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ETAR antagonist ZD4054 exhibits additive effects with aromatase inhibitors and fulvestrant in breast cancer therapy, and improves *in vivo* efficacy of anastrozole

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ABSTRACT

Purpose: Endothelin-1 (ET-1) and endothelin A receptor (ETAR) contribute to development and progression of breast carcinomas by modulating cell proliferation, angiogenesis, and anti-apoptosis. We investigated antitumoral effects of the specific ETAR antagonist ZD4054 in breast cancer cells and xenografts, and assessed antitumoral efficacy of the combinations of ZD4054 with aromatase inhibitors and fulvestrant.

Methods: Gene expression changes were assessed by quantitative real-time PCR. Cell proliferation was measured using alamarBlue®; migration and invasion assays were performed using modified Boyden chambers. Evaluating the antitumoral efficacy of ZD4054 *in vivo*, different breast cancer models were employed using nude mice xenografts.

Results: ZD4054 reduced ET-1 and ETAR expression in MCF-7, MDA-MB-231, and MDA-MB-468 breast cancer cells in a concentration-dependent manner. ZD4054 inhibited invasion by up to 37.1% (P=0.022). Combinations of ZD4054 with either anastrozole or letrozole produced significant reductions in migration of aromatase-overexpressing MCF-7aro cells (P<0.05). Combination of ZD4054 with fulvestrant reduced MCF-7 cell migration and invasion by 36.0% (P=0.027) and 56.7% (P<0.001), respectively, with effects significantly exceeding those seen with either compound alone. Regarding tumor volume reduction *in vivo*, ZD4054 (10 mg/kg) was equipotent to fulvestrant (200 mg/kg) and exhibited additive effects with anastrozole (0.5 mg/kg).

Conclusions: These data are the first indicating that selective ETAR antagonism by ZD4054 displays antitumoral activity on breast cancer cells *in vitro* and *in vivo*. Our data strongly support a rationale for the clinical use of ZD4054 in combination with endocrine therapies.

INTRODUCTION

Endothelin-1 (ET-1), a 21 amino acid peptide, is formed from inactive precursors by endothelin-converting enzyme-1 (ECE-1) and is secreted by endothelial, vascular smooth muscle and epithelial cells [1,2]. ET-1 signals *via* G-protein coupled endothelin receptors A (ET_AR) and B (ET_BR) [2,3], and is degraded by the metalloprotease neprilysin (NEP). ET-1 expression is increased in different human malignancies including ovarian, prostate and colorectal cancer [2]. In breast cancer, engagement of ET-1 to ET_AR activates tumor promoting mechanisms including proliferation, invasion, angiogenesis and anti-apoptosis [3]. Expression of the ET system is associated with poor outcome [4], increased vascular endothelial growth factor (VEGF) expression and higher vascularity of breast carcinomas [5], indicating a role in breast cancer angiogenesis. In contrast, ET_BR activation leads to ET-1 degradation and increased apoptosis [6]. Consequently, the ET system was proposed as a potential target for anticancer therapy [2,3]. Selective ET_AR antagonism inhibits hypoxia-induced breast cancer cell invasion [7], and ECE-1 inhibition decreases ET-1 secretion and invasion of breast cancer cells [8].

ZD4054 is an orally active specific ET_AR antagonist [9]. ZD4054 inhibits proliferation of ovarian carcinoma cells and tumour growth *in vivo* [6], enhances paclitaxel efficacy [10] and exhibits improved antitumoural activity when combined with EGFR inhibitor gefitinib [11]. In prostate cancer, ZD4054 has been evaluated for its efficacy on bone metastases in hormone-refractory prostate cancer (HRPC) [3, 12]. Results of phase II EPOC study have been presented at ECCO 14 (2007); respective data indicate improvement of overall survival in men with HRPC due to treatment with ZD4054, leading to a 45% reduction of risk of death compared to placebo [13].

Since 17 β -estradiol (E₂) exerts tumor promoting effects in estrogen receptor (ER) positive breast carcinomas, endocrine therapy remains an important therapeutic option in both premenopausal and postmenopausal women with ER-positive breast cancer. Most prevalent substance classes currently used are selective estrogen receptor modulators (SERMs, i.e. tamoxifen), ER downregulator fulvestrant, and aromatase inhibitors (AI, i.e. anastrozole, letrozole). We previously demonstrated an (unexpected) positive correlation between prognostically unfavourable ET system expression and prognostically favourable ER expression in breast cancer specimens [4]. Subsequently, E₂ was found to induce ET_AR expression in breast cancer cells *in vitro*, demonstrating a relevant mechanism of endocrine regulation of ET_AR expression [14]. These data indicate existence of an interaction between the ET system and E₂-mediated pathways. Therefore, combined inhibition of both E₂-activated pathways (using SERMs, anti-estrogens, or AIs) and ET-1/ET_AR-activated signal cascades (using ZD4054) may represent an efficient strategy for endocrine treatment of breast cancers. The objectives of this study were to investigate antitumoural effects of ZD4054 on breast cancer cells and to explore the relevance of combining established endocrine therapies with ZD4054 for future breast cancer therapy.

MATERIAL AND METHODS

Compounds. ZD4054, fulvestrant (Faslodex™) and anastrozole (Arimidex™) were kindly provided by AstraZeneca (Macclesfield, UK). Letrozole (Femara™) was kindly provided by Novartis Pharma AG (Basel, Switzerland). ET-1, tamoxifen, 17β-estradiol and Δ⁴-androstene-3,17-dione were obtained from Sigma (Steinheim, Germany).

Cell culture. MCF-7 cells [15] (ER-positive, PR-positive) and DU-4475 cells [16] (ER-negative, PR-negative) were maintained in RPMI 1640 containing 10% fetal calf serum (FCS), 1% glutamine and 1% penicillin/streptomycin. BT-474 cells [17] (ER-positive, PR-positive) were kept in RPMI 1640 supplemented with 20% FCS, 1% glutamine, 1% penicillin/streptomycin and 0.1% insulin. For experiments with AIs, MCF-7aro (*in vitro*) [18] and MCF-7-Ac1 (*in vivo*) [19] cells were used, which are stably transfected with the human aromatase gene *CYP19A1*. MCF-7aro and MCF-7-Ac1 cells were kept in RPMI 1640 containing 10% fetal calf serum (FCS), 1% glutamine, 1% penicillin/streptomycin and 600 μg/ml G418. MCF-7, MCF-7-Ac1 and BT-474 cells were kept in a humidified atmosphere at 37 °C of 5% CO₂. MDA-MB-231 and MDA-MB-468 cells [20] (both ER- and PR-negative) were maintained in DMEM High Glucose, containing 10% FCS, 1% glutamine and 1% penicillin/streptomycin in a humidified atmosphere at 37 °C of 7.5% CO₂. Cell culture reagents were obtained from Gibco (Karlsruhe, Germany). All cell lines used were proven to be ET_AR- and ET-1-positive by real-time PCR and ELISA. ET_AR-negative DU-4475 served as negative control.

Stimulation experiments. Cells were seeded into 6-well plates and incubated with the respective compound under cell culture conditions. Following incubation, cells were harvested by scraping, rinsed with phosphate buffered saline (PBS) and pelleted by centrifugation at 3000x g for 5 minutes. Total RNA was extracted using RNeasy-Protect Mini (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Each stimulation was performed in triplicate.

