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Molecular Evolution Assays Reveal Novel Targets and Mechanisms of Drug Resistance

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Erklärung

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Dedicated to My Mom

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1. INTRODUCTION

1.1 Breast Cancer

1.1.1 Breast cancer : an overview

The world health organization (WHO) reported that breast cancer is one of the leading cause of death and the most common cancer type amongst women worldwide in 2012 [1]. Moreover, breast cancer ranks as the fifth cause of death from cancer overall (522,000 deaths), is the most frequent cause of cancer death in women in less developed countries (324,000 deaths, 14.3% of total), and the second cause of cancer death in developed countries (198,000 deaths, 15.4%) after lung cancer. A recent study reported that breast cancer is leading in the estimated new cancer cases, and the second most common death cause of women suffering from cancer in the USA [2]. Therefore, research and development in cancer detection and therapy is urgently required to solve those problems.

Breast cancer occurs when breast cells start to grow uncontrollably. These cells can invade nearby tissues and spread throughout the body. Each type of tissue in the breast can form a cancer, but the cancer usually arises in the milk ducts or glands. Factors that influence the risk of breast cancer are the length of exposure to hormones (e.g. menstruation at an early age or late menopause), reproductive factors (e.g. no children and first pregnancy at an advanced age), dietary factors and lack of physical activity (e.g. obesity and dietary fat), radiation during breast development, hormone replacement therapy on chronic use, as well as congenital genetic factors associated with breast cancer like the presence of gene mutations [3].

By molecular characteristics breast cancer is classified into luminal, HER-2 positive, and triple negative subtypes [4]. The luminal subtype expresses estrogen receptor (ER). Approximately 70-75% of breast cancer cases express estrogen receptor alpha (ERα) [5], thus inhibiting estrogen signaling is an important target for breast cancer therapy. The HER-2 positive subtype expresses human epithelial growth factor receptor 2 (HER-2), therefore it responds well to anti HER-2 therapy. Her-2 overexpression is detected in approximately 15-23% of breast cancers patient [6]. The triple negative subtype does not express ER, progesterone receptor (PR), and HER2, therefore it is the most difficult one to treat. Moreover, the triple negative breast cancer subtype represents approximately 15% of breast cancer cases with high index of heterogeneity [7].

Breast cancer is a complex genetic disease due to the accumulation of various genetic abnormalities that affect gene expression [8]. The molecular mechanisms responsible for development of breast cancer is related to the expression of oncogenes such as c-myc, erbB2, and Ras [9], and mutations in the tumor suppressor gene BRCA1, BRCA2, and p53 [10]. Gene mutations can be caused by epigenetic changes or exposure to carcinogenic agents causing

DNA damage which then induces the initiation and malignant transformation of normal cells [9].

Hormonal factors also play an important role in the development of breast cancer. Stimulation of tissue growth by the estrogen participates in the pathogenesis of breast cancer [11]. Estrogen has several different signaling pathways. The first pathway is a direct genomic pathway, which is started by binding of estrogen to ER in the cytoplasm, continued with translocation of the complex (estrogen-ER) into the nucleus, dimerization of the complex, and binding of the complex to the DNA at the estrogen response element (ERE). In a second, indirect genomic pathway, the dimerized complex binds to the DNA through protein-protein interactions with other classes of transcription factors, e.g. NFkB, and GATA1. A third, non-genomic signaling pathway,started by binding of ER and estrogen in the cytoplasm, resulting in the activation of a kinase cascade. The fourth pathway is the ligand independent pathway, in which ER is activated via phosphorylation by their associated co-regulators including epidermal growth factor receptor (EGF), insulin-like growth factor receptor (IGFR) or human epidermal growth factor receptor 2 (Her2), thus leading to transcription of ER downstream targets in absence of estrogen [12].

The complexity of breast cancer mechanisms demand a comprehensive therapy. Surgery is the primary choice in breast cancer treatment. Surgery and radiotherapy can control local tumors in most patients, although more than 60% will die because of the disease if the tumor tissue spreads [13]. In the early stage of breast cancer, the disease is still localized in the mammary gland, and therefore local treatment is pursued through surgery or radiotherapy. In this case adjuvant therapy with chemotherapy or endocrine therapy is carried out in order to reduce the risk of re-emergence of the disease and to improve survival. The purpose of systemic chemotherapy in patients with advanced breast cancer and those who have had metastases is to maximize the control of symptoms, prevent serious complications, and prolong the patient's life expectancy [14]. Most of the patients will require multiple lines of therapy due to acquired resistance to chemotherapy [15].

1.1.2 Classical chemotherapy doxorubicin

Doxorubicin is an anthracycline antibiotic which is commonly used in breast cancer therapy [16]. The combination of docetaxel, doxorubicin and cyclophosphamide is administered as a standard adjuvant chemotherapy in patients before surgery [13]. Doxorubicin has several mechanisms of action on cellular level. First, doxorubicin inhibits topoisomerase II, resulting in inhibition of DNA replication. Doxorubicin is also able to intercalate with DNA, thus inhibiting DNA synthesis. Another study demonstrated that doxorubicin causes disturbance of the intracellular iron transport and disruption of intracellular ion balance. Excessive accumulation of intracellular iron catalyzes formation of free radicals semiquinone and reactive oxygen

species (ROS). Moreover, ROS induces damage in cellular elements, including DNA, either by direct action on DNA or by reaction with other cellular constituents to produce new ROS [17-21].

1.1.3 Tamoxifen, a selective estrogen receptor modulator

As approximately 70-75% of breast cancer cases express estrogen receptor alpha (ERα) [5], inhibition of the estrogen receptor is a promising strategy in breast cancer therapy. Selective estrogen receptor modulators (SERMs) are a diverse group of nonsteroidal compounds that function as agonists or antagonists for estrogen receptors (ERs) in a target gene and tissue dependent manner [22]. Tamoxifen emerged as the first SERM that is clinically applied in breast cancer treatment [23]. Tamoxifen is a triphenylethylene derivative that is metabolized by drug-metabolizing enzymes into active metabolites 4-hydroxytamoxifen (4-OHT) and endoxifen [24]. Tamoxifen blocks estrogen signaling in breast cancer cells, but increases endometrial stimulation and therefore increasing the risk of endometrial carcinoma at the same time [25].

1.2 Chemoresistance

Several advantages have been achieved for breast cancer treatment, including combinatorial treatment of chemotherapy and therapy with antibody and endocrine therapy, however chemoresistance is still a major obstacle in breast cancer treatment [26]. As few as 50% of patients may benefit from chemotherapy as a result of intrinsic or acquired chemoresistance [27], and 30% of women diagnosed with early breast cancer will progress or relapse with locally advanced or metastatic breast cancer [28]. Therefore, research on drug resistance formation and solutions to overcome drug resistance are urgently needed to achieve better efficacy of chemotherapeutic drugs.

Cancer cells use several mechanism to overcome the drug treatment. Overexpression of multidrug efflux pumps such as P-glycoprotein (encoded by MDR1), MRP1 (encoded by ABCC1) and ABCG2 (encoded by BCRP) is considered to be important in cancer chemoresistance [29-31]. Cancer cells may also develop resistance towards DNA-damaging chemotherapeutic agents by preventing DNA damage and enhancing DNA repair mechanism [32-34]. Other mechanisms are via alteration of drug metabolism, de-arrangement of intracellular signaling pathway, and modification of apoptotic signaling or increased drug detoxification [35]. At the cellular level, cancer stem cells (CSCs) and the induction of epithelial to mesenchymal transition (EMT) are responsible factors for relapse [36-38].

The known resistance mechanisms particularly of doxorubicin are decreasing the intracellular level of the drug by overexpressing efflux pumps [39] and increasing drug detoxification, by

upregulation of metabolism enzymes, e.g. glutathione S-transferase (GST) [40]. Doxorubicin resistant cells show reduced activity of topoisomerase II, a target of doxorubicin [41]. A recent studies showed that EMT and cancer stem cells are also factors causing doxorubicin resistance [42, 43].

Breast cancer cells develop resistance to tamoxifen via loss or modification of estrogen receptor expression, alterations in co-regulatory proteins and the regulation of survival, proliferation, cell cycle, inhibition of apoptosis by Bcl-2, autophagy, altered expression of miRNA and EMT [44]. Cancer stem cells can also account for tamoxifen resistance [45]. Another mechanism of tamoxifen resistance is associated to the activation of epidermal growth factor receptors (EGFR, HER2, FGFR), NFkB, and IGF-1, as well as the signaling pathway alterations of PI3K/Akt/mTOR [46, 47].

1.3 Salinomycin: A Promising Drug for Circumventing Multidrug Resistance

Salinomycin, an ionophore antibiotic, was introduced in 2009 as cancer stem cells targeting drugs [48]. It was shown that salinomycin efficiently reduces metastasis formation due to a decrease in migratory capacity of cancer cells [49]. Furthermore, salinomycin can inhibit cell survival, growth, migration and invasion on human non-small lung cancer cells [50]. Other studies showed that salinomycin induces cell death in breast cancer cells [51], ovarian cancer cells [52], as well as in head and neck cancer cells [53]. Moreover, salinomycin inhibited cell growth and cell proliferation in prostate cancer cells [54] and pancreatic cancer cells [55].

Studies of salinomycin overcoming drug resistance have also been conducted. Fuchs *et al*. showed that salinomycin overcomes ABC transporter-mediated multidrug and apoptosis resistance in human cancer cells [56, 57]. Salinomycin increases doxorubicin and etoposide cytotoxicity by enhancing DNA damage and by downregulating p21 [58], and elevates apoptosis in cisplatin-resistant colorectal cancer cells by accumulating reactive oxygen species [59]. A combination of salinomycin and trail circumvents trail resistance in glioblastoma cells [60]. It was previously shown that a combination of salinomycin and trastuzumab efficiently targets cancer stem cells and Her-2-positive cancer cells [61]. Moreover, a study by Kim *et al.* showed that combined treatment of salinomycin and doxorubicin enhances the cytotoxicity in multidrug resistant MCF-7/MDR human breast cancer cells by decreasing the efflux of doxorubicin [39].

1.4 Molecular Evolution Assay

Chemotherapy regimen in clinic is administered in sequential application of non–cross-resistant chemotherapy for a defined number of cycles [62]. The application might be either single treatment or a combination of several chemotherapies. For instance, combinational treatment of docetaxel and cisplatin, as first-line chemotherapy in patients with metastatic breast cancer, is performed every 3 weeks for 6 cycles [63]. Another clinical study on breast cancer patients administered 3-weekly courses of epirubicin followed by four 4-weekly cycles of cyclophosphamide, methotrexate and 5-fluorouracil or four 3-weekly courses of paclitaxel followed by four 3-weekly cycles of epirubicin and vinorelbine [63].

Many studies on cancer drug resistance use sequential drug treatment with an increase in concentration of the drugs [64-66]. Therefore, to mimic clinical application of sequential chemotherapy, Kopp *et al.* established an *in vitro* model named molecular evolution assay [67]. In the molecular evolution assay (Fig. 1), cells were sequentially treated with the same dose of chemotherapy for several cycles/rounds, followed by a recovery time. The recovery step is important to get closer to the real situation in the clinic. Moreover, Kopp *et al.* demonstrated for the first time that the molecular evolution assay of doxorubicin on BT474 epithelial breast cancer cells resulted in significantly more resistant cells within three treatment rounds [67]. In addition, cell morphology revealed a change towards an EMT phenotype. Thus, the assay is suitable to identify drug resistance formation, because genetic alteration in each round of drug treatment could be tracked and identified.

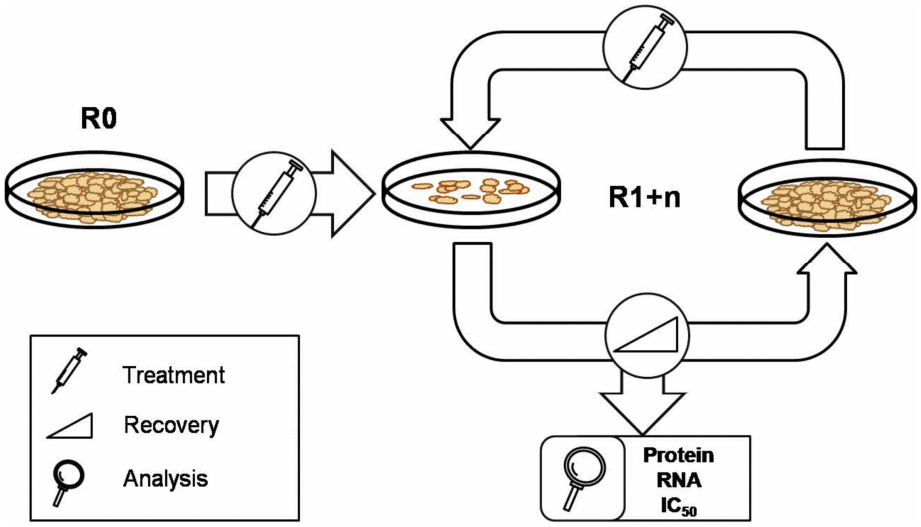


Fig. 1. Molecular evolution assay. Cells (80% of confluency) were treated with drugs for 72h. Medium was replaced with fresh medium after drug exposure, and cells were grown until recovery (80% of confluency). As soon as recovered, cells were splitted for protein and RNA analysis, as well as for the next sequential treatment and also seeded for cell viability assay. R0 represents the untreated control cell line (parental cells), whereas R1 and R1+n represent cells which treated for one times and (1+n) times, respectively.

1.5 Bone Morphogenetic Protein Receptor II (BMPR2) in Cancer Regulation

Cancer cells utilize very complex signaling pathways to control their cellular homeostasis. Transforming growth factor ß (TGF-ß) is one of the known signaling cascades maintaining a cancer phenotype. The bone morphogenetic proteins (BMPs), transforming growth factor (TGF)-β superfamily members, were originally identified as molecules regulating growth and differentiation of bone and cartilage, as well as teeth, skin and vascular formation [68-74]. Bone morphogenetic protein (BMP) type II receptor (BMPR2), a receptor for BMP ligands, is involved in the development of pulmonary arterial hypertension (PAH) and atherosclerosis [75-78]. Recent findings have revealed that BMPR2 also plays a role in cancer regulation.

1.5.1 Bone morphogenetic protein (BMPs) and BMP receptors (BMPRs)

Bone morphogenetic proteins (BMPs) are categorized into several groups i.e BMP2/4, BMP5/6/7/8, the growth and differentiation factor (GDF)5/6/7 and BMP9/10 [79] (Fig. 2). In order to start the signaling cascade, BMPs bind and interact with BMP receptors (BMPRs) [80]. The BMPRs consist of two types, type I and type II receptors. Type I includes activin receptor-like kinase 3 (ALK3, or BMPRIA) and ALK6 (BMPRIB), as well as the ALK1 and ALK2 group [81]. Type II receptors consist of BMPR2, activin receptor 2A (ACVR2A), and activin receptor 2B (ACVR2B) [73].

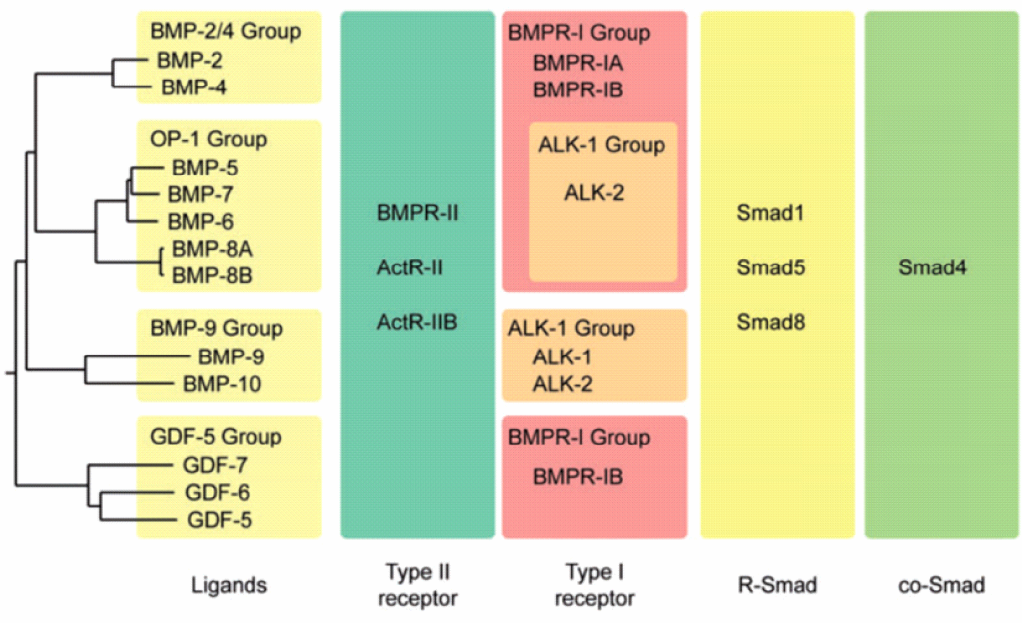


Fig. 2. Relationships between BMP ligands, type II and type I receptors, and Smad proteins in signal transduction. Concerning the binding of BMPs to type I receptors, BMP-2/4 bind to BMPR-IA and BMPR-IB, whereas BMP-6/7 bind strongly to ALK-2, and weakly to BMPR-IB. BMP-9/10 bind to ALK-1 and ALK-2, and GDF-5 preferentially binds to BMPR-IB. Adapted from Miyazono *et al*., 2010, *J Biochem*, 147(1): 35-51. © The Authors 2009. Published by Oxford University Press.

1.5.2 BMPR signaling pathway

BMP signaling is initiated by the binding of extracellular BMPs to heterodimeric BMP receptors (BMPRs) [82]. The type II receptor kinase is constitutively active in the absence of the ligand [73], but it cannot trigger BMP downstream signaling alone due to its poor affinity for BMP-binding [79]. In order to initiate signaling, BMPR2 needs interaction and complex formation with one of the type I receptors ACVR1, BMPR1A and BMPR1B. The interactions between BMPR2 and type I receptors depend on the sort of BMP which binds to the type I receptor [74]. Both receptors need to form a hetero-oligomeric (heterodimeric) complex to trigger the signaling pathway. Once phosphorylated by the type II receptor, heterodimeric complex activates downstream effectors via two different pathways, the smad dependent and/or smad independent pathway [82].

Activation of one of these pathways depends on the combination of oligomerization of type I and type II receptors (Fig. 3). The ligands can bind either to preformed type I-type II hetero-oligomeric complexes (PFC), to induce the smad dependent pathway, or to the type I receptor followed by recruiting the best fitting type II receptor and formation of a BMP-induced signaling complex (BISC), to activate the smad independent pathway [82-84]. The smad dependent pathway requires a complex formation between activated smad 1/5/8 and the common partner (co)-Smad 4 for signaling, thus translocating to the nucleus and regulating interaction with other transcription factors of the target genes [79]. In the smad independent pathway, activated BISC will then trigger mitogen-activated protein kinases (MAPKs) e.g. p38 MAPK, ERK, and JNK, PI3K/AKT or small Rho-like GTPases [73, 82]. X-linked inhibitor of apoptosis protein (XIAP) is a bridging protein between type I receptor and TGF-ß activated binding protein (TAB 1/2/3), an activator of MAPK TGF-ß activated tyrosine kinase 1 (TAK1) [85]. Accordingly, activation of TAK1 leads to activation of p38 via the smad independent pathway [83, 85, 86].

The type I and type II receptor are located in the cell surface, forming either homodimeric or heterodimeric complexes even in the absence of the ligand [83, 86]. Homodimeric complexes are stable, while heterodimeric complexes are transient [87]. Homodimeric complexes of type I receptor are rearranged in the presence of type II receptors, leading to PFC formation and smad dependent pathway [88]. Moreover, homodimeric BMPR complexes may limit excessive formation of heteromeric PFC in the absence of ligand [87].

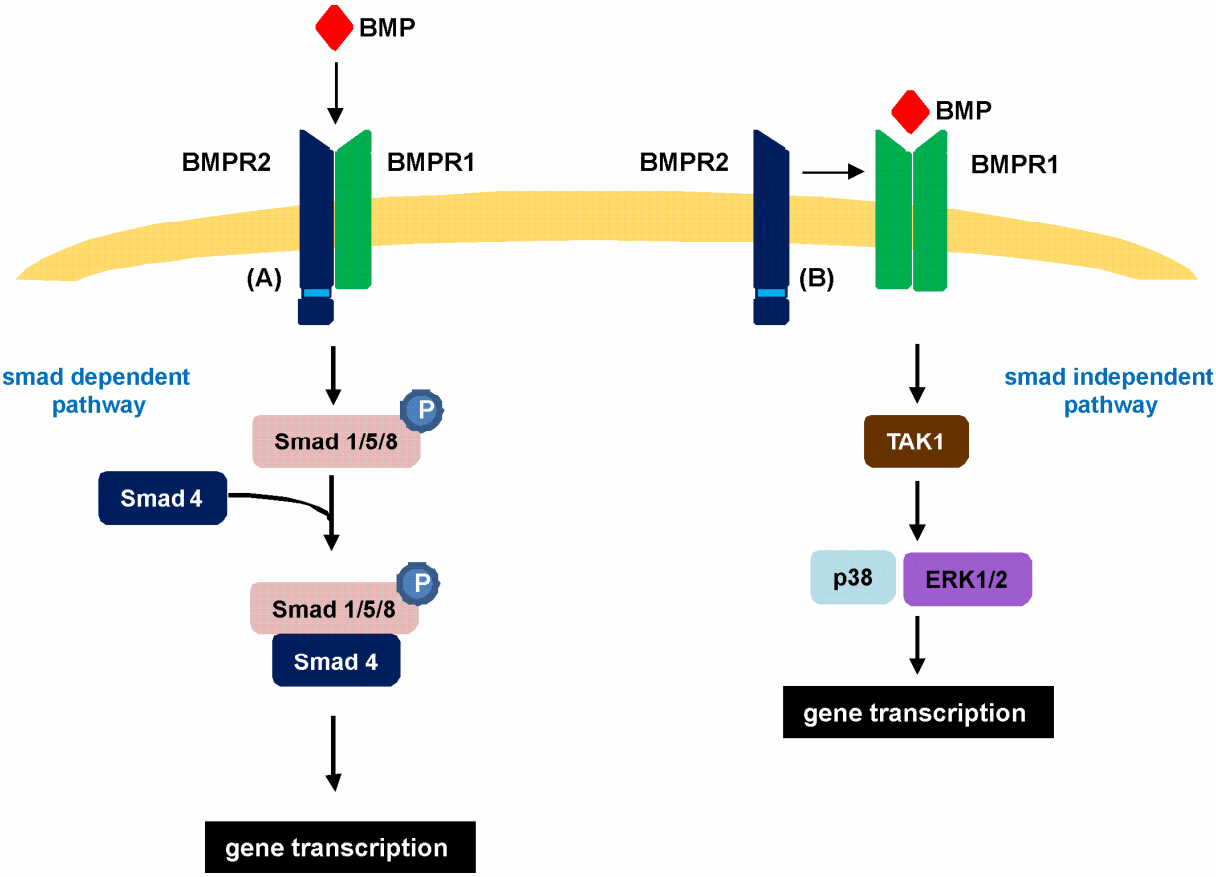


Fig. 3. Signaling pathway of BMPR2. BMP or the other ligands can bind either to (A) preformed type I-type II hetero-oligomeric complexes (PFC) to induce smad dependent pathway or to (B) the type I receptor continued with recruiting the best fitting type II receptor and formation of a BMP-induced signaling complex (BISC) to activate smad independent pathway. The smad dependent pathway requires a complex formation between activated smad 1/5/8 and common partner (co)-Smad 4 for the signaling, thus translocate to the nucleus and regulate interaction with other transcription factors of the target gene expression. In the smad independent pathway, X-linked inhibitor of apoptosis protein (XIAP) is a bridging protein between type I receptor and TGF-ß activated binding protein (TAB 1/2/3), an activator of MAPK TGF-ß activated tyrosine kinase 1 (TAK1), thus activation of TAK1 leads to activation mitogen-activated protein kinases (MAPKs) smad independent pathway and activation of gene transcription. Adapted from Nohe *et al.*, 2002, *J Biol Chem*, 277(7): 5330-5338. © by Baltimore, MD : American Society for Biochemistry and Molecular Biology

1.5.3 Role of BMPR2 in cancer

Cancer is also described as a disease caused by a damage of cell cycle regulation [89]. BMPR2 has shown either proliferative or anti-proliferative effects on cancer cells. Transient overexpression of dominant negative BMPR2 (DN-BMPR2) interfered with BMP2 inhibited cell proliferation, induced Smad1 signaling and G1 arrest on T47D breast cancer cells [90]. On the other hand, BMPR2 shows inhibition of cell proliferation on several cancer cells. *In vivo* xenograft of PC3M human prostate cancer cells transfected with dominant-negative BMPR2 (BMPR2-DN) showed faster tumor growth, indicating that loss of BMPR2 resulted in increase of tumorigenicity [91].

The balance between cell growth and death is important in homeostasis [92]. Disturbances of this balance can cause pathological disorders such as disturbances in embryogenesis, neural degenerative diseases, as well as the development of cancer. BMPR2 has been shown to either suppress or promote apoptosis in cancer. Inhibition of BMPR2 by transfecting a negative truncated mutant of the BMP receptor-II (tBMPR2) into Tca8113 oral squamous carcinoma cells induced apoptosis [93]. BMPR2 anti-apoptotic activities were shown in liver cancer cells. A study by Zeng *et al.* demonstrated that inhibition of BMPR2 with siRNA increases apoptosis in HepG2 liver cancer cells [94].

Cancer cells have the ability to invade and spread throughout the body via blood and lymphatic vessels in a process called metastasis [95]. BMPR2 could either induce or inhibit metastasis in cancer cells. A study on mammary tumor progression in mice showed that disruption of BMPR2 accelerated mammary carcinoma metastasis [96]. Moreover, mice expressing dominant-negative (DN) BMPR2 had a fivefold increase in lung metastasis [96]. In contrary, immunostaining on lower lip squamous cell carcinoma (LLSCC) showed that BMPR2 is expressed higher in metastatic cells than non-metastatic cells [97].

