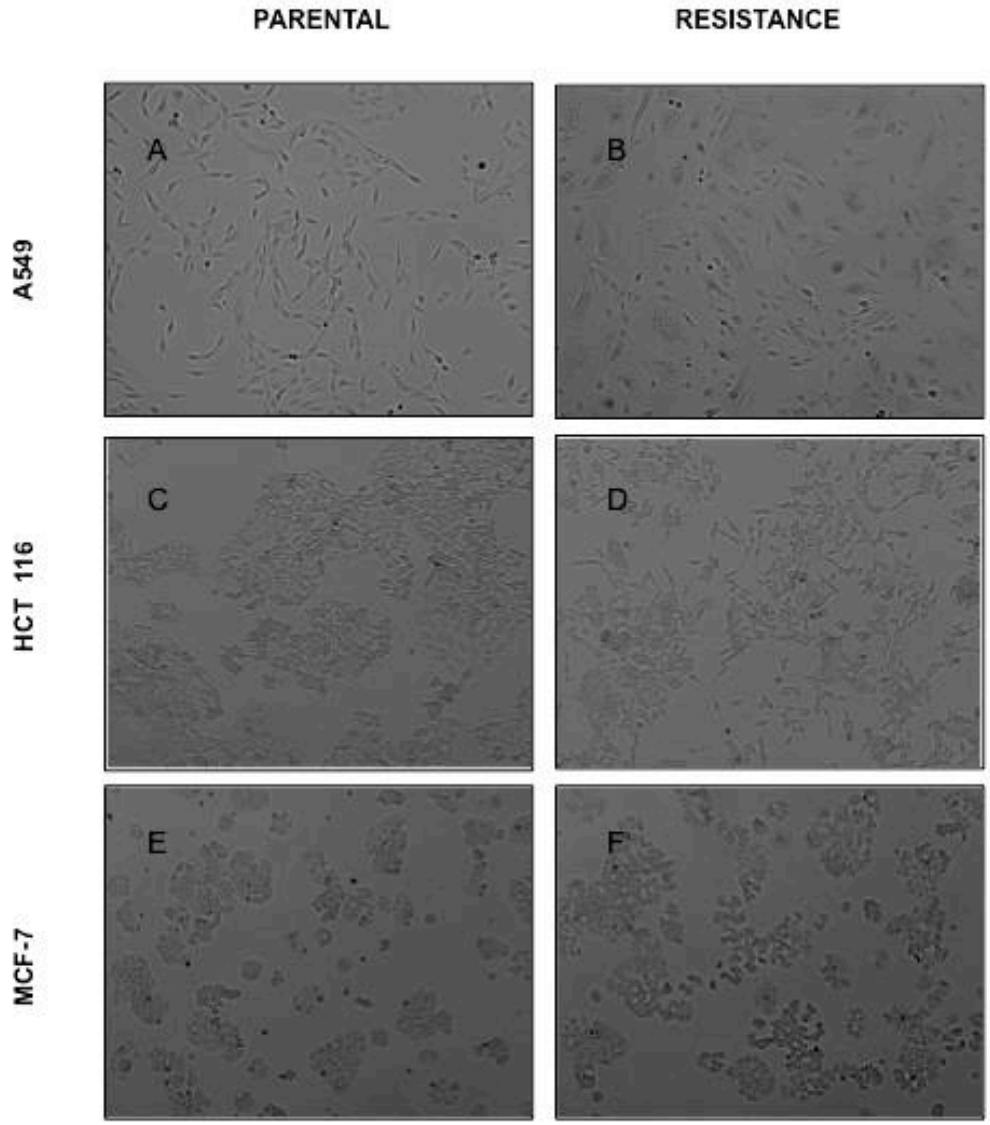


Figure 14. Morphology of lung, colon and breast cancer cell lines in two dimensions. Phase contrast pictures of parental (A, C and E) and resistance (B, D and F) cancer cell lines. A and B represented A549 lung cancer cell lines, C and D HCT 116 colon cancer cell line, and E and F MCF-7 breast cancer cell lines. Cells resistance to elisidepsin treatment had a phenotype more mesenchymal. Magnification 40X.

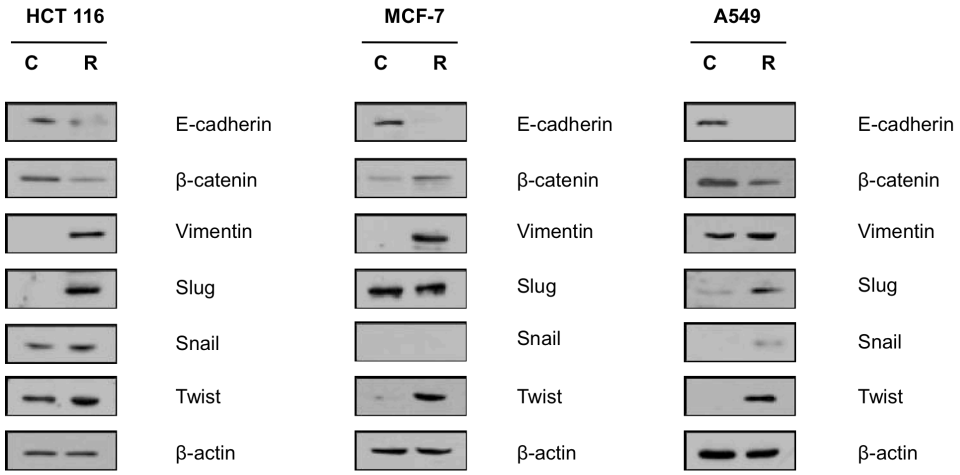


Figure 15. Acquired resistance to elisidepsin induces an EMT phenotype. Cells were lysed, proteins were extracted and western blots performed with an equal amount, 50 μg of protein, of cell lysate. Expression of epithelial (E-cadherin, β-catenin) and mesenchymal (vimentin, slug, snail, twist) associated proteins differentiates between elisidepsin sensitive and elisidepsin resistant cell lines. β-actin was used as an internal control. These western blots were performed by triplicate. C, control; R, resistance.

We then performed western blot analysis of the acquired resistance cancer cell lines and compared them to parental control cells. With this approach, we identified that the three different cancer cell types with acquire resistance to elisidepsin treatment changed their basal levels of EMT markers (figure 15). All resistance cell lines showed decreased E-cadherin and increased of vimentin and twist-1 expression. β-catenin expression was down-regulated in the resistance HCT 116 and A549 cancer cell lines but up-regulated in the MCF-7, while levels of slug and snail were up-regulated in the resistance cancer cell lines HCT 116 and A549 but no difference was found in the breast carcinoma MCF-7 cell line.

The role of the cell membrane as an important target of elisidepsin was studied in HCT 116 R, MCF-7 R and A549 R cell lines (cell lines that show specific resistance to elisidepsin). When treated with effective, high concentrations of elisidepsin, the resistant cells were completely insensitive, thus indicating that

might have acquired specific alterations in their cell membrane that rendered them resistant to drug treatment.

This study showed that continuous exposure of elisidepsin induces a down-regulation of epithelial markers in three different cancer cell types (lung, colon and breast). Loss of epithelial markers was further evidenced by the detection of morphological changes in the cells. The morphological changes observed after continuous long-term exposure of different cell types to elisidepsin suggest that the compound is able to modify the composition of the plasma membrane. This behavior is further accompanied by signaling changes, resulting in the upregulation of mesenchymal markers. All of these morphological and physiological events were confirmed in cells treated with and without elisidepsin.
Western blot was used to investigate the expression patterns of EMT-related proteins, including transcription repressors and this analysis confirmed that acquired resistance to elisidepsin is associated with a switch to EMT.

2.1.3 Implication of HER receptors into elisidepsin sensitivity

Since indirect evidence points to the involvement of HER RTK in the mechanism of action of elisidepsin, we compared the elisidepsin sensitivity of cell lines with the expression of HER receptors.
KF (the natural compound) was shown to be the third compound, among 49.000 receptor compounds, tested in the NCI database, showing the highest correlation between cytotoxicity and high HER2 mRNA expression levels; this information was generated in a study in which HER1, HER2 and TGF-α mRNA expression levels in the 60 cell lines comprising the NCI anticancer drug screen were analyzed and correlated by COMPARE analysis with drug sensitivity.

Based on this observation, we investigated the expression of endogenous HER family receptors by western blot in the panel of pancreatic and breast cancer cell lines studied in EMT (figure 16). The sensitivity to elisidepsin treatment was found to correlate with HER3 protein expression levels. Cell lines that were less sensitive to elisidepsin had no levels of HER3 in comparison with the sensitive cell lines, which expresses levels of this protein. Also the other members of HER family

were checked by western blot, but no correlations were found on them. Cell lines less sensitive to elisidepsin (MDA-MB-231, PANC-1 and MiaPaCa-2) do not present HER3 protein levels, while PANC-1 and MiaPaCa-2 cell lines present levels of HER1, HER2 and HER4. In the case of MDA-MB-231, it does not present levels of any of the HER family members.

The standard technique for the detection of predictive biomarkers of response in pathology is based on immunohistochemistry. For this reason we performed HER3 IHC staining in this panel of pancreatic and breast cancer cells and the same results were obtained as western blot (figure 17). Expressing HER3 protein cells were sensitive to elisidepsin treatment.

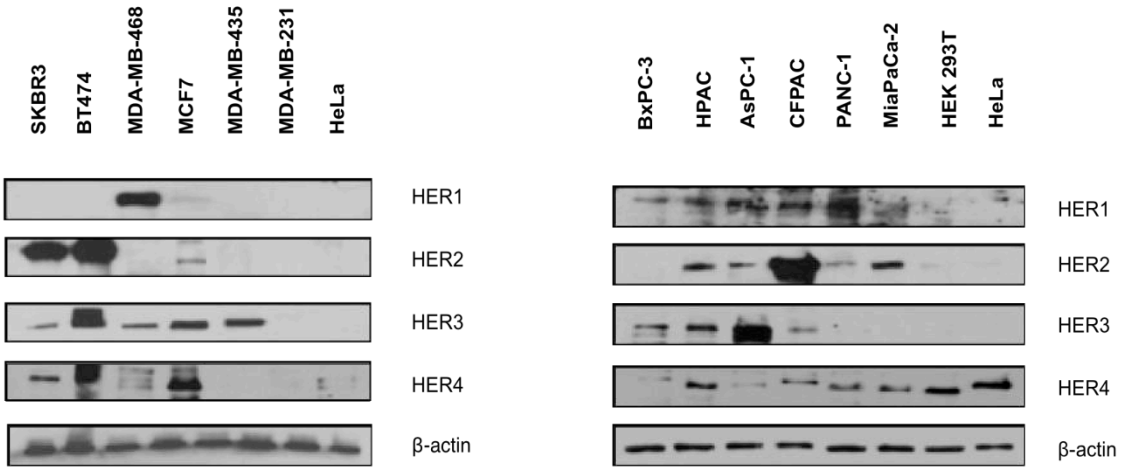


Figure 16. HER3 expression levels correlate with elisidepsin cell sensitivity. Basal expression levels of HER family members were analyzed by western blot and we found an association between HER3 expressions with elisidepsin sensitivity. Cell lines less sensitive (MDA-MB-231, PANC-1 and MiaPaCa-2) to elisidepsin do not present HER3 protein levels, while in the PANC-1 and MiaPaCa-2 cell lines present levels of other HER family members. No correlation was observed with HER1, HER2 and HER4 expression levels. These protein expression levels were analyzed in duplicate and 50µg of protein of cell lysate were loaded in each lane.

Furthermore, we wanted to study if the three elisidepsin resistant cancer cell lines (HCT 116 R, A549 R and the MCF-7 R) also present different expression

levels of HER family members and their direct signaling compared with their parental ones. Levels of protein markers studied were all different between sensitive versus resistant cell lines. Expression protein levels of pHER3, HER3 and HER4 were down-regulated in all resistance cancer cell lines and their downstream signaling (figure 18). Suppression of downstream signaling was similarly seen in HCT116 R and MCF-7 R cells, but only in the A549 R an up-regulation of pMAPK was seen identified, maybe due an increase of HER1 expression and the maintenance of HER2 expression levels. Also an increase of HER1 total levels was seen in HCT 116 R, while in MCF-7 R cancer cell line a decrease of all HER family members was obtained.

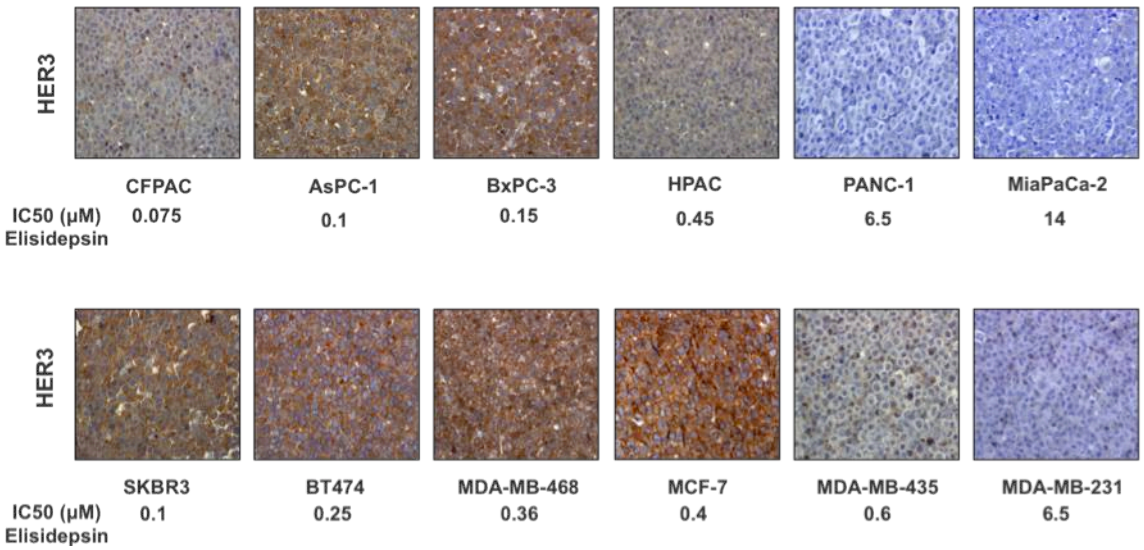


Figure 17. HER3 is a good predictive marker of elisidepsin sensitivity. Cell pellets were embedded in paraffin and IHC for HER3 was performed. The more elisidepsin sensitive cell lines presented significant HER3 levels. Magnification 20X.

These results showed that elisidepsin resistance acquired cells decreased all HER family expression levels except HER1 and those affect their direct signaling. This seems to be a likely possibility given that loss of epithelial markers are involved in resistance to elisidepsin.

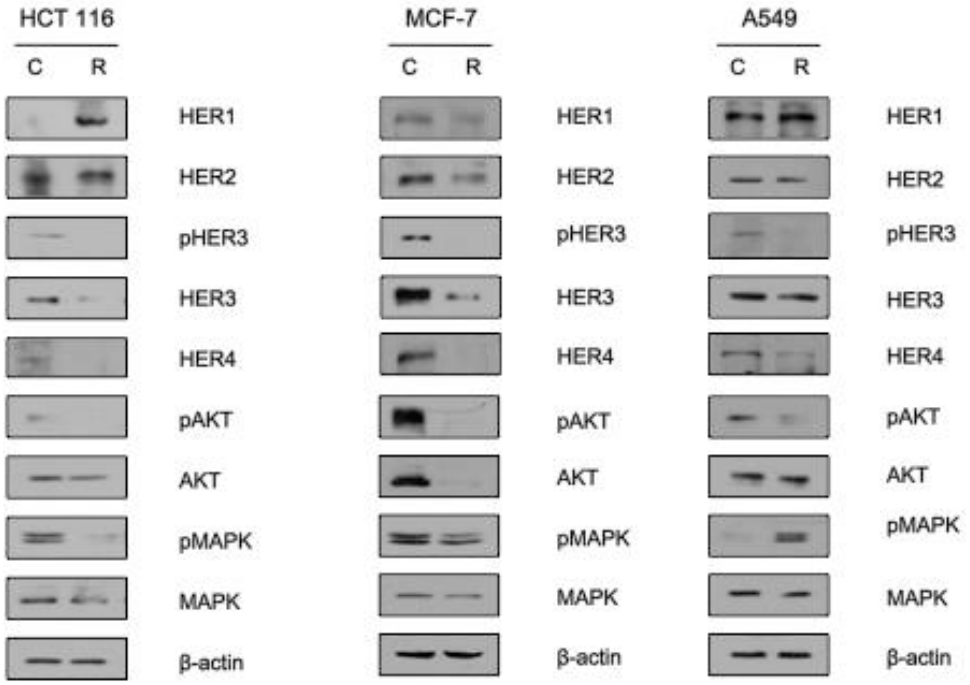


Figure 18. Elisidepsin induces down-regulation of HER3 and HER4 proteins. Western blot analysis of 50 μg of cell lysate of protein expression levels of pHER3, HER1, HER2, HER3, HER4, pAkt, pMAPK and their totals were analyzed. The membranes were stripped and reproved with anti-β-actin to verify equal protein loading. C, control; R, resistance.

In a parallel study, we compared the expression levels of all HER family members in our panel of cell lines and we found a correlation between HER3 expression and sensitivity to elisidepsin treatment. This finding is in agreement with previous observations in which a correlation between sensitivity to the parent compound elisidepsin and HER3 expression was observed in a panel of tumour cell lines (including NSCLC, ovarian and hepatic carcinomas) (Janmaat et al., 2005). With this data we can conclude that targeting HER3 expression could be in a valuable strategy to know when cells are going to be sensitive to elisidepsin treatment.

2.2 HER3

2.2.1 Effect of elisidepsin treatment on HER3 protein levels

Previous studies have reported a selective down-regulation of HER3 after cell exposure to the natural compound KF in a cell line expressing high levels of this receptor, SKBR3 (Janmaat et al., 2005). Western blot analysis was carried out to investigate potential differences in the expression levels of HER3 in MCF-7 breast cancer cell line, which expresses significant HER3 protein levels, with different exposure times and drug concentrations (figure 19).

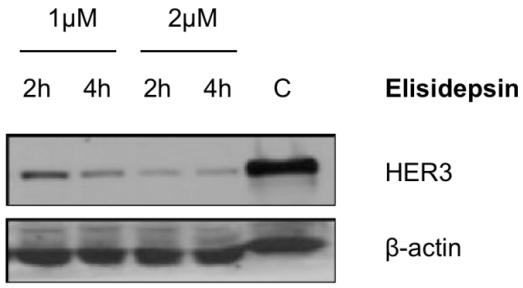


Figure 19. Elisidepsin induces down-regulation of HER3 protein. HER3 protein expression was analyzed by western blot on total cell lysates of MCF-7 cells and 50 μg of protein were loaded in SDS-PAGE. Effect of elisidepsin treatment on HER3 expression was analyzed on cells treated with 1 or 2 μM elisidepsin for a short period of time, 2 h and 4 h. A total cell lysate of MCF-7 cells was included as a positive control. The membranes were probed with β-actin to verify equal protein loading. C, control.

A 4 h exposure and 1 μM concentration of the drug were chosen as the ideal conditions for do next experiments, due the same results were obtained with a 2 μM dose and the possibility to spend less quantity of the drug for each experiment, to obtain results of higher specificity and selectively.

Next step was to determine if elisidepsin treatment could similarly affect the expression of other HER receptors. To this end, we did a time course of 2, 4 and 6 h treatment with 1μM of elisidepsin, and western blot analysis to investigate if the treatment induces the down-regulation of the HER family proteins in MCF-7 cell line. In contrast to the general down-regulation of HER family members observed after long-term exposure to elisidepsin (figure 18), a 4 h treatment with elisidepsin resulted in the selective down-regulation of HER3 and a maintenance of HER1 and

HER2 protein expression levels. Interestingly, in the case of the HER4 protein levels, we observed an initial up-regulation at 2 and 4 h post-treatment of the drug.

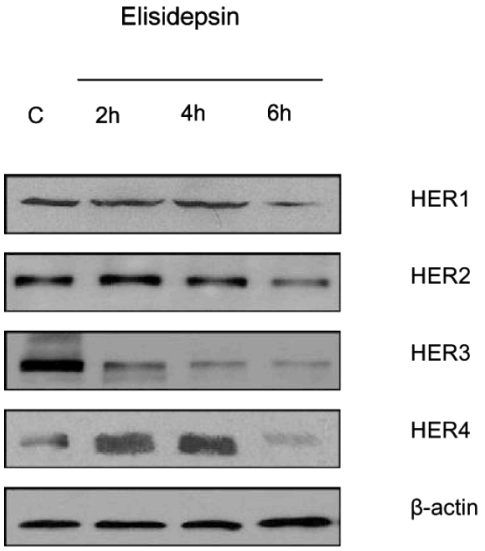


Figure 20. Down-regulation of HER family proteins expression upon 1 μM elisidepsin treatment. After treatment, cells were lysed and proteins were extracted and western blots performed with 50 μg of proteins. HER1, HER2 and HER3 were detected by western blot using corresponding antibodies. The membranes were stripped and reproved with anti-β-actin to verify equal protein loading. C, control.

Protein expression levels of all members of the HER family were found to be down-regulated after a 6 h treatment with elisidepsin (figure 20). This result clearly indicates that HER3 is the HER receptor most sensitive to treatment with elisidepsin in MCF-7 cells.

Since a down-regulation of HER3 protein expression was observed in MCF-7 cells upon elisidepsin treatment, we sought to determine if this compound also affected HER3 expression in a panel of human tumor cell lines with variable expression levels of this receptor, namely lung (A549, DV90 and HOP62), breast (MDA-MB-435, MDA-MB-231 and MCF-7) and colon (DLD1 and HT29) cancer cell lines (figure 21).

Cells were treated with 1 μM elisidepsin for 4 h and then lysed. HER3 receptor levels were down-regulated in the majority of cell lines analyzed after treatment with 1 μM elisidepsin (figure 22). Only two (MDA-MB-231 and HOP-62) out of eight cell lines tested maintained their HER3 expression levels after elisidepsin treatment.

Those differences in HER3 receptor levels in the analyzed cell lines prompted us to study the downstream signaling routes that link this receptor to proliferative responses, Akt and MAPK. Western blot with antibodies that recognized activated forms of Akt indicated that the resting levels of pAkt were lower or had disappeared more notably in the cells that had a down-regulation of HER3 after elisidepsin treatment than in the cells that had not (figure 22).

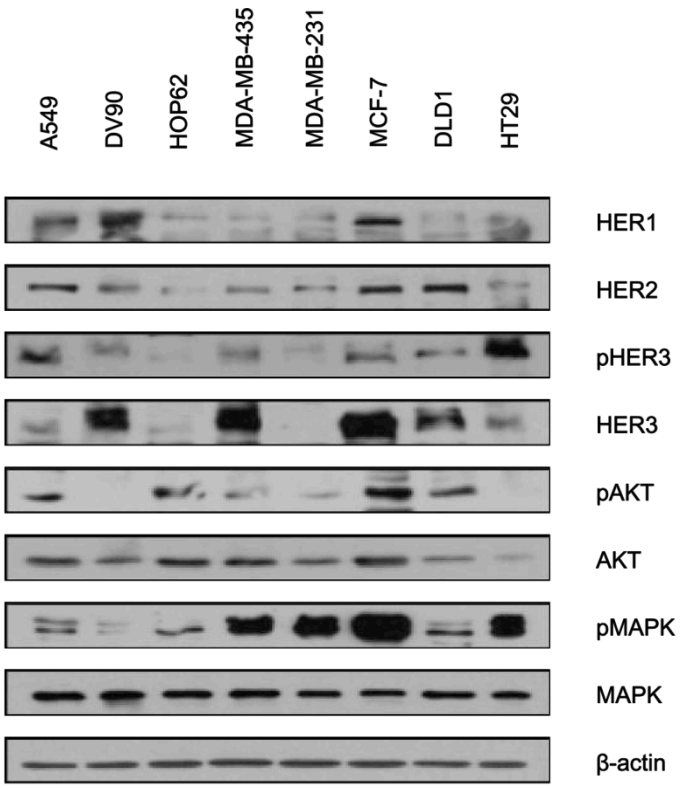


Figure 21. Study of a panel of cell lines. A total amount of 50 μg of protein extracts from 8 different cell lines were loaded in SDS PAGE gels and western blot analysis were performed against different antibodies regarding HER family, also pAkt and pMAPK activation. β-actin was used as an internal control to verify equal protein loading.

We also investigated the phosphorylation of MAPK. In contrast to pAkt results, pMAPK levels were not down-regulated in almost all cell lines. MAPK phosphorylation was up-regulated in HOP62 and MDA-MB-435 cell lines, and down-regulated in A549 and DV90 lung cancer cell lines, MCF-7 breast cancer cell line and DLD1 colon cancer cell line. MDA-MB-231 and HT29 cell lines maintained the same amount of pMAPK protein after treatment with the drug.

In contrast, total amounts of Akt and MAPK are not affected by elisidepsin treatment, except from the DLD1 colon cancer cell line that exhibited a decrease in

Akt protein expression levels. We also analyzed β-actin state to verify an equal amount of protein in each well.

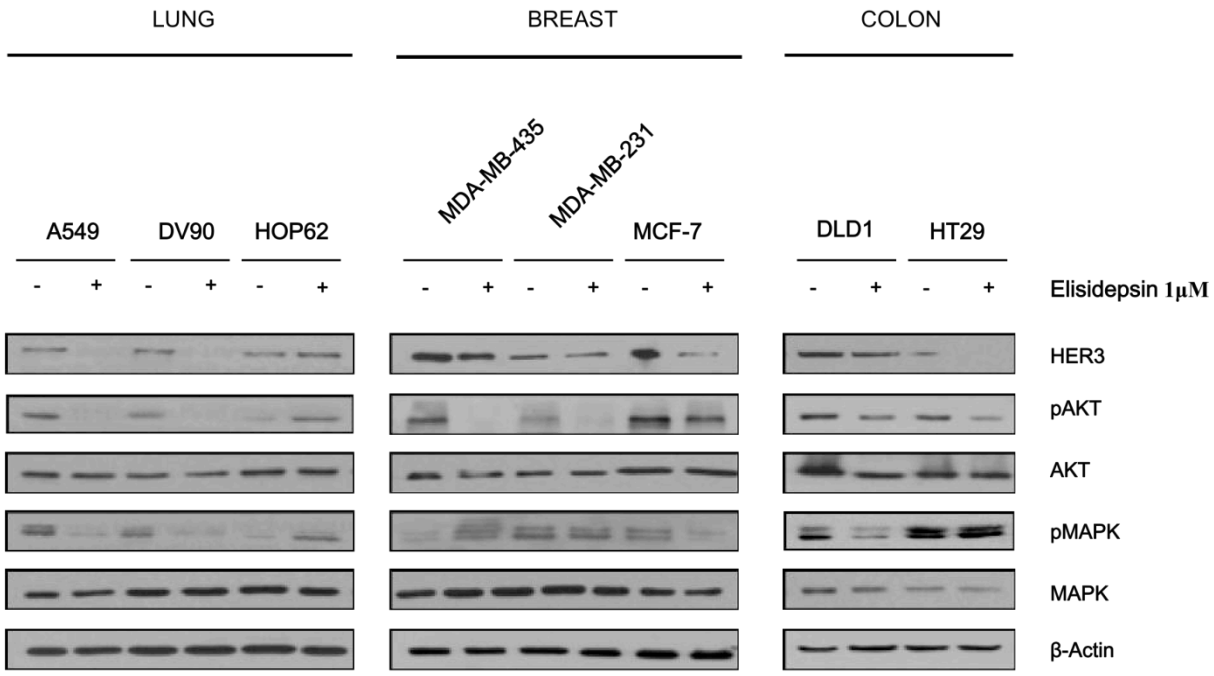


Figure 22. Effect of elisidepsin treatment on HER signaling pathways. All cell lines were seeded at 70% of confluence in 100 mm cell culture dishes, and 18 h later, were treated with 1 μM of elisidepsin for 4 h. After treatment, cells were lysed, proteins were extracted and western blots performed with 50 μg of protein, for each sample. In the control samples, the same amount of the dissolved product (DMSO/ethanol) without elisidepsin was added. Membranes were stripped and reproved with anti-β-actin and these were used as an internal control.

Recent studies had shown that the potent cytotoxic activity of elisidepsin is exerted very rapidly through insertion of the drug molecule into the plasma membrane and induction of drastic loss of membrane integrity (Molina-Guijarro et al., 2011). Due to this, next we investigated if the effects of elisidepsin treatment could lead the cells recover after its exposition. MCF-7 cancer cell lines were treated with 1 μM elisidepsin for 4 h and media were changed and proliferation was measured at different time points. More than 50% cells died after 4 h drug

treatment but after media changed cells could recover and proliferate again (figure 23).

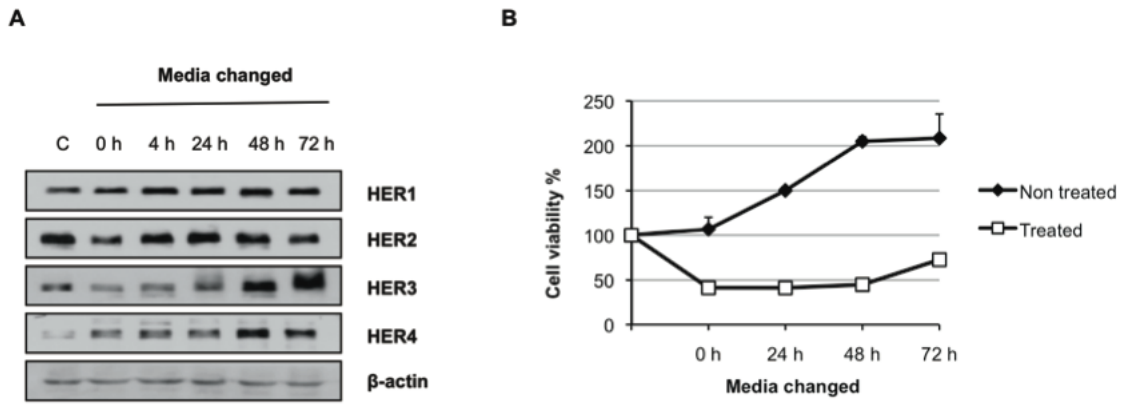


Figure 23. MCF-7 can recover after elisidepsin treatment. A) Cells were treated with 1 μM of elisidepsin for 4 h and then media was changed and waited for 4, 24, 48 and 72 h. HER protein expression levels were analyzed by western blot on total cell lysates of MCF-7 cells and 50 μg of protein were loaded in SDS-PAGE gels. The membranes were stripped and reproved with anti-β-actin to verify equal protein loading. B) Cells were treated with 1 μM of elisidepsin for 4 h elisidepsin and proliferation was measured by crystal violet in different time points. Results are mean ± SD of two independent experiments. C, control.

Furthermore, we studied in MCF-7 cells the protein expression levels of HER family members after treatment with 1 μM elisidepsin for 4 h, washing and changing of media. We obtained similar results than other experiments, a down-regulation of HER2 and HER3, maintenance of HER1 protein expression levels and an up-regulation of HER4 protein levels with an exposition of elisidepsin for 4 h.

The media change helped cells recover from elisidepsin effects and an increase of protein expression levels was seen in all HER family members. In the case of recovering HER2 and HER3 protein expression levels, HER2 protein levels were increased immediately after cells were washed (4 h), while for HER3 it was after 24 h. These data show that despite the fact that elisidepsin treatment induces into the cells drastic loss of membrane integrity, those cells that maintained alive after its treatment can recover and proliferate again.

Regarding HER family receptors, in the present study we observed an association between HER3 protein expression and sensitivity to elisidepsin treatment in a variety of cell lines. We observed a relatively rapid (2 and 4 h) specific down-regulation of HER3 upon elisidepsin treatment in the breast cancer cell line MCF-7, whereas the other HER family members were not affected. These data, obtained in a breast cell line model, stand in accordance with previous results obtained in a lung cancer model (Ling et al., 2009), supporting the hypothesis of a selective role of HER3 in the cellular response to this drug, although other authors proposed this HER role as a secondary process upon cell membrane alterations by elisidepsin treatment (Varadi et al., 2011).

HER3 is a major recruiter of PI3K; it often couples to other HER receptors to activate the PI3K-Akt pathway, consequently promoting the cancer phenotype (Prigent and Gullick, 1994, Burgering and Coffer, 1995). Using a broad panel of cell lines including lung, breast, and colon carcinoma, we analyzed the most important pathways downstream of this receptor and of the most transforming and mitogenic receptor complex of the HER family receptors (the HER2/HER3 heterodimers) (Holbro et al., 2003a, Alimandi et al., 1995, Wallasch et al., 1995), and evaluated the phosphorylation levels of Akt and MAPK in response to elisidepsin treatment. In these cell lines, we observed a down-regulation of HER3 in 6 out of 8 tested cell lines, confirming the previous data in MCF-7, this down-regulation being associated with a decrease in the levels of serine 473 phosphorylation in Akt in the same set of cell lines. These results are in agreement with previous results obtained in different cell lines with elisidepsin treatment (Janmaat et al., 2005), and due to this lower survival signaling, cell lines exhibit a cytotoxic response. Interestingly, the HOP62 cell line shows an up-regulation of pAkt upon elisidepsin treatment, and correlates with a less sensitive phenotype.
In contrast, the levels of pMAPK do not appear to predict cell viability response, because we did not observe significant differences after elisidepsin treatment in resistant (HOP62 and MDA-MB-231) and sensitive (MDA-MB-435 and HT29) cell lines. It is probable that other molecular alterations or crosstalk signaling pathways could activate pMAPK indirectly, but this phosphorylation does not reflect *in vitro* elisidepsin sensitivity.

In summary, these results indicate that elisidepsin treatment has an effect in HER3 protein levels and the downstream PI3K-Akt pathway coupled to this receptor in cell lines derived from different human tumor types. Elisidepsin decreases phosphorylated Akt levels and this reduction is associated with cytotoxicity in elisidepsin sensitive cell lines. Taking into account these data, the down-regulation of HER3 and pAkt levels could be a good predictive model to detect the *in vitro* response to elisidepsin treatment.

2.2.2 Study of HER3 mRNA levels after elisidepsin treatment

Furthermore we wanted to compare the elisidepsin sensitivity with HER3 mRNA levels in different cancer cell lines. The purpose was to know if HER3 mRNA levels could predict sensitivity or resistance to elisidepsin treatment. To carry out this study, cells were treated or not with 1 μM elisidepsin and then RNA extraction was performed.

Elisidepsin exposure induced several changes in mRNA expression of HER3 receptor in MCF-7 cancer cell lines (figure 24, A). Longer exposure of the drug induced a higher down-regulation of HER3 mRNA levels. This decreased of HER3 mRNA levels were not seen in other cancer cell lines studied. Three of four cancer cell lines had a down-regulation of HER3 mRNA levels after treatment with 1 μM elisidepsin for 4 h, but only one of them had a significant down-regulation of HER3 mRNA levels (BxPC-3, $p = 0.028$ with the Wilcoxon analysis test).

These data, obtained in the breast cancer cell line model MCF-7, stand in accordance with previous results obtained in protein expression levels, supporting the hypothesis of a specific down-regulation of HER3 upon elisidepsin treatment. Furthermore, other cell lines were chosen to study elisidepsin effect in HER3 mRNA levels with different HER3 expression levels (figure 17) but in this case sensitivity to elisidepsin treatment, and down-regulation of HER3 mRNA levels did not correlate with baseline expression levels. Although elisidepsin treatment decreased HER3 mRNA and protein expression levels our data indicate that elisidepsin is more effective in protein expression levels than in mRNA ones in the

cells studied.

