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COMBINATORIAL TREATMENT OF BREAST CANCER WITH SERMS AND SALINOMYCIN

KOMBINATIONSBEHANDLUNG VON BRUSTKREBS MIT SERMS UND SALINOMYCIN

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MASTER THESIS

DECLARATION

According to § 14 (8) of the examination regulation of the masters course "Pharmaceutical Sciences", I declare that this thesis is the result of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly.

The experimental work was done under the guidance of Hon.-Prof. Dr. h.c. Axel Ullrich at the Max-Planck-Institute for Biochemistry in the Department of Molecular Biology in Martinsried.

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(Ann-Katrin Sommer)

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ABSTRACT

According to several different associations breast cancer is still the second leading cause for cancer deaths in women. Therefore and also due to the fact that there is no curative therapy available up till now it is necessary to further investigate the molecular mechanisms of this disease as well as to develop new treatment options.

Therapy resistance also occurs in estrogen receptor α (ERα)-positive breast cancer types Luminal A and Luminal B which are the most frequent forms of breast tumors and are known to be treatable with endocrine therapy. Therefore it is important to elucidate different resistance mechanisms and options to intervene them. The monocarboxylic polyether antibiotic Salinomycin is able to overcome MDR-1 induced treatment resistance and to eradicate specifically CSCs thereby eliminating two of three possible resistance mechanisms (Fuchs et al., 2010; Gupta et al., 2009).

Therefore the aim of our study was to figure out whether Salinomycin is also able to hamper the third most common resistance mechanism, the ligand independent activation of ERα via human epidermal growth factor receptor 2 and 3 (Her2, Her3). As Salinomycin is not only an antibiotic but also an ionophore it is able to damage membrane integrity and thus may furthermore change receptor tyrosine kinase signaling. We investigated the effect of single and combinatorial treatment of Tamoxifen and Salinomycin on cell viability, protein and RNA-expression, cell morphology and mammosphere forming potential of monolayer and spherical breast cancer cells to figure out the optimal treatment option.

Our results clearly show that especially protein levels and the phosphorylation of Her2 and Her3 as well as their down-stream targets ERK 1/2, AKT and ERα are decreased upon Salinomycin treatment indicating that this ionophore inhibits the ligand independent activation of ERα.

Therefore this combinatorial treatment is a promising option to overcome all major mechanisms causing resistance to endocrine therapy and thus more efficiently eradicate ERα-positive breast tumor cells.

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1. INTRODUCTION

1.1 BREAST CANCER

According to the American Cancer Society breast cancer is still the second-most cause for cancer deaths in women in 2013. Moreover most new cancer cases diagnosed in women belong to breast cancer. This data was also confirmed by the International Agency for Research on Cancer for Europe (Ferlay J, 2010; Rick Alteri et al., 2013)

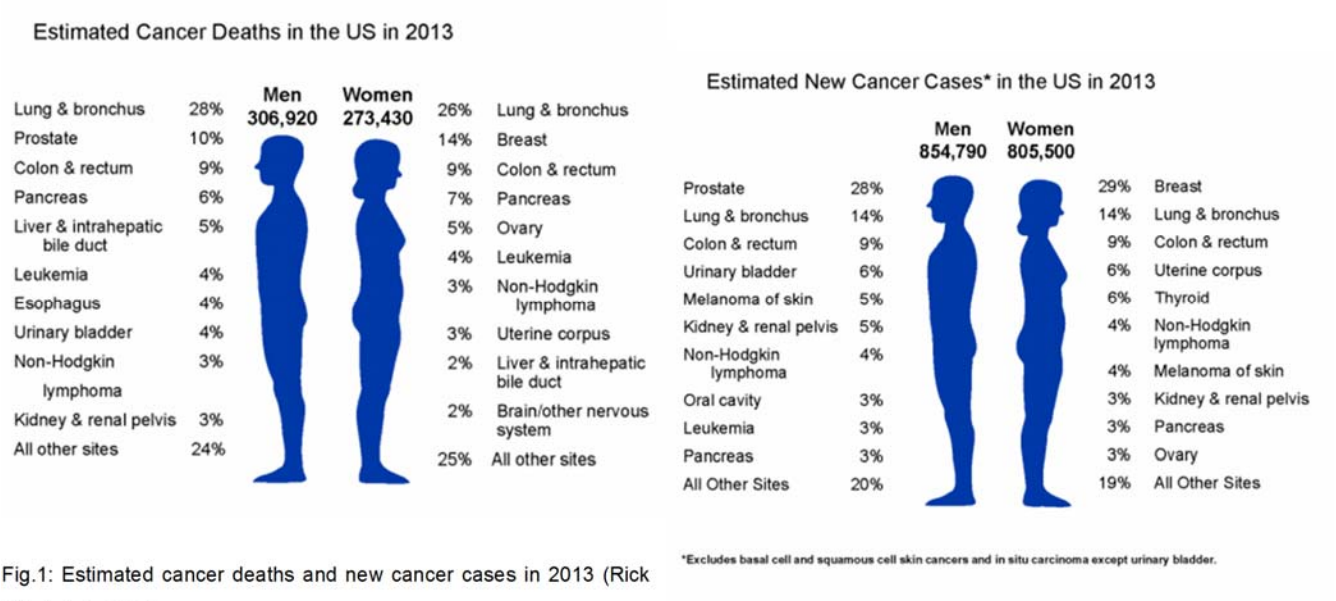


Fig.1: Estimated cancer deaths and new cancer cases in 2013 (Rick Alteri et al., 2013)

There are several different risk factors for breast cancer e.g. increasing age. According to the American cancer society 95% of breast cancer cases occur in women with 40 years of age and older whereas women between 75 and 79 years have the highest risk. Moreover post-menopausal obesity, alcohol and tobacco consumptions are also known to play an important role in the development of tumors in the breast. Additionally mutations in specific genes like BRCA1 and BRCA2 cause breast cancer and most important they are hereditary.

Thus, the breast cancer history in families has a great impact on the risk to develop tumors. It is also necessary to mention that every previous breast cancer case increases the probability to suffer once more from this disease.

A further risk factor is an increased endogenous estrogen level and oral contraceptives are also often discussed to augment the incidence for breast cancer. Nevertheless it was shown that in the absence of other risk factors oral contraceptives are not leading to breast cancer whereas hormone replacement therapy in post-menopausal women significantly contribute to a higher incidence for breast cancer (DeSantis, 2011).

Since there are several causes and several types of breast cancer, also several different therapy options and strategies exist. Basically breast cancer is usually treated in four different ways: surgery, radiation, chemotherapy and/or targeted therapy. Whereas the first three possibilities are very harmful, the targeted therapy can be less aggressive and more efficient especially in combination with one of the first options. To figure out the best therapy it is necessary to exactly characterize the tumor and thus group it to a specific class of breast cancer. Due to an official classification system and an appropriate therapy it is possible to eradicate cancer cells more efficiently and cause less side-effects (DeSantis, 2011).

1.2 Classification of different breast cancer types

In order to characterize different types of breast cancer and thus draw conclusions about prognosis as well as treatment options, clinicians use four different classes of breast cancer according to their gene expression profiles: Triple negative (basal like), Her2 overexpressing, Luminal A and B breast cancer whereas both last groups are the most common subtypes [Tab.1]

Type	Prevalence	Invasive-ness	5 years disease free survival	Progno-sis	Receptor	Targeted Treatment	Cell line resembling tumor type
Luminal A	50-60%	-	88,6%	++	ER+/PR+/ Her2-	Tamoxifen	MCF-7 T-47D
Luminal B	Ca. 20%	+	78,4%	+	ER+/PR+/ Her2+	Tamoxifen Lapatinib Trastuzumab	MCF-7/Her2 BT-474
Her2	Ca. 20%	++	66,0%	+	ER-/PR- /Her2+	Trastuzumab	SKBR3 MDA-MB-453
Triple negative	10-20%	+++	59,7%	-	ER-/PR- /Her2-	Paclitaxel Doxorubicin	MDA-MB-468

Table 1: Modified according to (Eroles et al., 2012; Holliday and Speirs, 2011; Natoli et al., 2013)

1.2.1 TRIPLE NEGATIVE BREAST CANCER (BASAL-LIKE)

This type of breast cancer is defined as estrogen receptor, Her2 and progesterone receptor negative. Moreover it is linked to poor prognosis, high invasiveness and short relapse free survival as well as overall survival. Due to lack of specific molecular markers the state of the art treatment nowadays is still very harmful and aggressive chemotherapy with doxorubicin or paclitaxel. Additionally breast cancer patients with BRCA1 and p53 mutations are grouped in this subtype. These alterations lead to accumulation of aberrant DNA-transcripts, genetic instability and increased invasive behavior.

Today also poly-ADP ribose-polymerase 1 (PARP1)-inhibitors are discussed as possible drugs targeting this type of breast cancer. Since PARP1 is responsible for repairing DNA double-strand breaks its inhibition causes cell deaths.

Nevertheless this type of breast cancer remains the most severe one and mutations in the BRCA1 genes are hereditary. Therefore it is necessary to develop a preventive therapy particularly because up to 20% of breast cancer patients suffer from this mutation (DeSantis, 2011).

1.2.2 HER2-OVEREXPRESSING BREAST CANCER

As the name already indicates this group of breast cancer is overexpressing the receptor tyrosine kinase, human epidermal growth factor receptor 2 (Her2) which belongs to the epidermal growth factor receptor (EGFR) family and was found by Axel Ullrich (Ullrich et al., 1984). This alteration occurs in approximately 20 to 25% of breast cancer cases and is thus the second most common subtype. In former times this group of tumor had an even worse prognosis than triple-negative breast cancer since Her2 is a driver for increased cell proliferation and decreased apoptosis rate (Slamon et al., 1987). In 1998 Trastuzumab, a monoclonal antibody against Her2 developed by Axel Ullrich in cooperation with Dennis J. Slamon was approved by the FDA for metastatic, Her2+ breast cancer which lead to increased disease-free survival, less invasiveness and a better prognosis in general. Moreover there are small-molecule competitive tyrosine kinase inhibitors like Lapatinib that blocks the EGFR- and Her2-pathway *via* inhibition of phosphorylation. It is approved by the FDA for ER+/PR+/Her2+ and Her2-overexpressing advanced breast cancer.

1.2.3 LUMINAL A

This class of breast cancer which belongs to the infiltrating lobular carcinoma is mainly characterized by high estrogen as well as progesterone receptor expression levels, low proliferation rate, no Her2 expression and thus a good prognosis in general. According to the increased estrogen receptor level anti-estrogen therapy is mainly used to treat this type of breast cancer nowadays. This includes aromatase inhibitors for postmenopausal women, selective estrogen recepetor modulators (SERMs) like Tamoxifen and estrogen receptor blockers like Fulvestrant.

Luminal A breast cancer is the most common subtype with 50-60% and the lowest relapse rate (ca. 30%) (Eroles et al., 2012).

1.2.4 LUMINAL B

The main difference of Luminal B breast cancer compared to Luminal A is the increased proliferation rate, more aggressive behavior and worse prognosis for cancer patients. This is due to the fact that Luminal B breast cancer often expresses lower levels of estrogen receptor but higher levels of Ki67 and Her2 which are both key players in cancer progression. Nevertheless there are also some cases classified as Luminal B which are neither expressing estrogen receptor nor Her2. Therefore this kind of tumor is involved in a higher relapse frequency and a shorter disease-free survival time. Thus treatment of this subtype remains a challenging task for clinicians and scientists (Creighton, 2012; Eroles et al., 2012).

This classification system is thus based on three different receptors: progesterone receptor, Her2 and ERα whereas ER-dependent breast cancer has the highest incidence [Tab.1]. Hence, its signaling plays an important role in breast cancer patients.

1.3 ESTROGEN RECEPTOR SIGNALING AND INTERVENTION MECHANISMS

1.3.1 ESTROGEN RECEPTOR SIGNALING

According to the current classification of different breast cancer types the estrogen receptor expression and endogenous estrogen levels are key factors for prognosis and treatment.

In general there are two different estrogen receptor types present in the human body: estrogen receptor α (ERα) and estrogen receptor β (ERβ) which are located on different chromosomes and possess different functions. ERβ apparently opposes the effects of ERα whereas both are steroid receptors and thus belong to the nuclear receptor family. Therefore the classical way to activate the ER-signaling cascade starts with ligand binding to the ER located in the cytoplasm. That leads to a translocation of the receptor towards the nucleus where it dimerizes and binds to DNA at specific estrogen response elements (ERE).

Subsequently the receptor recruits co-activators (CoA) or co-repressors (CoR) and thus activates or inhibits the transcription of down-stream genes. Finally, the ER-complex dissociates through translational modifications like methylation or acetylation and ubiquitylation of ERs either stops the signaling cascade or induces it [Fig2b].

Moreover ER is able to directly interact with DNA-bound transcription factors (TF) like cAMP response element-binding protein (CREB), nuclear factor κB (NF-κB) and p53 due to adjacent localization of ERE and the specific response elements of the transcription factor. Through this pathway estrogen also leads to the transcription of downstream genes of other transcription factors [Fig.2d].

Additionally, ER possesses a non-genomic mechanism of action. In this case ER is located at the cell membrane or in the cytoplasm where it is activated by ligand-binding. Furthermore the receptor-ligand complex interacts with steroid receptor co-activators (SRC), p53 as well as G proteins and thus activates protein kinase cascade through ERK1/2 and AKT. This causes the phosphorylation of transcription factors and thereby its translocation to the nucleus. Their binding to the response element induces the transcription of their target genes [Fig.2c].

Finally ER can also be activated in a ligand independent manner through growth factor receptors like epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR) or human epidermal growth factor receptor 2 (Her2). After binding of specific growth factors they are activating AKT and ERK serine/threonine kinase which phosphorylate the ER or CoA and thus lead to transcription of ER downstream targets in absence of estradiol. This mechanism is also discussed to be responsible for possible resistance to anti-estrogen treatment and is therefore of special interest in our study [Fig.2a] (Thomas and Gustafsson, 2011)

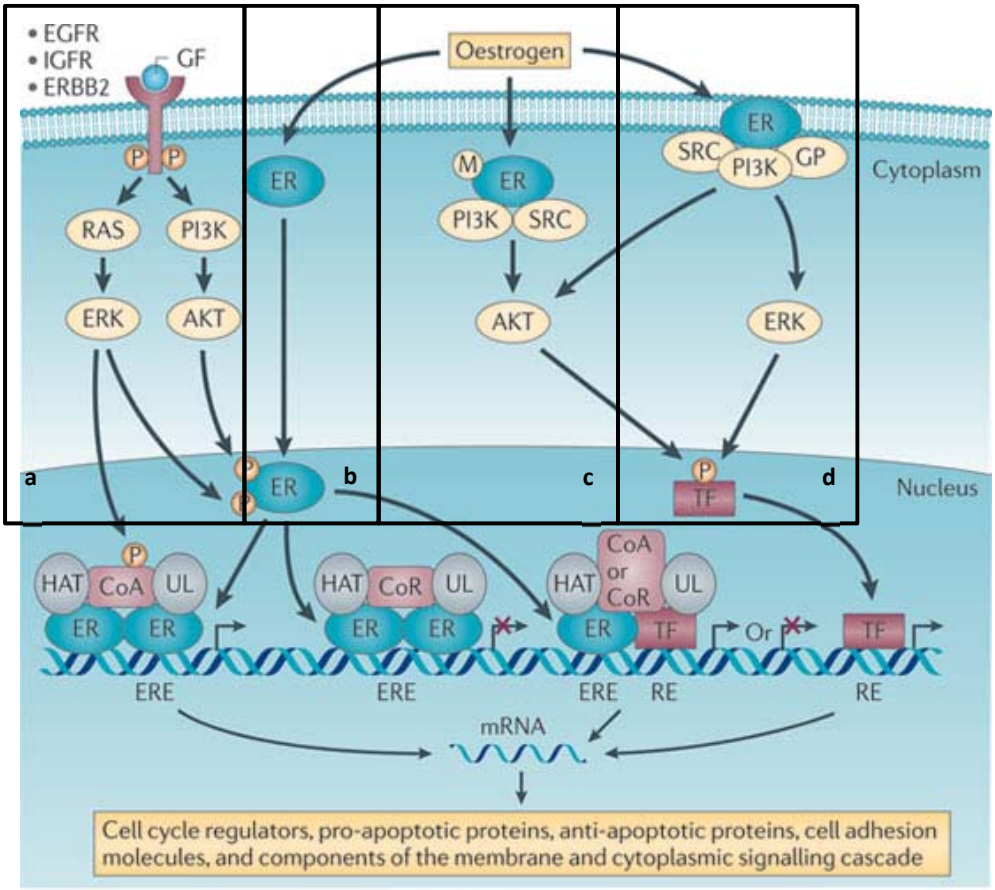


Fig.2: Different activation mechanisms of ERα (Thomas and Gustafsson, 2011)

a, ligand independent activation of ERα

b, classical activation of ERα

c, Non-genomic pathway

d, Activation by transcription factors

Since there are various ways how the ERα can be activated different mechanisms are needed to inhibit this signaling cascade in order to treat ERα+ breast cancer.