Quantitative real-time PCR. cDNA was prepared applying the Advantage RT-for-PCR kit (Clontech, Heidelberg, Germany). Quantitative real-time PCR was performed using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, USA) by using the default thermal cycling conditions (10 minutes at 95°C, and then 40 cycles of 15 seconds at 95°C plus 1 min at 60°C). For all reactions, standard concentrations of TaqMan™ Gene Expression Assays (β-actin: Hs_99999903_m1; ET-1: Hs_00174961_1; ET_AR: Hs_00609865_1; ECE-1: Hs_00154837_m1; NEP: Hs_00153510_m1) and Universal TaqMan™ PCR Mastermix (Applied Biosystems) were used. Quantification of gene expression was accomplished in triplicate for each single stimulation measuring the fractional cycle number at which the amount of expression reached the fixed threshold (cycle threshold, Ct). Relative gene expression levels were determined using the 2^{-ΔΔCt} method after normalization to β-actin and are expressed as relative quantification (RQ value).

Migration assay. Cell migration assays were performed in triplicate using Falcon® Cell Culture Inserts (BD Biosciences, Bedford, MA, USA). Cells (2.5 x 10⁴ per well) were added to the upper compartment in serum-free medium. Medium supplemented with 10% FCS and 25 ng/ml basic fibroblast growth factor (bFGF) was given to the lower compartment. For the time of incubation, the respective compound was added to both compartments. Cells then were allowed to migrate for 24 hours under routine conditions.

Subsequently, the medium was removed from the insert and cells on the upper side of the membrane were removed by gentle scraping. Cells on the lower surface were fixed with methanol, stained, and counted under the microscope at 40x magnification. Relative migration was expressed as a percentage of the cell number on compound-treated inserts compared to solvent-only treated controls.

Invasion assay. 0.5 ml of cell suspension in serum-free cell culture medium (5×10^4 cells/ml) was added to BD BioCoat[®] Matrigel Invasion Chambers (BD Biosciences, Bedford, MA, USA) with a layer of matrigel matrix serving as a reconstituted basement membrane *in vitro*. Medium containing 10% FCS and 25 ng/ml bFGF was given to the lower compartment. For test cells, the respective compound was added at 0.1 μ M to either compartment; for control cells, pure solvent was added. After 24h incubation, non-invading cells were removed from the upper surface by gentle scraping. Cells on the lower surface were fixed, stained and counted through the microscope at 40x magnification. At each triplicate membrane, cells in five different fields were counted. Relative invasion was expressed as percentage of the cell number on compound-treated inserts compared to control inserts.

Proliferation assay. For assessment of cellular metabolic activity ('proliferation'), alamarBlue[™] system was used as described previously [35]. 5×10^3 cells/170 μ l were seeded in triplicate into 96-well plates and incubated at 37 °C and 5% CO₂ for 24 hours to allow cell attachment. Subsequently, 10 μ l of ZD4054 stock solution (final concentrations 1 – 1000 nM) and 20 μ l of alamarBlue[™] substrate were added to each well. Cell-free wells containing medium were used as negative controls. The number of viable cells correlating with the magnitude of dye reduction was calculated according to the manufacturer's protocol. Fluorimetric determination of percentage of alamarBlue[™] substrate reduction was performed with a spectrophotometer at $\lambda=600$ nm and $\lambda=570$ nm in regular intervals between 2-32 hours after adding ZD4054. Data were corrected for background values of negative controls.

Tumour Cell Xenografts. Female athymic nude mice (CD-1 nu/nu; Charles River, Sulzfeld, Germany) were used to establish the breast cancer xenograft models. Breast cancer cells were washed with PBS and centrifuged at 1000 x g for 10 min at 4 °C. Prior to implantation, 5×10^6 MCF-7 cells were resuspended in 100 μ l Matrigel (BD Biosciences, Heidelberg, Germany) and 100 μ l RPMI. For MCF-7-Ac1 xenografts, 3×10^6 cells were resuspended in 100 μ l matrigel [21]; for MDA-MB-468 and DU-4475 xenografts, 1×10^7 cells were resuspended in 200 μ l PBS [16].

To generate tumour xenografts, cells were implanted subcutaneously (s.c.) in the right flank. MCF-7, MCF-7-Ac1, MDA-MB-468 cells were confirmed to be ET_AR-positive by PCR both prior to implantation and *ex vivo* after final tumor resection. ET_AR-negative DU-4475 served as a negative control [14]. For hormonal supplementation in a premenopausal breast cancer model, an E₂ pellet (SE-121, 0.72 mg/pellet, 60-day release; Innovative Research of America, Sarasota, FL, USA) was implanted in the breast fat pad [22]. Establishing a postmenopausal breast cancer model to evaluate AIs, mice were ovariectomized prior to tumor cell injection. From the day of ovariectomy, animals received daily s.c. injections of aromatase substrate Δ^4 -androstene-3,17-dione (4 mg/kg) to obtain relevant concentrations of E₂ precursors [21]. Tumor burden was determined by measuring the two perpendicular diameters of the implant every three days using a caliper. Tumor volumes (mm³) were calculated as volume = (length x width²)/2. Anastrozole was given once daily by s.c. injection (0.4 mg/kg) as aqueous solution of hydroxypropylcellulose (0.3%)

(Sigma) and NaCl (0.8%) (JT Baker, Phillipsburg, NJ, USA). Application of fulvestrant was conducted by weekly s.c. injection of 100µl Faslodex™ corresponding to a weekly dose of 200 mg/kg fulvestrant. ZD4054 was given orally once daily as an aqueous suspension (1% polysorbate 80, Sigma); ZD4054 dosages used were 10 and 30 mg/kg [10]. Animals of the control groups received vehicles only. Both control and treatment groups consisted of eight mice.

Statistical Analysis. For statistical analysis SPSS for Windows (Version 13.0) was used To determine anti-tumoral effects *in vivo*, ANOVA and Student *t* tests were performed. Correlations between tumor growth and treatment arm were tested by cross-tables applying Chi-square and Fisher`s exact test. Error bars represent standard deviation, and P values were generated by performing Student's t-test. A *P*-value of < 0.05 was considered to be statistically significant.

RESULTS

ZD4054 reduces ET system expression. In all breast cancer cells tested (MCF-7, BT-474, MDA-MB-231, MDA-MB-468), ZD4054 significantly reduced expression of the components of the ET system (**Figure 1**). For ET-1 expression, RQ values ranged from RQ=0.91 (MCF-7, 1 nM, n.s.) to RQ=0.68 (MDA-MB-468, 1000 nM, $P=0.002$). Within each cell line, no significant differences were observed between any ZD4054 concentrations (1–1000 nM). ZD4054-mediated reduction of ET_AR expression was concentration dependent; RQ values ranged from RQ = 1.03 (BT-474, 1 nM, n.s.) to RQ = 0.61 (MDA-MB-468, 1000 nM, $P<0.001$). Reduction of ECE-1 expression produced RQ values between RQ=0.81 (BT-474, 10 nM, n.s.) and RQ=0.47 (MDA-MB-231, 100 nM, $P=0.028$). While MDA-MB-231 cells were NEP-negative, ZD4054 reduced NEP expression in the other cell lines with RQ values ranging from RQ = 0.85 (BT-474, 10 nM, n.s.) to RQ = 0.43 (BT-474, $P<0.001$).

