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# Endothelin causes transactivation of the EGFR and HER2 in non-small cell lung cancer cells

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## Abstract

Endothelin (ET)-1 is an important peptide in cancer progression stimulating cellular proliferation, tumor angiogenesis and metastasis. ET-1 binds with high affinity to the ETA receptor (R) and $ET_BR$ on cancer cells. High levels of tumor ET-1 and $ET_AR$ are associated with poor survival of lung cancer patients. Here the effects of ET-1 on epidermal growth factor (EGF)R and HER2 transactivation were investigated using non-small cell lung cancer (NSCLC) cells. $ET_AR$ mRNA was present in all 10 NSCLC cell lines examined. Addition of ET-1 to NCI-H838 or H1975 cells increased EGFR, HER2 and ERK tyrosine phosphorylation within 2 min. The increase in EGFR and HER2 transactivation caused by ET-1 addition to NSCLC cells was inhibited by lapatinib (EGFR and HER2 tyrosine kinase inhibitor (TKI)), gefitinib (EGFR TKI), ZD4054 or BQ-123 ($ET_AR$ antagonist), GM6001 (matrix metalloprotease inhibitor), PP2 (Src inhibitor) or Tiron (superoxide scavenger). ET-1 addition to NSCLC cells increased cytosolic $Ca^{2+}$ and reactive oxygen species. ET-1 increased NSCLC clonal growth, whereas BQ123, ZD4054, lapatinib or gefitinib inhibited proliferation. The results indicate that ET-1 may regulate NSCLC cellular proliferation in an EGFR-and HER2-dependent manner.

### Keywords

endothelin; lung cancer; transactivation; EGFR; HER2

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## 1. Introduction

Endothelin (ET) peptides ET-1, ET-2 and ET-3 have 21 amino acids and two disulfide bonds. The structures of ET-2 and ET-3 differ by 2 and 6 amino acids, respectively, from ET-1 [20]. ET-1 is metabolized by neutral endopeptidase from a 212 amino acid prepro-ET-1 to big-ET-1 containing 38 amino acids. Big ET-1 is subsequently metabolized by endothelin converting enzyme (ECE-1) to ET-1 [41]. ET-1 is present in many human cancer cell lines derived breast, colon, lung, prostate and stomach cancer [1, 22, 31] ET-1 is overexpressed in breast, colorectal, lung and ovarian tumors [4, 7]. ET-1 is an important factor in tumor progression stimulating cellular proliferation, tumor angiogenesis and bone metastasis [4, 15, 30].

ET-1 and ET-2 but not ET-3 binds with high affinity to the $ET_AR$ [17]. In contrast, ET-1, ET-2 and ET-3 bind with high affinity to the $ER_BR$ [2]. BQ788 is a selective $ET_BR$ antagonist where BQ123, ZD4054 and atrasentan are selective $ET_AR$ antagonists [5]. The mitogenic effects of ET-1 are mediated by the $ET_AR$ [38]. When ET-1 binds to $ET_AR$, G proteins are activated resulting in stimulation of phospholipase C and D, phospholipase (PL) $A_2$, adenylylcyclase and/or guanylylcyclase [39]. Phospholipase C metabolizes phosphatidylinositol-4,5-diphosphate ($PIP_2$) to diacylglycerol (activates protein kinase C) and inositol-1,4,5-trisphosphate (elevates cytosolic $Ca^{2+}$) leading to Src activation (18]. ET-1 elevates cytosolic $Ca^{2+}$ in lung cancer cells and stimulates their proliferation [43]. ET-1 causes transactivation of receptor tyrosine kinases (RTK) such as the EGFR and PDGF receptor [13, 37, 40, 42]. The RTK activates the Ras, Raf, MEK,ERK pathway allowing phosphorylated ERK to enter the nucleus and increase expression of fos, jun and myc [6].

ET-1 mRNA was detected in 6/7 small cell lung cancer (SCLC) and 4/4 non-SCLC (NSCLC) cell lines [1]. NSCLC, which accounts for approximately 80% of the lung cancer cases is comprised of adenocarcinoma (Ad), large cell carcinoma (LC) and squamous cell carcinoma (Sq). ET-1 immunoreactivity was detected in 24 of 36 NSCLC biopsy specimens but only 2 of 12 SCLC tissues [14]. Similarly, big ET-1 was detected in lung cancer biopsy specimens and plasma [3]. ET-1 expression in lung tumors is associated with poor patient overall survival and decreased disease-free survival [7]. ET-1 may be an autocrine growth factor in lung cancer.

In this communication, the effects of ET-1 were investigated on non-small cell lung cancer (NSCLC) cells. It is unknown if the $ET_AR$ regulates EGFR and HER-2 transactivation in NSCLC. A hypothesis is that ET-1 regulates NSCLC cellular proliferation in an EGFR and HER2-dependent manner.

## 2. Materials and methods

### 2.1 Cell Culture

Human SCLC cell lines (ATCC, Manassas, VA) were cultured as floating aggregates and were split 1:1 weekly in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (FBS Invitrogen, Grand Island, NY). Human NSCLC cells (ATCC, Manassas, VA) were adherent and were treated with trypsin-EDTA after washing with PBS.

After detachment from the flask, the cells were treated with an equal volume of RPMI-1640 with 10% FBS. The cells were centrifuged at 1000 × g for 5 min and resuspended 1:20 in a T175 flask. The cells were mycoplasma free and were used when they were in exponential growth phase after incubation at 37°C in 5%CO2/95% air.

### 2.2 RT-PCR

Total RNA was isolated from frozen pellets of lung cancer cells. Total RNA was prepared using a RNeasy Mini Kit (Qiagen, Valencia, CA). RNA samples were treated with DNase Digestion (Qiagen, Valencia, CA) during preparation to remove contaminating DNA. Total RNA (1 μg) was reversetranscribed using a Super ScriptRM III First-Strand Synthesis SuperMix for the qRT-PCR (Invitrogen, Waltham, MA) according to the manufacturer's instructions for complementary DNA (cDNA) synthesis. One microliter of the RT reaction mix containing cDNA was amplified using EDNRA-S2 and EDNRBS1 PCR in a reaction mixture containing HotStar TaqR Master Mix Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Amplification was performed for the $ET_AR$ using the following: Forward primer is 5′TCTGTATGCCCTTGGTGTGC-3′ and the reverse primer is 5′CCACTTCTCGACGCTGCTTA-3′. For the $ET_BR$, the forward primer is 5′-CTTGCCATTGGCCATCACTG-3′ and the reverse primer is 5′-CCACTTCCCGTCTCTGCTTT-3′. Amplification for all PCR reactions included an initial cycle of 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min. After the final cycle, all PCR reactions were concluded with a 10 min extension at 72°C. The PCR products were analyzed on a 3% agarose gel and visualized by ethidium bromide staining.