1.3.2 AROMATASE INHIBITORS

Aromatase inhibitors like Anastrozole or Letrozole are approved for estrogen receptor positive mamma-carcinoma in postmenopausal women and are used as first line treatment for this indication. They are inhibiting the biosynthesis of endogenous estrogen from androgens and are orally applicable. As side-effect an increased risk for osteoporosis needs to be mentioned. Since in pre-menopausal women estrogen is mainly produced in ovaries it is only suitable to treat postmenopausal women [Fig.3] (Aktories, 2009).

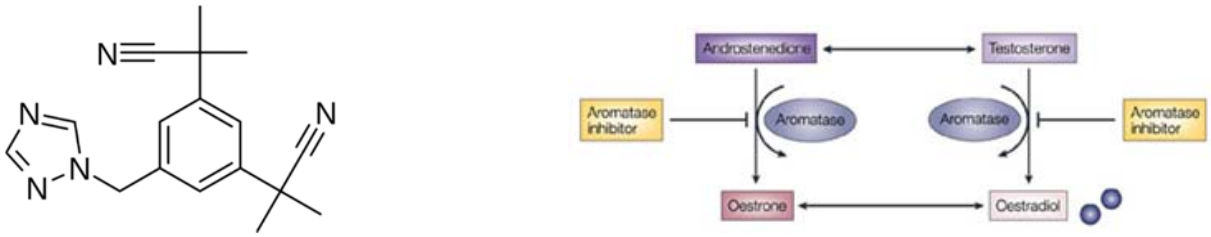


Fig.3: a,Chemical structure of Anastrozole (Plourde et al., 1994) , b, Mechanism of action of aromatase inhibitors (Johnston and Dowsett, 2003)

1.3.3 ESTROGEN RECEPTOR ANTAGONISTS

Another possible therapy for ERα+ breast cancer is an anti-estrogen treatment with drugs like Fulvestrant which is a selective estrogen receptor antagonist and thus decreases the amount of estrogen receptors. Therefore these kinds of drugs are also called selective estrogen receptors down-regulators (SERD) (Aktories, 2009). It is approved by the FDA for hormone-receptor positive breast cancer in post-menopausal women.

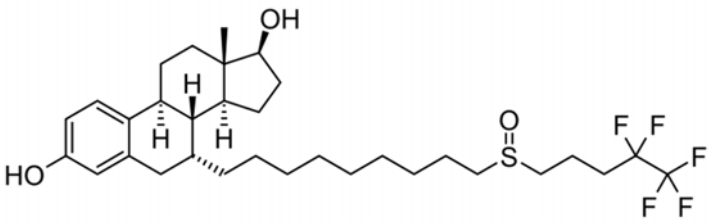


Fig.4: Chemical structure of Fulvestrant (Wakeling and Bowler, 1992)

1.3.4 SELECTIVE ESTROGEN RECEPTOR MODULATORS

Tamoxifen on the other hand is a selective estrogen receptor modulator (SERM) since it acts as a mixed agonist/antagonist on the estrogen receptor. Whereas it has an agonistic effect on estrogen receptor in bone tissue and thus prevents women from osteoporosis, it works as an antagonist on estrogen receptor in breast cancer tissue where it inhibits tumor growth in estrogen dependent breast cancer. There, its active metabolite hydroxytamoxifen is a competitive antagonist of the activation function 2 (AF-2) which is ligand dependent. AF-1 on the other hand is constitutively activated. In some tissues like bone or endometrium it is sufficient for the complete transcriptional capability if AF-1 is activated [Fig.6]. In breast cancer however both activation functions need to bind estradiol to develop their whole transcriptional activity. Moreover bound Tamoxifen leads to recruitment of co-repressors and thus inhibits transcription.

This compound is approved by the FDA since 1977 for early and advanced estrogen receptor positive breast cancer in pre- and postmenopausal women (Aktories, 2009).

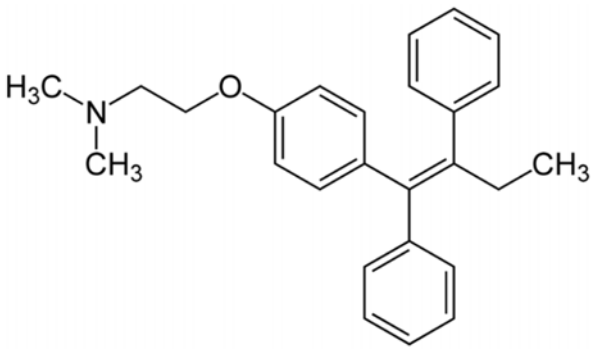


Fig.5: Chemical structure of Tamoxifen (Jordan, 2007)

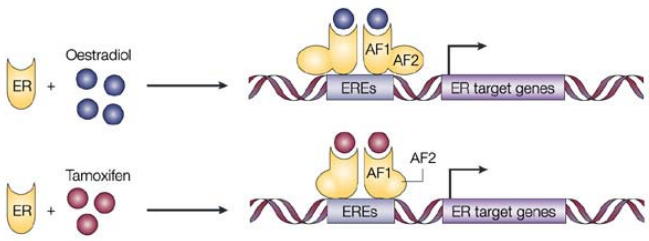


Fig.6: Mechanism of action of Tamoxifen (Johnston and Dowsett, 2003)

1.4 RESISTANCE DEVELOPMENT TO ENDOCRINE THERAPY

Although targeted treatment has many advantages, heterogeneity of tumors and the development of drug resistance remains the major obstacle in cancer therapy. Therefore single treatment with specific drugs is often not eradicating the tumor completely and cancer cells with different gene expression patterns survive. This fact causes relapse of breast cancer.

In estrogen receptor positive breast cancer there are three major reasons for resistance to anti-estrogen therapy and recurrence of tumors.

First of all a general resistance mechanism of cancer cells to overcome cytotoxicity is the overexpression of multidrug-resistance protein 1 (MDR-1). This glycoprotein belongs to the ATP-binding-casset (ABC) transporter superfamily and leads to increased efflux of chemotherapeutics, xenobiotics, immunosuppressive agents and steroids. The group of Teft et al. figured out that Endoxifen, the active metabolite of Tamoxifen, is a substrate of MDR-1 (Teft et al., 2011). They showed that MDR-1 deficient mice had increased Endoxifen concentrations in their brains. This indicates according to high expression levels of MDR-1 in the blood-brain barrier that Endoxifen usually is exported from brain by this ABC-transporter. Since MDR-1 is also overexpressed in many breast cancers it is one putative mechanism to overcome targeted treatment.

Another possibility to survive targeted therapy is the heterogeneity of breast tumors. Especially so called cancer stem cells (CSC) which display a small subpopulation in cancers are not affected by endocrine therapy since they are estrogen receptor negative. Further characteristics are high levels of CD44, low levels of CD24 and increased expression of ALDH, Vimentin as well as decreased E-cadherin levels (Charafe-Jauffret et al., 2010; Gupta et al., 2009). Moreover Nanog, Sox2 and OKT4 are specific stem cell markers and were shown to be overexpressed in CSC-like cells (Ben-Porath et al., 2008; Dontu, 2008). These cells are able to overcome chemotherapy as well as targeted therapy and thus give rise to new tumors which are often more resistant to previous treatment and show more aggressive as well as invasive behavior. Moreover they lead to a poor prognosis for breast cancer patients.

Finally, in ER-positive tumors the expression of growth factor receptors like EGFR, Her2 and Her3 has a huge impact on the sensitivity to endocrine therapy because of the ligand independent activation pathway of ERα [Fig.2a] (Garcia-Becerra et al., 2012; Nahta and O'Regan, 2012; Shou et al., 2004). It was shown that Tamoxifen treatment causes an increase in Her2 and Her3 levels, a mechanism of breast cancer cells to overcome SERM-induced inhibition of the classical activation pathway of ERα. Moreover Tamoxifen mimics the function of estradiol in ER+/Her2+ breast cancers like Luminal B and therefore activates the ERα-signaling cascade leading to increased proliferation rate (Pietras et al., 1995). The group of Moi *et al*. discovered furthermore that tumors of mice treated with Tamoxifen for 14 days had increased Her2 and Her3 levels compared to control (Moi et al., 2012). They showed a significant correlation between steroid receptor coactivators (SRCs) and Her2 as well as Her3. In breast cancer patients high levels of SRCs are leading to decreased disease-free survival time and resistance to endocrine therapy (McBryan et al., 2012; Redmond et al., 2009). These are possible reasons for the *de novo* resistance of Luminal B breast cancer to Tamoxifen treatment and acquired resistance in Luminal A breast cancer. Taken together this mechanism indicates that single SERM treatment is able to cause worse outcome for breast cancer patients. Therefore a combinatorial treatment with a drug inhibiting the ligand independent activation of ERα is necessary to overcome resistance to endocrine therapy.

1.5 SALINOMYCIN, A NOVEL DRUG TO OVERCOME TREATMENT RESISTANCE

According to recent publications Salinomycin is a novel drug to overcome MDR-1 mediated resistance and to eradicate CSC (Fuchs et al., 2010; Gupta et al., 2009). Therefore Salinomycin as additive to SERM treatment is a possible new approach in breast cancer treatment. It is isolated from *Streptomyces albus* and is already used in veterinarian medicine as antibacterial and anticoccidiosis drug (Danforth et al., 1977; Miyazaki et al., 1974). Moreover Salinomycin acts as a potassium ionophore in different biological membranes and promotes efflux of potassium as well as other monovalent alkali ions from mitochondria and cytoplasm (Mitani et al., 1975).

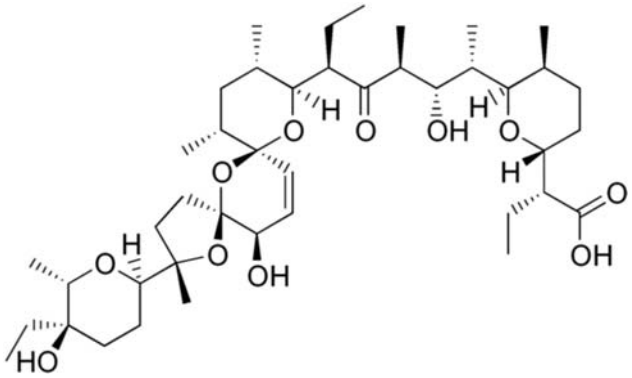


Fig.7: Chemical structure of Salinomycin (Miyazaki et al., 1974)

The group of Fuchs *et al.* discovered that Salinomycin is able to overcome apoptosis resistance and MDR-1 mediated multi-drug resistance in human leukemia stem cell-like KG-1a cells (Fuchs et al., 2010; Fuchs et al., 2009). They showed that Salinomycin induced apoptosis in this resistant cell line also in co-incubation with cyclosporine A, an inhibitor of ABC-transporters. Moreover they figured out that cultivation of these cells in Salinomycin for 12 weeks did not induce resistance to this ionophore. Therefore one could suggest that Salinomycin is a promising drug to overcome multi-drug resistance also in breast cancer.

Additionally, Gupta *et al.* figured out that Salinomycin selectively depletes CSC-like breast cancer cells (Gupta et al., 2009). They induced epithelial to mesenchymal transition (EMT) in HMLER breast cancer cells via inhibition of *CDH1* gene, which encodes E-cadherin a marker for epithelial cancer cells and sorted for $CD44^{high}/CD24^{low}$ cells. The amount was increased in EMT induced HMLER cells compared to normal ones indicating that they possess more CSC-like properties. Afterwards these cells were treated with different drugs whereas Salinomycin decreased this cell population and Paclitaxel, which is often used in breast cancer therapy, increased this population. Furthermore they discovered that Salinomycin treated cells showed less tumorsphere forming potential than control cells. They were also able to point out that Salinomycin pre-treated tumor cells are less likely to build tumors in mice, metastasize or build lung nodules than paclitaxel pre-treated cancer cells.

These are promising properties to overcome treatment resistance and therefore a new way to eradicate Luminal A and B breast cancer.

1.6 AIM OF THIS STUDY

Luminal A and B breast cancer is usually treated with Tamoxifen as first-line drug in order to inhibit the ERα signaling cascade and thus blocking cancer progression. Nevertheless it was shown that resistance to endocrine therapy prevents breast cancer from cell death and thus increases the relapse frequency. Two of the major resistance mechanisms to endocrine-therapy in Luminal A and B breast cancer can be addressed by the novel drug Salinomycin. Moreover its ionophoric properties are known to damage cell membranes and may thus also hamper the receptor tyrosine kinase signaling since they are located at the cell surface. MDR-1 as well as CD44 - both resistance marker, which are sensitive to salinomycin treatment - are located at cell surfaces and associated to cell membranes like the receptor tyrosine kinases Her2 and Her3. In these cases the ionophoric properties of Salinomycin seem to play an important role since it changes membrane gradients and may thus alter the function of membrane located receptors.

We chose different cell lines classified as Luminal A or Luminal B breast cancer since their only difference is the expression level of Her2 to investigate the effect of Salinomycin. Therefore cells were treated with Tamoxifen and Salinomycin in single as well as in combined manner to examine their influence on cell viability, on protein levels of Her2, Her3 as well as their downstream targets ERK 1/2, AKT and ERα and on RNA-levels of Her2, Her3 as well as ERα.

To further investigate the clinical impact of these drugs we also treated a more realistic tumor model so called mammospheres, spherical cancer cells that grow in suspension culture, which resemble the heterogeneity of tumors *in vivo*.

Thus the aim of our study was to figure out whether Salinomycin inhibts the Her2/Her3 crosstalk with ERα and has therefore a beneficial effect on ERα-positive breast cancer treatment in combination with Tamoxifen.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CELL CULTURE

Cell lines

Name	Origin	Type	Provider
MCF-7	Homo sapiens	Luminal A breast cancer	DKFZ
MCF-7/Her2	Homo sapiens	Luminal B breast cancer	Dennis J. Slamon (Pegram et al., 1997)
BT-474	Homo sapiens	Luminal B breast cancer	ATCC
T-47D	Homo sapiens	Luminal A breast cancer	ATCC

Media

Name	Supplements	Provider	Purpose
DMEM	20% FCS, 1% Sodium Pyruvate	lifeTechnologies™	MCF-7
RPMI	10% FCS, 1% Glutamine	lifeTechnologies™	MCF-7/Her2
RPMI	10% FCS, 1% Glutamine, 5µg Insulin	lifeTechnologies™	BT-474, T47D
Mammacult media DMEM/Hams	30% Glucose 1M Hepes Progesterone (4000x) Putrescine (100x) B-27 Growth supplement (100x) EGF 100 µg/ml FGF 100 µg/ml ITSS (1000x) Heparin 25mg/ml 7,5% NaHCO3	SigmaAldrich™ SigmaAldrich™ SigmaAldrich™ SigmaAldrich™ Gibco SigmaAldrich™ Roche SigmaAldrich™ Invitrogen Gibco	Mammosphere formation

Solutions

Name	Ingredients	Provider	Purpose
PBS	137 mM NaCl 27 mM KCl 80 mM Na_2HPO_4 1.5 mM KH_2PO_4 pH 7.4	SigmaAldrich™ SigmaAldrich™ SigmaAldrich™ SigmaAldrich™	Washing-Buffer
1x Trypsin		GE-Healthcare	Cell detaching
Glutamine		GE-Healthcare	Media supplement
Sodium Pyruvate		lifeTechnologies™	Media supplement
Fetal calf serum (FCS)		lifeTechnologies™	Media supplement
Polyhema		SigmaAldrich™	Mammosphere formation
DMSO		SigmaAldrich™	Drug solvent

Culture dishes

Size	Provider	Material
P15 adherent	BD Falcon	Polystyrene
P10 adherent	BD Falcon	Polystyrene
P6 adherent	BD Falcon	Polystyrene
P15 non-adherent	Greiner Bio-One	Polystyrene
P10 non-adherent	Greiner Bio-One	Polystyrene
P6 non-adherent	Greiner Bio-One	Polystyrene
96-well plate	BD Falcon	Polystyrene
96-well plate (black)	Corner	Polystyrene
12-well plate	BD Falcon	Polystyrene
6-well plate	BD Falcon	Polystyrene

2.1.2 TREATMENT

Drug	Solvent	Provider	Concentration
Tamoxifen	DMSO	SigmaAldrich™	10µM
Salinomycin	DMSO	SigmaAldrich™	0,5µM
Lapatinib	DMSO	Vichem Chemie Research Ltd.	5µM

