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A possible new target in lung-cancer cells: The orphan receptor, bombesin receptor subtype-3

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Abstract

Human bombesin receptors, GRPR and NMBR, are two of the most frequently overexpressed G-protein-coupled-receptors by lung-cancers. Recently, GRPR/NMBR are receiving considerable attention because they act as growth factor receptors often in an autocrine manner in different lung-cancers, affect tumor angiogenesis, their inhibition increases the cytotoxic potency of tyrosine-kinase inhibitors reducing lung-cancer cellular resistance/survival and their overexpression can be used for sensitive tumor localization as well as to target cytotoxic agents to the cancer. The orphan BRS-3-receptor, because of homology is classified as a bombesin receptor but has received little attention, despite the fact that it is also reported in a number of studies in lung-cancer cells and has growth effects in these cells. To address its potential importance, in this study, we examined the frequency/relative quantitative expression of human BRS-3 compared to GRPR/NMBR and the effects of its activation on cell-signaling/growth in 13 different human lung-cancer cell-lines. Our results showed that BRS-3 receptor is expressed in 92% of the cell-lines and that it is functional in these cells, because its activation stimulates phospholipase-C with breakdown of phosphoinositides and changes in cytosolic calcium, stimulates ERK/MAPK and stimulates cell growth by EGFR transactivation in some, but not all, the lung-cancer cell-lines. These results suggest that human BRS-3, similar to GRPR/NMBR, is frequently ectopically-expressed by lung-cancer cells in which, it is functional, affecting cell signaling/growth. These results suggest that similar to GRPR/NMBR, BRS-3 should receive increased attention as possible approach for the development of novel treatments and/or diagnosis in lung-cancer.

Keywords

BRS-3; Bombesin related peptides; Lung-cancer cells; Bombesin; Gastrin-releasing peptide; Neuromedin B

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Conflicts of interest

The authors have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.Org/10.1016/j.peptides.2018.01.016.

1. Introduction

Lung-cancer is a leading cause of global cancer death, responsible for 1.52 million deaths in 2012 [1]. Despite recent advances in treating lung-cancer, the 5-year survival rate of patients remains approximately 16% [40]. Therefore, the development of new therapeutic approaches is needed to increase the survival rates.

Human bombesin-receptor subtype-3 (BRS-3) is an orphan G-protein-coupled-receptor proposed to be classified in the bombesin-receptor (BnR) family, because of its high homology with the established human-BnRs, sharing with the gastrin-releasing peptide receptor (GRPR) 51% and with the neuromedin B receptor (NMBR), 47% amino-acid identities [24]. BRS-3 is widely distributed in the CNS, GI tract, pancreatic islets and other peripheral tissues, as well as in some tumors including pancreatic, ovarian, prostate and lung-cancer [24,40,49,52,58,70]. BRS-3, is reported, primarly in receptor-knockout studies, to be potentially involved in a wide number of physiological/pathophysiological processes such as energy metabolism [15,24], glucose homeostasis [24,30], lung-cancer metastasis formation [23], obesity and diabetes [18,51]. Because BRS-3 is an orphan-receptor whose natural ligand has not been described and, despite its sequence similarity to the other BnRs, it does not bind any natural bombesin-peptide (Bn) with high-affinity [24,30], the exploration of BRS-3's role in physiological/pathophysiological processes using selective ligands has been limited. Recently, a selective-agonist, MK-5046, and antagonist, Bantag-1, have been described providing pharmacological tools to study BRS-3's role in physiological/pathophysiological processes [40,44,49].

The established BnRs, GRPR and NMBR, are recently receiving increased attention because they are one of the G-protein-coupled receptor families most frequently overexpressed in many common tumors including lung-cancer[24]. It is reported that GRPR and NMBR are not only overexpressed in 25–100% of different types of lung-cancer, but have important growth effects on lung-cancer, often acting in an autocrine-manner [24,27,37,41,45,52,61]. In addition, activation of GRPR/NMBR enhance survival of lung-cancer cells exposed to EGFR tyrosine-kinase inhibitor (TKI) anti-cancer therapies such a gefitinib [27,37]. GRPR/NMBR-antagonists are being explored as novel therapies because they suppress lung-cancer angiogenesis [47], decrease growth and increase cellular death [27,35], as well as improve the cytotoxic potency of TKIs (gefitinib/erlotinib), either in EGFR-mutant or in EGFR-wild-type lung-cancers [27,35,37,41]. Furthermore, use of the GRPR/NMBR-overexpression is receiving increased attention to either image lung-cancers, or as a possible means to target cytotoxicity therapy to lung-cancer, as is being used in other cancers [45,49].

However, little it is known about BRS-3 in lung-cancer compared with GRPR/NMBR. A few studies report the frequent presence of BRS-3-mRNA in different types of lung-cancer [10,13,14,55,61,64]. However, quantitative comparisons with GRPR/NMBR or studies using more sensitive quantitative PCR methods have not been performed. In a few lung-cancer cell-lines, BRS-3 activates cell signaling cascades including phospholipase A, C and D activation, cytosolic-Ca^{2+} mobilization and ERK/MAPK, as well as stimulates growth via EGFR-transactivation by activation of matrix-metalloproteinases and generation of reactive oxygen species [38,43,44,49,55,71]. Furthermore, BRS-3-activation stimulates metastasis

formation and drug resistance in small-cell-lung-cancer (SCLC) [23]. These effects suggest that, similar to the established BnRs, GRPR/NMBR, the BRS-3-receptor could be playing an important role in lung-cancer pathophysiological processes. This could open novel approaches for lung-cancer treatment and diagnosis. To attempt to provide more detailed information in this area, we have investigated the presence of human BRS-3 in different lung-cancer cell-lines and compared expression to other BnR's, as well as explored the affect of BRS-3 activation on lung-cancer signaling and growth in a number of different lung-cancer cell lines.

Our results demonstrate that human BRS-3, similar to GRPR/NMBR, is ectopically-expressed by almost all lung-cancer cell lines, frequently with equal or higher expression than GRPR/NMBR. In these lung-cancer cells, BRS-3 is functional, affecting cell signaling and growth, in most cases, by transactivation of the EGFR. These results, combined with the recent availability of selective BRS-3 agonists/antagonists [18,31,49], suggest that similar to GRPR/NMBR [45,49], BRS-3 should receive increased attention as possible approach for the development of novel treatments and/or diagnosis in lung-cancer.

2. Materials and methods

2.1. Materials

All cell lines were obtained from the American Type Culture Collection (Rockville, MD), which uses cytochrome-C oxidase I gene analysis and the short tandem-repeat analysis to authenticate these celllines; Dulbecco's minimum-essential medium (DMEM), Roswell Park Institute medium 1× (RPMI 1640), Ham's F–12 K (Kaighn's) medium (F-K12), phosphate-buffered saline (PBS), fetal bovine serum (FBS), Dulbecco's phosphate buffer saline (DPBS), trypsin-EDTA 1×, penicillin/streptomycin, Novex®4-20% Tris-Glycine gel, and GENETICIN selective antibiotic (G418 Sulfate) were from Invitrogen/Gibco (Carlsbad, CA); gastrin-releasing peptide (GRP) and neuromedin B (NMB) were from Bachem (Torrance, CA); MK-5046[(2S)-1, 1, 1-trifluoro-2-[4- (1H-pyrazol-1-yl)phenyl]-3- (4-[[1-(trifluoromethyl)cyclopropyl]methyl]-1H-imidazol-2-yl)propan-2-ol] and Bantag-1 [Boc-Phe-His-4-amino-5-cyclohexyl-2, 4, 5-trideoxypentonyl-Leu- (3-dimethylamino)benzyl amide-N-methyl ammonium trifluoroacetate] were gifts from Merck, Sharp and Dohme (West Point, PA); [D-Tyr^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]Bn- (6–14) (peptide #1) and [D-Phe^{6}]Bombesin-(6–13)-methyl ester (ME) were gifts from D.H. Coy (New Orleans, LA); myo-[2-^{3}H]-Inositol 5 mCi (185MBq) was obtained from Perkin Elmer (Boston, MA); gefitinib (Tocris Bioscience, Bristol, UK), AG 1-X8 resin and 10×-Tris/Glycine/SDS was from Bio-Rad (Richmond, CA): bacitracin, sodium vanadate, triton X-100, deoxycholate, Tween®20, phenylmethylsulfonyl fluoride (PMSF), ethylene glycol tetra-acetic acid (EGTA), ethylene-diamine tetra-acetic acid (EDTA), sodium azide, RCH80267, U-73122hydrate, fat-free BSA, methanol and PD168368 were from Sigma-Aldrich (St. Louis, MO); monoclonal rabbit anti-α/β-tubulin, rabbit polyclonal EGF receptor (EGFR)-antibody, rabbit polyclonal anti-phosphorylated forms p44/42-MAP-Kinase (Thr202/Tyr204) and EGFR were from Cell Signaling Technology (Beverly, MA); purified mouse anti-phosphotyrosine was from BD Transduction Laboratories (San Jose, CA); goat anti-rabbit IgG (H + L) secondary antibody-horseradish peroxidase (HRP)-conjugated, protein-G

agarose, Supersignal Western Pico/Dura extended/Femto were obtained from Thermo Fisher Scientific (Rockford, IL); non-fat dry milk was from American Bio-analytical (Natick, MA); protease inhibitor tablets were from Roche (Basel, Switzerland); bovine serum-albumin fraction V (BSA) and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) were obtained from ICN Pharmaceutical Inc. (Aurora, OH). Human lung Total RNA were Zyagen (San Diego, CA) and human bronchial epithelial cell Total RNA was from ScienCell (Carlsbad, CA).

2.2. Cell culture

Balb-3T3-cells transfected with human GRPR (GRPR/Balb), human NMBR (NMBR/Balb) and human BRS-3-receptor (BRS-3/Balb), as well as NCI-H1299-cells transfected to over-express human BRS-3-receptor (BRS-3/H1299), were prepared and used as described previously [5–7,33,65]. GRPR/Balb, NMBR/Balb and BRS-3/Balb-cells were grown in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 300 mg/L G418 sulfate. BRS-3/H1299 were cultured in RPMI1640 containing 10% FBS, 1% penicillin/streptomycin and 300 mg/L G418 sulfate. NCI-H720-cells were grown in 50% DMEM and 50% F–12K mediums containing 5% FBS and 1% penicillin/streptomycin. A549 cells were cultured in F–12 K containing 10% FBS and 1% penicillin/streptomycin. NCI-H358, NCI-H460, NCI-H520, NCI-H838, NCI-H727, NCI-H69, NCI-H82, NCI-N417, NCI-H345 and NCI-H510 cells were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin. All the cells were incubated at 37 °C in a 5% CO_2/95% air.

2.3 Bombesin receptor (BnR) mRNA expression: reverse transcription and real time quantitative PCR

mRNA expression of BRS-3 receptor, GRPR and NMBR were initially assessed by the polymerase chain reaction (PCR), and then quantitated using quantitative-real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated from 3×10^6cells following the manufacturer's instructions with the RNeasy Mini-Kit (Qiagen, Valencia, CA, USA). RNA-samples were treated with DNase Digestion (Qiagen, Valencia, CA, USA) to avoid possible DNA contamination. Total RNA (1 μg) was reverse-transcribed into cDNA using a SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Waltham, MA) per the manufacturer's instructions. cDNA from RNA isolated, was amplified using the HotStarTaq® Master Mix Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Amplification conditions for PCR-reactions included an initial cycle of 95 °C for 15 min, followed by 35-cycles of denaturation at 94 °C for 30s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. After the final-cycle, all PCR-reactions concluded with a 10 min extension at 72 °C. The amplification conditions for qRT-PCR-reactions included an initial-cycle of 95 °C for 10 min, followed by 40-cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 30s. After the final-cycle, qRT-PCR-reactions concluded with a final-cycle with 3 steps of 95 °C for 1 min, 55 °C for 30 s and 95 °C for 30s. The primers used in both, PCR and qRT-PCR, were: for BRS-3, BRS-3-F (120): 5′-CAGAAGGATGGCTGTTCGGA-3′ and BRS-3-R (120): 5′-CAACTGCCTTGTATCTGTCAGC-3′; for GRP-R, GRPR-F (113): 5′-TACCTCTGTTGGGGTGTCTGT −3′ and GRPR-R (113): 5′-GGCAGATCTTCATCAGGGCAT −3′; for NMB-R, NMBR-F (131): 5′-

TATTTCCTCATACCACTTGC-3′; and NMBR-R (131): 5′-CGTTTCCGTGTTTCCATCTGT-3′; and for β-actin, human- β-actin-F (205): 5′-CCTCGCCTTTGCCGATCC-3′ and human-b-actin-R (205): 5;-GGAATCCTTCTGACCCATGC-3′. All primer sets were verified to yield only the product of the appropriate size, and no product was seen with genomic DNA or when RT was omitted. Furthermore, during each PCR, the following controls were included: water only, buffer only, no RT samples, melting curves reviewed and samples were examined on agarose gels to exclude the presence of other products or dimer interactions. To be certain that each of the primer pairs had comparable efficiencies serial dilutions up to 10^{10} fold were made of the GRPR, BRS-3 and NMBR Balb 3 cDNAs to determine their efficiencies. In each case the efficiencies were in the 90–100% range. To compare the sensitivities under similar PCT conditions of the nonquantitative PCT and qRT-PCR, serial dilutions of BRS-3 Balb 3 cDNA were made and analyzed. For the non-quantitative PCR, the PCR-products were analyzed on a 3% agarose gel, visualized by ethidium bromide staining and the intensity of the bands was measured using the Gene tools of Synegene program. qRT-PCR, PCR was performed using FastStrat Universal SYBR Green Master (ROX) (Roche, Indianapolis, IN). For each sample, gene expression was normalized with the housekeeping gene, β-actin, and expression-levels compared by the $2^{-\Delta\Delta Ct}$ method. The average of each BnR mRNA-levels obtained in each cell tested was expressed as fold higher/lower than the mRNA-levels of the NSCLC-cell-line NCI-H358, which was considered as 1, because all 3 BnR subtypes were well-expressed in this cell on qRT-PCR. The experiments were performed 3-times with each sample in triplicate. In both, PCR and qRT-PCR, total RNA from BRS-3/Balb, GRPR/Balb and NMBR/Balb was used as a positive control of BRS-3, GRPR and NMBR, respectively.

Primer design: The cDNA-sequences of human- β-actin and human-BnRs, BRS-3, GRPR and NMBR, were obtained from GenBank (accession number of BRS-3; NM_001727.1; GRPR: NM_005314.2; NMBR: NM_002511.2) and the primers were designed using the Primer-BLAST.