### 2.3 Receptor binding

For the radioreceptor assay, NCI-H838 or H1975 cells were placed in 24 well plates. $^{125}$I-ET-1 (2200 Ci/mmol) was prepared using 0.8 μg iodogen in 100 μl of 0.5 M $KH_2PO_4$ (pH 8.0), 10 μg ET-1 and 2 mCi $^{125}$I. After 6 min free $^{125}$I was removed after application to a Sep-Pak and washing with 5 ml of 0.1% trifluoroacetic acid (TFA). $^{125}$I-ET-1 was eluted with 5 ml of 60% acetonitrile in 0.1 %TFA. $^{125}$I-ET-1 was further purified by HPLC using a μBondapak column from Waters Corporation. The purified $^{125}$I-ET-1 was stored in 0.5% BSA at-20°C until use. Confluent cells were washed 3 times in SIT medium (RPMI-1640 containing $3 \times 10^{-8}$ M sodium selenite, 5 μg/ml bovine insulin and 10 μg/mltransferrin (Sigma-Aldrich, St. Louis, MO)). The cells were incubated in SIT buffer containing 0.25% bovine serum albumin and 250 μg/ml bacitracin (Sigma-Aldrich, St. Louis, MO) and $^{125}$I-ET-1 (100,000 cpm) added as well as various concentrations of ET-1, ET-3, ZD-4504, BQ123 or BQ788. After incubation at 37°C, for 30 min, free $^{125}$I-ET-1 was removed by washing 3 times in buffer and the cells which contained bound $^{125}$I-ET-1 dissolved in 0.2 N NaOH and counted in a gamma counter. The $IC_{50}$ was calculated for each unlabeled competitor.

### 2.4 Western Blot

The ability of ET-1 (Bachem Inc., Torrence, CA) to stimulate tyrosine phosphorylation of the EGFR, HER2 or ERK (p42/p44 MAP kinase) was investigated by Western blot. NCI-H838 or NCI-H1975 cells were placed in 10 cm dishes and when confluent they were placed

in SIT media for 3 hr. Routinely, NSCLC cells were treated with ZD4054 (ApexBio, Houston, TX), gefitinib (Tocris Bioscience, Bristol, UK), BQ123 (Tocris Brioscience, Bristol, UK), BQ788 (Tocris Bioscience, Bristol, UK), PP2 (Sigma-Aldrich, St. Louis, MO), GM6001 (Sigma-Aldrich, St. Louis, MO), Tiron (Sigma-Aldrich, St. Louis, MO) or no additions for 30 minutes. Then cells were incubated with 0.1 μM ET-1 for 2 min, washed twice with PBS and lysed in buffer containing 50 mM Tris.HCl (pH 7.5), 150 mM sodium chloride, 1% Triton X-100, 1% deoxycholate, 1% sodium azide, 1 mM ethyleneglycoltetraacetic acid, 0.4 M EDTA, 1.5 μg/ml aprotinin, 1.5 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride and 0.2 mM sodium vanadate (Sigma-Aldrich, St. Louis, MO). The lysate was sonicated for 5 s at 4°C and centrifuged at 10,000 × g for 15 min. Protein concentration was measured using the BCA reagent (Pierce Chemical Co., Rockford, IL), and 400 μg of protein was incubated with 4 μg of anti-phosphotyrosine (PY) monoclonal antibody (BD Biosciences), and 15 μl of immobilized protein G (Pierce Chemical Co., Rockford, IL) overnight at 4°C. The immunoprecipitates were washed 3 times with phosphate buffered saline and analyzed by sodium dodecyl sulfate/ polyacrylamide gel electrophoresis and Western blotting. Immunoprecipitates were fractionated using 4–20% polyacrylamide gels (Novex, San Diego, CA). Proteins were transferred to nitrocellulose membranes as described previously [29]. After washing the blot was incubated with enhanced chemiluminescence detection reagent for 5 min and exposed to Biomax XAR film (Carestream, Rochester, NY). The band intensity was determined using a densitometer.

Alternatively, 20 μg of cellular extract was loaded onto a 15 well 4–20% polyacrylamide gels. After transfer to nitrocellulose, the blot was probed with anti $PY^{1068}$-EGFR, anti EGFR, anti $PY^{204}$ERK, anti ERK, anti $PY^{1244}$-HER2, anti-HER2 or anti-tubulin (Cell Signaling Technologies, Danvers, MA).

### 2.5 Cytosolic $Ca^{2+}$.

NCI-H838 or H1975 cells were treated with trypsin-EDTA and harvested. After centrifugation the cells were resuspended in SIT medium ($2.5 \times 10^6$ cells/ml) containing Fura-2AM (Calbiochem, La Jolla, CA) at 37°C for 30 min [29]. The cells were centrifuged at 1000 × g for 5 min and resuspended at a concentration of $2.5 \times 10^6$/ml and 2 ml placed in a Quartz cuvette containing a stirbar. The excitation ratio was determined at 340 and 380 nm and the emission at 510 nm using a spectrofluorometer equipped with a magnetic stirring mechanism and temperature (37°C) regulated cuvette holder before and after addition of ET-1.

### 2.6 Reactive oxygen species

NCI-H838 cells were placed in black 96 well plates (30,000 cells/well) and cultured overnight. The cells were treated with 10 μM dichlorofluorescein diacetate (H2DCF) for 1 h and washed 3 times with serum-free SIT medium. Some of the cells were treated with 10 μM ZD4054 or 5 mM Tiron for 30 min and then stimuli such as 10 nM ET-1 or 10 μM $H_2O_2$ added. Fluorescence measurements were taken at the various times using an excitation wavelength of 485 nm and emission wavelength of 585 nm.

### 2.7 Proliferation

Growth studies in vitro were conducted using the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO) and clonogenic assays. In the MTT assay, A549, NCI-H727 or H838 cells were placed in SIT medium and various concentrations of ZD4054, lapatinib or trastuzumab (Sigma-Aldrich, St. Louis, MO) added. After 2 days, 15 μl of 0.1 % MTT solution added. After 4 h, 150 μl of dimethylsulfoxide was added and the optical density at 570 nm was determined. In the clonogenic assay, the effects of ET-1, BQ123, BQ788 and gefitinib were investigated on NCI-H838 cells. The bottom layer contained 0.5% agarose in SIT medium containing 5% FBS in 6 well plates. The top layer consisted of 3 ml of SIT medium in 0.3% agarose, ET-1, BQ788, BQ123 and/or gefitinib using $5 \times 10^4$ lung cancer cells. Triplicate wells were plated and after 2 weeks, 1 ml of 0.1% p-iodonitrotetrazolium violet was added and after 16 hours at 37°C, the plates were screened for colony formation; the number of colonies larger than 50 μm in diameter were counted using an Omnicon image analysis system.

### 2.8 Statistical analysis

The results are expressed as means ± S.D. Statistical significance of differences was performed by one-way or two-way repeated measures analysis of variance.