It is also known that transforming growth Factor-ß (TGF-ß) is involved in chemoresistance development by altering epithelial to mesenchymal transition (EMT), regulating stemness and increasing cancer cell survival protein [98-103]. BMPR2 is a receptor of TGF-β superfamily. Thus, the roles of BMPR2 in chemoresistance remain elusive.

1.6 Aim of Thesis

Important progress has been achieved for breast cancer treatment in recent years, including novel targeted therapies. However, classical chemotherapy is still one of the most frequently used in the clinics and thus, chemoresistance presents a major cause of breast cancer treatment failure. Complexity of cellular processes in breast cancer, results in the failure of the treatment due to lacking of signaling pathway knowledge. Therefore, research on drug resistance development and solutions to overcome drug resistance are urgently needed to achieve better efficacy of chemotherapeutic drugs.

The first aim of the thesis was to explore the potential of salinomycin, initially a cancer stem cell targeting drug, in circumventing drug resistance. The mechanism of salinomycin sequential treatment evading doxorubicin resistance was to be investigated. Additionally, activity and mechanism of salinomycin in sensitizing tamoxifen-resistant cells were also to be explored.

In the second part of the thesis, the aim was to explore the role of bone morphogenetic protein receptor II (BMPR2), a serine/threonine kinase and receptor of TGF-ß family member, in breast cancer chemoresistance. The potential use of BMPR2 as diagnostic marker and target for cancer therapy was further analyzed by several biological assay.

2. MATERIAL AND METHODS

2.1 Material

2.1.1 Drugs

Doxorubicin, salinomycin, tamoxifen and verapamil were purchased from Sigma Aldrich, Steinheim, Germany.

2.1.2 Cell culture

MCF-7, BT-474, MDA-MB 231, and MDA-MB 436 human breast cancer cells were purchased and cultured according to ATCC. MCF-7 cells were grown in DMEM medium high glucose (Gibco) supplemented with 20 % fetal calf serum (FCS) (Gibco) and 2 mM L-glutamine (Gibco) at 37°C and 5% CO_2. BT-474 cells were grown in RPMI 1640 medium (Gibco) supplemented with 10 % fetal calf serum (FCS) and 2 mM L-glutamine (Gibco) at 37°C and 5% CO_2. MDA-MB 231, and MDA-MB 436 cells were grown in L-15 Leibovitz's medium (Biochrom) supplemented with 10% FCS and 2mM glutamine at 37°C without CO_2. For estradiol stimulation experiment, MCF-7 cells were cultured in DMEM high glucose phenol red free (Gibco) supplemented with 20% charcoal stripped fetal bovine serum (Sigma-Aldrich) and 2 mM L-glutamine (Gibco). Cells were routinely tested and confirmed as mycoplasma free.

2.1.3 Oligonucleotides

pCPG-hCMVSCEP-BMPRII, encoded BMPR2 were purchased from imaGenes (Germany), Plasmid *pEGFP-N1* (Clontech Laboratories, Germany) or *pTRIPZ* (Thermo Scientific, Germany) were used as a scramble control. siRNA targeting BMPR2 (ON-TARGETplus SMARTpool Human BMPR2: 5'-3'), and siRNA-OFF-TARGET-siScramble were purchased from Thermo Scientific Dharmacon® (Germany). 3x-ERE-TATA-luc plasmid (#11354), EGFR-GFP plasmid (#32751) and HER2-GFP plasmid (#39321) were purchased from Addgene (Germany).

2.1.4 Primers

gene	UPL	sequence
ACVR2A	43	left primer : AAAGCCCAGTTGCTTAACGA
		right primer : TGCCATGACTGTTTGTCCTG
ACVR2B	45	left primer : TGTCAAGATCTTCCCACTCCA
		right primer : AGC AGGTTCTCGTGCTTCAT
BMPR2	5	left primer : CAGAGGCCTAATTCTCTGGATCT
		right primer : TCCTGATTTGCCATCTTGTG
BMPR1A	25	left primer :CTGTTTGGAGAAAATCAGAAGTACAG
		right primer : CTTCAACTCCCACTGCTTTCTT
BRCA1	8	left primer : GTACTTCTGCCTTTAAGCCACTTC
		right primer : CTCCAAATGTATCACTTTGTGC
MDR1	7	left primer : CAAGCATCTGCCAAAACCTC
		right primer : CTGGGTTTCCCCCTGTAAAT
BCRP1	56	left primer : TGGCTTAGACTCAAGCACAGC
		right primer : TCGTCCCTGCTTAGACATCC
SOX2	35	left primer : TTGCTGCCTCTTTAAGACTAGGA
		right primer : CTGGGGCTCAAACTTCTCTC
Oct4	35	left primer : AGCAAAACCCGGAGGAGT
		right primer : CCACATCGGCCTGTGTATATC
Nanog	69	left primer : ATGCCTCACACGGAGACTGT
		right primer : AGGGCTGTCCTGAATAAGCA
E-cadherin	35	left primer : CCCGGGACAACGTTTATTAC
		right primer : GCTGGCTCAAGTCAAAGTCC
ERα	17	left primer : ATCCACCTGATGGCCAAG
		right primer : GCTCCATGCCTTTGTTACTCA
GAPDH	45	left primer : TCCACTGGCGTCTTCACC
		right primer : GGCAGAGATGATGACCCTTTT

2.1.5 Antibodies

Primary antibody

Antigen	Catalogue No	Species	Manufacturer
BMPR2	6979S	rabbit	Cell Signalling Technology
pSmad 1/5/8	9511S	rabbit	Cell Signalling Technology
Smad 1/5/8	sc-6031-R	rabbit	Santa Cruz Biotechnology
pERK 1/2	9101R	Rabbit	Cell Signalling Technology
ERK 1/2	sc-93 R	Rabbit	Santa Cruz Biotechnology
p21	sc-397	Rabbit	Cell Signalling Technology
pγH2AX	9718	Rabbit	Cell Signalling Technology
XIAP	2045	Rabbit	Cell Signalling Technology
Actin	sc-1616-R	rabbit	Santa Cruz Biotechnology
Tubulin	T 9026	Mouse	Sigma Aldrich
GAPDH	14C10	Rabbit	Cell Signalling Technology
EGFR	ab30	Mouse	Abcam

Secondary antibody

Specification	Catalogue No	Species	Manufacturer
Peroxidase labeled anti-rabbit IgG (H+L)	PI-1000	Rabbit	Vector Laboratories
Peroxidase labeled anti-mouse IgG (H+L)	PI-2000	Rabbit	Vector Laboratories
Alexa Fluor® 488 labelled goat anti-rabbit IgG (H+L)	A-11034	Rabbit	Life Technologies
FITC labelled goat anti-mouse IgG (H+L)	A-11001	Mouse	Life Technologies

2.1.6 Animals

Female Rj:NMRI-Foxn1^{nu}/Foxn1^{nu} mice were purchased Janvier (Le Genest-St-Isle, France). The mouse strain has a mutation in the gene Foxn1 which caused thymic aplasia, resulting in development of immunodeficiency due to lack of thymus and hair follicle keratinization.

2.1.7 Kits

Name	Manufacturer
CellTiter-Glo®	Promega (Germany)
miRCURY RNA Isolation Kit	Exiqon, Denmark
Transcriptor High Fidelity cDNA Synthesis	Roche (Germany)
Light Cycler 480® Probes Master	Roche (Germany)
BCA-Assay	Thermo Fisher Scientific (USA)
OxiSelect™ Comet Assay Kit	Cell Biolabs (USA)
Caspase-Glo® 3/7 Assay System	Promega (Germany)

2.1.8 Instruments

Name	Manufacturer
Phase contrast microscope Axiovert 200	Carl Zeiss (Germany)
Fluorescence microscope Axiovert 200	Carl Zeiss (Germany)
SP 8 SMD confocal microscope	Leica (Germany)
TE200E spinning disc microscope	Yokogawa (Japan)
CyAnTM ADP	Dako cytomations (Germany)
LightCycler 480 system	Roche (Germany)
Luminometer Lumat LB960	Berthold (Germany)
FastPrep®-24 tissue	MP Biomedicals GmbH (Germany)
Thermal cycler	Thermo Fisher Scientific (USA)
Tecan spectra fluor plate reader	Tecan (Switzerland)
Mini-PROTEAN® Tetra Cell	Biorad (Germany)
Thermal Cycler	Thermoscientific (Germany)
NanoDrop 2000	Thermo Scientific

2.1.9 Software

Name	Manufacturer
Graph Pad Prism 5	Graph Pad Software (San Diego, USA)
FlowJo 7.6.5	FlowJo LLC (Oregon, USA)
Infinity capture	Lumenera Corporation (Canada)
AxioVision	Carl Zeiss (Germany)
Image J	NIH (USA)
CellProfiler	Broad Institute (USA)

2.2 Methods

2.2.1 General

2.2.1.1 Molecular evolution assay

Cells (80% confluency) were treated with drugs for 72 hours, as previously described [67]. Concentration of doxorubicin was 50 nM, while salinomycin were 500 nM for MCF-7 and BT-474 cells, and 50 nM for MDA-MB 231 and MDA-MB 436 cells. Medium was changed with fresh medium after drug exposure, and cells were grown until recovered (80% confluency again). As soon as recovered, cells then were splitted for RNA and protein analysis, cytotoxicity assay, and for the next sequential treatment. R0 represents the untreated control cell line (parental cells), whereas R1, R2, R3, R4, and R5 represent cells which are treated one, two, three, four and five times, respectively.

2.2.1.2 Cytotoxicity assay

For cytotoxicity assay, $3x10^3$ cells were seeded in 96 well plate (TPP) and incubated for 24 hours under suitable incubation condition. Cells were then treated with drugs for 72h, continued with Cell Titer-Glo® assay (Promega) as manufacturer's instruction.

2.2.1.3 RNA extraction and quantitative RT-PCR

Total RNA was extracted and isolated from cells or tissues using miRCURY™RNA Isolation Kit (Exiqon, Denmark). RNA was then reverse transcribed to cDNA from 1.0 μg of total RNA by using Transcriptor First Strand cDNA Synthesis Kit (Roche). Real time-PCR were then performed in Light Cycler®480 (Roche) using Master Probes Kit (Roche) and universal probe library (UPL) (Roche). All protocols were performed according to instruction from manufacturer. RT-PCR were performed using the following primers and UPLs. GAPDH was used as internal control. The results were analyzed using comparative threshold cycle (ΔCT method).

2.2.1.4 Protein extraction and western blotting

Protein was extracted from cells or tissues using lysis buffer containing Cell lysis solution® (Promega), protease inhibitor cocktail (Roche), and phosphatase inhibitor (New England Biolabs). Briefly, cells were washed three times with cold PBS, added with lysis buffer followed by 5 minutes incubation on ice, and harvested using cell scrapper. Supernatants were collected after cold centrifugation, and sample were then stored in -80°C until protein quantification. Protein were quantified using BCA Protein Assay Kit (Thermo Fisher Scientific). Equal

amounts of protein were resolved by SDS-PAGE (Bio-Rad), transferred into PVDF membrane (Bio-Rad) using Mini-PROTEAN® Tetra Cell (Bio-Rad), blocked with NET-gelatin for 1h, incubated with specific primary antibodies overnight at 4°C, and followed by 1h incubation of conjugated-peroxidase secondary antibody at room temperature. Antibody binding was then visualized using Lumi-Light^{PLUS} western blotting substrate (Roche). Film was developed in CP 1000 Film processor (AGFA). Quantification of western blot results were performed using ImageJ software.

2.2.1.5 Clonogenic survival assay

To perform clonogenic assay, 500 cells were seeded in 6-well-plate (TPP, Switzerland) and incubated under suitable condition for 24h prior to 72h treatment with various concentration of drugs treatment.Cells were then grown for 14 days, fixed and stained with para form aldehyde (PFA) containing crystal violet. Surviving colonies were analyzed by ColonyArea, as previously described [104].

2.2.2 Salinomycin circumvents drug resistance of breast cancer cells

2.2.2.1 Internalization of doxorubicin

Cells were treated with 10 μM of doxorubicin for 3h. Medium was changed with fresh medium 1h prior to image and FACS analysis. Intracellular doxorubicin level was analyzed with fluorescence microscope Axiovert 200 (Carl Zeiss, Germany). To performed FACS analysis, cells were prepared by trypsinization, followed by measurement on CyAn^{TM} ADP flow cytometer (DakoCytomation). Doxorubicin intracellular level were analyzed using FlowJo (FlowJo, LLC).

2.2.2.2 Generation of tamoxifen-resistant cells

MCF-7 tamoxifen-resistant cells (MCF-7 TAM-R6) were provided by Ann-Katrin Sommer (PhD student, Max Planck Institute of Biochemistry, Martinsried, Germany). MCF-7 TAM-R6 cells were generated by sequential treatment of 10 μM tamoxifen in 6 sequential rounds, as previously described [67].

2.2.2.3 Transfection

Plasmid transfection was performed either using K2® transfection system (Biontex Laboratories, Germany), or X-tremeGENE 9 DNA Transfection Reagent (Roche), as instructed by manufacturer.

2.2.2.4 Estrogen response element luciferase reporter assay

Cells were transfected with 3x-ERE-TATA-Luc plasmid (#11354) (Add gene) using K2® Transfection System (Biontex Laboratories GmbH), as instructed by manufacturer. At 36h after transfection, starvation with serum free medium was performed for 12h, followed by 12h treatment with 1 nM 17 ß-estradiol. Cells were then treated with 100 µL cell lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 2 mM DTT, 10% glycerol, 1% Triton X-100). Luciferase activity in the cell lysate was measured using a luciferase assay kit (100 µL Luciferase Assay buffer, Promega, Germany) and a Lumat LB960 luminometer (Berthold, Bad Wildbad, Germany).

2.2.2.5 Inhibition of EGFR recycling

Cells were seeded in 6-well plate, and incubated for 24h prior to stimulation with recombinant murine epidermal growth factor (mEGF) (Pepro Tech EC Ltd., UK) and treatment with salinomycin. Cells were collected with trypsinization, washed with PBS, resuspended in 0.1 % BSA and incubated with mouse anti-huEGFR antibody (Dako, Germany) for 1h at 4 °C. After washing with 0.1 % BSA, samples were stained with goat anti-mouse IgG1 antibody linked to FITC (Molecular Probes) for 1h at 4 °C. Samples were then transferred into flow cytometry tube and measured on CyAnTM ADP flow cytometer (DakoCytomation).

2.2.2.6 Spinning disk confocal microscopy

Imaging experiment was performed by Dr. Frauke Mickler (Postdoctoral fellow, Dept. of Physical Chemistry, LMU Munich). For all imaging experiments cells were seeded in 8 well chambered μ-Slides (ibiTreat, Ibidi GmbH) at a density of 7000 (3 days before imaging) or 10000 (2 days before imaging) cells per well.

To analyze EGFR and HER-2 trafficking, 24h after seeding, cells were transfected either with EGFR-GFP or HER2-GFP plasmid (Addgene) using the X-tremeGENE 9 DNA Transfection Reagent (Roche). Receptor GFP expressing cells were imaged 48-72h after transfection. To detect the effect of salinomycin on receptor trafficking, salinomycin was added at concentration of 6 μM 3h and 24h before imaging. For co-localization with lysosomes, 15 nM lysotracker deep red was added into the cells, followed by 1h incubation and medium change. In order to induce receptor endocytosis, 50 pmol/ml EGF was added to the cells 24h before measurement. To analyze calcium concentration and lysosome, cells were pre-treated with 6 μM salinomycin for 3h or 24h or kept without salinomycin treatment. 1h before imaging, 6 μM Fluo-3-AM and 15 nM Lysotracker deep red (both Molecular Probes, Life Technologies) were added to the cells. For imaging, medium was exchanged by marker-free CO_2 independent medium (with or without salinomycin).

Spinning disk confocal microscopy was performed on a setup based on the TE200E microscope and the Yokogawa spinning disk unit CSU10. Overlay images and LUT images were built in ImageJ. For co-localization analysis, the ImageJ macro JaCOP was applied. According to a manual threshold, the fluorescence signal in the red and green was selected. The fraction of green pixels overlapping red pixels was calculated. Results were shown as Manders coefficient, M1. Mean values of all evaluated cells (N=7-13 images) were presented together with the standard deviation.

2.2.3 BMPR2 promotes doxorubicin resistance

2.2.3.1 Transfection

Transfection was performed using Lipofectamine® 2000 (Invitrogen) for plasmid and Metafectene (Biontex) for siRNA, as instructed by manufacturers.
Cells were seeded for the intended use : in 6-well plates (for western blot), in 12 well-plate (for cell cycle analysis), in 48-well plates (for cell proliferation assay), and in 96-well plates (for cytotoxicity and caspase activity assay).
For the signalling experiment (western blot), 72h or 96h after transfection with plasmid or siBMPR2, respectively, cells were starved for 24h with serum free medium, continued with 30 min stimulation with 20 ng/mL BMP2 (Invitogen) and lysate preparation.

2.2.3.2 Cell proliferation assay

Cells were seeded in a 48-well-plate, and incubated under suitable condition. After 24h incubation, cells were transfected either with plasmid or siBMPR2, and controls as previously described. Starting from day one after transfection, cells were harvested by trypsinization, and counted using Bio-rad cell counter (Bio-Rad), as manufacturer's instruction.

2.2.3.3 Cell cycle analysis

Cells were seeded, transfected, and treated with doxorubicin as previously described. All floating cells were collected together with trypsinized cells, and incubated for 4h on ice in propidium iodide (Sigma) staining solution containing sodium citrate (Sigma) and triton X (Sigma), with intermittent shaking. Cells were then resuspended in PBS, transferred into a flow cytometry tube and measured on CyAn^{TM} ADP flow cytometer (DakoCytomation). Cell cycle were analyzed using FlowJo (FlowJo, LLC).

2.2.3.4 Comet assay

DNA damage was measured by the single cell electrophoresis comet assay under alkaline condition (OxiSelect™ Comet Assay Kit (3-Well Slides) (CellBiolabs, San Diego) as manufacturer's instruction. The results were analysed by OpenComet, as previously described [105]. DNA damage was expressed as tail moment.

2.2.3.5 Immunostaining

Cells were grown in 8 well chambered μ-Slides (ibiTreat, IbidiGmbH) and transfected as previously described. After 24h treatment with doxorubicin, cells were fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 0.2% Triton-X 100 in PBS, blocking with 10% FCS + 1% gelatin + 0.05 % Triton-X 100 in PBS for 1h in room temperature, first antibody incubation with phospho-Histone γH2AX (Ser139) (20E3) (#9718, Cell signaling) overnight at 4^{o}C, washing with PBS, and incubation with secondary antibody labeled with Alexa Fluor 488 (Invitrogen). Cells were counterstained with DAPI for 10 min. Images were obtained using a Leica SP 8 SMD confocal microscope (Germany) and image analysis was performed with the LAS AF software. Double strand break DNA damage was shown as number of speckle in the nucleus. Quantification of speckles was performed by CellProfiler, as previously described [106]. Immunostaining was performed together with Dr. Jonathan Garcia-Roman (Postdoctoral fellow, Pharmaceutical Biotechnology, LMU Munich).

2.2.3.6 Caspase-3/7 activity assay

To perform caspase assay, 5x10^{3} transfected cells and respective controls were seeded in 96 well plate (TPP) and incubated for 24h under suitable incubation condition. Cells were then treated with doxorubicin for 12h. The activity of caspase-3 and -7 was measured using Caspase-Glo® 3/7 Assay (Promega) as manufacturer's instruction.

2.2.3.7 Animal experiments

MDA-MB 231 cells (5x10^{6}) were mixed with matrigel (BD Bioscience) 1:1 and inoculated subcutaneously into the left flank of 6 weeks old female Rj:NMRI-nu (nu/nu) (Janvier, Le Genest-St-Isle, France). Experiments were started 30 days after tumor cell injection. Mice were treated with 5 mg/kg of doxorubicin (Sigma-Aldrich) weekly for 4 weeks intravenously. Tumors were homogenized in a tissue and cell homogenizer (FastPrep®-24, MP Biomedicals, USA) using cell culture lysis buffer (Promega) or miRCURY^{TM}RNA Isolation Kit (Exiqon, Denmark). Samples were then centrifuged at 14.000 rcf at 4 °C for 10 min to separate insoluble cell components. Protein and RNA samples were analyzed as previously described. The animal

experiments were performed by Johanna Busse (Veterinary MD student, LMU Munich) and Dr. Annika Herrmann (Pharmaceutical Biotechnology, LMU Munich). All animal procedures were approved and controlled by animal experiments ethical committee of Regierung von Oberbayern, District Government of Upper Bavaria, Germany, and carried out according to the guidelines of the German law of protection of animal life.

2.2.4 Statistical analysis

All data were analyzed using Prism 5.0 Software GraphPad. Statistical analysis was conducted using Student's t-test. * or ** or *** or **** indicates $p<0.05$ or $p<0.01$ or $p<0.001p<0.0001$, respectively.

3. RESULTS

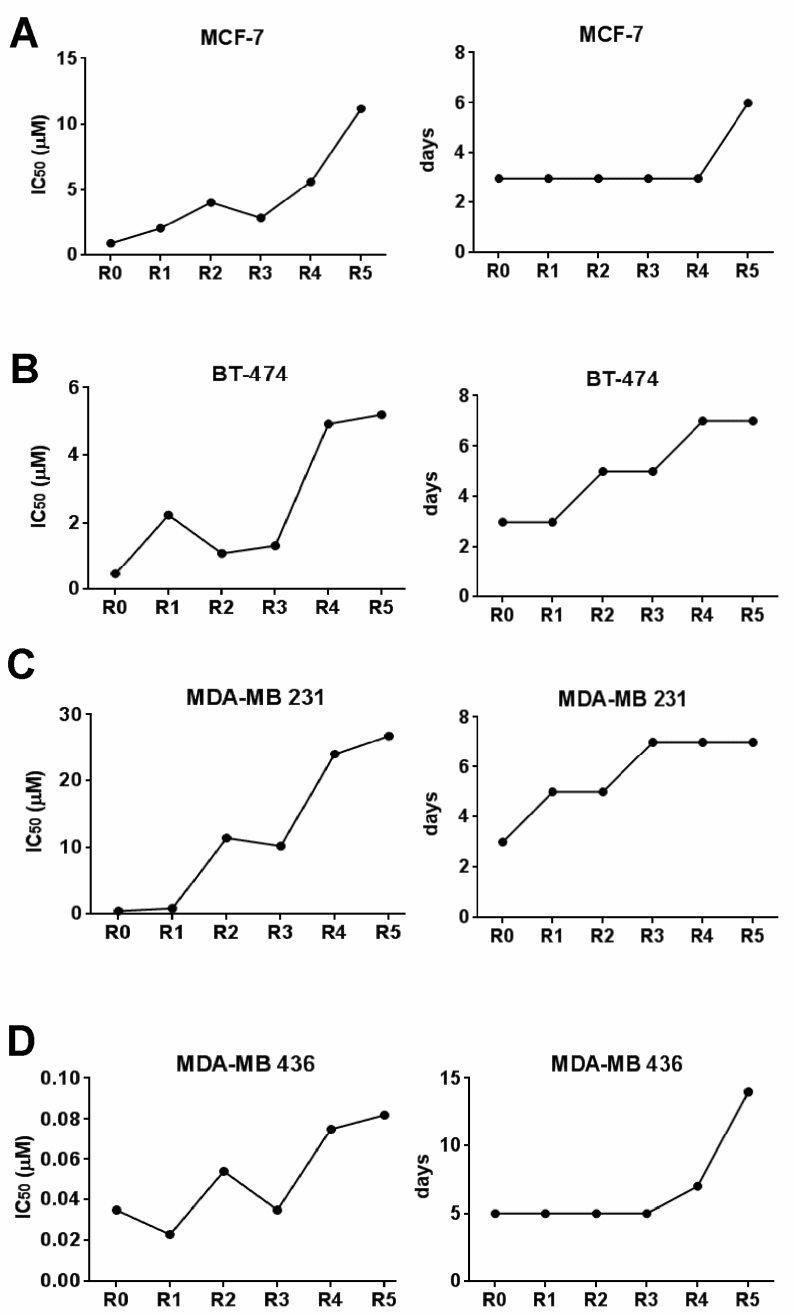
3.1 Salinomycin Circumvents Drug Resistance of Breast Cancer Cells

3.1.1 Sequential salinomycin pretreatment increases doxorubicin chemosensitivity of breast tumor cells

The discovery of salinomycin as anti-cancer stem cell drug provides progress in overcoming chemoresistance. Question arises whether treatment of salinomycin could also circumvent drug resistance by its sequential treatment.

3.1.1.1 Molecular evolution assay of salinomycin results in salinomycin-resistant cells

To mimic clinical application of chemotherapeutics *in vitro*, sequential treatment of salinomycin was performed in the epithelial breast cancer cell lines MCF-7 and BT-474, as well as in the mesenchymal breast cancer cell lines MDA-MB 231 and MDA-MB 436. Sequential treatment of salinomycin results in enhanced formation of salinomycin-resistant cells. The IC_{50} of salinomycin increased during the treatment cycles, both in epithelial MCF-7 (Fig. 4A) and BT-474 cells (Fig. 4B) as well as in mesenchymal MDA-MB 231 (Fig. 4C) and MDA-MB 436 (Fig. 4D) cells. Furthermore, the resistant cells also displayed slightly decreased proliferation compared to parental cells indicated by a longer time span to reach 80% confluence (Fig. 4A-D, middle-right panel). However, unlike in several molecular evolution assays of doxorubicin (Fig. S1, supplementary information), the growth retardation here is only modest.