2.4. Measurement of [^{3}H]-inositol phosphates ([^{3}H]IP)

[^{3}H]IP was determined as described previously [4,5,7,53,65]. Briefly, all adherent cells (NCI-H358, NCI-H460, NCI-H520, BRS-3-Balb-3T3, BRS-3-NCI-H1299, NCI-H727) were subcultured into 24-well-plates (5×10^5cells/ml) in regular propagation media and then incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. Then, the cells were incubated with 3μCi/ml of myo-[2-^{3}H] inositol in growth media supplemented with 2% FBS for an additional 24 h. After the incubation, the 24-well-plates were washed by incubating for 30 min, 37 °C with 1 ml/well of PBS (pH 7.0) containing 20 mM lithium chloride. The wash-buffer was aspirated and replaced with 500 μl of IP-assay-buffer containing 135 mM sodium chloride, 20 mM HEPES (pH 7.4), 2 mM calcium chloride, 1.2 mM magnesium sulfate, 1 mM EGTA, 20 mM lithium chloride, 11.1 mM glucose, 0.05% BSA (w/v) and incubated without (control) or with different peptides used for 60 min, at 37 °C. Cells which grew in suspension (NCI-H720, NCI-H69, NCI-H82, NCI-N417, NCI-H510), were centrifuged to removed RPMI-medium and incubated (2×10^6cell/ml) directly with myo-[2-^{3}H] inositol in RPMI with 2% FBS for 24 h [55,57]. The cells were then centrifuged to remove the myo-[2-^{3}H]-inositol, washed with wash-buffer containing 20 mM LiCl for 10 min, at 4 °C,

recentrifuged to removed wash-buffer and distributed to 5 ml tubes where they were incubated without (control) or with peptides in 500 μl of IP-assay buffer for 60 min at 37 °C. In both types of cells, the incubation was terminated by the addition of 1 ml of ice-cold 1% (v/v) hydrochloric acid in methanol. Total [^{3}H]IP was isolated by anion exchange chromatography as described previously [53,54,65]. Samples were loaded onto Dowex AG1-X8 anion exchange-resin columns, washed with 5 ml of distilled water to remove free [^{3}H]-inositol, then washed with 2 ml of 5 mM disodium-tetraborate/60 mM sodium formate solution to remove [^{3}H] glycerophosphoryl-inositol and after this 2 ml of 1 mM ammonium formate/100 mM formic acid solution was added to elute total-[^{3}H] IP. Each eluate was mixed with scintillation cocktail and measured for radioactivity in a scintillation β-counter.

2.5. Cytosolic Ca^{2+}

Cells with adherent growth were treated with trypsin-EDTA and harvested (2.5×10^6cells/ml). After centrifugation, the cells were resuspended in SIT-medium (RPMI-1640 containing 3×10^{-8}M sodium selenite, 5 μg/ml insulin and 10 μg/ml transferrin) [40,54,55] containing 1 μMFura-2 AM (Calbiochem, La Jolla, CA) at 37 °C for 30 min. Subsequently, the cells were centrifuged at 1000×g for 5 min, resuspended and 2 ml placed in a Quartz cuvette. The excitation-ratio was determined at 340- and 380-nm, and the emission at 510 nm using a QuantaMastera™400-Photon Technology International spectro-fluorometer. Cells which grew in suspension were centrifuged to remove the growth-medium, washed with PBS, centrifuged again, resuspended and incubated in SIT-medium ($3–4 \times 10^6$cells/ml) containing Fura-2AM at 37 °C for 30 min.

2.6 Western blotting

The ability of MK-546 to stimulate tyrosine phosphorylation of MAPK/ERK and EGFR was studied by Western blotting as previously described [43,44]. Briefly, all cells were washed with PBS and incubated with starvation-medium (DMEM or RPMI without FBS) for 3 h (BRS-3/Balb, NCI-H520, NCI-H720, NCI-H82) or 24 h for the rest of the cells, at 37 °C in a 5% CO2/95% air. After incubation, cells were treated with MK-5046 for 3 min and the reaction was stopped at 4 °C. In the case of inhibition studies, cells were treated with 20 nM of gefitinib for 30 min prior to stimulation for 3 min with MK-5046. Then, the cells were washed with PBS and lysed with lysis-buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium azide, 1 mM EGTA, 0.4 mM EDTA, 1 mM DTT, 0.4 mM sodium orthovanadate, 1 mM PMSF and 1 protease inhibitor tablet per 10 ml. Each lysate was sonicated for 5 s and after 5 min at 4 °C, centrifuged at 12, 200 rpm for 20 min at 4 °C; protein concentration was measured using the Bio-Rad protein-assay reagent (Hercules, CA). To investigate the phosphorylation of MAPK/ERK, 20 μg of protein from each cell lysate was loaded onto SDS-PAGE using 4–20% Tris-Glycine gels. To study EGFR-phosphorylation, 600 μg of protein was incubated in 200 μl of lysis-buffer with 4 μg of purified mouse anti-phosphotyrosine monoclonal-antibody and 30 μl of immobilized protein-G agarose, overnight, at 4 °C. The precipitates were washed 3-times with phosphate-buffered saline and the inmunoprecipitates loaded onto SDS-PAGE using 4–20% Tris-Glycine gels. For both studies, after electrophoresis, proteins were transferred to nitrocellulose membranes for 2 h. The membranes were then washed twice with washing-buffer (TBS plus 0.5% Tween®20) and incubated with primary antibody anti-P-p42/44

(Thr202/Tyr204) or anti-EGFR at 1:1000 dilution in washing-buffer + 5% BSA overnight at 4 °C, under constant agitation. After this incubation, membranes were washed twice in blocking-buffer (TBS plus 0.1% Tween® 20, 5% non-fat dry milk) for 5 min and then, incubated with HRP-conjugated secondary antibody (anti-rabbit) for 1 h at room temperature under constant agitation. Membranes were washed again twice in blocking-buffer for 5 min, and twice in washing-buffer 0.1% Tween®20 for 5 min. The membranes then, were incubated with chemiluminescence-detection reagents for 4 min and finally were exposed to G: BOX Chemi XX6 of Syngene (Frederick, MD). The intensity of the protein bands was measured using the Gene tools of Synegene program.

2.7. [^{3}H]-Thymidine-uptake

Briefly, [^{3}H]-thymidine uptake was performed as described previously [11,39]. Cells with adherent growth were plated (5×10^5cells/well) in 24-well-plates in their specific growth-medium (DMEM or RPMI containing 10% FBS and 1% penicillin/streptomycin). After 24 h, cells were washed with FBS and incubated for 24 h more in serum-free-medium (without FBS) with either MK-5046, gefitinib or both. Cells with suspension growth were directly incubated with serum-free-medium with MK-5046 added for 24 h. Six hours before the end of incubation, 1 μCi/ml of [^{3}H]-Thymidine was added to each well. Then cells were washed 2-times with 1 ml of ice-cold PBS and incubated with 1 ml of ice-cold 5% TCA for 30 min at 4 °C. TCA was aspirated and cells were washed with iced-cold PBS. Finally, cells were resuspended in 0.5 ml of 0.5N NaOH/0.5% SDS and placed in a scintillation vial and counted in a scintillation β-counter.

2.8. Statistical analysis

All results are expressed as a mean ± S.E.M from at least 5-experiments. Results were considered significant if they differed from control by $p < 0.05$ using Anova (nonparametric) or the unpaired- or paired-t-Test when performed with a specifically matched paired-control. Prism 6.0 was used for all statistical analysis.

3. Results

3.1. Expression of bn-receptor subtypes in human lung-cancer-cells

BRS-3, GRPR and NMBR expression was initially assessed by PCR using specific primers in 13 different lung-cancer cell-lines: 6 NSCLC-cells, 5 SCLC-cells and 2 carcinoid-cells. BRS-3 mRNA (Fig. 1) was detected in 8/13 (62%) cells: 3/6 NSCLC-cell-lines (NCI-H358, NCI-H520, NCI-H1299); 3/5 SCLC-cell-lines (NCI-H69, NCI-H82, NCI-N417); and 2/2 carcinoid-cell-lines (NCI-H720, NCI-H727). GRPRmRNA was detected in 10/13 (77%) of the cells: 5/6 NSCLC-cell-lines (NCI-H358, NCI-H520, A549, NCI-H838, NCI-H1299); 3/5 SCLC-cell-lines (NCI-H69, NCI-H345, NCI-H510); and in 2/2 carcinoid-cell-lines (NCI-H720, NCI-H727). Lastly, NMBRmRNA was found in 8/13 (62%) of cells (Fig. 1): 4/6 NSCLC-cell-lines (NCI-H358, A549, NCI-H838, NCI-H1299); 2/5 SCLC-cell-lines (NCI-H345, NCI-H510); and in 2/2 carcinoid-cell-lines (NCI-H720, NCI-H727). Most of the cell-lines tested expressed, at least, 2 of the 3 receptors, except in the case of the SCLC-NCI-H82 and SCLC-NCI-N417, which only expressed the BRS-3-receptor.

The mRNA-levels of human BnRs, was further characterized by quantifying the relative expression using qRT-PCR in each of the 13 human lung-cancer cell-lines. The average of each BnR mRNA-levels obtained in each cell tested was expressed as fold higher or lower than the mRNA-levels of the NSCLC-cell-line NCI-H358, which was considered as 1, because all 3 human BnR's were well-detected on qRT-PCR in this lung-cancer cell line. Using qRT-PCR, the BRS-3-receptor was expressed not only in the 8 cell-lines identified by nonquantitative-PCR (Fig. 1), but also in 3 other NSCLC-cell-lines (NCI-H460, NCI-A549, NCI-H838), and in the SCLC-cell-line, NCI-H510 (Fig. 2A). These results suggested that qRT-PCR in our analysis was more sensitive than non-quantitative PCR. This proposal was confirmed by analyzing serial dilutions of the BRS-3 BALB 3T3 cells by each method under identical conditions and the qRT-PCR was 300-fold more sensitive than non-quantitative PCR. The expression of the BRS-3-receptor was generally higher in SCLC-cells and carcinoid-cells than in NSCLC-cells. BRS-3-expression varied 3120-fold among the lung cancer cell lines (Fig. 2A). The highest levels of BRS-3-mRNA were in 2 SCLC-cell-lines (NCI-H69, NCI-N417) (38 ± 2 and 376 ± 10-fold greater, respectively). Five cell-lines showed BRS-3-mRNA levels greater than H358, while 4 had detectable mRNA levels less than BRS-3-mRNA expression shown in NCI-H358 cell-line. BRS-3-mRNA levels in normal lung were 2.5-fold less than found in NCI-358, and were not detectable in bronchial epithelial cells (data not shown). GRPR-expression also varied markedly in the different lung-cancer-cells (538-fold) and was detected by qRT-PCR in 11/13 cell-lines (Fig. 2B). Besides the 10 cell-lines that expressed GRPR in the nonquantitative-PCR conditions (Fig. 1), with qRT-PCR, GRPR expression was also detected in SCLC-NCI-H82. High levels of GRPR-mRNA were detected in 4/6 NSCLC-cells, in 2/5 SCLC-cells and in 1/2 carcinoid-cell-lines (Fig. 2B). qRT-PCR demonstrated that NMBRmRNA was expressed in 11/13 cell-lines, which included the 8 positive cells identified by nonquantitative-PCR (Fig. 1) and also the 3 SCLC-cell-lines (NCI-H69, NCI-H82, NCI-N417) (Fig. 2C). As it has seen with GRPR, NMBR is also widespread among the 13 different types of lung-cancer-cells, although the NMBR-mRNA-levels observed in general, were lower than in the case of GRPR. The NSCLC-NCI-H1299-cells showed the highest NMBR-mRNA-levels, while similar to GRPR, SCLC-NCI-H69-cells showed the lowest positive amount. Although most of the cells expressed at least 2 of the 3 BnRs, three (NCI-H69, NCI-H82, NCI-N417) of the four cells (NCI-H69, NCI-H82, NCI-N417) with the highest amount of BRS-3-mRNA (NCI-H69, NCI-H82, NCI-N417, NCI-520) had very low mRNA-levels of GRPR/NMBR (Fig. 2).

In terms of relative expression of the 3 BnR-subtypes: in 7/13 celllines the BRS-3-levels were the highest (H358, H460, H520, NCI-H69, NCI-H82, NCI-N417, NCI-H727); in 3/13 the GRPR levels were the highest (A549, H838, H345); and in 1/13 the NMBR-levels were the highest (NSCLC-H1299). H720-cells showed approximately the same amount of BRS-3 and GRPR, and H510-cells showed the same amount of GRPR and NMBR. In terms of the magnitude of difference in relative expression of the different BnRs, the lung-cancer cell-lines varied greatly with 2 cell-lines (NCI-H69, NCI-N417), having 864 ± 9- to 7082 ± 496-fold more BRS-3 than GRPR/NMBR, whereas 3 cell-lines (A549, H838, H345) had from 440 ± 10- to 1618 ± 107-fold greater GRPR than either NMBR/BRS-3, and 1 cell-line, H1299, had 154 ± 7-fold greater NMBR than either GRPR/BRS-3. Three of the 6 NSCLC-

cell-lines, 3/5 SCLC-cell-lines and l of 2 carcinoid-cell-lines, had either greater or similar amounts of BRS-3 to the GRPR/NMBR.

3.2. Stimulation of PLC by BRS-3 activation in human lung-cancer cells (Fig. 2, Tables 1, 2)

Previous studies have demonstrated that, similar to GRPR and NMBR, BRS-3-activation is primarily coupled to phospholipase-C (PLC) cascade activation, with stimulation of inositol phosphates generation (IP) and cytosolic-calcium $(Ca^{2+})_i$ release [24,40,44,55]. To determine whether the BRS-3 is biologically active in the lung-cancer cell lines and coupled to PLC-activation, each of BRS-3-qRT-PCR positive cells was first investigated for changes in cytosolic-calcium mobilization (Fig. 3, Table 1) after the addition of the BRS-3-selective-agonist, MK-5046 [44,60] (see Supplemental Table 1). Because the dose-response (DR)-curve for stimulation of IP by BRS-3-activation can be biphasic in some cells [44], we used two concentrations of MK-5046 (Table 1). In each lung-cancer cell-line, $(Ca^{2+})_i$ increased within seconds of MK-5046 addition (Fig. 3, Table 1), with the largest increase seen in the SCLC0NCI-H82 and BRS-3-transfected cells, Balb-3T3 and NCI-H1299 (Fig. 3B, D).