## 3. Results

### 3.1 $ET_AR$ and $ET_BR$ mRNA are present in NSCLC cells

$ET_AR$ and $ET_BR$ were investigated in SCLC and NSCLC cells. Figure 1 shows that $ET_AR$ PCR-products were present in all 10 NSCLC cells tested (NCI-H157 (Sq), H358 (Ad), H460 (LC), H520 (Sq), A549 (Ad), H838 (Ad), H1299 (LC), H1975 (Ad), H1650 (Ad) and SK-LU-1 (Ad) where $ET_BR$ mRNA was present in 8 NSCLC cell lines tested but not H358 or H1975. $ET_AR$ mRNA was present in 5 SCLC cells lines (NCI-H82, H209, H345, N417 and H510) but not H69. $ET_BR$ mRNA was detected only in SCLC cell lines NCI-H82 and H510 cell lines. $ET_AR$ and $ET_BR$ mRNA were detected in 2 lung carcinoid cell lines (NCI-H720 and H727) as well as a mesothelioma cell line NCI-H28. As a control, equal amounts on β-actin mRNA were detected in the cells. Densitometry analysis indicated that $ET_AR$ mRNA was highest in NCIH-358 and H1975, whereas $ET_BR$ mRNA was highest in SK-LU-1. In general, Ad, LC and Sq NSCLC cells were enriched in $ET_AR$ mRNA relative to $ET_BR$ mRNA.

### 3.2 $ET_AR$ binding sites are present in NSCLC cells

The ability of ET-1, ET-3, BQ123, BQ788 and ZD4054 to inhibit specific $^{125}$I-ET-1 binding to NSCLC cell lines was investigated. Figure 2A shows that ET-1 strongly but ET-3 weakly inhibited specific $^{125}$I-ET-1 binding to NCI-H838 cells ($IC_{50}$ = 0.2 and >1000 nM, respectively). Similarly, BQ123 and ZD4054 inhibited specific $^{125}$I-ET-1 binding with high affinity ($IC_{50}$ = 1 and 0.5 nM respectively) whereas BQ788 was less potent ($IC_{50}$ = 20 nM). Table I shows that the order of binding potency for both NCI-H838 and NCI-H1975 cells is ET-1 > ZD4054 = BQ123 > BQ788 > ET-3, gefitinib or lapatinib. The results indicate that ET-1 is primarily binding to $ET_AR$ on NCI-H838 and H1975 cells.

### 3.3 ET-1 increases cytosolic calcium which is blocked by $ET_AR$ antagonists

The ability of ET-1 to increase cytosolic $Ca^{2+}$ was investigated in NSCLC cells. Addition of 10 nM ET-1 to NCI-H1975 cells increased rapidly the cytosolic $Ca^{2+}$ within seconds from 150 to 180 nM (Fig. 2B, I). The cytosolic $Ca^{2+}$ slowly declined and return to baseline by 2 min. Addition of 1000 nM BQ123 did not alter the cytosolic $Ca^{2+}$ but blocked the ability of 10 nM ET-1 to increase $Ca^{2+}$ in NCI-H1975 cells (Fig. 2B, II). The effects of BQ123 were reversible in that addition of 1000 nM ET-1 increased the $Ca^{2+}$ (Fig. 2B, IV). Addition of 1000 nM BQ788 had no effect on cytosolic $Ca^{2+}$ and did not antagonize the ability of 10 nM ET-1 to increase $Ca^{2+}$ (Fig. 2B, III). Similar data were obtained using NCI-H838 cells (T. Moody, unpublished). The results indicate that the $ET_AR$ regulates cytosolic $Ca^{2+}$ in NSCLC cells.

### 3.4 ET-1 causes increases protein tyrosine phosphorylation in NSCLC cells

The ability of ET-1 to cause transactivation of the EGFR was investigated using NSCLC cells. Figure 3A shows that 2 min after addition of 1 nM ET-1 to NCI-838 cells, the EGFR tyrosine phosphorylation was moderately increased whereas 10 nM, 100 nM or 1000 nM ET-1 increased strongly EGFR transactivation. Figure 3B shows that 1 nM ET-1 increased EGFR tyrosine phosphorylation significantly to 149% whereas 10 nM, 100 nM or 1000 nM ET-1 increased EGFR tyrosine phosphorylation significantly to approximately 340%. The half maximal dose of ET-1 to increase EGFR tyrosine phosphorylation (ED50) was 3 nM. As a control, ET-1 had little effect on total EGFR. ET-1 addition to NCI-H838 cells at all doses tested increased tyrosine phosphorylation of ERK (Fig. 3A). ET-1 at a 1 nM dose significantly increased tyrosine phosphorylation of ERK to 161% whereas 10 nM, 100 nM or 1000 nM ET-1 increased significantly ERK tyrosine phosphorylation to approximately 190% (Fig. 3B). As a control, ET-1 had little effect on total ERK. The results indicate that ET-1 addition to NSCLC cells increases EGFR and ERK tyrosine phosphorylation in a dose-dependent manner.

### 3.5 ET-1 causes EGFR and HER2 transactivation

The effects of $ET_AR$ antagonists and TKI were investigated on EGFR and HER2 transactivation. Figure 4A shows that addition of 100 nM ET-1 to NSCLC cells increased significantly tyrosine phosphorylation of the EGFR and HER2. BQ123 in a dose-dependent manner inhibited the EGFR and HER2 transactivation caused by ET-1 addition to NSCLC cells. Figure 4B shows that 100 nM ET-1 increased EGFR and HER2 tyrosine phosphorylation significantly to 360 and 280%, respectively. BQ123 had little effect at 0.1 μM whereas 1 μM or 10 μM BQ123 significantly reduced EGFR and HER2 tyrosine phosphorylation that was regulated by the $ET_AR$. Similarly, ZD4054 inhibited whereas BQ788 had little effect on the ability of ET-1 to increase EGFR or HER2 transactivation (data not shown). Fig. 4C shows that 10 μg/ml but not 1 μg/ml gefitinib significantly reduced the ability of ET-1 to cause EGFR and HER2 transactivation. Fig. 4D shows that ET-1 significantly increased EGFR and HER2 phosphorylation by 355% and 308%, respectively and that 10 μg/ml gefitinib significantly inhibited the increase caused by ET-1. Similarly, lapatinib (EGFR and HER2 TKI) inhibited the ability of the $ET_AR$ to regulate EGFR and HER2 transactivation (data not shown). As a control, ET-1 had no effect on total EGFR or

HER2. The results suggest that the $ET_AR$ regulates the ability of the EGFR to form homodimers with itself or heterodimers with HER2.

### 3.6 Inhibitors of EGFR and HER2 transactivation caused by ET-1

The ability of other agents to impair the $ET_AR$ regulation of EGFR and HER2 tyrosine phosphorylation was investigated. Figure 5A shows that ET-1 addition to NSCLC cells significantly increased EGFR and HER2 phosphorylation which was inhibited by PP2 (Src inhibitor) or GM6001 (MMP inhibitor). Fig. 5B shows that ET-1 addition to NSCLC cells increased EGFR and HER2 phosphorylation significantly to 322 and 288%, respectively, and this increase was inhibited significantly by addition of PP2 or GM6001 to NSCLC cells. Figure 5C shows that ET-1 addition to NSCLC cells increased EGFR and HER2 tyrosine phosphorylation significantly to 348 and 275% significantly and that this increase was inhibited significantly by Tiron, a superoxide scavenger. As a control, PP2, GM6001 or Tiron had no effect on basal P-EGFR, EGFR, P-HER2 or HER2 (data not shown). Table II shows that addition of ET-1 or $H_2O_2$ to NSCLC cells increased reactive oxygen species (ROS). The increase in ROS caused by ET-1 or $H_2O_2$ was significantly inhibited by Tiron. The increase in ROS cause by ET-1, but not $H_2O_2$, was significantly inhibited by ZD4054. The results indicate that the $ET_AR$ regulates ROS produced by ET-1.