	IC_{50} (µM)	days to 80% confluency
R0	0.92	3
R1	2.06	3
R2	4.01	3
R3	2.84	3
R4	5.62	3
R5	11.2	6

	IC_{50} (µM)	days to 80% confluency
R0	0.47	3
R1	2.23	3
R2	1.09	5
R3	1.32	5
R4	4.92	7
R5	5.20	7

	IC_{50} (µM)	days to 80% confluency
R0	0.46	3
R1	0.86	5
R2	11.40	5
R3	10.20	7
R4	24.00	7
R5	26.78	7

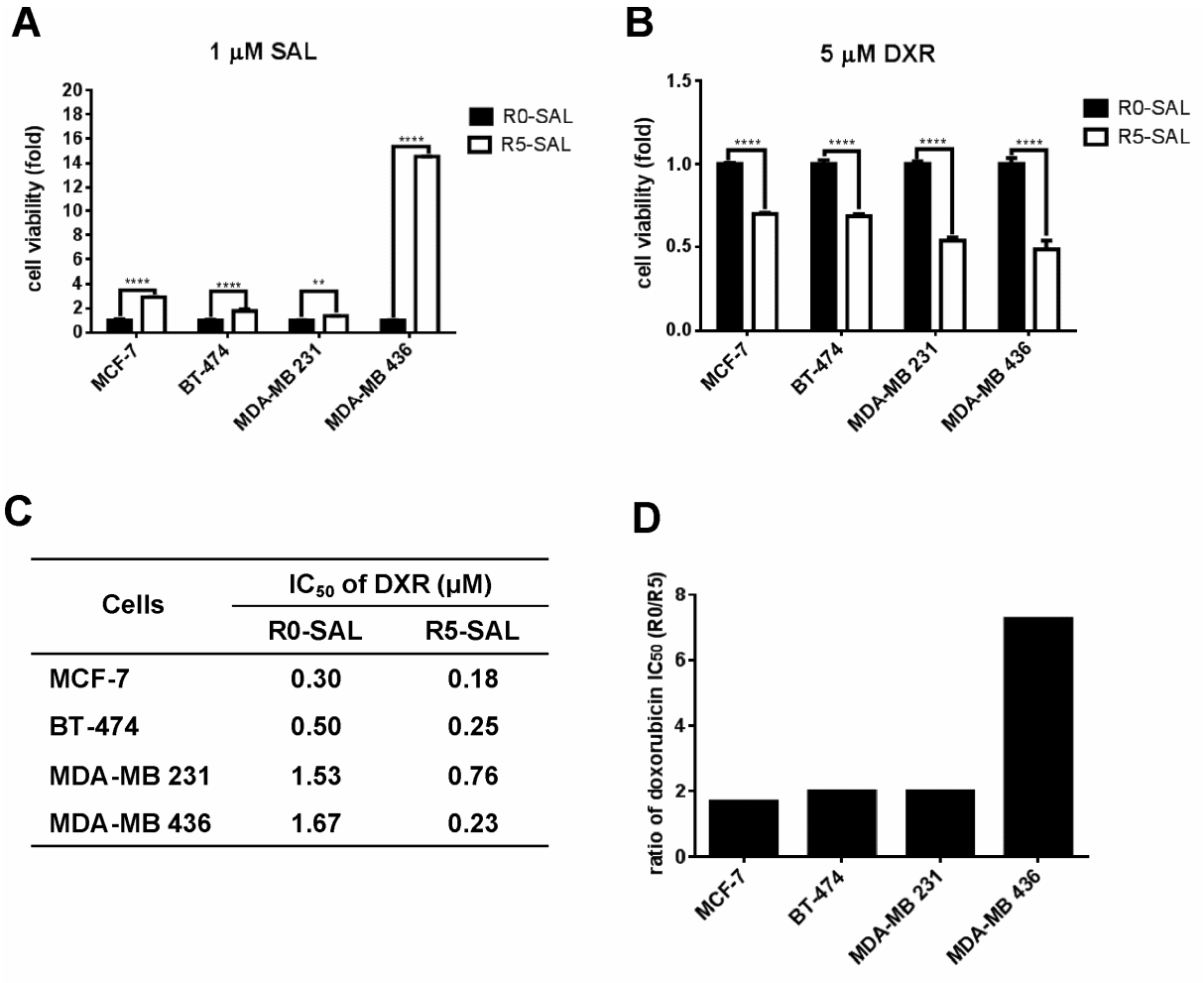
	IC_{50} (µM)	days to 80% confluency
R0	0.035	5
R1	0.023	5
R2	0.054	5
R3	0.035	5
R4	0.075	7
R5	0.082	14

Fig. 4. Molecular evolution assay reveals salinomycin-resistant cancer cells. IC_{50} of salinomycin during molecular evolution assay (left and right panel) and the day to reach an 80% confluency after cell splitting (middle and right panel). (A) MCF-7, (B) BT-474, (C) MDA-MB 231, (D) MDA-MB 436 cells. To determine IC_{50}, cells were treated with various concentrations of salinomycin for 72h. Cell viability was measured with CellTiter-Glo assay. Percentage was normalized to untreated cells. IC_{50} was analyzed with GraphPad. R0 represents the parental cells, whereas R1, R2, R3, R4 and R5 represent cells that are treated for one, two, three, four and five times, respectively.

3.1.1.2 Salinomycin resistant-cells show chemosensitivity to doxorubicin

The final increase in resistance was compared by analyzing the cytotoxicity of 1 μM salinomycin of parental (R0) and salinomycin-resistant cells (R5). In MCF7, BT474 and MDA-MB 231 an up to 3-fold increase was observed whereas MDA-MB 436 developed even higher

resistance (14-fold) (Fig. 5A). As salinomycin acts on other targets than classical chemotherapeutics, the cross-resistance to doxorubicin (5μM) was conducted by performing cytotoxicity assay on both resistant (R5) and parental cells (R0). The results revealed that all of the MCF-7, BT-474, MDA-MB 231 and MDA-MB 436 salinomycin-resistant cells (R5) became more sensitive to doxorubicin compared to the parental cells (R0) (Fig. 5B). When analyzing the IC_{50} for doxorubicin of R0 and R5 cells a 2- to 7-fold increase was found in doxorubicin sensitivity (Fig. 5C-D).



Cells	IC_{50} of DXR (μM)	
	R0-SAL	R5-SAL
MCF-7	0.30	0.18
BT-474	0.50	0.25
MDA-MB 231	1.53	0.76
MDA-MB 436	1.67	0.23

Fig. 5. Salinomycin-resistant cells show chemosensitivity towards doxorubicin. Susceptibility of MCF-7, BT-474, MDA-MB 231, and MDA-MB 436 R0 and R5 to (A) 1 μM of salinomycin and (B) 5 μM of doxorubicin. (C) IC_{50} of doxorubicin in R0 and R5 salinomycin resistant cells. (D) Ratio of doxorubicin IC_{50} (R0/R5 SAL). Cancer cells of R0 and R5 were treated with indicated concentrations of salinomycin or doxorubicin for 72h. Cell viability was measured with cell titer-glo assay. Percentage was determined to untreated cells. IC_{50} was analyzed with GraphPad. Results shown as fold in comparison with R0. Results shown as fold to parental cells (R0) and represent the average of three independent experiments (mean ± SD). Statistical analysis was conducted using Student's t-test. ** or **** indicates $p<0.01$ or <0.0001, respectively.

3.1.1.3 Decreased expression of drug efflux pump genes in salinomycin-resistant cells

A common mechanism in drug resistance is the upregulation and increased activity of efflux pumps, which play an important role in absorbing, distributing, and eliminating drugs from the cells. To determine their basal expression quantitative RT-PCR of the prominent efflux pump genes MDR1 and BCRP1 was performed in all parental cells (R0). MDR1 is highly expressed in BT-474, whereas BCRP1 is highly expressed in MDA-MB 231 cells (Fig. 6A). Furthermore, MCF-7 cells have the lowest efflux pumps gene expression among the analyzed cells. To check whether the sequential treatment of salinomycin influences the expression of drug efflux pumps, MDR1 and BCRP1 expression in salinomycin resistant cells (R5) was measured and compared to respective parental cells (R0). Both MDR1 and BCRP1 are downregulated in MCF-7 and BT474 resistant cells (Fig. 6B and 6E). In addition, MCF-7 salinomycin-resistant cells show a decrease in cancer stem cells markers SOX-2, Oct-4 and Nanog (Fig. S2, supplementary information). In MDA-MB 231 salinomycin-resistant cells, MDR1 is upregulated, and BCRP1 displayed decreased expression (Fig. 6D). MDR1 is downregulated, whereas BCRP1 is upregulated in BT-474 salinomycin-resistant cells (Fig. 6C). However, the basal BCRP1 expression in parental BT474 cells is originally very low. To sum up, a decreased expression of the two prominent drug efflux pumps was observed in the majority of salinomycin-resistant breast cancer cell lines.

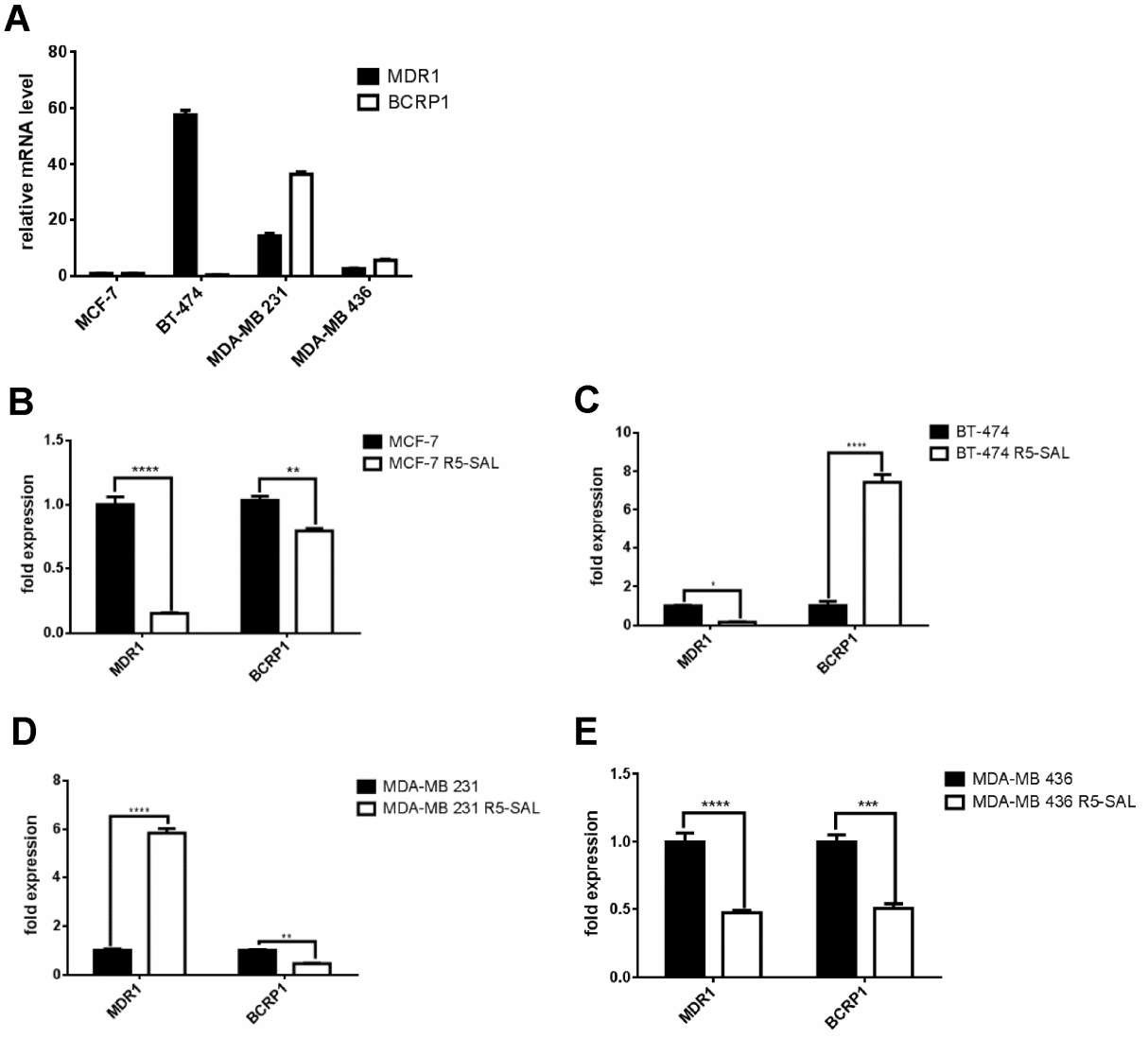


Fig. 6. Salinomycin-resistant cells show downregulation of efflux pump. MDR1 and BCRP1 expression in (A) MCF-7, BT-474, MDA-MB 231 and MDA-MB 436 parental cells (R0), (B) MCF-7, (C) BT-474, (D) MDA-MB 231, (E) MDA-MB 436 salinomycin resistant cells (R5) compared to R0. Gene expression is determined by quantitative RT-PCR. GAPDH was used as an internal control for quantitative RT-PCR. Results represent the average of three independent experiments (mean ± SD). Statistical analysis was conducted using Student's t-test. * or ** or *** or **** indicates $p<0.05$ or $p<0.01$ or $p<0.001$ or <0.0001, respectively.

3.1.1.4 Blocking the efflux pumps activity affects mostly parental cells

In order to analyze whether the decrease in efflux pump expression affects the pump activity, blocking of these pumps was performed with verapamil, a selective inhibitor, and cytotoxicity upon doxorubicin treatment was measured. In MCF-7 parental cells, addition of verapamil increased the doxorubicin induced cytotoxicity, whereas in salinomycin resistant cells addition of verapamil had almost no effect (Fig. 7A). Similar effects were also observed on BT-474 (Fig.

7B), MDA-MB 231 (Fig. 7C), and MDA-MB 436 (Fig. 7D). Thus, inhibition of the efflux pumps increases the doxorubicin-induced cytotoxicity only in parental (R0) cells.

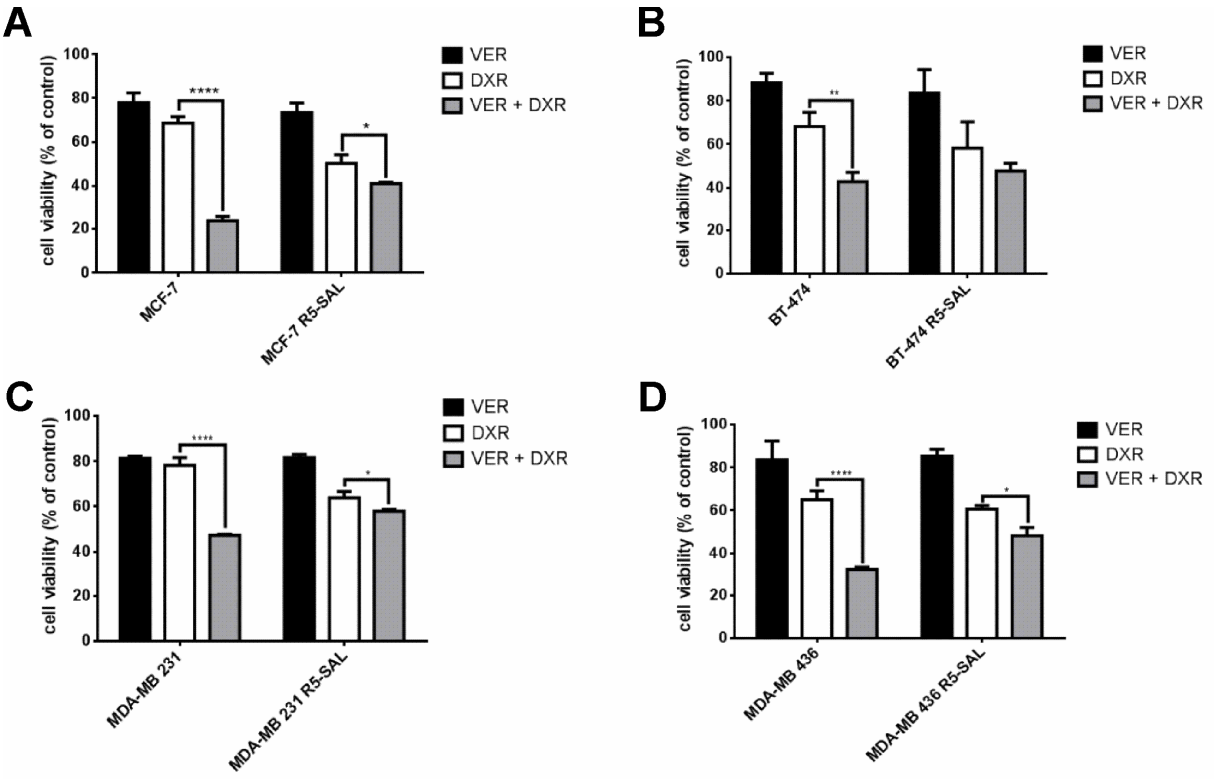


Fig. 7. Salinomycin-resistant cells show decreased in efflux pump activity Susceptibility of (A) MCF-7, (B) BT-474, (C) MDA-MB 231, and (D) MDA-MB 436 R0 and R5 salinomycin to doxorubicin single treatment or in combination with verapamil. Cancer cells of R0 and R5 were treated with respective IC_{50} of doxorubicin (as shown in Fig. 5C), with or without 10 μM of verapamil for 72h. Cell viability was measured with CellTiter-Glo assay. Percentage was determined to untreated cells. Results shown as fold to parental cells (R0) and represent the average of three independent experiments (mean ± SD). Statistical analysis was conducted using Student's t-test. *or **or **** indicates $p<0.05$ or $p<0.01$ or <0.0001, respectively.

3.1.1.5 Salinomycin-resistant cells show increased intracellular doxorubicin accumulation

As salinomycin-resistant cells show a downregulation of drug efflux pumps gene expression and their activity, intracellular drug accumulation was analyzed. Both the parental (R0) and salinomycin-resistant (R5) cells were treated with doxorubicin and the internalization of doxorubicin was measured by fluorescent microscopy (Fig. 8A), and FACS. An enhanced accumulation of doxorubicin was observed in MCF-7, BT-474, MDA-MB 436 salinomycin-resistant cells compared to parental cells (R0)(Fig. 8A, 8B and 8D). MDA-MB 231 salinomycin-resistant cells (Fig. 8C) showed no change in accumulation of doxorubicin compared to parental cells (R0), which is in line with the enhanced MDR1 expression and reduction in BCRP1.

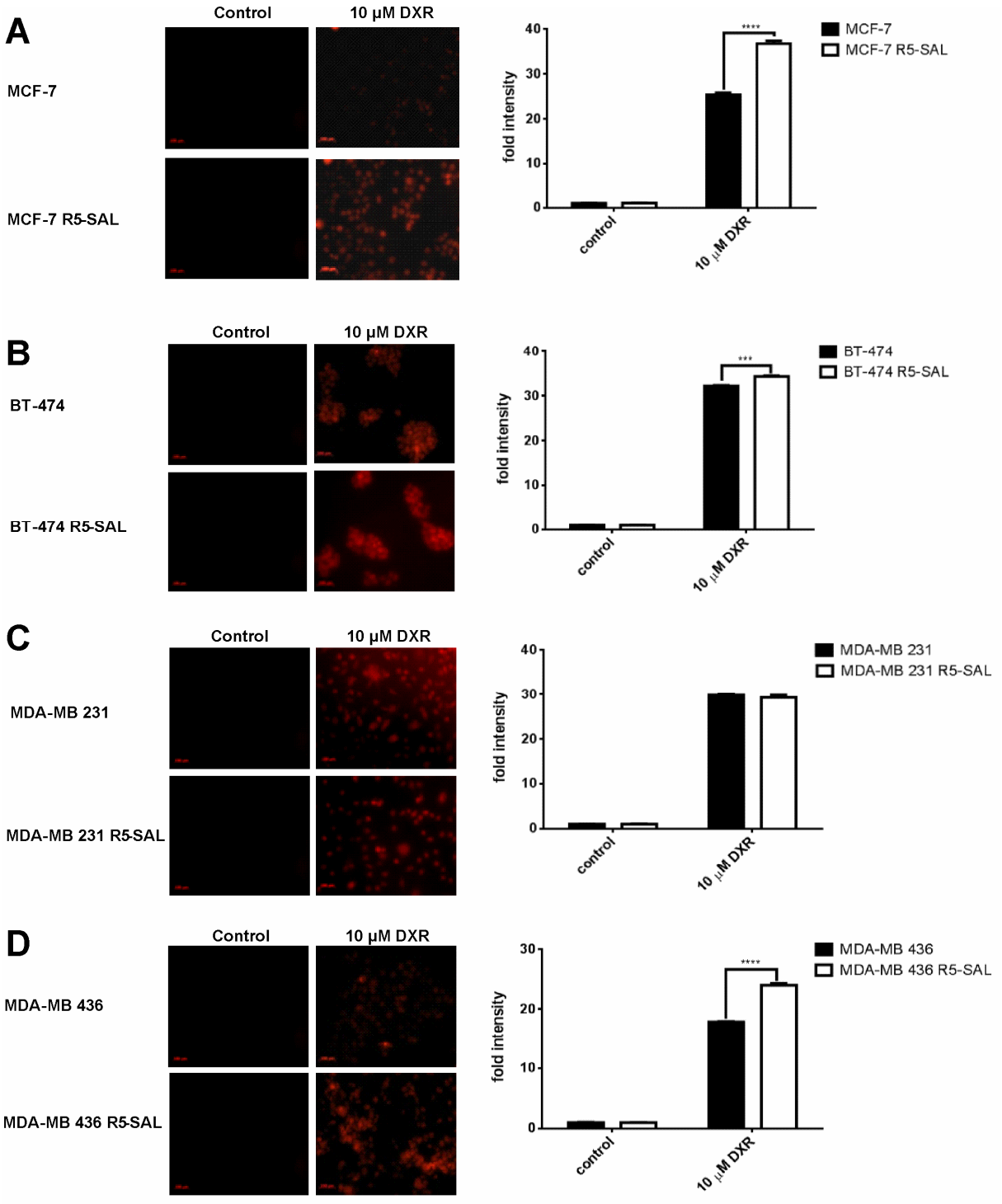


Fig. 8. Salinomycin-resistant cells show increased intracellular doxorubicin accumulation. Image analysis of intracellular doxorubicin (left panel) and FACS analysis in (A) MCF-7, (B) BT-474, (C) MDA-MB 231, and (D) MDA-MB 436 R0 and R5 salinomycin. Cells were treated with 10 μM of doxorubicin for 3h. Doxorubicin was removed from the medium 1h prior to image analysis and FACS. For flowcytometry, cells were collected with trypsinization, diluted in PBS and analyzed by FACS. Results are shown as relative of mean fluorescent to respective untreated controls and represent the average of three independent experiments (mean ± SD). Statistical analysis were conducted using Student's t-test. *** or **** indicates $p<0.001$ or <0.0001, respectively.

3.1.2 Salinomycin overcomes tamoxifen resistance of breast cancer cells

Breast cancer cells develop resistance to tamoxifen via several mechanism e.g. loss or modification of estrogen receptor expression, inhibition of apoptosis and activation of ligand independent signaling pathway. In this part of the thesis, the effects of salinomycin in overcoming tamoxifen resistance were explored.

3.1.2.1 Sequential tamoxifen treatment results in breast cancer resistant cells

To investigate tamoxifen resistance, MCF-7 breast cancer cells were sequentially treated with 10 μM of tamoxifen (Ann-Katrin Sommer, PhD student, MPI Munich). After 6 treatment cycles the sensitivity to tamoxifen was analyzed. In MCF-7 R6-TAM cells the IC_{50} value was significantly increased 2.8-fold (54 μM) compared to parental cells (R0), representing acquired tamoxifen resistance (Fig. 9A-B). A decrease in mRNA level of estrogen receptor alpha (ERα) was also detected in tamoxifen-resistant cells (R6) (Fig. 9C). As tamoxifen binds to ERα, one reason for resistance to endocrine therapy is therefore the loss of its target. The responsiveness of the ERα pathway to the inhibition with tamoxifen was further analyzed in MCF-7 TAM-R6. An ERE-luc reporter assay was used to detect estrogen signaling. Here, MCF-7 parental cells show a higher luciferase signal than MCF-7 R6-TAM after estradiol stimulation (Fig. 9D), indicating that parental cells activate the signaling pathway which is dependent of ERα, stronger than MCF-7 R6-TAM cells which have less ERα. E-Cadherin, an estradiol responsive gene, was selected to monitor ERα mediated gene transcription. Tamoxifen inhibition of ERα signaling after estradiol stimulation results in a decrease of E-Cadherin expression which was only detected in MCF-7 parental cells (Fig. 9E), indicating a decrease of the ERα signaling pathway and subsequent target gene transcription in tamoxifen-resistant cells. An increase in efflux pump expression and the presence of cancer stem cells (CSCs) are known as a reason for drug resistance. Hence, expression levels MDR1 and BCRP1 were analyzed by quantitative RT-PCR in parental (R0) as well as in tamoxifen-resistant cells (R6). Sequential tamoxifen treatment enhances the expression of both multiple drug resistance pumps causing an increased efflux of drugs (Fig. 9F). Moreover, increased expression of cancer stem cell markers Nanog and Oct-4 was observed in tamoxifen-resistant cells (Fig. 9G).