To further assess PLC activation, generation of inositol phosphates (IP) was investigated in each of the qRT-PCR-cell-lines positive for BRS-3 and in four BRS-3 negative cell lines [H345 and nontransfected BALB 3T3 cells, GRPR transfected and NMBR-transfected Balb 3T3 cells], and the positive control BRS3-transfected Balb 3T3 cells (Fig. 4; Tables 1 and 2; Supplemental Table 2). Changes in cytosolic Ca^{2+} were also investigated in two BRS-3 negative cell lines (supplemental Fig. 1). In all the non-BRS-3 containing cell lines, no stimulation with the BRS-3 specific agonist, MK-5046 was seen. However, in each case stimulation of [^{3}H]IP generation or changes in cytosolic calcium were seen with stimulation of other receptors on these cells (Supplemental Table 2, Supplemental Fig. 1). At least one of the concentrations of MK-5046 (10 nM, 100 nM), stimulated detectable IP-production in all the BRS-3 cell-lines except SCLC-NCI-H510 (Table 1). In this cell-line, the stimulation of IP by BRS-3 activation could not be detected (Table 1), even though changes in $(Ca^{2+})_i$ could be detected (Fig. 3). The greatest [^{3}H] IP-increase in IP-production (8-fold) was detected in BRS-3/H1299-cells (Table 1). There was a direct correlation between the magnitude of increase in [^{3}H]IP-generation and the BRS-3 mRNA amount from qRT-PCR with a regression curve of $y = 1.77x$, $r = 0.89$, $p = 0.0003$.

To examine the dose-response relationships for PLC activation, [^{3}H] IP-generation in 3 cell-lines were studied in more detail (Fig. 4). MK-5046 stimulated in BRS-3/H1299, NCI-H720 and NCI-H69 a concentration-dependent release of [^{3}H]IP with an EC_{50} of 0.37 ± 0.04 nM, 0.14 ± 0.05 nM and 3.29 ± 0.54 nM, respectively (Fig. 4A–C). The dose-response curves in NCI-H720 and NCI-H69 cells were biphasic (Fig. 4C). Supramaximal MK-5046 concentrations caused a 28% ($p < 0.05$) decrease in the [^{3}H]IP-release in H720 and 29% ($p < 0.001$) (Fig. 4B, C). In BRS-3/H1299 cells the dose-response curve was monophasic (Fig. 4A).

3.3. Effect of a BRS-3-antagonist and selective GRPR/NMBR agonists/antagonists on activation of PLC

The peptide-antagonist, Bantag-1, is highly selective for the BRS-3 [19,44,49] (see Supplemental Table 1). As shown in Figs. 1 and 2, many of the lung-cancer cell-lines tested possessed more than one BnR-sub-types. To be certain, MK-5046 was activating PLC via the BRS-3-receptor, the effect of Bantag-1 on this signaling pathway was determined, as well as the effect of other BnR-antagonists (Table 2).

The ability of Bantag-1 to inhibit the stimulation of [^{3}H]IP-production caused by both, the selective-agonist MK-5046 and the universal-nonselective-agonist, peptide#l [33,48], was examined in 4 celllines (NCI-H69, NCI-N417, NCI-H727, BRS-3/Balb) (Table 2). In addition, the ability of the selective NMBR-antagonist, PD16836 [16] (see Supplemental Table 1) and the potent GRPR-antagonist, [D-Phe^6] Bombesin-(6–13)-methyl ester (ME) [16,66,68] (see Supplemental Table 1) to inhibit IP-production stimulated by peptide #1, in these lung-cancer cells, was studied, since it was found that NCI-H69, as well as NCI-H727, expressed other BnRs besides BRS-3 (Table 2). Also, the ability of NMB, the selective-agonist of NMBR(see Supplemental Table 1), and GRP, a selective-agonist of GRPR [65] (see Supplemental Table 1), to stimulate PLC-activation in these cells was studied (Table 2). MK-5046 at 10 nM as well as peptide #1 at 10 nM caused a significant increase ($p < 0.01$) in [^{3}H]IP-generation in each of the 4 cell-lines (Table 2). However, NMB and GRP at 10 nM and 100 nM, activated PLC only in NCI-H727-cells (Table 2), which contain BRS-3, GRPR and NMBR (Fig. 1). Bantag-1 (100 nM), inhibited the [^{3}H]IP-generation stimulated by MK-5046 (10 nM) in all 4 cell-lines with a decrease in the [^{3}H]IP-production of 81% in NCI-H69, 99% in NCI-N417, 75% in NCI-H727 and 93% in BRS-3/Balb-cells (Table 2). Also, Bantag-1 (1000 nM), completely inhibited the stimulation of increases in cytosolic calcium by the BRS-3 agonist, MK-5046, in two cell lines (NCI-H727, H782, supplemental Fig. 2. To confirm that similar results were obtained with MK-5046 stimulation of increases in cytosolic Ca, we examined the ability of Bantag-1 to inhibit increases in $(Ca^{2+})_i$ in a small lung cancer cell line NCI-82, a nonsmall cell lung cancer cell line, NCI-H460 and a carcinoid cell line, NCI-H727, all of which showed strong stimulation by MK-5046 (Fig. 3). In each case Bantag-1 inhibited MK-5046 stimulation of increases in $(Ca^{2+})_i$.

Peptide #1 is a nonselective-agonist able to activate all 3 human BnRs [33,48,57,65]. Bantag-1 (100 nM) inhibited by 83% the [^{3}H]IP-production caused by peptide #1 (10 nM) in NCI-H69-cells, 68% in NCI-N417-cells and an 82% in BRS-3/Balb-cells. Neither the selective, NMBR-antagonist, PD16836, nor the selective, GRPR-antagonist, ME, inhibited the stimulation of IP caused by peptide #1, demonstrating it was via BRS-3 activation. Of the three inhibitors used, only the selective-BRS-3-antagonist Bantag-1 was able to inhibit the [^{3}H]IP-production in all the cells studied, supporting the conclusion that BRS-3 was functional in these cells (Table 2).

3.4. Selective BRS-3-agonist, MK-5046, stimulates p42/44-MAPK phosphorylation in human lung-cancer cells

BRS-3 activation in some tumor cells, as well as in some BRS-3-transfected-cells [43,44,71], stimulates MAPK-activation. To further investigate this in the present study, the

stimulation of the tyrosine phosphorylation of MAPK (Erkl/2 P-p42/44) with BRS-3 activation, was investigated in 9 BRS-3 positive lung-cancer-cell-lines and 2 BRS-3-transfected-cell-lines (Balb-3T3, NCI-H1299) (Table 1, Figs. 5 and 6).

MK-5046 (10, 100 nM) stimulated p42/44-MAPK phosphorylation in all the cell-lines (Table 1). MK-5046 significantly increased p42/44 tyrosine phosphorylation in the NSCLC lines NCI-H520 and NCI-H358 at 10 nM and 100 nM, and in NCI-H460 at 100 nM, with the same efficacy in the three of them (Fig. 5A–C). At 10 nM and 100 nM, MK-5046 also increased significantly the phosphorylation of p42/44 in the SCLC line NCI-H510 showing a biphasic response since p42/44 phosphorylation was 1.3-fold higher at 10 nM of MK-5046 than at 100 nM (Fig. 5C). MK-5046 stimulated p42/44 phosphorylation in a dose-dependent manner (Fig. 6) with high potency [BRS-3/H1299-cells (EC_{50}, 1.90 ± 0.16 nM); NCI-H727 (EC_{50}, 0.02 ± 0.01 nM); NCI-H720 (EC_{50}, 1.14 ± 0.57 nM); NCI-H69 (EC_{50}, 0.12 ± 0.01 nM); NCI-H82 (EC_{50}, 1.08 ± 0.16 nM); NCI-N417 (EC_{50}, 0.02 ± 0.01 nM)]. The dose-response curve in all the cells, except in the case of BRS-3/H1299-cells, was biphasic (Fig. 6). With supramaximal concentrations of MK-5046 a decrease in p42/44-phosphorylation was seen, which in NCI-H727-cells, represented a 49 ± 9% ($p < 0.0001$) decrease (Fig. 6B); in NCI-H720-cells (Fig. 6C), a 45 ± 8% ($p < 0.02$) decrease (Fig. 6D); in NCI-H82-cells (Fig. 6E), a 50 ± 7% ($p < 0.002$) decrease, while in the case of NCI-N417 (Fig. 6F) the decrease was 29 ± 6% ($p < 0.0001$). Lastly, BRS-3/H1299-transfected-cells showed a monophasic DR-curve, reaching the maximal stimulation (96 ± 7%) of p42/44-MAPK phosphorylation at 1 μM of MK-5046 (Fig. 6A).

3.5. Transactivation of EGFR by activation of BRS-3 and the effect of the EGFR inhibitor, gefitinib, in human lung-cancer cells

Recent studies report that activation of BRS-3, similar to other BnRs, can stimulate EGFR-transactivation [17,42,43,45]. Therefore, the ability of MK-5046 to stimulate EGFR-transactivation in the cell-lines was investigated by assessing EGFR-tyrosine (Tyr^{1068}) phosphorylation in 9 BRS-3-containing lung-cancer cell-lines and in 2 BRS-3-transfected-cell-lines, BALB-3T3 and NCI-H1299 (Table 3). MK-5046 stimulated the transactivation of EGFR in 6 of 11 cell lines tested (Table 3). At concentrations of 10 nM and 100 nM, MK-5046 caused a significantly increase of EGFR tyrosine phosphorylation in BRS-3/H1299, NCI-H727 and in NCI-N417 cell-lines (Fig. 7B, D and E, Table 3). In the SCLC line NCI-N417, MK-5046 increased significantly the phosphorylation of Tyr^{1068} of EGFR at 0.1 nM and 10 nM, showing a biphasic response, with stimulation 2.86–fold ($p < 0.05$) higher with 0.1 nM than 10 nM of MK-5046 (Fig. 7D, Table 3). In addition, MK-5046 increased significantly the EGFR phosphorylation at 10 nM or at 100 nM in NCI-H69 cells and in the NSCLC line NCI-H358, respectively (Fig. 7A, C, Table 3). However, MK-5046 did not stimulate the EGFR transactivation in the NSCLC lines NCI-H460 and NCI-H520, hBRS-3/Balb, carcinoid line NCI-H720 nor the SCLC line NCI-H82 (Table 3). In the carcinoid NCI-H727 cell line, gefitinib (20 nM) significantly inhibited ($p < 0.001$) the EGFR transactivation stimulated by MK-5046 (100 nM) (Fig. 7F).

3.6. Growth stimulation by BRS-3 agonist activation and effect of a EGFR inhibitor in lung-cancer cells

To study the ability of BRS-3 activation in lung-cancer cells to stimulate growth, an assessment of [^{3}H]-Thymidine-uptake assay was performed after addition of MK-5046. MK-5046 significantly increased the [^{3}H]-Thymidine-incorporation in the 11 different cell-lines tested; 9 BRS-3-containing lung-cancer-cell-lines and 2 BRS-3-transfected-cell-lines (Table 3). The increase of [^{3}H]-Thymidine-uptake stimulated by MK-5043 occurred in a dose-dependent manner in the 3 cell-lines investigated in detail (Fig. 8A) and its potency was high [NCI-H358 (EC_{50}, 0.52 ± 0.07 nM); NCI-H727 (EC_{50}, 0.31 ± 0.06 nM); NCI-H69 (EC_{50}, 0.27 ± 0.06 nM)]. In 2 of the cell-lines, NCI-H358 and NCI-H69, the dose-response curve was biphasic (Fig. 8A1, A3) with supramaximal MK-5046 concentrations causing in NCI-H358-cells, a 23% decrease (Fig. 8A1), and in NCI-H69-cells, an 87% decrease (Fig. 8A3). However, in NCI-H727-cells (Fig. 8A2) the MK-5046, growth dose-curve showed was monophasic. The stimulation of cell proliferation induced by MK-5046 was completely inhibited by the EGFR tyrosine kinase inhibitor, gefitinib (20 nM) in the NSCLC-NCI-H358 cell-line (Fig. 8B1) and the carcinoid-NCI-H727 cell (Fig. 8B2).

4. Discussion

Lung-cancer remains a leading cause of death worldwide [1] and is the leading cancer mortality in the United States, with approximately 160, 000 deaths in 2015 [2]. Although chemotherapy is the traditional method to treat lung-cancer, the low 5-yr survival rate after the treatment (16% [40]), has led to the development of other therapies including EGFR-tyrosine-kinase inhibitors (TKIs). Through the inhibition of EGFR, the TKIs inhibit cellular proliferation and induce apoptosis of lung-cancer cells [22,46,72,74]. Successful treatment with TKIs (i.e., gefitinib, erlotinib), is reported after failed chemotherapy in patients with advanced non-small cell lung-cancers with known EGFR-driver mutations [36,69,77]. Also, recently studies show possible benefits of EGFR-TKIs for patients previously treated with chemotherapy for NSCLC cells with an EGFR wild-type [62,72]. In the case of small cell lung-cancers, however, TKI benefit has not been seen [32]. Even though many patients initially respond well to TKI therapies, almost all develop EGFR-TKIs resistance [12,72]. Because of EGFR-TKIs resistance and the fact that TKIs are successful mainly in lung-cancers with EGFR mutations, which only represented at 10% of the cases, new therapeutic approaches are needed either alone or to improve the efficacy of TKIs by reducing cellular resistance and survival, but also to treat NSCLC EGFR wild-type and SCLC [27,32,35].

One area that is receiving increasing attention in lung-cancer, as well as other neoplasms, is the development of novel treatments using G protein-coupled receptors that are frequently overexpressed by the tumors [25,27,40,47,63,76]. The mammalian bombesin-receptor family (GRPR/NMBR), in the case of lung-cancer, is receiving considerable attention, because they are not only overexpressed in 25–100% of lung-cancer cells [24,45,52], but also have important growth effects, frequently functioning in an autocrine-manner [27,37,38,40,61]. Recent studies report that activation of BnRs in lung-cancer cells, as well as in other tumors, stimulates EGFR-transactivation through Src-dependent release of amphiregulin/TGFα, which results in increased survival of lung-cancer cells exposed to

EGFR-inhibitor anti-cancer therapies such as gefitinib [27,37,40,43]. Moreover, BnR-inhibitors can suppresses tumor angiogenesis [47], increase cellular death [27,35] and improve potency of TKIs, such as gefitinib, either in EGFR-mutant or in EGFR wild-type lung-cancer [27,35,40,41]. Furthermore, the overexpression of BnRs by lung-cancer cells is increasingly being used to allow targeted imaging of tumors overexpressing these receptors, as well as the possibility of targeted delivery of cytotoxic agents [28,34].