ZD4054 without effect on breast cancer cell proliferation. ZD4054 left breast cancer cell proliferation unchanged (**Figure 2**); the absence of any pro- or anti-proliferative effect was consistent for all breast cancer cell lines tested independent of the ZD4054 concentration used (1 nM - 1000 nM). Unaffected by single outliers (MCF-7 at ZD4054 (1 nM, 2 h); BT-474 at ZD4054 (100 nM, 2 h), an effect of ZD4054 on breast cancer cell proliferation can be excluded.

Differential effects of ZD4054 on breast cancer cell migration and invasion. ET-1 did not affect migration in all breast cancer cell lines (**Figure 3A**). ZD4054 too left the breast cancer cell migration unaffected, consistent for ZD4054 concentrations ranging from 1–1000 nM (data not shown). In contrast, both ET-1 and ZD4054 exhibited strong effects on breast cancer cell invasion (**Figure 3B**): ET-1 induced an increase of relative invasion, ranging from 177.5% (MCF-7, $P=0.041$) to 121.5% (MDA-MB-231, $P=0.003$). ZD4054 reduced invasion by 25.2% for MCF-7 ($P=0.031$), 29.8% for MDA-MB-468 ($P=0.003$), and 37.1% for MDA-MB-231 ($P=0.022$). Of note, this anti-invasive effect of ZD4054 was observed independent of the fact if assays were (data not shown) or were not (**Figure 3B**) stimulated with exogenous ET-1 (0.1 μM).

Combination of ZD4054 and anti-estrogens – effects on gene expression.

Combination with tamoxifen (**Figure 4A**). In MCF-7 cells, both E₂ and tamoxifen increased expression of ET-1, ET_AR and ECE-1. By simulating the clinical situation of a tamoxifen-treated patient (**Figure 4A**: E₂ plus tamoxifen), ET_AR and ECE-1 expression was reduced, indicating that the E₂-mediated induction of the ET system at least in part can be antagonized by tamoxifen. Compared to simulated tamoxifen therapy, co-incubation with tamoxifen and ZD4054 significantly reduced ET-1 expression: with RQ_{ET-1}=3.8 for E₂ plus tamoxifen and decreased values between RQ_{ET-1}=1.7 (1 nM) and RQ_{ET-1}=2.0 (1000 nM) due to additional ZD4054 treatment. Expression of ET_AR and ECE-1 remained unchanged. These results were confirmed in the respective assays for BT-474 cells (**Figure 4B**).

Combination with fulvestrant. Contrary to tamoxifen, fulvestrant did not significantly affect expression of the ET system (**Figure 4C,D**). Compared to simulated fulvestrant therapy in (postmenopausal) patients

(E₂ plus fulvestrant), co-incubation with ZD4054 reduced ET-1, ET_AR and ECE-1 expression below control level, thus marking another substantial difference from combination with tamoxifen.

Combination of ZD4054 and fulvestrant/aromatase inhibitors – effects on breast cancer cell migration. Fulvestrant and ZD4054 did not affect migration of MCF-7 cells (**Figure 5A**); however, combination of both compounds produced a significant anti-migratory effect (100% vs. 64%, $P=0.028$). Respective assays were conducted on MCF-7aro cells in steroid-depleted medium containing Δ^4 -androstene-3,17-dione (0.1 μ M) (**Figure 5B**). Both AIs did not influence cellular migration; however, combination of AIs with ZD4054 significantly reduced migration by 23.2% (ZD4054 plus anastrozole, $P=0.046$) and 32.7% (ZD4054 plus letrozole, $P=0.026$), respectively.

Combination of ZD4054 and anti-estrogens – effects on breast cancer cell invasion. Tamoxifen did not affect MCF-7 cells invasion, while it was reduced by fulvestrant (100% vs. 61.9%, $P=0.003$) (**Figure 5C**). Combination of tamoxifen with ZD4054 reduced invasion by 39.5% ($P<0.001$), combination of fulvestrant with ZD4054 reduced invasion by 60.8% ($P<0.001$). Compared to ZD4054 or fulvestrant (mono), invasion was significantly reduced by the combination ZD4054 plus fulvestrant. For the combination ZD4054 plus tamoxifen, the difference was only significant compared to tamoxifen and not compared to ZD4054. Since aromatase-transfected MCF-7 cells were only negligibly invasive, invasion assays were not performed.

Antitumoral efficacy of ZD4054 in vivo. The antitumoral effect of ZD4054 was investigated in two doses (10 mg/kg and 30 mg/kg) and two different breast cancer cell lines *in vivo*. Mean tumor volumes in MDA-MB-468 xenografted animals treated with either dose of ZD4054 were significantly smaller than in placebo-treated animals (**Figure 6A**). At day 30, mean tumor volumes of different treatment groups were 81.9 mm³ \pm 8 mm³ (ZD4054 10 mg/kg, $P<0.001$) and 71.4 mm³ \pm 10 mm³ (ZD4054 30 mg/kg, $P<0.001$) as compared to 239.3 mm³ \pm 21 mm³ in controls. While both doses were equipotent at the end of treatment, at initial tumor stages the higher dose of ZD4054 (30 mg/kg) was found to be superior to 10 mg/kg ZD4054. No effect of ET_AR antagonist ZD4054 was observed in ET_AR-negative DU-4475 xenografts (**Figure 6B**).

Antitumoral efficacy of the combinations ZD4054 plus fulvestrant and ZD4054 plus anastrozole. Results of the MDA-MB-468 xenograft trial investigating the effect of ZD4054 (10 mg/kg) plus fulvestrant (200 mg/kg) are given in **Figure 6C**. From day 22, tumor volumes of ZD4054 (mono)-treated animals were significantly smaller compared to that of control animals. Compared to fulvestrant mono, the combination ZD4054 plus fulvestrant was found to be equipotent; compared to ZD4054 mono, the combination ZD4054 plus fulvestrant was superior from day 22.

Combining ZD4054 with AI anastrozole, for the first time antitumoral efficacy of ZD4054 in an aromatase-overexpressing breast cancer xenograft model was proved (**Figure 6D**). While tumor volumes in control mice remained constant for the first weeks and initiated exponential growth from day 50, tumor

volumes in treatment groups decreased. All treatment groups were found to be equipotent, leading to tumor volumes significantly smaller than that of control animals (all $P < 0.001$). On day 75, mean tumor volumes were $121 \text{ mm}^3 \pm 39 \text{ mm}^3$ for ZD4054 at 10 mg/kg, $186 \text{ mm}^3 \pm 20 \text{ mm}^3$ for anastrozole (0.5 mg/kg) and $51 \text{ mm}^3 \pm 20 \text{ mm}^3$ for ZD4054 *plus* anastrozole compared to $342 \text{ mm}^3 \pm 29 \text{ mm}^3$ for controls. The differences between distinct treatments groups were not significant, however, differences in tumor growth kinetics were observed over the course of treatment. In early tumor stages, ZD4054 was superior to anastrozole: until day 50, mean tumor volume of the ZD4054 group was below that of anastrozole-treated animals. From day 22 to day 50, an additive effect of the combination ZD4054 *plus* anastrozole was observed: mean tumor volumes in the combination group were at any time significantly smaller than that in the anastrozole group. Compared to ZD4054 monotherapy, mean tumor volumes of the “ZD4054 plus anastrozole” treatment group were not significantly different, indicating that especially individuals treated with anastrozole would benefit from co-medication with ZD4054.