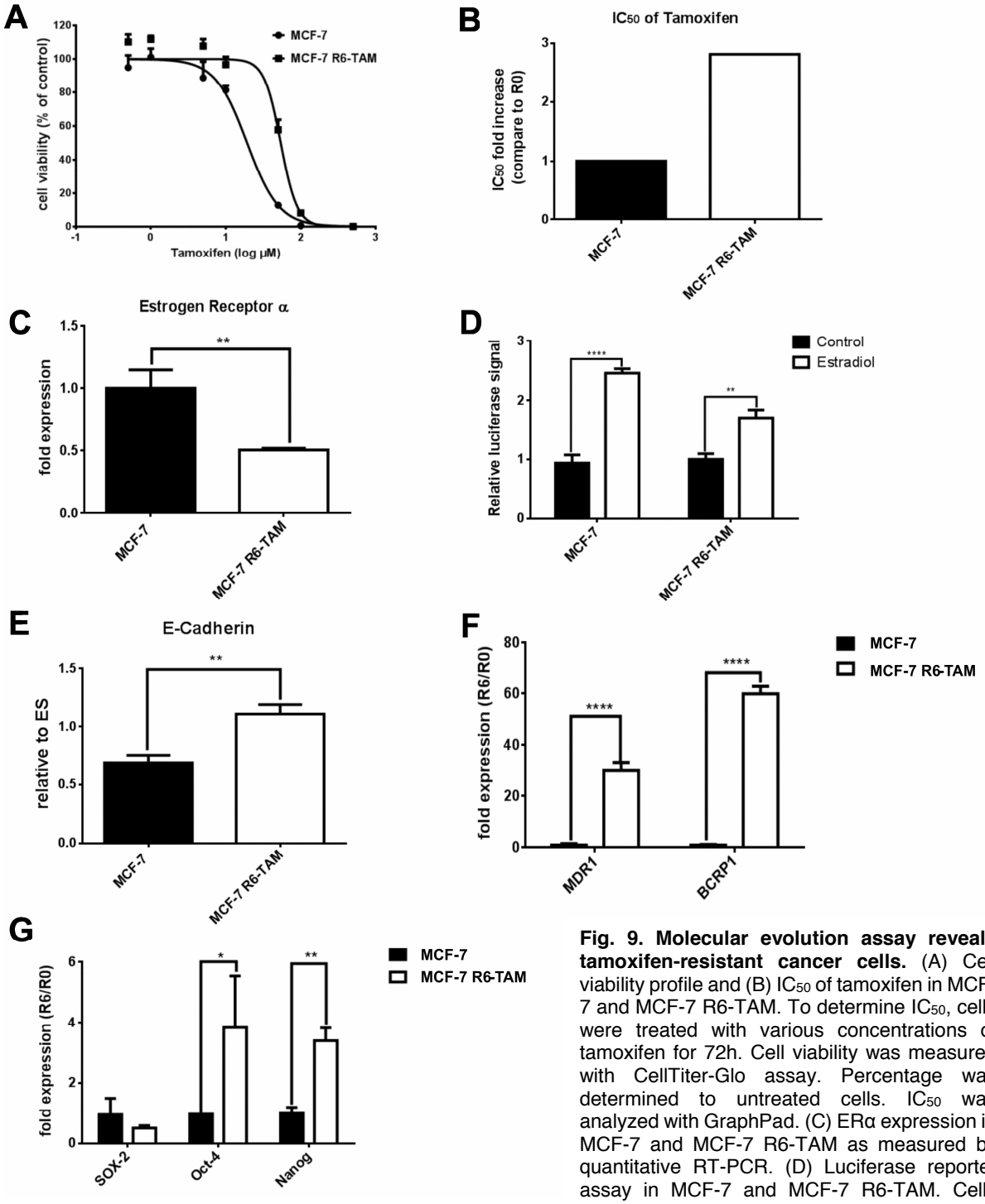


Fig. 9. Molecular evolution assay reveals tamoxifen-resistant cancer cells. (A) Cell viability profile and (B) IC_{50} of tamoxifen in MCF-7 and MCF-7 R6-TAM. To determine IC_{50}, cells were treated with various concentrations of tamoxifen for 72h. Cell viability was measured with CellTiter-Glo assay. Percentage was determined to untreated cells. IC_{50} was analyzed with GraphPad. (C) ERα expression in MCF-7 and MCF-7 R6-TAM as measured by quantitative RT-PCR. (D) Luciferase reporter assay in MCF-7 and MCF-7 R6-TAM. Cells were transfected with 3x-ERE-TATA-Luc plasmid. 36h after transfection cells were starved with serum free medium for 12h, followed by 1nM 17-ß estradiol treatment for 12h. Luciferase activity was normalized to the activity of the respective untreated control (n=5). (E) E-cadherin expression in MCF-7 and MCF-7 R6-TAM. Cells were seeded, starved with serum free medium for 12h, and treated with 1 nM 17-ß estradiol and 10 μM of tamoxifen for 12h. Cells were lysed and RNA was isolated. Gene expression is determined by quantitative RT-PCR. (F) MDR1 and BCRP1, and (G) SOX-2, Oct-4 and Nanog expression in MCF-7 and MCF-7 R6-TAM. Gene expression is determined by quantitative RT-PCR. GAPDH was used as an internal control for quantitative RT-PCR. Results represent the average of three independent experiments (mean ± SD). Statistical analysis was conducted using Student's t-test. * or ** or **** indicates $p<0.05$ or $p<0.01$ or <0.0001, respectively. Tamoxifen-resistant cells (MCF-7 TAM-R6) were generated by Ann-Katrin Sommer (PhD study, MPI Munich).

3.1.2.2 Salinomycin overcomes tamoxifen resistance

It was previously shown that salinomycin is a cancer stem cells targeting drug and can enhance doxorubicin cytotoxicity in doxorubicin-resistant cells [39, 48]. To investigate whether salinomycin can circumvent tamoxifen resistance, cell viability assay of salinomycin was performed in both parental and tamoxifen-resistant cells. The IC_{50} of salinomycin in parental cells was previously shown in figure 4A. Tamoxifen-resistant cells (R6) are four times more sensitive to salinomycin than its parental cells (R0) according to their IC_{50} values (Fig. 10A-B). Therefore the combination of both drugs works better in resistant cells than in parental cells (Fig. 10C-D). Resistance of tumor cells to a certain drug is not only reflected in short term cell survival, a long-term assay represents efficacy of drugs in a better way. Thus, a clonogenic assay was performed by incubating the cells for two weeks to recover after the 72h drug treatment (Fig. 10E-F). Tamoxifen-resistant cells displayed a higher number of colonies without any treatment indicating an increased proliferation rate. Furthermore, salinomycin treatment is more effective in tamoxifen-resistant cells (R6) than in parental cells. Taken together, combination of the drugs showed a better effect than the single treatment, even in the tamoxifen-resistant cell line.

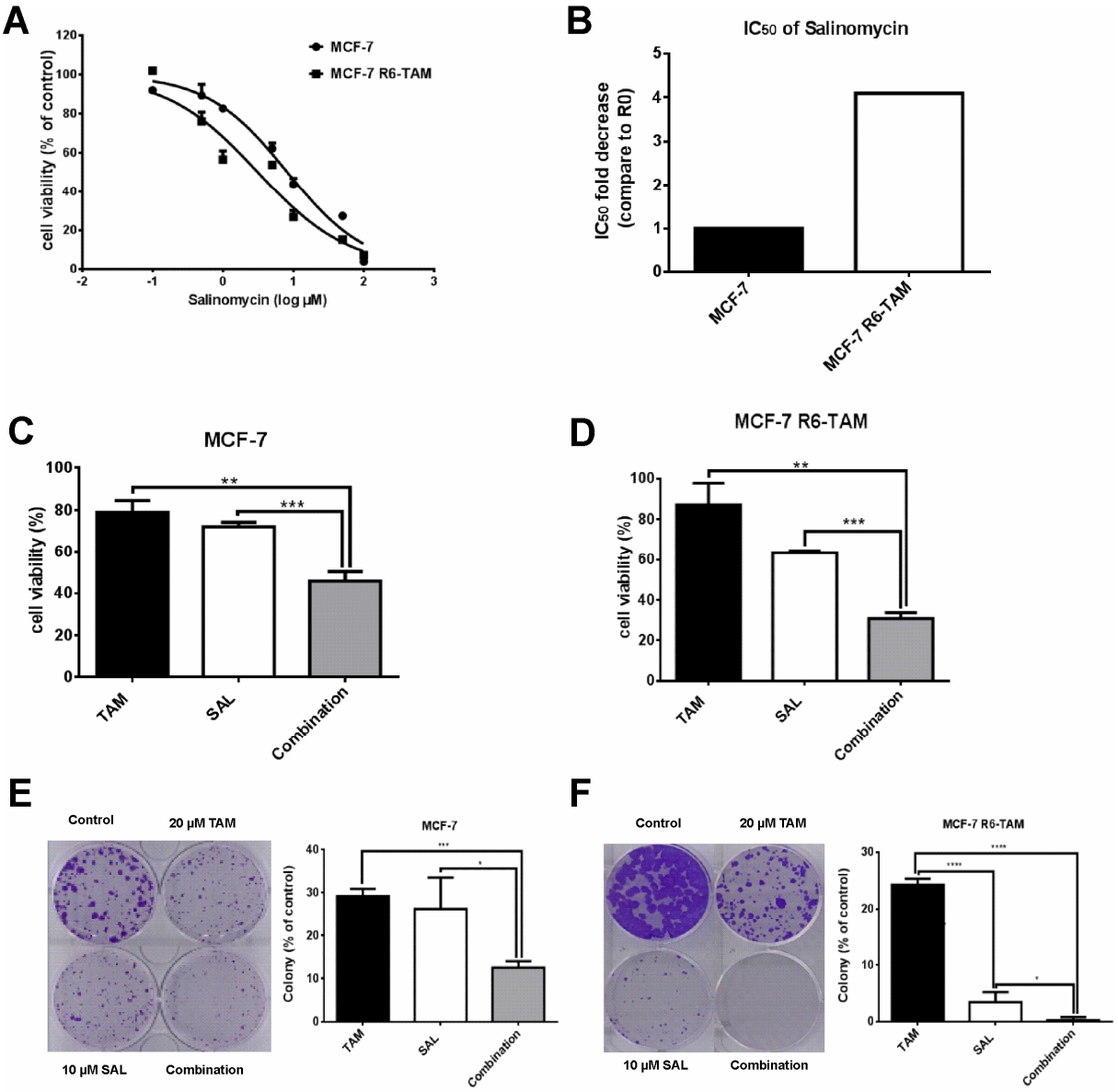


Fig. 10. Salinomycin overcomes tamoxifen resistance. (A). Cell viability profile and (B) fold increase of salinomycin IC_{50} (R6/R0). To determine IC_{50}, cells were treated with various concentrations of salinomycin for 72h. Combinational treatment of tamoxifen and salinomycin in (C) MCF-7 and (D) MCF-7 R6-TAM. Cells were treated with 10 μM tamoxifen, 0.5 μM salinomycin or combination of both for 72h. Cell viability was measured with CellTiter-Glo assay. Percentage was determined to the respective untreated cells. IC_{50} was analyzed with GraphPad. Clonogenic assay of (E) MCF-7 and (F) MCF-7 R6-TAM cells. Cells were treated with indicated concentration of tamoxifen, salinomycin and combination of both for 72h. The cells were subsequently cultivated for 2 weeks, and surviving clones were stained by crystal violet and analyzed by ColonyArea, Image J (right panel). Results represent the average of three independent experiments (mean ± SD). Statistical analysis was conducted using Student's t-test. * or ** or ***or**** indicates $p<0.05$ or $p<0.01$ or $p<0.001$ or <0.0001, respectively. Generation of tamoxifen-resistant cells (MCF-7 TAM-R6) and combinational treatment of tamoxifen and salinomycin in MCF-7 cells were performed by Ann-Katrin Sommer (PhD study, MPI Munich).

3.1.2.3 Salinomycin hampers the receptor tyrosine kinases (RTKs) recycling

One mechanism of tamoxifen resistance is associated with the activation of epidermal growth factor receptor (EGFR) family members [46, 47]. Therefore, to elucidate the mechanism of salinomycin interfering with the ligand independent activation of ERα via EGFR family members, the cellular distribution of EGFR and Her2 during salinomycin treatment was analyzed by spinning disk microscopy. To monitor the cellular fate of the RTKs, EGFR and Her2 with a GFP-tag were transfected into MCF-7 cells. A strong co-localization of these RTKs with the lysosomal marker lysotracker was detected suggesting an enhanced lysosomal degradation after 24h of salinomycin treatment (Fig.11A). When cells were stimulated with EGF the receptor disappeared from the membrane and co-localized with lysosomes. In contrast, single treatment of salinomycin in addition to lysosomal co-localization still displayed a cell surface staining indicating a balance between lysosomal degradation and *de novo* synthesis of these RTKs (Fig. 11B).

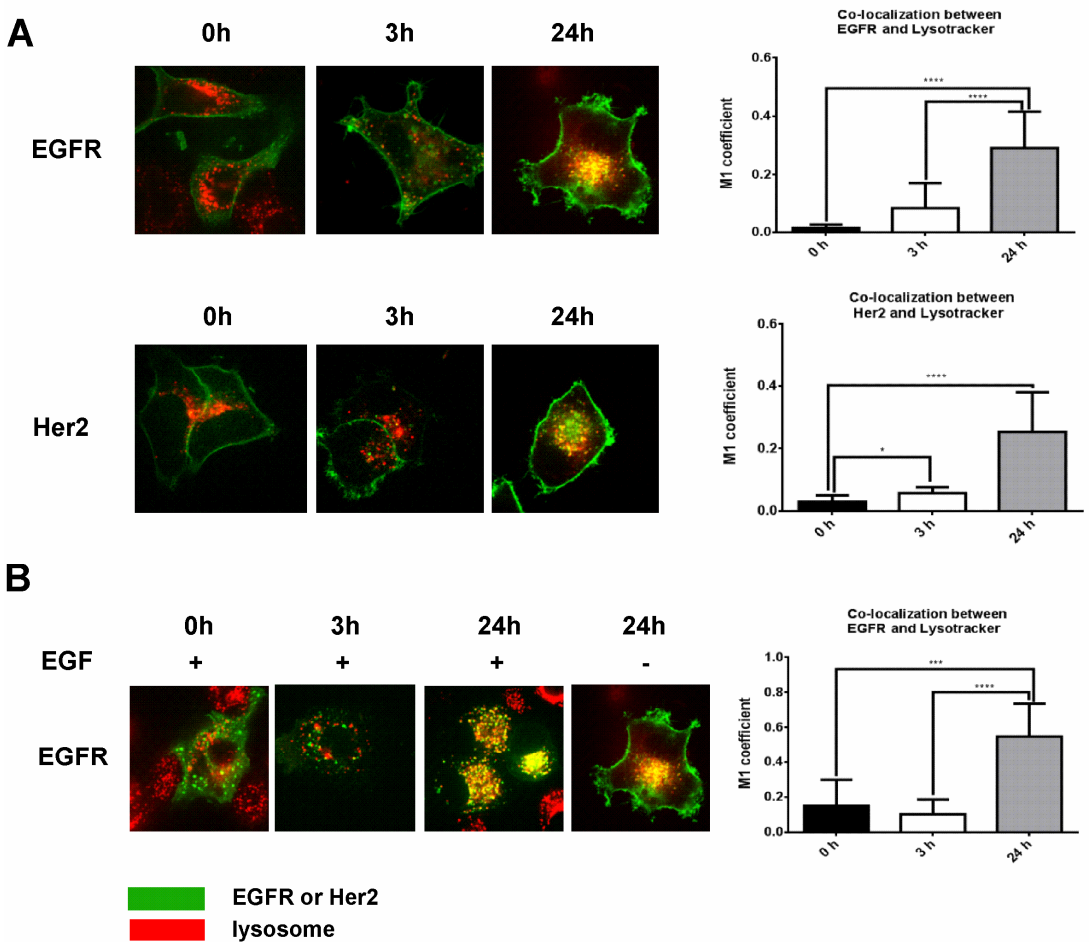


Fig. 11. Salinomycin induces accumulation of EGFR and Her2 in the lysosomes (A) without EGF stimulation, (B) with EGF stimulation. Cells were seeded in 8-well chambered μ-slides, incubated for 24h and transfected with EGFR eGFP or HER2 eGFP. 72h after transfection, 6 μM of salinomycin was added into the cells 3h and 24h before imaging. For co-localization with lysosomes, 15 nM lysotracker deep red was added into the cells, followed by 1h incubation and medium change. In order to induce receptor endocytosis, 50 pmol/ml EGF was added to the cells 24h before measurement. Image was acquired with spinning disk confocal microscopy. For imaging, medium was exchanged by marker-free CO_2 independent medium (with or without salinomycin). Analysis of image was performed according to a manual threshold, by selecting the fluorescence signal in the red and green. The fraction of green pixels overlapping red pixels was calculated. Results were shown as Manders coefficient, M1. Mean values of all evaluated cells (N=7-13 images) were presented together with the standard deviation. Statistical analysis was conducted using Student's t-test. * or ***or**** indicates $p<0.05$ or $p<0.001$ or <0.0001, respectively. Performed by Dr. Frauke Mickler (Dept. of Physical Chemistry, LMU Munich).

As RTKs are usually recycled and return to the cell surface upon ligand stimulation and internalization, EGFR level at the cell surface upon salinomycin treatment was analyzed by FACS. Stimulation with EGF induces internalization of the surface-EGFR followed by a transport back to the cell surface. Salinomycin hampers the recycling process and thus decreases the amount of active EGFR on the cell membrane (Fig. 12A). The effect of salinomycin treatment on the cytosolic calcium level was also investigated. This study showed that the intracellular calcium level is significantly increased upon salinomycin treatment (Fig. 12B). To sum up, salinomycin overcomes tamoxifen resistance by inhibiting of RTKs recycling.

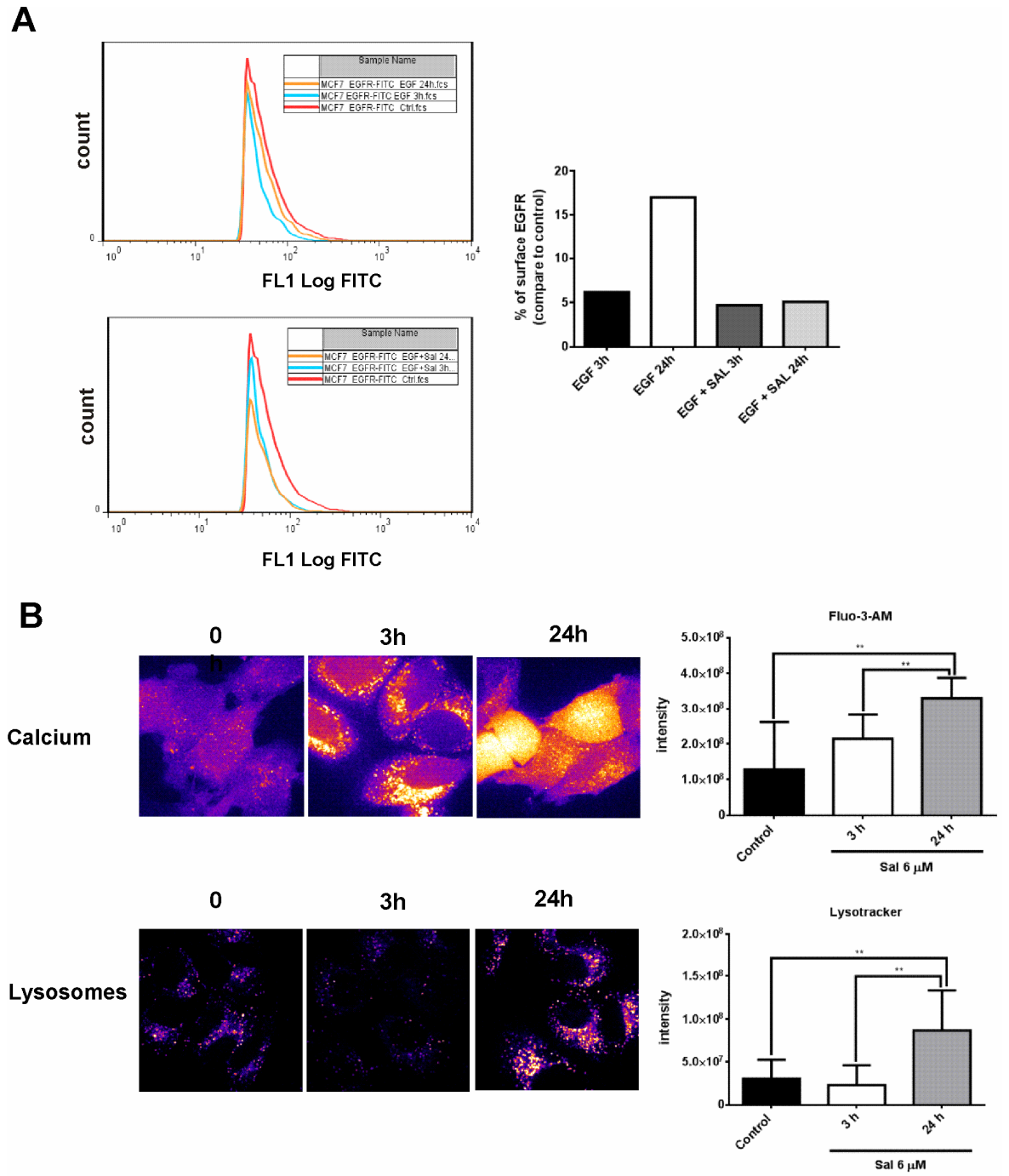


Fig. 12. Salinomycin hampers EGFR recycling and increase cytosolic calcium level. (A) Cell surface localization of EGFR is hampered by salinomycin. Cells were seeded in 6-well plate, incubated for 24 hours prior to stimulation with recombinant murine epidermal growth factor and treatment with 6 μM of salinomycin (Sigma, Germany). Cells were collected with trypsinization, incubated with mouse anti-huEGFR antibody and goat anti-mouse IgG1 antibody linked to FITC. Cells were then analyzed by CyAnTM ADP flow cytometer (DakoCytomation). (B) Ca^{2+} levels and intensity of lysosomes are elevated upon salinomycin treatment. To analyze calcium concentration and lysosome, cells were pre-treated with 6 μM salinomycin for 3h or 24h or kept without salinomycin treatment. 1h before imaging, 6 μM Fluo-3-AM and 15 nM Lysotracker deep red were added to the cells. For imaging, medium was exchanged by marker-free CO_2 independent medium (with or without salinomycin). Analysis of image was performed according to a manual threshold, by selecting the fluorescence signal in the red and green. The fraction of green pixels overlapping red pixels was calculated. Results were shown as Manders coefficient, M1. Mean values of all evaluated cells (N=7-13 images) were presented together with the standard deviation. Statistical analysis was conducted using Student's t-test. ** indicates $p<0.01$, respectively. Image analysis was performed by Dr. Frauke Mickler (Dept. of Physical Chemistry, LMU Munich).

3.2 BMPR2 Promotes Doxorubicin Resistance in Breast Cancer Cells

Acquired resistance of doxorubicin is one of the major obstacle in breast cancer treatment. Understanding of doxorubicin chemoresistance mechanism is urgently needed to improve its clinical outcome. Bone morphogenetic protein receptor type II (BMPR2) is a serine/threonine kinase and receptor of TGF-ß family. It is known that TGF-ß is a regulator in chemoresistance development by inducing epithelial to mesenchymal transition (EMT). In this part of the thesis, the roles of BMPR2 in promoting doxorubicin resistance was analyzed by several physiological assays.

3.2.1 Molecular evolution assay reveals increased expression of BMPR2 in chemoresistant cells

A molecular evolution assay of doxorubicin was performed using MCF-7 and MDA-MB 231 cells to mimic clinical application of doxorubicin in an *in vitro* model. After five sequential rounds of doxorubicin treatment, cytotoxicity analysis showed that both MCF-7 and MDA-MB 231 cells are getting more resistant to doxorubicin (Fig.13).

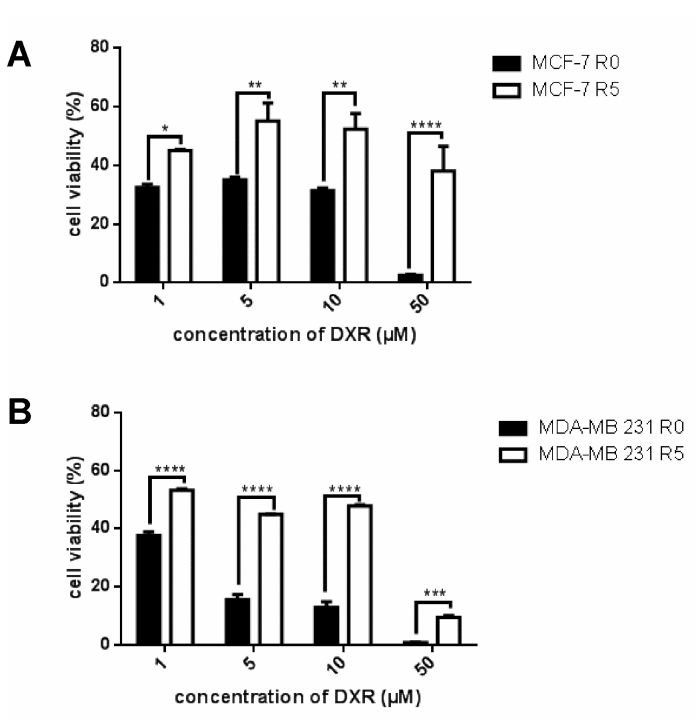


Fig. 13. Molecular evolution assay reveals doxorubicin-resistant cells. Susceptibility of (A) MCF-7, (B) MDA-MB 231 cells to doxorubicin. Cancer cells of R0 and R5 were treated with indicated concentrations of doxorubicin for 72h. Cell viability was measured with CellTiter-Glo assay. Percentage was determined to untreated cells. Results represent the average of three independent experiments (mean ± SD). Statistical analysis was conducted using Student's t-test. * or ** or ***or**** indicates $p<0.05$ or $p<0.01$ or $p<0.001$ or <0.0001, respectively.

To identify novel players in chemoresistance, a screening of candidates as markers of chemoresistance, cancer stem cells and EMT (Fig. 14) was performed by quantitative RT-PCR. BMPR2 was found as one of the interesting candidates for doxorubicin resistance, showing a 2.5-fold increase in gene expression.