### 3.7 Proliferation of NSCLC cells

The ability of $ET_AR$ antagonists and TKI to inhibit the proliferation of NSCLC cells was investigated. Fig. 6A shows that using the MTT assay, lapatinib inhibits the proliferation of NSCLC cells in a dose-dependent manner. For NCI-H727, H838 and A549 cells the $IC_{50}$ for lapatinib was 0.5, 1 and 2 μg/ml, respectively. Trastuzumab inhibited proliferation of NCI-H727, H838 and A549 cells with $IC_{50}$ values of 0.5, 1 and 2 μg/ml (Fig. 6B). For NCI-H727, H838 and A549 cells, the $IC_{50}$ for ZD4054 was 30, 50 and 45 μg/ml, respectively (Fig. 6C). The results indicate that HER2 inhibitors and $ET_AR$ antagonists inhibit the proliferation of NSCLC cells.

Using the clonogenic assay, 10 nM ET-1 increased significantly the clonal growth of NCI-H838 cells to 150% (Table III). In contrast, 1 μM BQ123 but not BQ788 reduced significantly NSCLC colony number in the presence or absence of exogenous ET-1. BQ123 and gefitinib reduced significantly the NSCLC colony number by 22 and 50%, respectively in the absence of exogenous ET-1. Addition of both BQ123 and gefitinib reduced significantly NSCLC colony number to approximately 10%. The results indicate that ET-1 stimulates, whereas BQ123 and gefitinib inhibit NSCLC proliferation.

## 4. Discussion

NSCLC patients are traditionally treated with combination chemotherapy, however, the five year survival rate is only 16% [19]. Approximately 13% of the NSCLC patients have EGFR mutations such as L858R and these patients initially respond to the TKI such as erlotinib or gefitinib [25, 32]. Subsequently NSCLC patients become resistant to erlotinib or gefitinib due to secondary EGFR mutations such as T790M [24]. It is important to increase the sensitivity of TKI in NSCLC patients with wild type EGFR or secondary T790M mutations.

Increased expression of HER2 occurs in approximately 20% of the NSCLC patients [26]. Breast cancer patients with overexpressed HER2 are treated with lapatinib (TKI) or Herceptin [trastuzumab; 16]. Ligands which activate HER2 have not been identified, whereas numerous ligands activate the EGFR such as amphiregulin, EGF, heparin binding EGF (HB-EGF) or TGFα [33]. Previously, we found that when GPCRs were activated, TGFα is released from NSCLC cells [29]. The TGFα activates the EGFR resulting in EGFR homodimers or EGFR-HER2 heterodimers [23].

$ET_AR$ mRNA is present in many lung cancer cells [1]. $ET_AR$ mRNA was significantly elevated in NSCLC biopsy specimens relative to normal lung tissue [7]. ET-1 or $ET_AR$ expression is associated with poor survival of NSCLC patients. In clinical studies, the $ET_AR$ antagonist atrasentan plus paclitaxelcarboplatin was well tolerated, however, efficacy and survival in NSCLC patients was similar to patients treated with chemotherapy alone [9]. Similarly, no differences were observed in overall survival of NSCLC patients treated with zibotentan (ZD4054) or zibotentan plus pemetrexed [10]. In contrast, ZD4054 is synergistic with gefitinib at inhibiting ovarian cancer growth and invasion [36]. Macitentan, a dual $ET_AR$ and $ET_BR$ antagonist, in combination with temozolomide inhibits glioblastoma growth in animal models [21]. The results suggest that $ET_AR$ regulates cancer proliferation and metastasis.

$ET_AR$ mRNA was detected in all NSCLC cell lines examined as well as most SCLC, lung carcinoids and a mesothelioma cell line (Fig. 1). Specific $^{125}$I-ET-1 binding to NSCLC cells was inhibited with high affinity by ET-1 and $ET_AR$ antagonists, but not ET-3 (Fig. 2A). These results suggest the $ET_AR$ predominates over $ET_BR$ in NSCLC cells. ET-1 addition to NSCLC cells increased cytosolic $Ca^{2+}$ from 150 to 180 nM, which was inhibited by BQ123 but not BQ788 (Fig. 2B). Similarly, ET-1 addition to NSCLC cell line SC-A1 increased cytosolic $Ca^{2+}$ by 32 nM and the increase caused by ET-1 was reversed by BQ123, U73122 (PLC inhibitor) and nifedipine [$Ca^{2+}$ channel blocker; 43]. These results demonstrate that the $ET_AR$ in NSCLC regulates PI turnover and the resulting $IP_3$ causes increased cytosolic $Ca^{2+}$.

In both normal and malignant cells, the $ET_AR$ regulates EGFR transactivation [13, 40]. Activation of the $ET_AR$ increases release of EGFR ligands such as HB-EGF [33]. The increase in EGFR transactivation caused by ET-1 addition to ovarian cancer cells is blocked by ZD4054 ($ET_AR$ antagonist) or gefitinib [TKI, 36]. Addition of ET-1 to NSCLC cells increased EGFR and HER2 transactivation which was inhibited by BQ123 or gefitinib (Fig. 4). ET-1 signaling stimulates ovarian cancer cellular proliferation via the scaffold protein β-arrestin [35]. β-Arrestin regulates Wnt signaling resulting in nuclear accumulation of β-catenin where it promotes histone deacetylase dissociation and the recruitment of p300 acetyltransferase [34]. This alters the expression of numerous genes such as ET-1, axin 2, MMP2 and cyclin D1. ET-1 signaling is impaired by siRNA to β-arrestin1 or the mixed $ET_AR/ET_BR$ antagonist macitentan [21]. It remains to be determined if ET-1 stimulates lung cancer proliferation in a β-arrestindependent manner.

EGFR transactivation caused by addition of ET-1 is dependent on generation of ROS, which may reduce essential disulfide bonds impairing phosphotyrosine. phosphatase activity [8].

Treatment of NSCLC cells with Nacetylcysteine (antioxidant) or Tiron (superoxide scavenger) inhibited the ability of ET-1 to cause EGFR or HER-2 transactivation.