DISCUSSION

This study provides a basis for a relevant role of specific ET_AR antagonist ZD4054 in future breast cancer therapy. While ZD4054 reduced mRNA expression of the ET system, individual differences between significant inhibitory effects on individual components of the ET axis were observed in four different breast cancer cell lines with varying ET system expression (Table I). The inhibitory effect is explained by the fact that the respective genes are controlled by transcription factors regulated by ET_AR activation: *via* ET_AR, ET-1 activates protein kinase C (PKC) and ras/raf/MEK/MAPK cascade, leading to an autostimulation of ET-1 gene expression [23]. Expression of ET_AR [24], ECE-1 [25] and NEP [26] are also induced by activated PKC. Thus, it can be expected that ET_AR antagonism reduces expression of the respective genes.

The role of the ET system for breast cancer cell proliferation appears to be only marginal, since ZD4054 did not affect proliferation, in contrast to results in other tumor entities: In lung, colorectal and ovarian carcinoma, a pro-proliferative effect of ET-1 could be abolished by selective ET_AR antagonism [6, 27, 28].

As MCF-7, MDA-MB-231 and MDA-MB-468 differ in their intrinsic ET system expression [14], results indicate that ET-1, independent of constitutive ET-1 or ET_AR expression is only of marginal relevance for breast cancer cell migration. In contrast, in prostate cancer and ovarian cancer cells, ET-1 induces migration [2,3], while it inhibits migration of lung cancer and neural stem cells [29, 30]. Cell migration is based on a complex set of intracellular changes, predominantly including remodelling of the actin cytoskeleton [31], depending on activation of protein tyrosine kinase Src, Ras, PKC, phosphatidylinositol-3-kinase, ERK and JNK [32]. Although most of these genes are regulated by ET-1 [2], in breast cancer cells ET-1 did not induce cellular migration. Investigations on the counter-regulatory mechanism by which ET-1 fails to induce migration will be part of subsequent studies.

In contrast, we could demonstrate a major role of the ET-1/ET_AR interaction for breast cancer cell invasion. ET-1 induced invasion, with the extent of induction being maximal in cell lines with poor invasiveness (MCF-7, MDA-MB-468) [33]. In highly invasive MDA-MB-231, different pro-invasive regulatory systems are induced or up-regulated already, including dependence receptors [34], CXCR4 [35] or matrix metalloproteases (MMPs) [36]. Therefore, the additional pro-invasive stimulus of ET-1 in these cells was relatively weak. ET-1 induced invasion was mediated by ET_AR, as it was inhibited by ZD4054. Of note, ZD4054 reduces invasion without exogenous ET-1, due to autocrine/paracrine secretion of ET-1 [2, 3], indicating a possible value of ZD4054 in a future anti-metastatic breast cancer therapy [37]. As transition to metastatic disease is associated with tumor-related death, anti-invasive efficacy of ZD4054 could mark a clinically important characteristic of ET_AR antagonists.

In ovarian and prostate cancer cells, ET-1 induces expression of MMPs and secretion of urokinase-type plasminogen activator (uPA), urokinase-type plasminogen activator receptor (uPAR) and plasminogen activator inhibitor-1 (PAI-1), facilitating extracellular matrix degradation [3, 38, 39]. Accordingly, selective ET_AR antagonism inhibits ET-1-mediated induction of MMPs and the uPA/PAI system [2, 3, 12]. In breast cancer cells, the pro-invasive effect of ET-1 could be a result of interactions between ET_AR downstream cascades and HIF-1 α signalling: as previously shown in melanoma and ovarian cancer cells,

ET-1 *via* HIF-1 α activation induces pro-invasive factors [2, 40]. In addition, engagement of ET-1 to ET_AR activates phospholipase D [41] which then induces pro-invasive signal cascades [42]. Moreover, induction of proto-oncogenes like *c-fos* and *c-jun* by ET-1/ET_AR interaction leads to an activation of genes encoding pro-invasive parameters [43, 44].

Both E₂ and tamoxifen increased mRNA expression of ET-1, ET_AR and ECE-1, while fulvestrant was without significant effect, indicating endocrine regulation of the ET system in breast cancer cells [14], similar to previous reports in prostate cancer cells [45]. However, in vascular endothelium, many studies have demonstrated the inhibition of ET-1 expression by estrogens [46, 47], a mechanism that may explain the well-known vasoprotective estrogen effects [47]. Fulvestrant in contrast to tamoxifen left gene expression of the ET system unaffected, supporting the perception of fulvestrant as the clinically superior compound. Different from the SERM tamoxifen, fulvestrant is an ER antagonist and ER downregulator without agonistic activity [48]. It is well-known that tamoxifen different from fulvestrant holds a considerable partial agonistic activity (PAA) on the ER [48,49], which easily explains the inducing effect of tamoxifen on the ET system, as the physiological ER agonist E₂ too enhanced gene expression of ET-1, ET_AR and ECE-1 [Fig. 4]. The different extent of ER mediated induction of ET system mRNA expression that is found due to E₂ or tamoxifen incubation again is well-founded in both compounds differing ER binding mechanisms and receptor affinities [50,51]. This differential mechanism of action may explain that tamoxifen only slightly reduced E₂-induced gene expression of the ET system, while this was completely abolished by fulvestrant. Another question addressed was if patients treated with tamoxifen or fulvestrant would benefit from co-medication with ZD4054. Compared to E₂ *plus* tamoxifen, addition of ZD4054 reduced ET-1 expression (MCF-7) and expression of all ET system components (BT-474), respectively. Comparing E₂ *plus* fulvestrant to E₂ *plus* fulvestrant *plus* ZD4054; in the latter, ET system expression was reduced even below control level. In view of the prognostic unfavorable relevance of ET system expression [10], combination of tamoxifen or fulvestrant with ZD4054 also seems to be clinically beneficial. In migration assays, ZD4054 or fulvestrant alone were without any effect. However, combination of ZD4054 with fulvestrant showed a synergistic antimigratory effect. An identical effect was observed when combining anastrozole or letrozole with ZD4054. However, endocrine regulation of breast cancer cell migration seems to be rather weak in different models of migration, too [52]. This is confirmed by our observations demonstrating that endocrine therapeutics (mono) were without significant effect and stimulation with E₂ (1–100nM) left migration of MCF-7 and MCF-7aro unaffected (data not shown).

ZD4054 significantly reduced breast cancer cell invasion. Combination of tamoxifen with ZD4054 reduced invasion to the same extent as did ZD4054 alone; in contrast, combination of fulvestrant with ZD4054 produced a strong, additive anti-invasive effect. Thus, the combination of fulvestrant with ZD4054 represents the most effective approach to inhibit breast cancer cell invasion, reducing the risk of systemic spread of tumor cells.