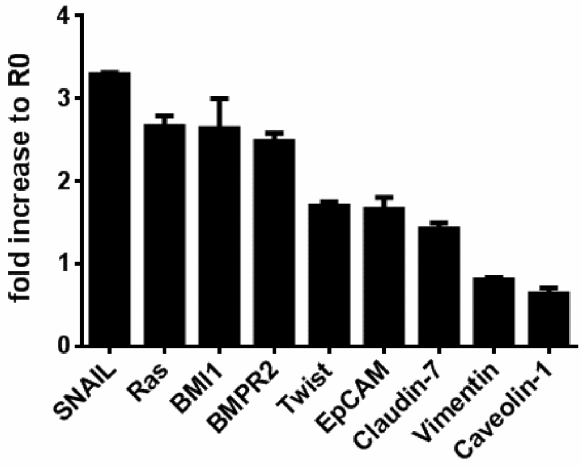


Fig. 14. Gene expression of chemoresistance marker in MCF-7 doxorubicin-resistant cells (R5) compared to the parental cells, as determined by quantitative RT-PCR. GAPDH was used as an internal control for quantitative RT-PCR. Results represent the average of three independent experiments (mean ± SD).

To further strengthen BMPR2 as a suitable target, the correlation between BMPR2 level and cancer drug resistance in an *in silico* analysis was investigated by an Oncomine database search [107-109]. As shown in figure 15A-B, doxorubicin-resistant cells showed the highest expression level of BMPR2 among several different analyzed cell types and tumors. Additionally, cancer cells also display an upregulation of BMPR2 after they were treated with several drugs (Fig. 15C).

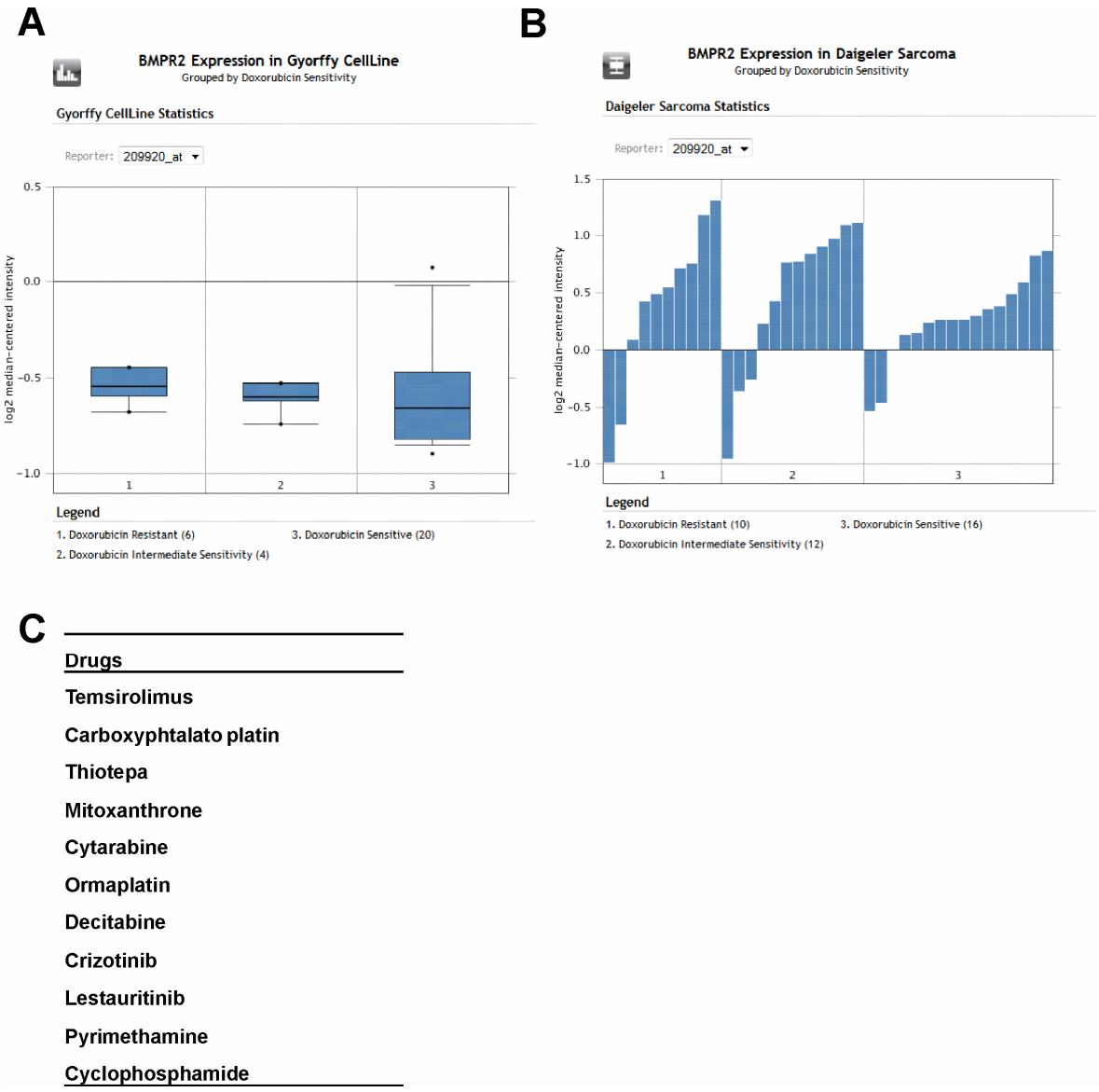


Fig. 15. *In silico* analysis of BMPR2 expression in chemoresistance studies published in the Oncomine database. (A) Analysis of BMPR2 expression in comparison to doxorubicin sensitivity in different cell lines. (B) A study by Daigeler *et al.* was analyzed regarding the BMPR2 expression and doxorubicin resistance in sarcomas. (C) Drugs that display an increased resistance when BMPR2 is upregulated. Analysis was performed with Oncomine.

Next, the change of the BMPR2 expression level during the rounds of the molecular evolution assay was analyzed. The results revealed that BMPR2 level increase during doxorubicin sequential treatment of doxorubicin in both MCF-7 and MDA-MB 231 cells (Fig. 16A-B).

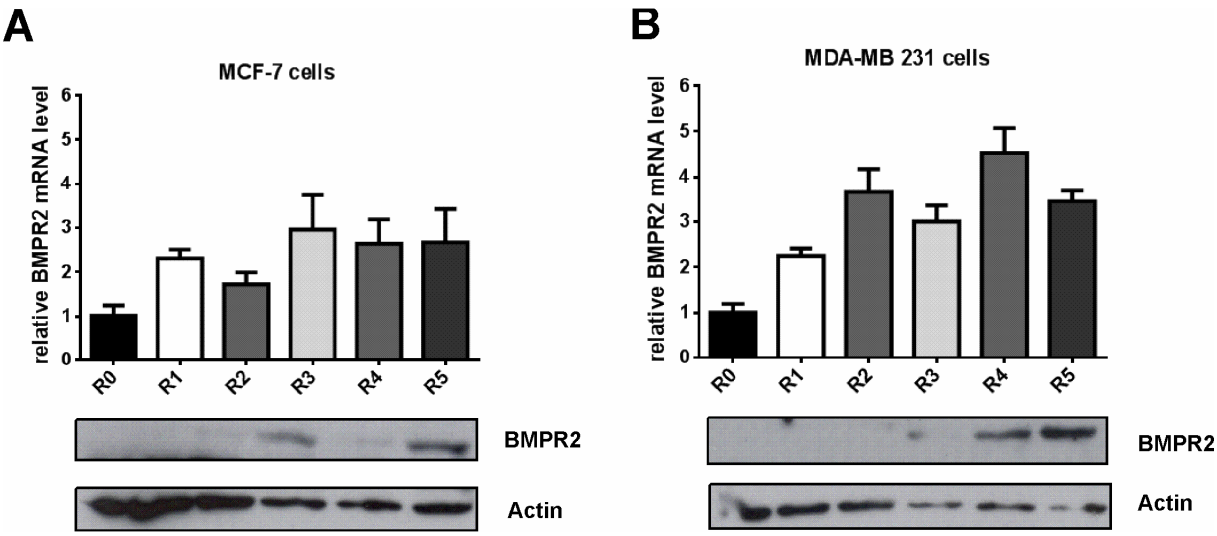


Fig. 16. BMPR2 expression during the treatment cycles of molecular evolution assay of doxorubicin in (A) MCF-7, (B) MDA-MB 231 cells as determined by quantitative RT-PCR (upper panel) and western blot (lower panel). GAPDH and Actin were used as internal control for q RT-PCR and western blot, respectively. Results represent the average of three independent experiments (mean ± SD).

3.2.2 Bone morphogenetic protein receptors expression in breast cancer cells

BMP receptors consist of two different types, type I and type II. To explore the abundance of both type I (Fig. 17A), and type II BMP-receptors (Fig. 17B), a panel of breast cancer cell lines, was analyzed by quantitative RT-PCR. BMPR2 was detected as the most abundant receptor among the BMPRs. BMPR2 expression level in the breast cancer cell panel was further analyzed by western blotting (Fig. 17C). BT-474 cells had the highest level of BMPR2, followed by MCF-7, MDA-MB 231 and MDA-MB 436 cells.

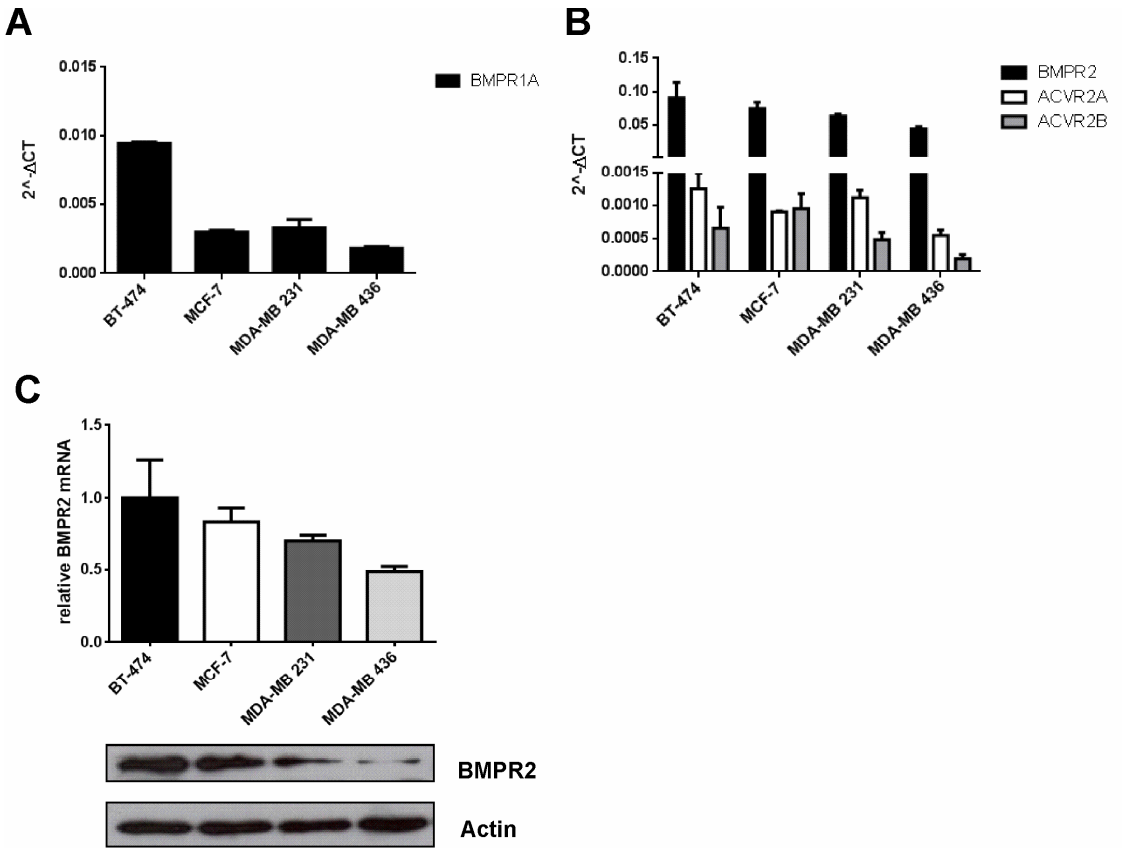


Fig. 17. Expression of BMP receptor in breast cancer cell panel. (A) Type I receptor. (B) Type II receptor. Gene expression level was determined by quantitative RT-PCR. (C) BMPR2 expression as determined by quantitative RT-PCR (upper panel) and western blot (lower panel). GAPDH and Actin were used as an internal control for quantitative RT-PCR and western blot, respectively. Results represent the average of three independent experiments (mean ± SD).

3.2.3 Modulation of BMPR2 mediates doxorubicin resistance

In vitro sequential treatment of doxorubicin decreased the sensitivity of breast cancer cells to doxorubicin and simultaneously increased BMPR2 expression. In order to explore the mechanism of BMPR2 in chemoresistance, both BMPR2 overexpression and inhibition of BMPR2 with siRNA were performed (Fig.18). Treatment with various concentration of doxorubicin was conducted 72h or 96h after transfection with BMPR2 or siBMPR2, respectively, and cytotoxicity was measured.

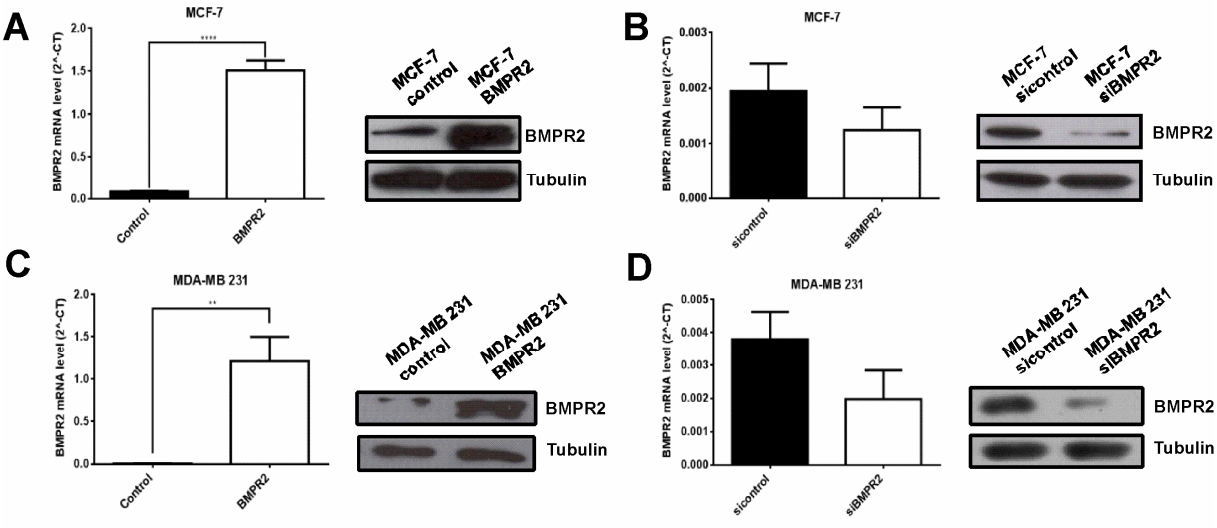


Fig. 18. Overexpression and inhibition of BMPR2 in several breast cancer cells. MCF-7 cells were transfected with (A) BMPR2, (B) siBMPR2, and controls. MDA-MB 231 cells were transfected with (C) BMPR2, (D) siBMPR2, and controls. 72h or 96h after transfection with BMPR2 or siBMPR2, respectively, cells were lyzed. Gene expression was determined by quantitative RT-PCR (left panel) and western blot (right panel). GAPDH and Tubulin were used as an internal control for quantitative RT-PCR and western blot, respectively. Results represent the average of three independent experiments (mean ± SD).Statistical analysis was conducted using Student's t-test.**** indicates $p<0.0001$.

Susceptibility of the cells towards doxorubicin was analyzed by propidium iodide staining (FACS) and a cytotoxicity assay (CellTiter-Glo). Analysis of the subG1 population in MCF-7 (Fig. 19A) and MDA-MB 231 (Fig. 19B) transfected cells indicated that overexpression of BMPR2 decreased the subG1 population, whereas knockdown of BMPR2 increased the subG1 population in both cells. Cytotoxicity of doxorubicin was then determined in the transfected cells which were stimulated with BMP2. The cytotoxicity assays revealed that transient overexpression of BMPR2 decreases the sensitivity of MCF-7, but not of MDA-MB 231 cells (Fig. 19C). Furthermore, inhibition of BMPR2 with siRNA, increases sensitivity of MCF-7, but not MDA-MB 231 cells (Fig. 19D).

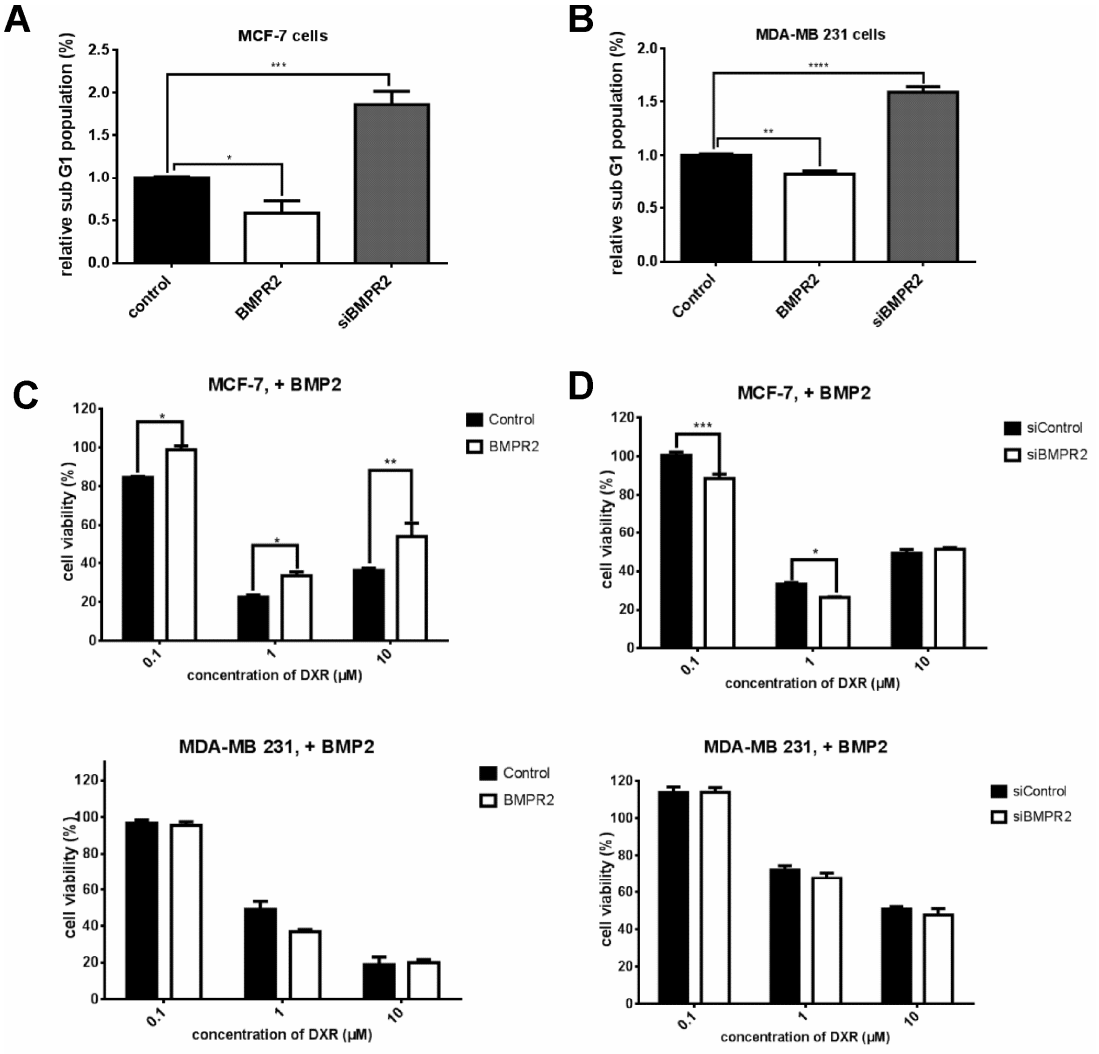


Fig. 19. Modulation of BMPR2 expression mediates doxorubicin resistance. subG1 population of (A) MCF-7 and (B) MDA-MB 231 cells with an overexpression of BMPR2 (BMPR2) or with reduced BMPR2 expression (siBMPR2) treated with doxorubicin (MCF-7 200 nM, MDA-MB 231 400 nM) for 72h. Cells were stained with propidium iodide and analyzed by FACS. MCF-7 and MDA-MB 231 cells, transfected with (C) BMPR2 or (D) siRNA against BMPR2 and respective controls, were stimulated with 20 ng/mL of BMP2 for 30 min and treated with 0.1, 1, and 10 μM of doxorubicin for 72h. Cell viability was measured with cell titer-glo assay. Results represent the average of three independent experiments (mean ± SD). Statistical analysis was conducted using Student's t-test. * or ** or ***or**** indicates $p<0.05$ or $p<0.01$ or $p<0.001$ or <0.0001, respectively.

To confirm whether BMPR2 affects cell survival upon treatment with doxorubicin in the long term, clonogenic survival assay was performed by growing the cells for 14 days after doxorubicin treatment. Surviving colonies were quantified by measuring colony area. In untreated cells, BMPR2 overexpression showed a decrease in colony formation, whereas inhibition of BMPR2 with siRNA increases colony formation (Fig. 20A-B). Colony area of doxorubicin treated group was normalized to the respective untreated control. As expected, in doxorubicin treated cells, transient overexpression of BMPR2 increased the surviving colony area, whereas inhibition of BMPR2 reduced the number of surviving colony area of both MCF-7 and MDA-MB 231 cells.

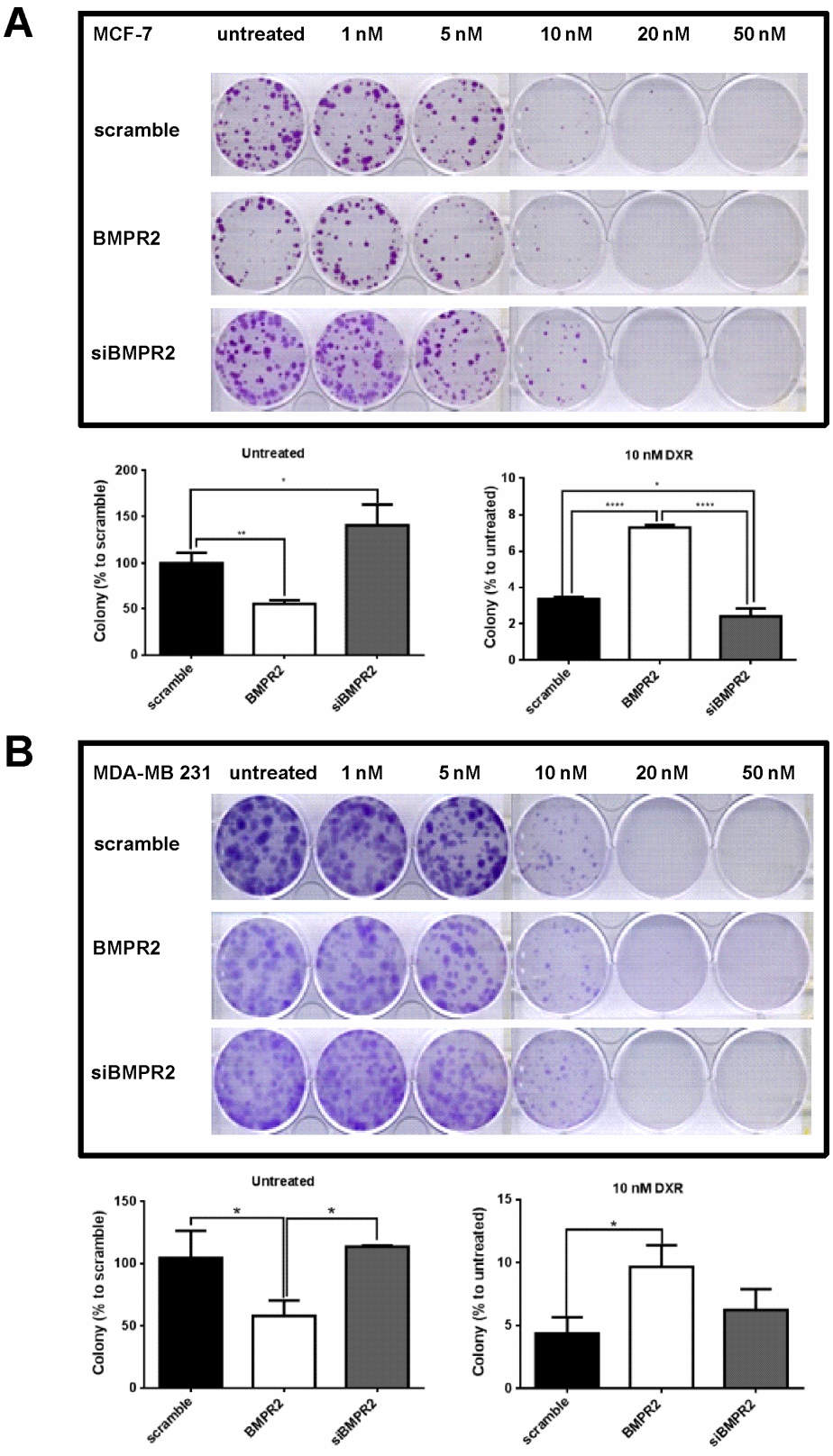


Fig. 20. Clonogenic assay of (A) MCF-7 and (B) MDA-MB 231 cells. Cells were transfected with BMPR2, siBMPR2 or scramble control and treated with doxorubicin (1, 5, 10, 20, 50 nM) for 72h. The cells were subsequently cultivated for 14 days, and surviving colonies were stained by crystal violet and analyzed by ColonyArea, Image J (lower panel). Results represent the average of three independent experiments (mean ± SD). Statistical analysis was conducted using Student's t-test. * or ** or ***or**** indicates $p<0.05$ or $p<0.01$ or $p<0.001$ or <0.0001, respectively.