2.1.3 QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

RNA-purification

Name	Provider
RNeasy® Mini Kit	Qiagen

cDNA-synthesis

Compound	Provider
K1-Primer: **5'-AAGCAGTGGTATCAACGCAGAGTACT(30)V N-3'** **(N = A, C, G, or T; V = A, G, or C)**	BD Bioscience Clontech
dNTPs (10mM)	Roche
DTT (10µM)	Sigma
AMV Reverse transcriptase	Roche
AMV Reverse transcriptase buffer	Roche
RiboLock® RNAse inhibitor	Roche
RNAse free water	Sigma-Aldrich™
TE-Buffer 10/0,1 **10 mM Tris/HCl pH 8.0** **0.1 mM EDTA pH 8.0**	Sigma-Aldrich™
TRIS (2N)	Sigma-Aldrich™
HCl (2N)	Sigma-Aldrich™
NaOH (1N)	Sigma-Aldrich™

cDNA-purification

Name	Provider
QIAquick® PCR purification kit	Qiagen

Primer

Primer	Sequence	Provider	Concentration
ER-alpha	F: 5'-ATCCACCTGATGGCCAAG-3' R: 5'-GCTCCATGCCTTTGTTACTCA-3'	Roche	10pM
Her2	F: 5'-CACATGACCCCAGCCCTCTACAGC-3' R: 5'-CACGGCACCCCCAAAGGCAAAAAC-3'	Eurofins MWG Synthesis GmbH	10pM
Her3	F: 5'-CTCCGCCTCAGCCTACCAGTT-3' R: 5'-TGCTCCGGCTTCTACACATTGACA-3'	Eurofins MWG Synthesis GmbH	10pM
hHPRT	F: 5´-TGACACTGGCAAAACAATGCA-3´ R: 5´-GGTCCTTTTCACCAGCAAGCT-3´	Eurofins MWG Synthesis GmbH	10pM
Cyclophilin A	F:5'-GGCAAATGCTGGACCCAACACAAA-3' R: 5'-CTAGGCATGGGAGGGAACAAGGAA-3	Eurofins MWG Synthesis GmbH	10pM

Kit

Name	Provider
Fast SYBR®Green Masster-Mix	Applied Biosystems

qPCR-System

Name	Provider
StepOnePlus™	Applied Biosystems

2.1.4 WESTERN BLOT

Primary antibodies

Antibody	Origin	Type	Dilution	Provider
ER alpha	Rabbit	Polyclonal	1:1000	Santa Cruz
p-Her2	Rabbit	Monoclonal	1:1000	Millipore
Her2	Rabbit	Polyclonal	1:1000	Millipore
p-Her3	Rabbit	Monoclonal	1:500	Cell signaling
Her3	Mouse	Monoclonal	1:1000	Millipore
p-AKT	Rabbit	Polyclonal	1:1000	Cell Signaling
AKT	Rabbit	Polyclonal	1:1000	Santa Cruz
p-ERK 1,2	Rabbit	Polyclonal	1:2000	Cell signaling
ERK 1,2	Rabbit	Polyclonal	1:2000	Santa Cruz
PARP	Rabbit	Polyclonal	1:1000	Cell signaling
Tubulin	Mouse	Monoclonal	1:10000	Sigma

Secondary antibodies

Antibody	Origin	Type	Dilution	Provider
Anti-mouse	Goat	Monoclonal	1:15000	Sigma
Anti-rabbit	Goat	Polyclonal	1:15000	Bio-Rad

Solutions

Agent	Ingredients/Provider
Protein Lyis-Buffer	50mM HEPES (N-(2-Hydroxyethyl) piperazine-N-(2-ethanesulfonic acid)) pH 7,5 150mM NaCl 1,5mM MgCl 1mM EGTA 10% (V/V) Glycerol 1% (V/V)Triton X 100 10mM $Na_4P_2O_7$ 100mM NaF 40µM Na_3VO_4 1µg/ml Aprotinin 1mM PMSF (Phenylmethanesulfonyl fluoride)
Lämmli-Buffer	100 mM Tris/HCl pH 6.8 3% SDS 45% Glycerol 0.01% Bromphenol blue 7.5% β-Mercaptoethanol
Blocking Solution/Washing Buffer NET-Gelatin	50 mM Tris/HCl pH 7.4 5 mM EDTA 0.05% Triton X-100 150 mM NaCl
Running-Buffer	25 mM Tris/HCl pH 7.5 200 mM Glycine 0.1% SDS
Blotting-Buffer	50 mM Tris/HCl pH 7.5 40 mM Glycine 20% Methanol 0.004% SDS
Marker	Page Ruler*Plus Prestained Protein Ladder
Ponceau solution	5% Ponceau S in 50% Trichloroacetic acid
Strip-Buffer	62.5 mM Tris/HCl pH 6.8 2% SDS 100 mM β-Mercaptoethanol
Lower-TRIS	1,5 mol TRIS/HCl pH 8,8 0,4% SDS
Upper-TRIS	0,5 mol TRIS/HCl pH 6,8 0,4% SDS
APS (Ammonium peroxodisulfate)	10% in water
Bovine serum albumin (BSA)	Thermo Scientific
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Sigma-Aldrich™

Kit

Name	Provider
Pierce BCA-Assay	Thermo Scientific
ECL/ECL Prime	GE Healthcare

X-Ray Film

Name	Provider
Amersham Hyperfilm™ ECL	GE-Healthcare

2.1.5 CellTiter-Glo® Assay

Name	Provider
CellTiter-Glo® Luminescent Cell Viabilty	Promega

2.2 METHODS

2.2.1 CELL CULTURE

2D cell culture

The different cell lines were cultured according to provider's protocol in growth media at 37°C and 5% CO_2. To harvest them the medium was removed, the cells were washed once with PBS and afterwards trypsinized with 1x Trypsin/EDTA.

After that the collected cells were transferred to a falcontube and centrifuged at 1200 rpm for 5 min. The supernatant was discarded, cells were resuspended in media and seeded in new plates.

3D cell culture

For mammosphere formation cells were seeded in non-adherent culture dishes and grown in mammacult medium for at least 72h. Mammospheres were collected and centrifuged at 1200 rpm for 5 min.

2.2.2 TREATMENT

2D cells were treated at a confluence of 80%, 3D after at least 72h. In 96-well plates as well as in 3D cell culture drugs were added without previous media change. In every other 2D cell culture media was changed before treatment.

As control we used the same amount of drug solvent as in the highest concentration.

2.2.3 QUANTITATIVE POLYMERASE CHAIN REACTION

RNA-lysis, RNA-purification, c-DNA synthesis and c-DNA purification

Cells were cultured in P15 adherent or non-adherent petridishes and treated at a confluence of 80% for 72h. In 2D conditions the medium was removed properly and cells were trypsinized without previous washing steps.

Mammospheres as well as 2D cells were then transferred to falcontubes and centrifuged at 1200 rpm for 5min. The supernatant was discarded, the tubes put on ice and the cell pellet resuspended in 350µl RLT-Lysisbuffer of the RNeasy® Minikit containing 1% β-Mercaptoethanol. After 10 min. the lysates were vortexed and transferred to Eppendorf caps. According to protocol 350µl 70% ethanol were added and the samples immediately put on the columns. After that the tubes were centrifuged at 13000xg for 30 sec and eluent was discarded.

The precipitated RNA was then washed once with 350µl RW1 buffer and afterwards 70µl DNAse-Mix containing RNAse-free water, RDD-buffer and RNAse-free DNAse was added to remove genomic DNA. Columns were centrifuged at13000xg for 30 sec. after 15 to 20 min. incubation on ice and again washed with 350µl RW1-buffer.

Finally the RNA samples were washed twice with 500µl RPE buffer and then eluted with 30µl RNAse-free water. The concentration was measured via Nanodrop. The samples were frozen at -80°C or directly used for c-DNA synthesis.

Therefore 1000ng total RNA were adjusted to a volume of 7,5 µl with RNAse-free water and 1 µl K1-primer was added. To anneal the primer the samples were heated to 70°C for 3 min., put on ice, vortexed and centrifuged. Afterwards deoxynucleotides (dNTPs), AMV reverse transcriptase, AMV reverse transcriptase buffer and dithiotreitol (DTT) were added. According to protocol samples were vortexed and heated at 42°C for 2h to create c-DNA. To stop this reaction 80µl Tris/EDTA 10/0,1 was added and samples were incubated at 72°C for 7 min. Additionally 10µl 1N NaOH were added to denature cDNA and the mixture was kept for 20 min. at 68°C.

After that the samples were neutralized with 5µl 2N HCl and 5µl 2M Tris-HCl. To purify the c-DNA the QIAquick® PCR purification kit was used. According to manufactory's protocol the columns were loaded with a mixture containing the c-DNA samples and 550µl PB-Buffer. After centrifugation the c-DNA was washed twice with PE-buffer and finally eluted with 30µl Elutionbuffer.

Quantitative polymerase chain reaction (qPCR)

The polymerase chain reaction is a standard method in molecular biology to exponentially amplify short DNA-sequences. The quantitative polymerase chain reaction (qPCR), also known as real-time polymerase chain reaction (RT-PCR) is based on the equal principle as standard PCR but is furthermore able to quantify DNA while amplifying it.

The PCR process consists of different steps: It starts with the denaturation of double-stranded DNA into two single strands at 95°C and continues with the annealing of primers – two oligonucleotides comprising 15 to 25 bases – to the complementary target DNA sequences at a lower temperature of 50 to 60°C. Finally during elongation a heat-resistant DNA-polymerase together with deoxynucleotides generate a new DNA strand complementary to the template strand.

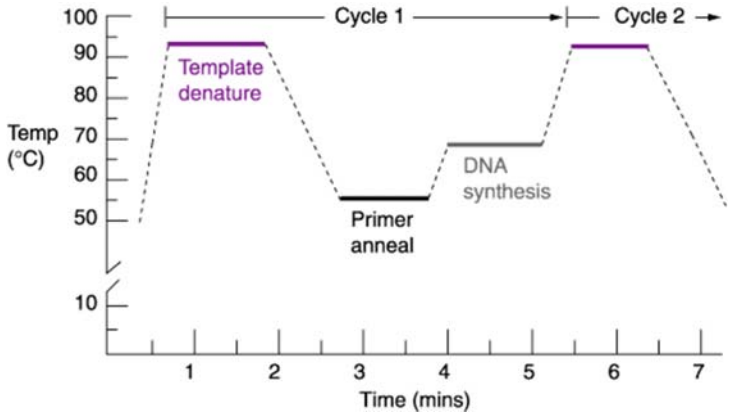


Fig.8: Principle of qPCR (arizona, 2001)

The common polymerase used in PCR is the Taq-Polymerase originating from the organism *Thermus aquaticus*, a thermophilic bacterium that lives in hot springs and reaches its working optimum at around 72°C. Because of that this is also the preferred elongation temperature. Thus the first amplifying cycle is finished and second one can be started.

To quantify the amplified c-DNA the SYBR® Green Master-Mix was used. SYBR® Green is asymmetrical cyanine dye that binds to double stranded DNA and thus forms a DNA-dye complex that emits green light.

Since the c-DNA double strands are separated during the heating step (template denature) there is no detectable signal in the beginning. After the primer anneal the polymerase chain reaction leads to an increasing amount of double stranded DNA and thus the emitted light can be measured (van der Velden et al., 2003b).

For this experiment we used a mixture containing 10 µl SYBR® Green Master-Mix, 0,5 µl forward primer as well as reverse primer, 3,3 ng c-DNA and 7 µl sterile water per sample which were analyzed in triplicates. We ran 40 cycles and the temperature protocol was as follows:

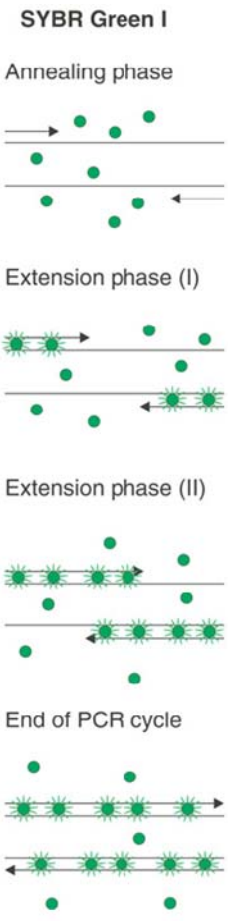


Fig. 9: Function of SYBR®-green (van der Velden et al., 2003a)

	Holding stage	**Cycling stage**		**Melt curve**		
	Step 1	Step1	Step2	Step1	Step2	Step3
Ramp rate [%]	100,0	100,0	100,0	100,0	100,0	
Temperature [°C]	95,0	95,0	60,0	95,0	60,0	95,0
Time [min.]	00:20	00:03	00:30	00:15	01:00	00:15
Temp. Increment [°C]						0,3

Tab.2: Temperature program of qPCR

To quantify the results the $2^{-\Delta\Delta CT}$-value was calculated whereas the ΔCT- value represents the difference of CT-values of gene of interest and housekeeping gene and the ΔΔCT-value the difference of ΔCT-values of treated sample and control sample. Thus the $2^{-\Delta\Delta CT}$-value represents the fold increase of gene expression.

2.2.4 Western Blot

Protein lysis

After treatment for 72h medium was discarded, monolayer cells were washed once with cold PBS (4°C) and finally 100 µl Protein-lysis-buffer were added to P15-dishes. The plates were incubated for 5 minutes on ice and afterwards cells were harvested with a scratcher. 3D cells were collected, transferred to a falcon tube and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded, the cell resuspended in protein-lysis-buffer and the tubes put on ice for 5 minutes. Finally the samples were centrifuged at 13000xg for 10 minutes to separate the protein-lysates from cell debris and after that frozen down at -20°C.

Concentration measurement

To quantify the protein concentration we used the BCA-Assay. This assay is based on the reduction of Cu^{2+} to Cu^{1+} by proteins. Therefore the protein forms a light blue chelat complex with the cupric ions in an alkaline environment containing sodium potassium tartrate. Afterwards the bicinchoninc acid (BCA) reacts with this complex and forms a purple complex containing 2 molecules BCA and one molecule cuprous ion. This complex is water soluble and exhibits a linear absorbance at 562 nm.

To calculate the protein concentration a BSA standard calibration curve with 0, 100, 200, 400, 600, 800, 1000, 1200 and 2000 µg/ml was necessary. Finally 30µg protein were taken for SDS-page, diluted 3:1 with lämmli-buffer and denatured for 5 min. at 95°C.

SDS-PAGE

To separate proteins a SDS protein acrylamide gel electrophoresis (PAGE) was used. This method is based on the separation of proteins according to their molecular weight since they are kept in a denature state. Therefore proteins got negatively charged by sodium dodecyl sulfate (SDS) and thus move to the anode through the acrylamide gel meshes. Dependent on their size small peptides can more easily penetrate the meshes whereas big proteins hardly move.

To focus the protein bands a stacking gel was used on top of the separation gel and a comb was inserted to create wells. After polymerization between to glass plates the comb was removed, gels were placed in an electrophoresis chambers and filled with running buffer. Then protein samples as well as marker were added and gels were run at 125 V for ca. 1,5h.

Separation gel: (10% Acryl amide)

- 8,1 ml H_2O
- 5 ml 4x Lower TRIS-Buffer
- 6,65 ml Acryl amide
- 15 µl TEMED
- 150 µl APS

Stacking gel:

- 4,7 ml H_2O
- 1,62 ml 4x Upper TRIS-buffer
- 0,9 ml Acryl amide
- 6,5 µl TEMED
- 65 µl APS

Blotting

In order to make proteins detectable by antibodies they were blotted to a nitrocellulose membrane via Wet Blot. Therefore a “sandwich” containing two buffer-soaked filter papers, the acryl amide gel and one nitrocellulose membrane was placed in a blotting chamber, the cooling unit was added and the chamber was filled with TransBlot-buffer. To transfer the proteins we used 400 mA for one hour. Due to the current negatively charged proteins are pushed out of the gel into the membrane from cathode to anode. The proteins are bound to the membrane by hydrophobic interactions and detected by Ponceau S staining.

Therefore the membrane was incubated with a solution of Ponceau S, a diazo dye, for a few minutes. Afterwards membranes were washed in blocking solution for one minute and cut according to the proteins of interest.

Blocking

For blocking unspecific antibody binding sites the membrane was incubated at least for 3 h in 1x NET-gelatin.

Detection

In order to detect the proteins the nitrocellulose membrane needs to be incubated with a specific primary antibody which binds to the protein of interest and afterwards with a horseradish peroxidase labeled secondary antibody that is able to bind the free Fc-fragment of the primary antibody. The horseradish peroxidase can then be detected by enhanced chemiluminescence (ECL) reagent mix on X-ray films.

Therefore we incubated nitrocellulose membranes with primary antibody diluted in 1xNET-Gelatin over night at 4°C. Afterwards the Blots were washed three times for 10 minutes with 1xNET-Gelatin and incubated with secondary antibody diluted in 1xNET-Gelatin for 1h. Finally the membrane was again washed three times for 10 minutes with 1xNET-Gelatin, incubated with ECL-reagent mix for one min. and films were developed with an exposure time between 10 sec and 20 min, depending on the protein expression.