In this study, ZD4054 for the first time was investigated in a breast cancer model *in vivo*. In ET_AR-positive MDA-MB-468 xenograft tumours, both doses (10 mg/kg and 30 mg/kg) were equipotent in inhibiting tumor growth with tumor volumes on day 30 being significantly smaller than that of controls;

however, in initial tumor stages the higher dosage was found to be superior. For response to treatment with ET_AR antagonists like ZD4054, existent (MDA-MB-468) or absent (DU-4457) ET_AR expression serves as a predictive parameter. The observation that both ZD4054 doses differ in their initial efficacy but are equipotent after day 30 confirms the perception that the ET system plays a major role in early tumor stages and initial tumorigenesis, providing the basis for a potential relevance of ET_AR antagonists in an adjuvant setting in breast cancer patients, which aims at preventing intravasation of primary tumor cells, and inhibiting engraftment of disseminated tumor cells at distant sites [53]. ZD4054 led to a reduction of tumour volumes in MDA-MB-468 xenografts, although it left both ET system expression and cellular proliferation *in vitro* unaffected. Most likely, these anti-tumoral effects *in vivo* may be explained by the fact that MDA-MB-468 xenograft tumors express both ETAR and ETBR; thus, selective ETAR antagonism will entail increased ETBR activation. This means that ETAR effects will be reduced, including diminished activity of the PAI-uPA system [12,54] HIF-1 α [7], phospholipase D [41] and different tumor promoting genes that are transcriptionally regulated by ETAR-induced c-fos/c-jun proto-oncogenes [43,44,55]. Moreover, antagonism at ETAR increases ET-1/ETBR interaction, thereby increasing both ET-1 clearance [56] and pro-apoptotic signalling pathways [57,58]. Taken together, these molecular effects may lead to a inhibition of tumor growth as demonstrated in this study, while leaving ET system expression and cellular proliferation *in vitro* unaffected. Therefore, verification of any specific mechanism explaining the anti-tumoral activity of ZD4054 *in vivo* needs to be subject to further studies. Comparing ZD4054 to fulvestrant *in vivo*, in early tumor stages fulvestrant therapy was superior to ZD4054, however, at the day of termination no significant difference between Fulvestrant, ZD4054 and Fulvestrant *plus* ZD4054 was existent. It is remarkable though, that treatment with ZD4054 was found as effective as the treatment with established fulvestrant. Combination of fulvestrant with ZD4054 was without additive or synergistic effects; however, in the xenograft model used therapeutic efficacy was assessed regarding tumor volumes only. To investigate possible benefits of the combination, future studies facing metastasis or progression-free survival have to be conducted. For the first time, we investigated the combination of ZD4054 with anastrozole in an appropriate postmenopausal breast cancer xenograft model. At the day of termination (day 75), we observed no significant differences between tumor volumes of different treatment groups ZD4054, anastrozole and ZD4054 *plus* anastrozole. Regarding reduction of tumor volumes ZD4054 was as effective as anastrozole, an established endocrine therapy in breast cancer. Nonetheless, in the course of the study differences between the treatment groups were observed: until day 50, tumor volumes in ZD4054-treated animals were smaller than that of the anastrozole group. Compared to anastrozole alone, we observed a clear benefit by combining anastrozole with ZD4054: from day 22 through day 50, tumor volumes of ZD4054 *plus* anastrozole were below that of anastrozole alone. This may indicate an improved efficacy of anastrozole treatment by co-medication with an ET_AR antagonist; the combination ZD4054 *plus* anastrozole seems to be superior to the combination of ZD4054 and fulvestrant.

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FIGURE LEGENDS

Figure 1.

Effect of ZD4054 (1 – 1000 nM) on the ET system expression in breast cancer cells, quantified by real-time PCR. (■) ET-1, (▨) ET_AR, (▩) ECE-1, (▧) NEP. (A) MCF-7, (B) BT-474, (C) MDA-MB-231, (D) MDA-MB-468. *, $P < 0.05$ compared to controls.

Figure 2.

Proliferation of breast cancer cells treated with ZD4054 (1 – 1000 nM). (A) MCF-7, (B) BT-474, (C) MDA-MB-231, (D) MDA-MB-468.

Figure 3.

Effect of ET-1 and ZD4054 on breast cancer cell migration (A) and invasion (B), relative to untreated control. (■) MCF-7, (▨) MDA-MB-468, (▩) MDA-MB-231. *, $P < 0.05$ compared to controls.

Figure 4.

Effect of E₂ and the combination „ZD4054 plus tamoxifen“ (A,B) and “ZD4054 plus fulvestrant” (C,D) on the expression on the ET system expression in breast cancer cells, quantified by real-time PCR. (■) ET-1, (▨) ET_AR, (▩) ECE-1, (▧) NEP. (A) MCF-7, (B) BT-474, (C) MCF-7, (D) BT-474. * (**), $P < 0.05$ ($P < 0.001$) compared to „E₂ + FULV“. +, $P < 0.05$ compared to „E₂“. E₂: 17β-estradiol (10 nM), FULV: Fulvestrant (0.1 μM).

Figure 5.

Effect of the combinations „ZD4054 plus fulvestrant“, “ZD4054 plus aromatase inhibitor“ on breast cancer cell migration (A,B), and of the combinations “ZD4054 plus tamoxifen” and “ZD4054 plus fulvestrant” on MCF-7 breast cancer cell invasion (C). (A) MCF-7 cells; *, $P < 0.05$ compared to untreated control. (B) MCF-7aro cells; *, $P < 0.05$ compared to untreated control. (C) Effect *, $P < 0.05$ compared to untreated control. All assays (A-C) were supplemented with E₂ (10 nM).

Figure 6.

Antitumoral effect of ZD4054 monotherapy (A,B) and effect of the combination of ZD4054 plus established endocrine therapies (C,D). (A) MDA-MB-468 xenograft, (B) DU-4475 xenograft. Mean tumor volumes at the respective measuring point, ± SEM. + (**): $P < 0.05$ ($P < 0.001$) for „ZD4054 (10 mg/kg)“, compared to control. * (**): $P < 0.05$ ($P < 0.001$) for „ZD4054 (30 mg/kg)“, compared to control. (C) ZD4054 plus fulvestrant, MDA-MB-468 xenograft. #, $P < 0.05$ for „ZD4054 10 mg/kg“, compared to control. + (**), $P < 0.05$ ($P < 0.001$) for „fulvestrant (200 mg/kg)“, compared to control. * (**): $P < 0.05$ ($P < 0.001$) for „ZD4054 (10 mg/kg) plus fulvestrant (200 mg/kg)“, compared to control. (D) ZD4054 plus anastrozole, MCF-7-Ac1 xenograft. # (**), $P < 0.05$ ($P < 0.001$) for „ZD4054 10 mg/kg“, compared to control. + (**), $P < 0.05$ ($P < 0.001$) for „anastrozole (0.5 mg/kg)“, compared to

control. * (**): $P < 0.05$ ($P < 0.001$) for „ZD4054 10 mg/kg plus anastrozole (0.5 mg/kg)“, compared to control.