3.2.4 BMPR2 regulates cell proliferation and cell cycle

As BMPR2 modulates colony formation, a cell proliferation assay was performed to investigate the role of BMPR2. The cell proliferation assay of transfected cells revealed that transient overexpression of BMPR2 decreases cell growth of both MCF-7 and MDA-MB 231 cells (Fig. 21A). *Vice versa*, inhibition of BMPR2 with siRNA increases cell proliferation of both cell lines (Fig.21B). The cell proliferation assay was also conducted in MDA-MB 231 parental cells (R0) and R5 treatment of doxorubicin. MDA-MB 231 doxorubicin-resistant cells (R5) showed a decrease in cell growth compared to the parental cells (R0) (Fig. 21C). Thus, the question arises whether inhibition of cell proliferation was induced through a cell cycle arrest. As shown in the cell cycle profile (Fig. 21A-B, right panel), overexpression of BMPR2 increased the accumulation of cells in G1 phase and reduced the number of cells in the S phase. Accordingly, inhibition of BMPR2 with siRNA decreased cell accumulation in G1 phase and increased cell number in S phase.

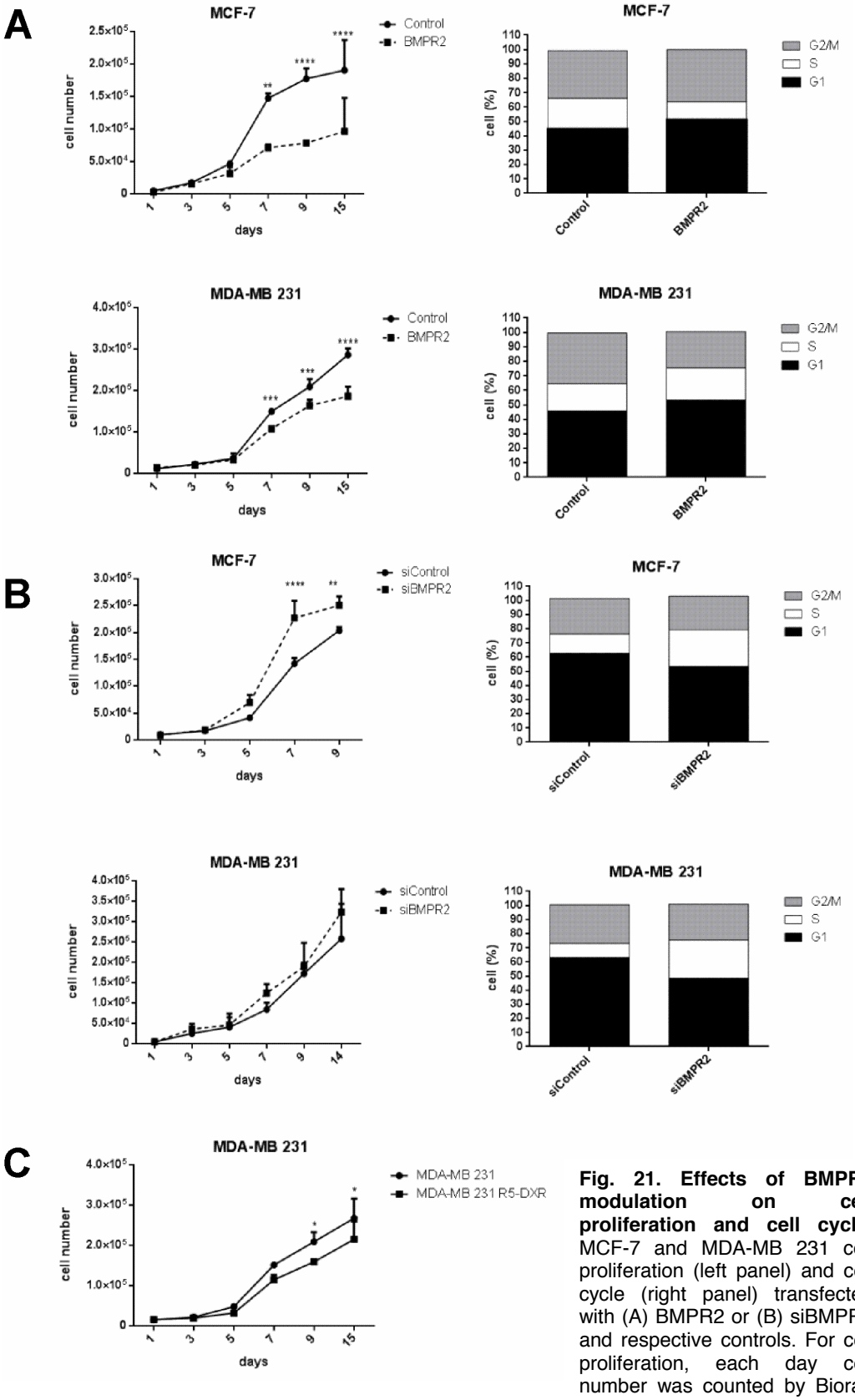


Fig. 21. Effects of BMPR2 modulation on cell proliferation and cell cycle. MCF-7 and MDA-MB 231 cell proliferation (left panel) and cell cycle (right panel) transfected with (A) BMPR2 or (B) siBMPR2 and respective controls. For cell proliferation, each day cell number was counted by Biorad cell counter. Cell proliferation profile was depicted against day of incubation. For the cell cycle profile, cells were stained with propidium iodide and analyzed by FACS. (C). Cell proliferation profile of MDA-MB 231 parental cells (R0) and MDA-MB 231 doxorubicin-resistant cells (R5). Cell number was counted by Biorad cell counter. Results represent the average of three independent experiments (mean ± SD). Statistical analysis was conducted using Student's t-test. * or ** or ***or**** indicates $p<0.05$ or $p<0.01$ or $p<0.001$ or <0.0001, respectively.

To explore the molecular mechanism of BMPR2 inducing chemoresistance, the signaling pathways were further analyzed. The BMPR2 signaling cascade proceeds via smad dependent and independent signalling pathways which regulate several cellular processes e.g. cell proliferation, cell cycle, and apoptosis. Activation of both smad dependent via phosphorylation of smad1/5/8 and independent pathways were observed in MCF-7 cells transfected with BMPR2 (Fig. 22A, upper panel). Expression level of p21, a G1 cell cycle inhibitor and also known as a target gene of the smad dependent pathway, was elevated in BMPR2 transfected cells. The activation of the smad independent pathway was indicated by the activation of ERK. Overexpression of BMPR2 in MDA-MB 231 cells (Fig. 22A, lower panel) also showed an activation of the smad dependent pathway, but the effect is not as strong as in MCF-7 cells. Stimulation with BMP2, activates both smad dependent and independent pathways also in control transfected cells.

No effect in smad dependent signaling was observed in MCF-7 transfected with siBMPR2 (Fig. 22B). Moreover, inhibition of BMPR2 activates smad independent signaling, indicated by increasing of activated ERK. In MDA-MB 231 cells transfected with siBMPR2 (Fig. 22B, lower panel), smad dependent pathway was downregulated showing a decrease of activated smad and p21. In addition, both smad dependent and independent pathway can be activated by BMP2 stimulation. To sum up, BMPR2 overexpression activates smad dependent and independent signaling by targeting p21 and ERK, respectively.

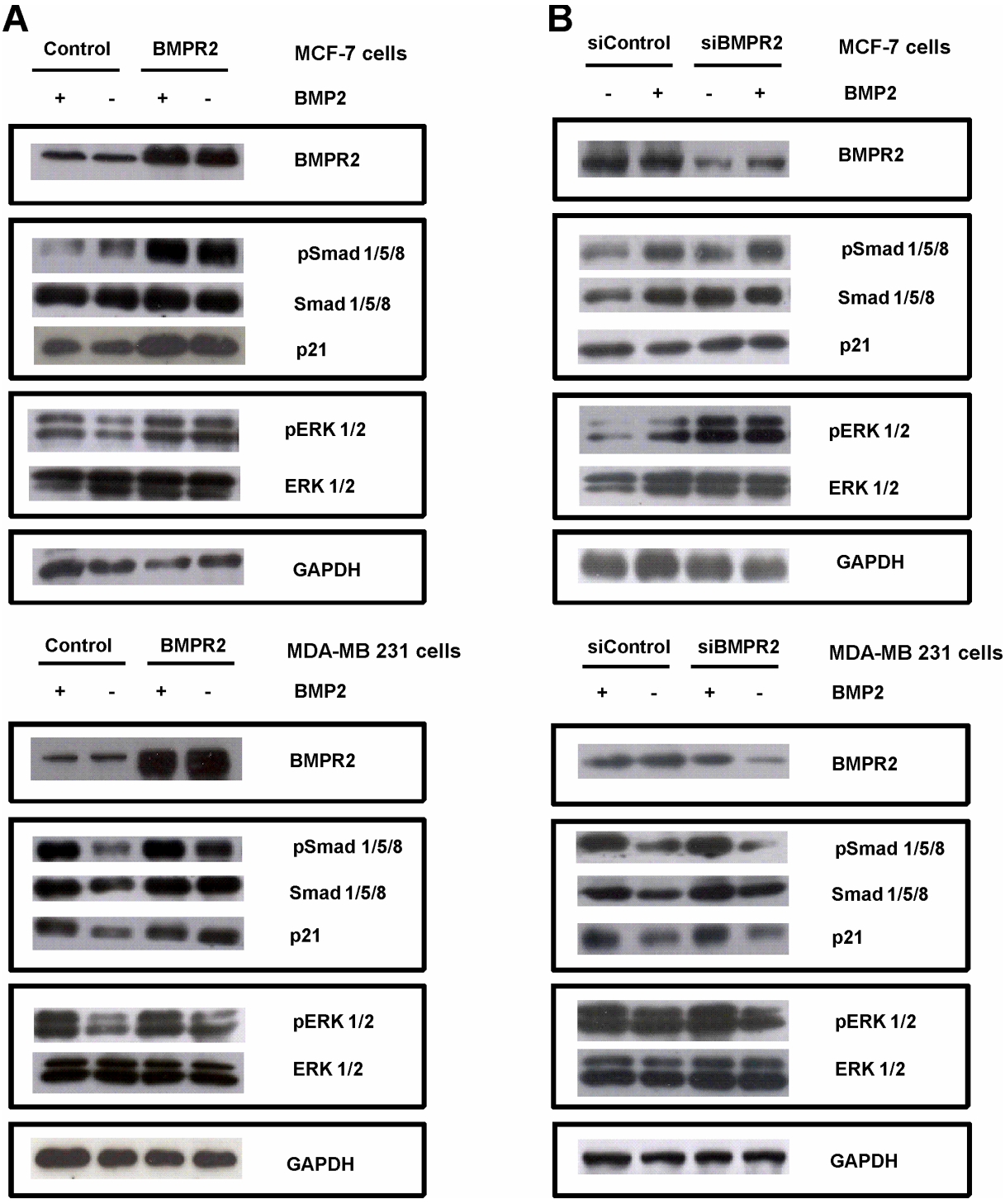


Fig. 22. Western blot analysis of BMPR2 modulation in MCF-7 and MDA-MB 231 cells. Cells were transfected with (A) BMPR2 or (B) siBMPR2 and respective controls, as described in material and methods. 72h or 96 after transfection with BMPR2 or siBMPR2, respectively, cells were starved for 24h with serum free medium, stimulated with 20 ng/mL of BMP2 for 30 min, and lyzed. Protein lysates were analyzed by western blot. GAPDH was used as an internal control.

3.2.5 BMPR2 enhances the DNA repair via regulation of BRCA1

One possible mechanism of BMPR2 inducing chemoresistance is by increasing DNA repair mechanisms. To confirm this hypothesis, a DNA-damage assay, the single cell electrophoresis comet assay, was performed in both BMPR2 and siBMPR2 transfected cells. Doxorubicin was used to induce DNA damage, which is demonstrated by the tail moment. Overexpression of BMPR2 reduced DNA damage (Fig. 23A), while inhibition of BMPR2 with siRNA enhanced DNA damage (Fig. 23B), indicated by increasing the tail moment in both MDA-MB 231 and MCF-7 cells.

In order to identify DNA damage on molecular level, a staining with pγH2AX, a double strand break DNA damage marker, was performed in BMPR2 and siBMPR2 transfected cells. The number of speckles per nuclei was quantified, representing a double strand break. Less DNA damage was measured when MCF-7 cells transfected with BMPR2 (Fig. 24A), whereas more DNA damage was observed when BMPR2 is inhibited by siRNA in both MCF-7 and MDA-MB 231 cells (Fig. 24B).

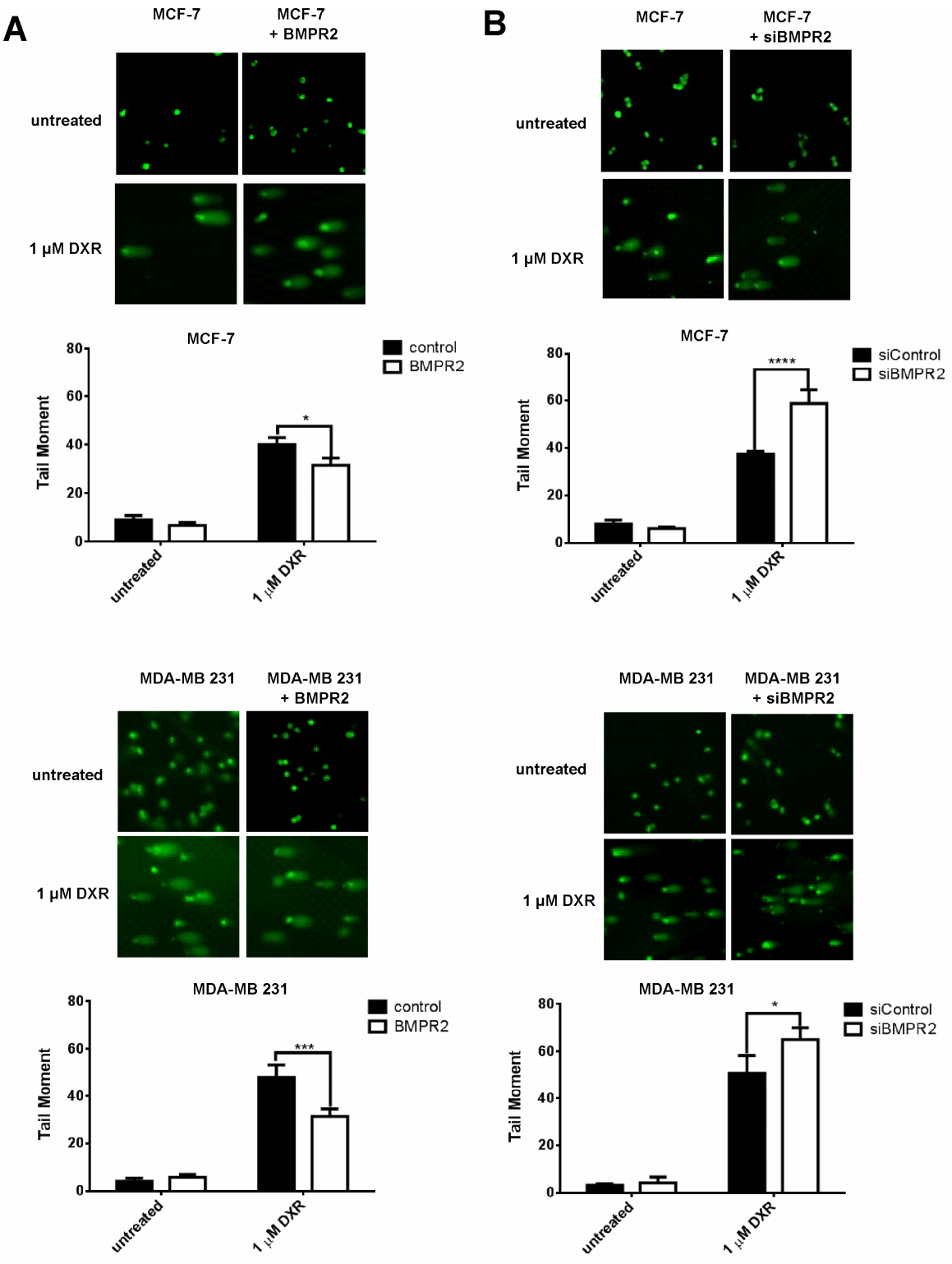


Fig. 23. Single cell comet assay of (A) BMPR2 (B) siBMPR2 transfected MCF-7 and MDA-MB 231 cells and respective controls after treatment with 1μM of doxorubicin for 24h. At least 100 cells were analyzed by OpenComet, ImageJ. DNA damage was quantified by measuring tail moment (lowe panel). Results represent mean±SEM. Statistical analysis was conducted using Student's t-test. * or***or **** indicates $p<0.05$ or $p<0.001$ or <0.0001, respectively.

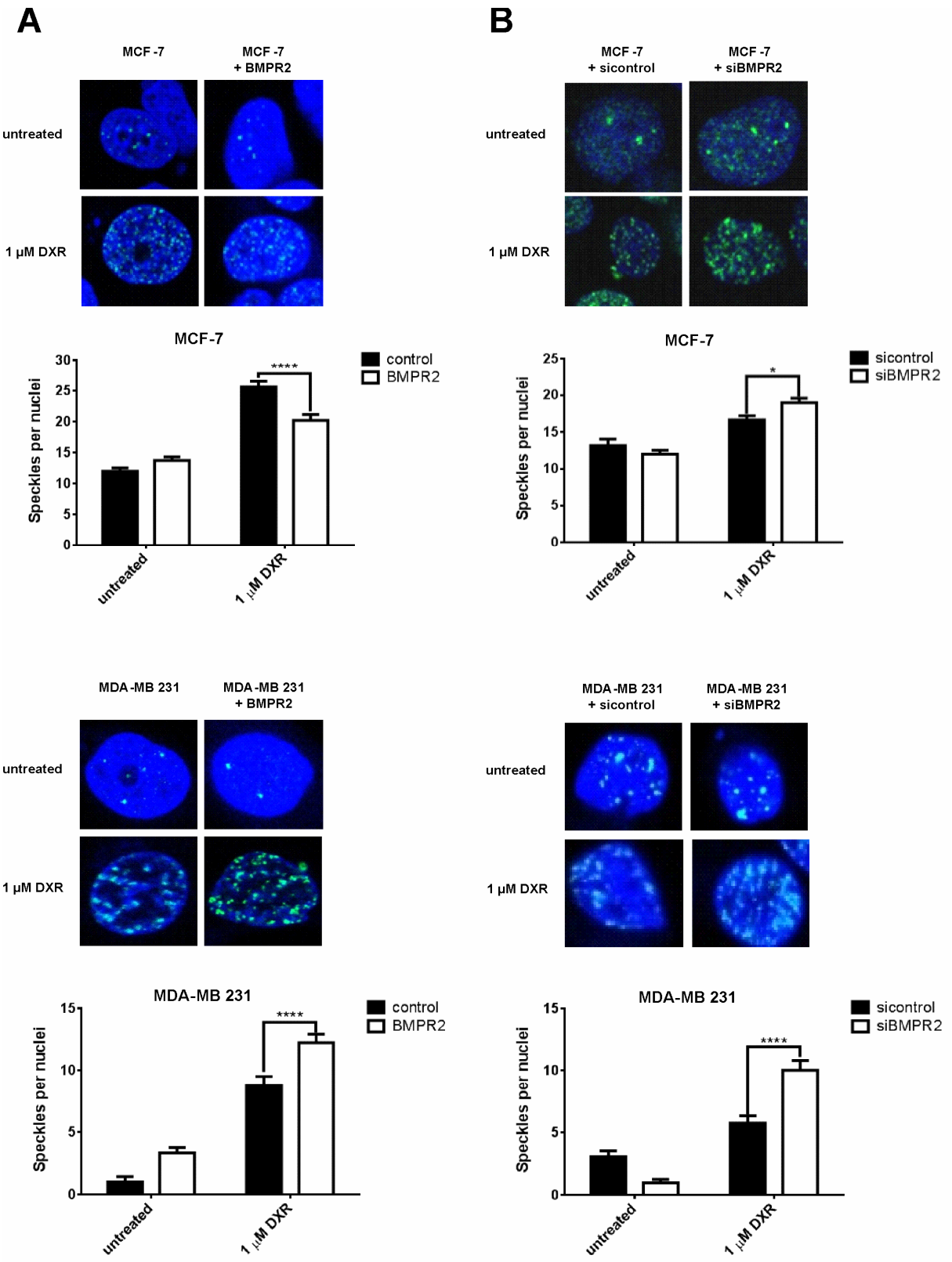


Fig. 24. Immunostaining of pγH2AX in (A). BMPR2, (B). siBMPR2 transfected MCF-7 and MDA-MB 231 cells and respective controls after treatment with 1μM of doxorubicin for 24h. Cells were stained for pγH2AX and counterstained with DAPI. Image were acquired with Leica SP 8 microscope (Leica Microsystem, Mannheim, Germany). At least 100 cells were analyzed by CellProfiler. Double strand breaks DNA damage was quantified by calculating number of speckles per nuclei (lower panel). Results represent mean ±SEM. Statistical analysis was conducted using Student's t-test. * or**** indicates p<0.05 or <0.0001, respectively. Immunostaining and analysis were performed by Dr. Jonathan Garcia-Roman (Pharmaceutical Biotechnology LMU).

Breast Cancer-1 (BRCA1), a regulator of DNA repair mechanisms, was recently shown to be a novel target gene of BMPR2 signaling in endothelial cells [110]. BRCA1 expression level was found increased during the molecular evolution assay (Fig. 25A-B). Level of BRCA1 was then measured in BMPR2 and siBMPR2 transfected cells. The results showed that BMPR2 overexpression enhanced BRCA1 expression, whereas inhibition of BMPR2 with siRNA decreased BRCA1 expression on both MCF-7 (Fig. 25C) and MDA-MB-231 cells (Fig. 25D). Moreover, treatment with doxorubicin induced higher expression of BRCA1 in BMPR2 overexpressing cells, and accordingly showed the opposite effect in siRNA BMPR2 transfected cells.

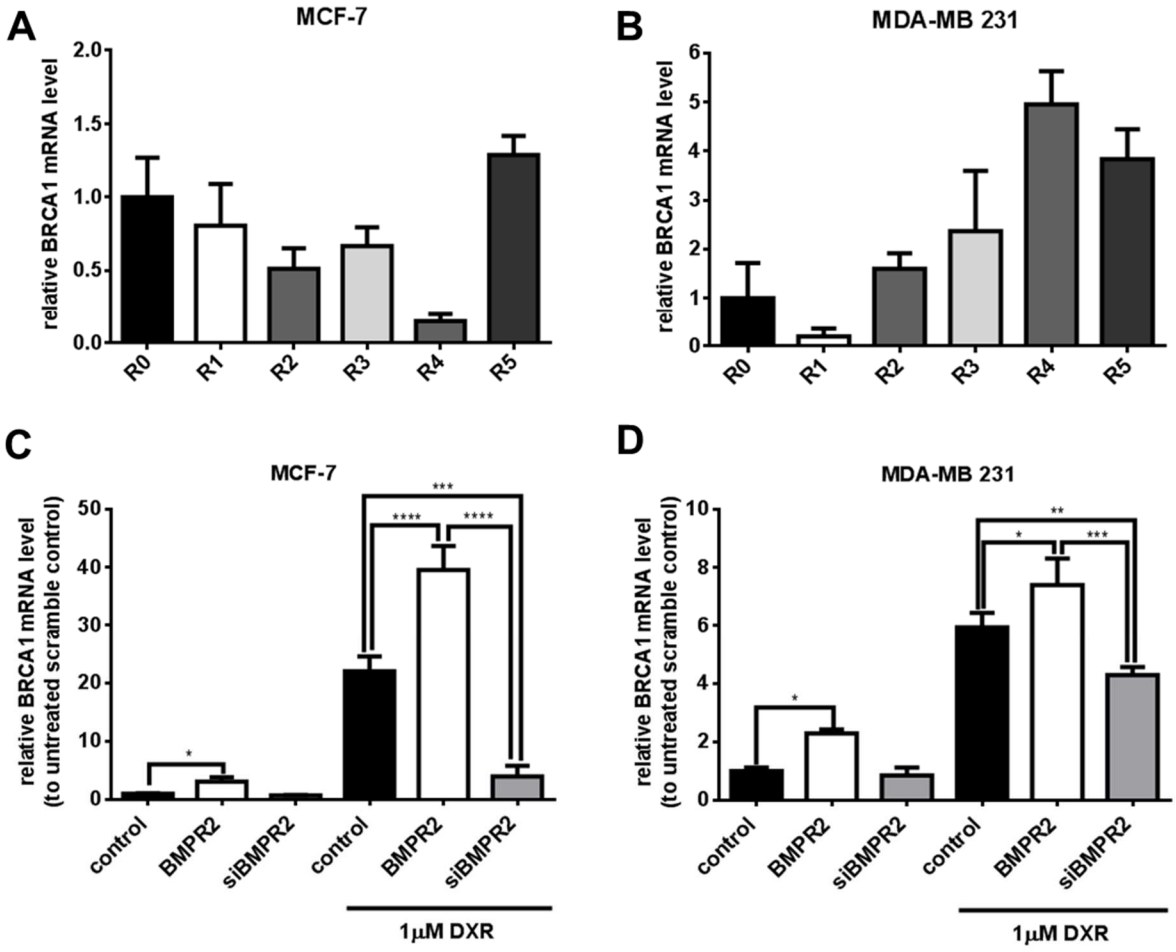


Fig. 25. BRCA1 expression level, during molecular evolution assay of doxorubicin on (A) MCF-7 and (B) MDA-MB 231 cells. BRCA1 expression level upon transfection BMPR2, siRNA against BMPR2 and control with or without treatment of 1μM doxorubicin for 24h on (C) MCF-7 and (D) MDA-MB 231 cells. BRCA1 expression was determined by quantitative RT-PCR. GAPDH was used as internal control. Results represent the average of three independent experiments (mean ± SD). Statistical analysis was conducted using Student's t-test. * or ** or ***or**** indicates $p<0.05$ or $p<0.01$ or $p<0.001$ or <0.0001, respectively.