ET-1 addition to ovarian cancer cells increased EGFR transactivation and ovarian cancer cellular proliferation which was inhibited by ZD4054 or gefitinib [36]. Gefitinib plus ZD4054 significantly increased apoptosis of ovarian cancer cells relative to gefitinib or ZD4054 alone. Treatment of ovarian cancer cells with ZD4054 or siRNA to the $ET_AR$ significantly increased E-cadherin expression, suggesting inhibition of the $ET_AR$ impairs epithelial to mesenchymal transitions [37]. Gefitinib and ZD4054 are synergistic at inhibiting ovarian cancer proliferation, invasion and VEGF production. BQ123 or gefitinib inhibited significantly NSCLC proliferation, whereas ET-1 stimulated the growth of NSCLC cells (Table III). Because BQ788 had little effect on NSCLC growth, the $ET_AR$ is important in NSCLC proliferation. Furthermore, the growth inhibitory effects of ZD4054 plus gefitinib are greater than either agent alone. It remains to be determined if gefitinib and $ET_AR$ antagonists synergistically inhibit NSCLC growth in vivo.

A surprising finding is that addition of ET-1 to NSCLC cells causes transactivation of HER2. The transactivation of the EGFR and HER2 caused by ET-1 addition to NSCLC cells is blocked by BQ123 ($ET_AR$ antagonist), gefitinib (EGFR TKI), PP2 (Src inhibitor), GM6001 MMP inhibitor) and Tiron (superoxide scavenger). The results suggest that ET-1 addition to lung cancer cells causes the formation of EGFR homodimers and EGFR/HER2 heterodimers. Preliminary data indicate that HER2 and EGFR mRNA is present in all NSCLC cell lines examined (P. Moreno, unpublished). HER2 is overexpressed in 20% and mutated in 2% of the lung cancer specimens examined [11]. Treatment of lung cancer patients who have HER2 mutations with trastuzumab resulted in a 51% overall response rate [27]. Afatinib, a second generation TKI, inhibits the growth of lung Ad patients with HER2 mutations [12]. It remains to be determined if $ET_AR$ antagonists or miRNA to ET-1 or the $ET_AR$ potentiate the ability of trastuzumab to inhibit lung cancer growth.

In summary, ET-1 addition to NSCLC cells increases EGFR, ERK and HER2 tyrosine phosphorylation as a result of activation of the $ET_AR$. ET-1 addition to NSCLC cells increases ROS leading to increased proliferation. The proliferation of NSCLC cells is inhibited by gefitinib or lapatinib (TKI) as well as BQ123 or ZD4054 ($ET_AR$ antagonist). The results suggest that ET-1 may stimulate NSCLC proliferation as a result of the formation of EGFR homodimers or EGFR-HER2 heterodimers.

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## Highlights

- ET-1 addition to NSCLC cells causes tyrosine phosphorylation of the EGFR, HER2 and ERK.
- ET-1 binds with high affinity to NSCLC and causes elevated cytosolic $Ca^{2+}$ which is blocked by the $ET_AR$ antagonists BQ123 and ZD4054.
- The ability of ET-1 to cause EGFR and HER2 transactivation is blocked by $ET_AR$ antagonists and gefitinib, a tyrosine kinase inhibitor.
- ET-1 stimulates the growth of NSCLC cells, whereas $ET_AR$ antagonists and gefitinib inhibit NSCLC proliferation.

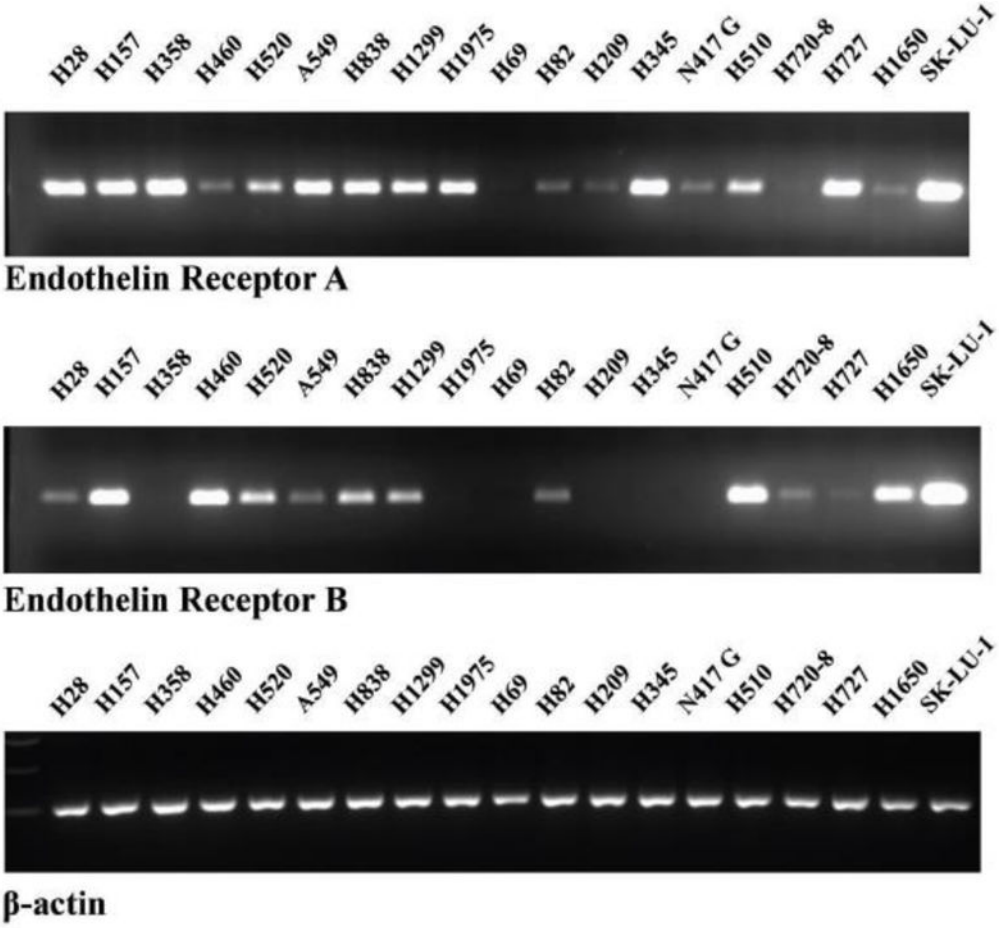


**Fig. 1.**

$ET_AR$ and $ET_BR$ mRNA. RNA was isolated from frozen pellets of lung cancer cells and cDNA prepared. PCR products were analyzed on a 3% agarose gel and visualized by ethidium bromide staining. The product size for the $ET_AR$, $ET_BR$ and β-actin control was 123, 116 and 205, respectively. This experiment is representative of 3 others.