The two established BnR subtypes, the gastrin–releasing peptide receptor (GRPR) and neuromedin B receptor (NMBR), have been well studied in lung-cancer and other cancers [21,38,45,49,59,70]. The orphan G protein-coupled receptor, BRS-3, is classified as a member of the bombesin-family of receptors, because of its high homology to GRPR/NMBR [24]. Although BRS-3 is reported to be widely distributed in both peripheral tissues and the CNS, as well as in some tumors [24,40,49,70], in general there is only limited information of this receptor in lung-cancer. Several studies report the present of BRS-3 in lung-cancers, and in a few lung cancer-cell lines, the activation o0f BRS-3 stimulates growth, involving activation of MAPK, phospholipase C activation and EGFR-transactivation [43,44]. Additional studies report that BRS-3 activation can promote metastasis formation and drug-resistance in SCLC cells [23]. The lack of information of BRS-3 is in part due to the fact that its natural ligand is unknown and also, until recently, in contrast to the GRPR/NMBR [9,20,49,67,68], there is a lack of selective agonists/antagonists. Recently both, selective agonists/antagonists of BRS-3, have been described, proving specific pharmacological tools to study its activation and its involvement in physiological/pathophysiological process [40,44,49]. Therefore, because of the importance of the established BnRs (GRPR/NMBR) and their ligands in affecting lung-cancer growth/signaling, their frequent overexpression in this tumor, as well as the increasing studies showing their presence in lung-cancer may lead to novel therapeutic approaches, we studied in detail BRS-3 expression comparing it to the expression of GRPR/NMBR and the effect of activation of the Bn-related orphan receptor, BRS-3 in a large number of different lung-cancer cell-lines.

A number of our results support the conclusions that BnRs are widely distributed in lung-cancer cells, that BRS-3 is more frequent and abundant in lung-cancer cells than previously reported, and that BnRs are abundant in each of the different subtypes of lung-cancer, but vary greatly in expression levels [24,35,52]. First, using nonquantitative-PCR, as used in all previous studies of lung-cancer BnR expression, we detected GRPR expression in 77% of the 13 lung-cancer cells and NMBR and BRS-3 expression in 62%. Second, using qRT-PCR for the first time in lung-cancer cells, we detected GRPR, NMBR and BRS-3 expression in higher frequency than using conventional non-quantitative PCR, as has been reported frequently with other assays in other cells [3,75]. A detailed analysis of serial dilutions of BRS-3 BALB 3T3 cDNA using both PCR methods confirmed that qRT-PCR had a 300-fold greater sensitivity in our assays than conventional nonquantitative PCR. In our study, GRPR, NMBR and BRS-3 expression using qRT-PCR, was detected in 85%, 85% and 92%, respectively, of the 13 lung-cancer cell-lines. Third, with qRT-PCR we found that in the 6 NSCLC cell-lines, 100% expressed BRS-3, 83% GRPR and 67% NMBR; among the 5 SCLC celllines, 80% expressed BRS-3, 80% GRPR and 100% NMBR; and that both carcinoid cells studied (100%) expressed BRS-3-receptor, GRPR and NMBR mRNA.

Fourth, with qRT-PCR, we found that in 50% of the SCLC and NSCLC cell-lines, as well as in lung carcinoid cells, the relative expression of BRS-3 was higher than GRPR/NMBR. Fifth, we found differences in expression levels of the three BnRs in the different lung-cancer subtypes, with BRS-3 expression levels varying by 3120-fold in the different lung-cancer cells, with the highest levels in SCLC cells. In contrast, the GRPR and NMBR expression varied by 538- and 60-fold, respectively, with the highest levels, in both cases, in NSCLC cells. Whereas the highest levels of BRS-3 tended to occur in the SCLC and carcinoid cell lines in contrast to GRPR where higher levels occurred in SCLC, NSLC and some carcinoids. However relative to each other's expression, in 54% of all the lung cancer cell lines, BRS-3 was the most abundant including in 50% of carcinoid and NSCLC cell lines and in 67% of SCLC cell lines. Our results have both similarities and differences from previous studies, which all used nonquantitative-PCR to examine BnR levels in lung-cancer. Our results differ from previous studies examining BRS-3 expression in lung-cancer cells, which report its presence in only 29% of 56 lung-cancer cell-lines (n = 6 studies) [10,13,14,55,61,64], whereas we found it in 62% by this PCR method. Our results are similar to previous studies with NMBR/GRPR expression in different subtypes of lung-cancer, which reported NMBR in 77% of 57 lung-cancer cells (n = 7 studies) [8,10,14,55,61,64,73] and GRPR in 66% of 61 different lung-cancer cell-lines examined (n = 7 studies) [8,10,14,55,61,64,73]. These results demonstrate that BRS-3, in contrast to previous studies, is as frequent in lung-cancer cells as the other BnRs, GRPR/NMBR and it is frequently present at high levels.

Our results support the conclusion that the BRS-3-receptor identified in the different lung-cancer cells is functional and its activation results in stimulation of the phospholipase C cascade inducing changes in cytosolic calcium and phosphoinositides, similar to reported in a few other native cells and in BRS-3-transfected cells [40,44,54,55,71]. This conclusion is supported by a number of findings. First, the selective, BRS-3 agonist, MK-5046, increased within seconds the $[Ca^{2+}]_i$ in 100% of BRS-3 positive lung-cancer cells, in a manner similar to that described previously in BRS-3-transfected cells [40,54]. These results are consistent with previous studies using nonselective agonists to activate BRS-3 in containing cells [23,40,54,71]. Second, MK-5046 stimulated phosphoinositide generation, which is a less sensitive measure of BRS-3 activation, in the 89% of the lung-cancer cells expressing BRS-3 and in the BRS-3-transfected cells. This MK-5046 stimulation of [^{3}H]IP production was characteristic of that seen in previous studies with MK-5046 in BRS-3-containing cells [44], in that it occurred in a dose-dependent manner with an EC_{50} in the nM range. That the changes in [^{3}H]IP generation seen with MK-5046 were due to BRS-3 activation was supported by its antagonism by the selective, BRS-3 antagonist, Bantag-1 [44], but not by ME, a selective GRPR, antagonist [26], or PD16836, a NMBR, selective antagonist [37]. Our results demonstrate that the BRS-3-receptor present in lung-cancer cells is coupled to the phospholipase C signaling cascade and functionally active.

Similar to GRPR/NMBR, recent studies have reported that BRS-3 activation can stimulate activation of the ERK/MAPK cascade, as well as cell proliferation, which is mediated in large part by transactivation of EGFR in BRS-3 transfected cells and a few other cells [40,43,44,50,71]. A number of our results demonstrate that similar signaling and growth effects are presented in the different subtypes of BRS-3 lung-cancer cells studied. First,

MK-5046 rapidly stimulated p42/44 tyrosine phosphorylation in the 100% of the BRS-3 containing lung-cancer cell-lines and in each of the BRS-3 transfected cells, and it occurred in a dose-dependent manner with an MK-5046 EC_{50} in the nanomolar range. These results are similar to previous studies using primarily nonselective, BRS-3 agonists, which reported p42/44 tyrosine phosphorylation in BRS-3 transfected cells, skeletal muscule cells and a few lung-cancer cells containing native BRS-3 [40,43,44,49,71]. Second, the BRS-3 selective agonist, MK-5046 stimulated EGFR-transactivation in 56% of the BRS-3 receptor lung-cancer cell-lines and in NCI-H1299 BRS-3-transfected cells. These results are compared with those previously observed using nonselective agonists to activate BRS-3-transfected cells, which resulted in EGFR tyrosine phosphorylation through the activation of endogenous TGFα release [40,43], similarly to reported with other BnRs [27,37]. However, our results show a difference from previous studies with nonselective BRS-3 agonists, in which it was proposed that activation of the ERK/MAPK cascade, seen with BRS-3 stimulation, occurs via EGFR-transactivation in BRS-3 transfected lung-cancer cells [40,43]. Our results suggest this is not the case in all BRS-3 containing lung-cancer cells, because in 45% of the native BRS-3 lung-cancer cell-lines, BRS-3 activation stimulated ERK/MAPK activation, but it did not stimulate EGFR-transactivation. Third, MK-5046 stimulates [^{3}H]-Thymidine incorporation in 100% of the BRS-3 containing lung-cancer cell-lines, as well as in the BRS-3 transfected cells. Similar to activation of phospholipase C and MAPK, the increase of [^{3}H]-Thymidine incorporation by MK-5046 occurred in a dose-dependent manner with a MK-5046 EC_{50} in the nanomolar range. Fourth, in 54% of the lung cancer cell-lines in which stimulation of BRS-3 activation resulted in EGFR transactivation, the growth-effect of BRS-3 in these lung cancer cell lines was due to the BRS-3 stimulated transactivation of EGFR. This was demonstrated by the ability of the EGFR tyrosine kinase inhibitor, gefitinib, to completely inhibit BRS-3 mediated EGFR transactivation and growth in two of these lung cancer cell lines showing EGFR transactivation. Our results are similar to findings using nonselective, BRS-3 agonists, which reported that BRS-3 activation could increase cellular growth in BRS-3 transfected cells and in NCI-H727 lung-cancer cells in an EGFR-dependent manner [40,43]. These results support the conclusion that stimulation of BRS-3 in different subtypes of lung-cancer cells can activate the ERK/MAPK signaling cascade and stimulate cellular proliferation/growth in an EGFR-dependent manner and EGFR-independent manner.

In summary, our results demonstrate that in lung-cancer cells, BRS-3 expression occurs more frequently than previously reported, that it occurs in all lung-cancer subtypes and that it is frequently highly expressed in the lung-cancer cells. The BRS-3 receptor in the lung-cancer cell-lines is functionally coupled to signaling cascades, activating phospholipase C and stimulating changes in cytosolic $[Ca^{2+}]_I$, as well as to generation of phophoinositides and MAPK activation. Activation of BRS-3 results in lung-cancer cell proliferation and this occurs associated via EGFR-transactivation in some, but not all, lung-cancer cells. These results suggest that BRS-3 should receive increased attention, similar to that occurring with GRPR and NMBR in lung-cancer cells, as a possible approach for the development of novel treatments and/or diagnosis. GRPR/NMBR are receiving increased attention because of their frequent overexpression in lung-cancer cells, which are being explored for tumor localization and targeted delivery of cytotoxic agents [29,45,56]. Furthermore, there is

interest in the receptors because of their effects as autocrine growth factors and on tumor angiogenesis, as well as their ability to enhance the therapeutic effects of TKIs such as gefitinib, resulting in reduced cellular resistance and tumor cell survival [35,40,47]. Our results suggest that BRS-3 should be also considered as a potential candidate with important clinical applications, similar to the other mammalian Bn receptors, GRPR/NMBR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BALB 3T3	mouse embryonic fibroblast cells
Bn	bombesin
BnR	bombesin receptors
BRS-3	bombesin receptor subtype-3
BSA	bovine serum albumin fraction V
CNS	central nervous system
DMEM	Dulbecco’s minimum essential medium
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinases
h	human
EC_{50}	concentration causing half-stimulation
FBS	fetal bovine serum
GPCR	G-protein-coupled receptor
GRP	gastrin-releasing peptide
GRPR	gastrin-releasing peptide receptor
IC_{50}	half maximal inhibitor concentration
IP	inositol phosphate
m	mouse
MAPK	mitogen activated protein kinases
MK-5046	nonpeptide BRS3 agonist

NMB	neuromedin B
NMBR	neuromedin B receptor
NSCLC	nonsmall cell lung cance
peptide #1	[D-Tyr^{6} β-Ala^{11}Phe^{13} Nle^{14}]Bn-(6–14)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PLC	phospholipase C
qPCR	quantitative real time PCR
r	rat
SCLC	small cell lung cancer

References

[1]. Stewart BW, Wild PJ, World Cancer Report 2014, WHO Press, Geneva, Switzerland, 2014.

[2]. Lung Cancer Fact Sheet 2015(American cancer Society). http://www.lung.org. (American Lung Association)] 2015 Ref Type: Generic.

[3]. Bastien P, Procop GW, Reischl U, Quantitative real-time PCR is not more sensitive than conventional PCR, J. Clin. Microbiol 46 (2008) 1897–1900. [PubMed: 18400914]

[4]. Benya RV, Fathi Z, Battey JF, Jensen RT, Serines and threonines in the gastrinreleasing peptide receptor carboxyl terminus mediate internalization, J. Biol. Chem 268 (1993) 20285–20290. [PubMed: 8397203]

[5]. Benya RV, Fathi Z, Pradhan T, Battey JF, Kusui T, Jensen RT, Gastrin-releasing peptide receptor-induced internalization, down-regulation, desensitization and growth: possible role of cAMP, Mol. Pharmacol 46 (2) (1994) 235–245. [PubMed: 8078487]

[6]. Benya RV, Kusui T, Pradhan TK, Battey JF, Jensen RT, Expression and characterization of cloned human bombesin receptors, Mol. Pharmacol 47 (1995) 10–20. [PubMed: 7838118]

[7]. Benya RV, Wada E, Battey JF, Fathi Z, Wang LH, Mantey SA, et al.,Neuromedin B receptors retain functional expression when transfected into BALB 3T3 fibroblasts: analysis of binding, kinetics, stoichiometry, modulation by guanine nucleotide-binding proteins, and signal transduction and comparison with natively expressed receptors, Mol. Pharmacol 42 (6) (1992) 1058–1068. [PubMed: 1336112]

[8]. Corjay MH, Dobrzanski DJ, Way JM, Viallet J, Shapira H, Worland P, et al., Two distinct bombesin receptor subtypes are expressed and functional in human lung carcinoma cells, J. Biol. Chem 266 (1991) 18771–18779. [PubMed: 1655761]

[9]. Coy DH, Taylor JE, Jiang NY, Kim SH, Wang LH, Huang SC, et al., Shortchain pseudopeptide bombesin receptor antagonists with enhanced binding affinities for pancreatic acinar and Swiss 3T3 cells display strong antimitotic activity, J. Biol. Chem 264 (1989) 14691–14697. [PubMed: 2475489]

[10]. DeMichele MA, Davis AL, Hunt JD, Landreneau RJ, Siegfried JM, Expression of mRNA for three bombesin receptor subtypes in human bronchial epithelial cells, Am. J. Respir. Cell Mol. Biol 11 (1994) 66–74. [PubMed: 8018339]

[11]. Di Florio A, Sancho V, Moreno P, Delle Fave GF, Jensen RT, Gastrointestinal hormones stimulate growth of Foregut Neuroendocrine Tumors by transactivating the EGF receptor, Biochim. Biophys. Acta 1833 (2013) 573–582. [PubMed: 23220008]

[12]. Fang X, Gu P, Zhou C, Liang A, Ren S, Liu F, et al., beta-Catenin overexpression is associated with gefitinib resistance in non-small cell lung cancer cells, Pulm. Pharmacol. Ther 28 (2014) 41–48. [PubMed: 23707949]

[13]. Fathi Z, Benya RV, Shapira H, Jensen RT, Battey JF, The fifth transmembrane segment of the neuromedin B receptor is critical for high affinity neuromedin B binding, J. Biol. Chem 268 (#20) (1993) 14622–14626. [PubMed: 8392057]

[14]. Fathi Z, Corjay MH, Shapira H, Wada E, Benya R, Jensen R, et al., BRS-3: novel bombesin receptor subtype selectively expressed in testis and lung carcinoma cells, J. Biol. Chem 268 (8) (1993) 5979–5984. [PubMed: 8383682]

[15]. Feng Y, Guan XM, Li J, Metzger JM, Zhu Y, Juhl K, et al., Bombesin receptor subtype-3 (BRS-3) regulates glucose-stimulated insulin secretion in pancreatic islets across multiple species, Endocrinology 152 (2011) 4106–4115. [PubMed: 21878513]

[16]. Gonzalez N, Mantey SA, Pradhan TK, Sancho V, Moody TW, Coy DH, et al., Characterization of putative GRP- and NMB-receptor antagonist's interaction with human receptors, Peptides 30 (2009) 1473–1486. [PubMed: 19463875]

[17]. Gonzalez N, Moody TW, Igarashi H, Ito T, Jensen RT, Bombesin-related peptides and their receptors: recent advances in their role in physiology and disease states, Curr. Opin. Endocrinol. Diabetes Obes 15 (2008) 58–64. [PubMed: 18185064]

[18]. Gonzalez N, Moreno P, Jensen RT, Bombesin receptor –subtype 3 as a potential target for obesity and diabetes, Exp. Opin. Ther. Targets 19 (2015) 1153–1170.