Table I: Summary of ZD4054-mediated effects on breast cancer cell lines

+ = significant effect of ZD4054; - = no effect; n.d. = not determined, * = MCF-7 (aro/Ac) in steroid hormone-dependent experimental settings

	MCF-7 *	BT-474	MDA-MB- 231	MDA-MB- 468	DU-4475
inhibition of gene expression, basal					
ET-1	-	+	+	+	n.d.
ETAR	+	+	+	+	n.d.
ECE-1	+	-	+	+	n.d.
NEP	+	+	n.d.	+	n.d.
inhibition of gene expression, E2 + fulvestrant					
ET-1	+	-	n.d.	n.d.	n.d.
ETAR	+	-	n.d.	n.d.	n.d.
ECE-1	+	-	n.d.	n.d.	n.d.
NEP	+	-	n.d.	n.d.	n.d.
change in gene expression, E2 + tamoxifen					
ET-1	+	+	n.d.	n.d.	n.d.
ETAR	-	+	n.d.	n.d.	n.d.
ECE-1	-	+	n.d.	n.d.	n.d.
NEP	-	+	n.d.	n.d.	n.d.
inhibition of proliferation, basal	-	-	-	-	n.d.
migration, basal	-	n.d.	-	-	n.d.
inhibition of migration, E2 + fulvestrant	+	n.d.	n.d.	n.d.	n.d.
inhibition of migration, E2 + AI	+	n.d.	n.d.	n.d.	n.d.
inhibition of invasion, basal	+	n.d.	+	+	n.d.
ET-1 mediated increase in invasion	+	n.d.	+	+	n.d.
inhibition of invasion, + fulvestrant	+	n.d.	n.d.	n.d.	n.d.
inhibition of invasion, + tamoxifen	+	n.d.	n.d.	n.d.	n.d.
inhibition of in vivo tumor growth, basal	+	n.d.	n.d.	+	-
inhibition of in vivo tumor growth, +	n.d.	n.d.	n.d.	+	n.d.

fulvestrant					
inhibition of in vivo tumor growth, + anastrozole	+	n.d.	n.d.	n.d.	n.d.