2.2.5 CellTiter-Glo® Assay

To detect the amount of viable cells among the treated ones a CellTiter-Glo® Luminescent Cell Viability Assay was performed. This kit uses the enzyme luciferase which needs ATP to generate a light signal. Since only cells with metabolic activity produce ATP the luminescence correlates with the number of viable cells.

Therefore we seeded cells in 96-well plates with black walls and clear bottom in 100µl medium. Afterwards 100µl CellTiter-Glo®-reagent mix were added to medium and incubated for 10 min. at room temperature. Finally plates were analyzed with a fluorescence detector.

2.2.6 MAMMOSPHERE FORMING POTENTIAL

In order to examine the mammosphere forming potential of cells after certain treatment we pre-treated cells with our compounds of interest for 72 h and afterwards seeded 10000 c/w in Polyhema-coated 96-well-plate in triplicates. After 96h incubation in absence of drugs at 37°C and 5% CO_2 microscope pictures were taken in a 50x magnification. Finally mammospheres were counted.

2.2.7 MOLECULAR EVOLUTION ASSAY

This assay was performed to detect changes in morphology after several treatment rounds. Therefore cells were seeded on 12-well plates in normal growth conditions and treated with our compounds of interest at a confluence of 80% for 72h. Afterwards media was changed and after recovering cells were divided 1:2. As far as they reached a confluence of 80% they were treated again with the same drug for 72h. This was repeated six times and pictures were taken directly after treatment.

2.2.8 STATISTICAL ANALYSIS

All data are presented as average + standard deviation. Student t-test (two-tailed distribution, two-sample equal variance (homoscedastic) test) was performed for statistical evaluation using Microsoft Excel. The data were compared to DMSO treated control and differences were considered as statistically significant at $p \leq 0{,}001$ ***, $p \leq 0{,}01$ ** and $p \leq 0{,}05$ *.

3. RESULTS

3.1 CHARACTERIZATION OF CELL LINES

For further experiments and also for clinical relevance it is necessary to clearly characterize the different cell lines we used in this study. This includes cell morphology as well as the expression pattern of our genes of interest.

Type	Receptor	Treatment	Cell line	Morphology	Mammosphere formation
Luminal A	ER+/PR+/Her2-	Tamoxifen	MCF-7 T47-D	Cobblestone shape	Many, small, rough surface
Luminal B	ER+/PR+/Her2+	Tamoxifen Lapatinib Trastuzumab	MCF-7/Her2 (Pegram et al., 1997) BT-474	More spindle-shaped	Few, big, smooth surface

Table 3: Characterization of cell lines

3.1.1 MORPHOLOGY

To investigate the morphology of different cell lines microscope picture were taken.

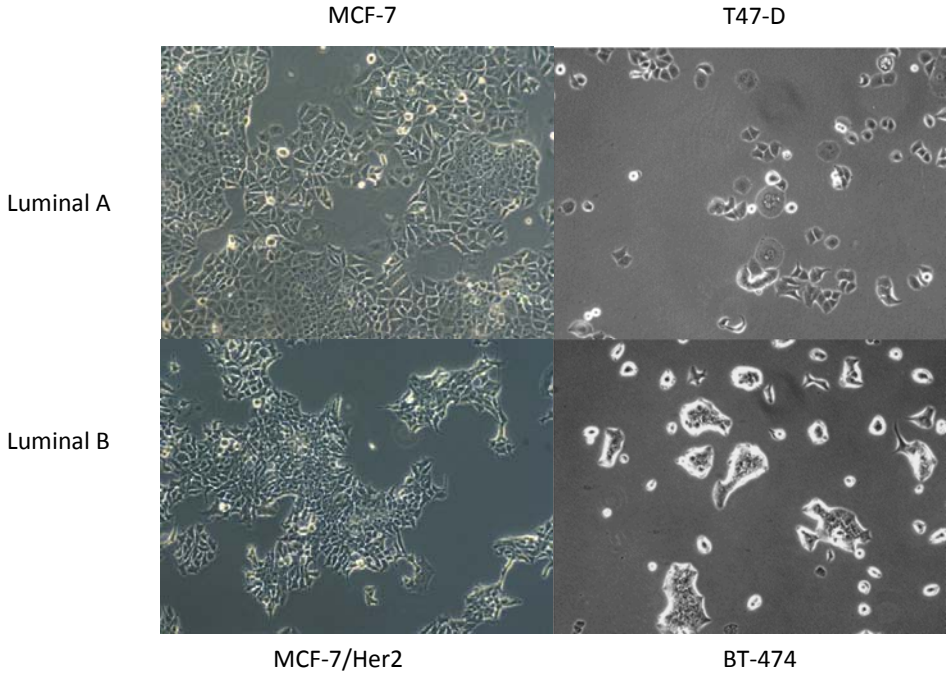


Fig.10: Microscope pictures of MCF-7, T47-D, MCF-7/Her2 and BT-474 in 100x magnification

These pictures show that both Luminal A breast cancer cell lines MCF-7 and T47-D possess a cobblestone like shape and grow in a dense layer. The Luminal B breast cancer cell lines MCF-7/Her2 and BT-474 however show more spindle shaped morphology, grow in clusters and are smaller compared to Luminal A breast cancer cell lines [Fig10].

3.1.2 CLASSIFICATION

Since the ligand independent activation pathway was of special interest in this study the expression levels of Her2, Her3 and ERα in our four cell lines were investigated by microarray analysis.

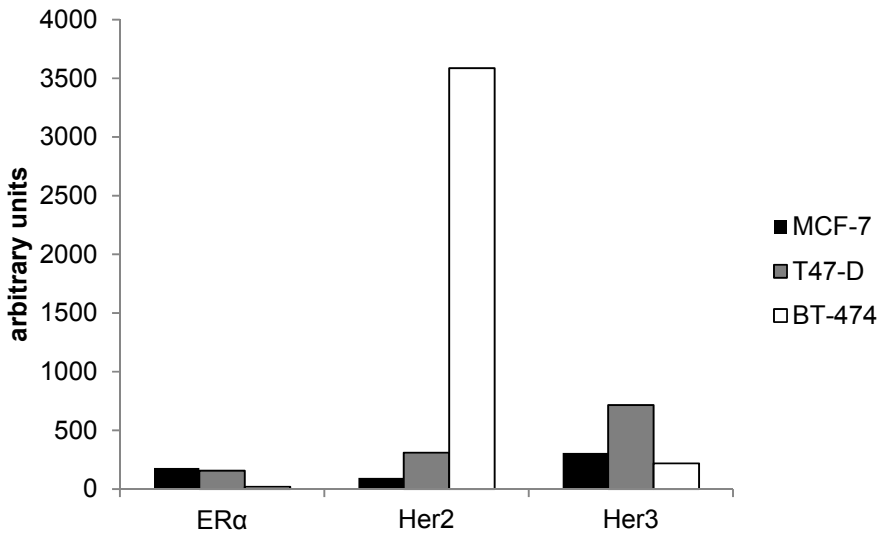


Fig.11: Expression of ERα, Her2 and Her3 in different cell lines. These data were a kind gift of Dr. Pjotr Knyazev

The array data show that ERα is highly expressed in Luminal A breast cancer cell lines. Moreover Her2 and Her3 are as well present in a high level in these cell lines whereas Her3 is even overexpressed in T47-D. BT-474, a Luminal B breast cancer cell line on the other hand expresses lower levels of ERα but Her2 is overexpressed. Also Her3 is highly expressed in this cell line. Therefore these cells display a model to investigate the ligand independent activation of ERα.

3.2 Effects of single Tamoxifen and Salinomycin treatment on monolayer cell lines

3.2.1 Salinomycin is more effective against Luminal B cancer cells than Luminal A

The cytotoxic effect of Salinomycin and Tamoxifen on Luminal A and B breast cancer cell lines is important for clinical relevance. Therefore the cell viability of breast cancer cells upon treatment was investigated.

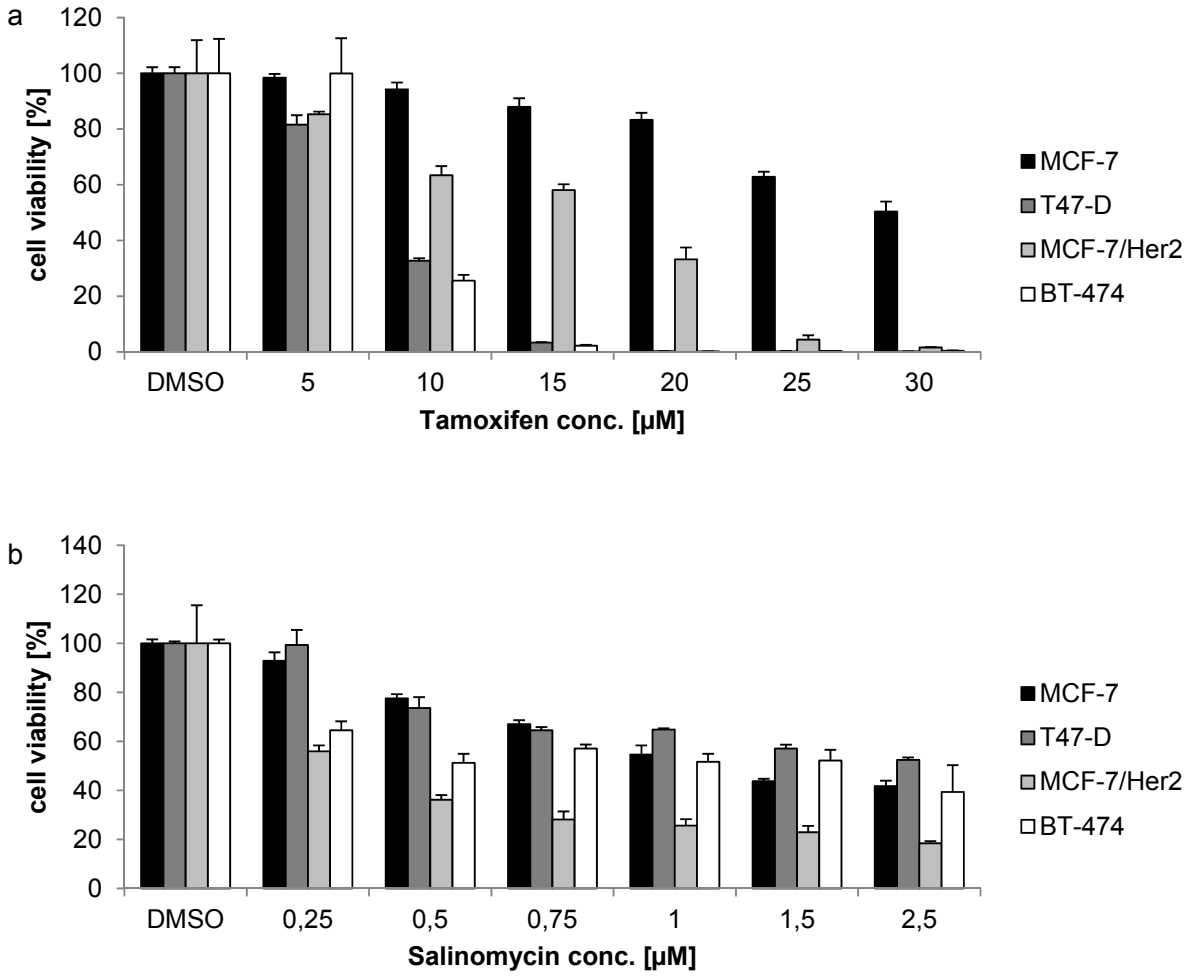


Fig.12: Cell viability measured by CellTiter-Glo® Assay after 72h treatment with a, Tamoxifen or b, Salinomycin. 2500c/w were seeded in 96-well plates and treated 24h after seeding for 72h.

After Tamoxifen treatment there is no significant difference between Luminal A and Luminal B cancer cells visible. Both MCF-7 and MCF-7/Her2 are rather resistant to Tamoxifen therapy whereas T47-D as well as BT-474 are more sensitive [Fig.12a].

On the other hand regarding the Salinomycin treatment both Luminal B cancer cell lines are more sensitive compared to Luminal A especially in the lower concentrations [Fig.12b].

3.2.2 Tamoxifen induces, Salinomycin blocks the ligand independent activation of ER-alpha

To investigate the effect of both drugs on the ligand independent activation of ERα a Western Blot and a qPCR analysis were performed. Since this pathway is driven by the receptor tyrosine kinases Her2 and Her3 their phosphorylation and expression status were of special interest. Moreover their down-stream targets ERK 1/2, AKT and ERα were investigated. It needs to be mentioned that MCF-7 and MCF-7/Her2 cells were analyzed at the same X-ray film and therefore the expression levels of different proteins are comparable. qPCR analysis was performed to examine changes in the RNA-level of ERα, Her2 and Her3 upon treatment and to figure out overlaps with Western Blot analysis.

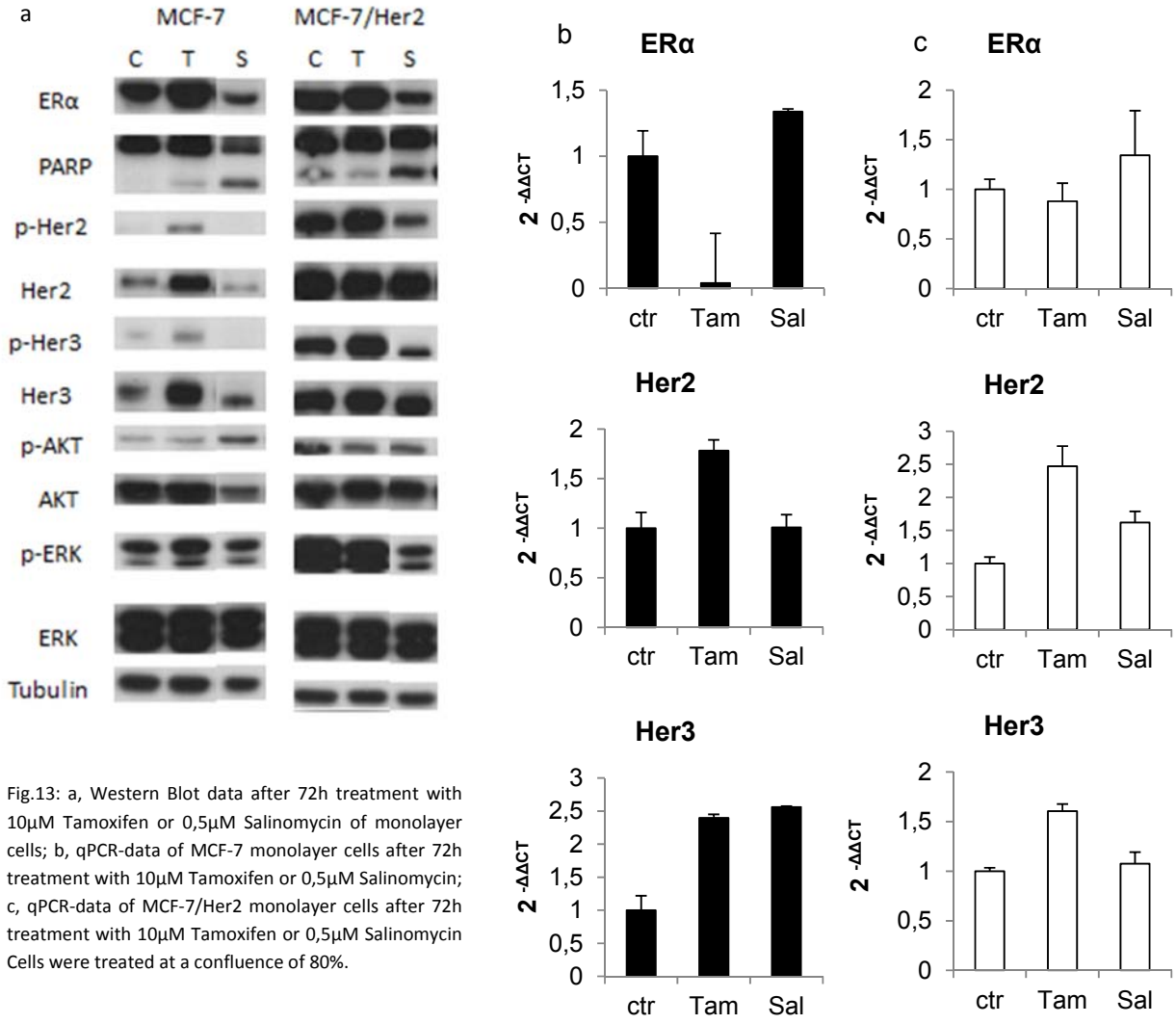


Fig.13: a, Western Blot data after 72h treatment with 10µM Tamoxifen or 0,5µM Salinomycin of monolayer cells; b, qPCR-data of MCF-7 monolayer cells after 72h treatment with 10µM Tamoxifen or 0,5µM Salinomycin; c, qPCR-data of MCF-7/Her2 monolayer cells after 72h treatment with 10µM Tamoxifen or 0,5µM Salinomycin Cells were treated at a confluence of 80%.