[19]. Guan XM, Chen H, Dobbelaar PH, Dong Y, Fong TM, Gagen K, et al., Regulation of energy homeostasis by bombesin receptor subtype-3: selective receptor agonists for the treatment of obesity, Cell Metab 11 (2010) 101–112. [PubMed: 20096642]

[20]. Heinz-Erian P, Coy DH, Tamura M, Jones SW, Gardner JD, Jensen RT, [D-Phe12] bombesin analogues: a new class of bombesin receptor antagonists, Am. J. Physiol 252 (1987) G439–G442. [PubMed: 2435173]

[21]. Hohla F, Schally AV, Targeting gastrin releasing peptide receptors: new options for the therapy and diagnosis of cancer, ABBV Cell Cycle 9 (2010) 1738–1741.

[22]. Hong D, Zhang G, Zhang X, Lian X, Pulmonary toxicities of gefitinib in patients with advanced non-small-cell lung cancer: a meta-analysis of randomized controlled trials, Medicine (Baltimore) 95 (2016) e3008. [PubMed: 26945426]

[23]. Hou X, Wei L, Harada A, Tatamoto K, Activation of bombesin receptor subtype-3 stimulates adhesion of lung cancer cells, Lung Cancer 54 (2006) 143–148. [PubMed: 16979789]

[24]. Jensen RT, Battey JF, Spindel ER, Benya RV, International Union of Pharmacology LVIII. Mammalian Bombesin Receptors: nomenclature, distribution, pharmacology, signaling and functions in normal and disease states, Pharmacol. Rev 60 (2008) 1–42. [PubMed: 18055507]

[25]. Kanashiro CA, Schally AV, Zarandi M, Hammann BD, Varga JL, Alterations of EGFR/HER, angiogenesis and apoptosis pathways after therapy with antagonists of growth hormone releasing hormone and bombesin in non-small cell lung cancer, Int. J. Oncol 30 (2007) 1019–1028. [PubMed: 17332943]

[26]. Lach E, Coy DH, Dumont P, Landry Y, gies JP, Gastrin releasing peptide-preferring bombesin binding sites in human lung, Eur. J. Pharmacol 265 (1994) 117–120. [PubMed: 7883024]

[27]. Liu X, Carlisle DL, Swick MC, Gaither-Davis A, Grandis JR, Siegfried JM, Gastrin-releasing peptide activates Akt through the epidermal growth factor receptor pathway and abrogates the effect of gefitinib, Exp. Cell Res 313 (2007) 1361–1372. [PubMed: 17349623]

[28]. Liu Z, Huang J, Dong C, Cui L, Jin X, Jia B, et al., 99mTc-labeled RGD-BBN peptide for small-animal SPECT/CT of lung carcinoma, Mol. Pharm 9 (2012) 1409–1417. [PubMed: 22452411]

[29]. Maina T, Nock BA, Kulkarni H, Singh A, Baum RP, Theranostic prospects of gastrin-releasing peptide receptor-radioantagonists in oncology, PET Clin 12 (2017) 297–309. [PubMed: 28576168]

[30]. Majumdar ID, Weber HC, Appetite-modifying effects of bombesin receptor subtype-3 agonists, Handb. Exp. Pharmacol (2012) 405–432. [PubMed: 22249826]

[31]. Majumdar ID, Weber HC, Biology and pharmacology of bombesin receptor subtype-3, Curr. Opin. Endocrinol. Diabetes Obes 19 (2012) 3–7. [PubMed: 22157398]

[32]. Mamdani H, Induru R, Jalal SI, Novel therapies in small cell lung cancer, Transl. Lung Cancer Res 4 (2015) 533–544. [PubMed: 26629422]

[33]. Mantey SA, Weber HC, Sainz E, Akeson M, Ryan RR, Pradhan TK, et al., Discovery of a high affinity radioligand for the human orphan receptor, bombesin receptor subtype 3, which demonstrates it has a unique pharmacology compared to other mammalian bombesin receptors, J. Biol. Chem 272 (41) (1997) 26062–26071. [PubMed: 9325344]

[34]. Marostica LL, de Barros AL, Silva JO, Lopes SC, Salgado BS, Chondrogiannis S, et al., Feasibility study with 99mTc-HYNIC-betaAla-Bombesin (7–14) as an agent to early visualization of lung tumour cells in nude mice, Nucl. Med. Commun 37 (2016) 372–376. [PubMed: 26629771]

[35]. Mattei J, Achcar RD, Cano CH, Macedo BR, Meurer L, Batlle BS, et al., Gastrin-releasing peptide receptor expression in lung cancer, Arch. Pathol. Lab. Med 138 (2014) 98–104. [PubMed: 24377816]

[36]. Melosky B, Review of EGFR TKIs in metastatic NSCLC, including ongoing trials, Front. Oncol. 4 (2014) 244.

[37]. Moody TW, Berna MJ, Mantey S, Sancho V, Ridnour L, Wink DA, et al., Neuromedin B receptors regulate EGF receptor tyrosine phosphorylation in lung cancer cells, Eur. J. Pharmacol 637 (2010) 38–45. [PubMed: 20388507]

[38]. Moody TW, Chan D, Fahrenkrug J, Jensen RT, Neuropeptides as autocrine growth factors in cancer cells, Curr. Pharm. Des 9 (2003) 495–509. [PubMed: 12570813]

[39]. Moody TW, Fuselier J, Coy DH, Mantey S, Pradhan T, Nakagawa T, et al., Camptothecin-somatostatin conjugates inhibit the growth of small cell lung cancer cells, Peptides 26 (2005) 1560–1566. [PubMed: 16112393]

[40]. Moody TW, Mantey SA, Moreno P, Nakamura T, Lacivita E, Leopoldo M, et al., ML-18 is a non-peptide bombesin receptor subtype-3 antagonist which inhibits lung cancer growth, Peptides 64 (2015) 55–61. [PubMed: 25554218]

[41]. Moody TW, Moreno P, Jensen RT, Neuropeptides as lung cancer growth factors, Peptides 72 (2015) 106–111. [PubMed: 25836991]

[42]. Moody TW, Nuche-Berenguer B, Nakamura T, Jensen RT, EGFR transactivation by peptide G protein-coupled receptors in cancer, Curr. Drug Targets 17 (2016) 520–528. [PubMed: 25563590]

[43]. Moody TW, Sancho V, Di Florio A, Nuche-Berenguer B, Mantey S, Jensen RT, Bombesin receptor subtype-3 agonists stimulate the growth of lung cancer cells and increase EGF receptor tyrosine phosphorylation, Peptides 32 (2011) 1677–1684. [PubMed: 21712056]

[44]. Moreno P, Mantey SA, Nuche-Berenguer B, Reitman ML, Gonzalez N, Coy DH, et al., Comparative pharmacology of bombesin receptor subtype-3, nonpeptide agonist MK-5046, a universal peptide agonist, and peptide antagonist Bantag-1 for human bombesin receptors, J. Pharmacol. Exp. Ther 347 (2013) 100–116. [PubMed: 23892571]

[45]. Moreno P, Ramos-Alvarez I, Moody TW, Jensen RT, Bombesin related peptides/receptors and their promising therapeutic roles in cancer imaging, targeting and treatment, Expert Opin. Ther. Targets 20 (2016) 1055–1073. [PubMed: 26981612]

[46]. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al., EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy, Science 304 (2004) 1497–1500. [PubMed: 15118125]

[47]. Park HJ, Kim SR, Kim MK, Choi KS, Jang HO, Yun I, et al., Neuromedin B receptor antagonist suppresses tumor angiogenesis and tumor growth in vitro and in vivo, Cancer Lett 312 (2011) 117–127. [PubMed: 21908103]

[48]. Pradhan TK, Katsuno T, Taylor JE, Kim SH, Ryan RR, Mantey SA, et al., Identification of a unique ligand which has high affinity for all four bombesin receptor subtypes, Eur. J. Pharmacol 343 (1998) 275–287. [PubMed: 9570477]

[49]. Ramos-Alvarez I, Moreno P, Mantey SA, Nakamura T, Nuche-Berenguer B, Moody TW, et al., Insights into bombesin receptors and ligands: highlighting recent advances, Peptides 72 (2015) 128–144. [PubMed: 25976083]

[50]. Ramos-Alvarez I, Moreno-Villegas Z, Martin-Duce A, Sanz R, Aparicio C, Portal-Nunez S, et al., Human BRS-3 receptor: functions/role in cell signaling pathways and glucose metabolism in obese or diabetic myocytes, Peptides 51 (2014) 91–99. [PubMed: 24220502]

[51]. Reitman ML, Dishy V, Moreau A, Denney WS, Liu C, Kraft WK, et al., Pharmacokinetics and pharmacodynamics of MK-5046, a bombesin receptor subtype-3 (BRS-3) agonist, in healthy patients, J. Clin. Pharmacol 52 (2012) 1306–1316. [PubMed: 22162541]

[52]. Reubi JC, Wenger S, Schumuckli-Maurer J, Schaer JC, Gugger M, Bombesin receptor subtypes in human cancers: detection with the universal radoligand (125)I-[D-TYR(6), beta-ALA(11), PHE(13), NLE(14)] bombesin(6–14), Clin. Cancer Res 8 (2002) 1139–1146. [PubMed: 11948125]

[53]. Rowley WH, Sato S, Huang SC, Collado-Escobar DM, Beaven MA, Wang LH, et al., Cholecystokinin-induced formation of inositol phosphates in pancreatic acini, Am. J. Physiol 259 (1990) G655–G665. [PubMed: 1699431]

[54]. Ryan RR, Weber HC, Hou W, Sainz E, Mantey SA, Battey JF, et al., Ability of various bombesin receptor agonists and antagonists to alter intracellular signaling of the human orphan receptor BRS-3, J. Biol. Chem 273 (1998) 13613–13624. [PubMed: 9593699]

[55]. Ryan RR, Weber HC, Mantey SA, Hou W, Hilburger ME, Pradhan TK, et al., Pharmacology and intracellular signaling mechanisms of the native human orphan receptor BRS-3 in lung cancer cells, J. Pharmacol. Exp. Ther 287 (1998) 366–380. [PubMed: 9765358]

[56]. Sancho V, Di Florio A, Moody TW, Jensen RT, Bombesin receptor-mediated imaging and cytotoxicity: review and current status, Curr. Drug Deliv 8 (2011) 79–134. [PubMed: 21034419]

[57]. Sancho V, Moody TW, Mantey SA, Di Florio A, Uehara H, Coy DH, et al., Pharmacology of putative selective hBRS-3 receptor agonists for human bombesin receptors (BnR): affinities, potencies and selectivity in multiple native and BnR transfected cells, Peptides 31 (2010) 1569–1578. [PubMed: 20438784]

[58]. Sano H, Feighner SD, Hreniuk DL, Iwaasa H, Sailer AW, Pan J, et al., Characterization of the bombesin-like peptide receptor family in primates, Genomics 84 (2004) 139–146. [PubMed: 15203211]

[59]. Schwartsmann G, DiLeone LP, Horowitz M, Schunemann D, Cancella A, Pereira AS, et al., A phase I trial of the bombesin/gastrin-releasing peptide (BN/GRP) antagonist RC3095 in patients with advanced solid malignancies, Invest. New Drugs 24 (2006) 403–412. [PubMed: 16505950]

[60]. Sebhat IK, Franklin C, Lo MC, Chen D, Jewell JP, Miller R, et al., Discovery of MK-5046, a potent, selective bombesin recptor subtype-3 agonist for the treatment of obesity, ACS Med. Chem. Lett 2 (2011) 43–47. [PubMed: 24900253]

[61]. Siegfried JM, Krishnamachary N, Davis AG, Gubish C, Hunt JD, Shriver SP, Evidence for autocrine actions of neuromedin B and gastrin-releasing peptide in non-small cell lung cancer, Pulm. Pharmacol. Ther 12 (1999) 291–302. [PubMed: 10545285]

[62]. Stinchcombe TE, The use of EGFR tyrosine kinase inhibitors in EGFR wild-type non-small-cell lung cancer, Curr. Treat. Options Oncol 17 (2016) 18. [PubMed: 26961971]

[63]. Sun L, Luo J, Mackey LV, Morris LM, Franko-Tobin LG, Lepage KT, et al., Investigation of cancer cell lines for peptide receptor-targeted drug development, J. Drug Target. 19 (2011) 719–730. [PubMed: 21830941]

[64]. Toi-Scott M, Jones CLA, Kane MA, Clinical correlates of bombesin-like peptide receptor subtype expression in human lung cancer cells, Lung Cancer 15 (1996) 341–354. [PubMed: 8959679]

[65]. Uehara H, Gonzalez N, Sancho V, Mantey SA, Nuche-Berenguer B, Pradhan T, et al., Pharmacology and selectivity of various natural and synthetic bombesin related peptide agonists for human and rat bombesin receptors differs, Peptides 32 (2011) 1685–1699. [PubMed: 21729729]

[66]. von Schrenck T, Wang LH, Coy DH, Villanueva ML, Mantey S, Jensen RT, Potent bombesin receptor antagonists distinguish receptor subtypes, Am. J. Physiol 259 (1990) G468–G473. [PubMed: 2169207]

[67]. Wang LH, Coy DH, Taylor JE, Jiang NY, Kim SH, Moreau JP, et al., Desmethionine alkylamide bombesin analogues: a new class of bombesin receptor antagonists with a potent antisecretory

activity in pancreatic acini and antimitotic activity in Swiss 3T3 cells, Biochemistry (Mosc) 29 (3) (1990) 616–622.

