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# SR48692 inhibits non-small cell lung cancer proliferation in an EGF receptor-dependent manner

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ABSTRACT

*Aims:* The mechanism by which SR48692 inhibits non-small cell lung cancer (NSCLC) proliferation was investigated.
*Main methods:* The ability of SR48692 to inhibit the proliferation of NSCLC cell lines NCI-H1299 and A549 was investigated in vitro in the presence or absence of neurotensin (NTS). The ability of NTS to cause epidermal growth factor receptor (EGFR) transactivation was investigated by Western blot using NSCLC cells and various inhibitors. The growth effects and Western blot results were determined in cell lines treated with siRNA for NTSR1.
*Key findings:* Treatment of A549 or NCI-H1299 cells with siRNA for NTSR1 reduced significantly NTSR1 protein and the ability of SR48692 to inhibit the proliferation of A549 or NCI-H1299 NSCLC cells. Treatment of A549 and NCI-H1299 cells with siRNA for NTSR1 reduced the ability of NTS to cause epidermal growth factor receptor (EGFR) transactivation. SR48692 or gefitinib (EGFR tyrosine kinase inhibitor) inhibited the ability of NTS to cause EGFR and ERK tyrosine phosphorylation. NTS transactivation of the EGFR was inhibited by GM6001 (matrix metalloprotease inhibitor), Tiron (superoxide scavenger) or U73122 (phospholipase C inhibitor) but not H89 (PKA inhibitor). NTS stimulates whereas SR48692 or gefitinib inhibits the clonal growth of NSCLC cells.
*Significance:* These results suggest that SR48692 may inhibit NSCLC proliferation in an EGFR-dependent mechanism.
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## Introduction

Neurotensin (NTS) (Carraway and Leeman, 1973) has potent growth effects in normal and neoplastic tissues (Evers, 2006). NTS is medullary thyroid carcinoma (Zeytinoglu et al., 1995) and small cell lung cancer (SCLC) cells (Moody et al., 1985). NTS is secreted from SCLC cells and binds with high affinity (Moody et al., 2003). The action of NTS is mediated by NTSR1 and NTSR2 as well as NTSR3, which has a single transmembrane domain and binds sortolin with high affinity (Betancur et al., 1998). SR48692 is a non-peptide NTSR1 antagonist (Gulley et al., 1993) which inhibits the proliferation of pancreatic, prostate and SCLC cells in vitro and in vivo (Moody et al., 2001; Valerie et al., 2011; Wang et al., 2011).

NTSR1 activation causes phosphatidylinositol (PI) turnover in a phospholipase C dependent manner (Dupouy et al., 2011). The inositol-1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG) released elevation of cytosolic $Ca^{2+}$ (Staley et al., 1989) and activate protein kinase (PK)C, respectively (Muller et al., 2011). The activation of ERK and PKD is dependent upon PKC activity (Guha et al., 2002; Kisfalvi et al., 2005). NTS activates Akt and NF-κB pathways leading to increased cellular survival (Hassan et al., 2004; Zhao et al., 2003) and inactivates glycogen synthase kinase leading to increased cyclin D1 expression (Wang et al., 2006). NTS causes tyrosine phosphorylation of focal adhesion kinase (FAK) (Leyton et al., 2002) and Src (Lee et al., 2001). NTS causes epidermal growth factor (EGF)R and ERK tyrosine phosphorylation in prostate cancer cells (Hassan et al., 2004). The results indicate that NTS causes tyrosine phosphorylation of numerous proteins (Servotte et al., 2006; Heakal et al., 2011).

The NTSR1 is present in several types of cancer. Reubi et al. (1999) found a high density of specific ($^{125}$I–Tyr$^3$)NTS binding sites in Ewing's sarcoma and medullary thyroid cancers. In non-small cell lung cancer (NSCLC), NTS and NTSR1 immunoreactivity are present in approximately 60% of lung adenocarcinoma biopsy specimens (Alfano et al., 2010). Patients with high NTSR1 had significantly lowers relapse-free survival than those with reduced NTSR1 levels. Similarly, high NTSR1 expression is associated with poor prognosis of patients with ductal breast cancer as well as head and neck squamous carcinomas (Dupouy et al., 2009; Shimizu et al., 2008). Treatment of mice containing NSCLC or colon cancer xenografts with the NTSR1 antagonist SR48692 reduced tumor growth (Moody et al., 2001; Maoret et al., 1999). These results suggest that NTSR1 may regulate the proliferation of numerous cancers.

The mechanism by which SR48692 inhibits NSCLC proliferation was investigated. Addition of siRNA to the NSCLC cells decreased significantly NTSR1 protein and decreased NTS transactivation of the EGFR and the ability of SR48692 to inhibit proliferation. The ability of NTS to cause EGFR tyrosine phosphorylation was inhibited by SR48692, gefitinib

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(EGFR TKI), GM6001 (matrix metalloprotease inhibitor), Tiron (superoxide scavenger) and U73122 (phospholipase C inhibitor). NTS stimulated, but gefitinib or SR48692 inhibited the clonal growth of NCI-H1299 cells. These results indicate that SR48692 inhibits the growth of NSCLC cells in an EGFR dependent mechanism.

## Main methods

### *Cell culture*

NSCLC NCI-H1299 or A549 cells, which contain NTSR1 and wild type EGFR, were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% heat-inactivated fetal bovine serum (Invitrogen, Grand Island, NY). The cells were split weekly 1/20 with trypsin-ethylenediaminotetraacetic acid (EDTA). The cells were mycoplasma-free and were used when they were in exponential growth phase after incubation at 37 °C in 5% $CO_2$/95% air.

