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Research Article

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.

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, primarily 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

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 cancer; 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

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(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 cell-lines; Dulbecco's minimum-essential medium (DMEM), Roswell Park Institute medium1X (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 1X, 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 10x-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–12 K 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