Regarding MCF-7 representing Luminal A it is visible in the Western Blot that Tamoxifen treatment induces the ligand independent activation of ERα: the phosphorylation and expression levels of both Her2 and Her3 are increased by Tamoxifen. Furthermore the phosphorylation rate of ERK 1/2 as a down-stream target is slightly augmented. Therefore also ERα is more expressed compared to control. Salinomycin on the other hand inhibits the escape mechanism via the ligand independent signaling pathway. Neither Her2 nor Her3 are phosphorylated upon treatment. Also the expression of both proteins is slightly decreased compared to control. Moreover ERα is less expressed in Salinomycin treated MCF-7 cells.

It has to be mentioned as well that protein levels of AKT are down-regulated whereas its phosphorylation is increased. Finally also PARP-cleavage, a marker for apoptosis, is increased after treatment with this antibiotic [Fig.13a].

Additionally the qPCR-results of MCF-7 indicate that Tamoxifen induces the ligand independent activation of ERα since the mRNA-levels of both Her2 and Her3 are increased. Nevertheless the mRNA-level of ERα is decreased upon Tamoxifen treatment and is thus not reflecting the protein status. Regarding the qPCR-data of Salinomycin treated MCF-7 cells it is visible that this drug has no effect on ERα and Her2. Her3 on the other hand is slightly up-regulated what is contradictory compared to protein levels [Fig.13b].

In MCF-7/Her2 cells both drugs have similar effects as in MCF-7. Regarding the Western Blot analysis Her2 and Her3 were found to be more phosphorylated upon SERM treatment compared to control. This indicates that Tamoxifen activates the ligand independent signaling pathway of ERα also in Luminal B cancer cells. Also Salinomycin shows a similar effect on these cells: the phosphorylation status of Her2 and Her3 are decreased upon treatment. Nevertheless the expression levels remain similar. Moreover the down-stream targets ERK 1/2 and ERα are affected by Salinomycin treatment: whereas ERK 1/2 is less phosphorylated, the protein levels of ERα are down-regulated. Finally also the amount of cleaved PARP is augmented in Salinomycin treated cells indicating that they are as well apoptotic [Fig.13a].

Moreover the qPCR-data confirm the Western Blot result. Also mRNA levels of Her2 and Her3 are slightly up-regulated upon Tamoxifen treatment. ERα on the other hand is not altered. Furthermore Salinomycin does not change the mRNA-level of Her2 and Her3 but is slightly up-regulating ERα indicating that this antibacterial drug is not hampering the RNA but protein expression [Fig.13c].

Since Salinomycin seems to antagonize the activation of the ligand independent signaling pathway of ERα, we investigated the effect of Tamoxifen and Salinomycin as a combinatorial breast cancer treatment. Additionally we examined the function of Lapatinib, a specific small-molecule inhibitor of Her2.

3.3 EFFECT OF COMBINATORIAL TREATMENT ON MONOLAYER CELLS

3.3.1 COMBINATORIAL TREATMENT ONLY BENEFICIAL IN LUMINAL A BREAST CANCER CELLS

Since the cytotoxicity of different combinatorial treatment options is important to figure out the optimal therapy, a cell viability assay was performed

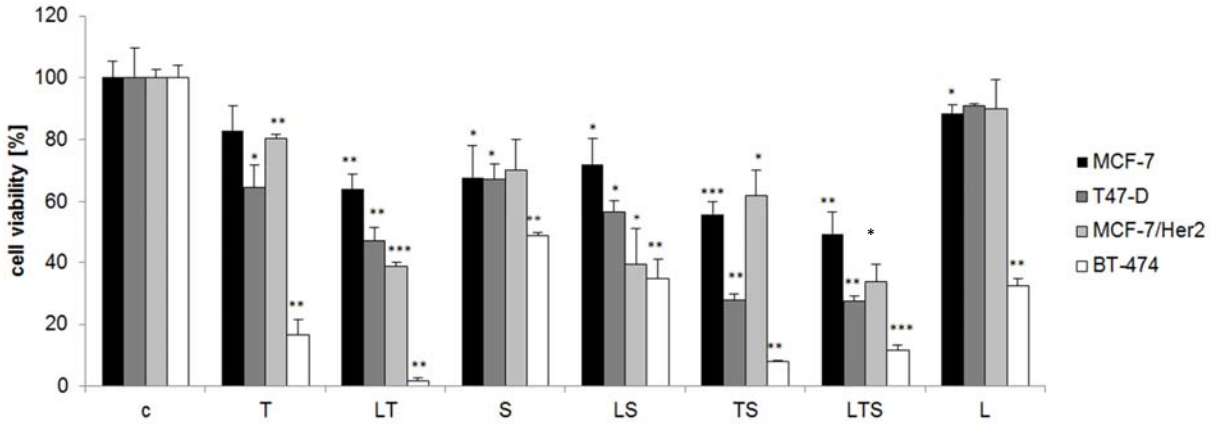


Fig.14: Cell viability measured by CellTiter-Glo®-Assay after 72h treatment with 10µM Tamoxifen, 0,5µM Salinomycin and 5µM Lapatinib. Lapatinib was added 2h before. 2500 c/w were seeded in a 96-well-plate..

Regarding both Luminal A breast cancer cell lines MCF-7 and T47-D it is obvious that the application of one single drug hardly shows a cytotoxic effect whereas the combination of Tamoxifen with Lapatinib or Salinomycin induces significant cell death. In comparison with other combinatorial treatment options the combination of Tamoxifen and Salinomycin has the most harmful effect on Luminal A breast cancer cells together with triple combination Lapatinib, Tamoxifen and Salinomycin.

The cell viability of Luminal B breast cancer cells MCF-7/Her2 and BT-474 on the other hand is already significantly decreased upon single Salinomycin treatment. In combination with Tamoxifen this antibiotic only has an additional effect on BT-474. The most prominent reduction in cell viability induces the triple combination with Lapatinib, Tamoxifen and Salinomycin in both Luminal B breast cancer cells [Fig.14].

3.3.2 Combination of Tamoxifen and Salinomycin effectively blocks ligand independent signaling

The effect of combinatorial treatment on the ligand independent activation of ERα via the receptor tyrosine kinases Her2 and Her3 as well as their down-stream targets was investigated by Western Blot and qPCR.

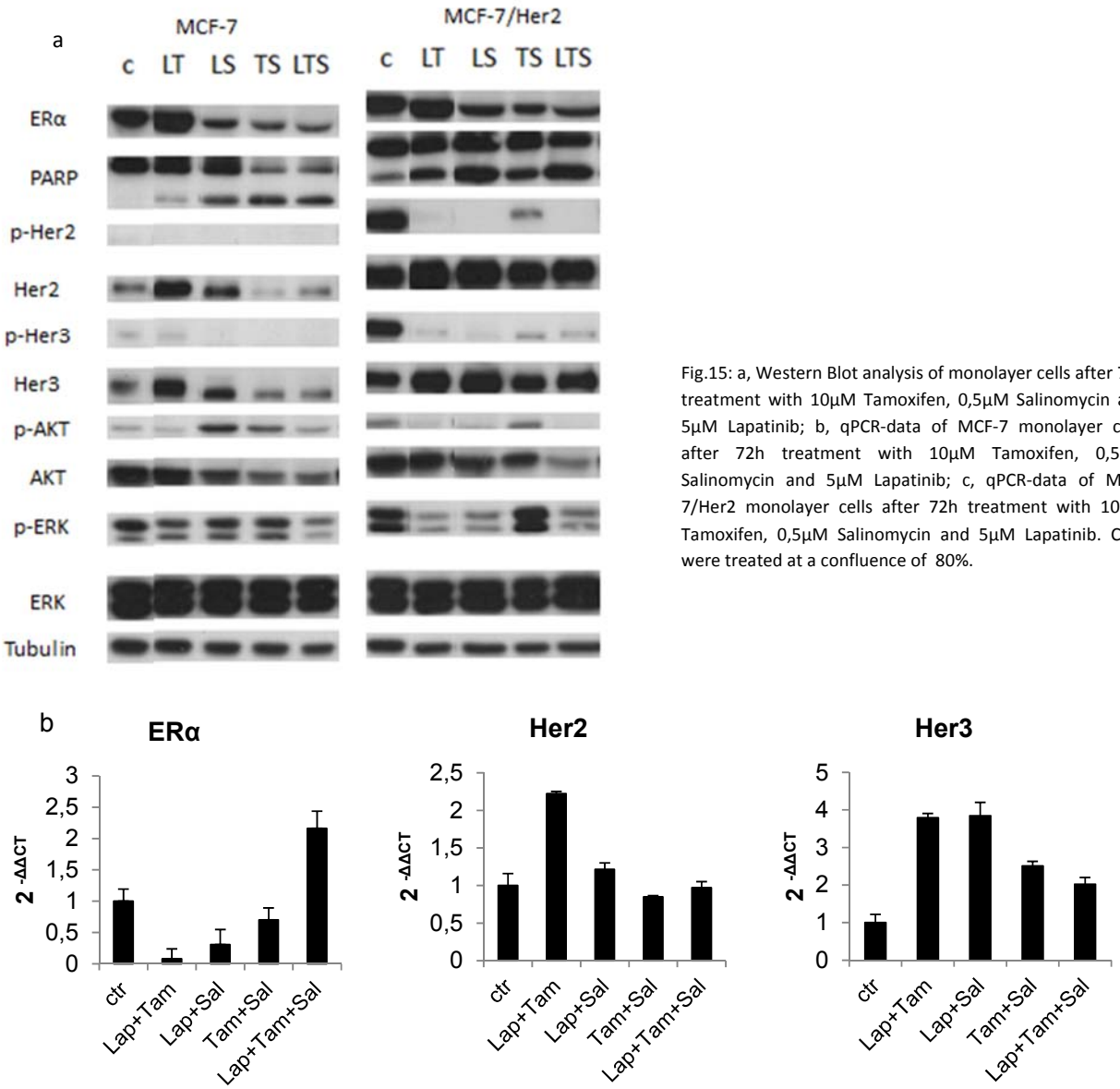
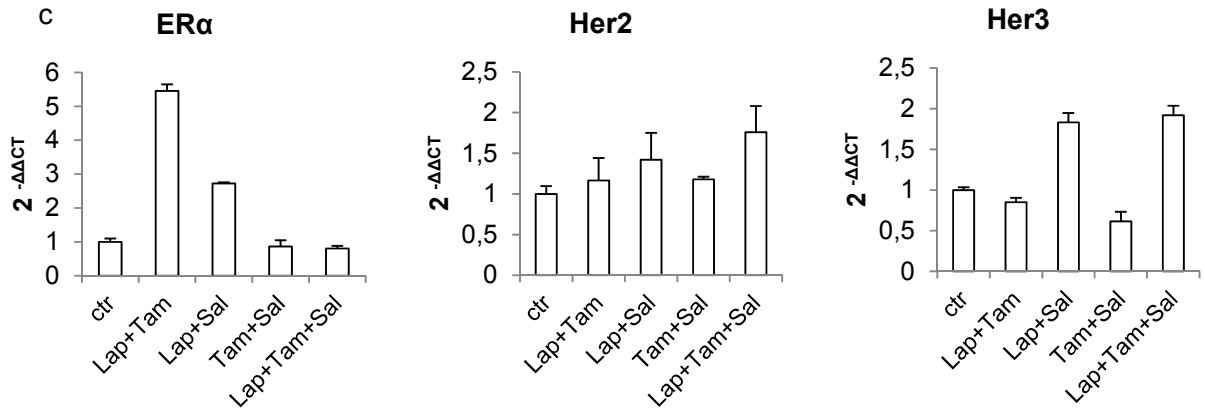


Fig.15: a, Western Blot analysis of monolayer cells after 72h treatment with 10µM Tamoxifen, 0,5µM Salinomycin and 5µM Lapatinib; b, qPCR-data of MCF-7 monolayer cells after 72h treatment with 10µM Tamoxifen, 0,5µM Salinomycin and 5µM Lapatinib; c, qPCR-data of MCF-7/Her2 monolayer cells after 72h treatment with 10µM Tamoxifen, 0,5µM Salinomycin and 5µM Lapatinib. Cells were treated at a confluence of 80%.



First of all it is obvious that Tamoxifen in combination with Salinomycin is significantly reducing the protein levels of Her2 and Her3 in a Luminal A breast cancer cell line. The combination of Lapatinib with Tamoxifen or Salinomycin on the other hand increased the expression of both proteins. Regarding the down-stream targets of both receptor tyrosine kinases the combination of Tamoxifen with Salinomycin induces a down-regulation of AKT expression whereas its phosphorylation status is increased upon treatment. Nevertheless only the triple combination of Lapatinib, Tamoxifen and Salinomycin reduces the phosphorylation of ERK 1/2 but does not alter the protein levels. Moreover it is visible in MCF-7 that combinations with Salinomycin decreased the expression of ERα on protein level. This effect seems to be induced by Salinomycin since this effect was also determined upon single treatment. Finally also the PARP-cleavage is increased after combinations with Salinomycin indicating a higher apoptosis rate [Fig.15a].

Also on RNA-level the combination of Tamoxifen and Salinomycin slightly reduces the amount of Her2. Her3 on the other hand is up-regulated. Nevertheless the expression of both receptor tyrosine kinases is mostly increased upon Lapatinib plus Tamoxifen treatment. Additionally the ERα levels are decreased after any combinatorial treatment besides the triple combination of Lapatinib, Tamoxifen and Salinomycin.

This is partly reflecting the changes in protein level: for Lapatinib in combination with Salinomycin as well as Tamoxifen in combination with Salinomycin this qPCR-result confirms the Western Blot data. Both left combinations show contradictory effects on RNA compared to protein levels [Fig.15b].

The most prominent effect on a Luminal B breast cancer cell line is induced by combinations with Lapatinib. Upon treatment with this specific receptor tyrosine kinase inhibitor phosphorylation of Her2 and Her3 as well as their down-stream targets ERK 1/2 and AKT is blocked completely. Nevertheless the protein levels of Her2 and Her3 are increased after treatment. Tamoxifen in combination with Salinomycin on the other hand causes a decrease in phosphorylation of Her2 and Her3 as well but does not change their expression level in comparison to control. However ERK 1/2 and AKT are not influenced by this combination in MCF-7/Her2 cell line. Furthermore the Western Blot data show that ERα is less expressed upon Salinomycin treatment like in MCF-7 and also the amount of cleaved PARP is increased, mainly after Salinomycin in combination with Lapatinib [Fig.15a].

These results are also broadly confirmed by qPCR-analysis. Regarding the changes in Her2 and Her3 only Tamoxifen in combination with Lapatinib does not induce an increase in RNA-levels. The ERα expression profile however shows a different pattern compared to Western Blot analysis. Whereas the mRNA-level of ERα was significantly higher in Lapatinib+Tamoxifen and Lapatinib+Salinomycin treated cells, protein levels were not changed or reduced. Also Tamoxifen in combination with Salinomycin as well as the triple combination were not decreasing the mRNA-level of ERα [Fig.15c].

3.4 Molecular Evolution Assay

For clinical application of these drugs the effect of several treatment rounds on morphology and cell viability needs to be investigated. Therefore cells were treated, recovered and were again treated (Kopp et al., 2012)

3.4.1 TAMOXIFEN IS NOT ERADICATING LUMINAL A AND LUMINAL B CANCER CELLS

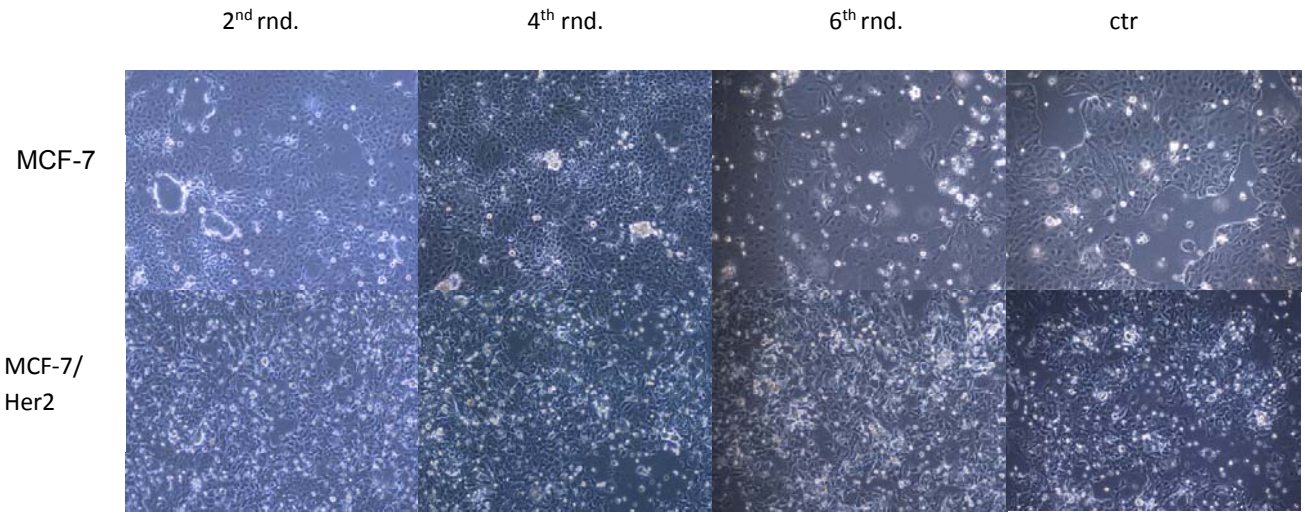


Fig.16: Microscopic pictures of MCF-7 and MCF-7/Her2 monolayer cells in 100x magnification after 2nd, 4th and 6th treatment (72h) round with 10µM Tamoxifen as well as control

These pictures show that both cell lines are hampered and morphology changed after 6th treatment rounds with Tamoxifen. Also the amount of floating cells, indicating cell death, was increased upon treatment. Nevertheless MCF-7 as well as MCF-7/Her2 cells recovered.