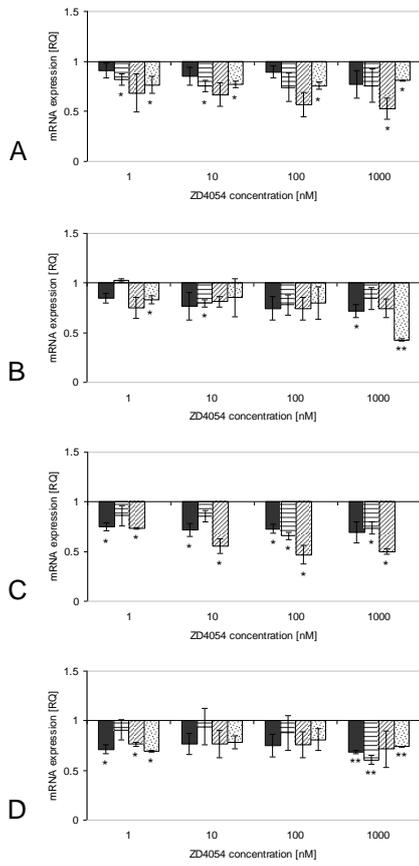


Fig. 1

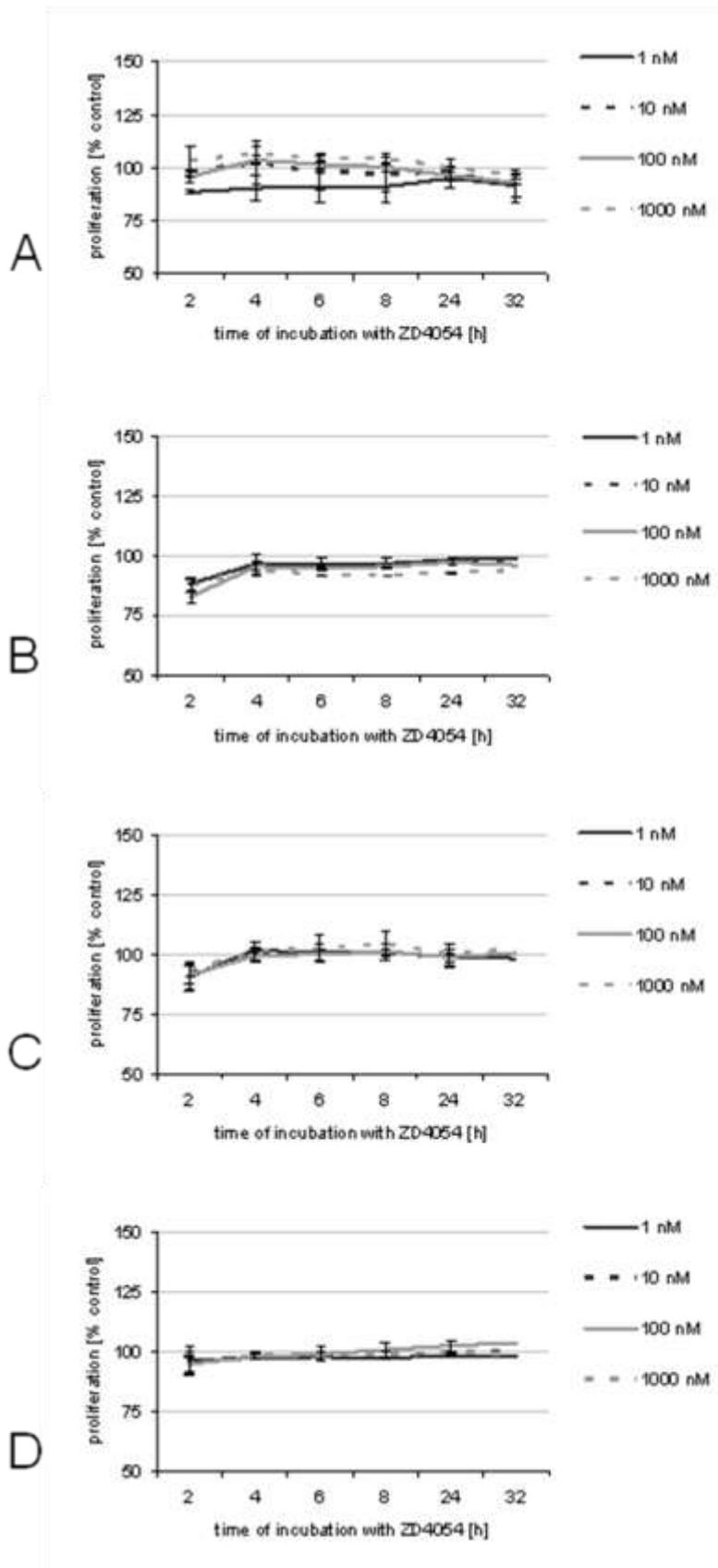


Fig. 2

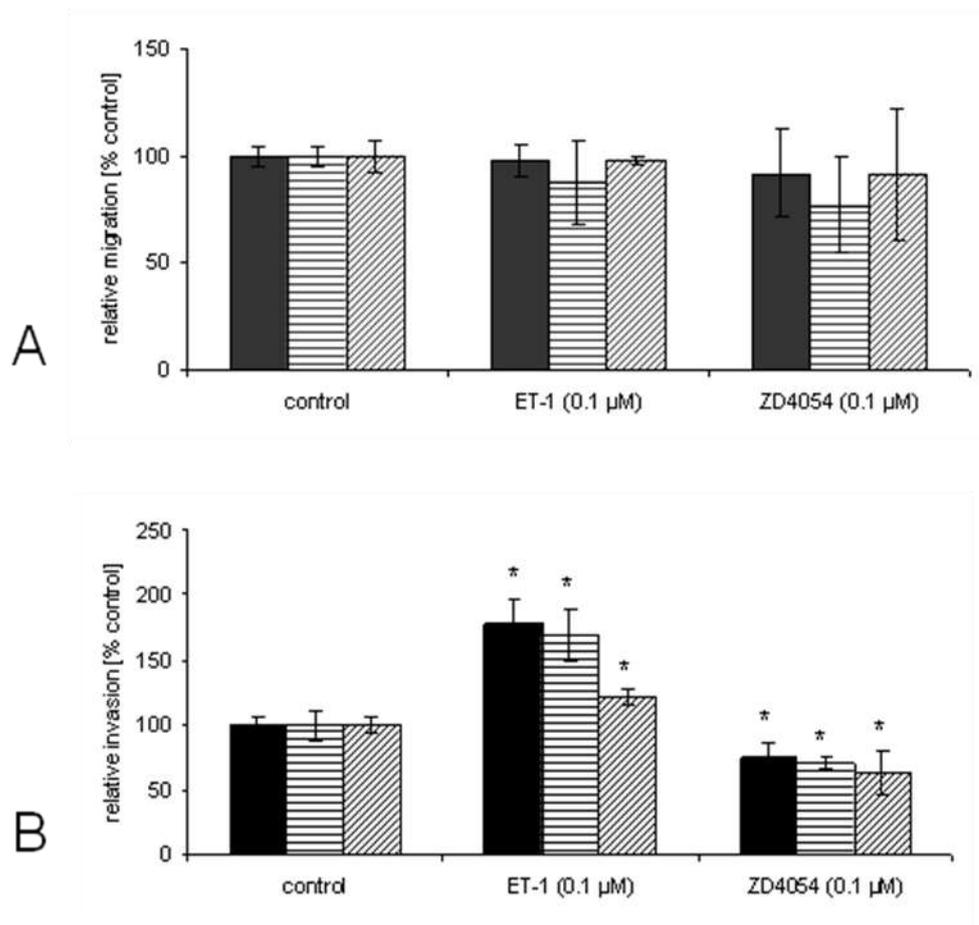


Fig. 3