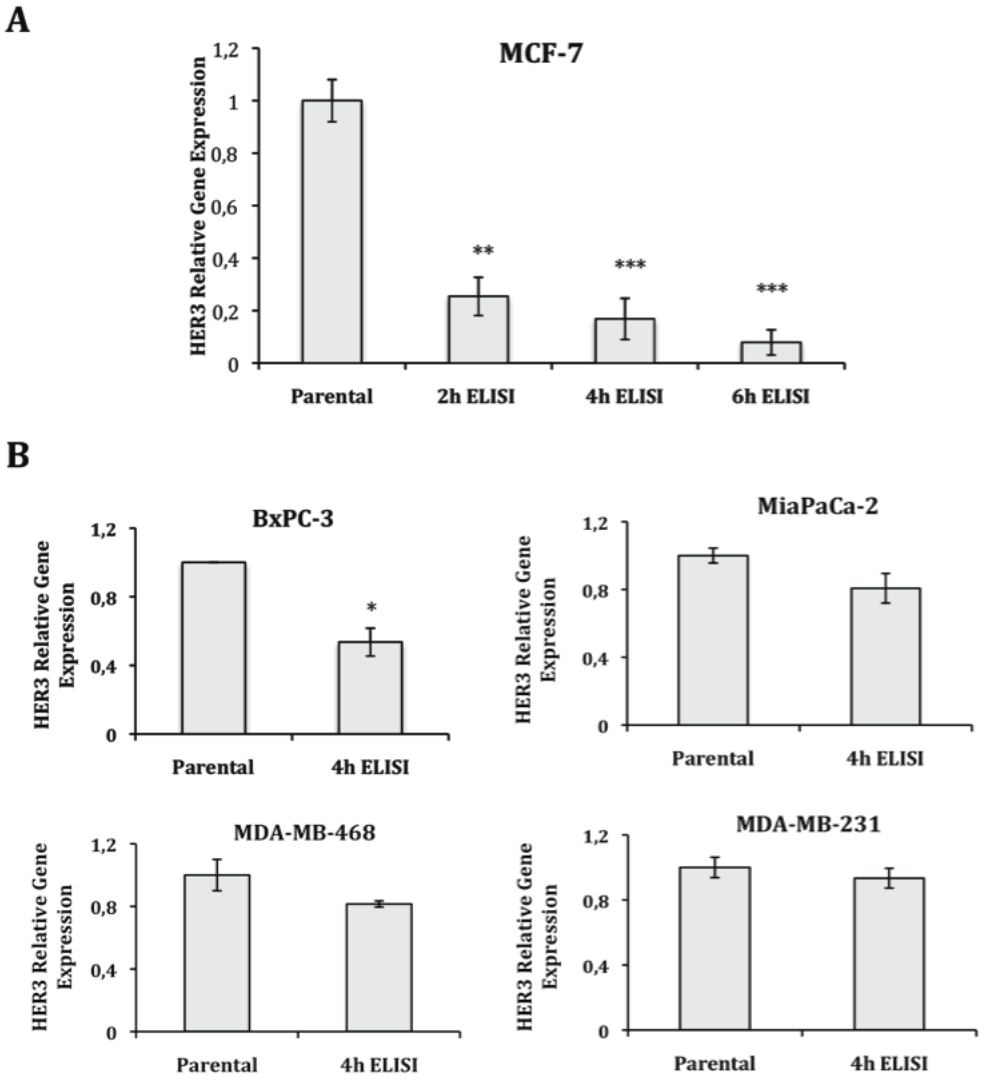


Figure 24. Elisidepsin decreased HER3 mRNA expression. Study of HER3 mRNA expression in breast and pancreatic cancer cell lines. A) MCF-7 cancer cell lines treated with 1μM elisidepsin for 2, 4 and 6 h. B) Two pancreatic (BxPC-3 and MiaPaCa-2) and two breast (MDA-MB-468 and MDA-MB-231) cancer cell lines were treated with 1 μM elisidepsin for 4 h. Results are mean ± SD of two independent experiments; *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$. ELISI, Elisidepsin.

This could be due because elisidepsin treatment induces damage of mitochondria, endoplasmatic reticulum, and the plasma membrane, while the nuclear membrane is preserved and no DNA damage is detected, although the cell nucleus shows irregular clumping of chromatin (Janmaat et al., 2005).

2.2.3 Regulatory factors of HER3

The mechanism by which HER3 is down-regulated by elisidepsin remains to be clarified. Elisidepsin induced down-regulation of HER3 is not caused by inhibited synthesis of the receptor because treatment of MCF-7 cells with the protein synthesis inhibitor CHX (50 μM) for 4 h did not affect HER3 expression levels (figure 25 A). These data thus indicate that HER3 down-regulation is caused by degradation rather than inhibition of protein synthesis. HER3 protein can be ubiquitinated by the ubiquitin ligase Nrdp1 and subsequently degradated by proteasomes (Diamonti et al., 2002). This phenomenon appears to be, at least in part, proteasome dependent since co-administration of the proteasome inhibitor MG132 do prevent HER3 loss expression compared to elisidepsin alone. However, elisidepsin induced HER3 degradation was not proteasome mediated, because treatment of cells with the proteasome inhibitor MG132 failed to protect from elisidepsin induced HER3 depletion (figure 25 B).

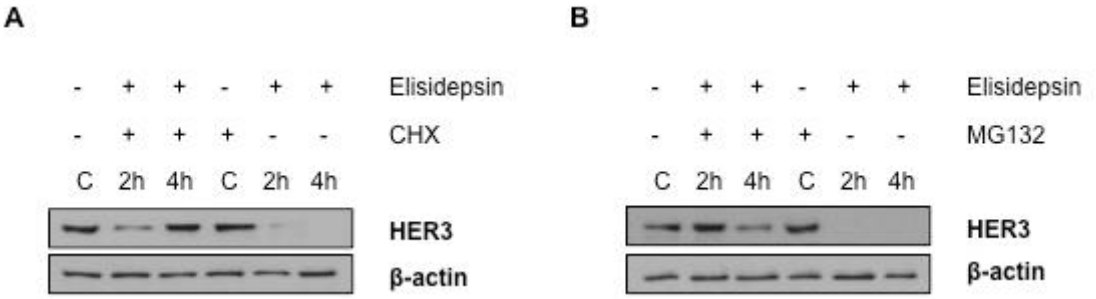


Figure 25. Elisidepsin does not affect HER3 stability by regulating its degradation. HER3 degradation was analyzed in culture, at elisidepsin-treated MCF-7 cells in presence or absence of the protein synthesis inhibitor CHX (A) and the proteasome inhibitor MG132 (B) for 2 and 4 h. β-actin was used as an internal control to verify equal protein loading. C, control; CHX, cyclohexamide.

On the other hand, to study if elisidepsin mediates the internalization of HER3 and subsequently targets it for degradation, similarly to EGF mediated internalization of the HER1 (French et al., 1995) we analyzed Nrdp1 and USP8 protein expression levels in different cells treated with 1 μM elisidepsin. As mentioned above, Nrdp1 is an ubiquitin ligase of HER3 protein, while USP8 is a

deubiquitinating enzyme that markedly enhances Nrdp1 stability (Cao et al., 2007). As shown in figure 26, the presence of elisidepsin resulted in a diminution of the basal levels of Nrdp1 in two cell lines (HPAC, MDA-MB-468), an elevation in two cell lines (MCF-7, BxPC-3) and a maintenance of the basal levels of Nrdp1 in three of the cancer cell lines studied (MiaPaCa-2, PANC-1, SKBR3) (figure 26 A and B). In the case of USP8, elisidepsin treatment induced a down-regulation of USP8 protein expression levels, as expected, in HPAC cell lines, but no levels in the other protein extracts were detected.

To explore this possibility further, we performed an experiment in MCF-7 cells treating them with NRG-1β, elisidepsin or both. The increase in Nrdp1 correlated with an NRG-1β-induced increase in USP8 levels. This could explain that Nrdp1 could play its role in HER3 expression and degraded it by proteasome. However no differences were observed in Nrdp1 levels at 2 h of elisidepsin treatment.

These observations indicate that Nrdp1 and USP8 seem not to be involved in the mechanism by which elisidepsin mediates the internalization of HER3.

Overexpression of HER3 can occur through loss of regulatory controls at the transcriptional, translational or protein stability levels (Yen et al., 2006, Folgiero et al., 2007). Indeed, loss of the ubiquitin ligase Nrdp1 has been associated with HER3 overexpression (Yen et al., 2006) and ZNF217 gene expression was found to contribute to HER3 overexpression in breast cancer (Krig et al., 2010). The ZNF217 gene is amplified in a variety of tumor types (Kallioniemi et al., 1994, Iwabuchi et al., 1995, Schlegel et al., 1995) and it target genes upstream of the PI3K-Akt pathway.

On the basis of these findings, we expect that cells treated with elisidepsin may have changes in their ZNF217 expression, at least in part due to decreases in HER3 levels and associated Akt signaling. We examined the expression of ZNF217 in normal and treated pancreatic and breast cancer cell lines (figure 27). RNA lysates were collected at 4 h after treatment. Endogenous ZNF217 mRNA expression was elevated in treated cells, but HER3 mRNA was not (figure 24).

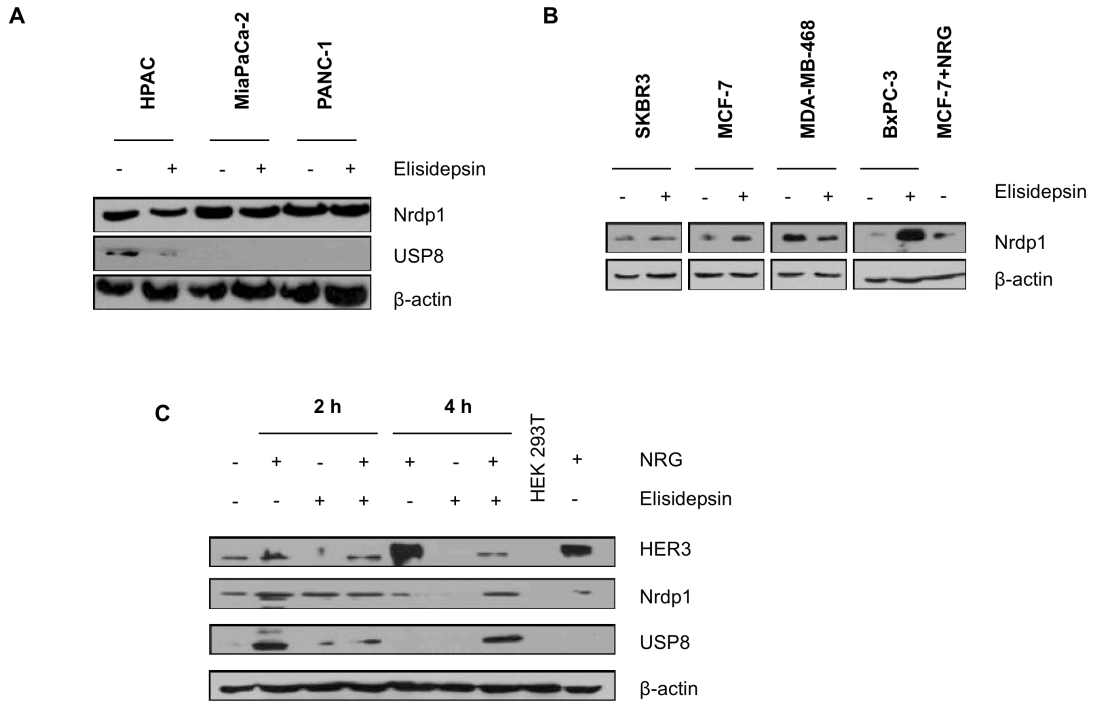


Figure 26. Nrdp1 and USP8 implication in elisidepsin treatment. Cells were treated with 1 μM of elisidepsin for 4 h or/with 100 nM NRG-1β for 30 min and cells were lysed, proteins were extracted and western blots performed with an equal amount, 50 μg of protein, of cell lysate in three pancreatic cancer cell lines (A), in three breast carcinoma cell lines and one pancreatic cancer cell line (B), and in MCF-7 cell line (C). Nrdp1, USP8 and HER3 were detected by western blot using corresponding antibodies. The membranes were stripped and reproved with anti-β-actin to verify equal protein loading.

These results indicate that elisidepsin treatment affects HER3 expression levels but no ZNF217 expression, as we thought it would happen. The ectopic expression of ZNF217 was sufficient to augment HER3 expression in HMECs cancer cell line and silencing ZNF217 to reduce HER3 transcript and protein expression (Krig et al., 2010). In addition, Krig et al. showed that ZNF217 expression is required for the maintenance of robust HER3 expression in breast cancer cells and that these gain and loss of function studies indicate that ZNF217 acts upstream of HER3 expression. Taken together, the observations illustrated in

figure 27 point that elisidepsin is likely to bind HER3 receptor because we did not see an effect on its upstream factor, ZNF217, and cells after drug treatment have a diminution of HER3 expression levels, whereas no effect was observed in other HER family members. However, more studies are required to know the mechanism of action of elisidepsin and how it affects HER3 in the cells.

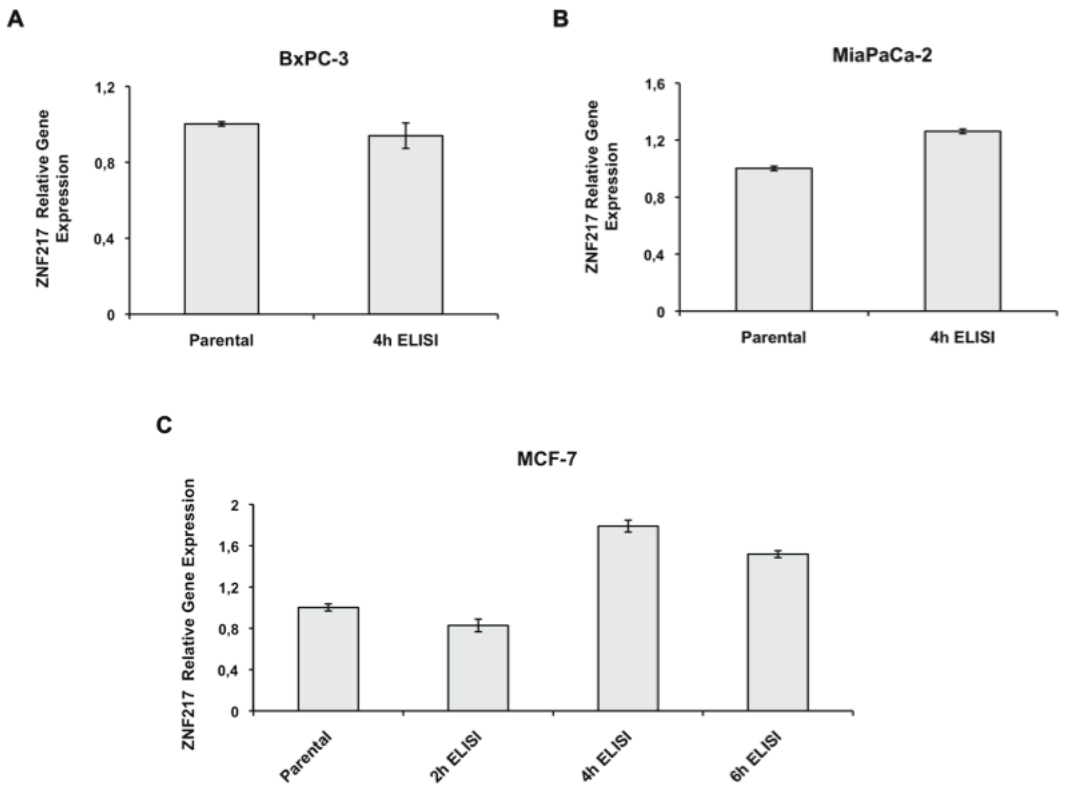


Figure 27. Elisidepsin increases ZNF217 mRNA expression. Study of HER3 mRNA expression in pancreatic (A and B) and MCF-7 breast (C) cancer cells. Lysates were collected at several time points after 1 μM elisidepsin treatment and analyzed for qRT-PCR. An increase of ZNF217 levels was observed in MiaPaCa-2 and MCF-7 cells when treated with elisidepsin, while no differences were observed in BxPC-3 cell line. ZNF217 relative gene expression is shown for a representative experiment performed in triplicate. ELISI, elisidepsin.

2.2.4 Implication of NRG-1β into elisidepsin sensitivity

A down-regulation of HER3 protein and mRNA expression was observed in different cells upon elisidepsin treatment. As this effect seems to be very selective to this receptor we explored if the activation of HER3 could have a different effect on cells within elisidepsin treatment.

For this purpose, first we used MCF-7 cancer cell line and performed a time-course experiment treating the cells with elisidepsin, NRG-1β or the combination and analyzing their pHER3 and total HER3 levels (figure 28). We found that pHER3 protein levels are reduced in just 30 min with elisidepsin treatment and that reduction is inhibited partially when treated in combination with NRG-1β. In contrast, NRG-1β binding hyperactivates a pathway responsible for the maintenance of basal HER3 levels (figure 28 B).
It has been reported that HER3 can become down-regulated in response to growth factors. These studies characterize for the first time a pathway involved in ligand-induced HER3 down-regulation, as NRG-1β treatment augments HER3 protein degradation pathways (Cao et al., 2007). Accordingly with these results, we have observed that NRG-1β stimulates HER3 loss expression in MCF-7 human breast tumor cells at 8 h.

Next, we performed cell viability assays to examine if elisidepsin cytotoxicity depends on the activation of HER3 expression, by stimulating the cells with NRG-1β. Its overexpression is associated to promote survival of epithelial and other cell types through interaction with the HER receptors and to induce the expression of VEGF and stimulate angiogenesis (Russell et al., 1999, Yen et al., 2000, Ritch et al., 2003). Only slight overall differences in elisidepsin sensitivity were found between MCF-7 (figure 29 A) and SKBR3 (figure 29 B) breast cancer cells treated with NRG-1β versus control cells, but not in their IC_{50}. Elisidepsin action was independent of NRG-1β expression. This result was confirmed by the finding that cell lines expressing high (SKBR3, BxPC3), intermediate (MDA-MB-468, MDA-MB-435 and MCF-7) or low (MDA-MB-231) levels of HER3 protein showed no significant differences in elisidepsin sensitivity after NRG-1β stimulation (figure 29).

Preclinical and translational studies have indicated that NRG-1β may modulate the response to certain agents used in the treatment of breast cancer, as trastuzumab (Yuste et al., 2005). In our study, treatment of MCF-7 with the specific ligand of activation of HER3 receptor, NRG-1β causes a slight tendency to maintain pHER3 and HER3 expression levels to elisidepsin treatment, suggesting that activation of this receptor may play a role in the modulation of the cellular response.

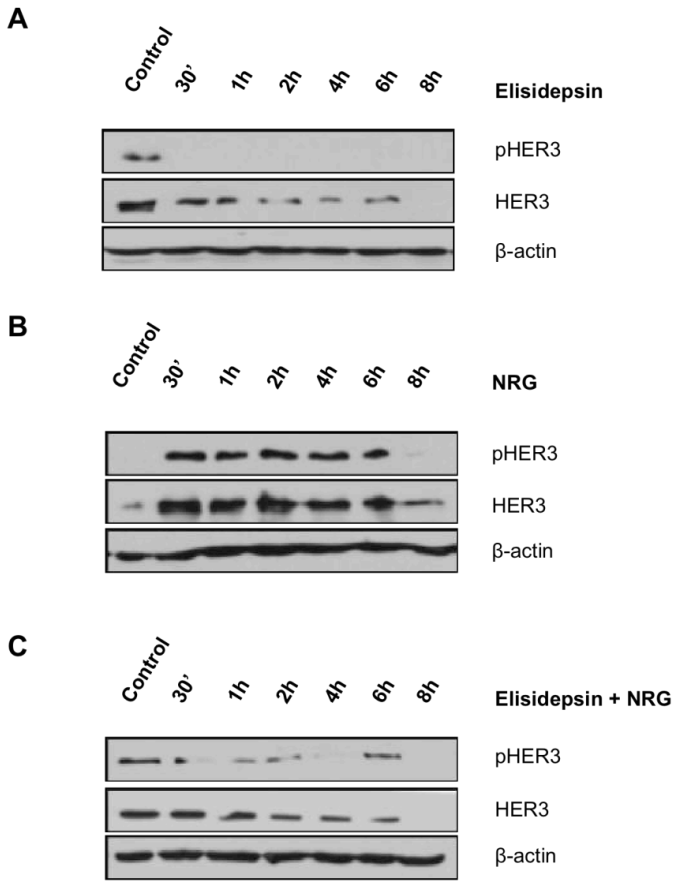


Figure 28. Elisidepsin induces pHER3 down-regulation in MCF-7 cells. MCF-7 cells were treated with and without 100 nM NRG-1β for 20 min and then treated with and without 1 μM elisidepsin for various times. Fifty micrograms of cell lysates were blotted with anti-pHER3 and HER3 and β-actin was used as an internal control to verify equal protein loading.

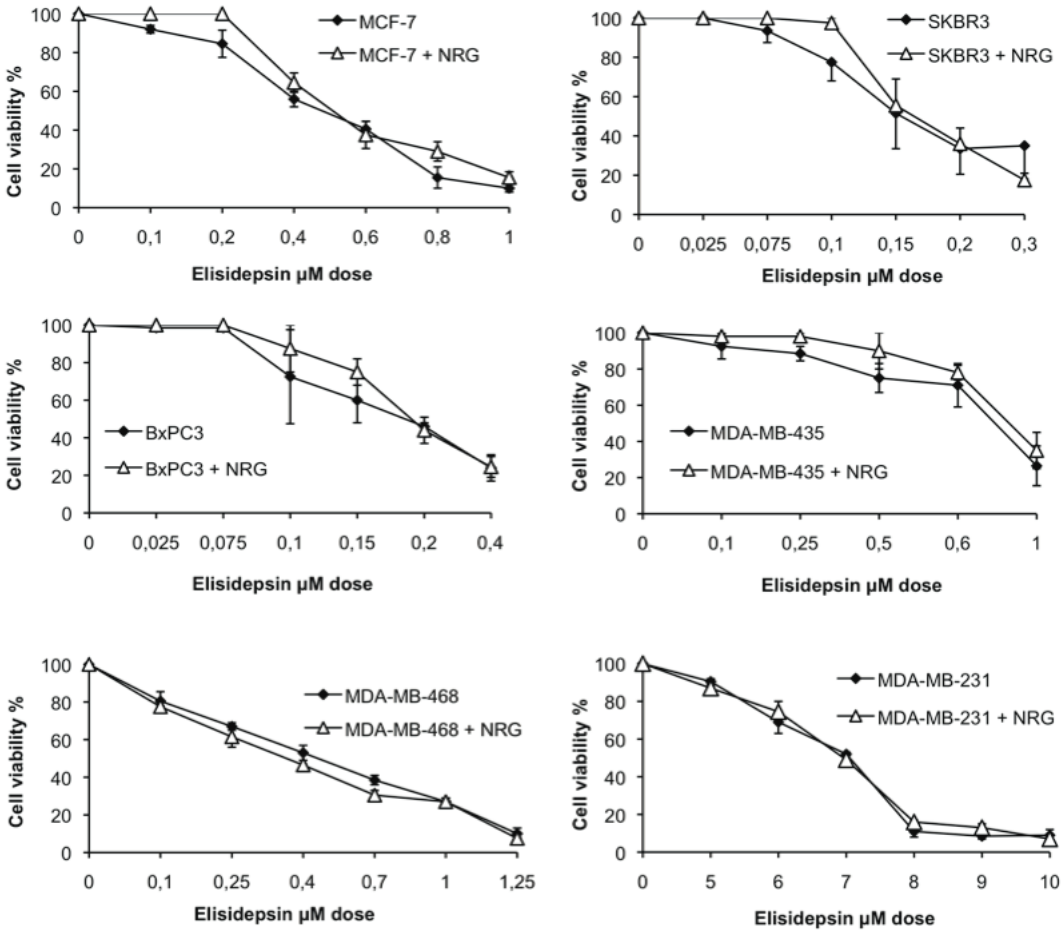


Figure 29. Effect of NRG-1β on elisidepsin cytotoxic activity. Cells were incubated with elisidepsin at the indicated doses for 72 h and the levels of cell viability were determined in crystal violet assays. Values corresponding to percentage of treated cells are shown (mean ± SD of triplicates obtained in two independent experiments). Empty triangles, cells treated with 100 nM NRG-1β for 30 min and then treated with 1 μM elisidepsin; solid circles, cells treated with 1 μM elisidepsin in MCF-7 (A), SKBR3 (B), BxPC-3 (C), MDA-MB-435 (D), MDA-MB-468 (E) and MDA-MB-231 (F).

On the other hand, exogenous addition of NRG-1β did not affect elisidepsin sensitivity in cell viability assays, so the expressions of HER3, NRG-1β and elisidepsin effect were regulated possibly by different pathways. Although the structure of NRG-1β is clear, the mechanism through which NRG-1β expression

and its release are regulated has not yet to be completely elucidated. These data suggest that activation of HER3 receptor does not play a role in modulating the cellular response to treatment with elisidepsin for 72 h.

2.2.5 Modulation of HER2 and HER3 change elisidepsin cell lines sensitivity

Malignant cells commonly possess overactivated signal-transduction cascades that provide potential selective targets of antitumor drugs (Blume-Jensen and Hunter, 2001). Since in this study we saw that elisidepsin inhibits HER3 (figure 20, 22, 24) and high expression of the HER3 receptor tyrosine kinase is prevalent in tumor cells, including cancers of the breast, ovary and prostate (Koumakpayi et al., 2006, Ciardiello et al., 1991, Friess et al., 1995, Kountourakis et al., 2006, Lemoine et al., 1992a, Lozano et al., 2005, Naidu et al., 1998, Tanner et al., 2006), we investigated if modulation of protein expression levels of HER3 receptor correlate with the sensitivity to elisidepsin in a panel of tumor cell lines with variable expression of this receptor. Furthermore, like HER2/HER3 heterodimer is the strongest known transforming and mitogenic receptor complex (Citri and Yarden, 2006, Holbro et al., 2003a, Wallasch et al., 1995) we also studied the effect on elisidepsin treatment in the modulation of HER2 expression levels.

To examine this experimentally, we utilized an shRNA construct to stably attenuate HER2 and HER3 expression in a panel of cell lines (figures 30 and 31). Stable clones of HER2 shRNA, HER3 shRNA and LUC shRNA vector-transfected (control) cells were selected and examined for expression of HER2 and HER3, respectively. As expected, levels of HER2 and HER3 were significantly reduced in transfected cells, but not in those containing pLKO LUC shRNA vector alone, indicating that the attenuation of HER2 and HER3 were not due to non-specific effects of introducing shRNA into the cells. Once checked, cell viability assays were performed to analyzed elisidepsin sensitivity in those cells generated. Figures 30 and 31 show that cells that have moderate levels of HER2 and HER3 and we knockdown its expression showed loss of sensitivity to elisidepsin treatment

versus the control cell lines. Cell viability differences between shRNA HER2 and control cells were not significant as were shRNA HER3 cell lines.

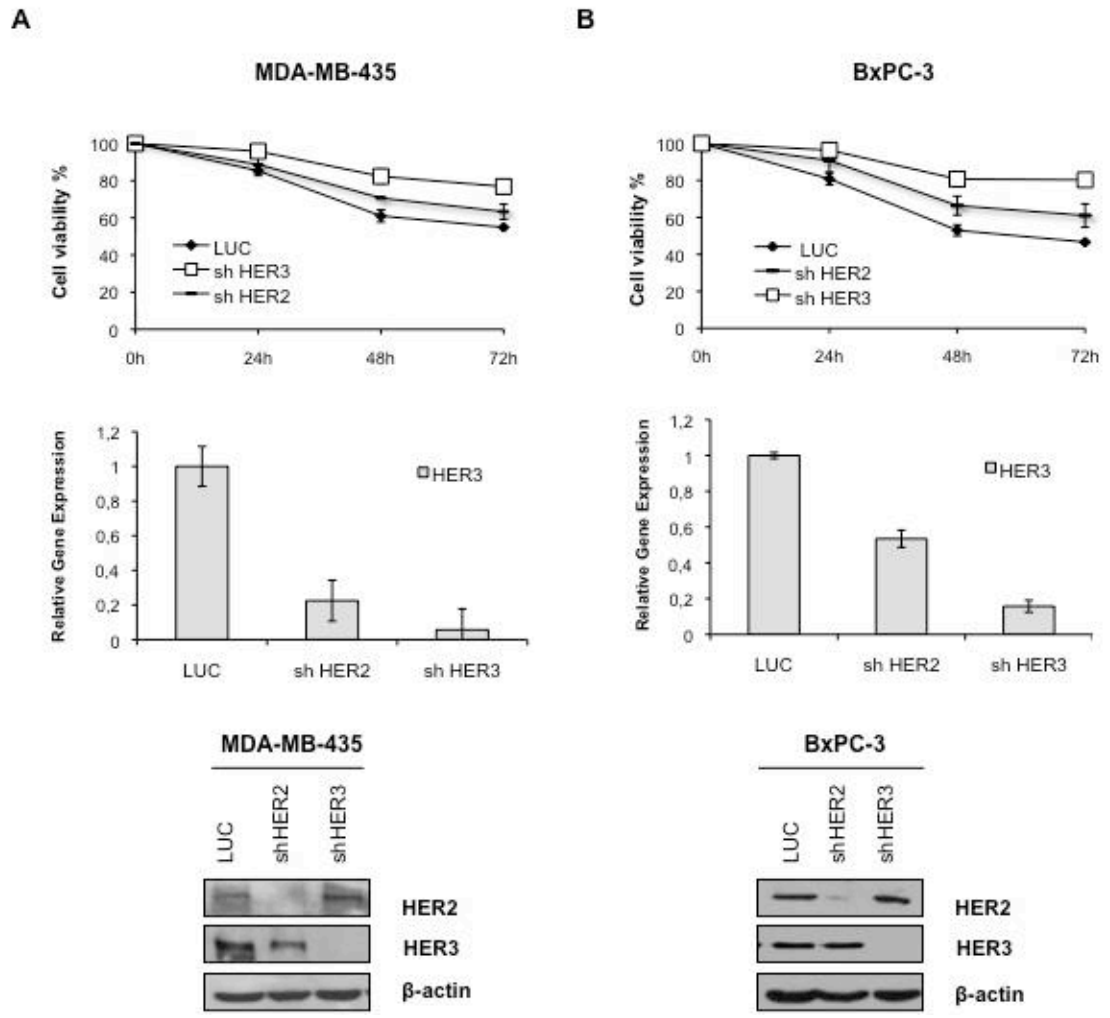


Figure 30. HER2 and HER3 inhibition reduces cell sensitivity to elisidepsin. Stable MDA-MB-435 (A) and BxPC-3 (B) cells were generated with pLKO shRNA LUC, shHER2 and shHER3. Upper panel, the IC_{50} for elisidepsin was used in the different cell lines generated for 72 h and cell viability percentage was calculated. Measurement of knockdown activity of HER3 was validated by qRT-PCR (mRNA levels, medium panel) and western blot (low panel). Fifty micrograms of protein lysates were probed with the indicated antibodies. Reproving with β-actin was used to control for loading. Results are mean ± SD of three experiments performed in triplicates; *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$. ELISI, Elisidepsin.

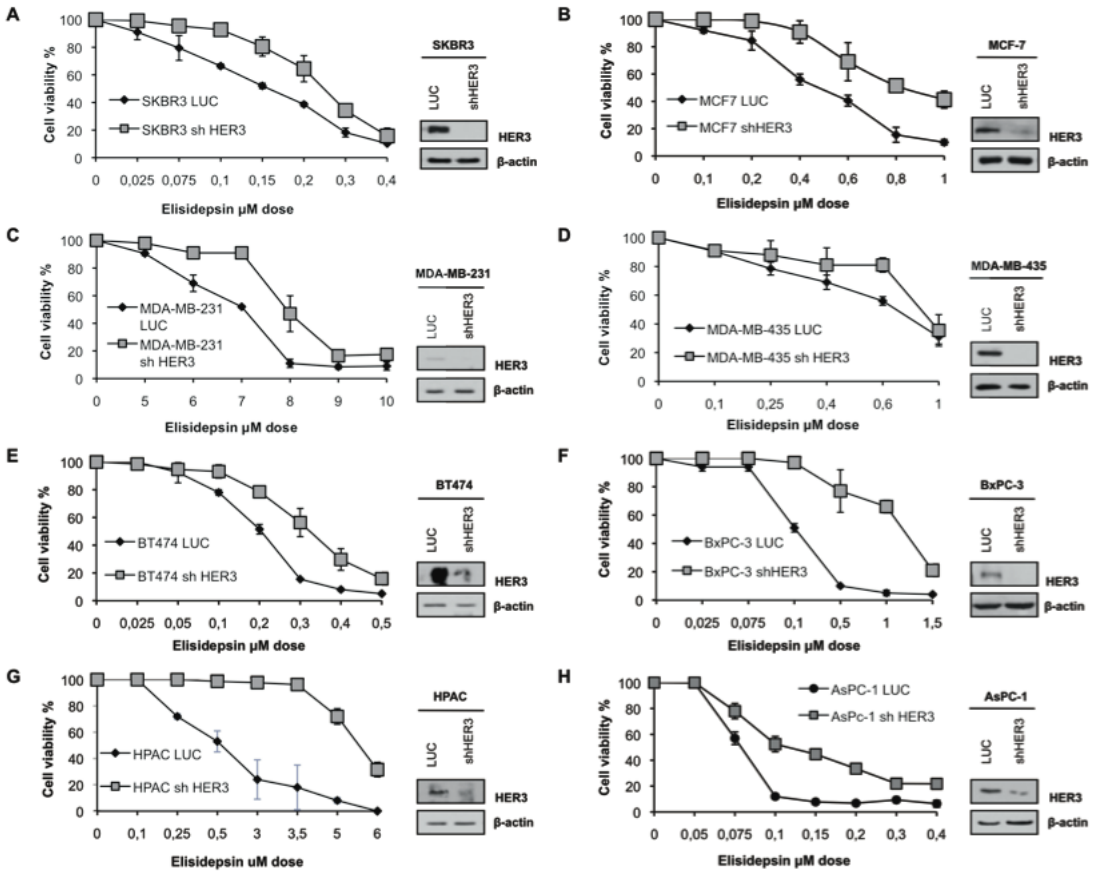


Figure 31. Loss of HER3 decreases the sensitivity for elisidepsin treatment. Cell viability after treatment with various concentrations of elisidepsin was determined in down-regulation of HER3 versus LUC vector at 72 h in SKBR3 (A), MCF-7 (B), MDA-MB-231 (C), MDA-MB-435 (D), BT474 (E), BxPC-3 (F), HPAC (G) and AsPC-1 (H). Mean, S.D., and IC_{50} values are shown from three independent experiments. Before performing the viability experiments all cell lines were checked by western blot to confirm their levels of HER3 expression with 50 µg of protein.

To investigate whether ectopic HER2 and HER3 expression affects the sensitivity of low expressing cells for those receptors to elisidepsin treatment, cells were transfected with cDNA, encoding HER2 or HER3. Treatment of the different cells generated with elisidepsin resulted in increased cell sensitivity. In comparison to control cells co-transfected with a LUC vector, a decreased percentage of cell viability was noted in HER2 co-transfected cells (figure 32).

Similar results were obtained when cells where up-regulated by HER3, cells automatically became more sensitive to the drug (figure 33). Altogether, the results suggest that ectopic HER2 and HER3 expression sensitizes these cells for elisidepsin treatment.

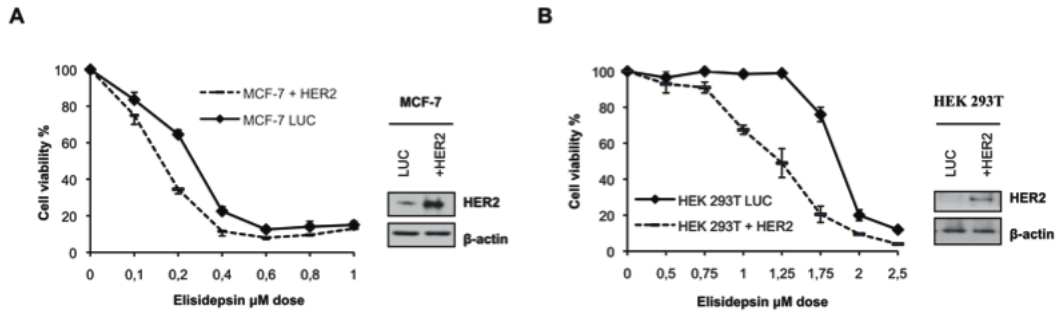


Figure 32. Ectopic HER2 expression augments the sensitivity of cells to elisidepsin. MCF-7 (A) and HEK 293T (B) were transfected with pIRES HER2 and then their sensitivity to elisidepsin and HER2 expression were checked by western blot analysis (50 μg). Cells were cultured and incubated with elisidepsin at the indicated doses for 72 h and cell viability percentage was calculated by crystal violet and corresponds to the mean ± SD of three independent experiments. A reduction of the IC_{50} concentration of elisidepsin compared with LUC vector-transfected cells was found.