3.2.6 BMPR2 modulates apoptosis by upregulating XIAP

Cancer cells modify apoptotic signaling to circumvent the effects of chemotherapy. X-linked inhibitor of apoptosis protein (XIAP) is a bridging protein between type I receptor and TGF-ß activated binding protein (TAB 1/2/3), an activator of MAPK TGF-ß activated tyrosine kinase 1 (TAK1) [85]. Therefore, the XIAP expression in BMPR2 and siRNA against BMPR2 transfected cells was measured by western blot. As shown in figure 26A, an increase in XIAP expression was detected in MCF-7 cells transfected with BMPR2, and the expression was even higher after stimulation with BMP2. However, increased expression of XIAP in MDA-MB 231 is only detected when cells transfected with BMPR2 and stimulated with BMP2 (Fig. 26A, lower panel). Inhibition of BMPR2 with siRNA leads to downregulation of XIAP in both MCF-7 and MDA-MB 231 cells (Fig. 26B).

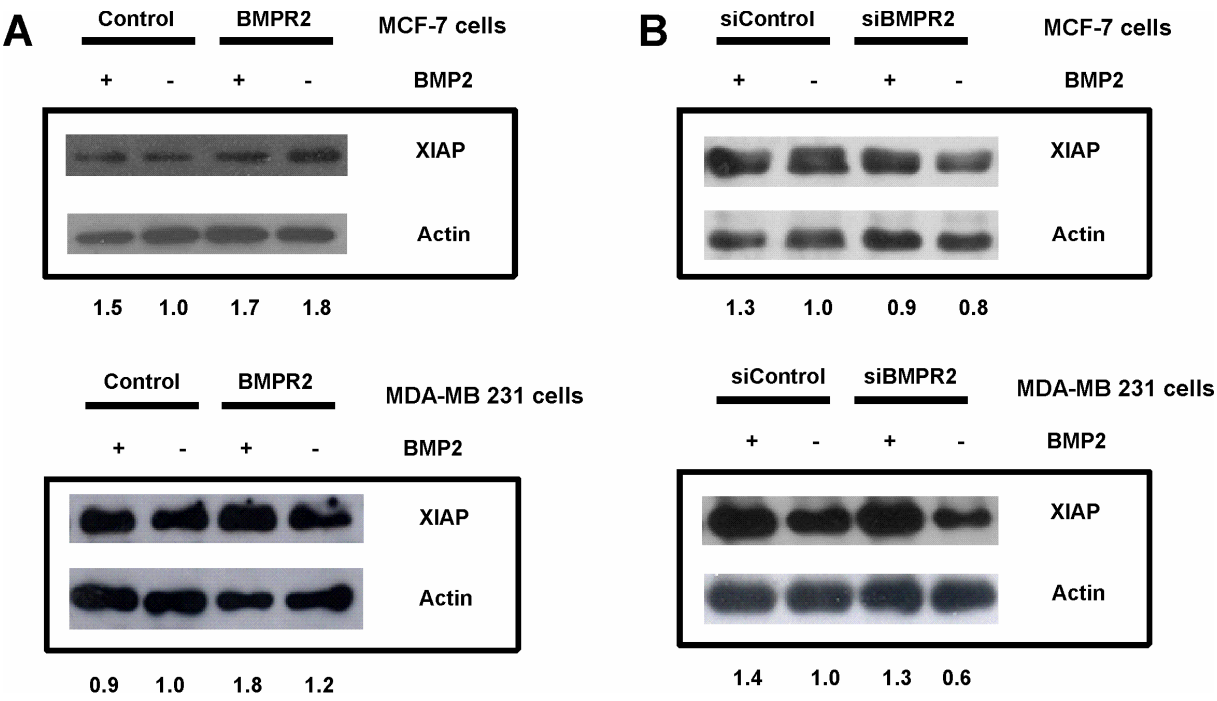


Fig. 26. Western blot analysis of XIAP in MCF-7 and MDA-MB 231 cells transfected with (A) BMPR2 or (B) siBMPR2 and controls as described in material and methods. 72h after transfection, cells were starved for 24h with serum free medium, stimulated with 20 ng/mL of BMP2 for 30 min, and lyzed. Protein lysates were analyzed by western blot. Actin was used as an internal control. Quantification of XIAP was performed with Image J.

As XIAP is an inhibitor of caspases, the activity of caspase-3/7 was then measured in both BMPR2 and siBMPR2 transfected cells. Doxorubicin was used to induce apoptosis. Here, overexpression of BMPR2 in MDA-MB 231 cells decreased the caspase-3/7 activity (Fig. 27A). *Vice versa,* caspase-3/7activity increased when BMPR2 is inhibited by siRNA (Fig. 27B). This effect was demonstrated only in MDA-MB 231 cells, because MCF-7 cells do not express caspase-3.

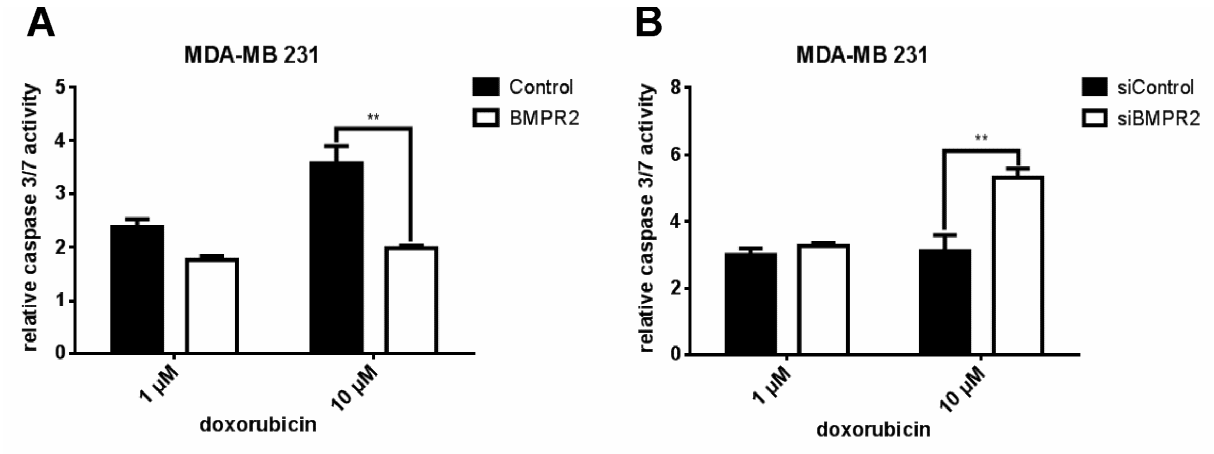


Fig. 27. Caspase-3/7 activity of (A) BMPR2, (B) siBMPR2 transfected MDA-MB 231 transfected cells, and controls, treated with 1 and 10 μM doxorubicin for 12 h. Caspase activity was determined by Caspase-Glo®-3/7 Assay. Caspase activity was shown as relative to untreated cells. Results represent the average of three independent experiments (mean ± SD). Statistical analysis was conducted using Student's t-test. ** indicates $p<0.01$.

3.2.7 *In vivo* sequential treatment of doxorubicin results in the BMPR2 upregulation

In vitro sequential treatment of doxorubicin demonstrated that BMPR2 is upregulated during molecular evolution assay. To verify the hypothesis that sequential treatment of doxorubicin enhances BMPR2 expression, an *in vivo* study was performed in a xenograft mouse model. Doxorubicin was administered four times, once per week in mice bearing MDA-MB 231 cells subcutaneously. As expected, a significant reduction in tumor growth was detected in the doxorubicin treated group (Fig. 28A,B). Western blot analysis confirmed an increase of BMPR2 (Fig. 28C), p21 (Fig. 28D), and XIAP (Fig. 28E) in doxorubicin treated mice. To sum up, doxorubicin treatment *in vivo* induces upregulation of BMPR2.

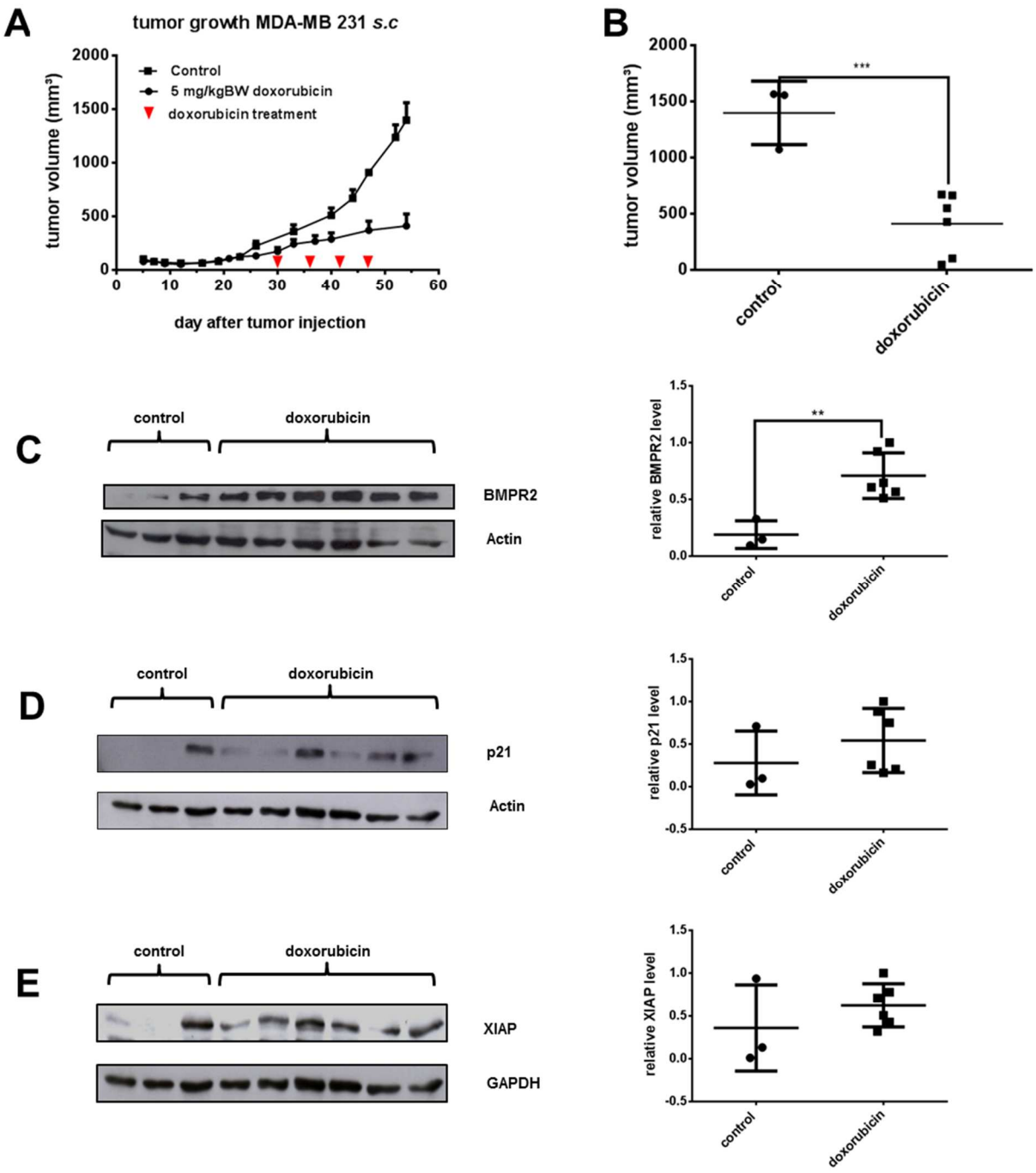


Fig. 28. *In vivo* molecular evolution assay of doxorubicin increases BMPR2 level.
(A) Tumor growth of subcutaneous MDA-MB 231 tumors. 30 days after the tumor was injected into the flank, mice were treated with 5 mg/kg of doxorubicin weekly for 4 weeks intravenously. Tumor growth was monitored using a caliper, and depicted as tumor volume (mm3). (B) After 60 days, tumor volumes are represented as tumor volume ± SD. Western blot analysis of (C) BMPR2, (D) p21, and (E) XIAP in animal tumors. ß-Actin or GAPDH were used as internal control. Quantification was performed by ImageJ. Results represent as mean ± SD. Statistical analysis was conducted using Student's t-test. ** or *** indicates $p<0.01$ or $p<0.001$, respectively. Animal experiments were performed by Johanna Busse and Dr. Annika Herrmann (Pharmaceutical Biotechnology, LMU).

4. DISCUSSION

4.1 Cancer Drug Resistant Cells Obtained from Molecular Evolution Assays

Chemoresistance is still a major threat to a successful cancer therapy. To investigate novel mechanisms and drugs circumventing therapy resistance, sequential treatment of salinomycin or doxorubicin or tamoxifen was performed in epithelial and mesenchymal breast cancer cells, which generated drug resistant cells. Many studies on cancer drug resistance utilize continuous drug treatment with increasing concentrations of the drugs [65, 66, 111, 112]. However, clinical study on patients with non-resectable colorectal cancer suggested that sequential use of the same cytotoxic drugs is more effective than combination therapy [113]. In the present study, breast cancer cells were treated with the same concentration of drugs, on the one hand with classical chemotherapeutic drug doxorubicin, and on the other hand with salinomycin, a novel anti cancer stem cell drug, and followed by recovery period after the treatment. This procedure was performed for 5-6 treatment cycles. These settings have the advantage to mimic the application process of chemotherapeutics in the clinics.

4.2 Salinomycin Circumvents Drug Resistance of Breast Cancer Cells

4.2.1 Sequential salinomycin pretreatment increases doxorubicin chemosensitivity of breast tumor cells by downregulating efflux pumps

The resistance formation in the molecular evolution assay with salinomycin exhibits no linear increase of the IC_{50} for salinomycin. One could conclude that the resistance formation here is based on the principle of clonal selection. According to the clonal evolution theory, tumor cells display heterogeneity and genetic instability. When treated with chemotherapy a selection pressure is applied and some of the cells will survive the treatment. These cells will form a new polyclonal cell population with various genetic predispositions [114]. After each round of the molecular evolution assay, different cell population might have survived and thus no linear increase in the IC_{50} was detected in this study. Additionally, many different resistance mechanism possibly exist in parallel caused by the clonal selection in the molecular evolution assay. For example the decrease of cell proliferation indicates that salinomycin treatment eradicates fast growing cell populations. Unlike in several molecular evolution assays of doxorubicin the growth retardation here is only modest. A recent study by Kopp *et al.* also showed that sequential treatment of mesenchymal breast cancer cells with salinomycin resulted in resistance formation by mesenchymal to epithelial transition (MET) [115].

Interestingly, salinomycin-resistant cells exhibit increased sensitivity to doxorubicin in both, epithelial and mesenchymal tumor cells. As it was reported that combinatorial treatment, i.e. simultaneous treatment, of salinomycin with doxorubicin, cisplatin or etoposide increased the cytotoxicity [58, 59], this study hypothesize that in the molecular evolution assay the sensitivity for other drugs was increased as well.

The most important drug resistance mechanism is the upregulation and increased activity of ABC-transporters (ATP binding cassette transporter) [116]. Among these efflux pumps in charge of decreasing intracellular drug level are p-glycoprotein (encoded MDR1 gene), breast cancer resistance protein (encoded by BCRP1 gene), and multidrug resistance associated proteins (encoded by MRP genes) [65, 117-119]. The ABC transporter family consists of membrane proteins that translocate a variety of substrates across extra- and intracellular membranes [65]. They play an important role in absorbing, distributing, and eliminating drugs from the cells [120]. Hence, the upregulation of efflux pumps decreases intracellular drug accumulation and increases drug resistance so called multiple drug resistance (MDR).

Several multiple drug resistance reversing agents modify cell membrane fluidity and increase cell membrane permeability [121]. Salinomycin is able to circumvent multidrug resistance by acting as ionophore. Salinomycin, a transmembrane K^+ ionophore is embedded in biological membranes, and thus by changing membrane integrity it is able to inhibit the activity of drug efflux pumps. Additionally, salinomycin disturbs the intracellular balance of ions by increasing the potassium level [122, 123]. In this study, MDR1 and BCRP1 are downregulated after sequential salinomycin treatment.

The decrease of the expression of multidrug resistance proteins can occur via a direct and indirect mechanism (Fig. 29). On the one hand, salinomycin was found to be an anti-cancer stem cell drug. One of the hallmarks of cancer stem cells is an increase of efflux pump activity. That is why cancer stem cells are thought to be present in the side population. Salinomycin is eradicating this population and thereby increasing the non-cancer stem cell fraction with low efflux pump expression. Cancer stem cells markers SOX-2, Oct-4 and Nanog were downregulated in MCF-7 salinomycin-resistant cells (R5-SAL), thus supporting the hypothesis that by cyclic drug treatment the offspring of these efflux pump-positive cancer stem cells also die out over time. However, whether the killing of cancer stem cells and their offspring accounts for such a strong effect in gene expression of efflux pumps and eventually doxorubicin sensitivity, remains to be elucidated. On the other hand, efflux pumps might be important for the direct execution of salinomycin cytotoxicity as they regulate the intracellular balance of molecules.

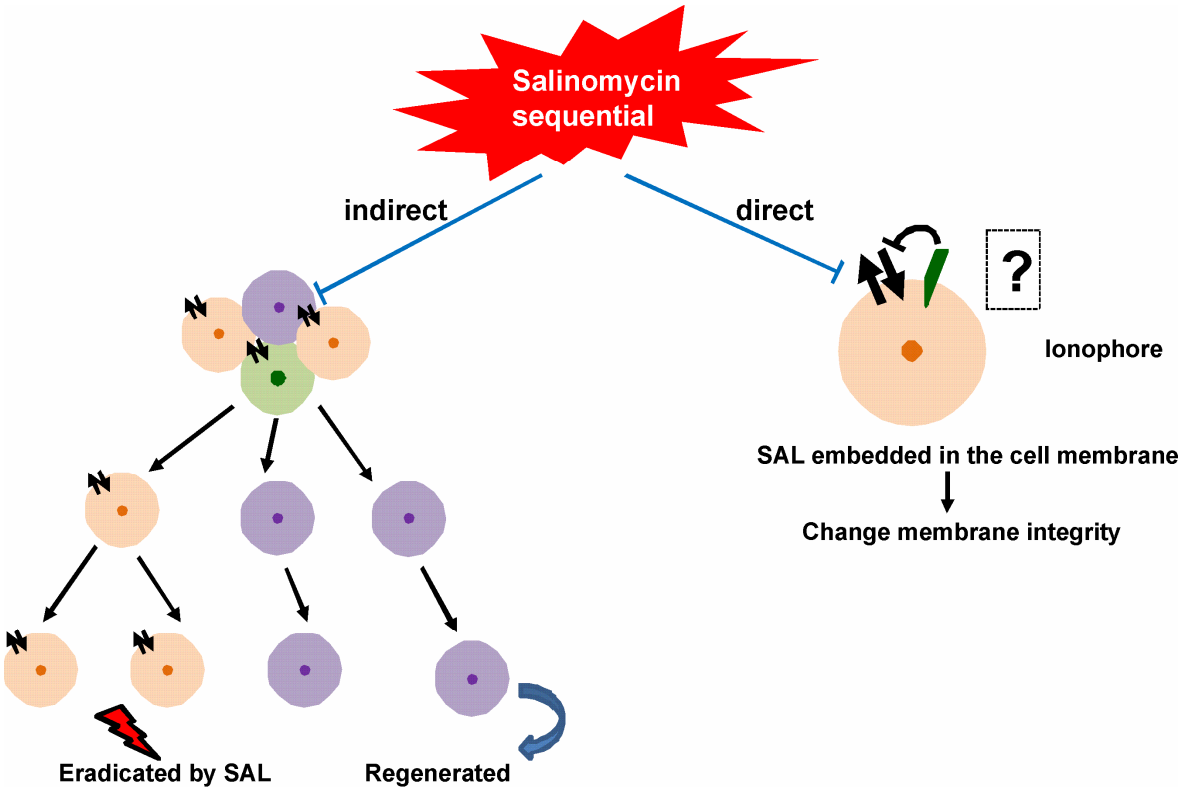


Fig. 29. Model of salinomycin decreased drug efflux pump. On the one hand, salinomycin is eradicating cancer stem cell population which overexpress efflux pumps, and therefore increasing the non-cancer stem cell fraction with low efflux pump expression. On the other hand, salinomycin is also embedded in biological membranes, and thus by changing membrane integrity it is able to inhibit the activity of drug efflux pumps.

In contrast to the breast cancer cell lines MCF7 and MDA-MB 436, where MDR1 and BCRP1 expression was decreased, an upregulation of some of these proteins in BT-474 and MDA-MB 231 cells is observed. BCRP1 is increased 7- fold in BT-474. However, the BCRP1 level in BT-474 parental cells is very low compared to the other cells, thus the upregulation in salinomycin-resistant cells did not influence the efflux pump activity substantially, as shown by blocking the pump activity with verapamil.

Doxorubicin is a substrate of p-glycoprotein [65], while verapamil acts as competitive inhibitor by specific binding to p-glycoprotein [124], thereby preventing doxorubicin efflux across the cell membrane. In contrast to BT474, MDA-MB 231 salinomycin resistant cells displayed a 6-times higher increase in MDR1 which resulted in increased efflux activity. The increased doxorubicin sensitivity is therefore due to a different mechanism. Since ABC transporters are downregulated in salinomycin-resistant cells, an enhanced doxorubicin accumulation was observed. Doxorubicin induces DNA damage by generating malondialdehyde-DNA adducts induced by free radicals [19]. Hence, increased DNA damage in salinomycin resistant cells is hypothesized. These findings suggest salinomycin treatment of chemoresistant tumors is an option in order to resensitize them.

In conclusion, this study demonstrated that sequential salinomycin treatment generates salinomycin-resistant cells and increases susceptibility to doxorubicin by downregulation of MDR1 and BCRP1 gene expression. However, due to the complexity of the genetic changes based on clonal selection during the molecular evolution assay, a comprehensive study e.g. next generation sequencing or a proteomics approach would further increase our understanding of chemoresistance mechanisms and improve clinical application of chemotherapeutics.

4.2.2 Salinomycin overcomes tamoxifen resistance of breast cancer cells by inhibiting RTKs recycling

The luminal subtype is the most common breast cancer cases with an incidence of 70-75 % [5]. Due to its dependency on ERα signaling, inhibition of the signaling with tamoxifen and other SERMs is considered as a frontline treatment. However, in about 20-30% of these cases, tamoxifen therapy fails because of existing or acquired resistance [46]. In this study, in collaboration with A.-K. Sommer (PhD student, MPI, Munich), sequential treatment of tamoxifen was performed in MCF-7 breast cancer cells. A decrease in ERα expression, increase in efflux pump genes expression, cancer stem cell marker and the activation of ligand-independent signaling were identified in tamoxifen-resistant cells.

Loss or modification in ERα expression is one of the mechanisms related to tamoxifen resistance [44]. Therefore, to maintain the proliferation, tamoxifen-resistant cells activate alternative pathways independent from the genomic cascade, including activation of ERα by epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 (IGF-1) receptor [12, 46]. Epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases, including EGFR/ErbB1/HER1, ErbB2/HER2/Neu ErbB3/HER3, and ErbB4/HER4 [125].

The ligand independent activation of ERα by EGFR family members and other RTKs plays an important role in tamoxifen resistance development [126-128]. A previous study showed upregulation of RTKs in tamoxifen-resistant cells [129, 130]. Moreover, tumors of patients with acquired tamoxifen resistance showed significantly higher EGFR levels compared to sensitive breast cancer samples [131]. This findings, further highlight the impact of the EGFR-family in breast cancer.

In tamoxifen-resistant cells upregulation of both cancer stem cells marker and ABC transporter efflux pumps was observed. As previously shown, salinomycin is able to eradicate cancer stem cells and to overcome ABC mediated drug resistance [56, 59, 132], and thereby, on the one hand salinomycin is able to overcome tamoxifen resistance by those mechanisms. On the other hand, salinomycin can also interfere with the ligand independent activation of ERα. A previous study by A.K. Sommer showed that salinomycin reduces the expression and

phosphorylation of Her2 and Her3 and thus is able to block their activation by tamoxifen treatment [133].

In this present study, FACS analysis showed that EGFR as well as Her2 disappear from cell surface and spinning disk microscopy revealed that both accumulate in lysosomes followed by degradation. Boehmerle *et al.* discovered that increased intracellular calcium levels induced by salinomycin are responsible for a pre-mature fusion of endosomes with lysosomes in neurons [134]. In order to verify this effect in breast cancer cells, the intracellular calcium levels was investigated in in vitro system and determined a significant elevation upon salinomycin treatment.

Combined targeting of ERα and RTKs is an effective solution to overcome tamoxifen resistance. Modulating ERα with SERMs i.e tamoxifen not only inhibits the genomic estrogen signaling pathway, but also activates the ligand independent pathway by upregulating RTKs. In this study, combined treatment of tamoxifen and salinomycin showed beneficial effect in both tamoxifen-sensitive and -resistant cells, even in the low dose. Salinomycin not only prevents resistance development to endocrine therapy but also eradicates already resistant breast cancer cells. Sommer demonstrated that combined treatment of tamoxifen and salinomycin reduces the expression levels of ERα as well as all EGFR- family RTKs, indicating inhibition of the ligand independent signaling which is turned on after single treatment of tamoxifen [133]. Combined treatment of tamoxifen and salinomycin is beneficial because tamoxifen blocks the genomic pathway, while salinomycin inhibits the ligand independent pathway of the estrogen signaling. This findings provide a novel treatment strategy for luminal subtype breast cancer patient in order to overcome tamoxifen resistance.

4.3 BMPR2 Promotes Doxorubicin Resistance in Breast Cancer Cells

This part of the thesis demonstrates that sequential treatment of doxorubicin induces MCF-7 and MDA-MB 231 doxorubicin-resistant cells. To explore the mechanism of doxorubicin chemoresistance, screening for drug resistance marker was performed, showing BMPR2 to be one of the interesting candidates. Consequently, BMPR2 upregulation was observed during the round of molecular evolution assay.