3.4.2 COMBINATION OF TAMOXIFEN AND SALINOMYCIN ERADICATES BOTH CELL LINES

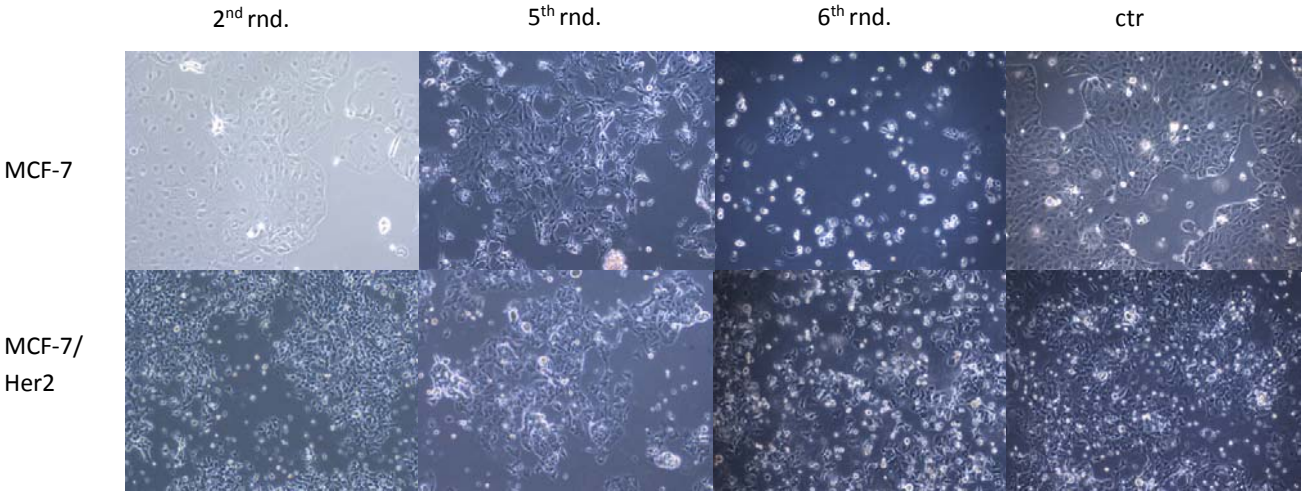
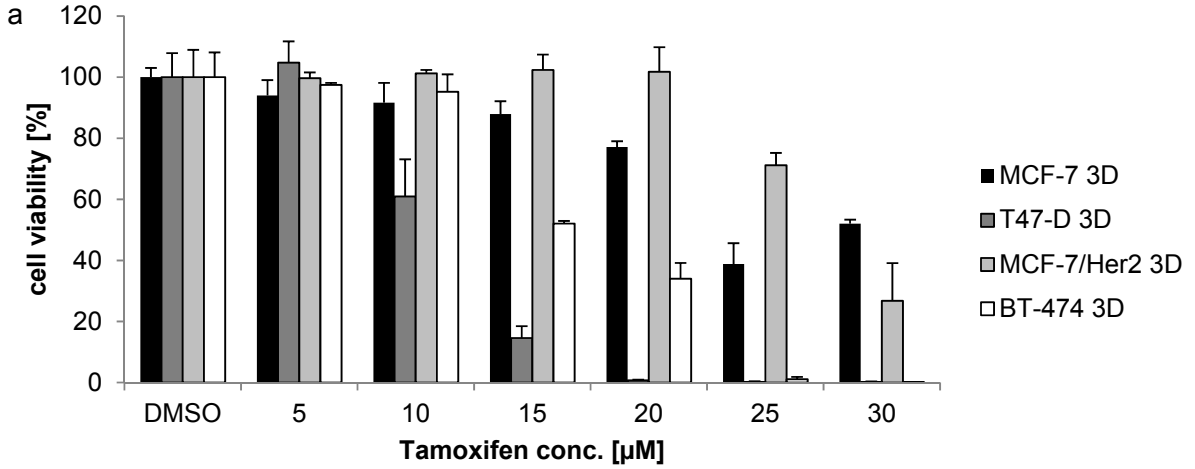


Fig.17: Microscopic pictures of MCF-7 and MCF-7/Her2 monolayer cells in 100x magnification after 2nd, 5th and 6th treatment (72h) round with 10µM Tamoxifen in combination with 0,5µM Salinomycin as well as control

Regarding these pictures it is obvious that Tamoxifen in combination with Salinomycin harms both cell lines already after the second treatment round and cell morphology changed. The most prominent effect is visible after the fifth and the sixth treatment round: After five treatment rounds MCF-7 changed their morphology completely: from a cobblestone-like shape they became spindle-shaped. After another treatment round MCF-7 lost their capability to attach to the petri-dish and were floating. MCF-7/Her2 changed their morphology as well after five treatment rounds and became more spindle shaped however the differences are not as obvious as in MCF-7. A further treatment round caused increased cell death as well as detachment from each other. Thus MCF-7/Her2 cells lost their capability to grow in clusters. Finally after seven treatment rounds cells were also detached from the plastic surface of the petri-dish and became apoptotic (data not shown).

3.5 Effect of Tamoxifen and Salinomycin treatment on Mammospheres

Since *in vivo* tumors are not properly represented by monolayer cells we moreover analyzed the effect of single and combinatorial treatment on mammospheres which are a more realistic tumor-model. These spheroids mimic the heterogeneity of human breast cancer and are therefore important for clinical relevance.



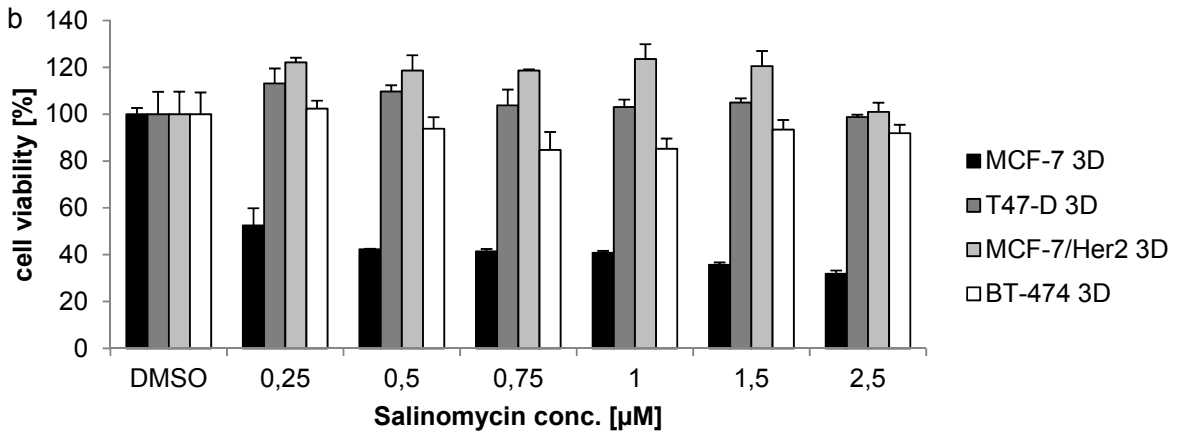


Fig.18: Cell viability, measured by CellTiter®-Glo of MCF-7, T47-D, MCF-7/Her2 and BT-474 mammospheres after 72h treatment with a, Tamoxifen or b, Salinomycin. 20000 c/w were seeded in polyhema-coated 96-well plates and treated 72h later.

These results show that Luminal B breast cancer cells in 3D are more resistant to Tamoxifen treatment compared to Luminal A especially in lower concentrations. Moreover we determined that both types of spherical cell lines were more resistant to endocrine therapy compared to 2D cell lines [Fig.12a, 18a].

Salinomycin on the other hand induces significant cell death especially in mammospheres of MCF-7. These spheroids were also more sensitive to Salinomycin compared to monolayer cells. Nevertheless mammospheres of other cell lines showed increased resistance to this antibiotic compared to 2D cells since they were not affected by any concentration of Salinomycin [Fig.12b, 18b].

To examine the effect of Salinomycin and Tamoxifen on the ligand independent activation of ERα, a Western Blot and a qPCR analysis were performed upon 72h treatment of mammospheres. Since the major key-players in this resistance mechanism are the receptor tyrosine kinases Her2 and Her3 as well as their down-stream targets their expression and activation was of special interest in this study.

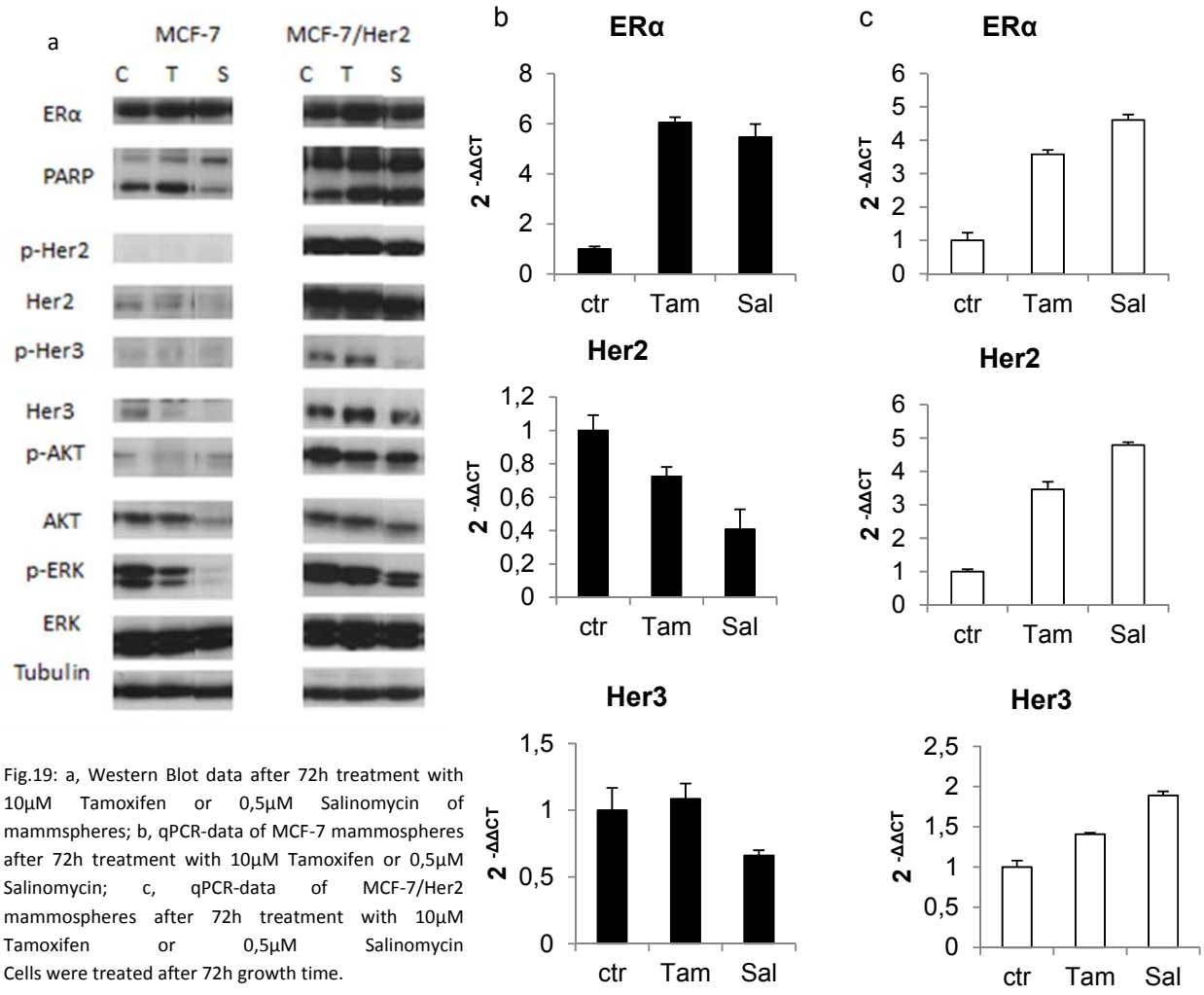


Fig.19: a, Western Blot data after 72h treatment with 10µM Tamoxifen or 0,5µM Salinomycin of mammspheres; b, qPCR-data of MCF-7 mammospheres after 72h treatment with 10µM Tamoxifen or 0,5µM Salinomycin; c, qPCR-data of MCF-7/Her2 mammospheres after 72h treatment with 10µM Tamoxifen or 0,5µM Salinomycin Cells were treated after 72h growth time.

The Western Blot analysis shows that upon Salinomycin treatment phosphorylation and expression of Her2 as well as Her3 were significantly reduced in MCF-7 spheroids. Moreover also their down-stream targets ERK 1/2 and AKT are affected by this antibiotic. Whereas the protein level of AKT is decreased after Salinomycin treatment, ERK 1/2 is less phosphorylated. The PARP-cleavage however was reduced by Salinomycin compared to control. Tamoxifen on the other hand only induces a slight reduction in ERK 1/2 phosphorylation and an increase in PARP-cleavage [Fig.19a].

These data are furthermore confirmed by qPCR. Also on RNA-level Her2 and Her3 are reduced after Salinomycin treatment. ERα on the other hand is significantly up-regulated by Tamoxifen as well as Salinomycin and is thus not reflecting the protein level [Fig.19b].

Also in MCF-7/Her2 spheroids the ionophore Salinomycin induces a decreased Her3 phosphorylation. Moreover ERK 1/2 is as well less phosphorylated upon Salinomycin treatment. Nevertheless phosphorylation and expression level of Her2 and AKT are not altered. Finally it is also detectable that the amount of cleaved PARP is increased upon Salinomycin as well as Tamoxifen treatment [Fig.19a].

However these results are not reflected by qPCR in MCF-7/Her2 mammospheres. Both ERα as well as Her2 are significantly enhanced on RNA-level upon treatment with Tamoxifen or Salinomycin. Finally also the expression of Her3 is slightly up-regulated by both compounds [Fig.19c].

Since the single treatment of mammospheres shows that Salinomycin is inhibiting the ligand independent activation of ERα the effect of different combinations on spheroids was investigated. Therefore also the cytotoxicity is of big clinical impact.

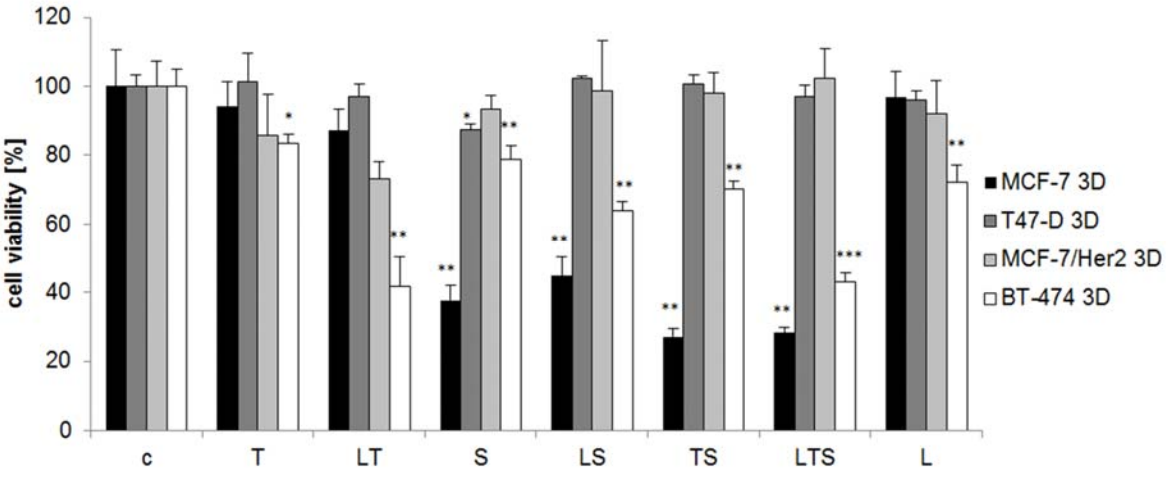


Fig.20: Cell viability measured by CellTiter®-Glo Assay of MCF-7, T47-D, MCF/7-Her2 and BT-474 mammospheres 72h after combinatorial treatment. 20000 c/w were seeded in polyhema-coated 96-well plates .and treated 72h later

It is visible that all different combinations only cause cell death in MCF-7 and BT-474 spheroids. MCF-7/Her2 and T47-D however were resistant to every combinatorial treatment option. In MCF-7 mammospheres cell viability was significantly decreased by Salinomycin and its combinations with Tamoxifen and/or Lapatinib. Nevertheles this combinatorial treatment only shows a negligible additional effect compared to single Salinomycin treatment in MCF-7.