Furthermore, we chose 14 human cell lines from different types of cancer of our study (comprising pancreas, breast, lung, colon and kidney) and compiled their IC_{50} cell viability assays. In order to evaluate HER3 protein expression levels and correlate them with the sensitivity of the cell lines to elisidepsin treatment, we performed different analysis based on western blot (data not shown) and immunoprecipitation in 14 cell lines (figure 34 B). Cell lines that were less sensitive to elisidepsin had lower or no levels of HER3 in comparison with the sensitive cell lines which expressed higher levels, the results being statistically significant (figure 34 C).

The mechanisms involved in the down-regulation of HER3 have been explored previously with elisidepsin by other authors with inconclusive results (Suarez et al., 2003, Janmaat et al., 2005, Ling et al., 2009) and therefore still

remain unknown. Nevertheless, we explored whether basal levels of HER3 protein could be a predictor marker to elisidepsin.

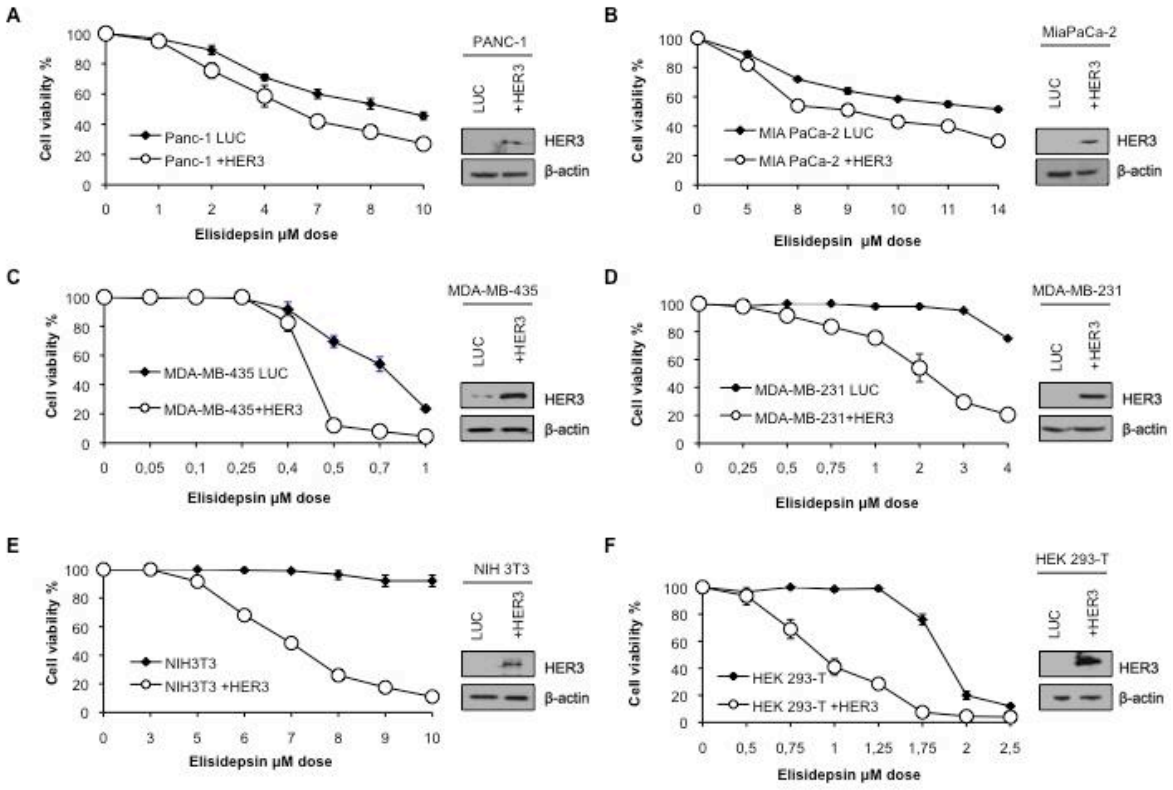


Figure 33. Up-regulation of HER3 increases elisidepsin sensitivity. Stable cell lines with an up-regulation of HER3 expression (with the pIRES HER3) were generated, checked by western blot with 50 µg of protein and cell viability assays were performed in PANC-1 (A), MiaPaCa-2 (B), MDA-MB-435 (C), MDA-MB-231 (D), NIH 3T3 (E) and HEK 293-T (F). Monolayers were stained following the crystal violet procedure 72 h after initial elisidepsin treatment and survival was estimated as percentage of staining over that of untreated cultures and corresponds to the mean ± SD of triplicates of three experiments.

Importantly we have observed that cell lines with low basal levels of HER3 receptor are less sensitive to elisidepsin treatment, whereas in cells with high HER3 basal levels there is a correlation with elisidepsin sensitivity. No correlation was observed with HER1, HER2 and HER4 expression levels (figure 21).

Furthermore, with those experiments we show here that loss of HER3 exerted a significant protective effect against the cytotoxicity of elisidepsin, while loss of HER2 expression attenuated it partially. That could happen because when we knockdown in those cells the levels of HER3 we had a down-regulation too of HER2 basal levels by qRT-PCR analysis and only in one cell line by western blot. No difference was found in the opposite situation, with a knockdown of HER2. Therefore, we can speculate that this effect of loss of HER3 on the fluidity of the lipid bilayer avoids the effects of elisidepsin treatment.

In fact, HER3 significantly increases cell sensitivity in all cell lines studied. Also, the role of HER3 as an important target of elisidepsin was subsequently studied and sensitivity to elisidepsin in a panel of tumor cell lines was found to significantly correlate with HER3 protein expression levels ($p=0.015$), supporting previous indications that HER3 could be a good predictive marker of elisidepsin sensitivity.

In summary, there is solid evidence that sensitivity to elisidepsin compound correlates with HER3 receptor expression. However, it remains to be elucidated why elisidepsin affect HER3 and why its effect depends on HER3 expression.

2.2.6 Study of a possible interaction between elisidepsin and HER3

Based on the last experiments where HER3 modulation influences the effect on elisidepsin treatment, we considered HER3 would not only be a good predictive marker of elisidepsin sensitivity, but it could be a target of the drug. NRG-1β studies showed us that elisidepsin does not compete with the specific ligand of HER3, which means that does not bind to the extracellular part of this or share the common epitope with NRG-1β.

On the other hand, we have seen in a time-course drug treatment causes loss of phosphorylation of HER3 levels in very short time. Following this line of reasoning and with the collaboration of an expert in modeling, (Dr. Verma from Institute of Molecular and Cellular Biology, Singapore, Malaysia), we postulated that elisidepsin is a candidate molecule to bind to tyrosine kinase domain of HER3 receptor, through which it could block several of its functions.

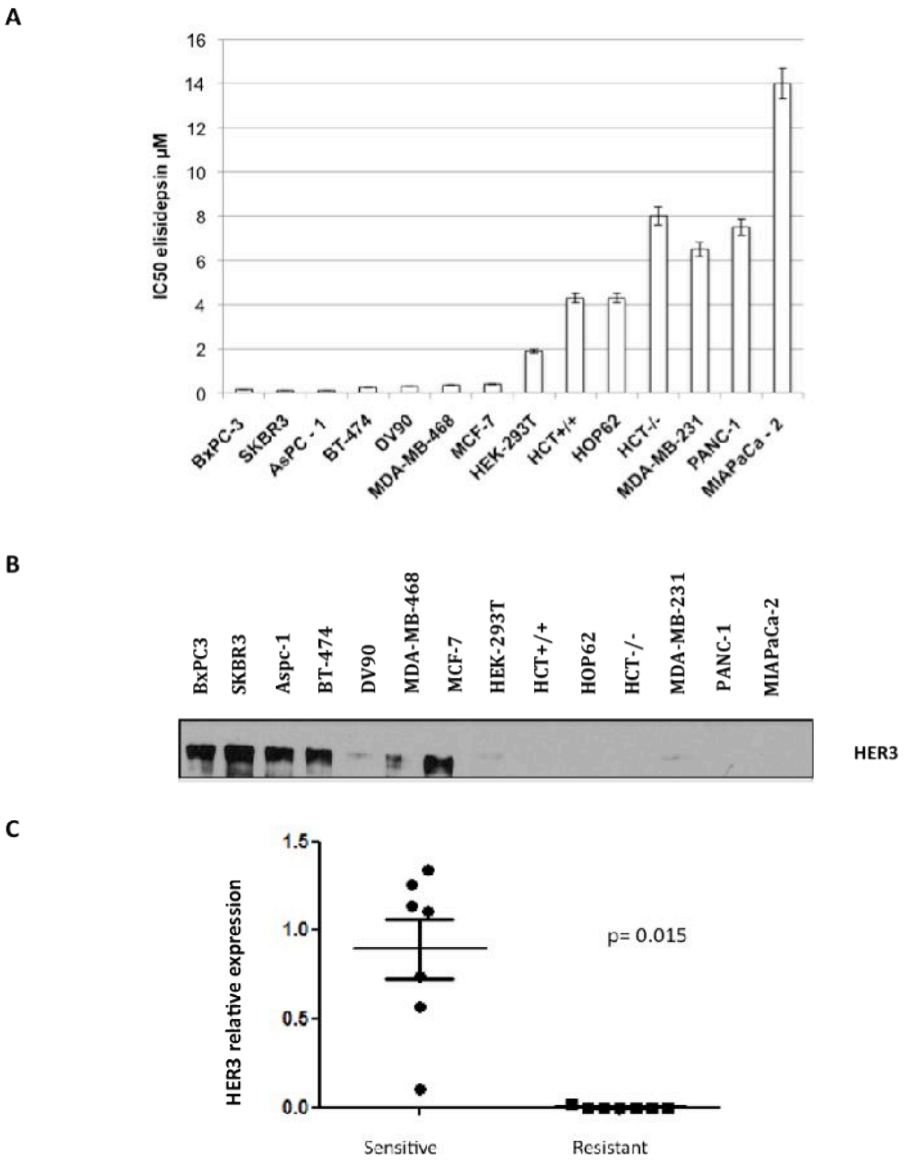


Figure 34. HER3 expression correlates with elisidepsin cell sensitivity. A) Cell viability assay upon 72 h treatment with different concentrations of elisidepsin. Bars indicate IC_{50} values (± SD) for three independent experiments per cell line. B) Total HER3 protein expression in different cell extracts was detected by western blot after immunoprecipitation with 1 µg of HER3 antibody. C) Levels of HER3 protein were quantified from western blot analysis by densitometry. The graph represents the HER3 relative expression in cell lines, classified as sensitive (IC_{50} < 1 µM) and less sensitive (IC_{50} > 1 µM) to elisidepsin. We obtained a satistical significance *p*=0.015 with the Wilcoxon analysis test.

To find out if elisidepsin subcellular localization is associated with HER3 receptor, we considered the study of the compound by microscopy marked with a fluorescent molecule such as Oregon Green and proceeded to evaluate and compare the presence of the drug in a cell line overexpressing HER3 wild type form (WT) versus a HER3 truncated form (without part of its carboxy terminal, see material and methods, where is the kinase domain). To this end, we worked with a model that expresses low levels of HER3 to see the effect and the differences between their basal conditions and with the up-regulation of each HER3 form. The cell line chose was HEK 293T and cells were transiently co-transfected with cDNA, encoding HER3 WT, HER3 truncated form or an empty vector, to be used as a control of transfection, all of them containing a Flag epitope for easy protein detection.

For immunofluorescence analysis, the transfected HEK 293T cells were placed on coverslips and incubated with anti-flag or treated with fluorescently labeled elisidepsin analog (elisidepsin-Oregon Green 488) (figure 35 A), to observe its localization in the cells. Surprisingly, when observed with microscopy, cells with HER3 truncated form did not show any significant fluorescence in the plasma membrane or elsewhere. Only a fluorescent labeling was observed in HER3 WT overexpressing cells. The lack of fluorescence in the plasma membrane in the parental cells could be due to the fact elisidepsin co-localized with the receptor in its activated form in the membrane. HER3 truncated form, which does not have all the cytoplasmatic region of the receptor, is not associated with elisidepsin's presence in the plasma membrane, and so the co-localization could be associated to the fragment that differentiates both constructs (the receptor tyrosine kinase domain of HER3).

Also, we checked the localization of HER3 in cells overexpressing HER3 WT and HER3 truncated form with flag antibody to be sure that HER3 is localized in the membrane of the cells and could be activated or be functional. In both situations HER3 expression was found in the cell membrane (figure 35 A).

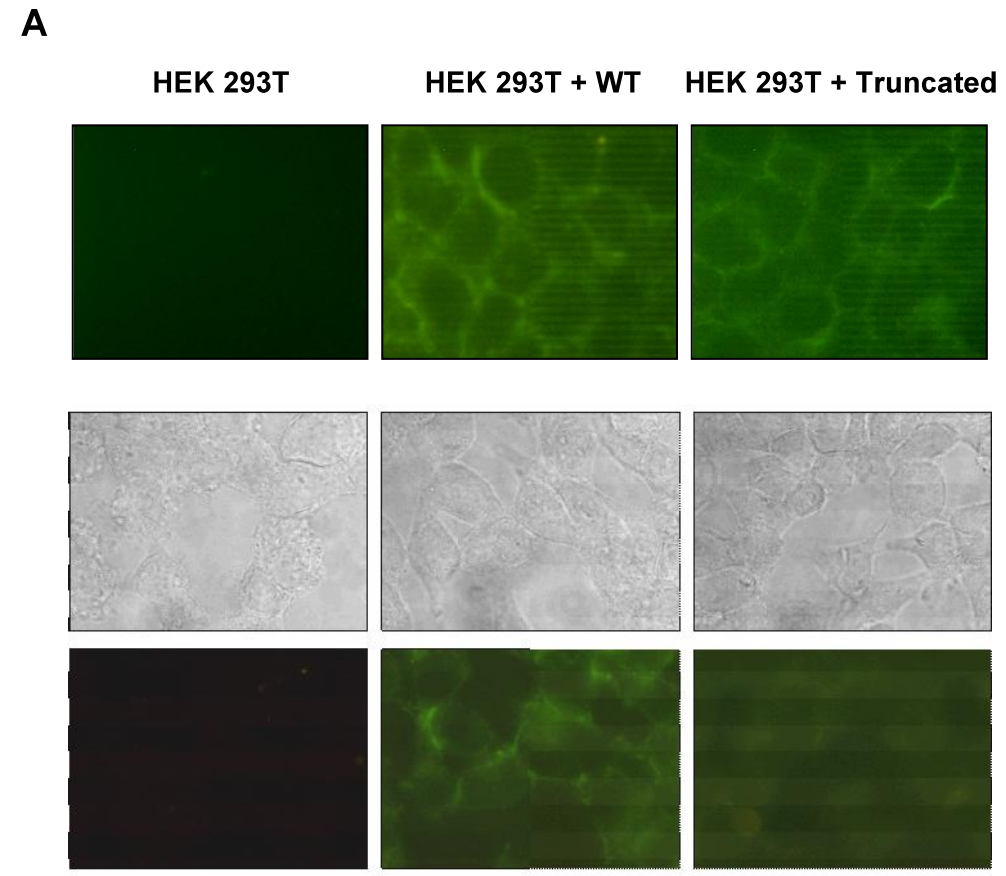


Figure 35. Elisidepsin subcellular localization is associated with HER3 receptor. A) Localization studies in HEK 293T cells overexpressing HER3 WT, HER3 truncated form or an empty vector with anti-flag antibody (upper) and treated with fluorescently labeled 10 nM elisidepsin (OG), 10 min (down). Magnification 100x. B) Top, HEK 293T cells transiently co-transfected with HER3 WT, HER3 truncated form or an empty vector for 36 h were treated with 1 μM elisidepsin and then cross-linked. Immunoprecipitation of cross-linked cells with OG and reveale with HER3 antibody and the opposite were performed. Down, HEK 293T cells overexpressing HER3 WT, HER3 truncated form or an empty vector were

incubated with elisidepsin at the indicated doses for 72 h and the levels of cell viability were determined in crystal violet assays. Values corresponding to percentage of treated cells are shown (mean ± SD of triplicates obtained in three independent experiments). OG, Oregon Green; IP, immunoprecipitation; WB, western blot.

To further investigate the localization of the drug we performed a cross-linking assay to analyze if elisidepsin binds to HER3 receptor.

HEK 293T cells were transiently co-transfected with HER3 WT, HER3 truncated form or an empty vector (36 h), treated with elisidepsin 10 min and then cross-linking assay was performed. Immunoprecipitation of cross-linked HER3 and Oregon Green confirmed HER3 interaction with elisidepsin and the receptor (figure 35 B). In line with the results in transient transfected cells, western blot analysis revealed interaction of HER3 carboxy terminal and elisidepsin.

Also, a cross-linking assay was done in MCF-7 cell line, which present moderate levels of HER3 and a binding between HER3 and the drug was observed as well (figure 36).

Furthermore, this interaction between HER3 carboxy terminal and elisidepsin was confirmed by cell viability assay (figure 35 B, down). In comparison to control cells, an increased percentage of cell death was noted in HER3 co-transfected cells upon elisidepsin treatment, while no difference was observed in HER3 truncated expressing cells. Again, a sensitization of the drug was obtained with an up-regulation of HER3 versus the parental cell line.

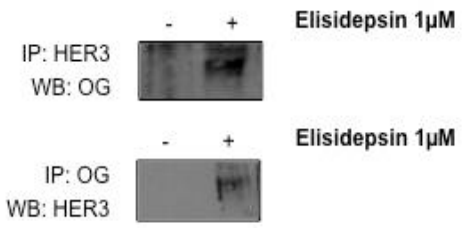


Figure 36. Elisidepsin binds to HER3. MCF-7 cells were used to perform a cross-linking assay after treatment with fluorescently labeled 1 μM elisidepsin (OG) for 10 min. Immunoprecipitation of cross-linked cells with OG, revealed for HER3 and the opposite were performed. IP, immunoprecipitation; WB, western blot and OG, Oregon Green.

Elisidepsin is a novel marine compound with a potent cytotoxic activity in various tumor cell lines. Its mechanism of action remains poorly understood, although several targets have been proposed to be involved in the cellular response to elisidepsin treatment, such as fatty acid containing ceramides, FA2H, lipid rafts, lysosomes and HER receptors (Garcia-Rocha et al., 1996, Hama et al., 2000, Janmaat et al., 2005, Shilabin et al., 2007, Herrero et al., 2008). In yeast models, resistance to elisidepsin was associated to the deficiency of FA2H indicating that the presence of the sphingolipid should be important for the maintenance of a membrane conformation required for drug activity (Nyholm et al., 1990, Herrero et al., 2008). However, A549 R cells did not present any alteration in the expression of FA2H (Molina-Guijarro et al., 2011). Also, it was demonstrated that elisidepsin appears capable of disrupting lysosome membranes within certain target cells, thereby initiating apoptosis (Garcia-Rocha et al., 1996, Shilabin et al., 2007). However, the nature and characteristics of the cytotoxic effect of elisidepsin and mechanisms of cell death induction remain to be elucidated. Although elisidepsin could interact with the lipid bilayer, it is unlikely that it will form pores because the molecule is too small to span the whole length of the lipid bilayer. (Kourie and Shorthouse, 2000, Sewell et al., 2005). On the other hand, other groups have reported that elisidepsin interferes with different signaling pathways such as HER1, HER2, HER3, TGF-α or PI3K-Akt (Janmaat et al., 2005, Shilabin et al., 2007, Ling et al., 2009, Ling et al., 2011).

The data presented here may have important clinical relevance. We demonstrate that elisidepsin is active *in vitro* against cells derived from various tumor types, including breast and pancreas. At clinical relevant concentrations, our finding that HER3 expression levels correlated with sensitivity of cell lines to elisidepsin suggests HER3 as a predictive marker for elisidepsin sensitivity. Furthermore, down-regulation of pHER3, HER3 expression or inhibition of Akt or downstream events could serve as surrogate markers for elisidepsin activity.
The drug sensitivity also proved to be dependent on HER3 expression. Modulation of its expression produces changes in elisidepsin sensitivity in a panel of cell lines. Besides, cross-linking assays discovered a binding between the elisidepsin compound and HER3 receptor allowing us to answer the above questions and

understand the reasons why elisidepsin affects HER3 and why its effect depends on its expression. These findings suggest that HER3 may be a potential marker for screening patients that will be sensitive to elisidepsin treatment. However, it remains to be elucidated how elisidepsin binds HER3. We think that elisidepsin could bind to tyrosine kinase domain of HER3 receptor, through which it could block several of its functions as we observed in our experiments and act as a TKI, because it inhibits catalytic function of HER3 protein which is essential for oncogenic function, and their mechanism of action does not presume a requirement for ligand binding or other extracellular interactions.

More studies are needed to fully characterize the anti-tumor action of elisidepsin including the identification of its primary target(s), the precise molecular mechanism of cytoplasmic organelle damage, or the basis for its preferential effect on tumor cells.

3. Study of HER3 implication in breast tumorigenesis

Breast cancer is the most common cancer type occurring in women world-wide. During the last 25 years, the technological progress that has characterized both genetic and molecular biology has led to a greater understanding of the events underlying the normal tissue development as well as malignant transformation. This level of knowledge has enabled the identification of molecular players responsible for cell growth, survival, motility, and transformation that may serve as therapeutic targets. The HER family receptors have been proposed as a rationale target for cancer therapy, because many types of human cancers are characterized by deregulation of its family of transmembrane tyrosine kinases (Holbro et al., 2003b, Hynes and MacDonald, 2009). In some cancers, overexpression and hyperactivity of individual HER family members are linked with the pathogenesis of these malignancies (Hynes and Lane, 2005), which are consequently “addicted” to this pathway to sustain their proliferation and survival.

Up-regulation of HER3 has been described to decrease the sensitivity to HER inhibitors (Wheeler et al., 2008, Wheeler et al., 2009, Gijsen et al., Engelman et al., 2007) and HER3-derived downstream signaling has been linked to cancer etiology and progression (Sithanandam and Anderson, 2008).

3.1 Mutational analysis of the extracellular domain of HER3 gene in breast carcinomas

Co-expression of both HER3 and HER2 is seen in many tumors, including breast cancer (Travis et al., 1996, Naidu et al., 1998). In this disease, the formation of HER2/HER3 heterodimers may be crucial for the aggressive phenotype of cancers with HER2 amplification, and may contribute to intrinsic and acquired resistance to therapy (Stern, 2008, Menendez and Lupu, 2007). HER2 is the preferred dimerization partner for the other HER family receptors and this partnership creates opportunities for improving efficacy of HER-targeted pharmaceuticals, by interfering with coupling of HER2 to HER3 through dimerization inhibitors, and by use of therapeutic compounds that target Akt-dependent pathways (Graus-Porta et al., 1997).

Deregulated HER signaling is associated with malignant transformation. HER signaling can be altered via a number of mechanisms (Yarden and Sliwkowski, 2001) including ligand overexpression, overexpression of the normal or constitutively mutated HER receptor, and defective HER receptor internalization degradation (Yarden and Sliwkowski, 2001). HER3 is expressed at abnormally high levels in several tumor types and although mutations in the kinase domain of HER family members have been reported in some cancers (Kang et al., 2006, Tsugu et al., 1997, Gorgoulis et al., 1992, Soung et al., 2006), activating HER3 mutations have not been found, maybe because has a kinase domain that lacks several key conserved and catalytically important amino acid residues.

Despite this, in 1994 (Guy et al., 1994) it was found that HER3 has an active ligand binding domain, and one or more of its ligands can establish an autocrine loop that drives uncontrolled cell growth (Hansen et al., 2006). For this reason we consider important to study the mutational status of HER3 extracellular domain in

a series of breast cancer patients for find possible mutations in this domain that could explain a hyperactivation of this receptor.

For this purpose, the extracellular domain of HER3 was divided into four parts so it could be easier to amplify and to study in detail the domain by Sanger sequencing. We evaluated a series of 32 breast cancer patients.
Cases were primary breast carcinomas diagnosed in 2008 at Vall d'Hebron University Hospital. Among the patients examined we have a median age of 65 years (range, 32-84). Most cases were invasive ductal carcinomas (90.10%), grade II (54.41%), with a tumor size between 20 and 50 mm (pT2, 65.23%), and HER2 amplification (16%).

In our study all cases were negative for HER3 mutation in its extracellular domain. Although HER family members are often overexpressed, amplified or mutated in many forms of cancer, making them important therapeutic targets, we did not find any mutation in HER3 receptor in a series of breast cancer patients.

3.2 HER3 expression in breast cancer patients

Since HER3 expression in breast cancer, is associated with other HER receptors with kinase activity (mainly that of HER2) and its presence has been associated with the development of resistance to anti-HER therapies (Erjala et al., 2006, Sergina et al., 2007) we thought it could be useful to study the receptor expression and amplification in patients samples. Then, specific antibody obtaining was one of our first requisites to study its activation and expression. For this reason collaboration with Research Cancer center of Salamanca University-CSIC from Atanasio's Pandiella laboratory begun, where they generated an antibody HER3 specific for its intracellular domain (HER3-R114)

The antibody operation and its specificity were checked with immunoprecipitation and western blot experiments (figure 37). Next, the antibody was confirmed that it works perfectly in patients' samples by IHC, so it could be an antibody useful to study the role of HER3 in breast cancer.

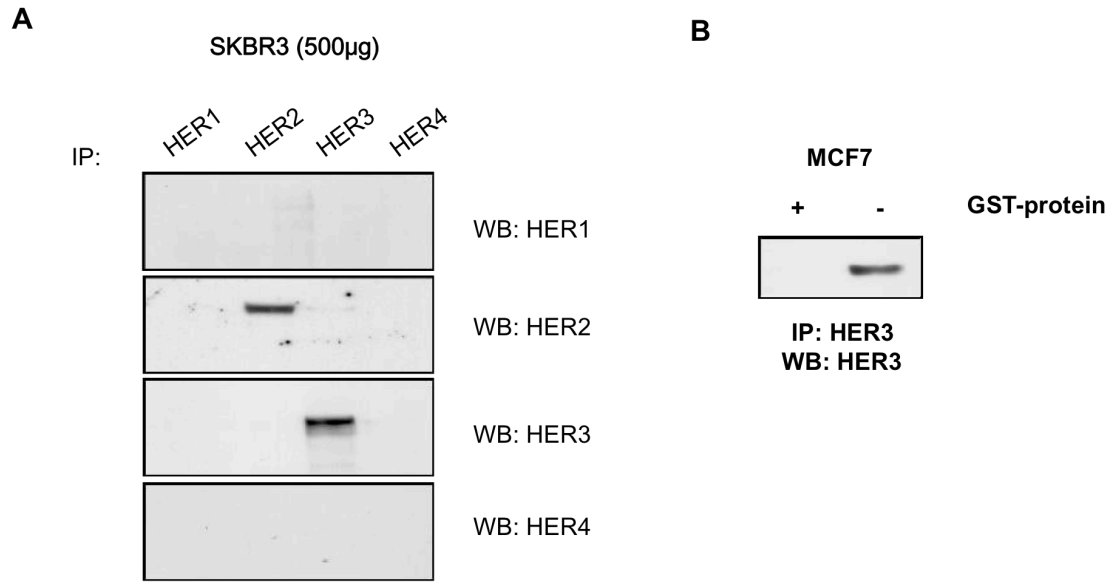


Figure 37. Specificity of anti-HER3 antibody. A) SKBR3 HER2 overexpressing cells from human breast carcinoma were lysed, and HER receptors were immunoprecipited with specific antibodies which include previously mentioned anti-HER3 antibody. Blots were probed with the same antibodies. Anti-HER3 antibody could only recognize HER3 receptor in immunoprecipitation and western blot. No cross-reactivity of anti-HER3 antibody was detected with HER1, HER2 or HER4 receptors. B) The anti-HER3 R114 antibody is a rabbit polyclonal purified antibody. It was generated against a fusion protein with GST (ab70456, Abcam) that was used in this experiment to compete. To perform the immunoprecipitation moreover the protein extract, the protein G sepharose and the antibody we added 5 µg of the fusion protein. These experiments were performed by Dr. Sánchez-Martín and Dr. Pandiella (IBMCC). IP, Immunoprecipitation; WB, western blot.

A classification of breast cancers has been defined using IHC to analyze patterns of protein expression in tumor sections. This pathological classification employs a limited number of protein biomarkers to classify breast cancers into luminal A, luminal B, HER2 enriched or triple negative. Luminal tumors are characterized by their expression of ER and PR, tagging these tumors for therapies that impede the activation of those receptors. Genomic studies have complemented this pathologic classification by confirming the pathologic subtypes, and the

addition of the claudin-low, and normal breast-like groups (Brenton et al., 2005, Callagy et al., 2003, Jacquemier et al., 2005, Nielsen et al., 2004, Herschkowitz et al., 2007). Gene expression profiling revealed that within the ER positive-related tumors, two subtypes, luminal A and luminal B, could be distinguished that vary markedly in gene expression and prognosis (Sorlie et al., 2001). Conversely, hormone receptor–negative breast cancer comprised two distinct subtypes, the HER2 subtype and the basal-like subtype (Sorlie et al., 2001, Sorlie et al., 2003). These subtypes differ in biology and behavior, and show a poor outcome.

Importantly, the role of HER3 in these molecular subtypes of breast cancer has not been defined. To assess it, we evaluated HER3 expression by IHC in one hundred and forty-seven cases of primary breast carcinomas diagnosed between 2008 and 2009 at Vall d'Hebron University Hospital and compared it with the IHC profiles ER, PR, HER2, Ki-67 and p53, and with different clinical and pathological variables (histological type, grade, age, tumor size and lymph node status).

Patient ages ranged from 30 to 90 years with a mean age of 63 years. Histologic type, grade, age, tumor size, lymph node status, hormone receptors (ER/PR+), Ki-67, p53 and HER2 are shown in table 11. Most cases were invasive ductal carcinomas (88.44%), grade II (48.23%), and with a tumor size between 20 and 50 mm (pT2, 53.06%).

HER3 expression was analyzed by IHC in all breast cancer tissues and HER3 membrane and cytoplasmatic staining was evaluated separately because different expression pattern was observed (figure 38). Figure 39 shows the different localization of HER3 staining observed in our series and its evaluation. A weak expression (1+) was found in normal adjacent tissue. Total HER3 was detected in 21% of cases. Co-expression of HER3 in the membrane and cytosol was observed in 3% of the patients, whereas the membrane and cytoplasmatic staining alone was detected in 14 and 4%, respectively. All HER3 positive cases were analyzed by FISH, and none of them was found to be positive (figure 38).

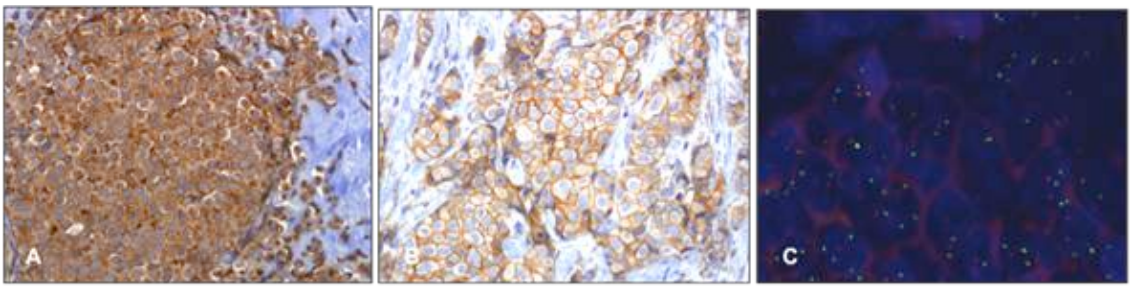


Figure 38. HER3 expression in breast carcinomas. HER3 expression was analyzed by IHC and HER3 cytoplasmatic (A) and membrane (B) staining was evaluated separately because different patron expression was observed. Magnification 40x. C) HER3 FISH was performed in all HER3 positive cases. Magnification 100x.

Correlation between HER3 expressions with the aforementioned biomarkers was analyzed using Chi-Square and Kruskal-Wallis statistical tests. The results of their assessment of associations are shown in table 12.

Expression levels of HER3 did not correlate with clinical variables, such as age, tumor size, HER2 amplification (FISH+), p53 status and presence of lymph node metastasis (p=0.697, p=0.059, p=0.258, p=0.519, p=0.896, respectively) but significantly correlated with HER2 staining (IHC), ER and PR (p=0.033, p<0.001 and p=0.013 respectively) positively. Finally, Ki-67 expression and histological grade negatively correlated with total HER3 expression levels (p=0.007 and p=0.046, respectively).

When we evaluated HER3 membrane staining, a significant association was found between HER3 staining and tumor size and ER expression levels (p=0.016 and p=0.004, respectively). No other significant correlations with prognostic indicators such as PR, HER2, histological grade and Ki-67 expression, HER2 amplification, age (p=0.124, p=0.646, p=0.769, p=0.357, p=0.406, and p=0.284, respectively) were observed. HER3 cytoplasmatic expression levels were also analyzed and positively correlated with ER and PR staining (p=0.005 and p=0.032, respectively). However, cytoplasmatic HER3 expression inversely correlated with Ki-67 staining (p=0.001). Although an inverse association has been found between high expression of total HER3 staining and histologic grade, only a trend was observed with HER3 cytoplasmatic staining (p=0.054, data not shown).

FEATURE	N (%)
Histologic type	
Apocrine	1 (0.68%)
Cribiform	1 (0.68%)
Ductal	130 (88.44%)
Lobular	9 (6.12%)
Micropapilar	1 (0,68%)
Mucinous	2 (1.63%)
Neuroendoncrine	1 (0.68%)
Papilar	2 (1.32%)
Histologic Grade[a]	
I	13 (8.84%)
II	71 (48.23%)
III	62 (42.18%)
Age of patient	
<50 years	35 (16.20%)
≥50 years	112 (76.19%)
Tumor size	
pT1 (<20mm)	54 (36.73%)
pT2 (20-50mm)	78 (53.06%)
pT3 (>50mm)	15 (10.20%)
pN	
0	76 (47.80%)
1	48 (30.19%)
2	17 (10.69%)
3	18 (11.32%)
Hormone receptors	
ER+ (Estrogen receptors)	123 (83.67%)
PR+ (Progesterone receptors)	112 (76.19%)
Ki67	
>15%	94 (63.95%)
≤15%	53 (36.05%)
p53	38 (28.60%)
Her2/neu	
Amplified	33 (22.45%)
Not amplified	114 (77.55%)

[a] According to the Scarff Bloom Richardson (SBR) classification

Table 11. Characteristics of the breast tumors.