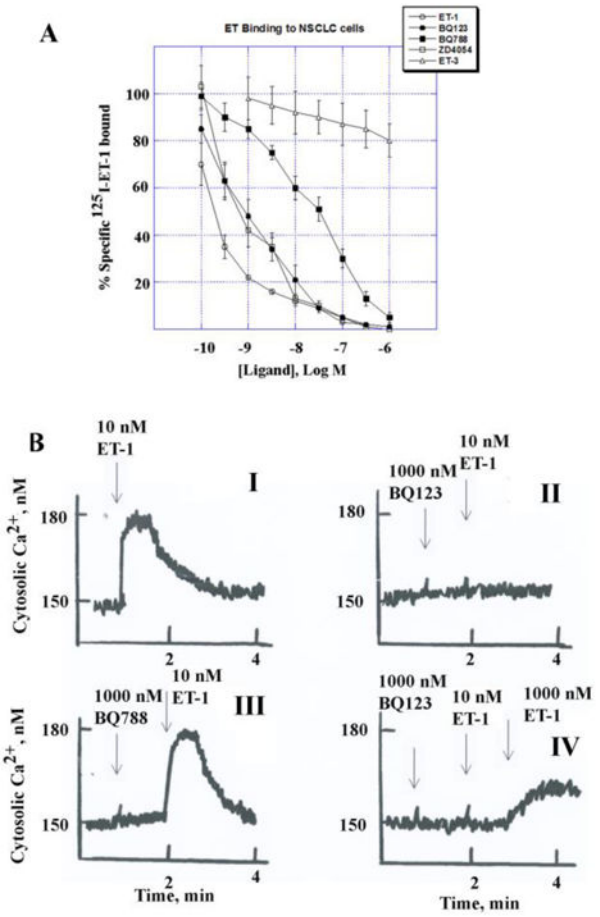


**Fig. 2.**

Binding and cytosolic $Ca^{2+}$. (A) Specific $^{125}$I-ET-1 binding to NCI-H838 cells for 30 min was inhibited by increasing doses of unlabeled ET-1(○), BQ123 (•), BQ788 (■), ZD4054 (□) and ET-3 (A). The mean value ± S.D. of 3 determinations each repeated in duplicate is indicated. (B) The ability of ET-1 to alter cytosolic $Ca^{2+}$ was investigated in Fura-2AM loaded NCI-H1975 cells. (I) ET-1, 10 nM, increased transiently the $Ca^{2+}$ from 150 to 180 nM. The increased caused by ET-1 was antagonized by 1000 nM BQ123 (II) but not 1000 nM BQ788 (III). The block caused by BQ123 was reversed by 1000 nM but not 10 nM ET-1 (IV). This experiment is representative of 3 others.

Sequence homologies of ET-1 and ET-3 are underlined and shown below.

ET-1 CSCSSLMDKECVYFCHLDIIW

ET-3 CTCFTYKDKECVYYCHLDIIW

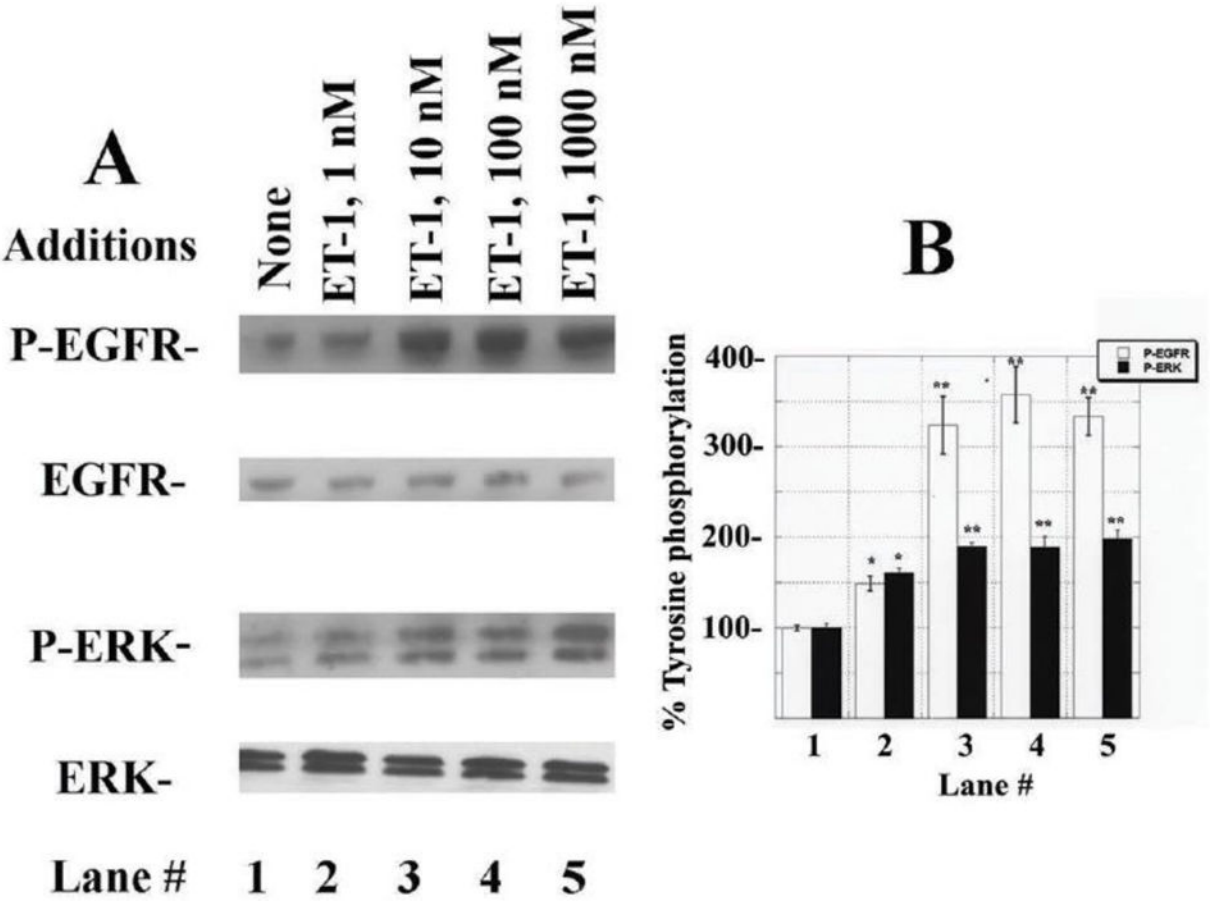


**Fig. 3.**

EGFR and ERK phosphorylation. (A) Addition of 1 nM ET-1 to NCI-H838 cells for 2 min moderately increased EGFR and ERK tyrosine phosphorylation, whereas 10, 100 or 1000 nM ET-1 strongly increased EGFR and ERK phosphorylation. (B) The mean % EGFR (□) and ERK (■) tyrosine phosphorylation ± S.D. is indicated for 3 determinations as a function of ET-1 concentration; $p < 0.05$, $^{*}$; $p < 0.01$, $^{**}$ relative to control by ANOVA.