### *Receptor binding*

NCI-H1299 cells were plated in 24 well plates. When the cells were confluent, they were rinsed 2 times with PBS and placed in PBS containing 0.1% bovine serum albumin (BSA) and 100 ug/ml bacitracin. ($^{125}$I–Tyr$^3$)NTS (0.1 nM) was added in the presence or absence of NTS analogs (NTS, NTS$^{8-13}$, Ac-NTS$^{8-13}$ and NT$^{1-8}$) (Bachem, Torrence CA). After 30 min at 37 °C, the cells were washed 3 times in PBS containing 0.1% BSA. The cells containing bound radiolabeled NTS were counted in a LKB gamma counter.

### *Western blot*

The ability of NTS to stimulate tyrosine phosphorylation of EGFR was investigated by Western blotting. NCI-H1299 or A549 cells were cultured in 10 cm dishes. When a monolayer of cells formed they were placed in SIT media for 3 h. Routinely, NSCLC cells were pre-treated with SR48692, gefitinib (Tocris Bioscience, Ellisville, MO), GM6001, Tiron or U73122 (Sigma-Aldrich, St. Louis, MO) for 30 min. Then cells were treated with 100 nM NTS 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 (Thermo Scientific, Rockford, IL), and 600 μg of protein was incubated with 4 μg of anti-phosphotyrosine (PY) monoclonal antibody, 4 μg of goat anti-mouse immunoglobulin IgG and 30 μl of immobilized protein G (Thermo Scientific, 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 and the membranes were blocked overnight at 4 °C using blotto [5% non-fat dried milk in solution containing 50 mM Tris/HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM sodium chloride, 0.05% Tween 20 and 0.02% sodium azide (Sigma-Aldrich, St. Louis, MO)] and incubated for 16 h at 4 °C with 1 μg/ml anti-EGFR antibody (Cell Signaling Technologies, Danvers, MA) followed by anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (Upstate Biotechnologies, Lake Placid, NY). The membrane was washed for 10 min with blotto and twice for 10 min with washing solution (50 mM Tris/HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM sodium chloride, 0.05% Tween 20 and 0.02% sodium azide (Sigma-Aldrich, St. Louis, MO)). The blot was incubated with enhanced chemiluminescence detection reagent for 5 min and exposed to Kodak XAR film. The intensity of the bands 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-ERK, anti-ERK, anti-PY$^{402}$PYK2, anti-PY$^{416}$Src, anti-PY$^{654}$β-catenin or anti-tubulin (Cell Signaling Technologies, Danvers, MA).

### *siRNA*

A549 and NCI-H1299 cells were cultured in 6 well plates. When the cells were confluent, they were washed in 1 ml of siRNA transfection medium followed by addition of 20 pmol NTSR1 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA.) in 100 μl of siRNA transfection medium. After 6 h at 37 °C, 1 ml of RPMI-1640 with 10% fetal bovine serum was added. After 72 h, the cells were washed in PBS and the protein lysate prepared for Western blot. The Western blots were probed with NTSR1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

### *Reactive oxygen species*

NCI-H1299 or A549 cells were placed in 96 well plates (30,000 cells/well) and cultured overnight. The cells were treated with 10 μM dichlorofluoresceindiacetate for 1 h and washed 3 times with serum free SIT medium. Some of the cells were treated with 5 mM Tiron for 30 min and then stimuli such as 0.1 μM NTS 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.

### *Proliferation assays*

In the MTT assay, A549 or NCI-H1299 cells were placed in 96 well plates ($10^5$ cells/well) in SIT media (100 μl). Varying concentrations of SR48692 (Tocris Biosciences, Ellisville MO) were added. After 3 days, 15 μl of MTT (1 mg/ml) was added. The following day 150 μl of DMSO was added and the absorbance determined at 540 nm using an ELISA reader.

In the clonogenic assay, NCI-H1299 cells were treated with 10 nM NTS in the presence or absence of SR48692 and/or gefitinib. The bottom layer contained 0.5% agarose in SIT medium containing 5% FBS in 6 well plates in 2 ml. The top layer consisted of 2 ml of SIT medium in 0.3% agarose (Lonzo, Rockford, ME), $5 \times 10^4$ NCI-H1299 cells, SR48692 and/or gefitinib. Triplicate wells were plated and after 2 weeks, 1 ml of 0.1% p-iodonitrotetrazolium violet (Sigma-Aldirch, St. Louis, MO) was added and after 16 h 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.

### *TGFα ELISA*

NCI-H1299 cells in 24 well plates were washed in 0.25 ml of SIT media. The cells were incubated with inhibitors such as 10 μM GM6001 for 30 min, followed by addition of 100 nM NTS for 5 min. The supernatant was collected and assayed for TGFα by ELISA (R & D; Minneapolis, MN).

## Results

### *NTSR1 knock-down and effects of SR48692 on NSCLC cells*

NTSR1 was investigated in NSCLC cells by Western blot and receptor binding. Table 1 shows that specific $^{125}$I–Tyr$^3$-NTS binding to NCI-H1299 cells was inhibited with high affinity by NTS, NTS$^{8-13}$, Ac-NTS$^{8-13}$ but not NTS$^{1-8}$ with $IC_{50}$ values of 4, 10, 7 and >2000 nM, respectively. Also, SR48692 inhibited specific binding of radiolabelled NTS to NCI-H1299 cells ($IC_{50}$ value of 205 nM), whereas levocabastine, a NTSR2 agonist,