To investigate the role of BMPR2 in acquired doxorubicin resistance, two different cell lines, epithelial MCF-7 and mesenchymal MDA-MB 231 were used. Modulation of BMPR2 expression in both cells line was performed by overexpressing BMPR2 which reduced the sensitivity to doxorubicin, and inhibiting BMPR2 with siRNA which enhanced susceptibility to doxorubicin. A reduction in cell growth was observed during the molecular evolution assay of doxorubicin, therefore cell proliferation and cell cycle profile were also analyzed in both MCF-

7 and MDA-MB 231 cells upon BMPR2 or siBMPR2 transfection. Overexpression of BMPR2 reduces cell proliferation and induces cell cycle arrest, whereas inhibition of BMPR2 increases cell proliferation.

To further explore the molecular mechanism of BMPR2 inducing chemoresistance, BMPR signaling pathways were investigated in both BMPR2 and siBMPR2 transiently transfected cells. BMPR2 signalling consists of 2 different pathways i.e the smad dependent and the independent pathway [73]. The smad dependent signaling pathway involves phosphorylation of smad1/5/8, complex formation of activated smad1/5/8 with co-smad4, and translocation of the complex to the nucleus to modulate gene transcription [82-84]. The smad independent pathway utilizes MAP kinase pathway for its signaling cascade [73, 82].

Activation of both smad dependent and independent pathways of BMP signaling in BMPR2 transfected MCF-7 cells was observed in this study. In MDA-MB 231 cells, overexpression of BMPR2 activated only the smad dependent pathway, and treatment with BMP2 stimulated both the smad dependent and the independent pathway. Cells develop resistance mechanism by modulating cell cycle and cell proliferation. It is known that p21, an inhibitor of cyclin-CDK in G1 phase of cell cycle is a common target of all TGF-ß superfamily pathways via the smad dependent mechanism [135]. A previous study by Kim *et al.* on bladder cancer cells showed that BMPR2 overexpression leads to restoration of BMP signaling and decrease in tumor growth [136]. In this present study, an increase in p21 upon overexpression of BMPR2 was observed in MCF-7 cells which have wild type p53 gene. The p53 acts as a tumour suppressor gene and regulates transcription of p21 [137]. p53 is also supposed to interact with smad2 and smad4 and therefore has a direct interlinkage to the smad dependent signalling pathway [138, 139]. MDA-MB 231 cells have a mutant p53 gene [140], therefore intense expression of p21 upon transfection of BMPR2 could not be observed.

Activation of the smad independent pathway in BMPR2 transfected MCF-7 cells was observed by increase in activated ERK1/2. In this study, ERK activation induces cell cycle arrest rather than cell proliferation. In general, activated ERK can regulate the expression of cyclin D1, a regulator of cell cycle, and can also regulate the post translational regulation of the assembly of cyclin D-CDK4/6 complexes, thereby induces cell cycle progression [141]. However, a study by Park *et al.* on primary hepatocytes showed that activated ERK increases the phosphorylation of the transcription factors Ets2, C/EBPalpha, and C/EBPbeta, which rapidly enhance transcription of the p21 promoter via multiple Ets- and C/EBP-elements within the enhancer region, thus resulting in increase of p21 expression and induction of cell cycle arrest [142]. Accordingly, activation of the smad independent pathway in the present study, might also increase p21 and cell cycle arrest via the same or similar mechanism. However, this hypothesis remains to be investigated.

In MCF-7 cells inhibition of smad dependent pathway by siBMPR2 can be counter balanced by p53 inducing p21 expression, however this mechanism does not occur in MDA-MB 231 cells which have a mutant p53 gene. Inhibition of BMPR2 in MCF-7 cells enhances ERK activation which stimulates cell proliferation. However, no change in ERK activation was observed in MDA-MB 231 transfected with siBMPR2. MDA-MB 231 cells have mutant KRAS gene [110], which constitutively induces Ras activating ERK and PI3K/AKT pathways [143], and thereby the smad independent pathway cannot be inhibited by siBMPR2. Taken together, BMPR2 upregulation results in p21 inducing cell cycle arrest, thus provides tumor cells with more time to modulate DNA repair mechanisms.

Cancer cells may develop chemoresistance towards DNA-targeting chemotherapeutic agents by regulating DNA damage and DNA repair mechanisms [32-34]. A recent study on pulmonary arterial hypertension (PAH) by Li *et al.* showed that BMPR2 expression is tightly linked to DNA damage control, and moreover breast cancer 1 (BRCA1) was identified as a novel target of BMPR2 and a novel modulator of PAECs homeostasis [42]. In addition, in the presence of BMPR2, DNA damage can be repaired by BRCA1-related pathways, thus maintaining vascular homeostasis. In the present study DNA damage upon treatment with doxorubicin was observed in both BMPR2 and siBMPR2 transfected cells by single cell comet assay and measuring double strand break DNA damage marker γH_2AX. The results revealed less DNA damage in BMPR2 transfected cells and more DNA damage in siBMPR2 transfected cells. Upregulation of BRCA1 was detected during the round of molecular evolution assay. Additionally BRCA1 upregulation upon BMPR2 overexpression was observed in MCF-7 and MDA-MB 231 cells, and the level of BMPR2 was induced upon doxorubicin treatment. In contrary, inhibition of BMPR2 with siRNA reduced BRCA1 expression. Taken together, these findings suggest BMPR2 upregulation leads to an increased in DNA repair mechanisms by upregulating BRCA1.

Cells can also circumvent drug treatment by enhancing anti-apoptotic mechanisms. It is known that XIAP is a part of the BMP signaling pathway and a positive regulator, linking the BMP receptors withTAB1-TAK1, a member of the MAP kinase kinase kinase family [85]. XIAP inhibits either initiator caspase-9, or effectors caspase-3/7 in the apoptosis pathway [144]. DNA damage or other events that result in the induction of apoptosis can lead to ubiquitination and auto-ubiquitination of XIAP [145]. A previous study by Liu *et al.* suggested that activation of BMPR2 stabilizes XIAP by preventing ubiquitination and subsequent degradation [146, 147]. Furthermore, inhibition of BMPR2 with siRNA induces apoptosis by destabilization XIAP, and induces autophagy via XIAP-mdm2-p53 pathway [148].

This present study demonstrates that XIAP is upregulated upon BMPR2 overexpression, and downregulated upon inhibition by siBMPR2. The expression of XIAP is even higher upon stimulation with BMP2. Moreover, XIAP upregulation leads to decrease in caspase activity,

while XIAP downregulation enhances caspase activity. Accordingly, overexpression of BMPR2 in doxorubicin-resistant cells enhances the anti-apoptotic mechanism by stabilizing XIAP, but the exact mechanism requires further studies.

Sequential treatment of doxorubicin in a xenograft mouse model showed a significant reduction in tumor growth. Moreover, upregulation of BMPR2, p21 and XIAP was also observed in doxorubicin treated mice. In summary, this study findings show an increase in BMPR2 expression upon sequential doxorubicin treatment both *in vitro* and *in vivo*. The mechanism of BMPR2 promoting chemoresistance by modulation of proliferation, cell cycle, DNA repair and apoptosis is depicted in Fig. 30. BMPR2 undergoes signaling via both smad dependent and independent pathways. The smad dependent pathway induces p21 inhibiting cell cycle and cell proliferation, as well as BRCA1 upregulating DNA repair. The smad independent signaling increases XIAP stabilization leading to inhibition of apoptosis. Taken together, these pathways enhance cell survival upon DNA-damaging drugs.

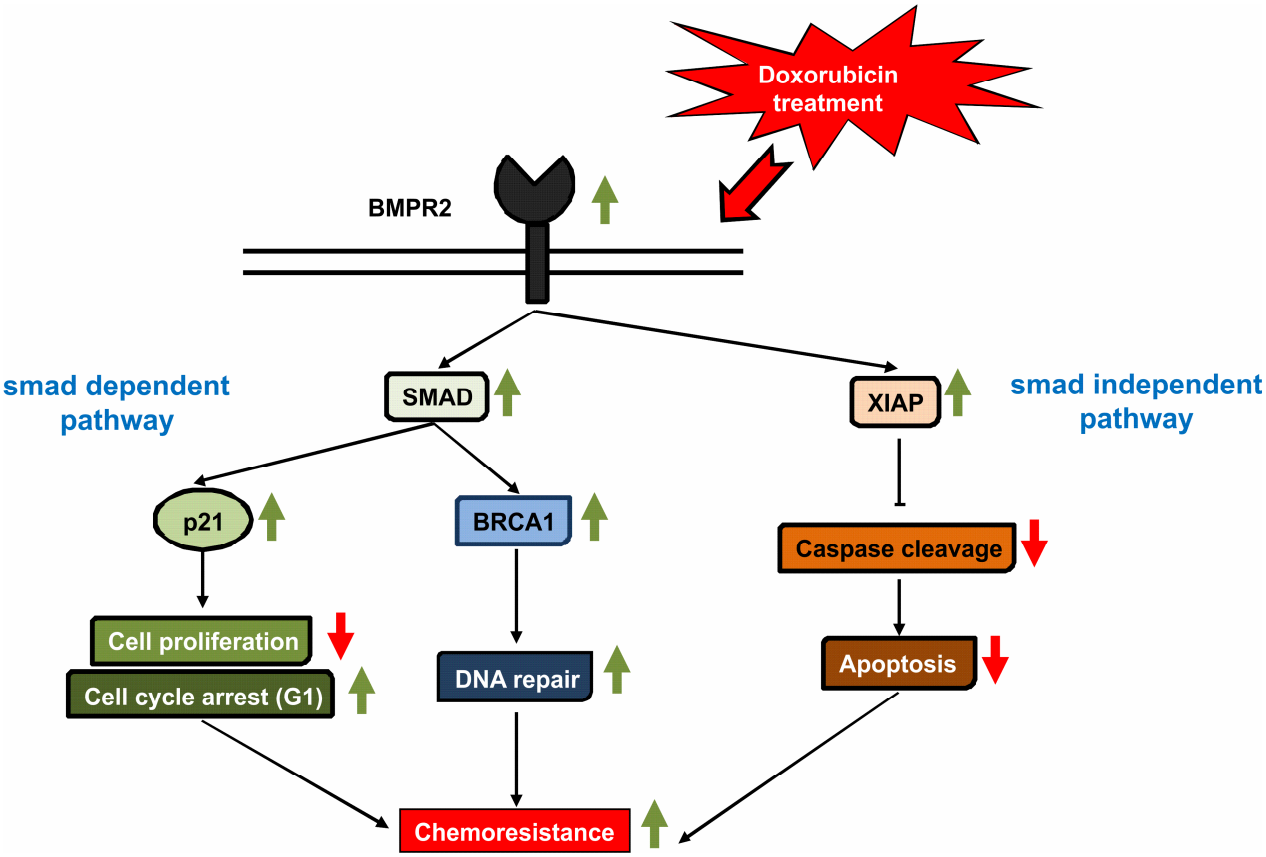


Fig.30. Model of BMPR2 mediated chemoresistance. Chemotherapeutic treatment of tumor cells induces a selection of chemoresistant cells. These cells display an increased level of BMPR2. The BMPR2 downstream signaling results in two pathways which are important for the formation and prolongation of chemoresistance. On the one hand, cells are able to evade the toxic stress of chemotherapeutics by arresting cell cycle via p21 and upregulating the DNA repair enzyme BRCA1. On the other hand BMPR2 is able to induce anti apoptotic effects by stabilizing XIAP. The increased signaling of BMPR2 thus facilitates the repair of DNA damage and survival of tumor cells.

Some studies on compounds or gene therapeutic approaches which modulate BMPR2 signaling have been conducted to treat dysregulation of BMP signaling in several physiological processes. On the one hand, adenovirus-mediated BMPR2 gene delivery to the pulmonary vascular endothelial cells using *in vivo* models of PAH showed therapeutic potential for upregulation of BMPR2 [149, 150]. On the other hand, adenovirus-mediated siRNA BMPR2 delivery in *in vivo* model promises a candidate for osteolysis treatment [151]. Development of a small molecule targeting BMP signaling has also been established. FK506 (tacrolimus) binds FK-binding protein-12 (FKBP12), a repressor of BMP signaling which release FKBP12 from type I receptors activin receptor-like kinase 1 (ALK1), ALK2, and ALK3, and activates the smad dependent and smad independent signaling pathways, thus normalizing BMP signaling in PAH [76]. Nevertheless, targeting BMPR2 in cancer therapy remains elusive.

This present study provides new insights into the role of BMPR2 in doxorubicin chemoresistance of breast cancer cells. The use of BMPR2 inhibitor may enhance efficacy of chemotherapeutics and overcome drug resistance. Moreover, targeted therapy on either the smad dependent or independent signaling pathways is also an interesting topic for future cancer drug development.

5. SUMMARY

Important progress has been achieved for breast cancer treatment in recent years, however, classical chemotherapy is still one the most frequently used in clinics, and therefore chemoresistance is still a major challenge for successful breast cancer therapy. As a result of intrinsic or acquired chemoresistance, tumors will progress or relapse with locally advanced or metastatic breast cancer. Accordingly, research on drug resistance formation and solution to overcome drug resistance is urgently needed to achieve better efficacy of chemotherapeutic drugs. In this present study, clinical application of chemotherapy is mimicked by an *in vitro* model called molecular evolution assay. Cells were sequentially treated with the same dose of drugs (doxorubicin or salinomycin) for several cycles/rounds, followed by a recovery time.

The discovery of salinomycin as anti-cancer stem cell drug provides progress in overcoming chemoresistance. However, a study on whether long term application of salinomycin is able to abolish drug resistance remained elusive. In this study, sequential treatment of salinomycin was performed in MCF-7 and BT-474 epithelial breast cancer cells as well as MDA-MB 231 and MDA-MB 436 mesenchymal cells and the gene expression of multiple drug resistance (MDR) genes was further analyzed. Here, sequential treatment of salinomycin generated resistance in all cell lines and increased the chemosensitivity towards doxorubicin. Drug efflux pump gene expression and the pump activity of MDR1 and BCRP1 were downregulated in these cells. Moreover, intracellular drug accumulation was increased compared to the respective parental cells. These findings suggest a novel treatment option for multiple drug resistant tumors by sensitizing these tumors via salinomycin pretreatment.

The luminal subtype of breast cancer is the most common breast cancer cases, and due to its dependency of estrogen signaling, inhibition of the signaling with tamoxifen is considered as the firstline treatment. However, cells develop resistance to tamoxifen by activating ligand independent signaling pathway. In this part of the thesis, sequential treatment of tamoxifen on MCF-7 cells generated tamoxifen-resistant cells. Upregulation of efflux pumps and cancer stem cell marker, but downregulation of ERα gene expression was observed in tamoxifen-resistant cells. Here, salinomycin circumvents tamoxifen resistance by inhibiting ligand independent pathway. Therefore combinational treatment of tamoxifen and salinomycin is a novel strategy to improve clinical efficacy of tamoxifen.

Doxorubicin is the most commonly used chemotherapy in clinic, and therefore exploring chemoresistance mechanism of doxorubicin is urgently needed to improve its clinical outcome. Bone morphogenetic protein receptor type II (BMPR2) is a serine/threonine kinase and receptor of TGF-ß family. BMPR2 can regulate bone, teeth, and vascular formation, as well as kidney regulation and hypertension. In this part of the thesis, BMPR2 was identified as a promising target of doxorubicin resistance. This study demonstrates that BMPR2 promotes

doxorubicin chemoresistance in breast cancer cells via regulating cell proliferation and cell cycle, DNA repair, and apoptosis. Furthermore, increased BMPR2 expression in doxorubicin-resistant breast cancer cells was confirmed in a xenograft mouse model. These findings show a novel role of BMPR2 in breast cancer chemoresistance. Antagonizing BMPR2 action might improve clinical outcome of breast cancer patients by sensitizing resistant tumor cells to doxorubicin.

In this thesis many options to circumvent chemoresistance were found. Further studies are needed to validate these novel treatment options.

6. APPENDIX

6.1 Supporting Information Chapter 3.1.1

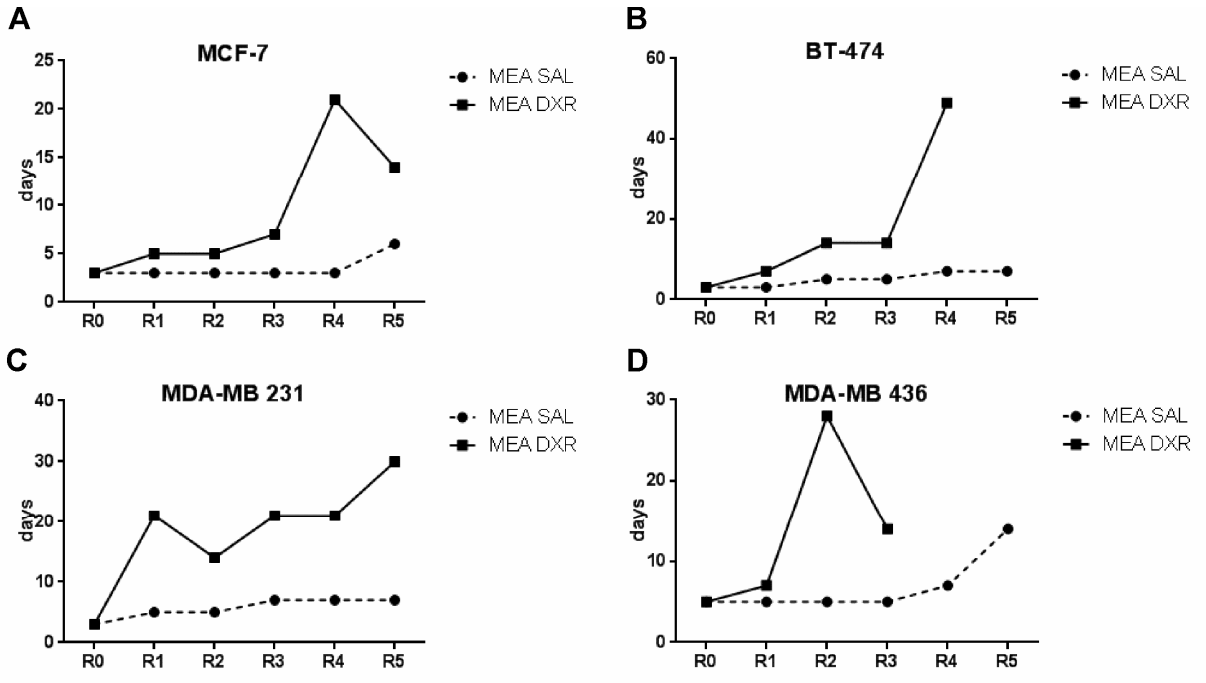


Fig. S1. The time to reach an 80% confluency after cells splitting during the molecular evolution assay of salinomycin (SAL) and doxorubicin (DXR). (A) MCF-7, (B) BT-474, (C) MDA-MB 231, (D) MDA-MB 436 cells.

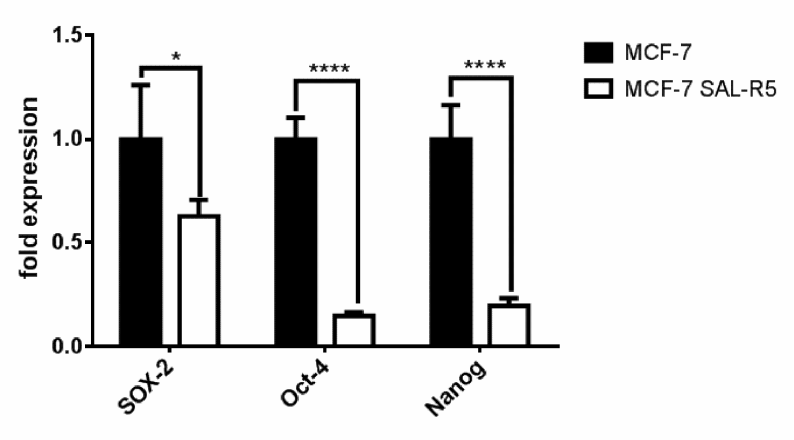


Fig. S2. Molecular evolution assay of salinomycin in MCF-7 cells results in downregulation of cancer stem cells marker gene expression. SOX-2, Oct-4 and Nanog expression in MCF-7 and MCF-7 R5-SAL were determined by quantitative RT-PCR. GAPDH was used as an internal control for quantitative RT-PCR. Results represent the average of three independent experiments (mean ± SD). Statistical analysis was conducted using Student's t-test. * or ** or ***or**** indicates $p<0.05$ or $p<0.01$ or $p<0.001$ or <0.0001, respectively.

6.2 Abbreviations

ABC	ATP binding cassette
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BISC	BMP-induced signaling complex
cDNA	Complementary DNA
CSCs	Cancer stem cells
DAPI	4',6-Diamino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid
dNTP	Desoxynukleosid-triphosphate
DTT	Dithiothreitol
DXR	Doxorubicin
e.g.	exempli gratia (for example)
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-mesenchymal transition
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
G	Gap
M	Mitosis
MDR	Multiple drug resistance
MET	Mesenchymal to epithelial transition
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PAGE	Polyacrylamide gel electrophoresis
PAH	Pulmonary arterial hypertension
PBS	Phosphate buffered saline
PFA	Para-formaldehyde
PFC	Preformed type I-type II heteromeric receptor complex
PI	Propidium iodide
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
R	Round
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RPMI	Rosewell park memorial institute
RT	Reverse transcriptase
RTKs	Receptor tyrosine kinases
S	Synthesis
SAL	Salinomycin
SD	Standard deviation
SDS	Sodiumdodecylsulfate
SEM	Standard error of the mean
siRNA	Small interfering RNA
TAM	Tamoxifen
TE	Trypsin/EDTA
TEMED	N,N,N′,N′-Tetramethylethan-1,2-diamine
UPL	Universal probe library
WHO	World health organization

6.3 Genes and Proteins

ABC	ATP binding cassette
ACVR	Activin receptor
AKT	Protein kinase B
ALK	Activin like kinase
BCRP	Breast cancer resistance protein
BCRP1	Breast cancer resistance protein
BMP	Bone morphogenetic protein
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BRCA1	Breast cancer 1
CDK	Cyclin-dependent kinase
co-smad	Common-mediator Smad
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERK	Extracellular-signal-regulated kinase
ERK	Extracellular signal-regulated kinases
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDF	Growth differentiation factor
GDF	Growth and differentiation factor
GST	Glutation S-transferase
H2AX	H2A histone family, member X
Her2	Human epidermal growth factor receptor
IGF-1R	Insulin-like growth factor 1 receptor
I-smad	Inhibitory Smads
JNK	C-Jun N-terminal kinases
MAPK	Mitogen-activated protein kinase
MDR1	Multidrug resistance protein 1
Oct-4	Octamer-binding transcription factor 4
p21	Protein p21
p38 MAPK	p38 Mitogen-activated protein kinases
p53	Protein p53
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
Rho	Ras homologue
R-smad	Receptor-regulated Smads
smad	Small mothers against decapentaplegic

SOX-2	SRY (sex determining region Y)-box 2
TAB	TGF-ß activated binding protein
TAK1	TGF-ß activated tyrosine kinase 1
TGF-ß	Transforming growth factor beta
XIAP	X-linked inhibitor of apoptosis

7. PUBLICATIONS

7.1 Original Articles

Kopp F, **Hermawan A**, Oak PS, Herrmann A, Wagner E, Roidl A. Salinomycin treatment reduces metastatic tumor burden by hampering cancer cell migration. *Mol Cancer*, 2014.**13**: p. 16.

Kopp F, **Hermawan A**, Oak PS, Ulaganathan VK, Herrmann A, Elnikhely N, Thakur C, Xiao Z, Knyazev P, Ataseven B, Savai R, Wagner E, Roidl A. Sequential Salinomycin Treatment Results in Resistance Formation through Clonal Selection of Epithelial-Like Tumor Cells. *Transl Oncol.* 2014, 7(6):702-11.

Sommer A*, **Hermawan A***, Mickler F, Knyazev P, Bräuchle C, Wagner E, Ullrich A, Roidl A, Tamoxifen resistance of breast cancer cells is antagonized by Salinomycin treatment, submitted. (*equal contributors)

Hermawan A, Wagner E, Roidl A, Consecutive salinomycin pretreatment reduces drug efflux pump expression and chemoresistance of breast tumor cells against doxorubicin, research article, submitted.

Hermawan A, Kopp F, Busse J, Herrmann A, Garcia-Roman J, Ljepoja B, Wagner E, Roidl A, Bone morphogenetic protein receptor II (BMPR2) promotes doxorubicin-resistance in breast cancer cells, research article, in preparation.

Hermawan A, Wagner E, Roidl A, Emerging role of bone morphogenetic protein receptor II (BMPR2) in cancer, review article, in preparation.

7.2 Posters

Sommer A[#], **Hermawan A**, Mickler F, Knyazev P, Bräuchle C, Wagner E, Ullrich A, Roidl A, Tamoxifen resistance can be overcome by salinomycin treatment, 23rd Biennial Congress of the European association for cancer research (EACR), Munich, Germany, 5-8 July 2014.

Ljepoja B[#], Garcia-Roman J, Bruno N, **Hermawan A**, Kopp F, Wagner E, Roidl A, miR-27a is a functional biomarker for tamoxifen treatment of luminal A/B breast tumors, 23rd Biennial Congress of the European association for cancer research (EACR), Munich, Germany, 5-8 July 2014.

Sommer A, **Hermawan A[#]**, Mickler F, Knyazev P, Bräuchle C, Wagner E, Ullrich A, Roidl A, Tamoxifen resistance can be overcome by salinomycin treatment, Interact Munich, Germany, 5-6 March 2015.

Hermawan A[#], Kopp F, Busse J, Herrmann A, Garcia-Roman J, Ljepoja, Wagner E, Roidl A, Bone morphogenetic protein receptor II (BMPR2) promotes doxorubicin-resistance in breast cancer cells, EACR-AACR-SIC Special Conference, Florence, Italy, 20-23 June 2015.

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