[68]. Wang LH, Coy DH, Taylor JE, Jiang NY, Moreau JP, Huang SC, et al., Des-Met carboxyl-terminally modified analogues of bombesin function as potent bombesin receptor antagonists, partial agonists, or agonists, J. Biol. Chem 265 (26) (1990) 15695–15703. [PubMed: 1697594]

[69]. Watanabe H, Tamura T, Kagohashi K, Takayashiki N, Kurishima K, Satoh H, et al., Successful treatment of EGFR-mutated non-small cell lung cancer with reduced-dose gefitinib: a case report, Exp. Ther. Med 10 (2015) 386–388. [PubMed: 26170967]

[70]. Weber HC, Regulation and signaling of human bombesin receptors and their biological effects, Curr. Opin. Endocrinol. Diabetes Obes 16 (2009) 66–71. [PubMed: 19115523]

[71]. Weber HC, Walters J, Leyton J, Casibang M, Purdom S, Jensen RT, et al., A bombesin receptor subtype-3 peptide increases nuclear oncogene expression in a MEK-1 dependent manner in human lung cancer cells, Eur. J. Pharmacol 412 (2001) 13–20. [PubMed: 11166731]

[72]. Yang H, Wang R, Peng S, Chen L, Li Q, Wang W, Hepatocyte growth factor reduces sensitivity to the epidermal growth factor receptor-tyrosine kinase inhibitor, gefitinib, in lung adenocarcinoma cells harboring wild-type, Oncotarget 7 (2016) 16273–16281. [PubMed: 26919104]

[73]. Yang HK, Scott FM, Trepel JB, Battey JF, Johnson BE, Kelley MJ, Correlation of expression of bombesin-like peptides and receptors with growth inhibition by an anti-bombesin antibody in small-cell lung cancer cell lines, Lung Cancer 21 (1998) 165–175. [PubMed: 9857994]

[74]. Yu SY, Liu HF, Wang SP, Chang CC, Tsai CM, Chao JI, Evidence of securinmediated resistance to gefitinib-induced apoptosis in human cancer cells, Chem. Biol. Interact 203 (2013) 412–422. [PubMed: 23523951]

[75]. Zemtsova GE, Montgomery M, Levin ML, Relative sensitivity of conventional and real-time PCR assays for detection of SFG Rickettsia in blood and tissue samples from laboratory animals, PLoS One 10 (2015) e0116658. [PubMed: 25607846]

[76]. Zhang Q, Bhola NE, Lui VW, Siwak DR, Thomas SM, Gubish CT, et al., Antitumor mechanisms of combined gastrin-releasing peptide receptor and epidermal growth factor receptor targeting in head and neck cancer, Mol. Cancer Ther 6 (2007) 1414–1424. [PubMed: 17431120]

[77]. Zhang Y, Sheng J, Yang Y, Fang W, Kang S, He Y, et al., Optimized selection of three major EGFR-TKIs in advanced EGFR-positive non-small cell lung cancer: a network metaanalysis, Oncotarget 7 (2016) 20093–22108. [PubMed: 26933807]

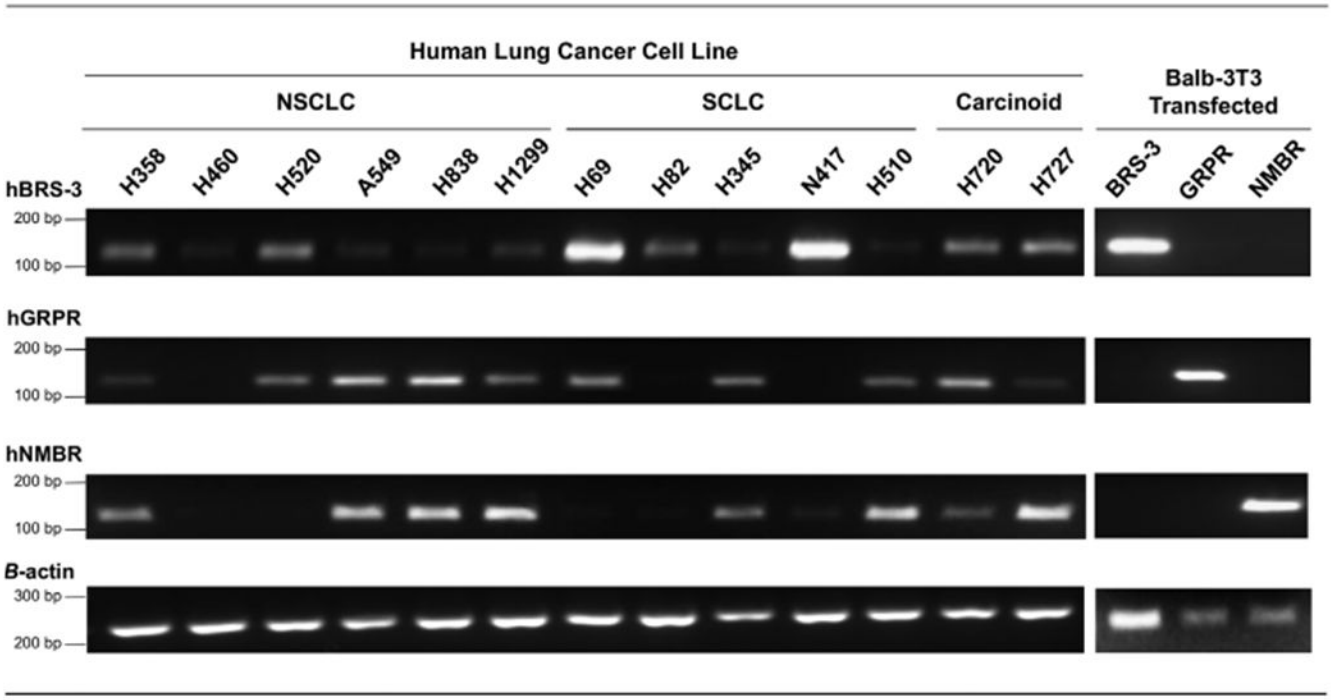

Fig. 1.

Detection of human BRS-3 receptor, GRPR and NMBR expression by PCR in 13 different human lung-cancer cell types. The mRNA was isolated from 6 NSCLC, 5 SCLC and 2 carcinoid cells (3×10^6 cells), and it was reverse transcribed into cDNA that was amplified using gene-specific primers for each bombesin receptor (BnR) and for β-actin (Product size: BRS-3, 120 bp; GRPR, 113 bp; NMBR, 131 bp and β-actin, 205 bp). The PCR products were analyze in 3% agarose gel and visualized by ethidium bromide staining. Total mRNA from transfected cells (hBRS-3, GRPR and NMBR) was used as control of each receptor and mRNA from β-actin was used as a PCR control in the lung-cancer cells. Note that most cell lines express at least one of the three BnRs. Each result is representative of three other experiments in which new cDNA was used.

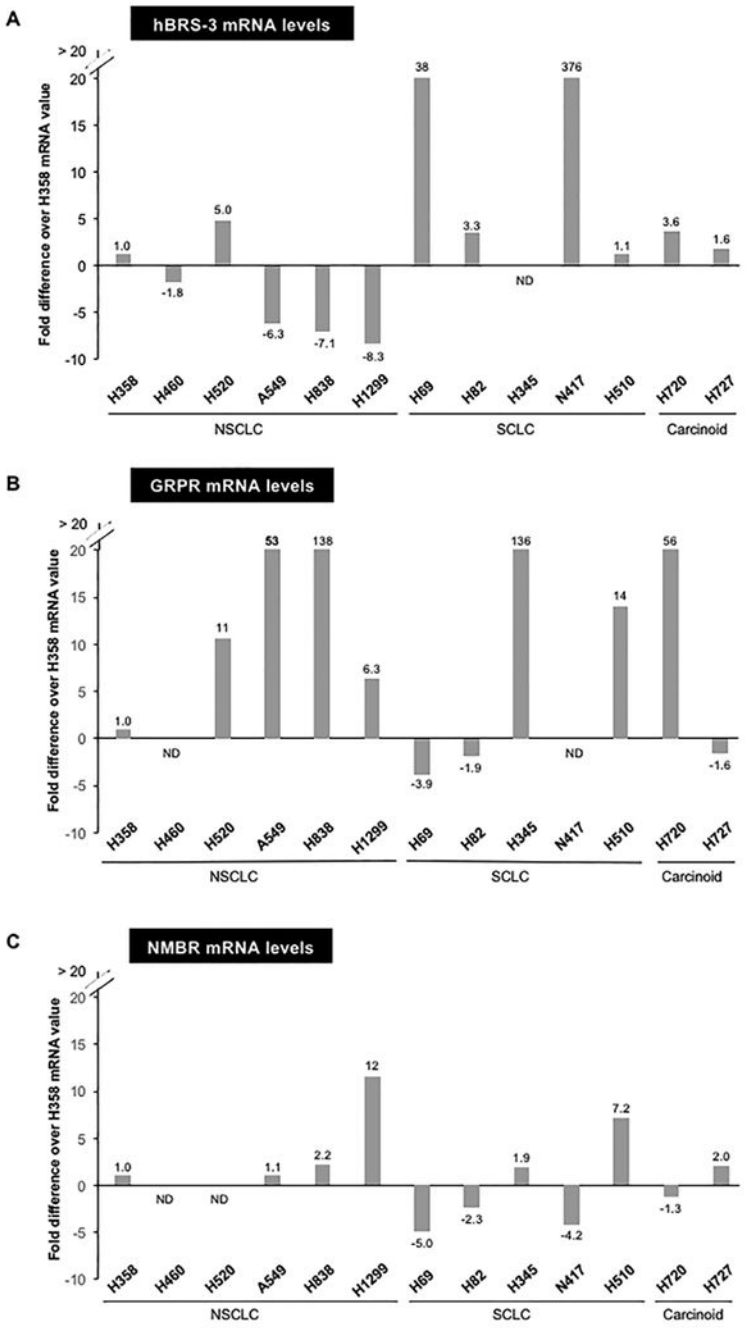

Fig. 2.

Analysis of human BRS-3 receptor, GRPR and NMBR mRNA levels by quantitative RT-PCR In 13 different human lung cancer cell types. Results from qRT-PCR In the 13 different human lung cancer cell lines tested, were normalized with the housekeeping gene β-actin and expressed as fold Increase over the positive mRNA-value of the NSCLC-NCI-H358 cell-line, which expressed each BnR subtype, using the $2^{-\Delta\Delta Ct}$method. NCI-H358 positive mRNA-level for BRS-3 (A) was 31.02 ± 0.39 Ct with a β-actin ratio of 18.27 ± 0.58 Ct. In the case of GRPR (B), NCI-H358 mRNA level was 31.07 ± 0.39 Ct and the β-actin was

18.50 ± 0.68 Ct. The value of NCI-H358 mRNA level for NMBR (C) was 30.84 ± 0.56 Ct with a β-actin ratio of 18.41 ± 0.69 Ct. Numbers in parenthesis show the fold increase and ND indicates cell lines in which the indicated BnR sub-type was not detectable. The results are from at least six experiments and in each experiment the data points were determined in triplicate and new cDNA was used in each experiment.

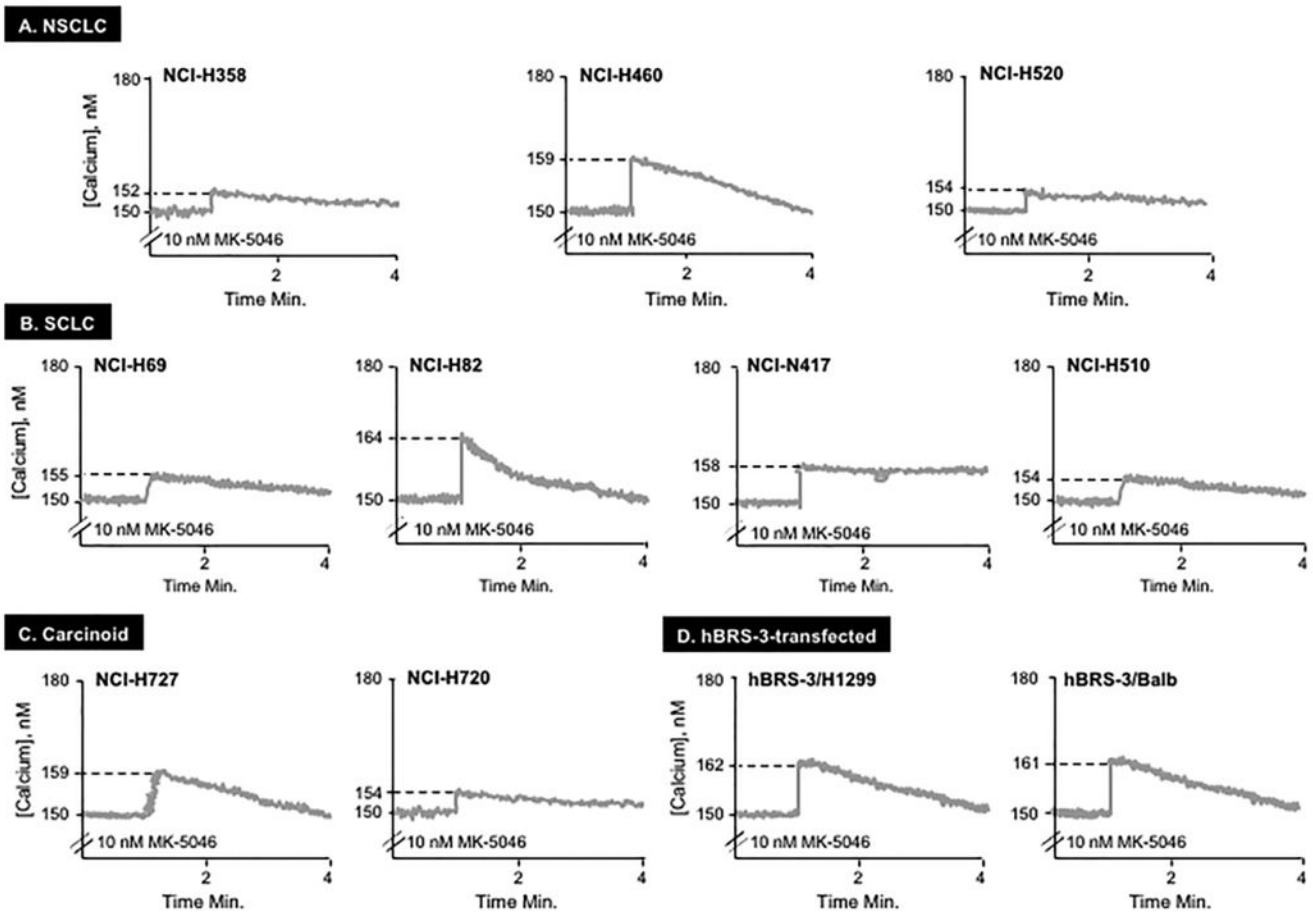

Fig. 3.