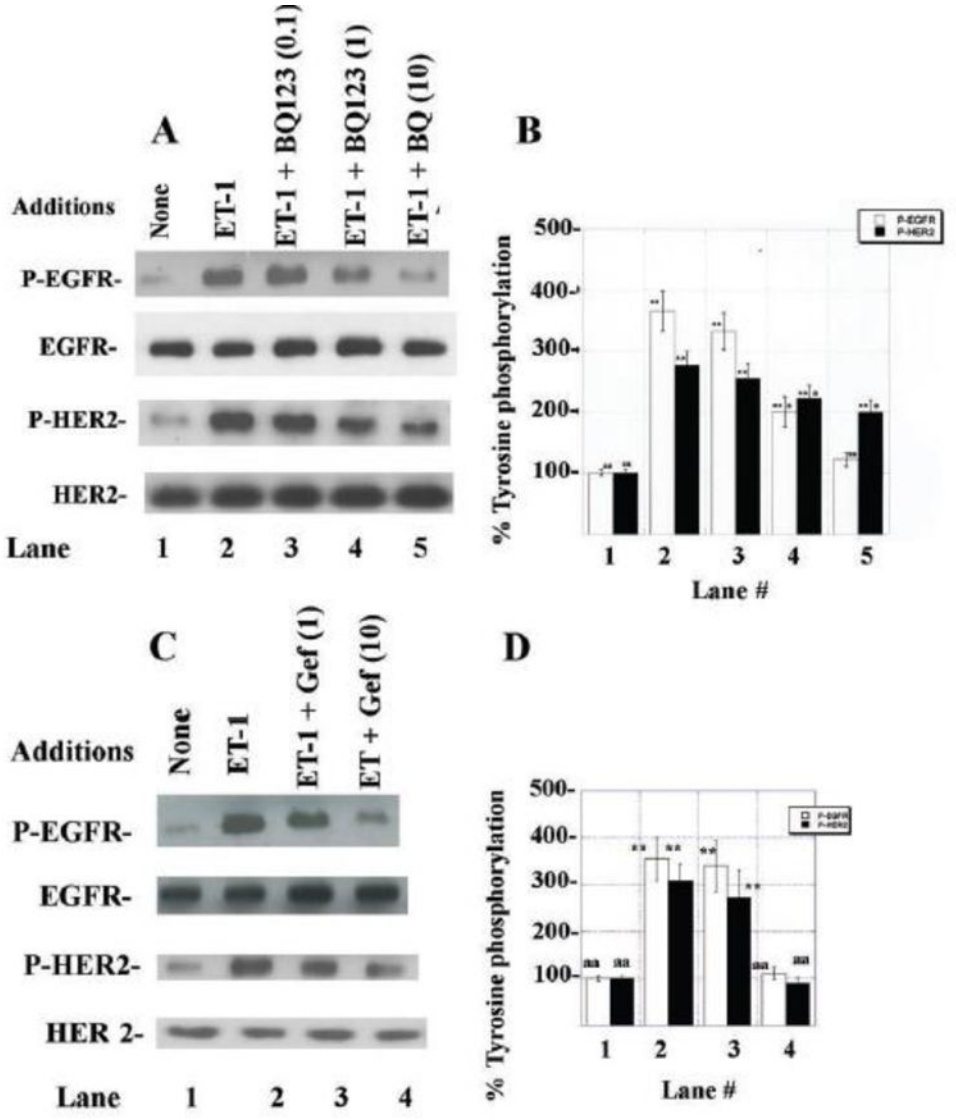


**Fig. 4.**

EGFR and HER2 tyrosine phoshorylation. (A) ET-1 (100 nM) was added to NCI-H838 cells for 2 min in the presence or absence on increasing doses of BQ123. (B) The mean % EGFR (□) and HER2 (■) tyrosine phosphorylation ± S.D. of 3 determinations are indicated. (C) ET-1 (100 nM) was added to NSCLC cells in the presence or absence on increasing doses of gefitinib. (D) The mean % EGFR (□) and HER2 (■) tyrosine phosphorylation ± S.D. of 3 determinations is indicated; $p < 0.05$, $^{*}$; $p < 0.01$, $^{**}$ relative to control; $p < 0.05$, $^{a}$; $p < 0.01$, $^{aa}$ relative to ET-1; by ANOVA.