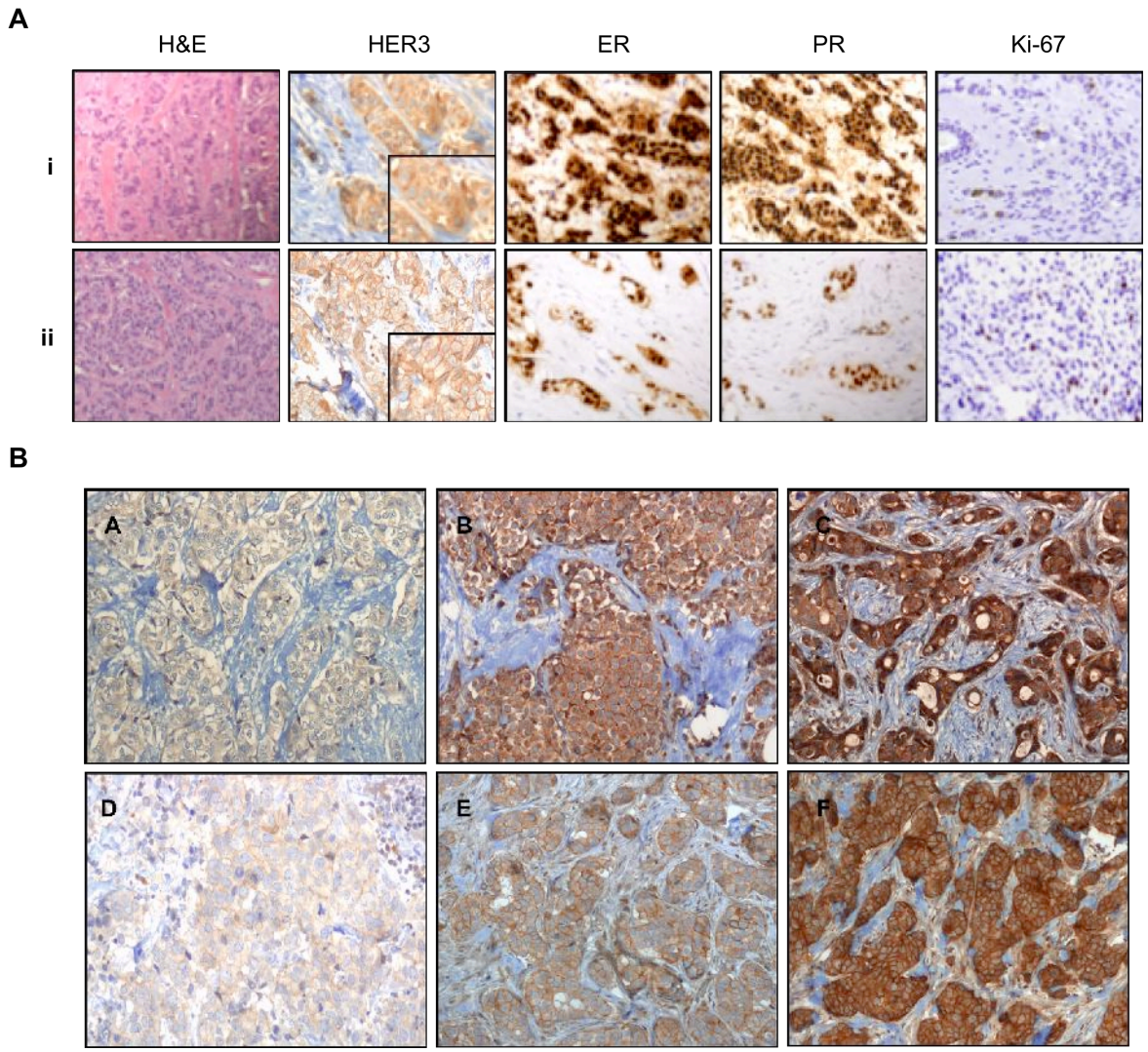


Figure 39. Expression levels of HER3, ER, PR and Ki-67 in breast carcinomas. A) Immunostaining for H&E, HER3, ER, PR and Ki-67 expression in 2 solid tumors of the breast, which present different pattern of HER3 protein expression. i) HER3 cytoplasmatic positive staining. ii) HER3 membrane positive staining. Magnification 20x. B) Representative examples of different expression levels of HER3 IHC with cytoplasmatic (A-C) and membrane staining (D-F). A and D, weak expression of HER3; B and E, moderate HER3 expression; C and F, strong staining for HER3. Magnification 40x.

On the other hand, protein expression levels of HER3 were also analyzed by western blot in 43 breast cancer tissues (29% of the series). HER3 expression was also analyzed by qRT-PCR and a significant number of frozen tissues were available to do the RNA extraction. Here we analyzed a total of 32 samples (22% of the series) that corresponded all of them to the same that used to analyze the protein expression of HER3 by western blot and were the same that we used for the mutational analysis. As shown in figure 40, some tumors displayed higher levels of HER3 than others by either IHC, western blot or qRT-PCR (figure 41).

The HER3 protein expression levels quantified by IHC were significantly concordant with the levels obtained by western blot ((p=0.009), figure 40) and the HER3 mRNA levels measured by qRT-PCR (p=0.0254 and p=0.048, respectively, figure 41).

	Total	**Membrane**	**Cytoplasm**
Tumor Size	p=0.059	p=0.016*	p=0.526
Lymph node metastasis	p=0.896	p=0.456	p=0.459
HER2	p=0.033*	p=0646	p=0.055
HER2 amplified	p=0.258	p=0.406	p=0.878
Estrogen Receptors	p<0.001*	p=0.004*	p=0.005*
Progesterone Receptors	p=0.013*	p=0.124	p=0.032*
Ki-67	p=0.007*, [1]	p=0.357	p=0.001*, [1]
p53	p=0.519	p=0.725	p=0.116

* Statistically significant (p≤0.05)
[1] Inverse correlation

Table 12. Statistical significance of biomarkers with HER3 staining.

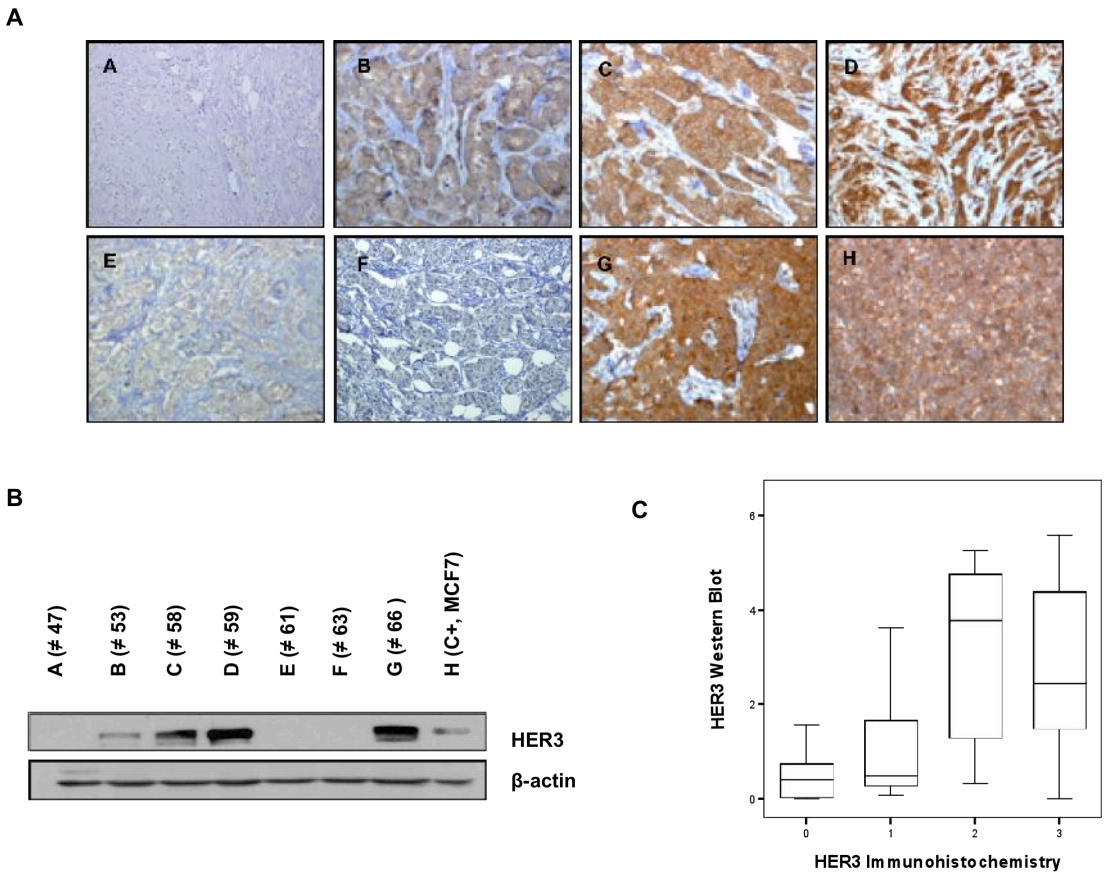


Figure 40. Correlation between HER3 expression by immunohistochemical analysis and western blot. A) Representation of different HER3 expression levels by IHC in some solid tumors of the breast (*A*-*G*). The breast carcinoma cell line MCF-7 was used as a positive control (*H*). Magnification 20x. B) The same breast carcinoma tumors that we performed the IHC above were lysed, and HER3 receptor protein expression was analyzed by western blot in 8% SDS-PAGE gel. β-actin was used as an internal control to verify equal protein loading. C) Association of HER3 expression by western blot with HER3 IHC staining in 43 patients with primary breast carcinomas. Levels of HER3 protein were quantified from western blot analysis by densitometry and were compared with IHC evaluation by Kruskal-Wallis test. HER3 expression by western blot correlated with HER3 IHC staining and we obtained a statistical significance (p=0.009).

Given the observed correlation between HER3 protein expression levels by IHC and hormone receptors (ER and PR) in the tested samples, we sought to determine if this result could be reproduced *in vitro*. We therefore investigated whether ectopic overexpression of HER3 in MDA-MB-231, MDA-MB-435 (triple negative breast cancer), MiaPaCa-2 (pancreas) and HEK 293T (human embryonic kidney) cell lines affects hormone receptors levels.

Cell lines overexpressing HER3 had higher levels of ER compared with the parental cell lines. No differences were noticed between the overexpression of HER3 and PR expression levels (figure 42.)

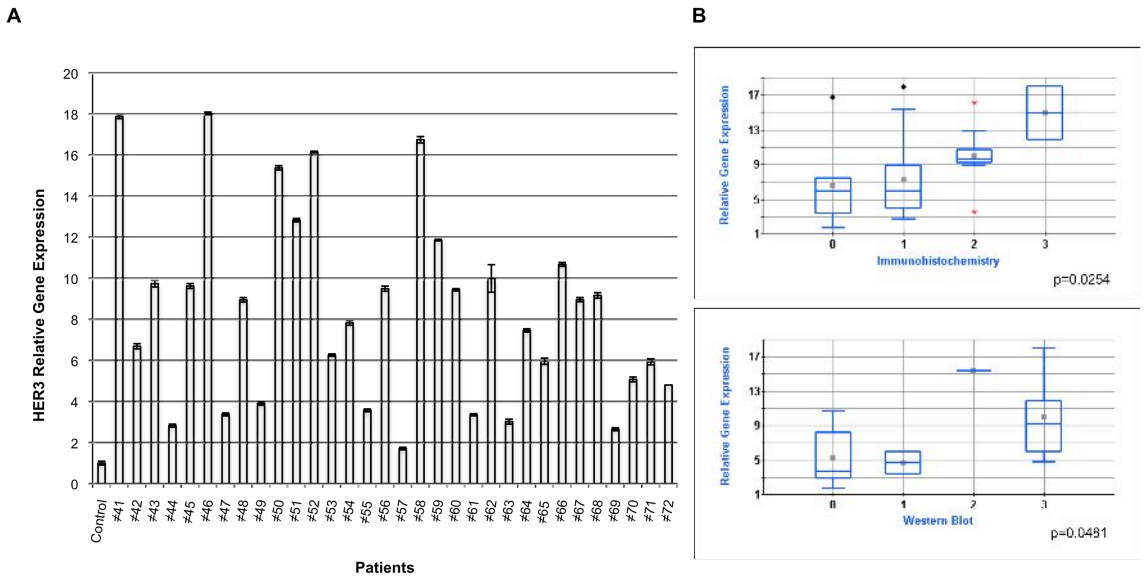
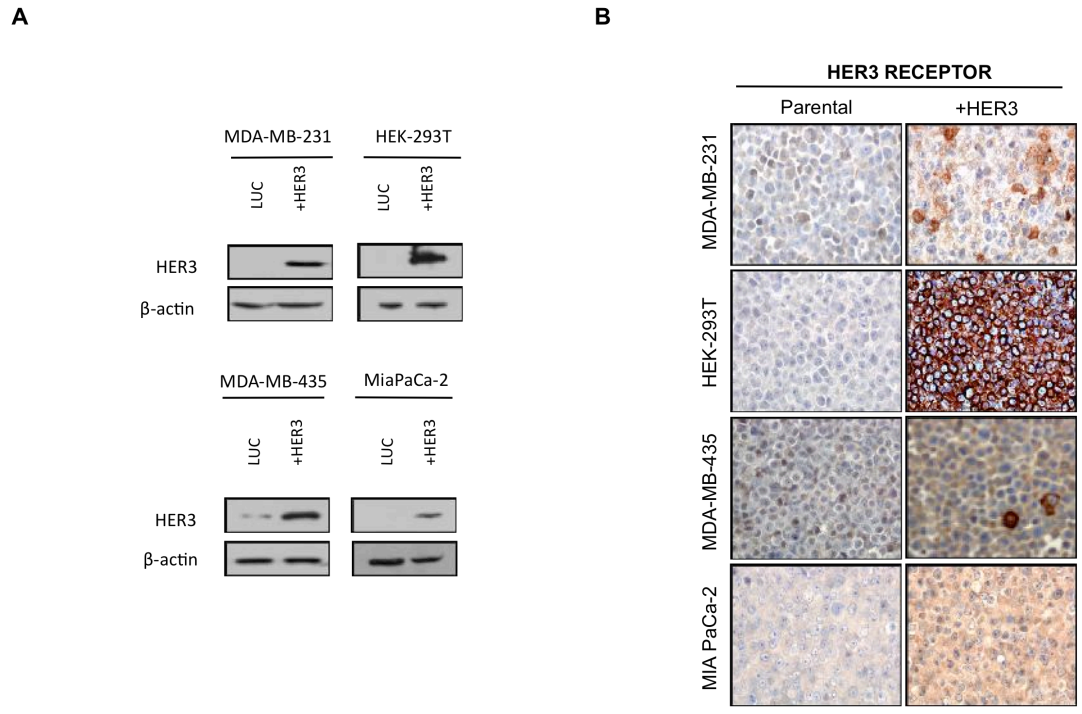


Figure 41. HER3 expression levels by different techniques in breast cancer patients. A) HER3 expression was analyzed by quantitative real-time PCR in 32 breast carcinomas. A commercial pool of human breast total RNA was used as control. An increase of HER3 was seen in all the cases studied. Error bars, SD of triplicates. Each experiment was done at least twice. B) Relationship between HER3 relative gene expression with HER3 protein expression in 32 patients with primary breast carcinomas. Top, association between HER3 relative gene expression with HER3 protein expression by IHC. The association was significant with the Kruskal-Wallis test, p=0.0254. Down, correlation between HER3 relative gene expression with HER3 protein expression by western blot (Kruskal-Wallis test, p=0.0481).

Breast carcinomas have been classified into luminal, HER2 enriched and triple negative subtype, depending on their IHC patterns of protein expression of ER, PR and HER2. Expression of HER3 was found mainly in breast cancer luminal subtype, followed by HER2 enriched molecular type.

In accordance with the literature, the HER2/HER3 heterodimer has been shown to be the most transforming and mitogenic receptor complex of the HER family (Holbro et al., 2003a, Alimandi et al., 1995, Wallasch et al., 1995). In our series, we observed a correlation between the expression of total HER3 and total HER2 but only a trend between total HER3 and HER2 amplification. Thus, we included a larger series of patients with amplification of HER2 to determine the role of HER3 in patients with HER2 amplification. A total of 67 new HER2 amplified patients were added to the list of HER2 positive tumors, totaling 100 HER2 positive tumors. Of them, 10% overexpressed HER3 (3+). Furthermore, we analyzed in this subset of patients their correlation with ER, PR and Ki-67. HER3 total expression, HER3 membrane staining and HER3 cytoplasmatic localization was correlated positively with ER (p=0.004, p=0.027, p=0.014, respectively) but not with the other parameters, data not shown.

These results show that HER3 expression is not always linked to HER2 expression because it is predominantly found in the molecular luminal subtype, which does not have overexpression of HER2.



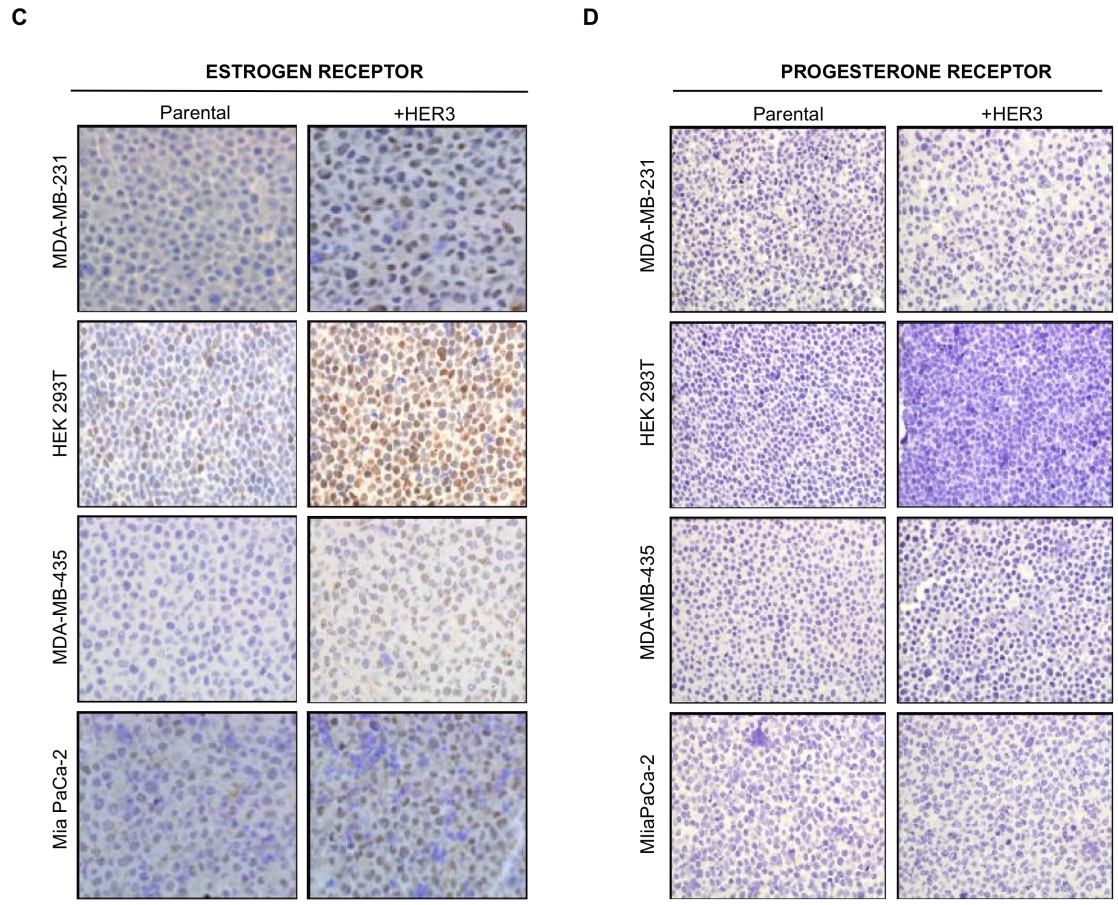


Figure 42. Effect of HER3 on hormonal receptor expression of stably transfected cells. A) Four HER3 overexpressing cell lines from different cancer cell types were generated by transfection and selection with 1 µg of pIRES-Hyg-HER3. In all cases, ectopic expression of HER3 was confirmed by western blot, using β-actin expression as a loading control. B) HER3 expression was also analyzed by IHC, in all cell lines corroborating the same results. C) ER expression levels were analyzed by IHC in the same panel of tumor cell lines where HER3 have been previously analyzed. Cell lines overexpressing HER3 had higher levels of ER in comparison with the parental cell lines in all cases. D) PR expression in cell lines with overexpression of HER3. PR protein expression levels were also analyzed in the cancer cell lines with overexpression of HER3. There was no effect on the tested cell lines with the PR expression when they were transfected with HER3.

Increasing evidence indicates that HER3 plays an important role in the genesis and progression of cancer. Not only HER3 expression in different tumors is increasingly being reported, but expression of HER3 has also been linked to resistance to anti-HER therapies using small molecule tyrosine kinase inhibitors. Furthermore, HER3 overexpression has been found to be an independent predictor of survival in some, (Witton et al., 2003, Bieche et al., 2003) but not in all studies (Travis et al., 1996). Moreover, co-expression of HER2 and HER3 is a poor prognostic indicator (Wiseman et al., 2005).

The aim of this section was to compare IHC profiles of primary breast carcinomas with HER3 staining to assess the role of HER3 in breast cancer. In this study, we show that HER3 expression was high in hormone positive tumors. The prevalence of HER3 expression as judged by analysis of protein levels by IHC (Barnes et al., 2005, Naidu et al., 1998) has been reported to be between 49-53% and we found an expression of HER3 (3+) in the 21% of the series analyzed. HER3 is frequently overexpressed in breast cancer and is found in the absence of gene amplification or mutation (Lemoine et al., 1992a). In our series we were unable to detect HER3 amplification in HER3 positive tumors.

HER3 membrane and cytoplasmatic staining were evaluated separately and different associations were found. Three studies (Naidu et al., 1998, Travis et al., 1996, Witton et al., 2003) found no significant association with HER3 and ER status but another (Knowlden et al., 1998) found that HER3 expression positively correlated with ER. Despite these results we observed a positive relationship between HER3 and ER expression in both scenarios. HER3 membrane expression was positively correlated with tumor size and ER. On the other hand, we found that HER3 cytoplasmatic staining positively correlated with hormone receptors (ER, PR) and was inversely associated with Ki-67 expression.

Expression of HER3 was found mainly in luminal and also was found in HER2 enriched molecular subtype. These results show a HER3 role independent to HER2 and not always are linked. Also we observed a presence of HER3 positive

cytoplasmatic expression staining in a better prognostic group, because it correlated positively with hormone receptors and inversely with Ki-67, than HER3 positive membrane expression.

In summary, the aim of this work was to emphasize the role of HER3 in breast cancer and determine a new valuation of HER3 staining. We think that is important do an evaluation of HER3 with membrane and cytoplasmatic staining separately because they may play different roles in the genesis of breast cancer. ER positive patients are normally considered to represent a good prognostic group and thus the interaction between HER and ER expression may be the key to the clinical management of this group. Further work will also be needed to evaluate HER3 as a marker for breast cancer and to allow identification of patients who may not respond to existing HER directed therapies.

3.3 Analysis of fulvestrant sensitivity in MCF-7 breast cancer cell line

Estrogens are known to regulate the proliferation of breast cancer cells and to alter their cytoarchitectural and phenotypic properties, but the gene networks and pathways by which estrogenic hormones regulate these events are only partially understood. More than 80% of breast tumors express ER establishing the hormonal therapy as one of the most common therapies for breast cancer patients. The expression of ER is used to predict which patients will benefit from hormone therapy, while loss of ER expression is associated strongly with poor prognosis in breast cancer patients (Dati et al., 1990). Anti-hormonal therapy has proved to be highly successful in the treatment of ER-positive breast cancer; however, resistance to these agents remains a significant clinical problem, with many patients either gaining no benefit or relapsing during therapy (Nicholson et al., 2007).

An inverse correlation between the HER2 and ER signaling pathways has been demonstrated in breast cancer cells (Adnane et al., 1989, Warri et al., 1991), while we observed a positive relationship between HER3 and ER expression in breast cancer patients and in breast cancer cell lines (figure 43). Those ER positive cells

presented levels of HER3, while the negative ones did not and when a non-ER positive cell line was transfected with HER3 an up-regulation of ER was observed. Furthermore, we analyzed this relationship in the breast cancer cell line MCF-7 and studied the downstream signaling routes that link this receptor to proliferative responses, Akt and MAPK. Cells transfected with HER3 had an up-regulation of pHER3, pAKT, pER levels and their total proteins but not of pMAPK or pS6 levels indicating *de novo* a correlation between those two parameters.

Numerous preclinical and clinical studies have established that increased expression of two members of the HER receptor family, HER1 and HER2, plays a central role in the acquisition of resistance to anti-hormonal therapies (Knowlden et al., 2003, Gutierrez et al., 2005, Nicholson et al., 2003, Nicholson et al., 1993, Nicholson et al., 2007). Up-regulation of HER1 and HER2 has been reported to be an early response to anti-hormone treatment in ER-positive breast cancer cell lines (Yarden et al., 2001, Gee et al., 2003, Hurtado et al., 2008); however, despite a number of preclinical findings reporting improved magnitude and duration of response with combined of targeting of HER1, HER2 and ER signaling (Johnston, 2010), translation of these findings into the clinical setting has proved largely disappointing, with a large number of patients gaining little or no benefit from such combination therapies. Further studies have now revealed an array of candidate genes with potential involvement in anti-hormone resistance, including the other members of the HER receptor family, HER3 and HER4. Indeed, a role for HER3, rather than HER4 has been supported, with increased activation of HER3 being reported in acquired tamoxifen and fulvestrant (ICI 182780) resistant MCF-7 cells (Frogne et al., 2009, Ghayad et al., 2010).

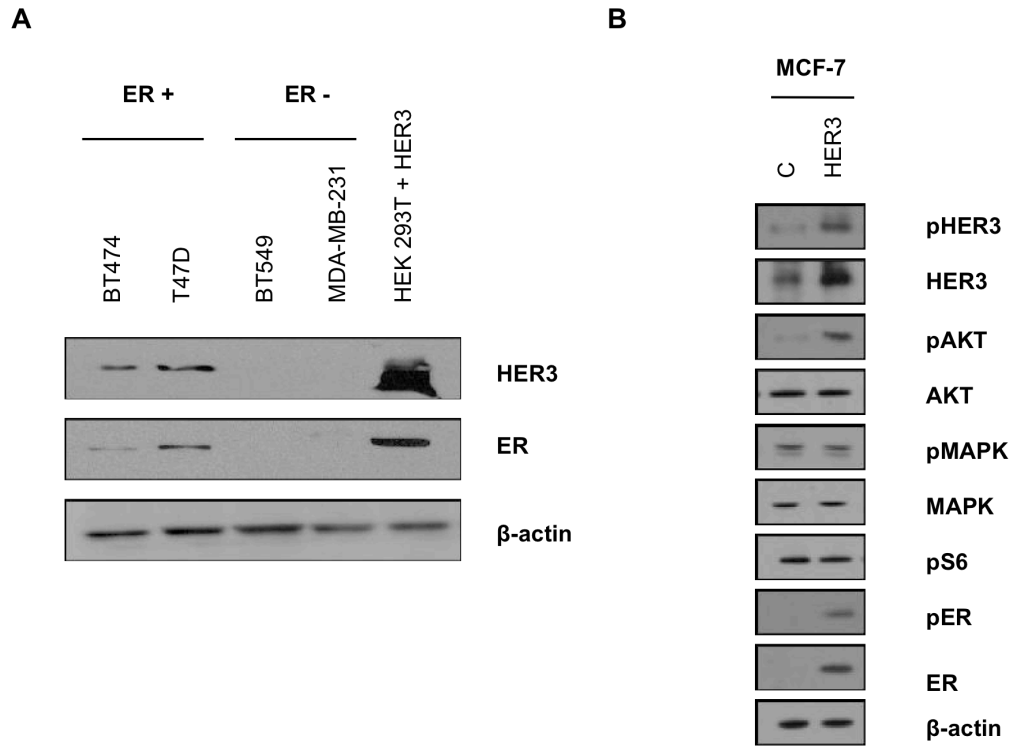


Figure 43. ER expressing cells have HER3 protein expression levels. A) Two breast cancer cell lines which naturally express ER and two breast cancer cell lines ER negative were analyzed by western blot for their level of HER3 expression. B) MCF-7 ER positive cell line was transfected with HER3 (pCEFL HER3) and checked HER3 downstream protein expression levels by western blot. β-actin was used as an internal control to verify equal protein loading. C, control; WT, wild type.

In the present study, we have examined the acute inductive capacity of the pure anti-ER fulvestrant on HER3 receptor expression in the MCF-7 ER-positive breast cancer cell line, with and without HER3 down-regulation and assessed the effect of ligand activation of this receptor on anti-hormone response with NRG-1β. For this purpose cells were treated with fulvestrant for 12 h and/or one week in presence or absence of NRG-1β for 20 min and the following results were obtained.

First, fulvestrant consistently inhibited ER of all cell lines demonstrating a greater sensitivity in MCF-7 shHER3 than MCF-7 cells to this pure anti-ER drug treatment (figure 44). Second, ligand activation of HER3 with NRG-1β and

fulvestrant has more growth-inhibitory activity than the anti-ER alone. Third, we demonstrate that fulvestrant treatment induces the expression of both HER2 and HER3 receptors, resulting in enhanced sensitivity to the action of NRG-1β.

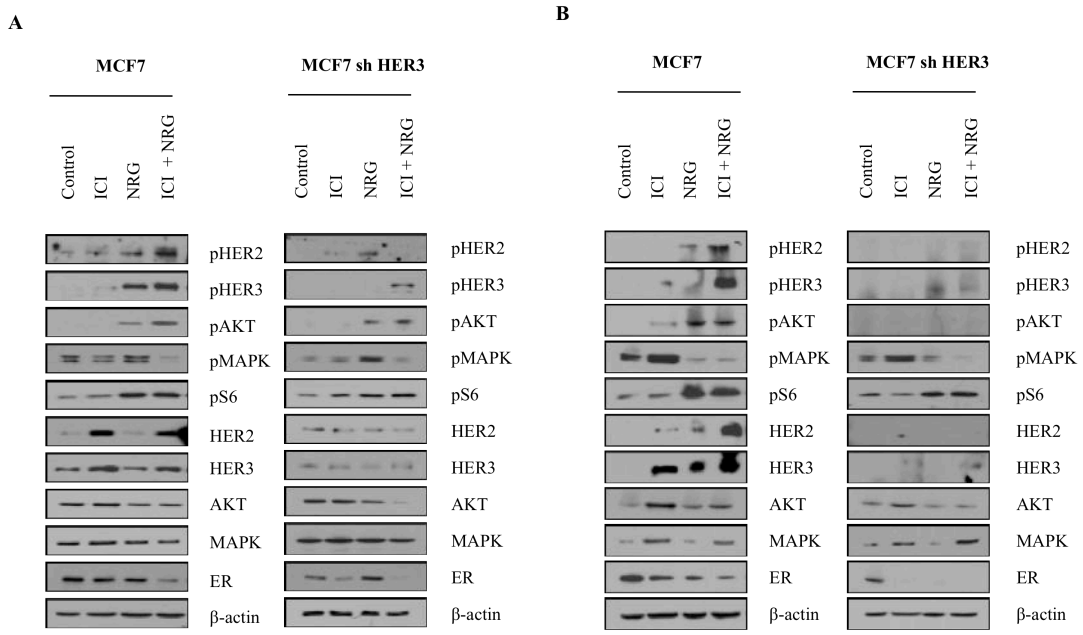


Figure 44. ICI 182780 and NRG-1β treatment in MCF-7 cell lines. Cells were starved and treated with 1 μM ICI 182780 and 100 nM NRG-1β for 12 h (A) and/or for 7 days (B). After treatment, cells were lysed and proteins were extracted and western blots performed. A total cell lysate of MCF-7 cells was included as a positive control. The membranes were probed with β-actin to verify equal protein loading. ICI, ICI182780 or fulvestrant; NRG, NRG-1β.

These findings clearly indicate the potent anti-ER activity of fulvestrant. However, alongside this potent acute growth-inhibitory activity of fulvestrant, there was also clear evidence of the inductive capacity of this agent, with increased HER3 protein expression in these cells and more consistently at 7 days post anti-ER treatment. In addition to enhancing total protein expression levels, fulvestrant treatment also promoted basal HER3 phosphorylation even when cells have a shRNA of this receptor.

Indeed, the enhanced level of HER3 phosphorylation in the MCF-7 cell line was associated in both experiments with an increased activation of PI3K-Akt signaling but not in the MAPK pathway, whilst in the MCF-7 shHER3 cell line, the up-regulation of pHER3 was associated with increased Akt activity within 72 h but not following 7 days anti-ER treatment. This induction of HER3 signaling by fulvestrant, as previously suggested for HER1 and HER2, may provide these cells with a further input into signaling pathways that could potentially allow cells to survive the initial action of the anti-ER and ultimately provide a resistance mechanism. It is possible that fulvestrant plays an active role in limiting their own activity, through an ability to promote expression of the HER3 cell growth promoter.

On the other hand, it has been reported that NRG activates the HER3 and HER4 receptors in a direct fashion, whereas activation of HER2 receptor is indirect (Carraway et al., 1994, Sliwkowski et al., 1994). Activation of HER2 is achieved presumably by trans-phosphorylation and/or through the induction of HER2/HER3/HER4 receptor heterodimerization (Carraway and Cantley, 1994) and the consequent constitutive phosphorylation of tyrosine residues is probably one important element in human breast carcinoma progression.

Next, we performed colony formation and cell viability assays to examine if fulvestrant cytotoxicity depends on the activation of HER3 expression, by stimulating the cells with NRG-1β. MCF-7 cells were infected with lentivirus encoding shLUC or shHER3, and fourteen days after selection, colony formation and cell viability assays were analyzed upon fulvestrant and/or NRG-1β treatment.

We observed that fulvestrant reduces transformation cell capacity in function of HER3 expression levels and its activation by NRG-1β. In normal MCF-7 cell line the addition of NRG-1β prevented fulvestrant induced transformation cell availability (figure 45 A). This effect was not observed in absence of HER3 expression (figure 45 B). To determine if these differences were significant we analyzed the results with non-parametric Wilcoxon statistical test, which were obtained significant differences for MCF-7 ($p = 0.028$) but not for the MCF-7 shHER3 cell line ($p = 0.068$).

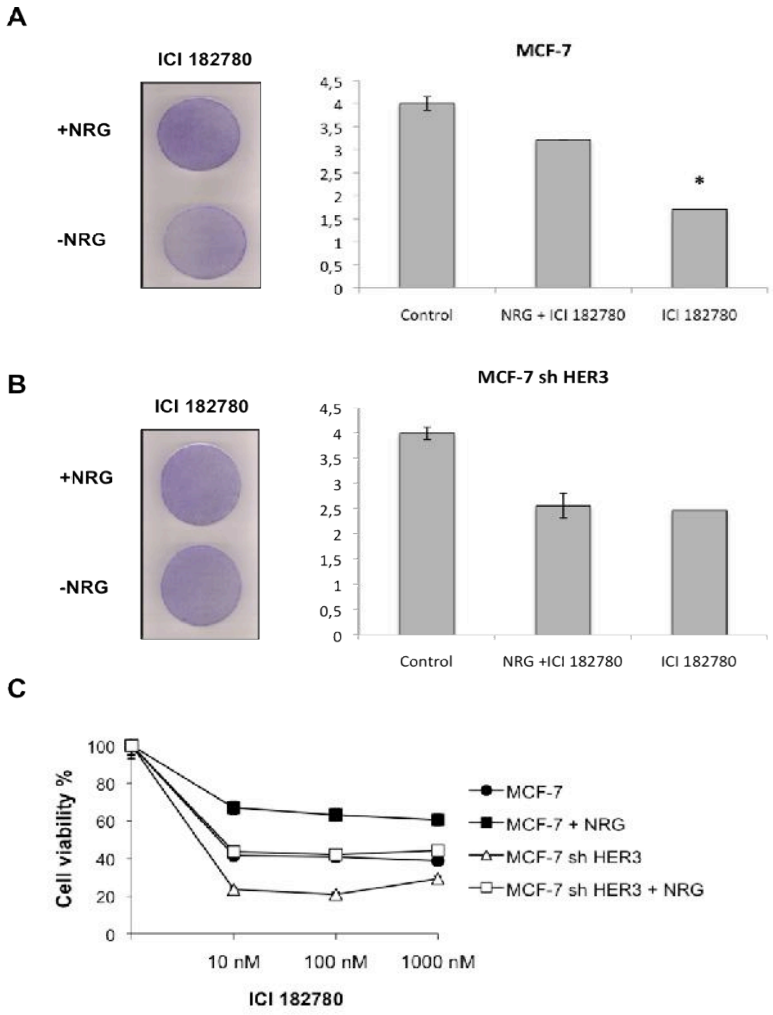


Figure 45. HER3 stimulation protects cells from fulvestrant effect. Stable MCF-7 cells were generated with pLKO shRNA LUC and shHER3. Cells were seeded at the same density as to perform colony formation assays of MCF-7 (A), MCF-7 shHER3 cells (B) or cell viability assays of both (C). For colony formation assays cells were treated with 1 μM fulvestrant and 100 nM NRG-1β for 72 h, and each cell type was plated in duplicate for each experiment. C) Cell viability induced by various concentrations of fulvestrant was determined in down-regulation of HER3 versus LUC vector at 72 h in MCF-7 cell line with and without 100 nM NRG-1β stimulation. Results are mean ± SD of three experiments performed in triplicates.
NRG, NRG-1β ; ICI 182780, fulvestrant. * Statistically significant ($p \leq 0.05$).