In BT-474 spheroids on the other hand combinatorial treatment is more harmful compared to single treatment. The most prominent reduction in cell viability was induced by the triple combination Lapatinib, Tamoxifen and Salinomycin [Fig.20].

Moreover changes in the receptor tyrosine kinase signaling upon combinatorial treatment play an important role in the ligand independent activation of ERα and were therefore investigated by Western Blot and qPCR.

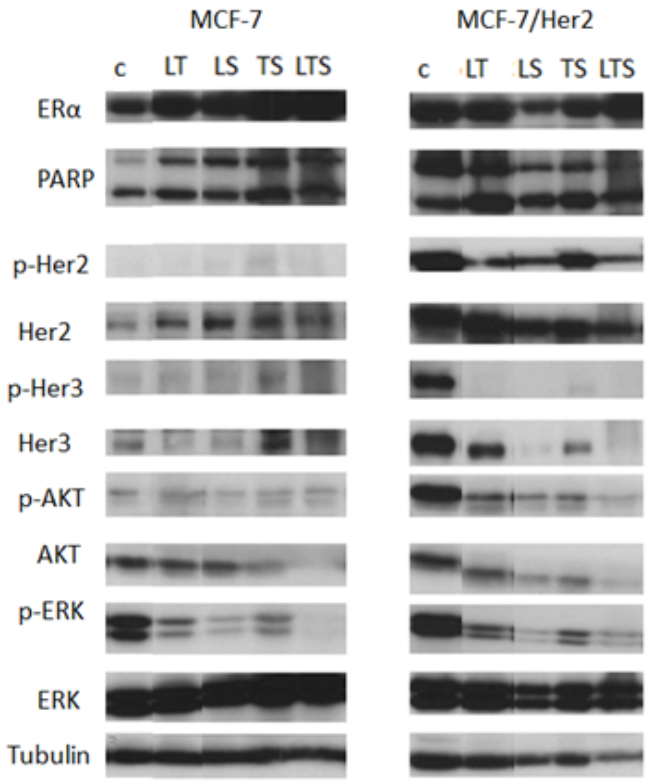
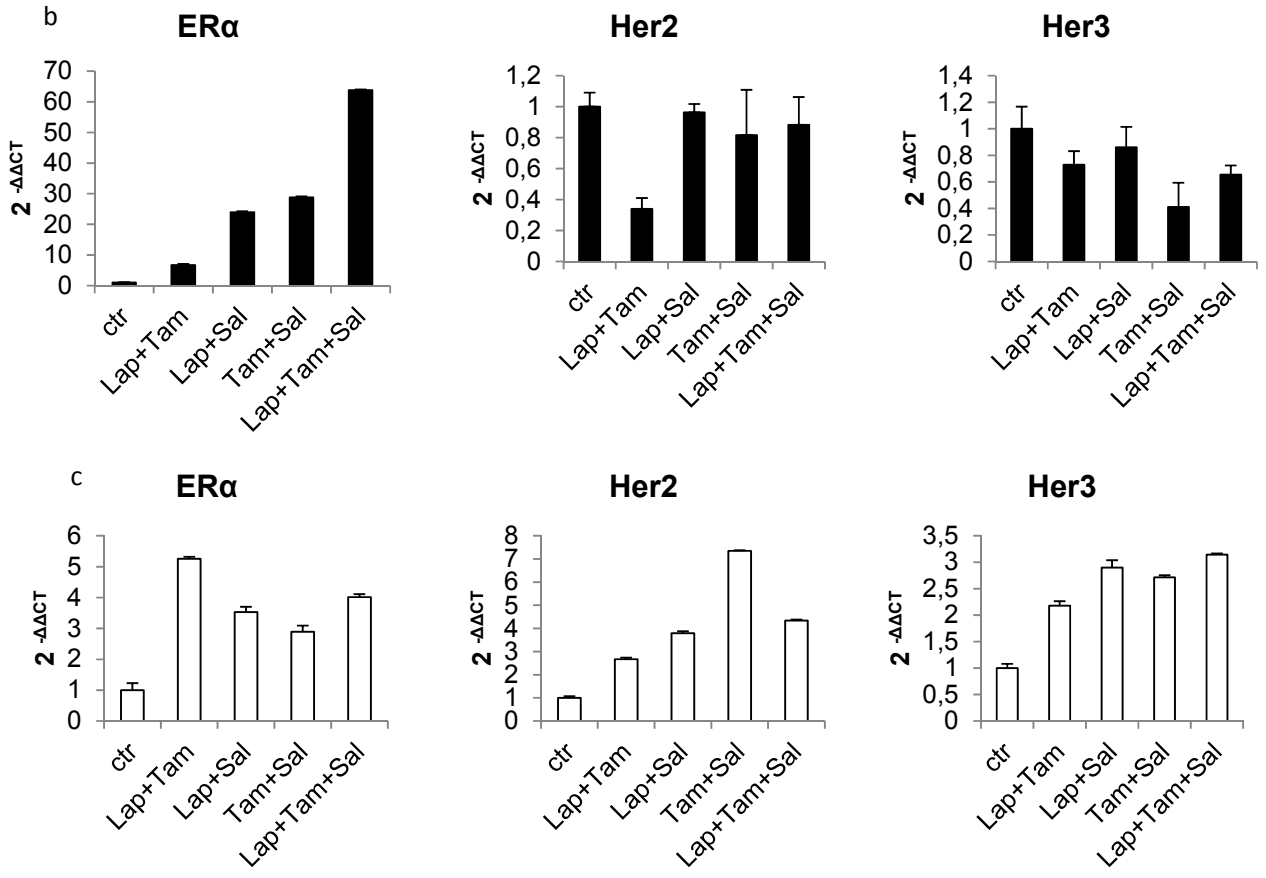


Fig.21: a, Western Blot analysis of mammospheres after 72h treatment with 10µM Tamoxifen, 0,5µM Salinomycin and 5µM Lapatinib; b, qPCR-data of MCF-7 mammospheres after 72h treatment with 10µM Tamoxifen, 0,5µM Salinomycin and 5µM Lapatinib; c, qPCR-data of MCF-7/Her2 mammospheres after 72h treatment with 10µM Tamoxifen, 0,5µM Salinomycin and 5µMLapatinib. Cells were treated 72h after seeding.



The Western Blot data of MCF-7 mammospheres show that only Her3 is down-regulated after Lapatinib+Tamoxifen and Lapatinib+Salinomycin treatment. The expression of Her2 however is up-regulated upon every combinatorial therapy. Also protein levels of Her3 are increased by Tamoxifen+Salinomycin as well as the triple-combination. Nevertheless the expression of down-stream target AKT is reduced after combinatorial treatment with Tamoxifen and Salinomycin as well as the triple combination. Moreover the phosphorylation of ERK 1/2 is decreased upon treatment whereas the most prominent reduction is induced by Lapatinib in combination with Salinomycin or the triple combination. ERα on the other hand is significantly up-regulated by every combinatorial treatment option. Finally also the PARP-cleavage was increased after Tamoxifen+Salinomycin treatment as well as the triple combination [Fig.21a].

Regarding the qPCR-results it is visible that RNA-levels of Her2 and Her3 are not directly correlated with protein levels. Whereas Western Blot data show that Her2 is slightly up-regulated upon treatment, it is significantly down-regulated by Lapatinib in combination with Tamoxifen on RNA-level. The other combinations did not change Her2 expression. Moreover the qPCR-results show that Her3 is slightly down-regulated upon treatment. The clearest reduction was induced by Tamoxifen in combination with the ionophore Salinomycin. Nevertheless the qPCR-analysis of ERα confirms the Western-Blot data. Also on RNA-level ERα is significantly increased by different treatment combinations [Fig.21b].

MCF-7/Her2 spheroids were affected differently by various drug combinations. The Western Blot data show that Her2 is less phosphorylated by different Lapatinib combinations. Also its expression is reduced by Lapatinib+Salinomycin, Tamoxifen+Salinomycin and the triple combination. Moreover the phosphorylation of Her3 is inhibited as well upon combinatorial treatment and its protein level is also decreased whereas the most prominent reduction is induced by Lapatinib in combination with Salinomycin and the triple combination. Furthermore the down-stream targets AKT and ERK 1/2 are clearly influenced by the different drug combinations. The phosphorylation as well as the expression level of AKT is significantly decreased upon combinatorial treatment. Also ERK 1/2 is less phosphorylated whereas the protein level is only slightly decreased after Lapatinib+Salinomycin treatment.

Additionally the ERα level was reduced by this combination as well as Tamoxifen in combination with Salinomycin. Finally the amount of cleaved PARP was increased by Lapatinib+Tamoxifen [Fig.21a].

However these results are not confirmed by qPCR-analysis. Both Her2 and Her3 are significantly up-regulated upon combinatorial treatment, Her2 especially by Tamoxifen and Salinomycin. Also the mRNA-level of ERα is clearly increased after 72h treatment with different combinations [Fig.21c].

3.6 EFFECT OF SINGLE AND COMBINATORIAL TREATMENT ON MAMMOSPHERE FORMATION

The formation of mammospheres is a physiological capability of breast cancer cells and indicates the ability to build tumors in a human body. Moreover it is a widely accepted assay to investigate the effect of different drugs on cancer stem cells.

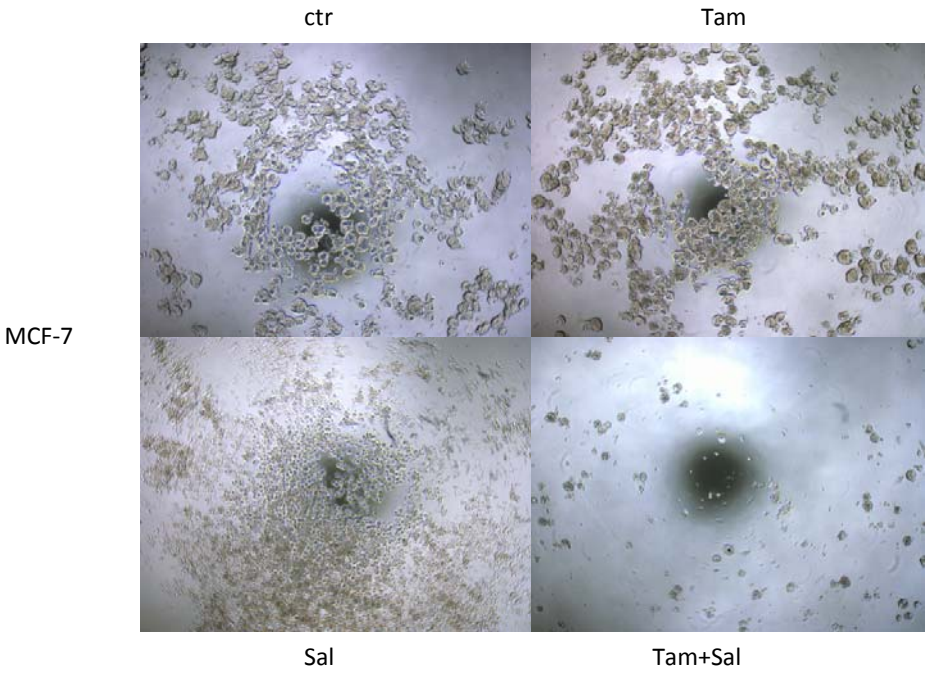


Fig.22: Microscopic pictures in 50x magnification of MCF-7 mammospheres after 72h pre-treatment and 96h growth time. 10000 c/w were seeded in polyhema-coated 96-well plates.

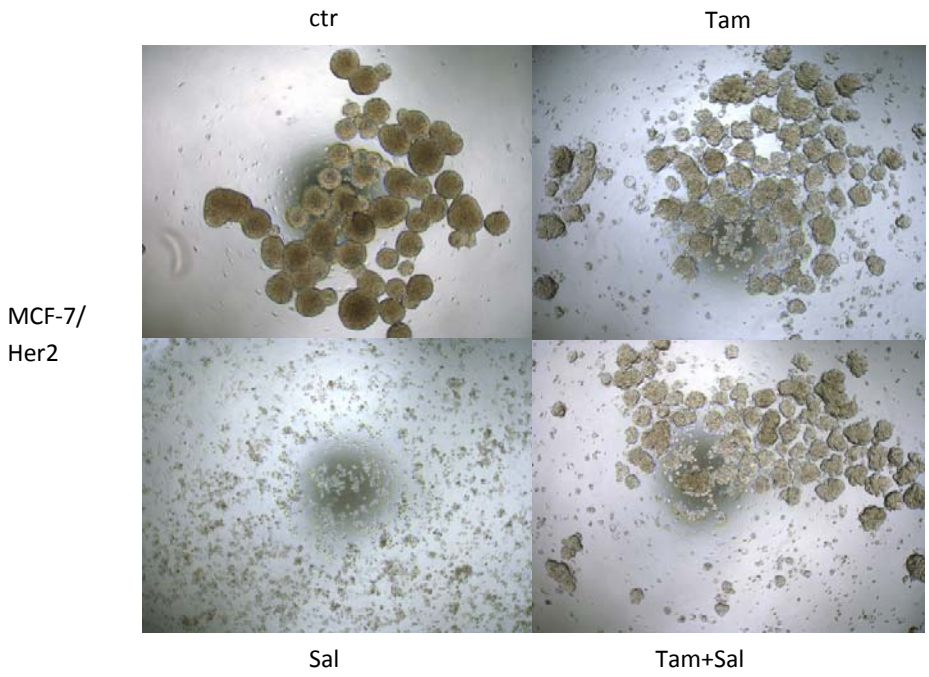


Fig.23: Microscopic pictures in 50x maginification of MCF-7/Her2 mammospheres after 72h pre-treatment and 96h growth time

These pictures show that mammosphere formation is not inhibited by single Tamoxifen treatment in Luminal A breast cancer cell line. Salinomycin on the other hand blocked the formation of spheroids completely but does not eradicate the cells. In contrast the combination of both drugs decreased mammosphere formation significantly and also inhibits cell growth [Fig.22].

Luminal B breast cancer cells however behave different. First of all it is visible that MCF-7/Her2 mammospheres have a different shape compared to MCF-7. Whereas MCF-7 form many, small spheroids, MCF-7/Her2 build few and big mammospheres. Moreover Tamoxifen treatment induced a change in morphology of these spheroids but does not inhibit formation of mammospheres or eradicate cells. Nevertheless Salinomycin caused in MCF-7/Her2 as well a complete inhibition of spheroid formation like in MCF-7. The additional application of Tamoxifen does not hamper cells but seem to protect MCF-7/Her2 from Salinomycin effect. Therefore mammosphere formation is still possible [Fig.23].

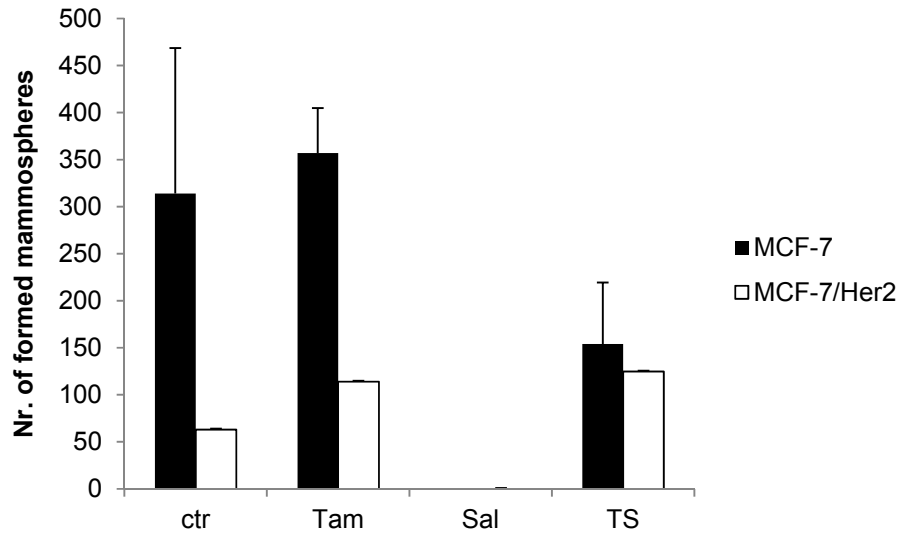


Fig.24: Mammosphere formation upon 72h pre-treatment with 10µM Tamoxifen, 0,5µM Salinomycin or the combination

Finally also the amount mammospheres differs between Luminal A and Luminal B breast cancer cell lines. MCF-7 form more but smaller spheroids, whereas MCF-7/Her2 only build few but big ones. Moreover the mammosphere forming potential of both cell lines were not altered significantly upon single Tamoxifen treatment. Nevertheless Salinomycin treated cells were not able to build spheroids. The combinatorial treatment however affects Luminal A and Luminal B breast cancer cell lines in a different way: Whereas the mammosphere forming potential of MCF-7 is decreased about 50%, it is not altered in MCF-7/Her2 compared to single Tamoxifen treatment.