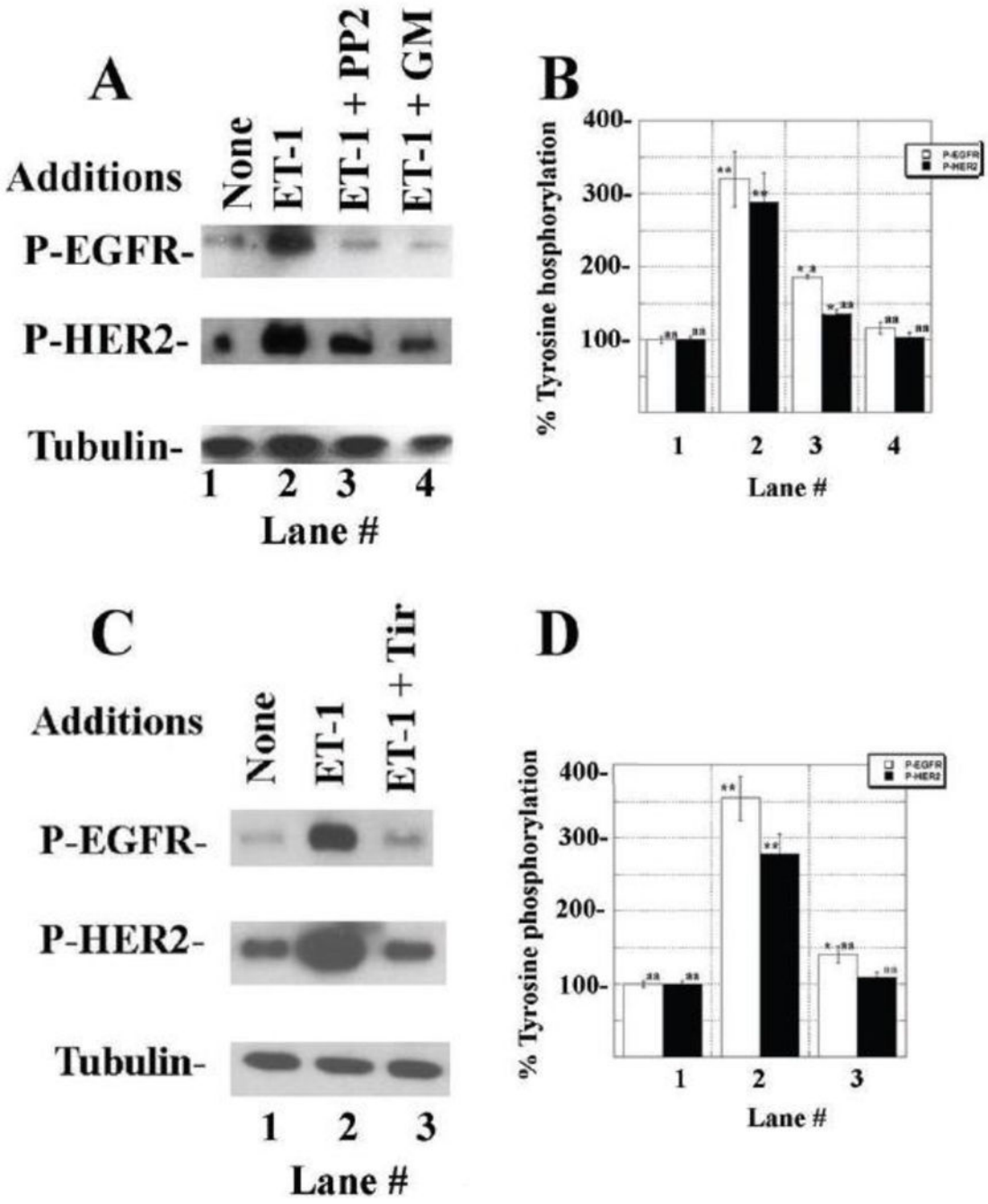


**Fig. 5.**

Effect of inhibitors on EGFR and HER2 transactivation. (A) PP2 (10 μM) and GM6001 (10 μM) inhibit the ability of ET-1 (100 nM) to increase EGFR and HER2 tyrosine phosphorylation in NCI-H838 cells after 2 min. (B) The mean % EGFR (□) and HER2 (■) tyrosine phosphorylation ± S.D. of 3 determinations is indicated. (C) Tiron (5 mM) inhibits the ability of ET-1 to increase EGFR and HER2 tyrosine phosphorylation in NCI-H1975 cells. (D) The mean % EGFR (□) and HER2 (■) tyrosine phosphorylation ± S.D. is

indicated; $p < 0.05$, *; $p < 0.01$, ** relative to control; $p < 0.05$, [a]; $p < 0.01$, [aa] relative to ET-1; by ANOVA.

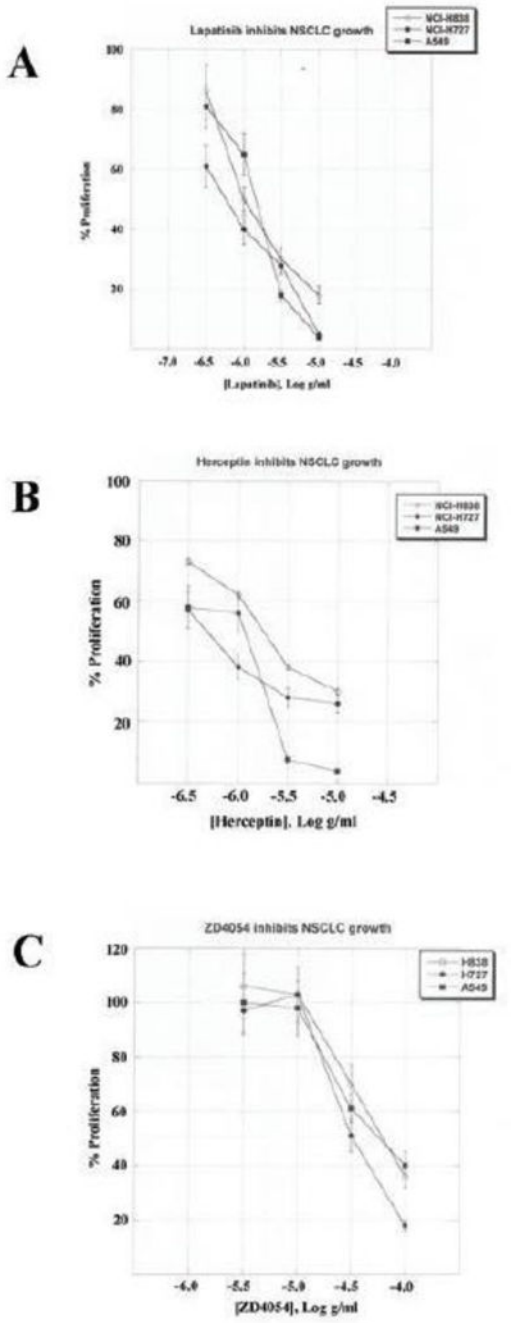


**Fig. 6.**

NSCLC proliferation. Using the MTT assay the growth of A549 (■), H727 (•) and H838 (○) cells is indicated as a function of trastuzumab, lapatinib and ZD-4054 concentration. The mean value ± S.D. of 8 determinations is indicated. This experiment is representative of 2 others.

## Table I

Binding of ligands

| Addition | IC50, nM | |
|---|---|---|
| | **NCI-H838** | **NCI-H1975** |
| ET-1 | 0.2 ±0.03 | 0.4 ± 0.1 |
| ZD4054 | 0.5 ± 0.1 | 0.7 ± 0.2 |
| BQ123 | 1 ± 0.2 | 1.5 ± 0.03 |
| BQ788 | 20 ± 2 | 25 ± 3 |
| ET-3 | >10,000 | >10,000 |
| Gefitinib | > 10,000 | >10,000 |
| Lapatinib | >10,000 | >10,000 |

Binding of $^{125}$I-ET-1 to NCI-H838 and H1975 cells was determined as a function of ligand concentration. The mean $IC_{50}$ ± S.D. of 3 determinations is indicated.

## Table II

Reactive oxygen species

| Addition | % Fluorescence intensity |
|---|---|
| None | 100 ± 5 *aa,bb* |
| ET-1, 100 nM | 162 ± 23*bb* |
| ET-1 + Tiron, 5 mM | 113 ± 8*aa,bb* |
| ET-1 + ZD4054, 10 μM | 116 ± 9*aa,bb* |
| $H_2O_2$, 10 μM | 401 ± 37*aa* |
| $H_2O_2$ + Tir | 171 ± 12*bb* |
| $H_2O_2$ +ZD4054 | 351 ± 22*aa* |
| Tir | 99 ± 7*aa,bb* |
| ZD4054 | 105 ± 6*aa,bb* |

The relative fluorescence was determined 0.5 hr after the addition of ET-1 or H2O2 to H2DCF labeled NCI-H838 cells. Tiron or ZD4054 was added 0.5 h before the addition of ET-1 or $H_2O_2$. The mean value ± S.D. of 8 determinations is indicated. This experiment is representative of 3 others;

*aa* < 0.01 relative to ET-1;

*bb* p <0.01 relative to $H_2O_2$ by ANOVA.

## Table III

Colony number.

| Addition | Colony number |
|---|---|
| None | 32 ± 3[a] |
| BQ123, 1000 nM | 25 ± 2[*aa] |
| BQ788, 1000 nM | 35 ± 4[a] |
| Gefitinib, 1 ug/ml | 16 ± 2[**aa] |
| BQ123 + Gefitinib | 3 ± 1[**aa] |
| ET-1, 10 nM | 48 ± 5[*] |
| ET-1 + BQ123 | 37 ± 2[a] |
| ET-1 + BQ788 | 46 ± 4[*] |
| ET-1 + Gefitinib | 32 ± 3[aa] |
| ET-1 + BQ123 + Gefitinib | 15 ± 1[*aa] |

The mean number of NCI-H838 colonies ± S.D. of 3 determinations is indicated. This experiment is representative of 2 others; $p < 0.05$,

[*] $p < 0.01$,

[**] relative to control; $p < 0.05$,

[a] $p < 0.01$,

[aa] relative to ET-1 by ANOVA.