Indeed, differences in fulvestrant sensitivity were found between MCF-7 and MCF-7 shHER3 in presence and absence of NRG-1β (figure 45 C) when we performed a cell viability assay. MCF-7 parental cells were less sensitive to anti-ER treatment than cells with a shRNA of HER3. Furthermore, differences between these cells and NRG-1β stimulation were also found. NRG-1β stimulation induces increased cell viability.

The results presented here demonstrate that stimulation of NRG-1β protects cells from fulvestrant treatment and that HER3 has the potential to play a central role in the development of resistance to this anti-hormonal therapy *in vitro*.

The ability of anti-hormones to inhibit growth of ER-positive breast cancer cells has principally been attributed to the ability of these agents to block the transcriptional activity of the ER and prevent activation of genes responsible for mediating cell cycle progression. Resistance to anti-ER therapies limits the efficacy of these compounds in ER positive breast cancer and several mechanisms of resistance have been proposed. Expression of HER1 and/or HER2 has been linked to resistance (Newby et al., 1997, Shou et al., 2004) and cross-talk between the ER and HER1 and HER2 signaling pathways has been described in cancer cell lines (Benz et al., 1992, Pietras et al., 1995, Tang et al., 1996). Interestingly, increased functioning of the PI3K-Akt route has also been linked to resistance to anti-hormonal therapies.

As expected, fulvestrant was a potent growth inhibitor in the ER-positive breast cancer cell line MCF-7 through its ability to suppress ER. However, fulvestrant alone or more potently the simultaneous induction of fulvestrant with a NRG-1β enriched environment, provide a mechanism for these cells, to promote re-expression of HER2 and HER3 and perhaps its downstream signaling, and ultimately drive fulvestrant-resistant cell growth. Such signaling may allow cells to evade inhibition during the drug-responsive phase, as targeting this HER receptors in combination with fulvestrant could suppress the residual signaling activity and greatly improve and extend the growth-inhibitory action of this anti-hormone in the cells as Gee and colleagues demonstrated with gefitinib (Gee et al., 2003).

Furthermore, the anti-ER effect was higher when cells do not present moderate HER3 levels.

Altogether, the current findings demonstrate that targeting ER signaling with the pure anti-ER fulvestrant can both suppress and induce protein expression in MCF-7 cells. Thus, although fulvestrant has potent, acute growth-inhibitory activity in ER-positive breast cancer cells, its ability to rapidly induce and sensitize cells to growth factors, such as NRG-1β may serve to reduce and ultimately limit its inhibitory activity. Indeed, such a rapid induction of these proliferative genes may provide a mechanism of de novo endocrine resistance in situations in which NRG expression in the tumor microenvironment is high. However, it should also be noted that all of these studies were performed in a single cell line and that further studies in other ER-positive cell lines are required to fully support this proposition.

Importantly, these findings also indicate that NRG-1β signaling via HER3 can provide a potent mechanism of resistance to such combination therapies and may provide an explanation for the disappointing results of clinical studies in which the combination strategy of anti-ER alongside an anti-HER1 or anti-HER2 agent was examined (Normanno et al., 2009, Johnston, 2010).

Since HER3 signals through this route it will be interesting to evaluate whether patients expressing higher levels of HER3 also express elevated Akt phosphorylation at Thr308 or Ser473, two sites that reflect increased functioning of Akt. These findings, together with the precedent that persistent activation of HER3 results in escape from growth inhibition induced by HER TKIs should represent the bases of additional clinico-pathological studies to analyze the value of HER3 as a predictive marker in anti-hormonal therapies (Sergina et al., 2007).

Furthermore, HER3 is essential in HER2 driven tumorigenesis and may have clinical relevance for tumor aggressiveness. Patients with expression of both proteins (HER2 and HER3) may benefit from anti-HER3 targeted therapy. Clinical studies examining a group of tamoxifen treated, ER positive breast cancer patients have demonstrated that HER2 and HER3 positive patients relapsed on tamoxifen

with a higher incidence than HER receptor negative patients (Tovey et al., 2005). These results support the hypothesis that HER3 up-regulation may play a role in tumor aggressiveness in ER positive breast tumors.

In addition, the ability of HER3 through sustained activation of Akt to circumvent both hormonal and TKI based therapies presents an important clinical problem where HER3 therapeutic targeting is warranted.

4. Combination therapy with elisidepsin and fulvestrant

Most of the studies in breast cancer have been focused on identifying genes overexpressed in this type of tumor (Jiang et al., 2002, Knappskog et al., 2011) or patterns of gene expression associated with clinical outcome or prognosis (Goodwin et al., 2012, Ahmed et al., 2011), classification of primary tumors (Perou et al., 2000, Prat and Perou, 2011), tumor aggressiveness (Zajchowski et al., 2001, Amin et al., 2010), or responses to chemotherapy or drug resistance (Sotiriou et al., 2002, Hutcheson et al., 2011). Focusing in tumor resistance to drugs, genetic heterogeneity and the cellular dynamics are thought to be the cause of it but combinations of non-cross-resistant treatment regimens might prevent its recurrence.

Therapeutic strategies that target multiple components within the HER network might be beneficial. As it applies to drug resistance, inhibition of HER2 phosphorylation by TKIs targeting HER1 and HER2 in HER2 positive breast cancer cells is followed by feedback up-regulation of activated HER3, thus limiting the inhibitory effect of HER TKIs (Sergina et al., 2007, Amin et al., 2010, Garrett et al., 2011). Integration of anti-HER drugs with conventional anti-cancer chemotherapy and radiotherapy has been shown to improve outcome and overcome drug resistance (Hynes and Lane, 2005, Citri and Yarden, 2006, Ardavanis et al., 2008).

In light of this, we have initiated a feasibility study to investigate the efficacy of fulvestrant treatment in what are traditionally ER positive samples with elisidepsin, which it binds to HER3. We hypothesized that sustained and complete inhibition of HER3 and its output to PI3K-Akt is required for the maximal anti-

tumor effect of ER inhibitors. We demonstrate herein that inhibition of the ER with fulvestrant results in time-dependent up-regulation of the HER3 protein using both breast cancer cell lines (figure 44). These results imply HER3 expression is under negative regulation by signaling of the ER network and these studies point to a central role for HER3 in the survival of HER2 or ER positive cells that potentially limit the full activity of HER2 or ER antagonists.

We tested the ER positive breast cancer cell line MCF-7 with fulvestrant or the combination of it with elisidepsin, NRG-1β or both and cell viability was measured by crystal violet assays (figure 46). Inhibition of ER with fulvestrant sensitized ER positive MCF-7 cells and perhaps their cell viability. Similar results were observed with elisidepsin compound. On the contrary, when cells were stimulated with NRG-1β a cell viability increase was observed. The combination of fulvestrant, NRG-1β and elisidepsin was markedly more effective than fulvestrant and NRG-1β but the same as elisidepsin and NRG-1β.

The reemergence of pHER3 and HER3 in fulvestrant treated cells also suggested that, in addition to HER1 and HER2, an other kinase is an early response to anti-hormone treatment in ER-positive breast cancer cell lines and maybe the cause of why a large number of patients gaining little or no benefit from HER1, HER2 and ER signaling combination (Johnston, 2010). Moreover, we found that HER3 activation up-regulates pER and ER expression in the MCF-7 cell line. Also, a correlation between HER3 and ER expression in a series of breast cancer patients was observed, while HER1 are HER2 expressions pertained in a molecular subtype independent of luminal (ER positive) breast carcinomas.

We hypothesized that by blocking the compensatory reengagement of HER3, combined targeting of ER and HER3 will be synergistic against ER-dependent tumors. The lack of effect of ER overexpression raises the possibility of combined treatments of anti-hormones, as fulvestrant with other drugs and of the use of elisidepsin as a treatment in patients developing resistance to conventional drugs mediated by ER. Treatment of hormone resistant breast tumors is a clearly unmet medical need, and investigation of new treatment options is warranted.

Elisidepsin has been extensively studied and it is a well-tolerated compound, with preliminary evidence for antitumor activity in NSCLC, particularly in patients with squamous histology. Additionally, elisidepsin may be useful against tumors with HER3 expression, that usually linked to high malignancy and bad prognosis (Tanner et al., 2006, Hayashi et al., 2008, Gespach, 2011) such as breast tumors.

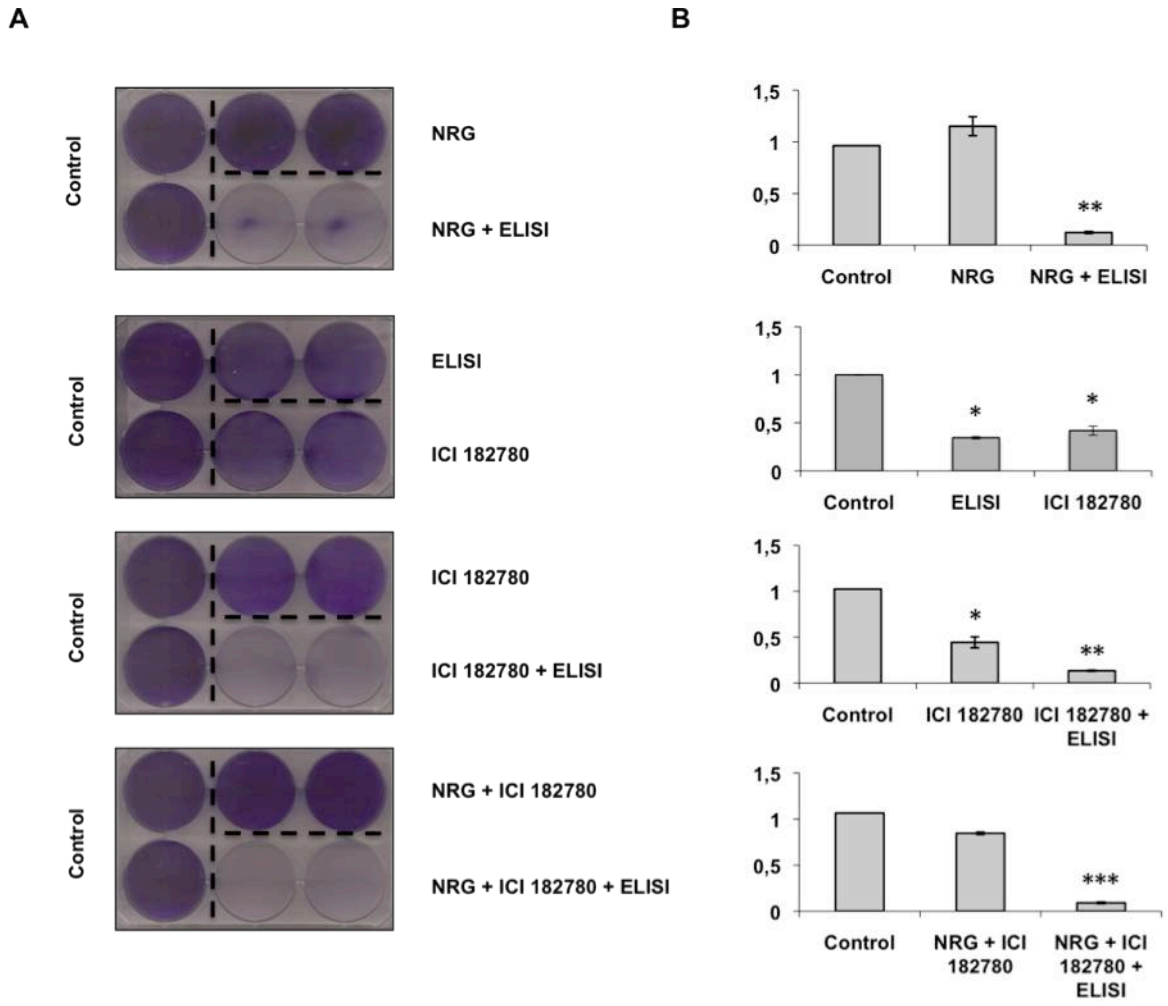


Figure 46. Combination of fulvestrant with elisidepsin and NRG-1β. Cells were plated overnight and then treated for 72 h at a fixed ratio of doses that corresponded to 1 μM fulvestrant, 1 μM elisidepsin and 100 nM NRG-1β for each drug alone. Cell viability was measured by a crystal violet assay for optical density using a spectrophotometer (A). Graphics show each treatment alone, the combination of fulvestrant with elisidepsin, NRG-1β and both (B). Results are mean ± SD of at least three independent experiments in different days; *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$**.** ELISI, Elisidepsin; NRG, NRG-1β; ICI 182780, fulvestrant.

Conclusions

1. Elisidepsin, a novel antitumor agent of marine origin, which is currently under clinical investigation, showed potent cytotoxic activity against a broad spectrum of cancer cell lines (pancreas, breast, head and neck, lung, colon, ovary and prostate) and against embryonic fibroblasts.

2. Elisidepsin in combination with the chemotherapeutic agents, CDDP, TAX, and gemcitabine showed a synergistic effect into the lung, breast, colon and pancreas cancer cell lines, independently of the sensitivity to elisidepsin treatment of each cell line.

3. Sensitivity of pancreatic and breast cancer cell lines to elisidepsin positively correlates with an epithelial phenotype. Furthermore, continuous exposure to elisidepsin induced a down-regulation of epithelial markers in three different cancer cell types (lung, colon and breast), while acquired resistance to elisidepsin is associated with a switch to EMT.

4. Elisidepsin had an effect in HER3 mRNA, protein levels and the downstream PI3K-Akt pathway. It induced down-regulation of phospho-HER3 and HER3 expression levels. Combination of elisidepsin treatment and HER3's specific ligand, NRG-1β caused a slight tendency to maintain pHER3 and HER3 expression levels. In contrast, in cell viability assays elisidepsin activity was independent of NRG-1β expression.

5. The sensitivity to elisidepsin was found to correlate with HER3 protein expression levels, but not with the other members of HER family, neither its regulatory factors, Nrdp1, USP8 and ZNF217. Cell lines that were less sensitive to elisidepsin had low or no levels of HER3 in comparison with the sensitive cell lines, which expressed higher levels of this protein.

6. Elisidepsin sensitivity was also proven to be dependent on HER3 expression in cell viability assays. Down-regulation of HER3 expression exerted a significant protective effect, while ectopic HER3 expression significantly sensitized cells for elisidepsin treatment in all cell lines studied. This effect was not observed when a HER3 truncated form was overexpressed.

7. Co-localization of elisidepsin in HER3 overexpressing HEK 293T cells was detected, but not in cells expressing the HER3 truncated form. Immunoprecipitation assays of cross-linked HER3 and elisidepsin confirmed HER3 carboxy terminal binding with elisidepsin.

8. HER3 expression in human tumors samples was evaluated by IHC and 21% of them were positive, but none of them were found to be amplified or mutated. HER3 expression (independently of its localization) was found to correlate with ER expression levels. Moreover, cell lines with ectopic HER3 expression levels up-regulate their ER expression levels compared with the parental ones.

9. Fulvestrant treatment consistently inhibited ER expression levels, demonstrating a greater activity in MCF-7 shHER3 than MCF-7 cell lines. Moreover, ligand activation of HER3 had increased growth-inhibitory activity with fulvestrant than the anti-ER alone.

10. Fulvestrant treatment induced the expression of both HER2 and HER3 receptors, resulting in enhanced activity with NRG-1β stimulation. Apart from enhancing total protein expression levels, fulvestrant treatment also promoted basal HER3 phosphorylation.

11. Differences in fulvestrant sensitivity were found to be depended on HER3 expression levels in MCF-7 cells in cell viability assays. NRG-1β stimulation induced increased cell viability and protected cells with fulvestrant treatment versus those without.

12. Combined treatment of tumor breast cancer cell lines with fulvestrant and elisidepsin drugs gives rise to an enhancement of the reduction in cell viability. This synergy can be attributed to simultaneous blockade of ER and HER3-PI3K-Akt pathways, suggesting a new therapeutic approach in breast cancer.

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Appendix

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RSK4 inhibition results in bypass of stress-induced and oncogene-induced senescence

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p90 Ribosomal S6 kinase (RSK) 4 is a serine–threonine kinase that belongs to the p90RSK family. RSK4 has been proposed as a tumor suppressor gene, related with anti-invasive activity, inhibition of the RAS–mitogen-activated protein kinase (MAPK) pathway and induction of senescence. Despite the related findings, little is known about RSK4 effectors. In human tumors, RSK4 is downregulated even in some benign lesions, such as colon adenomas and breast papillomas, indicating that RSK4 inhibition could be an early event in cellular transformation. For cells to achieve immortality and transformation, it is believed that they must override senescence. In the present study, we found that when RSK4 is inhibited *in vitro* using short hairpin RNA technology, cells can bypass stress-induced senescence and oncogene-induced senescence: normal human fibroblasts grew following oxidative stress, induction of DNA damage and $KRAS^{V12}$ or $BRAF^{E600}$ overexpression. To investigate the RSK4 effectors, we used short hairpin RNA or inhibitor molecules against major senescence mediators. We found that RSK4-induced senescence is mediated through p21, but is independent of p16, p38MAPKs and induction of reactive oxygen species, delimiting RSK4 signaling. These data support the importance of RSK4 for regulating senescence and indicate that downregulation of this kinase could be an important element in facilitating cell transformation.

Introduction

Senescence is a permanent growth arrest activated by normal cells in response to various stress stimuli. Senescence was first described by Hayflick (1) decades ago as a phenomenon that occurred in cultured cells as a consequence of progressive telomere shortening after numerous cell divisions. The phenomenon was called 'replicative senescence'. Subsequently it was observed that other stress stimuli, such as DNA damage, exposure to reactive oxygen species (ROS), and oncogene activation can induce 'premature senescence' in a telomere-independent manner (2). Several recent reports have demonstrated that oncogene-induced senescence occurs *in vivo* in human tumors and in mouse models (3–6) and acts as a protective mechanism against cancer.

Many signals that induce senescence converge on the p19ARF–p53 and p16INK4a–Rb pathways, but utilize different intermediaries. P16 activates the Rb pathway by inhibiting Cyclin D/cyclin-dependent kinase (CDK) 4,6 and p19 activates p53 by inhibiting MDM2; p53 can also be activated by phosphorylation performed by the ataxia telangiectasia mutated (ATM)/ATM and Rad3-related (ATR) (Rad3-related) and/or CHK1/CHK2 proteins. These two pathways can be connected through p21, which is activated by p53; p21, in turn, can activate Rb by inhibiting CycE/Cdk2. Once Rb is activated, it can inactivate the transcription factor E2F. In some senescent cells, E2F target genes are silenced by Rb-dependent chromatin reorganization into discrete foci that are termed senescence-associated-heterochromatin foci (7). Despite the connection between these two pathways, there are cell type-specific and species-specific differences in the way cells respond when one or the other pathway mediates a senescence response. For example, experimental disruption of telomeres activates only the p53 pathway in mouse cells and both the p53 and the p16–Rb pathways in human cells (8).

Telomeres consist of repetitive DNA elements at the end of linear chromosomes that protect them from degradation or recombination. Due to the inability of the replication machinery to copy the end of linear molecules, telomeres become shorter at every round of replication. When telomeres are shortened under a minimum threshold, a DNA damage response (9) is activated through ATM, ATR and CHK1-2, which in turn, activate p53, thereby contributing to either apoptosis or replicative senescence (10). In the case of oncogene-induced senescence, several pathways involving activation of tumor suppressor genes have been described. One model proposes that some oncogenes, such as *RAS* or *STAT5*, cause DNA damage (11), which could occur through ROS generation (12) or through excessive replication due to a sustained oncogenic signal (13,14). Consistent with the hypothesis that oncogene activation can produce an accumulation of ROS, ROS also induces senescence via a kinase cascade involving p38 and its effector p38-regulated/activated protein kinase, which can phosphorylate and activate p53 (15). Although oncogene activation induces a hypermitogenic signal, it has been proposed that the activation is rapidly shut down by a negative feedback signaling network that acts as the true senescence trigger (5). The mechanisms described above are not necessarily exclusive, and depending on the genetic background and tissue type, the proteins connecting oncogene activity to the tumor suppressors p53 and/or pRb might be different. Identification and characterization of new genetic components of the senescence pathways will provide a better understanding of the molecular mechanisms of senescence.

In a previous report, we found that the serine–threonine kinase, p90 ribosomal S6 kinase (RSK) 4, might have an important role regulating senescence and immortalization in colon and renal carcinomas (16). RSK4 belongs to the p90RSK family, which is composed of six members identified by the presence of two non-identical and active kinase domains (17). This family includes two protein groups: RSK1–4 are activated by extracellular signal-regulated kinase (ERK) 1 and 2, and are more closely related to each other than the mitogen-stress activated kinases 1 and 2, the other two members of this family.

RSK1–3 are the central mediators of the ERK–mitogen-activated protein kinase (MAPK) pathway in the regulation of cell division, survival and differentiation via phosphorylation of numerous intracellular proteins including transcription factors and coregulators, such as CREB, CBP, p300, c-Fos, IκBα and ERα. Nevertheless, RSK4, the last member of this kinase family identified, has important features that clearly distinguish it from other RSK proteins, involving an inhibitory function in cell proliferation. Depletion of RSK4 seems to suppress p53-dependent cell cycle arrest and reduces messenger RNA expression of the cyclin-dependent inhibitor p21. Myers *et al.* (18) identified RSK4 as an inhibitor of the RAS–MAPK pathway because of its ability to disrupt mesoderm formation. Another study describes anti-invasive and anti-metastatic activities of RSK4 in breast cancer cell lines both *in vitro* and *in vivo* (19). In our previous study, we observed that RSK4 was downregulated in colon and renal human carcinomas, as well as in early stages of tumorigenesis, for example in colon adenomas. When RSK4 was overexpressed, senescence was induced, and its depletion

Abbreviations: CDDP, cisplatin; ERK, extracellular signal-regulated kinase; hTERT, human telomerase reverse transcriptase; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; PD, population doubling; pRS, pRetroSuper; RSK, p90 ribosomal S6 kinase; SA-β-gal, senescence-associated β-galactosidase; shRNA, short hairpin RNA.

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increased the lifespan of human diploid fibroblasts. Endogenous RSK4 was also increased when senescence was induced. We found that RSK4-mediated senescence was dependent on Rb but not on p53. In the present study, we report that RSK4 inhibition overrides premature senescence in response to oxidative stress, DNA damage and oncogene activation. We also show that RSK4 mediates senescence through p21 activation.

Material and methods

Cell culture and reagents

The TIG3 and TIG3 p16-null human diploid fibroblasts immortalized with human telomerase reverse transcriptase (hTERT) used in this study were kindly donated by Dr D.Peeper (The Netherlands Cancer Institute, Amsterdam). Cells were grown in Dulbecco's modified Eagle's medium (Cambrex, Barcelona, Spain) supplemented with 10% fetal calf serum (Labclinic, Barcelona, Spain) and antibiotics (Cambrex). The p38 MAPK inhibitor SB203580 was obtained from Calbiochem (Darmstadt, Germany). *N*-acetyl-L-cysteine (NAC) and hydrogen peroxide (H_2O_2) were purchased from Sigma (Taufkirchen, Germany).

Cisplatin and hydrogen peroxide (H_2O_2) resistance curves

For Cisplatin (CDDP) and H_2O_2 resistance curves, cells were seeded at low density and treated for 2 h with 200 μM of H_2O_2 or for 6 h with 2 μg/ml of CDDP. After treatment, fresh medium was added and cells were maintained in culture for 3, 6 or 9 days. At these times, cells were washed with phosphate-buffered saline, fixed in 10% formalin and rinsed with distilled water. Cells were stained with 0.1% crystal violet (Sigma) for 30 min, rinsed thoroughly and dried. Cell-associated dye was extracted with 2 ml 10% acetic acid and the relative cell number was determined reading the optical density at 590 nm. A value of 100% was assigned to cells stained just before the treatment. All points within each experiment were determined in duplicate and each experiment was done at least twice with similar results.

Growth curves

Two days after selection, cells were counted and seeded in duplicate every 4 days. Population doublings (PDs) were determined by the following formula: PD = Log (Nf/Ni)/Log2, where Nf is the number of cells counted and Ni is the number of cells seeded. Cumulative PD numbers represent the sum of PDs from previous passages. Each curve was done at least twice with similar results, and each time point was determined in duplicate.

Vectors and plasmid construction

The pWZL-RSK4 blasticidin vector was constructed by cloning the respective complementary DNA fragment of *RSK4* from pLPCX-RSK4 into the retroviral vector pWZL. The retroviral short hairpin RNA (shRNA) vectors pRetroSuper (pRS)-shp21 and pRS, as well as the pBabe-KRAS^{V12} vector and pWZL vector were kindly donated by Dr A.Carnero (Centro Nacional de Investigaciones Oncológicas, CNIO, Spain). pMSCVblast-BRAF^{E600} and pMSCVblast empty vector were kindly donated by Dr D.Peeper. These vectors contain a resistance gene against blasticidin. The retroviral vectors, pLPCX-RSK4 and pLPCX-green fluorescence protein, and the lentiviral vectors, pLKO.1-shNonTarget (pLKO.1-shNT) and pLKO.1-shRSK4, were generated as described previously (16).

Lentivirus-based shRNA transduction

Lentivirus-based transduction was carried out using a packaging cell line, HEK293T, by cotransfection of pCMV-dR8.91 dvpr (kindly donated by D.Trono, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) and VSV-G (Clontech, Saint-Germain-en Laye, France). After two consecutive virus infections, cells were selected with the indicated antibiotic.

Retrovirus-based gene transduction

Retrovirus-based gene transduction was carried out using a packaging cell line (GP-293; Clontech) according to the manufacturer's instructions. After two consecutive virus infections, cells were selected with the indicated antibiotic.

Western blot

Subconfluent cells were lysed in a lysis buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid pH 7.5; 150 mM NaCl; 1% Triton X; 1 mM ethylenediaminetetraacetic acid and 10% glycerol) in the presence of protease and phosphatase inhibitors for 30 min at 4°C. After the lysates were cleared by centrifugation, protein concentrations were determined using the Bradford assay (Bio-Rad Protein Assay, Munich, Germany). About 25–50 μg of protein was denatured and resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad). The primary antibodies used were anti-RSK4 (sc-17178, diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-actin (CP01, diluted 1:7000; Calbiochem), anti-p21^{WAF1} (MS-891, diluted 1:500; Neomarkers, Fremont, CA), anti-p16 (DB018, diluted 1:200; Deltabiolabs, Gilroy, CA), anti-KRAS (sc-30, diluted 1:200; Santa Cruz Biotechnology) and anti-BRAF (sc-5284, diluted 1:500; Santa Cruz Biotechnology). The secondary antibodies used were donkey anti-goat IgG-horseradish peroxidase (sc-2020, diluted 1:5000; Santa Cruz Biotechnology), sheep anti-mouse IgG-horseradish peroxidase (NA9310, diluted 1:3000; Amersham Pharma-Biotech, Dreieich, Germany) and donkey anti-rabbit IgG-horseradish peroxidase (NA9340, diluted 1:2000; Amersham Pharma-Biotech). Bound antibodies were visualized with an enhanced chemiluminescence detection kit (Amersham Pharma-Biotech). For the p21 band intensities, proteins were quantified by densitometry with the Image J software (version 1.42q, National Institutes of Health, Bethesda, MD), normalized to the intensity of β-actin in each sample and expressed in arbitrary densitometric units.

Analysis of senescence

Senescence-associated β-galactosidase (SA-β-gal) activity was determined with the senescence β-galactosidase kit (Cell Signaling, Beverly, MA), following the manufacturer's instructions. To quantify SA-β-gal activity, at least 500 cells were counted in five random fields using a standard light microscopy.

Colony formation assay

Cells were plated at 7.5×10^4 cells per well in six-well dishes or at 1.7×10^5 in 60 mm plates and cultured for 14 days prior to fixation with 4% paraformaldehyde (Sigma–Aldrich, Taufkirchen, Germany). After fixation, cells were washed with phosphate-buffered saline and stained with 0.1% crystal violet (Sigma) for 30 min, rinsed thoroughly and dried. Each cell type was plated in duplicate for each experiment.

RNA extraction and quantitative reverse transcription–polymerase chain reaction

Total RNA was isolated from cells with the RNeasy Mini Kit (Qiagen, Germantown, MD) following the manufacturer's instructions. Random primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) were used to carry out complementary DNA synthesis from 1.5 μg of total RNA. RSK4 expression was detected using the Taqman Gene Expression Assay (Hs00179523_m1; Applied Biosystems, Carlsbad, CA). The endogenous control PPIA (4326316E; Applied Biosystems) was used as the reference gen. An ABI PRISM 7000 instrument (Applied Biosystems) was used to perform the relative quantification analysis, and data were analyzed with the 7000 Sequence Detection Software, v.1.2.3 (Applied Biosystems). The polymerase chain reaction cycling program consisted of denaturing at 95°C for 10 min and 40 cycles at 95°C for 15 s and annealing and elongation at 60°C for 1 min. The reactions were done in triplicate.

Results

RSK4-induced senescence in TIG3 and TIG3 p16-null human diploid fibroblasts

It is known that human diploid fibroblasts in culture accumulate proteins related with senescence, such as p16 and p21, when they reach the limit of their finite lifespan (20). In a previous study, we reported that RSK4 overexpression induced a senescence-like phenotype in the IMR90 human diploid fibroblast line (16). In order to separate the RSK4-induced senescence from lifespan-based events and to clarify the relationship between RSK4 and senescence, we decided to work with the hTERT-expressing fibroblasts, TIG3 and TIG3 p16 null. hTERT expression enables human diploid fibroblasts to bypass replicative senescence without affecting premature senescence (4,20,21).

The RSK4 gene was transduced into these cell lines via retroviral infection. Six days postselection, we observed dramatic morphologic changes characteristic of senescence (2) together with an increment in SA-β-gal activity (Figure 1A). This phenotype was independent of p16, as it was also observed in TIG3 p16-null cells. As was described previously (16), RSK4 induced an increase in p21 expression in both cell lines (Figure 1B).

RSK4 inhibition conferred resistance to stress-induced senescence

In a previous study, we found that RSK4 inhibition delays replicative senescence in IMR90 cells (16). To determine whether RSK4 could also confer resistance to stress-induced senescence, we infected TIG3

cells with the lentiviral vector pLKO.1 encoding shRNA against RSK4 (shRSK4) and against a non-target sequence (shNT). Following cell selection, we checked RSK4 inhibition by quantitative real time reverse transcription–polymerase chain reaction (supplementary Figure 1 is available at *Carcinogenesis* Online). Because subcytotoxic treatments are able to induce senescence (22), selected cells were seeded at low density and treated with 200 μM of H_2O_2 for 2 h or 2 μg/ml of CDDP for 6 h. Subsequently, cells were incubated with fresh medium and the relative cell number was measured every 3 days. After either treatment, control cells stopped cell proliferation (its relative cell number did not increase during 9 days) (Figure 2A) and 73% of cells after H_2O_2 treatment or 68% of cells after CDDP treatment were positive for the senescence marker SA-β-gal (Figure 2B). Nevertheless, cells with RSK4 inhibition resumed proliferation after either treatment and the number of positive cells for SA-β-gal was reduced to 30% after H_2O_2 treatment or 15% after CDDP treatment (Figure 2A and B), indicating that cells lacking RSK4 are more resistant to stress-induced senescence.

RSK4 inhibition conferred resistance to oncogene-induced senescence

Many genes trigger oncogene-induced senescence, and several reports have now demonstrated that mutations in *K-RAS*, *B-RAF*, *PTEN* and *NF1* can trigger cell senescence *in vivo* (3–6,23–25). Once, we had observed that RSK4 inhibition could reduce stress-induced senescence, we investigated whether RSK4 inhibition could also override oncogene-induced senescence. We infected TIG3 and TIG3 p16-null cell lines with lentiviral vector pLKO.1 encoding shRNA against RSK4 (shRSK4) or against a non-target sequence (shNT). After selection, cells were reinfected with a retroviral vector coding for $KRAS^{V12}$ or a control. Six days postselection, we analyzed SA-β-gal activity in

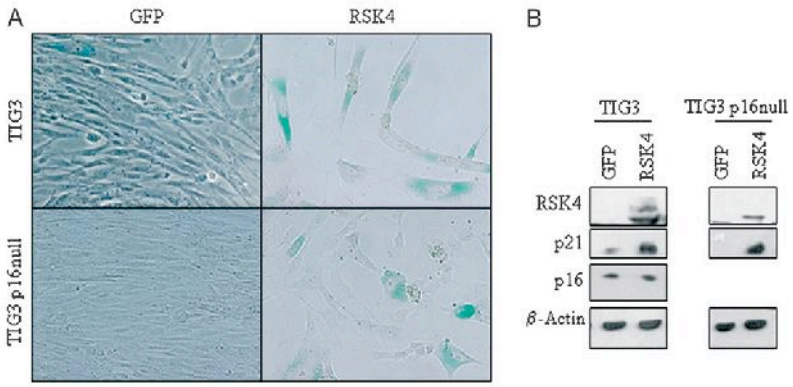

Fig. 1. RSK4 induces senescence in TIG3 and TIG3 p16-null human diploid fibroblasts. (**A**) TIG3 and TIG3 p16-null cells were infected with retrovirus encoding green fluorescence protein or RSK4. After selection, cells were seeded at the same density, and cell morphology and SA-β-gal activity were analyzed 6 days later. Images are all at the same magnification (×20). (**B**) Four days after selection, whole cell lysate from each cell line was assessed by immunoblotting for levels of RSK4, p21 and p16. Equal loading was verified by β-actin.

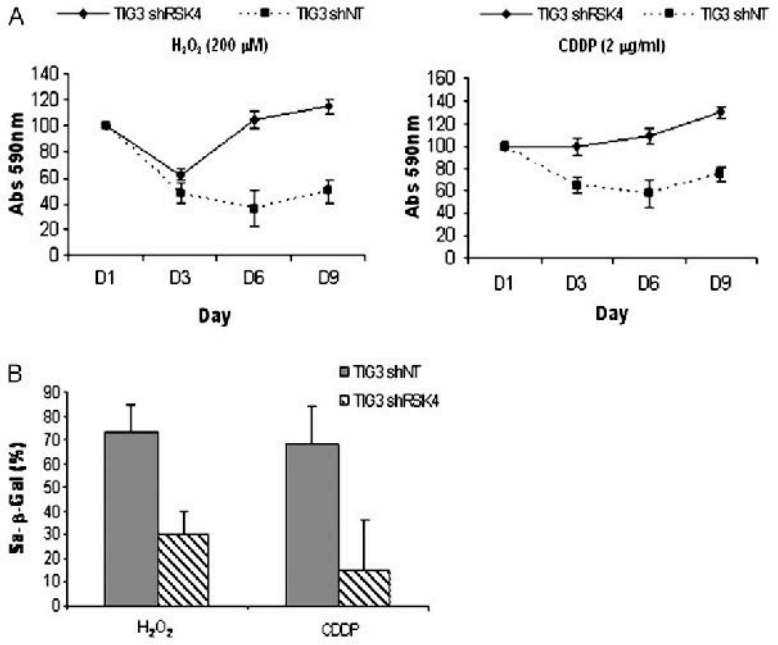

Fig. 2. RSK4 inhibition prevents stress-induced senescence. (**A**) H_2O_2 and CDDP resistance curves of TIG3-expressing pLKO.1-shNT (TIG3 shNT) or pLKO.1-shRSK4 (TIG3 shRSK4). After treatments, cells were maintained in culture with fresh medium. At day 3, 6 and 9 after treatments, cells were stained with crystal violet and the relative cell number was determined reading the optical density at 590 nm. A value of 100% was assigned to cells stained just after treatment. (**B**) Analysis of SA-β-gal activity in TIG3 shNT or TIG3 shRSK4 cells 9 days after H_2O_2 or CDDP treatment.

cells that had been infected with pLKO.1 shNT vector. In our cell models, KRAS^{V12} only induced senescence in TIG3, and not in cells lacking p16 (TIG3 p16-null) (supplementary Figure 2 is available at *Carcinogenesis* Online). This effect has been described previously in other fibroblasts, like Leiden or Q34 fibroblast (26,27).