Stimulation of changes in cytosolic Ca2+ by the BRS-3 selective agonist, MK-5046 in various BRS-3 containing lung cancer cells. Results are shown with 3 NSCLC cell lines (A), 4 SCLC cell lines (B), 2 carcinoid cell lines (C) and 2 hBRS-3 transfected cells (D). All the cells ($2.5 \times 10^6 - 4 \times 10^6$ cells/ml) were loaded with 1 μM Fura-2AM and the cytosolic Ca2+ was determined after the addition of 10 nM of the nonpeptide agonist MK-5046. The results are representative of at least five experiments.

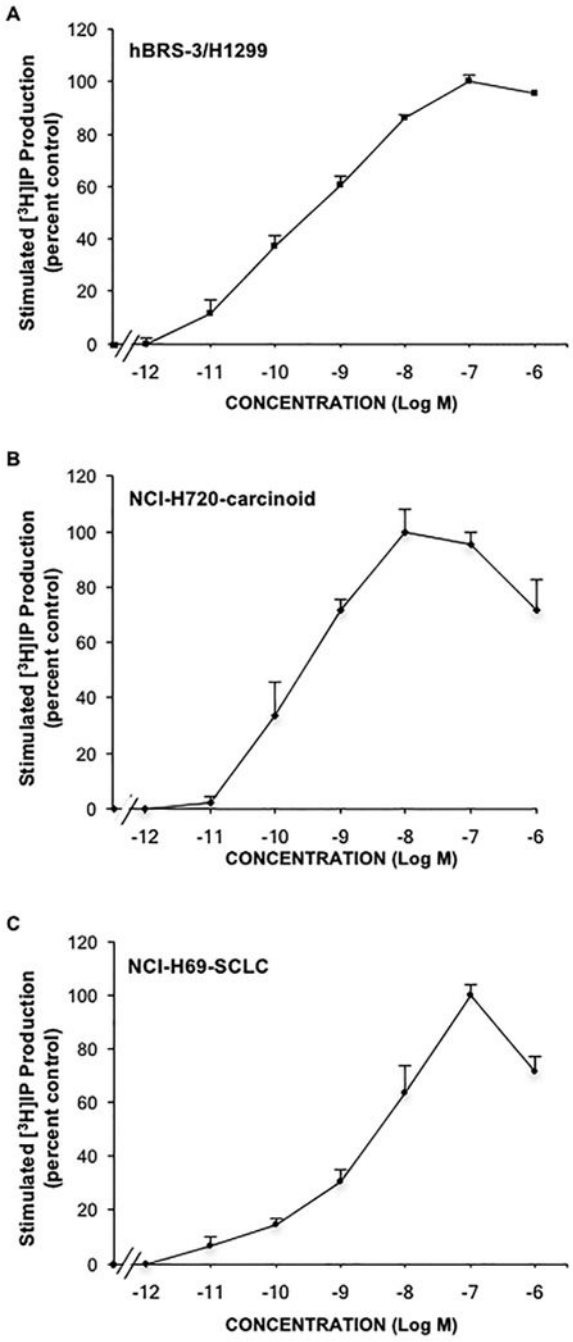

Fig. 4.

Ability of the BRS-3 agonist, MK-5046, to stimulate [3H]-Inositol phosphate ([3H]IP) generation in three lung-cancer cell lines. Results are shown with the NSCLC cell NCI-H1299 transfected with hBRS-3 receptor (A), in the carcinoid cell NCI-H720 (B) and in the SCLC cell NCI-H69 (C). After loading the cells with 3μCi/ml of myo-[2–3H] inositol, each cell type was incubated with the indicated concentrations of MK-5046 for 60 min at 37 °C. The results are expressed as the percentage of increase over control (no treatment with MK-5046). The [3H]IP measurement was determined as described in Materials & Methods.

The results are the mean ± S.E.M. from at least five experiments and in each experiment the data points were determined in duplicate. In the hBRS-3-NCI-H1299 cells (A), the maximal stimulated [3H]IP value by 100 nM of MK-5046 was 10,144 ± 1310 dpm and the control value was 1169 ± 88 dpm (n = 21). In NCI-H720 cells (B), the maximal stimulated [3H]IP value was 375 ± 31 dpm reached at 10 nM of MK-5046 and the control value was 268 ± 23 dpm (n = 10). In NCI-H69 cells (C), the maximal stimulated [3H]IP value by 100 nM of MK-5046 was 834 ± 66 dpm and the control value was 177 ± 27 dpm (n = 8).

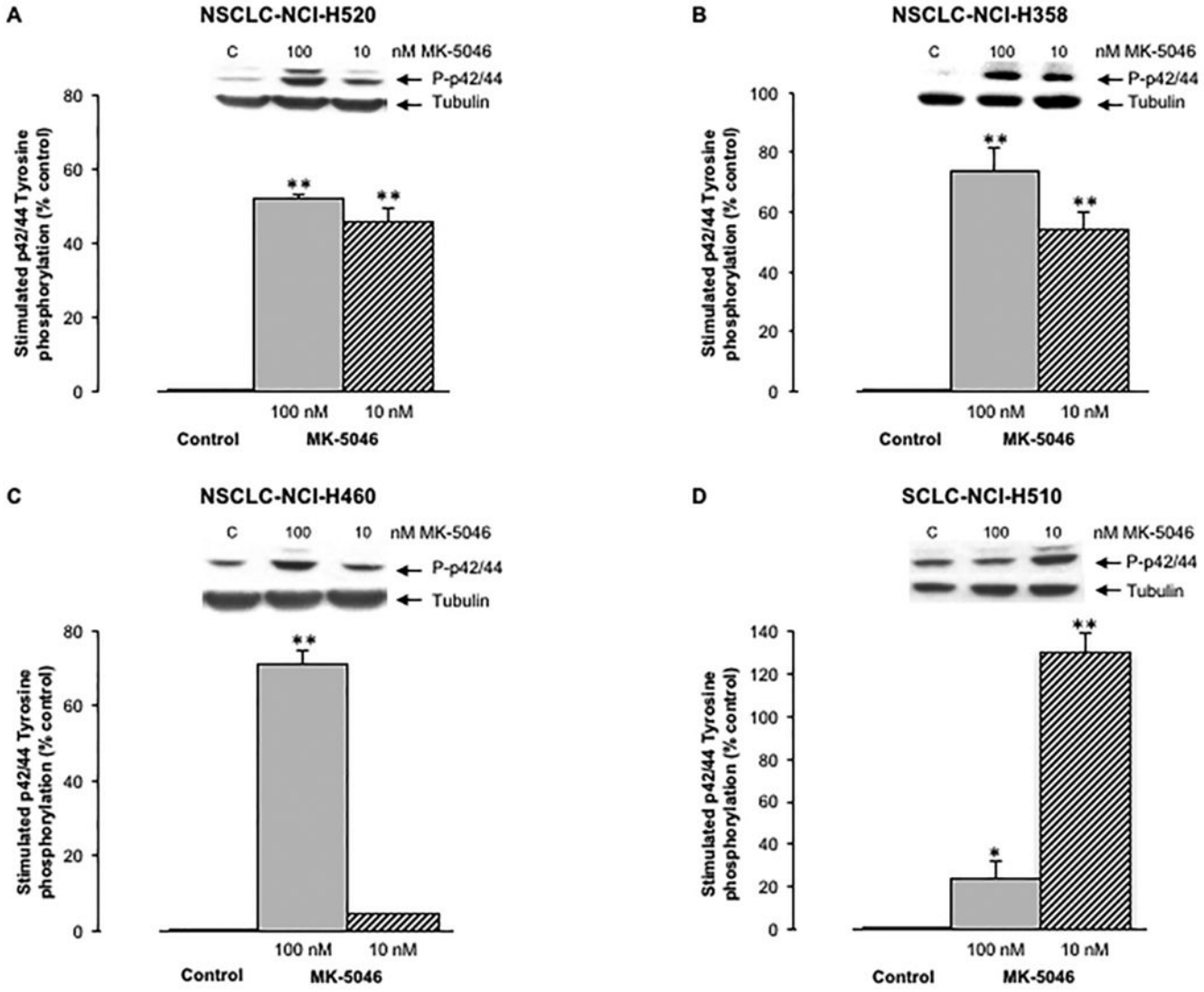

Fig. 5.

Stimulation of p42/44 MAPK phosphorylation by MK-5046 in 4 different hBRS-3 lung cancer cells. Result are shown with NSCLC-NCI-H520 (A), NSCLC-NCI-H358 (B), NSCLC-NCI-H460 (C) and SCLC-NCI-H510 (D). Representative p42/44 Western blots of the human lung cancer cells treated with 10 nM and 100 nM of MK-5046 for 3 min at 37 °C, are shown in the top of each panel. In the bottom of each panel are shown the mean ± S.E.M. from at least five experiments and each experiment was determined in duplicate. Results are expressed as the percentage of increase over control (no treated with MK-5046). *$p < 0.01$ vs control. **$p < 0.0001$ vs control.

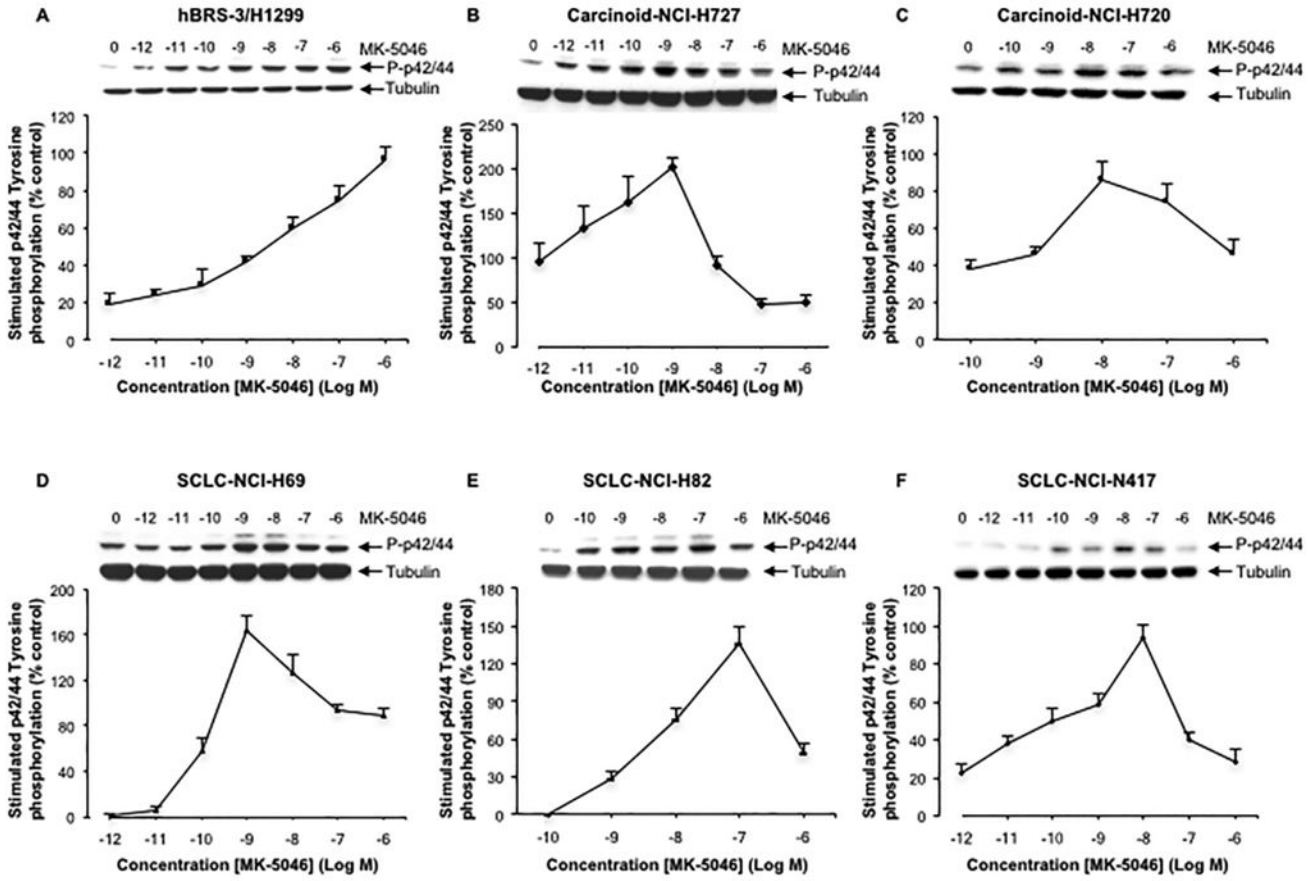

Fig. 6.

Dose-response of p42/44 MAPK phosphorylation stimulated by MK-5046 in various BRS-3 positve cell lines. Hown are resluts from hBRS-3-transfected NCI-H1299 cells (A), in 2 carcinoid cell lines, NCHI-H727 and NCI-H720 (B & C), and in 3 different SCLC cell lines, NCI-H69, NCI-H82 and NCI-N417 (D–F). In each panel, at the top, is shown a representative Western blot of the activation of p42/44 in each of the cells treated with different MK-5046 concentrations. In the bottom of each panel are shown the mean ± S.E.M. from at least five experiments and each experiment was determined in duplicate. Results are expressed as the percentage of increase over control (not treated with MK-5046).