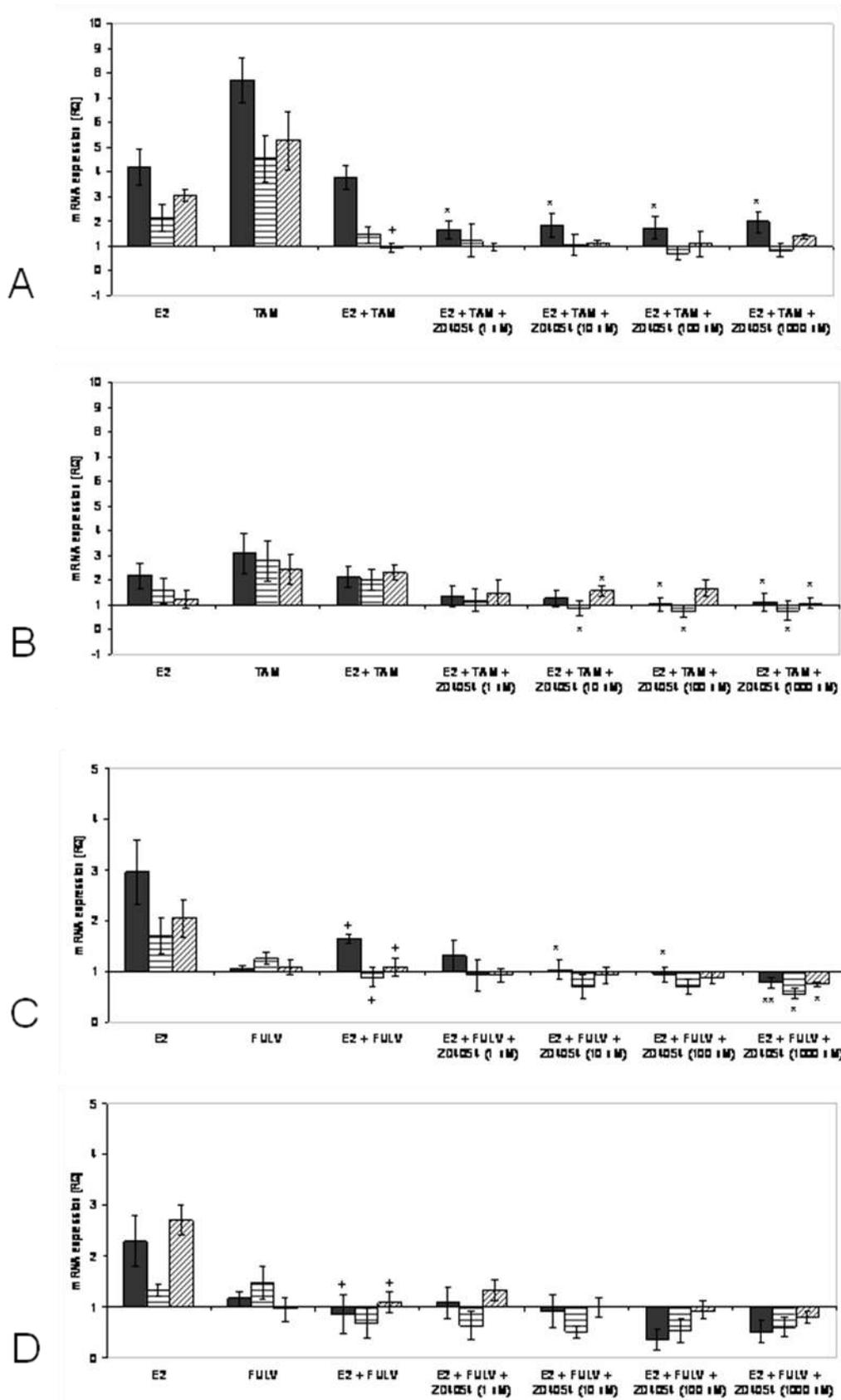


Fig.4

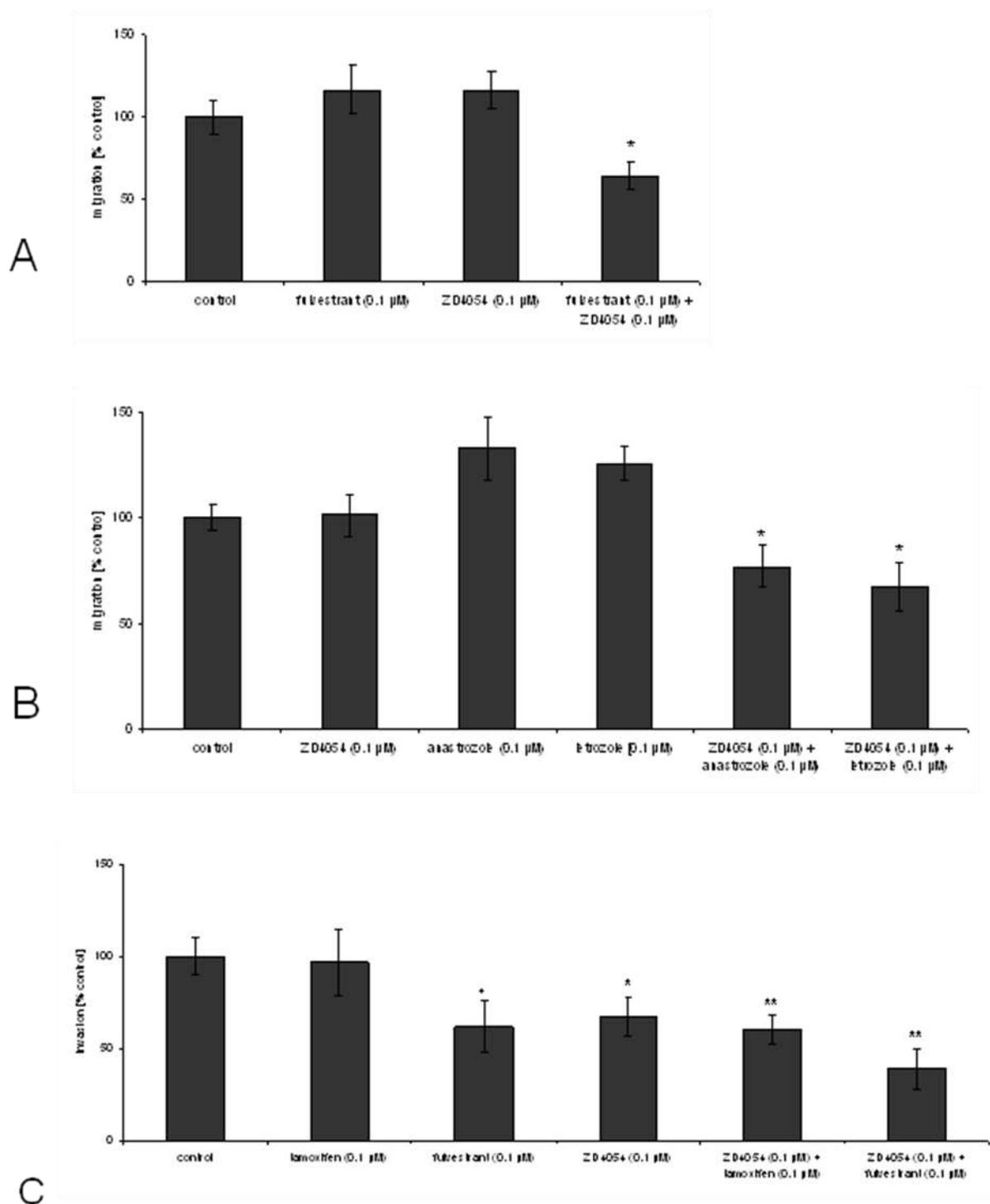


Fig. 5

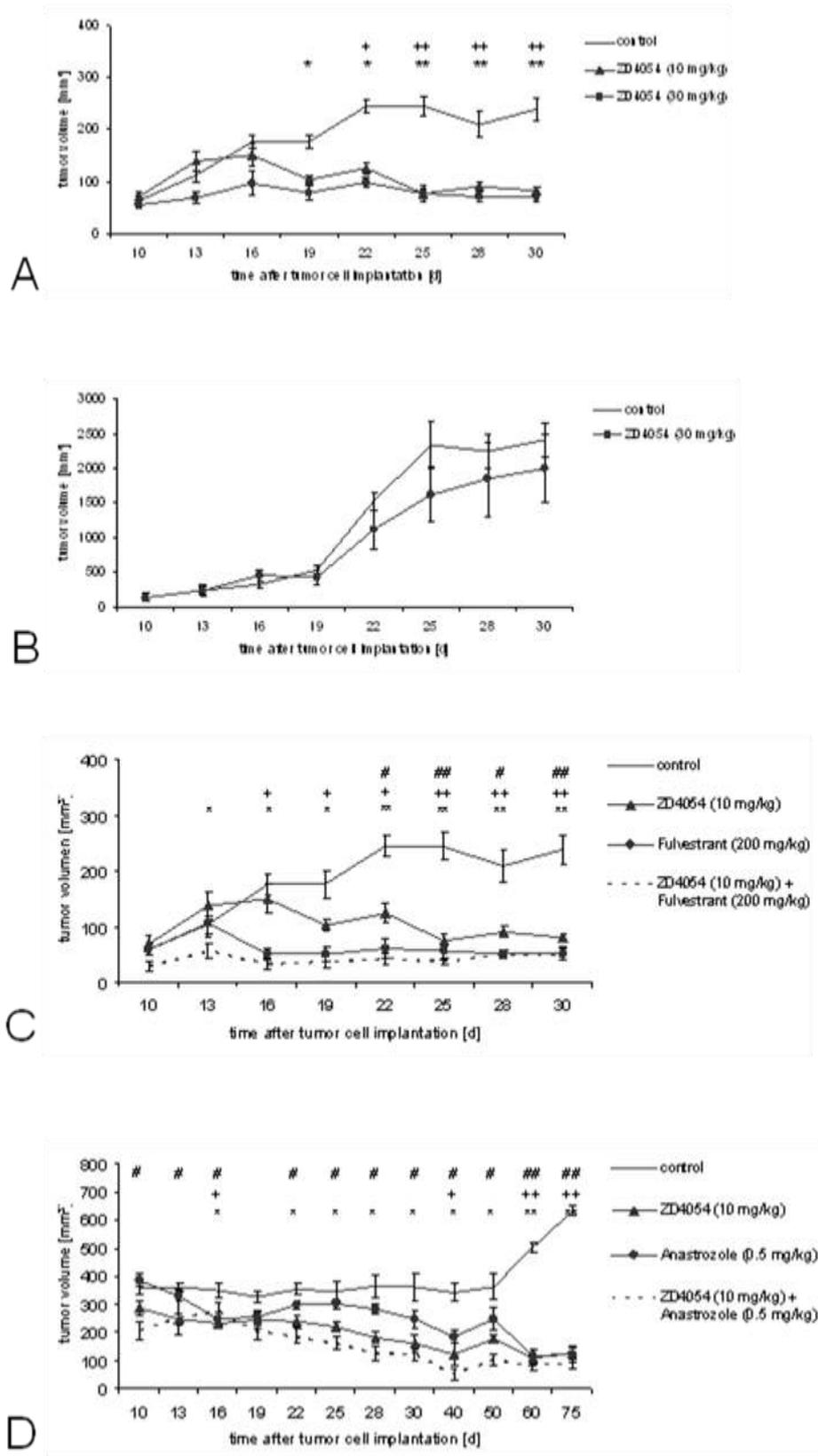


Fig. 6