The growth curve analysis on the TIG3 cell line showed that KRAS^{V12}-induced senescence was bypassed when RSK4 was inhibited (Figure 3A). Fourteen days after selection, KRAS, p16 and p21 expression was analyzed by western blot (Figure 3B). We observed that KRAS induced an increment of p21 expression that was not produced in cells with RSK4 inhibition, which were still proliferating in spite of KRAS^{V12} overexpression. This observation suggests that KRAS^{V12}-induced senescence was bypassed in cells with RSK4 inhibition and that this fact could be due to the lack of p21 expression when RSK4 is inhibited.

BRAF is a downstream RAS effector that is also commonly mutated in human tumors. To test whether RSK4 inhibition could also override BRAF-induced senescence, we overexpressed BRAF^{E600} in TIG3 and TIG3 p16-null cells that had been previously infected and selected with lentiviral vectors coding for shNT and shRSK4. First, RSK4 expression was measured by reverse transcription–polymerase chain reaction in these cells (supplementary Figure 3 is available at

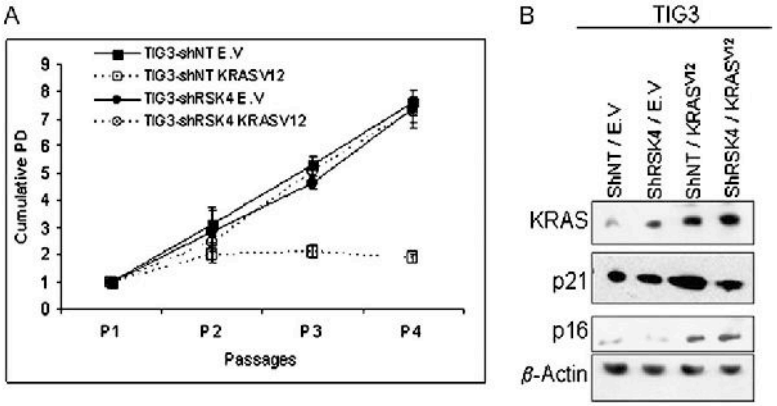

Fig. 3. RSK4 inhibition overrides KRAS-induced senescence. (**A**) TIG3 cells were infected with lentivirus encoding pLKO.1-shRSK4 or pLKO.1-shNT vectors. After selection, cells were reinfected with control vector (empty vector or E.V) or pBabe-KRAS^{V12} vector (KRAS^{V12}). Two days after selection, cells were counted and seeded every 4 days in duplicate and PD were calculated. (**B**) Western blot analysis of the indicated proteins. Equal loading was verified by β-actin.

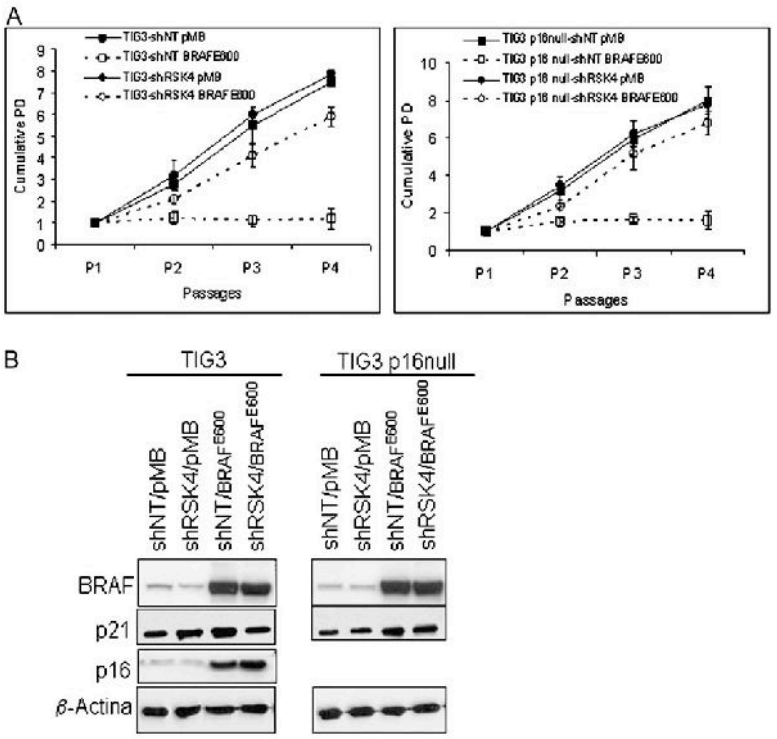

Fig. 4. RSK4 inhibition overrides BRAF-induced senescence. (**A**) TIG3 and TIG3 p16-null cells were infected with lentivirus encoding pLKO.1-shRSK4 or pLKO.1-shNT vector. After selection, cells were reinfected with control vector (pMB vector) or pMB-BRAF^{E600} vector. Two days after selection, cells were counted and seeded every 4 days in duplicate and PDs were calculated. (**B**) Western blot analysis of the indicated proteins. Equal loading was verified by β-actin.

Carcinogenesis Online). Although BRAF is an effector of RAS, $BRAF^{E500}$ can induce senescence in p16-null cells (4). Two days after $BRAF^{E600}$ selection, we seeded cells at low density and performed growth curves. RSK4 inhibition partially rescued $BRAF^{E500}$-induced senescence in both TIG3 and TIG3 p16-null (Figure 4A). BRAF, p16 and p21 expression were analyzed by western blot (Figure 4B). We observed that p16 was induced by BRAF independently of RSK4, nevertheless, we did not observe a clear increment of p21 expression induced by BRAF. These observations indicate that $BRAF^{E600}$-induced senescence was bypassed in cells with RSK4 inhibition despite p16 increase.2

RSK4-induced senescence is independent of p38MAPK

Some oncogenes induce senescence by a buildup of DNA damage caused by oncogene driven accumulation of ROS. ROS can trigger senescence via a kinase cascade involving p38MAPK, and it has been described that oncogenic RAS induced senescence in normal fibroblasts is mediated by activation of the RAF/MEK/ERK and p38 MAPK pathways (15). To test whether RSK4 induced senescence is due to an accumulation of ROS or is mediated by p38MAPK, we overexpressed RSK4 in TIG3. At day 0 postselection, we seeded cells at low density and treated them with a control solvent (dimethyl sulfoxide), with the p38 MAPK inhibitor SB203580, or with the ROS scavenger *N*-acetyl-L-cysteine (NAC). After 14 days, colony formation assays showed that neither SB203580 nor NAC were able to inhibit RSK4 induced senescence (Figure 5A). RSK4 and p21 expression were analyzed by western blot (Figure 5B).

p21 inhibition overrided RSK4-induced senescence

Several reports have shown that RSK4 overexpression induces p21 expression and that RSK4 inhibition prevents p21 accumulation (16,19,28). In our previous study, we found that RSK4 induced senescence is dependent on Rb expression (16). Rb is mainly activated by p16. Nevertheless, RSK4 induced senescence even in TIG3 p16 null cells (Figure 1). P21 is a CDK inhibitor that can also activate Rb; hence, to determine whether RSK4 induced senescence is mediated by p21, we infected TIG3 and TIG3 p16-null cells with a retroviral vector coding for shRNA against p21 or an empty vector. Once cells were infected and selected, we reinfected them with a control vector or a vector coding for RSK4. Two days after selection, we seeded cells at low density, and 14 days later, we analyzed colony formation (Figure 6A) and RSK4 and p21 expression by western blot (Figure 6B). In both cell types, p21 inhibition partially prevented RSK4-induced senescence. Moreover, p21 band intensities were quantified by densitometry and normalized to the intensity of β actin in each sample (Figure 6C).

Discussion

The last member of the p90RSK family discovered was RSK4 (29). Since its discovery, RSK4 has been described as a component of the p53 pathway (28) and an inhibitor of the RAS–MAPK pathway (18). Two recent reports have highlighted the importance of RSK4 in tumorigenesis, with descriptions of its contribution to regulating cell senescence and invasion (16,19).

RSK4 is an unusual RSK in that it has very low expression levels and appears to be fully phosphorylated and activated in unstimulated cells (30). In humans, RSK4 is downregulated in some tumors, such as colon and renal carcinomas, as well as is some benign lesions, such as colon adenomas and benign breast papillomas (16,31). RSK4 overexpression has been related to senescence in human fibroblasts and in colon carcinoma cells with *CDKN2A* mutation and no p53 expression (16). The aim of this study was to elucidate the role of RSK4 in regulating senescence by analysing the effect of its inhibition *in vitro*. To achieve this objective, we used a human diploid fibroblast cell line, TIG3, immortalized with the catalytic subunit of telomerase (hTERT). hTERT lengthens the life of fibroblasts without affecting the signals that activate senescence in response to non-telomeric stress stimuli (4,20,21). As was previously seen in normal IMR90 human fibroblasts, we found that RSK4 also induced senescence in our fibroblast model. As was expected, RSK4 overexpression induced SA-β-gal activity, stopped proliferation and increased p21 expression. This phenotype was also observed in TIG3 p16 null cells, supporting the concept that RSK4 induced senescence is p53 (16) and p16 independent.

In the last years, mouse models have yielded evidence that cell senescence has a tumor suppressor role. Genetic deletion of senescence regulatory genes, such as *Cdkn2a* and *Tp53*, is related with progression to malignant stages (6,25,32). To investigate whether RSK4 inhibition could also be related with malignancy, we generated two cell models without RSK4 expression using shRNA technology. It is known that a wide variety of anticancer agents induce senescence-like morphological changes and SA-β-gal expression even in tumor cells (22). H_2O_2 and CDDP subcytotoxic treatment can induce senescence by accumulation of ROS and induction of DNA damage, respectively (22). To test whether RSK4 is an important factor mediating this response, we treated cells expressing shRNA against RSK4 with H_2O_2 and CDDP and observed that RSK4 inhibition conferred resistance to both stress induced senescence treatments. This finding indicates that tumors with low RSK4 expression may be more resistant to anticancer drugs, although further investigation is needed to confirm this hypothesis.

Once, we saw that RSK4 inhibition conferred a proliferative advantage, delaying senescence induction, we wondered whether if it could also confer resistance to oncogene induced senescence. Since its first description over a decade ago, several reports have now demonstrated that oncogene-induced senescence functions to suppress tumor development by preventing progression of benign lesions in the absence of additional co-operating mutations (33,34). The finding that RSK4 inhibition also conferred resistance to $KRAS^{V12}$ and $BRAF^{E600}$-induced senescence, indicates that this little known gene may also play an important role in oncogene induced senescence. This observation, together with clinical data, which has shown that RSK4 is downregulated in premalignant lesions, could indicate that RSK4 inhibition is a

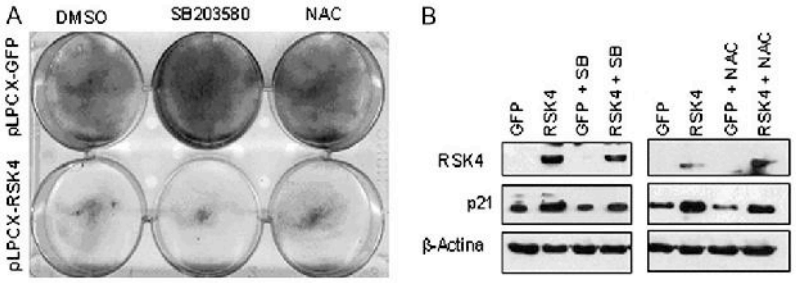

Fig. 5. RSK4-induced senescence is not mediated by p38MAPK. (**A**). Colony formation assay of TIG3 cells transduced with pLPCX-green fluorescence protein or pLPCX-RSK4 and treated with control solvent (dimethyl sulfoxide), p38 inhibitor SB203580 (8 uM) or NAC (1 mM). Cells were seeded on day 0 postselection in the presence of drugs and were provided with fresh medium containing drug every day for 2 weeks before they were fixed and stained. (**B**) Western blot analysis of the indicated proteins. Equal loading was verified by β-actin.

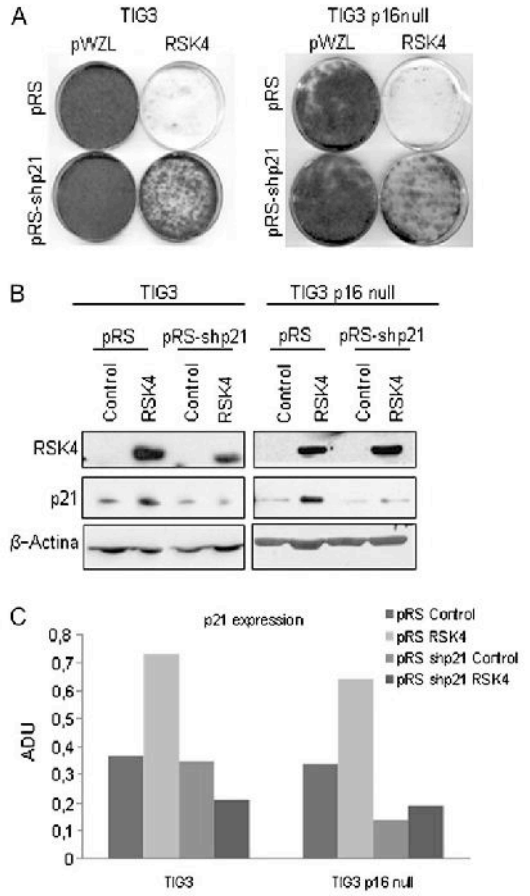

Fig. 6. RSK4-induced senescence is mediated by p21. (**A**) Colony formation assay of TIG3 and TIG3 p16-null cells transduced with an empty vector (pRS) or a vector containing shRNA against p21 (pRS-shp21). Once selected, cells were reinfected with a second empty vector (pWZL) or pWZL-RSK4 vector. Two days after selection, cells were seeded at low density and maintained in culture for 2 weeks before they were fixed and stained. (**B**) Western blot analysis of the indicated proteins. Equal loading was verified by β-actin. (**C**) For the p21 band intensities, proteins were quantified by densitometry, normalized to the intensity of β-actin in each sample and expressed in arbitrary densitometric units.

prerequisite that allows cells to proliferate after oncogenic alterations occur, such as *KRAS* and *BRAF* mutations.

Despite the advances in our understanding of senescence, there is no unified model that integrates all the current findings. DNA damage, replicative stress, ROS, heterochromatin formation and negative feedback signaling have been proposed as factors that promote senescence in response to oncogene mutations (33). RAS-induced senescence can be activated by an accumulation of ROS and through the p38 MAPK pathway (33). Treatment with antioxidant agents or the p38 MAPK inhibitor SB203580 can prevent RAS-induced senescence (12,35). Since RSK4 inhibition also prevents RAS-induced senescence, we wondered whether RSK4 induced senescence is mediated by ROS generation and the p38 MAPK pathway. To test this hypothesis, we overexpressed RSK4 in the presence of the ROS scavenger NAC or the p38 MAPK inhibitor SB203580 and saw no effect, indicating that RSK4-induced senescence was independent of ROS induction and the p38 MAPK pathway.

Although several mechanisms can regulate senescence, in all cases, the signals function through Rb and p53, using different intermediates. RSK4 was first described as a mediator of p53-dependent cell cycle arrest (28). However, RSK4 can induce senescence even in the absence of p53, and RSK4 expression increases even in p53 null cell lines after different stress stimuli, indicating that RSK4 can also function in a p53-independent way (16). Previous findings indicated that RSK4 regulates senescence mainly through the classical p16/Rb pathway (16), but p16 inhibition did not prevent RSK4 senescence induction in this study, which suggests that RSK4 does not regulate Rb through this pathway. It has been proposed that RSK4 could regulate p21 expression, even at the messenger RNA level, although it is not known if RSK4 regulates p21 at the transcriptional level or if it stabilizes p21 messenger RNA (28). In this study, we found that RSK4-induced senescence is dependent on p21 expression, since inhibition of p21 allowed proliferation in cells with RSK4 overexpression. Since p21 is a major CDK inhibitor that can also activate Rb, we propose that RSK4 takes part in senescence by regulating p21 in a p53-independent manner, and that p21 induces senescence through Rb hypophosphorylation. The existence of signaling pathways that activate senescence independently of p53 and p16 would be a double barrier to cellular transformation. In our cell model of $KRAS^{V12}$-induced senescence, we observed that p21 was not overexpressed in response to $KRAS^{V12}$ in cells lacking RSK4, indicating that p21 expression could be dependent of RSK4. Nevertheless, p21 inhibition did not suffice to completely prevent the RSK4 induced phenotype in TIG3. Moreover, p21 is not considered a major regulator of oncogene induced senescence, and although it is overexpressed in human fibroblasts in response to RAS (2,36,37), p21 inhibition does not prevent H RAS induced senescence (37). In the case of BRAF, p21 is not upregulated after BRAF overexpression in different cell models (4,38,39). Since RSK4 inhibition sufficed to partly prevent $BRAF^{E600}$ induced senescence, it is possible that RSK4 could regulate factors other than p21 that participate in senescence. BRAF overexpression upregulates IGFBP7 synthesis and secretion, which regulates senescence in a feedback negative way, inhibiting the BRAF/MEK/ERK pathway (40). A similar negative feedback mechanism has been proposed to occur in RAS-induced neurofibromas (5). In the present study, we investigated the relationship between RSK4 and p21 and observed that p21 is necessary for RSK4-induced senescence, even though RSK4 has been described as an inhibitor of the RAS–MAPK pathway (18); thus, this role of RSK4 could also be important for oncogene-induced senescence regulation.

In this study, the effect of RSK4 inhibition *in vitro* is reported. Although the exact mechanisms behind the action of RSK4 remain unknown, RSK4 seems to be an important regulator of senescence, mainly through p21, and its downregulation may confer a proliferative advantage. Further study of RSK4 is needed; deeper understanding of the RSK4 pathway could allow its manipulation in the design new therapeutic strategies for cancer.

Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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ErbB3 expression predicts sensitivity to elisidepsin treatment: *In vitro* synergism with cisplatin, paclitaxel and gemcitabine in lung, breast and colon cancer cell lines

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Abstract. Irvalec® (elisidepsin trifluoroacetate, PM02734) is a novel marine-derived cyclic peptide belonging to the Kahaladide family of compounds, currently in clinical trials with preliminary evidence of antitumor activity. Previous studies have shown a correlation between elisidepsin sensitivity and expression of the ErbB3 receptor in a panel of NSCLC cell lines. We have studied the effect of elisidepsin on the ErbB3 pathway, characterizing the expression of all members of the ErbB (HER) family of receptors and their main downstream signaling effectors, such as Akt and MAPK. Interestingly, we observed a downregulation of ErbB3 upon elisidepsin treatment that correlates with a reduction in the Akt phosphorylation levels in the most sensitive cell lines, whereas ErbB3 levels are not affected in the less sensitive ones. Also, we observed that the basal levels of ErbB3 protein expression show a significant correlation with cell viability response against elisidepsin treatment in 14 different cell lines. Furthermore, we analyzed the combination of elisidepsin with different chemotherapeutics agents, such as cisplatin, paclitaxel and gemcitabine, in a panel of different breast (MDA-MB-435, MDA-MB-231 and MCF7), lung (HOP62, DV90 and A549) and colorectal cancer cell lines (DLD1 and HT29). IC_{50} values for the different drugs were tested. We observed a synergistic effect in all cell lines tested with any chemotherapeutic agent. More importantly, the two *in vitro* elisidepsin-resistant cell lines (MDA-MB-231 and HOP62) presented a synergistic effect in combination with cisplatin and paclitaxel, respectively. These results provide a rationale for further development of these combinations in an ongoing clinical trial.

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Introduction

Nowadays, the prognosis of most advanced carcinomas remains poorly understood and the search of new drugs is crucial. Recent clinical data obtained with new EGFR inhibitors in lung tumors harboring EGFR mutations are promising but the complex genetic background of most of these tumors and the redundancy of genetic drivers indicates that combination with other antitumor agents is needed. In this regard, the search of chemical compounds obtained from marine organisms could be an interesting new approach. Several compounds were found in some previous screening with a high antitumor activity such as Yondelis® in sarcomas and more recently in ovarian cancer (1).

Irvalec® (elisidepsin trifluoroacetate, PM02734), a novel synthetic marine-derived antitumor agent belonging to the Kahalalide family of peptides originally isolated from the Hawaiian marine mollusk Elysia rufescens (2), is currently undergoing phase II clinical trials.

Elisidepsin has shown a potent *in vitro* cytotoxic activity toward several epithelial cell lines (3) and this activity is markedly higher in tumorigenic vs. normal cell lines (4,5). In addition, elisidepsin shows statistically significant *in vivo* antitumor activity in several human cancer cell lines xenografted into mice. Based on these observations, and in view of its acceptable low clinical toxicity profile, elisidepsin has been selected for clinical development (6). In phase I trials Irvalec was shown to be safe, well tolerated and with evidence of activity in patients with solid tumors (7-9).

In vitro treatment of tumor cells with elisidepsin induces necrotic cell death by inducing rapid and severe membrane damage, a process that appears to involve 2-hydroxy fatty acids located at the cell membrane (10).

Interestingly, previous *in vitro* studies with the natural parent compound Kahalalide F (KF) have shown that KF inhibits the tyrosine kinase activity of HER2/neu, blocks EGFR and inhibits the expression of TGF-α (11). The sensitivity to KF in a panel of tumor cell lines, including non-small

cell lung cancer (NSCLC), breast, ovarian and hepatic carcinomas was found to correlate with ErbB3 protein expression levels, whereas no correlation was observed with the expression levels of the other family members (12). KF induces a downregulation of this receptor, subsequently reducing the levels of phospho-Akt, resulting in a significant reduction of the cellular survival rate.

In vitro and *in vivo* synergism has been described when combining elisidepsin with a specific small tyrosine kinase inhibitor of EGFR (erlotinib) in NSCLC (13). Because human carcinomas harbor multiple genetic alterations, the combination of different antitumor agents is essential in most cases. Classical antitumor agents such cisplatin (CDDP), gemcitabine and paclitaxel (TAX) are frequently used in carcinomas with unpredictable results. The combination with synergistic drugs could be a relevant approach to increase the rate of tumor responses.

CDDP is a typical DNA-damaging agent with the ability to induce crosslinking between inter and intra DNA strands (14). CDDP is also known for its ability to induce apoptosis, although other mechanisms have been reported to induce cell death (15). CDDP therapy is used in several types of tumors such as NSCLC, testicular and ovarian cancer. TAX belongs to a family of drugs that, through the blocking of microtubule formation, interfere with the normal progression of the cell cycle. The drug has therefore been successfully used in the treatment of breast, lung, melanoma and ovary tumors (16,17). Gemcitabine is an anticancer nucleoside that is an analog of deoxycytidine. This compound has been widely used in pancreatic and metastatic breast cancer. These chemotherapeutic agents have shown a limited effect, and in recent years, a huge number of combined studies with other compounds have been proposed (18-20).

In this study we sought to characterize the cytotoxic effect of elisidepsin in a panel of human lung, breast and colon carcinoma cell lines, and subsequently a combination of elisidepsin with CDDP, TAX and gemcitabine. Here we show that elisidepsin exposure induced downregulation of ErbB3 protein expression, thus inhibiting the PI3K-Akt signaling pathway in most cell lines, but only partially affecting the MAPK pathway, indicating that the drug severely affects the ErbB3 signaling pathway. We provide further evidence that *in vitro* sensitivity to elisidepsin correlates with ErbB3 protein expression. Cell lines with high levels of ErbB3 receptor were found to be the most sensitive to elisidepsin.

Finally, the combination of elisidepsin with CDDP, TAX and gemcitabine showed a synergistic effect in almost all cell lines tested, regardless of their genetic background or their sensitivity to each drug alone. Our observations suggest a clinical use of elisidepsin, and its combination with CDDP, TAX or gemcitabine, may improve the efficiency in the chemotherapy currently used in different types of cancer.

Materials and methods

Chemicals. Elisidepsin was obtained from PharmaMar (Madrid, Spain) as a dry powder to be reconstituted with dimethyl sulfoxide (DMSO, Sigma Chemical Corp.)/ethanol (1:1) as a 1 mM stock solution, and kept in aliquots at -20˚C. CDDP, TAX or gemcitabine were obtained from the Vall d'Hebron University Hospital (Barcelona, Spain). Drug dilutions were freshly prepared before each experiment.

Cells and cell culture. Cell lines were obtained from the American Type Culture Collection, except for the DV90 cell line, purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. The HCT116 $p53^{-/-}$ was kindly provided by Dr Francisco Real (Centro Nacional de Investigaciones Oncológicas, CNIO, Madrid). The following cell lines were maintained in RPMI-1640 with 4 mM L-glutamine: DV90, HOP62 (lung carcinoma), MDA-MB-231, MCF-7, SKBR3, MDA-MB-435 (breast carcinoma) and AsPC-1, BxPC-3 (pancreas carcinoma). The following were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine and 4.5 g/l of glucose: DLD1, HT29, HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$ (colon carcinoma), MDA-MB-468 (breast carcinoma), PANC-1, MIAPaCa-2 (pancreas carcinoma) and the human embryonic kidney 293T (HEK 293T) cancer cell line. A549 (lung carcinoma) was maintained in Ham's F-12 medium supplemented with 1 mM L-glutamine. Finally, DMEM:Ham's F12 (1:1 mixture) supplemented with 1 mM L-glutamine was used to maintain BT-474 (breast carcinoma). All cell lines were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES and were cultured in a 37˚C humidified atmosphere containing 95% air and 5% CO_2.

Cell growth assay. Cells were plated overnight at a density of 50,000 cells/well in 24-well plates in 1 ml of medium. At least 3 wells were used for each condition. Cell lines were treated with various concentrations of elisidepsin, CDDP, TAX or gemcitabine for 72 h as single agents. Cell viability was measured by a crystal violet assay. Briefly, after each treatment, cells were fixed in 1% glutaraldehyde for 20 min, washed twice in PBS 1X, stained with 0.1% crystal violet for 30 min and then washed with abundant deionized water. Colorant was recovered with 5% acetic acid and optical density was measured at 590 nm using an ELISA plate reader.

Analysis of combined drug effects. Cells were plated in 24-well plates as described above. After overnight incubation at 37˚C, the attached cells were treated for 72 h at a fixed ratio of doses that corresponded to 0.125, 0.25, 0.5, 1 and 2 times the individual IC_{50} values of elisidepsin in combination with CDDP, TAX and gemcitabine. Cell survival fractions were determined by crystal violet assay and the combination indexes (CI) were analyzed by the median effect method of Chou and Talalay by using CalcuSyn software (version 2.1, Biosoft, Cambridge, UK) (21). CI<1, CI=1, CI>1 indicate synergism, additive effect and antagonism, respectively. The study was repeated three independent times and representative data are shown.

Western blot analysis. Just prior to use, cultured cells for western blotting were scraped in lysis buffer containing 20 mM Tris HCl (pH 8.0), 137 mM NaCl, 2 mM EDTA, 10% glicerol, 1% NP-40, 0.1 M sodium pyrophosphate, 20 mM β-glycerophosphate, 1 mM DTT, 20 mM NaF, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 1 μg/ml aprotinin. Lysates were centrifuged and supernatants were collected for protein concentration determination by

the Bradford protein assay reagent (Bio-Rad) method. Equal amounts of protein were separated by 8% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) gels, electrophoresed at 100 V and electroblotted onto polyvinylidene difluoride membranes (Millipore) at 0.4 A at room temperature. Blots were blocked in 5% dried milk solution for 1 h at room temperature and probed overnight with antibodies. After blocking, membranes were probed with primary antibodies against ErbB1 (F4, Sigma), ErbB2 (CB11, BioGenex), ErbB3 (2F12, NeoMarkers), p-ErbB3 (21D3), ErbB4 (111B2), p-Akt (587F11), Akt (#9272), p-MAPK (#9101), β-actin (#A5060) (Cell Signaling) and MAPK (C-14, Santa Cruz Biotechnology), upon treatment with 1 μM elisidepsin for 4 h. After incubation with horseradish peroxidase-conjugated secondary antibodies, antigen-antibody complexes were visualized using enhanced chemiluminescence (Amersham Biosciences). Western blot analyses were repeated in independent conditions at least twice; representative blots are shown.

Immunoprecipitation. Cells were treated and collected in lysis buffer. Extracts were precleared and the soluble fraction was incubated with anti-ErbB3 (1 mg/sample) overnight at 4˚C. The following day, extracts were incubated for 45 min in the presence of protein G Sepharose 4 Fast Flow (17-0618-01, Amersham Pharma-Biotech), centrifuged at 12,000 x g for 20 sec and then washed three times with 1 ml in the same lysis buffer. The pellet was suspended in 30 μl sample reducing buffer (1% SDS, 100 mM DTT, 50 mM Tris, pH 7.5). Then immunocomplexes were resuspended in loading buffer and loaded onto 8% SDS-PAGE gels. Quantification of autoradiograms was performed by using Image J software (version 1.41o, National Institutes of Health, Bethesda, MD), normalized to the intensity of β-actin in each sample, and expressed in arbitrary densitometric units.

Results

Elisidepsin downregulates ErbB3 protein level. Previous studies have reported a selective downregulation of ErbB3 after cell exposure to the natural compound KF in a cell line expressing high levels of this receptor SKBR3. We sought to determine if elisidepsin treatment could similarly affect the expression of other ErbB receptors. To this end, we did a time course of 2, 4 and 6 h treatment with 1 μM of elisidepsin, and western blot analysis to seek if the treatment induces the downregulation of the ErbB family proteins in MCF-7 breast cancer cell line, which expresses moderate ErbB3 protein levels.

Protein expression levels of all members of the ErbB family were analyzed and all were found to be downregulated after 6 h of treatment with elisidepsin (Fig. 1). Unlike other ErbB receptors, the downregulation of the expression of ErbB3 protein level was seen as early as 2 h. Interestingly, in the case of the ErbB4 protein levels, we observed an initial upregulation at 2 and 4 h post-treatment. This result clearly indicates that ErbB3 is the ErbB receptor most sensitive to treatment with elisidepsin in MCF-7 cells.

ErbB3, p-Akt and p-MAPK are downregulated upon elisidepsin treatment. Since downregulation of ErbB protein expression was observed in MCF-7 cells upon elisidepsin treatment, we sought to determine if this compound also affected ErbB3 expression in a panel of human tumor cell lines with variable expression levels of this receptor, namely lung (A549, DV90 and HOP62), breast (MDA-MB-435, MDA-MB-231 and MCF-7) and colon (DLD1 and HT29) cancer cell lines.

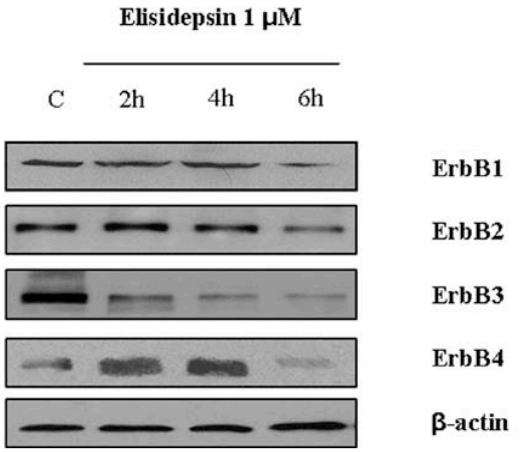

Figure 1. Downregulation of the expression of ErbB family proteins upon elisidepsin treatment. MCF-7 cells were seeded at 70% confluence, and after 24 h cells were treated at 2, 4 and 6 h with 1 μM elisidepsin. After treatment, cells were lysed and proteins were extracted and quantified by Bradford assay. Protein (50 μg) was loaded in SDS-PAGE gels. The four ErbB receptors were detected by western blot assays using corresponding antibodies. The membranes were stripped and reprobed with anti-β-actin to verify equal protein loading.

Cells were treated with 1 μM elisidepsin for 4 h and then lysed. ErbB3 receptor levels were downregulated in the majority of cell lines analyzed after treatment with 1 μM elisidepsin. Only two (MDA-MB-231 and HOP-62) out of eight cell lines tested maintained their ErbB3 expression levels after elisidepsin treatment.

Those differences in ErbB3 receptor levels in the analyzed cell lines prompted us to study the downstream signaling routes that link this receptor to proliferative responses, Akt and MAPK. Western blot analysis with antibodies that recognized activated forms of Akt indicated that the resting levels of p-Akt were lower or had disappeared more notably in the cells that had a downregulation of ErbB3 after elisidepsin treatment than in the cells that had not (Fig. 2A).

We also investigated the phosphorylation of MAPK. In contrast to p-Akt results, p-MAPK levels were not downregulated in most of the cell lines. MAPK phosphorylation was upregulated in HOP62 and MDA-MB-435 cell lines, and downregulated in A549 and DV90 lung cancer cell lines, MCF-7 breast cancer cell line and DLD1 colon cancer cell line. MDA-MB-231 and HT29 cell lines maintained the same amount of p-MAPK protein after treatment with the drug.

In contrast, total amounts of Akt and MAPK are not affected by elisidepsin, except for the DLD1 colon cancer cell line that exhibited a decrease in Akt protein expression levels. We also analyzed β-actin state to verify an equal amount of protein in each well.

These results indicate that elisidepsin affected ErbB3 protein levels, the downstream pathway PI3K-Akt, and in

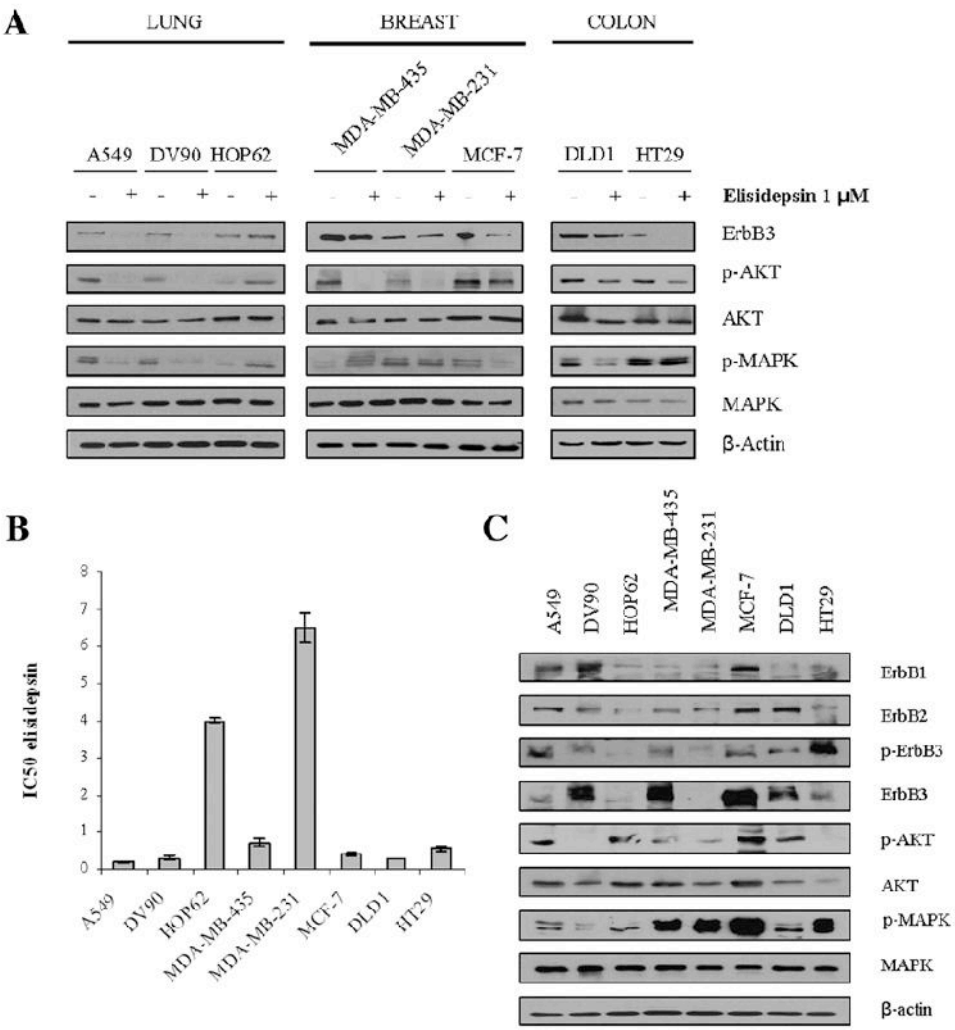

Figure 2. Effect of elisidepsin treatment on ErbB signaling pathways. (A) All cell lines were seeded at 70% of confluence in 100-mm cell culture dishes, and 18 h later, were treated with 1 μM of elisidepsin for 4 h. After treatment, cells were lysed, proteins were extracted and western blot analysis performed with 50 μg of protein, for each sample. In the control samples, the same amount of the dissolved product (DMSO/ethanol) without elisidepsin was added. Membranes were stripped and reprobed with anti-β-actin and these were used as an internal control. (B) Representation of cell viability upon 72-h treatment with different concentrations of elisidepsin in a panel of 8 different cell lines. Bars indicate IC_{50} values (±SD) for 3 independent experiments per cell line. (C) A total amount of 50 μg of protein extracts from 8 different cell lines were loaded in SDS-PAGE gels and western blot analyses were performed against different antibodies regarding HER family, also p-Akt and p-MEAK activation. β-actin was used as an internal control to verify equal protein loading.

some cases also the MAPK route in cell lines derived from different human tumor types.