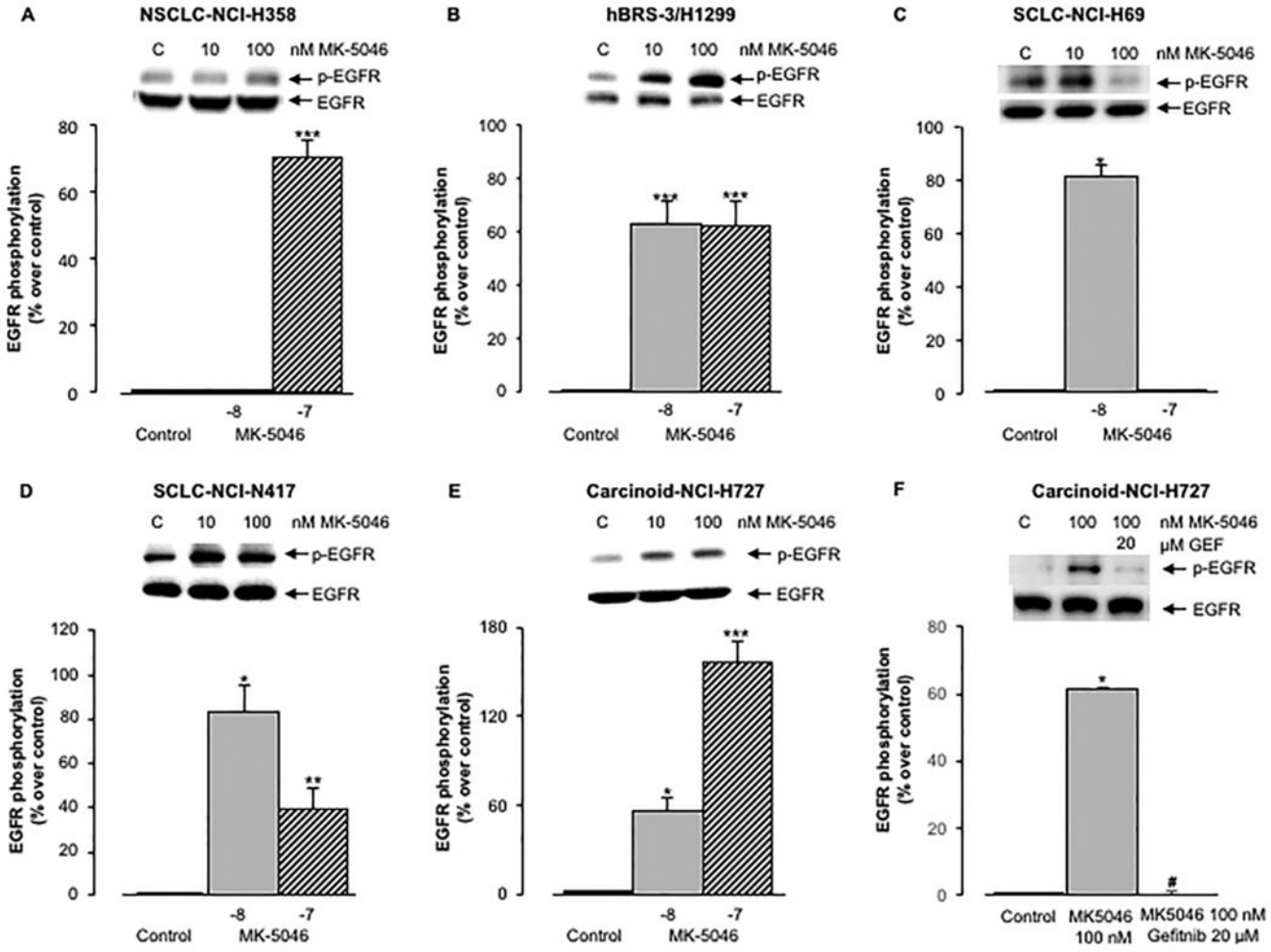

Fig. 7.

EGFR transactivation stimulated by MK-5046 (A–E) in 5 different human lung cancer cells and the ability of gefitinib to inhibit the EGFR transactivation stimulated by MK-5046 (F). Representative Western blots of EGFR-tyrosine phosphorylation stimulated by MK-5046 are shown at the top of each panel. At the bottom of each panel are shown the mean ± S.E.M. from at least five experiments and each experiment was determined in duplicate. Results are expressed as the percentage of increase over control (no treated with MK-5046). Panel F show the results on MK-5046 stimulated EGFR transactivation after preincubation with the EGFR tyrosine kinase inhibitor, gefitinib. *$p < 0.01$ vs control; **$p < 0.001$ vs control; ***$p < 0.0001$ vs control; $^{\#}p < 0.001$ vs MK-5046.

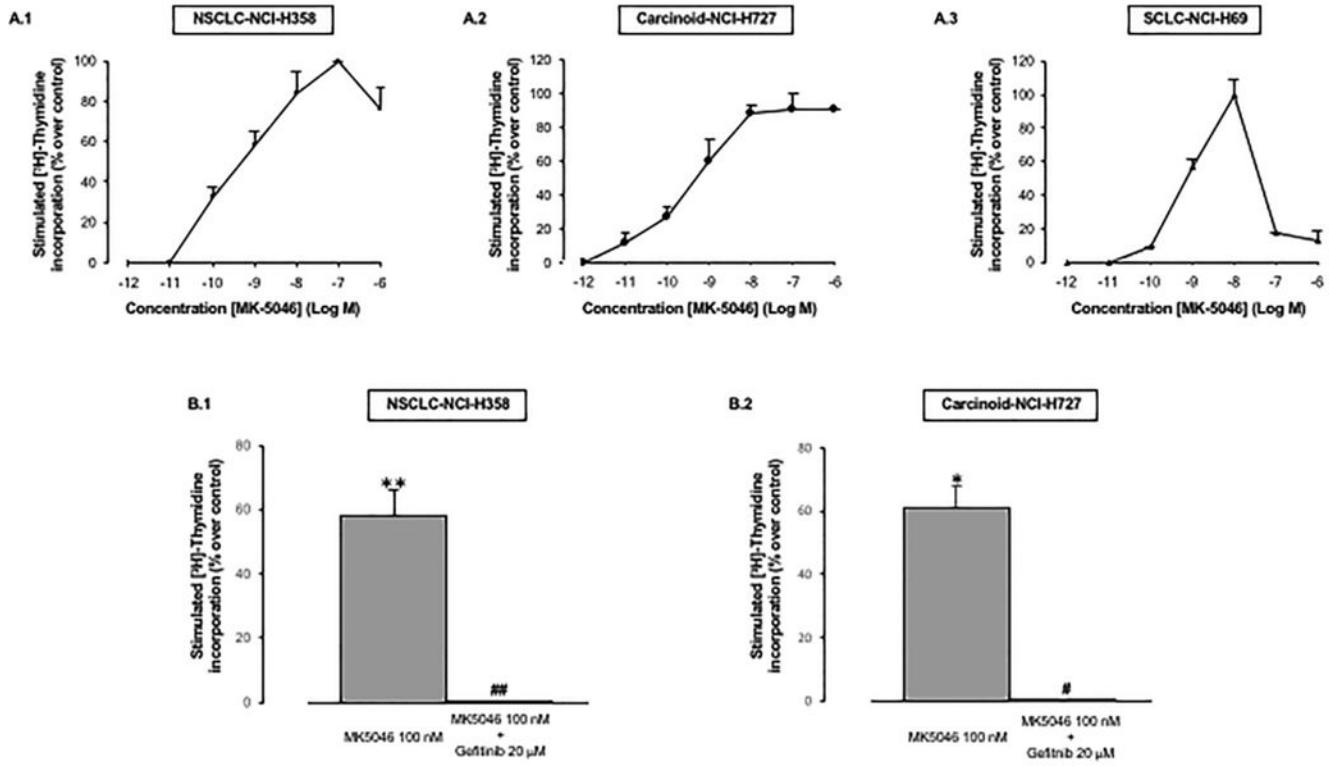

Fig. 8.

Ability of MK-5046 to stimulate cell growth in a dose-dependent manner (Panel A) and effect of gefitinib on MK-5046 stimulated growth (Panel B) in various lung-cancer cell lines. In panel A, the cells were incubated for 24 h in serum-free medium with different MK-5046 concentrations (1 pM to 1 μM), while in panel B, cells were incubated for 24 h in 10 nM of MK-5046 and 20 μM of gefitinib. Cell growth was evaluated by [^{3}H]-Thymidine uptake as described in Materials and Methods. Results are the mean ± S.E.M. from at least six experiments each experiment was determined in duplicate. Results in both panels are expressed as the percentage of increase over control (no treated either with MK-5046 or gefitinib). *$p < 0.05$ vs control; **$p < 0.005$ vs control; $^{\#}p < 0.005$ vs MK-5046; $^{\#\#}p < 0.0005$ vs MK-5046.

Table 1

Ability to activate phospholipase C causing [^{3}H]IP production, Ca^{2+} release and MAPK phosphorylation by the selective BRS-3 agonist, MK-5046, in human lung-cancer cells.

	[^{3}H]IP (Exp/control)		Ca^{2+} release (% over basal)	P-p42/44 (Exp/control)	
	MK-5046 [10 nM]	**MK-5046 [100 nM]**	**MK-5046 [10 nM]**	**MK-5046 [10 nM]**	**MK-5046 [100 nM]**
NSCLC					
NCI-H358	1.50 ± 0.15**	1.02 ± 0.10	1.65 ± 0.43*	2.33 ± 0.34*	2.82 ± 0.29***
NCI-H460	1.63 ± 0.18*	1.24 ± 0.16	5.77 ± 1.41**	1.07 ± 0.16	3.38 ± 0.46***
NCI-H520	1.84 ± 0.36*	1.00 ± 0.18	2.60 ± 0.40*	1.88 ± 0.24**	2.32 ± 0.21***
BRS-3 transfected					
NCI-H1299	7.63 ± 0.77***	8.97 ± 0.42***	8.04 ± 0.55***	1.54 ± 0.28	2.16 ± 0.34**
Balb-3T3	7.50 ± 0.68***	7.96 ± 0.47***	7.43 ± 1.22**	1.85 ± 0.21***	1.41 ± 0.24*
Carcinoid					
NCI-H727	2.59 ± 0.22***	1.46 ± 0.20*	6.26 ± 1.43*	2.41 ± 0.34**	1.73 ± 0.20*
NCI-H720	1.64 ± 0.22*	1.55 ± 0.41	2.40 ± 0.28**	2.53 ± 0.39**	2.36 ± 0.55**
SCLC					
NCI-H69	3.38 ± 0.17***	4.81 ± 0.44***	3.09 ± 0.67**	1.95 ± 0.18***	1.75 ± 0.17***
NCI-H82	2.09 ± 0.33*	1.61 ± 0.32	9.00 ± 1.11***	1.50 ± 0.10***	2.16 ± 0.26***
NCI-N417	4.31 ± 1.03**	3.94 ± 1.00**	4.40 ± 0.62***	1.64 ± 0.25**	1.03 ± 0.10
NCI-H510	1.12 ± 0.05	1.00 ± 0.05	2.94 ± 0.52**	2.22 ± 0.13***	1.70 ± 0.26*

Data, mean ± SEM, are expressed as percentage of the change of $[Ca^{2+}]_I$ caused by addition of 10 nM MK-5046 or as value of [^{3}H]-IP or P-p42/44 in the experimental samples divided by control (not MK-5046 treated). Data are from 5 to 18 separated experiments.

Abbreviations: NSCLC (non-small cell lung-cancer); SCLC (small cell lung-cancer); Exp/cont (Experimental sample/control).

* $p < 0.05$.

** $p \leq 0.01$.

*** $p \leq 0.0001$ *vs* control.

Table 2

Ability of various bombesin receptor antagonists, Bantag-1 (BRS-3), PD168368 (NMBR) and ME (GRPR), to inhibit phospholipase C and [^{3}H]IP production in human lung-cancer

	[^{3}H]IP (DPM)			
	SCLC		Carcinoid	BRS-3 transfected
	NCI-H69	NCI-N417	NCI-H727	Balb-3T3
Control	177 ± 27	660 ± 87	143 ± 21	2487 ± 185
MK-5046				
10 nM	597 ± 29***	2850 ± 571***	371 ± 32***	18657 ± 1698***
MK-5046				
10 nM				
+ Bantag	256 ± 56$^{\#\#}$	678 ± 82$^{\#\#}$	215 ± 25$^{\#\#}$	3697 ± 1064$^{\#\#\#}$
100 nM				
Peptide $^{\#}$1				
10 nM	680 ± 73***	2131 ± 244***	235 ± 25**	16341 ± 1071***
Peptide $^{\#}$1				
+ Bantag	265 ± 35$^{\#\#}$	1125 ± 154$^{\#\#\#}$	176 ± 27	4980 ± 503$^{\#\#\#}$
100 nM				
+ ME 1 μM	476 ± 128	2513 ± 662	189 ± 56	16107 ± 100
+ PD 1 μM	471 ± 121	2176 ± 518	157 ± 48	15006 ± 769
NMB				
100 nM	186 ± 20	706 ± 132	278 ± 48*	2697 ± 720
1 μM	187 ± 37	707 ± 137	178 ± 30	2652 ± 838
GRP				
100 nM	180 ± 19	881 ± 204	254 ± 40*	2519 ± 744
1 μM	200 ± 41	724 ± 138	164 ± 32	2537 ± 769

Data, mean ± SEM, are expressed as DPM from 5 to 32 separated experiments. Abbreviations: NSCLC (non-small cell lung-cancer): SCLC (small cell lung-cancer); ME ([D-Phe^6]Bombesin-(6–13)-methyl ester); peptide #1 ([D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]Bn-(6–14).

$^{\#}p < 0.05$.

$^{*}p < 0.05$.

**p≤0.01.

$^{***}p \leq 0.0001$ *vs* control.

$^{\#\#}p \leq 0.01$.

$^{\#\#\#}p \leq 0.0001$ *vs* BRS-3 agonist (MK-5046 or peptide #1).

Table 3

Ability of the h-BRS-3 agonist, MK-5046, to stimulate ^{3}H-Thymidine uptake and the EGFR transactivation in human lung-cancer cells.

	EGFR transactivation (Exp/control)		[^{3}H]-Thymidine Incorporation (Exp/control)	
	MK-5046 [10 nM]	**MK-5046 [100 nM]**	**MK-5046 [10 nM]**	**MK-5046 [1 μM]**
NSCLC				
NCI-H358	NS	1.60 ± 0.18***	1.20 ± 0.05**	1.15 ± 0.06*
NCI-H460	NS	NS	1.29 ± 0.07*	1.07 ± 0.07
NCI-H520	NS	NS	1.27 ± 0.10*	1.07 ± 0.14
BRS-3-transfected				
NCI-H1299	3.39 ± 0.79**	3.76 ± 0.99*	1.41 ± 0.06**	1.27 ± 0.11*
Balb-3T3	NS	NS	1.00 ± 0.04	1.24 ± 0.07*
Carcinoid				
NCI-H727	1.57 ± 0.09***	1.96 ± 0.31**	1.41 ± 0.18*	1.47 ± 0.16**
NCI-H720	NS	NS	1.15 ± 0.04*	1.17 ± 0.03*
NSCLC				
NCI-H69	1.81 ± 0.05**	1.00 ± 0.09	1.36 ± 0.09*	0.76 ± 0.07
NCI-H82	NS	NS	1.08 ± 0.08	1.17 ± 0.05*
NCI-N417	1.90 ± 0.26*	1.31 ± 0.05**	1.29 ± 0.08*	1.26 ± 0.07*
NCI-H510	1.99 ± 0.23**	2.36 ± 0.32* (0.1 nM)#	1.39 ± 0.04***	1.36 ± 0.10*

Data, mean ± SEM, are expressed as value of P-EGFR or [^{3}H]-thymidine incorporation in the experimental samples divided by control (not MK-5046 treated). Data are from 5 to 16 separated experiments.

Abbreviations: NSCLC (non-small cell lung-cancer); SCLC (small cell lung-cancer); NS (no stimulation).

MK-5046 0.1 nM.

* $p \leq 0.05$.

** $p \leq 0.01$.

*** $p \leq 0.0001$ *vs* control.