4. DISCUSSION

The investigated classes of breast cancer, Luminal A and B, are ERα positive and are usually well treatable with endocrine therapy i.e. Tamoxifen, a selective estrogen receptor modulator. Nevertheless resistance to anti-estrogen treatment occurs and increases the relapse frequency. In ERα three major resistance mechanisms are known to induce worse prognosis for breast cancer patients: Multidrug resistance protein-1 (MDR-1) overexpression, existence of cancer stem cells and the ligand independent activation of ERα. According to recent publications the novel drug Salinomycin, an ionophoric antibiotic, is able to overcome both MDR-1 mediated drug resistance as well as to eradicate cancer stem cells (Fuchs et al., 2010; Gupta et al., 2009). Therefore Salinomycin addresses two major resistance mechanisms in Luminal A and B breast cancer and is thus a new approach in breast cancer therapy.

Since it was also shown that single Tamoxifen treatment induces the ligand independent activation of the ERα via the receptor tyrosine kinases Her2 and Her3, it is necessary to find an additional drug to improve the outcome of breast cancer patients (Shou et al., 2004).

As Salinomycin is known to overcome two resistance mechanisms in ERα-positive breast cancer, the combination of Tamoxifen and Salinomycin is a promising treatment option. Nevertheless it was of special interest if Salinomycin and the combination with Tamoxifen are moreover able to block the ligand independent activation of ERα and would therefore overcome all three major resistance mechanisms.

This would be a huge progress in breast cancer therapy and we therefore focused in this study on the effect of Salinomycin and the combination with Tamoxifen on the ligand independent activation of ERα.

4.1 COMBINATORIAL TREATMENT INHIBITS THE LIGAND INDEPENDENT ACTIVATION OF ER-ALPHA

The results of our different experiments show that Salinomycin hampers the ligand independent activation of ERα since phosphorylation and expression of Her2 and Her3, the key factors in this signaling cascade, are decreased upon Salinomycin treatment in both Luminal A and B breast cancer cell lines. Also the down-stream targets ERK 1/2 and AKT which proceed the receptor tyrosine kinase signaling to ERα are negatively affected by Salinomycin therapy [Fig.13].

Regarding the effect of Salinomycin on cell viability it turned out that this antibiotic decreases metabolic activity in both Luminal A and Luminal B breast cancer cells. However, Luminal B cell lines were more sensitive to this therapy [Fig.12]. As these cell lines are dependent on the Her2 signaling which is the major difference between Luminal A and B breast cancer, these results indicate that Salinomycin inhibits the receptor tyrosine kinase signaling cascade.

Single Tamoxifen treatment caused an increase in Her2 and Her3 expression levels and therefore our results confirm the discovery of Shou *et al*. (Shou et al., 2004): SERMs induce resistance to endocrine therapy by activation of ERα through the ligand independent signaling cascade. Since both increased receptor tyrosine kinase expression as well as ERα levels cause poor prognosis in breast cancer patients this effect is problematic in breast cancer therapy [Fig.13].

To overcome this difficulty Tamoxifen needs to be combined with a drug antagonizing these resistance mechanisms to improve breast cancer therapy. According to the effect of single Salinomycin treatment on Luminal A and B breast cancer, this antibiotic is useful additive to SERM treatment. Also Lapatinib, a specific small molecule receptor tyrosine kinase inhibitor was investigated, since Her2 and Her3 play an important role in the ligand independent activation of ERα.

Our results show that the combination of Tamoxifen and Salinomycin has a beneficial effect on breast cancer therapy. This is indicated by the fact that SERM treatment no longer induces the ligand independent activation of ERα as far as it is combined with Salinomycin. Nevertheless Luminal A and B cell lines behave different. In Luminal A breast cancer the combinatorial treatment decreases the expression of Her2 and Her3 whereas their levels are unaltered in Luminal B [Fig.15]. Since the receptor tyrosine kinases are overexpressed in Luminal B breast cancer the treatment duration needs to be longer than 72h to reduce their expression. However the phosphorylation which is necessary to activate the down-stream signaling cascade is reduced in both Luminal A and B breast cancer cell lines.

The investigation of cytotoxicity induced by combinatorial treatment shows that the combination of Tamoxifen and Salinomycin only has an additive effect on Luminal A breast cancer cells compared to single treatment. The cell viability of MCF-7/Her2 on the other hand was increased upon combinatorial therapy in comparison to single Salinomycin treatment [Fig.14] indicating that Tamoxifen prevents this cell line from death. The group of Dowset showed that SERMs act as agonists at the ERα in ER+/Her2+ breast cancer like Luminal B which are therefore *de novo* resistant to Tamoxifen treatment (Dowsett, 2001). Moreover the agonistic effect of SERMs increases the survival rate of breast cancer and blocks thus the mechanism of action of Salinomycin.

Finally it is also necessary to mention that the pro-drug Tamoxifen is a competitive mixed agonist/antagonist and has a 100 times lower affinity at the ERα compared to estradiol (Aktories, 2009). Therefore it needs to be metabolized in the human body by CYP3A4 and CYP2D6 to develop its full activity. Since breast cancer cells are not able to metabolize SERMs *in vivo* and *in vitro* effects are not comparable. Moreover one has to keep in mind that by investigating Tamoxifen in cell culture fetal calf serum already contains estradiol and may thus inhibit the function of this SERM. It was also shown that phenol red which is included in normal growth media mimics estradiol and may influence the effect of Tamoxifen as well (Berthois et al., 1986).

4.2 NO RESISTANCE DEVELOPMENT BY SEVERAL TREATMENT ROUNDS WITH TAMOXIFEN AND SALINOMYCIN

The molecular evolution assay is an important experiment to investigate the clinical impact of different drugs. Acquired resistance to endocrine therapy often occurs in clinical application since not eradicated cancer cells recover and are less sensitive to SERM treatment.

Therefore we investigated cell morphology after several treatment rounds in order to figure out whether Tamoxifen or Tamoxifen in combination with Salinomycin induces the creation of resistant cell populations or if breast cancer cells can be eradicated completely.

The microscopic pictures show that single Tamoxifen treatment hampered Luminal A monolayer cells more than Luminal B since they became more flat indicating senescence. But nevertheless both recovered after at least 72h and were not eradicated by six treatment rounds [Fig,16, 17].

The combinatorial treatment with Tamoxifen and Salinomycin however not only hampered cells but completely changed their morphology. We determined that after five treatment rounds the effect on MCF-7 was most prominent since they lost their original morphological properties and became spindle-shaped. Moreover these cells were not able to recover properly and to reestablish their cobblestone like morphology (data not shown). After a further treatment round they lost their ability to attach to the surface of the cell culture dish and were floating. This indicates on the one hand side that these cells will underwent apoptosis but on the other hand this is a hint that Salinomycin treatment hampers membrane integrity and therefore cells are no longer able to grow on plastic surface.

Also the morphology of MCF-7/Her2 cells was more altered by combinatorial therapy than single Tamoxifen treatment. However they are not as sensitive as MCF-7 monolayer cells. Even after five treatment rounds their original morphology has not changed significantly, they just became slightly more spindle shaped.

Nevertheless after a further application of both drugs for 72h only big, single and also some floating cells were left. Increasing size and detachment of culture dish surfaces are indications for apoptosis. Therefore these are further hints that Tamoxifen together with Salinomycin is able to eradicate Luminal B breast cancer cells. Moreover an additional treatment round caused as well detachment of MCF-7/Her2 cells from petri-dishes (data not shown). Finally MCF-7/Her2 cells usually form clusters and therefore it is uncommon that they grow in single cells after six treatment rounds. This effect combined with the detachment of plate surfaces moreover leads to the hypothesis that also in Luminal B breast cancer cell lines the ionophore Salinomycin induces damage of cell membranes.

Nevertheless the morphology of both cell lines did not change significantly between 2nd and 4th treatment round and were therefore not shown. Since cells were able to recover during these treatment rounds no irreversible changes in cell physiology were induced. However after four treatment rounds sensitive cells were eradicated and surviving ones tried to escape cell death. Therefore they needed to change their morphological properties which was not sufficient to overcome combinatorial treatment with Tamoxifen and Salinomycin.

4.3 COMBINATORIAL TREATMENT IS ONLY EFFECTIVE ON LUMINAL A MAMMOSPHERES

A further step towards clinical application is the treatment of more realistic tumor models like mammospheres, a spherical suspension culture of breast cancer cells. This cell system displays the heterogeneity of *in vivo* tumors.

In general Tamoxifen and Salinomycin as single and combined treatment induced the same effect in mammospheres as in monolayer cell culture. Regarding the Western Blot results Salinomycin and its combinations hampered the ligand independent activation of ERα. Phosphorylation and expression of involved genes like Her2 and Her3 as well as their downstream targets are decreased upon treatment [Fig.19, 21].

Nevertheless only in spheroids of MCF-7 and BT-474 Salinomycin and different combinations show cytotoxicity whereas the cell viability of other mammosheres was not affected. One possible reason is that in spheroids cells at the surface are more affected compared to cells in the center. Therefore shape and size of mammospheres are important properties influencing the drug sensitivity. Since especially MCF-7/Her2 breast cancer cells form big spheroids with smooth surface it is possible that Salinomycin was not able to penetrate into the center and cells survived [Fig.18, 20].

The mammosphere forming potential of Luminal A and B breast cancer cells differed upon combinatorial treatment. Whereas both cancer cell lines were similarly affected by single Tamoxifen and Salinomycin, their combination was only beneficial in the case of Luminal A breast cancer cells. Luminal B cancer cells however formed equal mammospheres, regarding amount, size and shape, as after single Tamoxifen treatment. That indicates again that SERMs prevent ER+/Her2+ breast cancer cells from Salinomycin effect which caused inhibition of mammosphere formation [Fig.22, 23].

On the other hand one has to keep in mind that cells were pre-treated with different drugs and no drug was present during 96h mammosphere formation. Thus another possibility why MCF-7/Her2 spheroids are not affected by the combinatorial treatment is, that these cells were able to recover since they were not hampered by this combination and formed spheroids.

Basically it has to be mentioned that spherical suspension culture is more complex compared to monolayer cell culture. One general issue is the time to generate mammospheres which also depends on the cell line. There are different groups that let spheroids grow for five days and longer (Gupta et al., 2009; Guttilla et al., 2012; Oak et al., 2012). Therefore it would be possible that 72h for mammosphere formation was not enough to create a stable system. Moreover the different assays we used are established for monolayer cell culture and were slightly modified to analyze spheroids. Due to the fact that there are little data available about investigation of mammospheres it is possible that various methods need to be further optimized for proper results.

5. SUMMARY

In this study we figured out that Luminal B breast cancer cells were more affected by single Salinomycin treatment than Luminal A cell lines indicating that this antibiotic hampers the receptor tyrosine kinase cascade being the only difference between both breast cancer classes.

This was further confirmed by Western Blot and qPCR analysis which show that Her2 and Her3 as well as their down-stream targets ERK1/2 and AKT were less phosphorylated and expressed upon Salinomycin treatment.

We discovered moreover that also the combinatorial treatment of Luminal A and B breast cancer cell lines with Tamoxifen and Salinomycin inhibited the signaling cascade of Her2 and Her3 and therefore as well the ligand independent activation of ERα.

Additionally, the molecular evolution assay shows that several treatment rounds with Tamoxifen and Salinomycin did not create drug resistant cell clones but eradicated Luminal A and B breast cancer cells.

Furthermore, these effects were also determined in mammospheres, a spherical *in vitro* tumor model. Single Salinomycin treatment as well as the combination with Tamoxifen blocked the Her2/Her3 mediated signaling cascade and therefore the ligand independent activation of ERα.

Finally, we found that Luminal A and B breast cancer cells lost their ability to form mammospheres upon by Salinomycin treatment. The combinatorial treatment with Tamoxifen however had only an additional effect on Luminal A breast cancer cells.

Taken together our results show that the ligand independent activation of ERα is inhibited by Salinomycin treatment. Moreover, in combination with Tamoxifen, Salinomycin hampered the Her2/Her3 induced signaling cascade. Therefore one major problem in endocrine therapy, the ligand independent activation of ERα by Tamoxifen, can be solved by combinatorial treatment. However, this is not the only benefit of this therapy.

Salinomycin is moreover able to overcome MDR-1 mediated drug resistance and to eradicate CSC. Thus, this antibiotic can address all three major resistance mechanisms in SERM therapy (Fuchs et al., 2010; Gupta et al., 2009).

Therefore the combinatorial treatment of Luminal A and B breast cancer with Tamoxifen and Salinomycin is a novel approach in breast cancer therapy and one step ahead towards circumvention of resistance to endocrine therapy.

Since these breast cancer classes have the highest prevalence with around 70% the combined application of Tamoxifen and Salinomycin might decrease relapse frequency and therefore improves the prognosis for many breast cancer patients.

6. FUTURE PERSPECTIVE

Since our discovery represents the base of a new approach in breast cancer therapy further experiments are needed to proceed the combinatorial treatment towards clinical application.

Besides some *in vitro* studies like the alternating application of Salinomycin and Tamoxifen as well as the influence of several treatment rounds on the ligand independent activation of ERα, there are two major experiments needed to investigate the clinical impact of combinatorial treatment.

First of all it is necessary to examine human breast cancer tissue upon Tamoxifen treatment to figure out whether this SERM induces the ligand independent activation of ERα as well in humans. The investigation of tumors being resistant to endocrine therapy is important to increase the knowledge about resistance mechanisms in humans.

The next step towards clinical application is then to test our drugs and their combination in *in vivo* models. On the one hand side it is necessary to figure out if Tamoxifen and Salinomycin pre-treatment of breast cancer cells alter their ability to form tumors in mice. This is an indicator for metastasis and is therefore an important factor in treatment of breast cancer. On the other hand mice resembling Luminal A or Luminal B breast cancer need to be treated with Tamoxifen and Salinomycin as well as their combination to mimic their effect on humans. Of special interest are tumor size upon treatment, metastasis formation as well as possible side-effects caused by our therapy.

If these results moreover confirm the benefit of a combinatorial breast cancer treatment with Tamoxifen and Salinomycin, this combination is a promising, new therapy option to overcome resistance in ERα tumors.

7. LIST OF FIGURES

8. ABBREVIATIONS

Abbreviations	Definition
°C	Degree Celsius
µM	Micromolar
AI	Aromatase inhibitor
AKT/PKB	Protein kinase b
ALDH1	Aldehyde dehydrogenase
APS	Ammonium peroxodisulfate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
c/w	cells per well
cDNA	Complementary deoxyribonucleic acid
conc.	concentration
CT	Cycle threshold
ctr/c	control
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTPs	deoxynucleotides
DTT	dithiotreitol
ECL	Enhanced chemoluminescence
EDTA	Ethylenediaminetetraacetic acid
e.g.	Exempli gratia, for example

EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-regulated kinases
ERα	Estrogen receptor alpha
FCS	Fetal calf serum
FDA	Food and Drug Administration
Fig.	Figure
h	hours
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Her2	Human epidermal growth factor receptor 2
Her3	Human epidermal growth factor receptor 3
i.e.	Id est, that means
Lap/L	Lapatinib
mA	Milliampere
MDR-1	Multidrugresistance protein 1
min.	minutes
mRNA	Mesenger RNA
ng	Nano Gramm
p-…	Phospho-…
PAGE	Polyacrylamide gel electrophorese
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphor buffered saline
Polyhema	Poly(2-hydroxyethyl methacrylate)

qPCR	Quantitative polymerase chain reaction
PR	Progesterone receptor
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
Sal/S	Salinomycin
SDS	Sodium dodecyl sulfate
Sec.	Second
SERD	Selective estrogen receptor down-regulator
SERM	Selective estrogen receptor modulator
Sox2	SRY (sex determining region Y)-box 2
Tam/T	Tamoxifen
TEMED	Tetramethylethylenediamine
Temp	Temperature
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
V	Volt

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