ErbB3 expression levels correlate with elisidepsin cell sensitivity. We performed cell viability assays in a panel of cell lines to analyze if there was a correlation between the down-regulation of ErbB3 protein expression and cell sensitivity to elisidepsin. Cells were treated with increasing concentrations of the compound for 72 h. IC_{50} values for elisidepsin, as measured by crystal violet assays using a spectrophotometer, ranged from 0.2 to 6.5 μM within the panel of cell lines (Fig. 2B).

The cells that have an IC_{50} value <1 μM were considered sensitive, while the rest were considered less sensitive to the drug. HOP62 and MDA-MB-231 cell lines were the only cell lines that had an IC_{50} value >1 μM (4 and 6.5 μM, respectively). The other cell lines were catalogued as sensitive to the drug (IC_{50} value from 0.2 to 0.6 μM) and, as we described above, all of them presented downregulation of the ErbB3 receptor. We did not observe any relationship between HER1 and HER2 expression levels and elisidepsin sensitivity (Fig. 2C).

To analyze if there is a correlation between ErbB3 expression levels and elisidepsin sensitivity we performed cell viability assays in a larger panel of cell lines. We chose 14 human cell lines from different types of cancer (comprising pancreas, breast, lung, colon and kidney cell lines). IC_{50} values for elisidepsin ranged from 0.075 to 14 μM within the panel of cell lines (Fig. 3A).

In order to evaluate the ErbB3 protein expression levels and correlate them with the sensitivity of the cell lines to

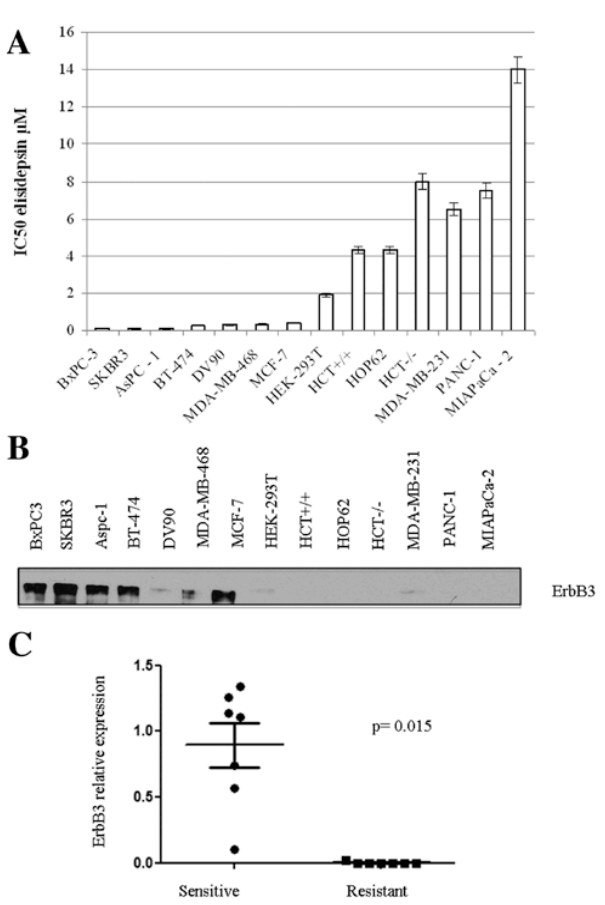

Figure 3. ErbB3 expression correlates with elisidepsin cell sensitivity. (A) Cell viability assay upon 72-h treatment with different concentrations of elisidepsin in 14 human cell lines. Bars indicate IC_{50} values (±SD) for 3 independent experiments per cell line. (B) Total ErbB3 protein expression in different cell extracts was detected by western blot analysis after immunoprecipitation with 1 µg of ErbB3 antibody. (C) Levels of ErbB3 protein were quantified from western blot analysis (data not shown) by densitometry. The graph represents the ErbB3 relative expression in cell lines, classified as more sensitive (IC_{50} <1 µM) and less sensitive (IC_{50} >1 µM) to elisidepsin. We obtained a statistical significance p=0.015 with the Wilcoxon analysis test.

elisidepsin, we performed different analysis based on western blot analysis (data not shown) and immunoprecipitation in 14 cell lines (Fig. 3B). Cell lines that were less sensitive to elisidepsin had lower or no ErbB3 in comparison with the sensitive cell lines which expressed higher levels, the results being statistically significant, p=0.015 in a Wilcoxon test (Fig. 3C). In summary, we observed a marked correlation between ErbB3 protein expression and cell sensitivity to elisidepsin in a panel of 14 cell lines.

Combinational studies of elisidepsin with CDDP, TAX or gemcitabine. After seeing a correlation between the sensitivity and downregulation of ErbB3 protein after the treatment of elisidepsin we defined the sensitivity to the first panel of 8 cell lines studied performing cell viability assays with different compounds used routinely in conventional chemotherapeutic treatments, namely CDDP, TAX and gemcitabine. The conditions of these experiments were the same as the cell viability assays for elisidepsin. The values of the different compounds ranged from 2.64 to 24.75 µM of CDDP, from 7 to 40 mM of TAX and from 0.004 to 2 µM of gemcitabine, data not shown.

DLD1 cells were the most resistant to CDDP and the MDA-MB-435 cell line the most sensitive one. Regarding the TAX treatment also the DLD1 cell line together with HOP62 cell line were the more resistant to it and the A549 cells the most sensitive. For the gemcitabine treatment, DLD1 cell line was again the most resistant and the A549 cells the most sensitive. For all three compounds the DLD1 cell line was the most resistant, in contrast to the elisidepsin treatment, which was one of the most sensitive (0.3 µM). Next we wanted to determine the potential synergism of the combination of elisidepsin with other antitumor agents such as CDDP, TAX and gemcitabine, performing different combinational drugs assays.

The combination of elisidepsin and CDDP was synergistic in all cell lines except in the HT29 colon adenocarcinoma cell line (Table I). Several cell lines present synergistic effect with high doses such as DV90, HOP62, MCF-7, whereas others cell lines have the same effect a low doses of each drug such as MDA-MB-231 (Fig. 4). However, in the breast cancer cell line MDA-MB-435 we observed synergism at x0.25 and x2 times

Table I. *In vitro* combination of elisidepsin and cisplatin.

Cell line	Origin	CI (at 0.125xIC_{50})	CI (at 0.25xIC_{50})	CI (at 0.5xIC_{50})	CI (at 1xIC_{50})	CI (at 2xIC_{50})
A549	Lung	4.89	1.14	1.06	**0.75**	1.21
DV-90	Lung	1.32	1.03	1.4	**0.48**	**0.88**
HOP-62	Lung	38.88	2.12	3.64	**0.85**	**0.75**
MDA-MB-435	Breast	1.42	**0.72**	1.28	2.28	**0.66**
MDA-MB-231	Breast	**0.72**	0.6	**0.66**	**0.91**	1.6
MCF-7	Breast	1.82	1.39	1.32	3.45	**0.72**
DLD-1	Colon	**0.63**	**0.79**	1.1	**0.6**	**0.82**
HT-29	Colon	1.76	1.22	1.28	1.97	1.1

CI, combination indexes. CI <1, CI=1, CI >1 indicate synergism, additive effect and antagonism, respectively.

Table II. *In vitro* combination of elisidepsin and paclitaxel.

Cell line	Origin	CI (at 0.125xIC$_{50}$)	CI (at 0.25xIC$_{50}$)	CI (at 0.5xIC$_{50}$)	CI (at 1xIC$_{50}$)	CI (at 2xIC$_{50}$)
A549	Lung	**0.78**	**0.81**	0.93	1.35	1.65
DV-90	Lung	**0.73**	**0.85**	0.92	1.13	1.73
HOP-62	Lung	**0.41**	**0.39**	**0.63**	**0.86**	1.03
MDA-MB-435	Breast	**0.73**	**0.72**	1.35	2.36	0.78
MDA-MB-231	Breast	15.14	0.92	1.17	2.27	2.81
MCF-7	Breast	0.81	1.28	1.39	1.05	1.14
DLD-1	Colon	0.55	0.75	1.42	2.54	0.89
HT-29	Colon	0.94	1.11	1.33	0.93	1.18

CI, combination indexes. CI <1, CI=1, CI >1 indicate synergism, additive effect and antagonism, respectively.

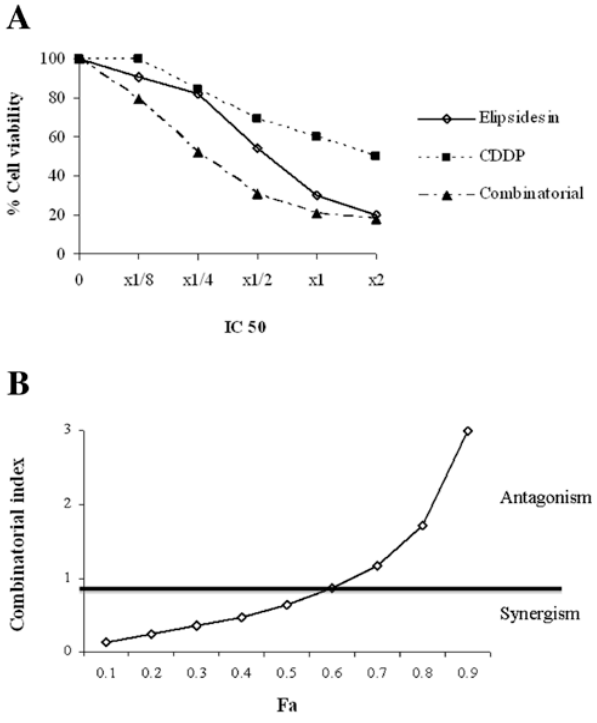

Figure 4. Combination of elisidepsin with CDDP, TAX and gemcitabine. (A) MDA-MB-231 cells were plated overnight at a density of 50,000 cells/well in 24-well plates in 1 ml of medium, and then treated for 72 h at a fixed ratio of doses that corresponded to 0.125, 0.25, 0.5, 1 and 2 times the individual IC$_{50}$ values for each drug alone. Cell viability was measured by a crystal violet assay for optical density using a spectrophotometer. (B) Isobologram representation of CI (combination index) vs. fractional effect analyzed by software CalcuSyn in MDA-MB-231 cancer cell line. CI <1 and CI >1 indicate synergism and antagonism, respectively.

the individual IC$_{50}$ values for the compounds. DLD1 cell line presented synergism with elisidepsin and CDDP at a broad range of doses of both drugs.

In contrast, the combination of elisidepsin and TAX showed synergism in all cell lines (Table II). Several cell lines, exhibit the same tendency of synergism with TAX and CDDP such as MDA-MB-435 and DLD1. Whereas other cell lines such as A549 and DV90 had synergism at low doses and also in the HOP62 cell line at a broad range of doses of the combinatorial drugs.

The combination results of elisidepsin and gemcitabine in the panel of different cell lines are presented in Table III. In contrast to the previous combinational assays this is the only one with a synergistic effect in all cell lines in at least one of the fixed ratio of doses. All lung carcinoma cell lines have a synergism with gemcitabine at low doses. Moreover, all breast carcinoma cell lines and colon cancer cell line DLD1 have a synergism in a broad range of concentrations, whereas for the other colon carcinoma cell lines, synergism was observed in HT29 only to have synergism in the highest doses tested.

Drug combination using each of these three drugs with elisidepsin has shown more efficacy than the monotherapy alone. Interestingly, the above combination was found to be synergistic even in cell lines less sensitive to elisidepsin, such as HOP62 and MDA-MB-231 cell lines.

Discussion

Elisidepsin is a novel marine compound with a potent cytotoxic activity in various tumor cell lines. In addition, identical results were obtained in several xenograft studies, supporting the use of this compound in different I and II clinical trials.

The mechanism of action of this compound remain poorly understood, although several targets have been proposed to be involved in the cellular response to elisidepsin treatment, such as fatty acid containing ceramides, FA2H, lysosomes, lipid rafts and epithelial growth factor receptors (10,12,22,23). Although elisidepsin could interact with the lipid bilayer, it is unlikely that it will form pores because the molecule is too small to span the whole length of the lipid bilayer. A minimum of 20 amino acids is required for this action (24) and elisidepsin contains only 14. Recently, ErbB receptors have been proposed as predictive markers of *in vitro* sensitivity to elisidepsin (12,13)

Regarding HER family receptors, in the present study we also observed an association between ErbB3 protein expression and sensitivity to elisidepsin treatment in a variety of cell lines. We observed a relatively rapid (2 and 4 h) specific downregulation of ErbB3 upon elisidepsin treatment in the

Table III. *In vitro* combination of elisidepsin and gemcitabine.

Cell line	Origin	CI (at 0.125xIC$_{50}$)	CI (at 0.25xIC$_{50}$)	CI (at 0.5xIC$_{50}$)	CI (at 1xIC$_{50}$)	CI (at 2xIC$_{50}$)
A549	Lung	**0.42**	**0.63**	**0.85**	1.20	1.15
DV-90	Lung	2.46	2.81	**0.62**	**0.81**	**0.92**
HOP-62	Lung	1.17	**0.80**	**0.68**	1.29	1.58
MDA-MB-435	Breast	**0.17**	**0.24**	**0.43**	**0.78**	1.17
MDA-MB-231	Breast	1.69	**0.60**	**0.64**	**0.94**	1.19
MCF-7	Breast	9.27	1.71	1.46	**0.89**	**0.76**
DLD-1	Colon	1.59	**0.57**	**0.62**	**0.90**	1.17
HT-29	Colon	5.06	1.60	1.32	1.12	**0.91**

CI, combination indexes. CI <1, CI=1, CI >1 indicate synergism, additive effect and antagonism, respectively.

breast cancer cell line MCF-7, whereas the other ErbB family members were not affected. These data, obtained in a breast cell line model, agree with previous results obtained in a lung cancer model (13), supporting the hypothesis of a selective role of ErbB3 in the cellular response to this drug, although other authors proposed this ErbB role as a secondary process upon cell membrane alterations by elisidepsin treatment (35).

The lower tyrosine kinase activity of ErbB3 receptor prompts heterodimerization with other HER receptors and ErbB2/ErbB3 heterodimers have been shown to be the most transforming and mitogenic receptor complex of the ErbB family (25-27). These observations suggest that cancer cell lines driven by a member of the ErbB receptor system often couple with ErbB3 to activate the PI3K/Akt pathway, consequently promoting the cancer phenotype (28,29). Akt is a major downstream target of receptor tyrosine kinases that signals via PI3K. Using a broad panel of cell lines including lung, breast, and colon carcinoma, we analyzed the most important pathways downstream of this heterodimer and evaluated the phosphorylation levels of Akt and MAPK in response to elisidepsin treatment. In these cell lines, we observed downregulation of ErbB3 in 6 out of 8 tested cell lines, confirming the previous data in MCF-7, this downregulation being associated with a decrease in the levels of serine 473 phosphorylation in Akt in the same set of cell lines. These results are in agreement with previous results obtained in different cell lines with KF (12), and due to this lower survival signaling, cell lines exhibit a cytotoxic response. Interestingly, the HOP62 cell line shows up-regulation of p-Akt upon elisidepsin treatment, and correlates with a less sensitive phenotype. Taking into account these data, the downregulation of ErbB3 and p-Akt levels could be a good predictive model to detect the *in vitro* response to elisidepsin.

In contrast, the levels of p-MAPK do not appear to predict cell viability response, because we did not observe significant differences after elisidepsin treatment in resistant (HOP62 and MDA-MB-231) and sensitive (MDA-MB-435 and HT29) cell lines. It is probable that other molecular alterations or crosstalk signaling pathways could activate p-MAPK indirectly, but this phosphorylation does not reflect *in vitro* elisidepsin sensitivity.

A variety of alterations could be involved in the less sensitive cell line MDA-MB-231, such as the low presence of other possible potential predictive markers of elisidepsin treatment like fatty acid synthase (FAS) (30) or the presence of signaling pathways independent of this drug such as EGFR or Src (31,32).

The mechanisms involved in the downregulation of ErbB3 have been explored previously with KF by other authors with inconclusive results (5,12,13) and therefore still remain unknown. Nevertheless we explored whether basal levels of ErbB3 protein could be a predictor maker to elisidepsin. Importantly we have observed that cell lines with low basal levels of ErbB3 receptor are less sensitive to elisidepsin, whereas in cells with high ErbB3 basal levels there is a correlation with elisidepsin sensitivity, supporting previous indications that ErbB3 could be a good predictive marker of elisidepsin sensitivity; further studies are necessary to confirm this observation in different *in vitro* and *in vivo* models.

In order to enhance the cytotoxic effect of elisidepsin and other anticancer drugs, we studied whether the combination of elisidepsin with other current chemotherapeutic agents could be effective in elisidepsin less sensitive cell lines. In this regard, we have tested the cellular effect of elisidepsin combined with CDDP, TAX and gemcitabine. The combination of elisidepsin with CDDP showed synergism in all tested cell lines except for the colorectal cell line HT29. In contrast, the combination of elisidepsin with TAX and gemcitabine showed synergism in all cell lines studied. In our panel of cell lines, the MDA-MB-231, MCF-7 and HT29 were the least synergistic with TAX and the HT29 the least synergistic with gemcitabine. HOP62 and MDA-MB-435 were the most synergistic in combination of elisidepsin with TAX and gemcitabine, respectively.

Based on these data, we conclude that the combination of elisidepsin with CDDP, TAX or gemcitabine could be an effective and viable therapeutic option to be evaluated in several *in vivo* studies and provide a rationale for further development of these combinational treatments in future clinical trials. The combination of elisidepsin with any of the chemotherapeutic agents shows a synergistic effect in different cell lines. The most probably rationale is that elisidepsin treatment could affect cells on the lipidic bilayer membrane, preferentially containing high levels of ErbB3 receptor, and this could enhance the activity of the different tested drugs (CDDP, TAX or gemcitabine). In this regard, tumors that harbor overexpression of ErbB3 could be good

candidates to perform this type of combinational studies, such as metastatic breast or lung tumors (33,34).

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New evaluation of HER3 expression in human breast carcinomas; association with estrogen receptor but not with progesterone receptor status.

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Short Running Title: Association of HER3 and ER expression in human breast carcinomas

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Abstract

Aims: HER3 is a member of the HER family of tyrosine kinase receptors. The role of HER signalling in breast cancer has focused primarily on HER1 and HER2, but it is becoming increasingly clear that HER3 also has an important role in the development of resistance to different anti-HER treatments, but its role in the breast cancer prognosis is currently unknown.

Methods and Results: We analyzed HER3 expression in 147 cases of breast carcinomas by Immunohistochemistry, of which 29% were by western blot, and 22% by quantitative real-time PCR. Two different patterns of HER3 expression were observed (membrane and cytoplasmatic) and were evaluated separately. Compared to normal breast tissue, 21% of the cases showed an overexpression. Cytoplasmatic expression of HER3 was correlated with low Ki-67 and with higher hormone receptors, while predominantly membrane expression correlated with estrogen receptor status and tumour size but not with progesterone receptor status and Ki-67 immunostaining. Interestingly, overexpression of HER3 in different tumour cell lines confirmed the relationship between HER3 and estrogen receptor expression but not with progesterone receptor expression.

Conclusions: Our results provide evidence that HER3 expression is associated with estrogen receptor status, and show two different patterns of expression suggesting a different role in breast cancer prognosis.

Introduction

Many types of human cancers are characterized by deregulation of the human epidermal growth factor receptor (HER) family of transmembrane tyrosine kinases (1, 2). The HER family includes four members: HER1, HER2, HER3 and HER4; characterized by their homology to the avian erythroblastosis virus transforming protein (v-erb-B) (3-6). In some cancers, overexpression and hyperactivity of individual HER family members are linked with the pathogenesis of these malignancies (7), which are consequently "addicted" to this pathway to sustain their proliferation and survival.

HER3 stands out among this family as the only member that has low catalytic activity (8). It is activated by binding to ligands Neuregulin-1 and Neuregulin-2 (9-11), and signal transduction pathways activation occurs through formation of dimers with any member of the HER family, although HER2 is the preferred dimerization partner (12-14). HER3, having six binding sites for the p85 SH2 adapter subunit of PI3K, is the major activator of PI3K among HER receptors (15). Upregulation of HER3 has been described to decrease the sensitivity to HER inhibitors (16-19) and HER3-derived downstream signaling has been linked to cancer etiology and progression (20).

Coexpression of both HER3 and HER2 is seen in many tumors, including breast cancer (21, 22). In this disease, the formation of HER2/HER3 heterodimers may be crucial for the aggressive phenotype of cancers with HER2 amplification, and may contribute to intrinsic and acquired resistance to therapy (23, 24). This partnership creates opportunities for improving efficacy of HER-targeted pharmaceuticals, by interfering with coupling of HER2 to HER3 through dimerization inhibitors, and by use of therapeutic compounds that target Akt-dependent pathways.

A classification of breast cancers has been defined using immunohistochemistry (IHC) to analyze patterns of protein expression in tumor sections. This pathological classification employs a limited number of protein biomarkers to classify breast cancers into luminal A, luminal B, HER2 enriched or triple negative. Luminal tumors are characterized by their expression of estrogen (ER) and progesterone receptors (PR), tagging these tumors for therapies that impede the action of those receptors. Genomic studies have complemented this pathologic classification by confirming the pathologic subtypes, and the addition of the claudin-low, and normal breast-like groups (25-29). Gene expression profiling revealed that within the ER positive-related tumors, two subtypes, luminal A and luminal B, could be distinguished that vary markedly in gene expression and prognosis (30). Conversely, hormone receptor–negative breast cancer comprised two distinct subtypes, the HER2 subtype and the basal-like subtype (30, 31). These subtypes differ in biology and behavior, and show a poor outcome.

Importantly, the role of HER3 in these molecular subtypes of breast cancer has not been defined. To assess this relevant growth factor receptor, we evaluated HER3 expression by IHC, quantitative RT-PCR and Western blot in primary breast carcinomas diagnosed between 2008 and 2009 at Vall d'Hebron University Hospital and compared it with the IHC profiles ER, PR, HER2, Ki-67 and p53, and with different clinical and pathological variables (histological type, grade, age, tumor size and lymph node status). In our series of 147 breast tumors we detected HER3 protein overexpression in 21% of cases. Interestingly, cytoplasmatic HER3 expression correlated with low cell proliferation and with higher hormone receptors while predominantly membrane HER3 cell expression correlated with ER and tumor size but not with PR and Ki-67.

Materials and methods

Patients

Firstly, breast tissue specimens with carcinoma from 147 patients diagnosed between 2008 and 2009 were obtained from the tumor bank at the Pathology Department of Vall d'Hebron University Hospital (Barcelona, Spain).

Secondly, in order to analyze the role and levels of HER3 in HER2 positive tumors, we studied another set of 67 breast carcinomas positive for HER2 (either FISH+ or herceptest 3+) obtained from the same source. All tumors were histologically examined to confirm the diagnosis of carcinoma, using light microscopy and conventional hematoxylin and eosin (H&E) stain. A portion of the biopsied samples was quickly frozen and stored at –80ºC immediately after surgery. The remainder of the tumor was fixed in neutral formalin and embedded in paraffin. The Ethics Committee of Vall d'Hebron University Hospital approved all the procedures used in the study.

Antibodies and immunohistochemistry

Immunohistochemical staining using the avidin-biotin-peroxidase technique was performed for each antibody. Five- micrometer-thick sections were cut from the tissue specimens and placed on poly-L-lysine–coated glass slides. Sections were deparaffined by xylene and rehydrated in graded alcohol. Endogenous peroxidase was blocked by immersing the sections in 0.1% hydrogen peroxidase in absolute methanol for 20 min. For antigen retrieval, the tissue sections were heated in a pressure cooker in citric acid monohydrate 10 mM, pH 6.0, for 5 min, and then incubated with the primary antibody at room temperature. IHC was performed with the EnVision system (Dako, Glostrup, Denmark) except anti-HER3 IHC that was with Benchmark XT (Ventana Medical Systems, Inc, Tucson, AZ).

The primary antibodies and dilutions used were: anti-HER3 (generated by Dr. Pandiella (IBMCC, Salamanca, Spain), 1:75), anti-Ki-67 (Dako, prediluted), anti-HER2 (Dako, prediluted), anti-ER (Novocastra, Bannockburn, IL, 1:50), anti-PR (Novocastra, prediluted) and anti-p53 (clone DO-7, Ventana Medical Systems, prediluted). The incubation time for all antibodies was 60 min with the exception of p53, which was incubated for 16 min. All slides were hematoxylin counterstained, dehydrated, and mounted. Omitting the primary antibody performed negative controls. Specificity of HER3 polyclonal antibody is shown in Supplementary Figure 1.

Immunohistochemistry evaluation

All cases were evaluated by two pathologists (VP and SRC). Hormone receptors (ER and PR) were evaluated taking into account the percentage of positive cells and intensity of the staining, which was assessed semi-quantitatively according to the ASCO (American Society of Clinical Oncology) and CAP (College of American Pathologists) guidelines for hormone receptor evaluation in breast cancer (32). Cases were considered as ER and/or PR positive if ≥1% was seen. For Ki-67, only strong nuclear staining was considered as positive, and then the percentage of positive neoplastic cells was calculated. HER2 was evaluated according to the ASCO and CAP guidelines (33). When an inconclusive HER2 result was found (2+), FISH was carried out (Pathvysion, Vysis Inc., Downers Grove, IL) according to the manufacturer's instructions to confirm HER2 amplification.

HER3 IHC was performed in whole sections from formalin fixed paraffin embedded blocks and cases were evaluated as follows: 0 (no expression), 1 (weak expression or moderate staining in <10% of neoplastic cells), 2 (moderate staining in >10% neoplastic cells) and 3 (strong staining). HER3 membrane and cytoplasmatic

staining was evaluated separately. Cases were then considered as positive (if score 3) or negative (if score 0, 1 or 2). Discordant cases were discussed using a multiheaded microscope. When a HER3 positive result was found (3+), FISH was carried out (ZytoVision GmbH, Bremerhaven, Germany) according to the manufaturer's instructions to confirm if its positivity was due to amplification.

Statistical analysis

Statistical studies were performed with the Statistical Package for the Social Sciences (SPSS 15.0; SPSS, Inc, Chicago, IL). Categorical variables were analyzed by cross- tabulation and differences were evaluated by the χ^2 test. The Kruskal-Wallis or Mann Whitney U tests were used to seek associations between the parameters analyzed. Statistical significance was set at a two-tailed *P* value of ≤ 0.05.

RNA extraction and quantitative RT-PCR

Total RNA was isolated from tumor tissue with the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Random primers and SuperScript II reverse transcriptase (Invitrogen) were used to carry out cDNA synthesis from 1.5 μg of total RNA. HER3 expression was detected using the Taqman Gene Expression Assay (Hs00951444_m1; Applied Biosystems). An ABI PRISM 7000 instrument (Applied Biosystems) was used to do the relative quantification analysis, and data were analyzed with the 7000 Sequence Detection Software, v.1.2.3 (Applied Biosystems). The PCR cycling program consisted of denaturing at 95ºC for 10 min and 40 cycles at 95ºC for 15 s, and annealing and elongation at 60ºC for 1 min. The reactions were done in triplicate. Previously, a Taqman Human Endogenous Control Plate (Applied Biosystems) was done to determine which endogenous controls showed less variation between normal

and tumoral tissue. POLR2A (Hs00172187_m1; Applied Biosystems) was chosen and the FirstChoice® Human Breast Total (AM6952, Applied Biosystems) as an internal control. Target and reference genes showed similar, nearly 100% amplification efficiencies (data not shown). Therefore, the ΔΔCT method was appropriate for relative gene expression analysis.

Western blot analysis

Lysates were obtained from cell lines and tissues. Tissues were ground and sonicated in lysis buffer (20mM Tris HCL (pH 8), 137 mM NaCl, 2mM EDTA, 10% Glycerol, 1% NP-40), in the presence of protease and phosphatase inhibitors. Subconfluent cells were lysed in the same buffer. After clearing the lysates by centrifugation, protein concentrations were determined using the Bradford assay (Bio-Rad Protein Assay, Munich, Germany). Equal amounts of protein were separated by 8% SDS-PAGE and transferred to PVDF membranes (Bio-Rad). The primary antibodies used were: anti-HER3 (diluted 1:3000) and anti-actin (A5060, Sigma-Aldrich, St Louis, MO, USA; diluted 1:1000) was used as the loading control. The secondary antibodies used were: donkey anti-rabbit IgG-HRP (NA9340, Amersham Pharma-Biotech, Uppsala, Sweden; diluted 1:2000) Bound antibodies were visualized with an enhanced chemiluminescence detection kit (Amersham Pharma-Biotech). Quantification of autoradiograms was performed by using Image J software (version 1.41o, National Institutes of Health, Bethesda, MD), normalized to the intensity of β-actin in each sample, and expressed in arbitrary densitometric units.

Cells and Cell Culture

Cell lines were obtained from the American Type Culture Collection. The following cell

lines were maintained in RPMI 1640 with 4mM of L-glutamine: SKBR3, MDA-MB-231 (breast carcinoma) and MDA-MB-435 (melanoma). In contrast, MIA PaCa-2 (pancreas carcinoma) and the human embryonic kidney 293T (HEK 293T) cancer cell line were maintained in DMEM with 4mM of L-glutamine and 4.5 g/L of glucose. All the cell lines were supplemented with 10% fetal bovine serum, 100U/mL of penicillin, 100 μg/mL of streptomycin and 10mM of HEPES and were cultured in a 37°C humidified atmosphere containing 95% air and 5% CO_2.

Cell transfection

pIRES-Hyg-HER3 and the pIRES-LUC were kindly donated by Dr. Scaltriti (Vall d'Hebron University Hospital Research Institute, Barcelona, Spain). The pIRES-LUC was used as a control for transfection. Both vectors contain hygromycin resistance.

To generate cell lines that stably express HER3 and LUC, constructs were transfected in cell monolayers at approximately 60% confluency with Jet-Pei (Polyplus-Transfection, Illkirch, France) according to manufacturer's protocols. Transfection was repeated the next day and 24 h after the second transfection, medium supplemented with hygromycin (Sigma-Aldrich) was added, and cells underwent selection for 10 days to eliminate untransfected cells.

Results

Clinico-pathological features

One hundred and forty-seven cases of primary breast cancer diagnosed between 2008 and 2009 were examined to study the protein expression pattern of HER3. Patient

ages ranged from 30 to 90 years with a mean age of 63 years. Histologic type, grade, age, tumor size, lymph node status, hormone receptors (ER/PR+), Ki-67, p53 and HER2 are shown in Table 1. Most cases were invasive ductal carcinomas (88.44%), grade II (48.23%), and with a tumor size between 20 and 50 mm (pT2, 53.06%).

HER3 immunohistochemistry expression

HER3 expression was analyzed by IHC in all breast cancer tissues and HER3 membrane and cytoplasmatic staining was evaluated separately. Figure 1 shows the different localization of HER3 staining observed in our series. A weak expression (1+) was found in normal adjacent tissue. Total HER3 was detected in 21% of cases. Co-expression of HER3 in the membrane and cytosol was observed in 3% of the patients, whereas the membrane and cytoplasmatic staining alone was detected in 14 and 4%, respectively. All HER3 positive cases were analyzed by FISH, and none of them was found to be positive.

Correlations between expression profiles and clinical and pathological variables

HER3 expression was evaluated in correlation with both clinico-pathological features (histological grade, age, tumor size, lymph node status) and molecular biomarkers (ER, PR, HER2, p53 and Ki-67). Correlation between HER3 expressions with the aforementioned biomarkers was analyzed using Chi-Square and Kruskal-Wallis statistical tests. The results of their assessment of associations are shown in Table 2.

Expression levels of HER3 did not correlate with clinical variables, such as age, tumor size, HER2 amplification (FISH+), p53 status and presence of lymph node metastasis (p=0.697, p=0.059, p=0.258, p=0.519, p=0.896, respectively) but

significantly correlated with HER2 staining (IHC), ER and PR (p=0.033, p<0.001 and p=0.013 respectively) positively. Finally, Ki-67 expression and histological grade negatively correlated with total HER3 expression levels (p=0.007 and p=0.046, respectively).

When we evaluated HER3 membrane staining, a significant association was found between HER3 staining and tumor size and ER expression levels (p=0.016 and p=0.004, respectively). No other significant correlations with prognostic indicators such as PR, HER2, histological grade and Ki-67 expression, HER2 amplification, age (p=0.124, p=0.646, p=0.769, p=0.357, p=0.406, and p=0.284, respectively) were observed. HER3 cytoplasmatic expression levels were also analyzed and positively correlated with ER and PR staining (p=0.005 and p=0.032, respectively). However, cytoplasmatic HER3 expression inversely correlated with Ki-67 staining (p=0.001). Although an inverse association has been found between high expression of total HER3 staining and histologic grade, only a trend was observed with HER3 cytoplasmatic staining (p=0.054, data not shown).

Evaluation of HER3 expression: correlation between IHC, Western blot and qRT-PCR

Protein expression levels of HER3 were also analyzed by Western blot in 43 breast cancer tissues (29% of the series). HER3 expression was also analyzed by qRT-PCR and a significant number of frozen tissues were available to do the RNA extraction. Here we analyzed a total of 32 samples (22% of the series) that corresponded all of them to the same as used to analyze the protein expression of HER3 by Western blot. As shown in Figure 2, some tumors displayed higher levels of HER3 than others by either IHC, Western blot or qRT-PCR (Supplementary Figure 2).

The HER3 protein expression levels quantified by IHC were significantly concordant with the levels obtained by Western blot ((p=0.009), Figure 2) and the HER3 mRNA levels measured by qRT-PCR (p=0.0254 and p=0.048, respectively, Supplementary Figure 3).

HER3 upregulates ER expression levels

Given the observed correlation between HER3 protein expression levels by IHC and hormone receptors (ER and PR) in the tested samples, we sought to determine if this result could be reproduced *in vitro*. We therefore investigated whether ectopic overexpression of HER3 in MDA-MB-231 (triple negative breast cancer), MDA-MB-435 (melanoma), MIA PaCa-2 (pancreas) and HEK 293T (human embryonic kidney) cell lines affects hormone receptors levels.

Cell lines overexpressing HER3 had higher levels of ER compared with the parental cell lines (Figure 3). No differences were noticed between the overexpression of HER3 and PR expression levels (Supplementary Figure 4).

HER3 expression is not dependent of HER2 expression

Breast carcinomas have been classified into luminal, HER2 enriched and triple negative subtype depending their IHC patterns of protein expression of ER, PR and HER2. Expression of HER3 was found mainly in breast cancer luminal subtype, follow by HER2 enriched molecular type.

In accordance with the literature, the HER2/HER3 heterodimer has been shown to be the most transforming and mitogenic receptor complex of the HER family (13, 34, 35). In our series, we observed a correlation between the expression of total HER3 and

total HER2 but only a trend between total HER3 and HER2 amplification. Thus, we included a larger series of patients with amplification of HER2 to determine the role of HER3 in patients with HER2 amplification. A total of 67 new HER2 amplified patients were added to the list of HER2 positive tumors, totalling 100 HER2 positive tumors. Of them, 10% of the tumors overexpressed HER3 (3+). Furthermore, we analyzed in this subset of patients their correlation with ER, PR, Ki-67. HER3 total expression, HER3 membrane staining and HER3 cytoplasmatic localization correlated positively with ER (p=0.004, p=0.027, p=0.014, respectively, data not shown).

These results show that HER3 expression is not always linked to HER2 expression because it is predominantly found in the molecular luminal subtype, which does not have overexpression of HER2.

Discussion

Increasing evidence indicates that HER3 plays an important role in the genesis and progression of cancer. Not only HER3 expression in different tumors is increasingly being reported, but expression of HER3 has also been linked to resistance to anti-HER therapies using small molecule tyrosine kinase inhibitors. Furthermore, HER3 overexpression has been found to be an independent predictor of survival in some, (36, 37) but not all studies (21). Moreover, coexpression of HER2 and HER3 is a poor prognostic indicator (38).

The aim of this study was to compare IHC profiles of primary breast carcinomas with HER3 staining to assess the role of HER3 in breast cancer. In this study, we show that HER3 expression was high in hormone positive tumors. The prevalence of HER3 expression as judged by analysis of protein levels by IHC (22, 39) has been reported to be between 49-53% and we found an expression of HER3 (3+) in the 21% of the series

analyzed. HER3 is frequently overexpressed in breast cancer and is found in the absence of gene amplification or mutation (40). In our series we were unable to detect HER3 amplification in HER3 positive tumors

HER3 membrane and cytoplasmatic staining were evaluated separately and different associations were found. Three studies (21, 22, 36) found no significant association with HER3 and ER status but another (41) found that HER3 expression correlated with ER positivity. Despite these results we observed a positive relationship between HER3 and ER expression in both scenarios. HER3 membrane expression was positively correlated with tumor size and ER. On the other hand, we found that HER3 cytoplasmatic staining positively correlated with hormone receptors (ER, PR) and was inversely associated with Ki-67 expression.

Expression of HER3 was found mainly in luminal and also was found in HER2 enriched molecular subtype. These results shown a HER3 role independent to HER2 and not always are linked. Also we observed a presence of HER3 positive cytoplasmic expression staining in a better prognostic group, because it correlated positively with hormone receptors and inversely with Ki-67, than HER3 positive membrane expression, that correlated with ER and tumor size breast cancers (42).

Resistance to anti-ER therapies limits the efficacy of these compounds in ER positive breast cancer and several mechanisms of resistance have been proposed. Expression of HER1 and/or HER2 have been linked to resistance (43, 44) and cross talk between the ER and HER1 and HER2 signaling pathways has been described in cancer cell lines (45-47). Interestingly, increased functioning of the PI3K/Akt route has also been linked to resistance to anti-hormonal therapies. Since HER3 signals through this route it will be interesting to evaluate whether patients expressing higher levels of HER3 also express elevated Akt phosphorylated at Thr308 or Ser473, two sites that

reflect increased functioning of Akt. These findings, together with the precedent that persistent activation of HER3 results in escape from growth inhibition induced by HER tyrosine kinase inhibitors (TKIs) should represent the bases of additional clinico-pathological studies to analyze the value of HER3 as a predictive marker in anti-hormonal therapies (48).

Furthermore, HER3 is essential in HER2 driven tumorigenesis and may have clinical relevance for tumor aggressiveness. Patients with expression of both proteins (HER2 and HER3) may benefit from anti-HER3 targeted therapy. Clinical studies examining a group of tamoxifen treated, ER positive breast cancer patients have demonstrated that HER2 and HER3 positive patients relapsed on tamoxifen with a higher incidence than HER receptor negative patients (49). These results support the hypothesis that HER3 upregulation may play a role in tumor aggressiveness in ER positive breast tumors.

The purpose of this paper was to emphasize the role of HER3 in breast cancer and determine a new valuation of HER3 staining. We think that is important do a valuation of HER3 with membrane and cytoplasmatic staining separately because they may play different roles in the genesis of breast cancer. ER positive patients are normally considered to represent a good prognostic group and thus the interaction between HER and ER expression may be key to the clinical management of this group. Further work will also be needed to evaluate HER3 as a marker for breast cancer and to allow identification of patients who may not respond to existing HER directed therapies. In addition, the ability of HER3 through sustained activation of Akt to circumvent both hormonal and TKI based therapies presents an important clinical problem where HER3 therapeutic targeting is warranted.

Acknowledgements

We are grateful to the tumor banks of Hospital Vall d'Hebron and Xarxa del Banc de Tumors de Catalunya for providing the tumor samples. This work was supported by grants from Fundación Mutua Madrileña (FMM2009/02), and Caja Navarra (Ref. CAN2009-16662). Support for this work was also obtained from the Cancer Center Network Program from the ISCIII (RD06/0020/0041 and RD06/0020/0104).

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Tables

Table 1. Characteristics of the breast tumors

FEATURE	N (%)
Histologic type	
Apocrine	1 (0.68%)
Cribiform	1 (0.68%)
Ductal	130 (88.44%)
Lobular	9 (6.12%)
Micropapilar	1 (0.68%)
Mucinous	2 (1.63%)
Neuroendoncrine	1 (0.68%)
Papilar	2 (1.32%)
Histologic Grade[a]	
I	13 (8.84%)
II	72 (48.98%)
III	62 (42.18%)
Age of patient	
<50 years	35 (16.20%)
≥50 years	112 (76.19%)
Tumor size	
pT1 (<20mm)	54 (36.73%)
pT2 (20-50mm)	78 (53.06%)
pT3 (>50mm)	15 (10.20%)
pN	
0	42 (47.70%)
1	29 (33.00%)
2	9 (10.20%)
3	8 (9.10%)
Hormone receptors	
ER+ (Estrogen receptors)	123 (83.67%)
PR+ (Progesterone receptors)	112 (76.19%)
Ki-67	
>15%	94 (63.95%)
≤15%	53 (36.05%)
p53	38 (28.60%)
Her2/neu	
Amplified	33 (22.45%)
Not amplified	114 (77.55%)

[a] According to the Scarff Bloom Richardson (SBR) classification

Table 2. Statistical significance of biomarkers with HER3 staining

	Total	**Membrane**	**Cytoplasm**
Tumor Size	p=0.059	p=0.016*	p=0.526
Lymph node metastasis	p=0.896	p=0.456	p=0.459
HER2	p=0.033*	p=0646	p=0.055
HER2 amplified	p=0.258	p=0.406	p=0.878
Estrogen Receptors	p<0.001*	p=0.004*	p=0.005*
Progesterone Receptors	p=0.013*	p=0.124	p=0.032*
Ki-67	p=0.007*, [1]	p=0.357	p=0.001*, [1]
p53	p=0.519	p=0.725	p=0.116

* Statistically significant ($p \leq 0.05$)
[1] Inverse correlation

Figure legends

Figure 1. Expression levels of HER3, ER, PR and Ki-67 in breast carcinomas

A) Immunostaining for H&E, HER3, ER, PR and Ki-67 expression in 2 solid tumors of the breast, which present different pattern of HER3 protein expression. i) HER3 cytoplasmatic positive staining. ii) HER3 membrane positive staining. Magnification 20x.

B) Representative examples of different expression levels of HER3 IHQ with cytoplasmatic (A-C) and membrane staining (D-F).A and D, shows a weak expression of HER3; B and E, express moderate levels of HER3 ; C and F, shows a strong staining for HER3. Magnification 40x.

Figure 2. Correlation between HER3 expression by immunohistochemical analysis and Western blot

A) Representation of different HER3 expression levels by IHC in some solid tumors of the breast (A-G). The breast carcinoma cell line MCF-7 was used as a positive control (H). Magnification, 20x.

B) The same breast carcinoma tumors that we performed the IHC aboved were lysed, and HER3 receptor protein expression was analyzed by Western blot in 8% SDS-PAGE gel. β-actin was used as an internal control to verify equal protein loading.

C) Association of HER3 expression by Western blot with HER3 IHC staining in 43 patients with primary breast carcinomas. Levels of HER3 protein were quantified from Western Blot analysis by densitometry and were compared with IHC evaluation by Kruskal-Wallis test. HER3 expression by Western blot

correlated with HER3 IHC staining and we obtained a statistical significance (p=0.009).

Figure 3. Effect of HER3 on ER expression of stably transduced cells.

A) Four HER3 overexpressing cells from different cancer cell types were generated by transfection and selection with 1 μg of pIRES-Hyg-HER3. In all cases, ectopic expression of HER3 was confirmed by Western blot, using β-actin expression as a loading control. HER3 expression was also analyzed by Immunohistochemistry in all cell lines corroborating the same results.

B) ER expression levels were analyzed by Immunohistochemistry in the same panel of tumor cell lines where HER3 have been previously analyzed. Cell lines overexpressing HER3 had higher levels of ER in comparison with the parental cell lines in all cases.

Figure 1

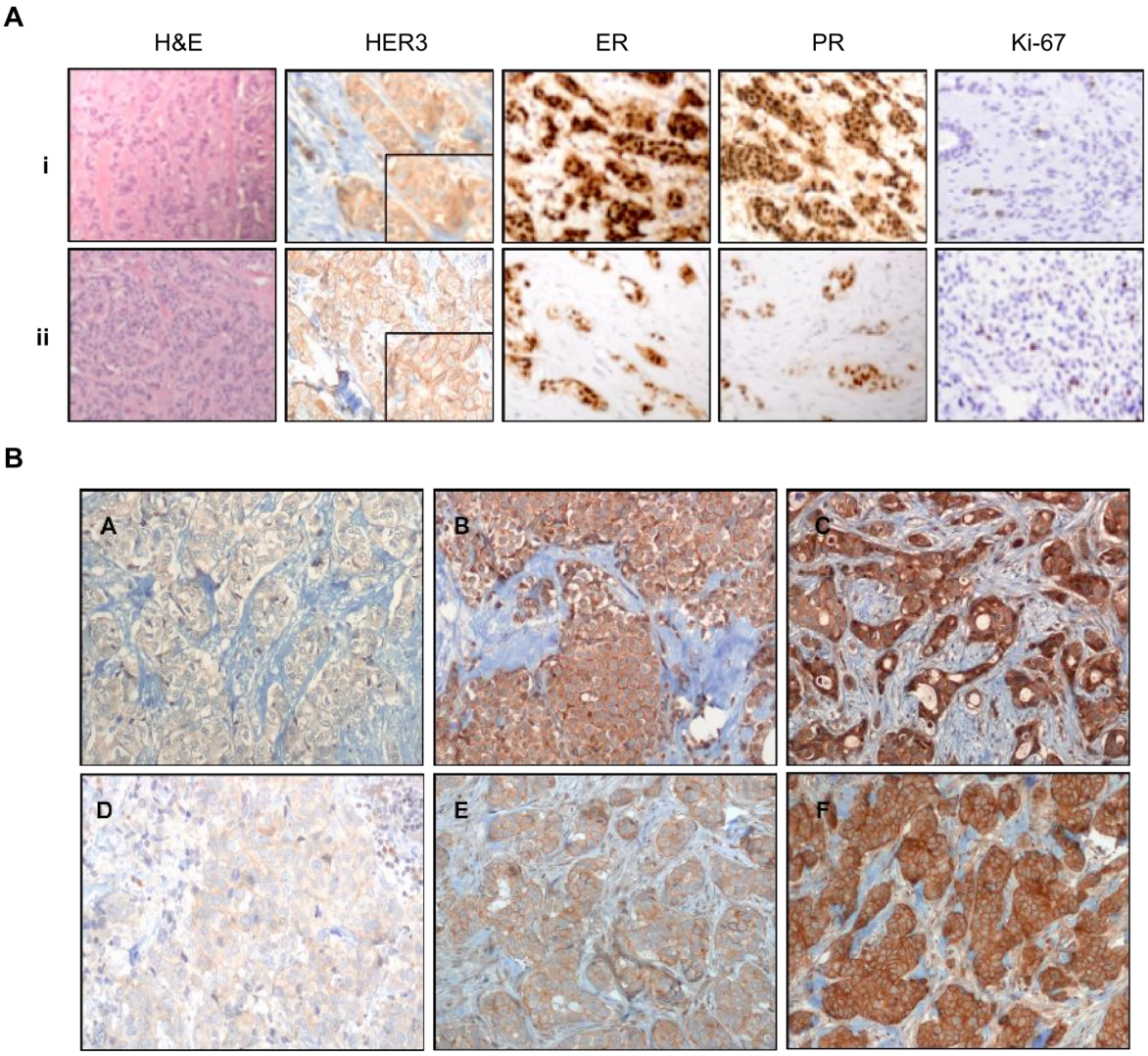
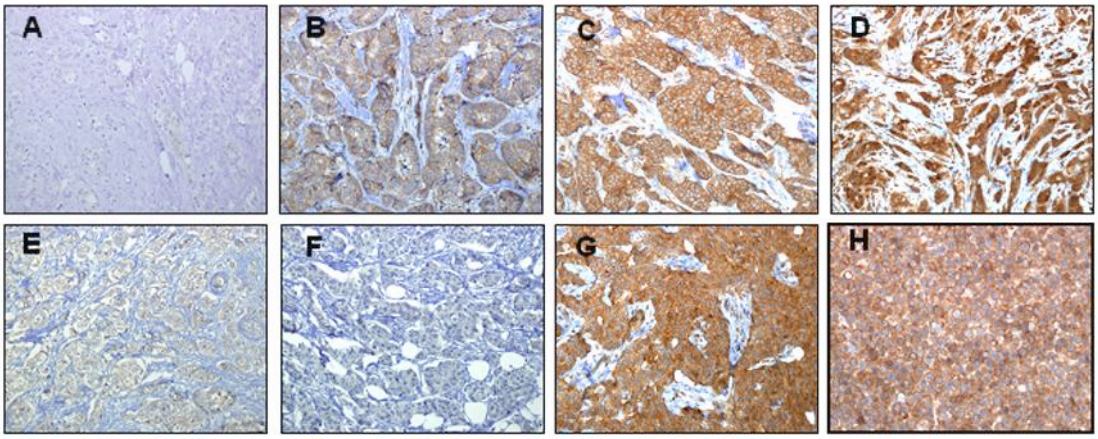
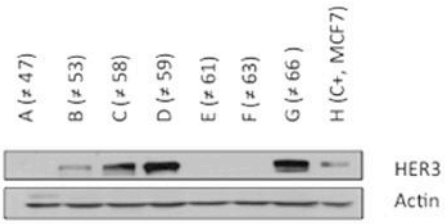


Figure 2

A


A
B
C
D
E
F
G
H

B


A (≠ 47)
B (≠ 53)
C (≠ 58)
D (≠ 59)
E (≠ 61)
F (≠ 63)
G (≠ 66)
H (C+, MCF7)
HER3
Actin

C

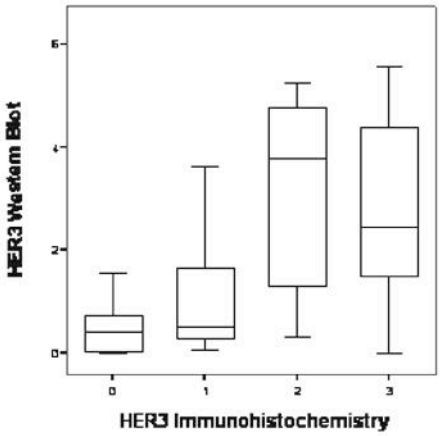

HER3 Western Blot
HER3 Immunohistochemistry
0
1
2
3

Figure 3

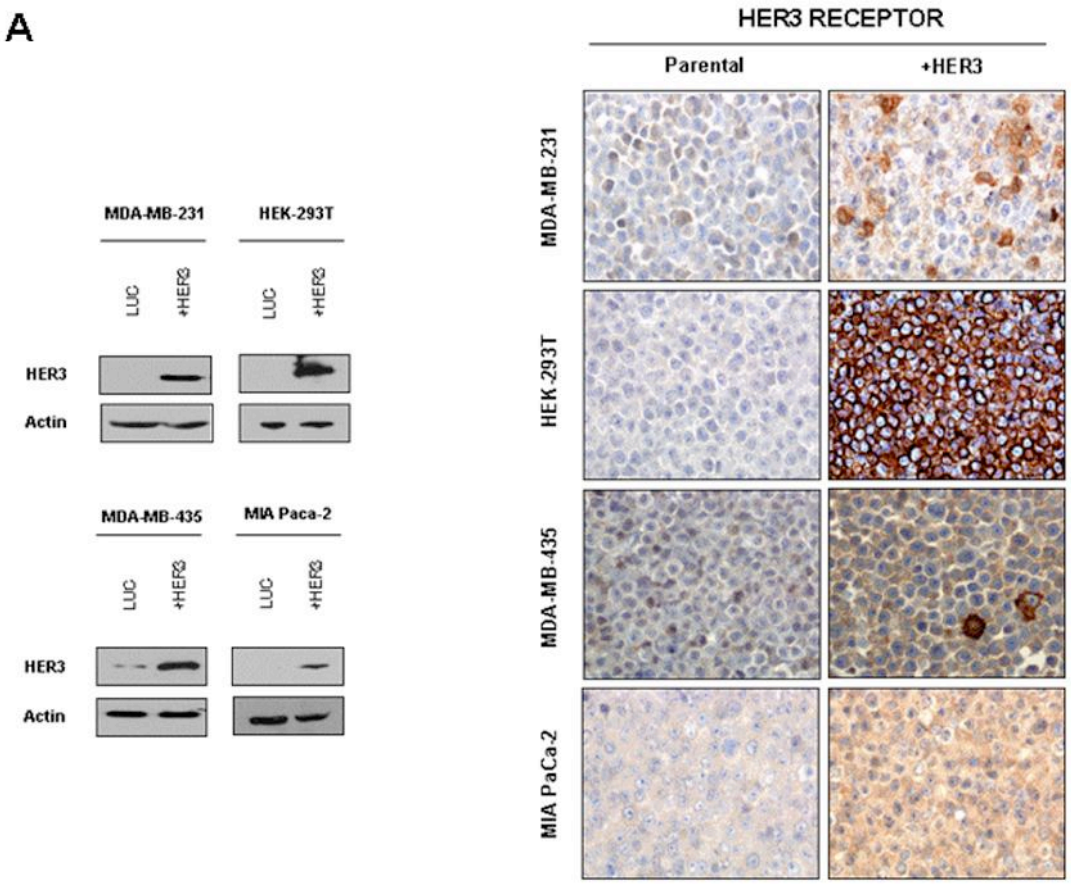
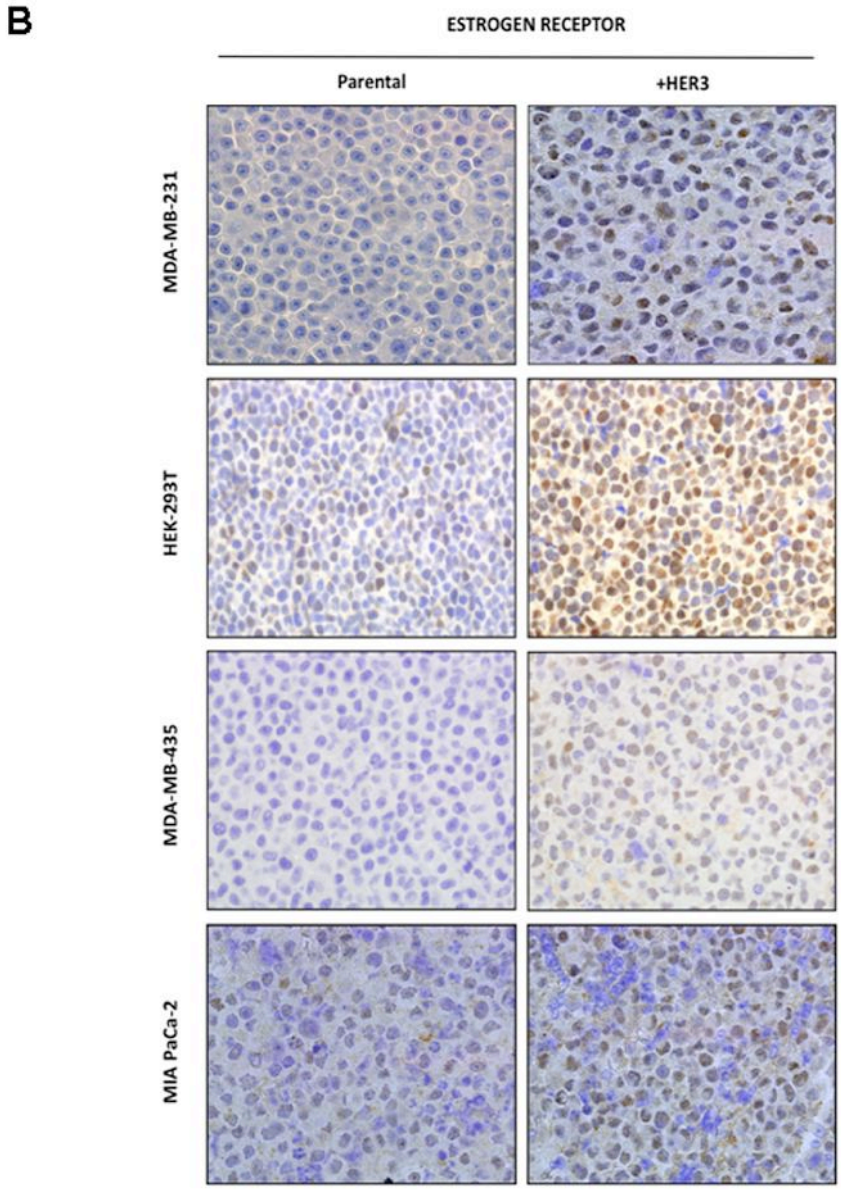

A
MDA-MB-231
HEK-293T
LUC
+HER3
HER3
Actin
MDA-MB-435
MIA Paca-2
HER3 RECEPTOR
Parental
+HER3
MDA-MB-231
HEK-293T
MDA-MB-435
MIA PaCa-2

Figure 3


B
ESTROGEN RECEPTOR
Parental
+HER3
MDA-MB-231
HEK-293T
MDA-MB-435
MIA PaCa-2

Supplementary Figure Legends

Supplementary Figure 1. Specificity of anti-HER3 antibody

SKBR3 HER2 overexpressing cells from human breast carcinoma were lysed, and HER receptors were immunoprecipited with specific antibodies which include previously mentioned anti-HER3 antibody. Blots were probed with the same antibodies. Anti-HER3 antibody could only recognize HER3 receptor in immunoprecipitation and Western blot. No cross-reactivity of anti–HER3 antibody was detected with HER1, HER2 or HER4 receptors**.**

Supplementary Figure 2. HER3 relative gene expression in breast cancer patients

HER3 expression was analyzed by quantitative real-time PCR in 32 breast carcinomas. A commercial pool of human breast total RNA was used as control. An increase of HER3 was seen in all the cases studied. Error bars, SD of triplicates. Each experiment was done at least twice.

Supplementary Figure 3. Relationship between HER3 relative gene expression with HER3 protein expression in 32 patients with primary breast carcinomas.

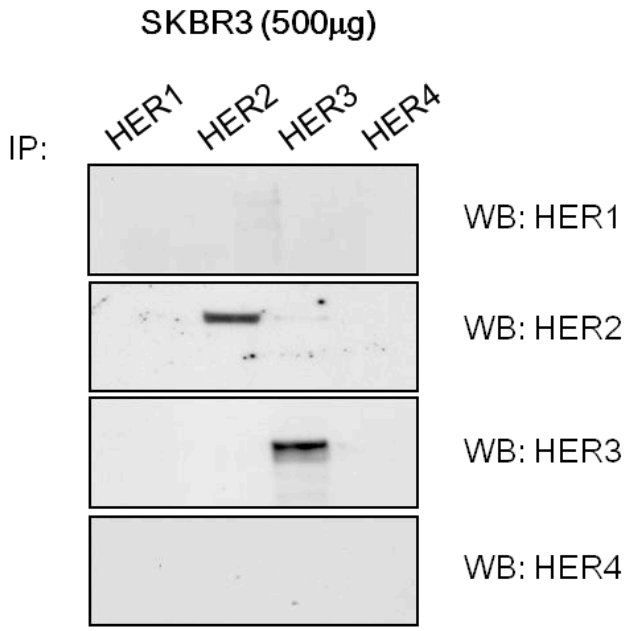
A) Association between HER3 relative gene expression with HER3 protein expression by IHC. The association was significant in the Kruskal-Wallis test, p=0.0254.

B) Correlation between HER3 relative gene expression with HER3 protein expression by Western blot (Kruskal-Wallis test, p=0.0481).

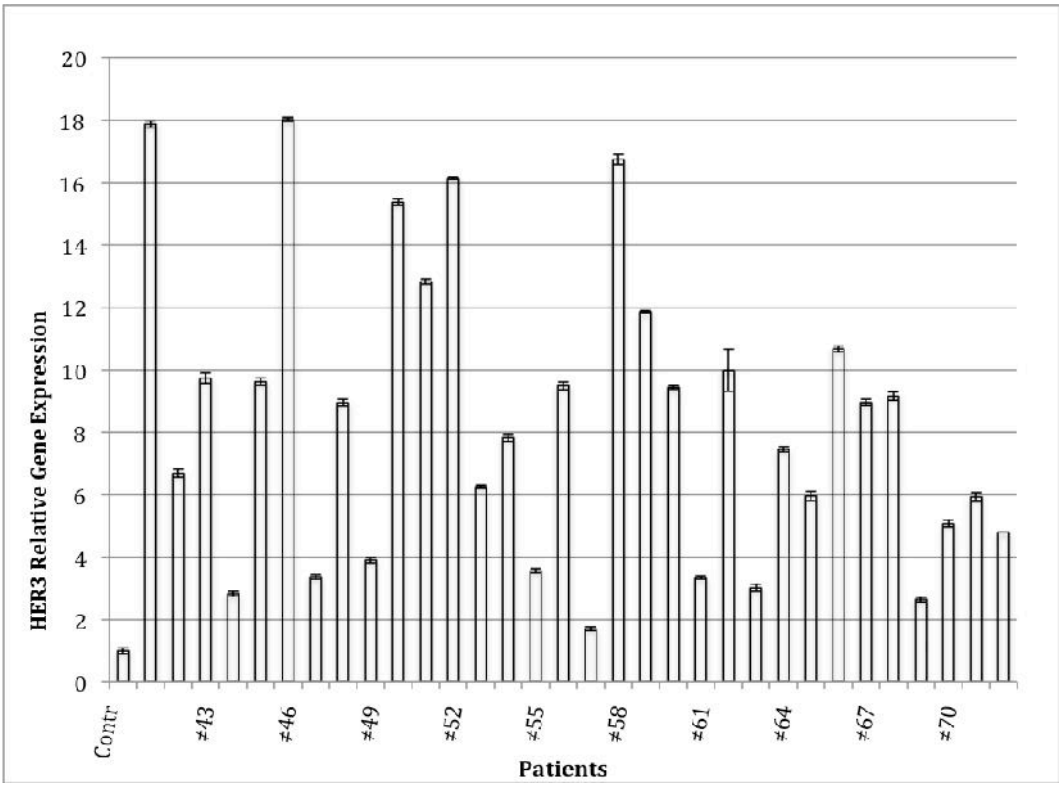
Supplementary Figure 4. PR expression in cell lines with overexpression of HER3

PR protein expression levels were also analyzed in the cancer cell lines with overexpression of HER3. There was no effect on the tested cell lines with the PR expression when they were transduced with HER3.

Supplementary Figure 1.

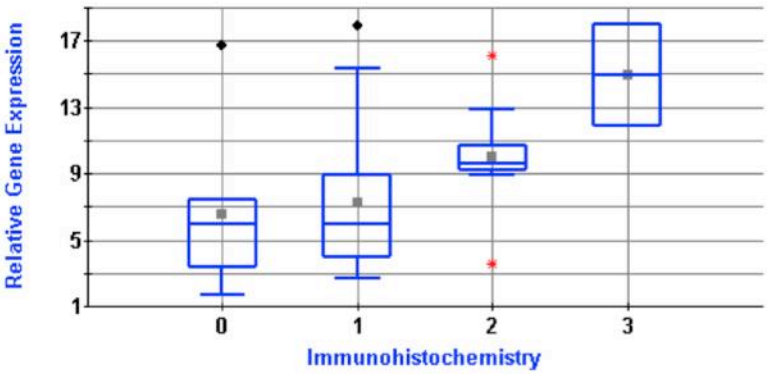


Supplementary Figure 2.



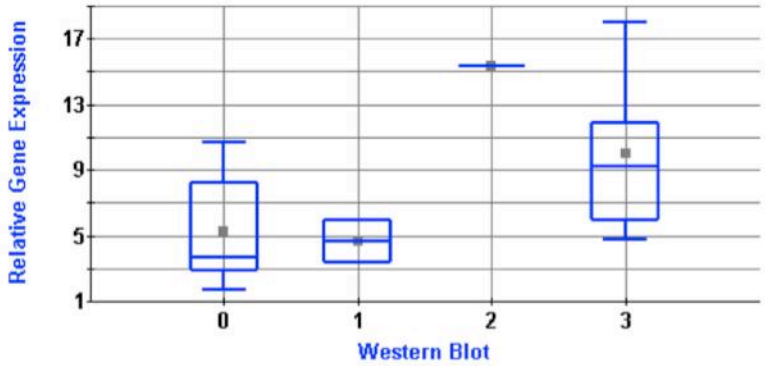
Supplementary Figure 3.

A



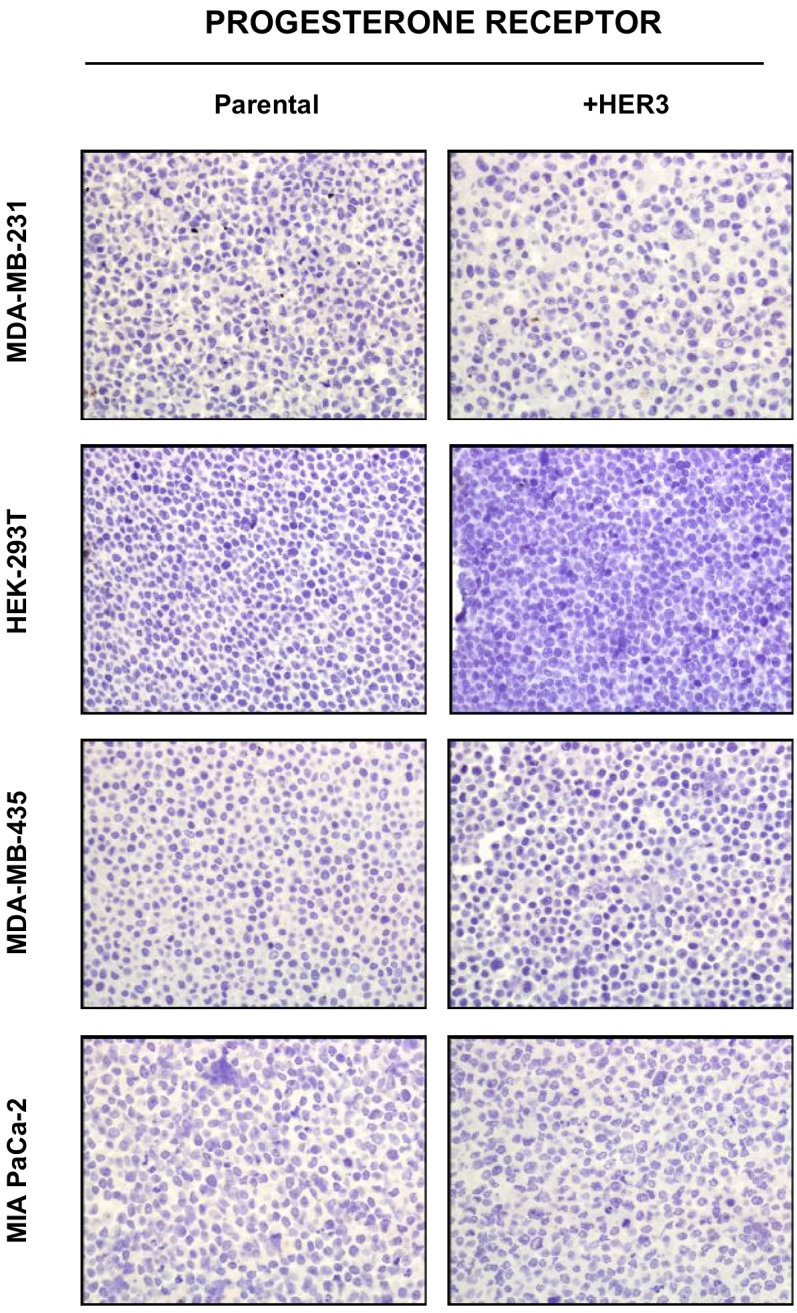
p=0.0254

B



p=0.0481

Supplementary Figure 4.



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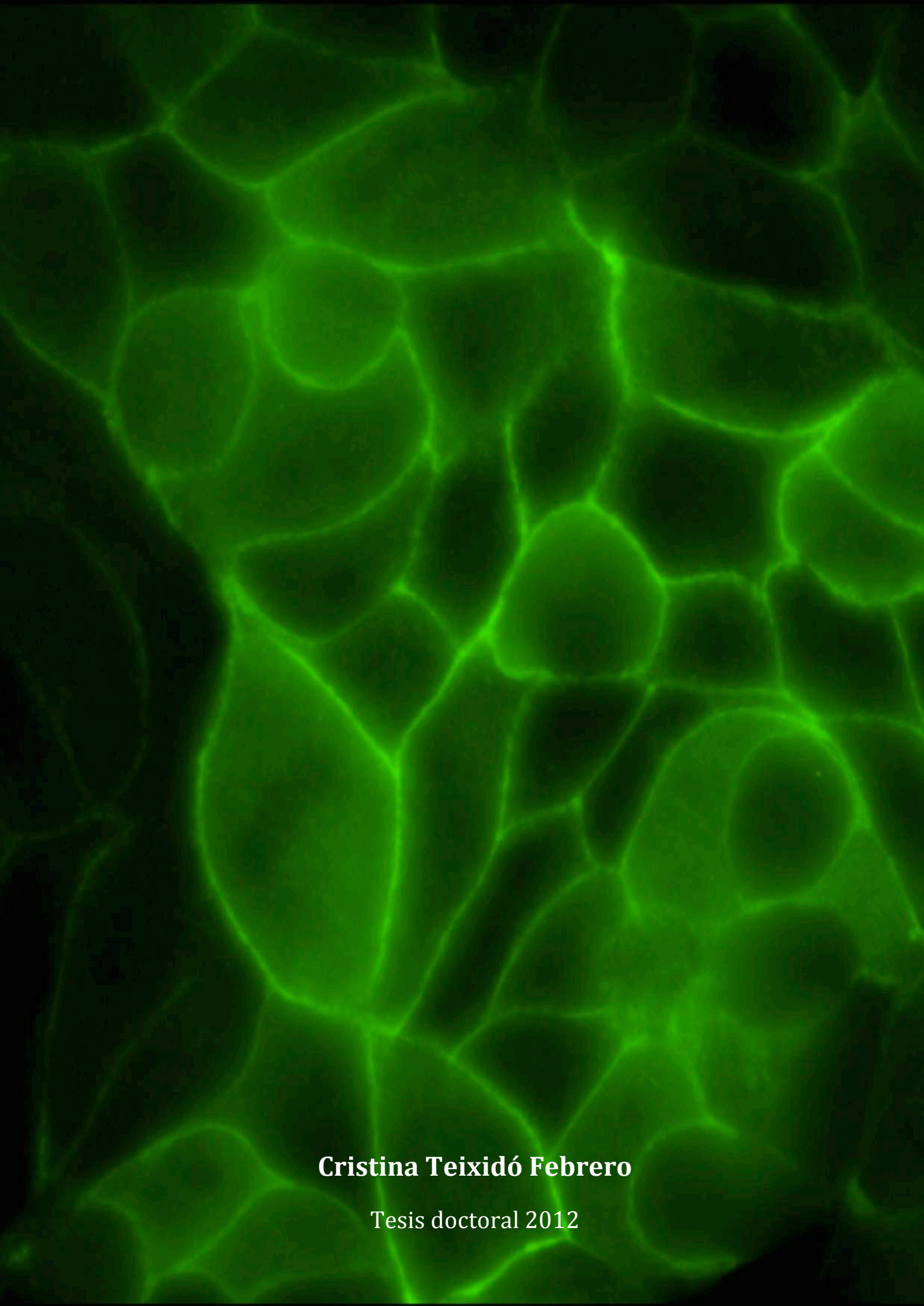

Cristina Teixidó Febrero

Tesis doctoral 2012