BOMBESIN RECEPTORS REGULATE EGFR TRANSACTIVATION IN CANCER

Terry W. Moody

Department of Health and Human Services, National Institutes of Health, National Cancer Institute, Center for Cancer Research, Office of the Director, Bethesda, MD USA (moodyt@mail.nih.gov)

Correspondence to:

Dr. Terry Moody
Office of the Director, CCR, NCI
9609 Medical Center Drive, Rm. 2W340
Bethesda, MD 20892 USA
Email; moodyt@mail.nih.gov
240-276-7785

Abstract—Bombesin (BB)-like peptides are autocrine growth factors for small cell lung cancer (SCLC), a neuroendocrine tumor. BB-like peptides are present in and secreted from SCLC cells, where they bind to cell surface G protein coupled receptors (GPCR). When the BB_2R is activated it interacts with a G-protein (Gq) causing signal transduction mechanisms which lead to increased cellular proliferation. The growth of SCLC cells is inhibited by BB_2R antagonists. In non-SCLC (NSCLC) cells, but not SCLC cells, the receptor tyrosine kinase (RTK) for epidermal growth factor (EGF) predominates. Addition of NMB to NSCLC cells, causes tyrosine phosphorylation of the EGFR through a process called transactivation. The EGFR transactivation caused by NMB addition to NSCLC cells is inhibited by BB_1R antagonists or EGFR tyrosine kinase inhibitors (TKI) such as gefitinib or erlotinib. BB_1R antagonists are synergistic with gefitinib at inhibiting NSCLC growth. The BB_1R may regulate the growth of NSCLC, an epithelial tumor, in an EGFR-dependent manner.

Keywords—bombesin, EGF receptors, transactivation, tyrosine kinase inhibitors, bombesin receptor antagonists, lung cancer

INTRODUCTION

Bombesin (BB)-like peptides are biologically active in the central nervous system (CNS) and periphery. The BB-like peptides bind to G protein coupled-receptors (GPCR) and cause signal transduction. The 27 amino acid gastrin releasing peptide (GRP) binds with high affinity to the GRP (BB_2) receptor (R), whereas the 10 amino acid neuromedin B (NMB) binds with high affinity to the NMB (BB_1) R. Structurally related to the BB_1R and BB_2R is the orphan bombesin receptor subtype 3 (BRS-3) which binds neither GRP nor NMB with high affinity. The BB_2R is overexpressed in 92% of SCLC, 76% of NSCLC, breast cancer, pancreatic cancer, prostate cancer, head/neck cancer, and brain cancers (Reubi *et al.,* 2002; Mattei *et al.,* 2014). The proliferation of these cancers is stimulated by BB or GRP but inhibited by BB_2R antagonists (Moody *et al.,* 1995). The BB_1R is overexpressed in 55% SCLC, 67% NSCLC, intestinal carcinoids, colon cancer, prostate cancer and glioblastoma and the proliferation is stimulated by NMB and inhibited by BB_1R antagonists (Jensen and Moody 2006). BRS-3 is overexpressed in 44% SCLC, 35% bronchial carcinoids prostate cancer, pancreatic cancer and pituitary adenomas (Fathi *et al.,* 1993) and the growth is inhibited by BRS-3 antagonists (Moody *et al.,* 2015). The BBR family containing BB_1R, BB_2R and BRS-3 are overexpressed in numerous cancers, especially lung cancer.

Synthetic agonists and antagonists are available for BB_1R, BB_2R and BRS-3. Table I shows that the 14 amino acid BB and 27 amino acid GRP are agonists which bind with high affinity to the BB_2R, whereas NMB is a 10 amino acid agonist which binds with high affinity to the BB_1R. BB, GRP and NMB have sequence homology at the C-terminal. PD168,368 is a nonpeptide BB_1R antagonist, whereas BW2258U89 is a peptide BB_2R antagonist. MK-5046 is a nonpeptide agonist for BRS-3 whereas Bantag-1 is a peptide antagonist for BRS-3. In contrast, bombesin agonist (BA) 1 is a universal agonist for BB_1R, BB_2R and BRS-3. The signal transduction mechanisms for BB $_1R$, BB_2R and BRS-3 are similar. The GRCRs interact with Gq activating phospholipase (PL) C which causes metabolism of PIP_2 to IP_3, which elevates cytosolic Ca^{2+} and diacylglycerol (DAG) which activates protein kinase (PK) C. The selective agonists and antagonists can be used to determine the physiological role of the BB_1R, BB_2R and BRS-3.

Table I. BB_1R, BB_2R and BRS-3 ligands

Ligand	IC_{50}, nM		
	BB_1R	BB_2R	BRS-3
BB	50	1	>10,000
BA1	1.5	0.3	2.5
GRP	250	0.2	>10,000
NMB	0.5	60	>10,000
Bantag-1	>10,000	>10,000	2
BW2258U89	>10000	10	>10,000
MK-5046	>10,000	>10,000	18
PD168,368	30	>10,000	>10,000

The IC_{50} (nM) to inhibit specific ^{125}I-BA1 binding to cells transfected with human BB_1R, BB_2R or BRS-3 is indicated. The structures of the ligands are shown below. Sequence homologies are relative to BB are underlined.

BB Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

GRP Val-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-Leu-Thr-Lys-
Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

NMB Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2

BA1 D-Tyr-Gln-Trp-Ala-Val-βAla -His-Phe-Nle-NH_2

BW2258U89 3-Phenylpropanoyl-His-Trp-Ala-Val-DAla-His-DProψPhe-NH_2

Bantag-1 [Boc-Phe-His-4-amino-5-cyclohexyl-2,4,5-trideoxyupentonyl-Leu-(3-dimethylamino)benzylamide N-methylammonium trifluoroacetate]

MK-5046 [(2S)-1,1,1-trifluoro-2-[4-(1H-pyrazol-1-yl)phenyl]-3-(5{[1-(trifluoromethyl)cyclopropyl]methyl}-1-imidazo-2-yl_propan-2-ol]

PD168368 (S)-a-methyl-a-[[[(4-nitrophenyl)amino]carbonyl]amino]-N-[[1-(2pyridinyl)cyclohexyl]methyl]-1H-indole-3-propanamide]

1. Lung cancer EGFR

NSCLC, which kills 130,000 U.S. patients annually, is treated with combination chemotherapy, however, the 5 year survival rate is only 16%. In 2004, EGFR tyrosine kinase inhibitors (TKI) were found to be effective in NSCLC patients whose tumors have certain EGFR mutations. Short in-frame deletions (exon 19) or the L858R mutation in exon 21 activate the EGFR tyrosine kinase (Shepherd *et al.,* 2005). Erlotinib or gefitinib are small molecule TKI that bind with high affinity to the mutant EGFR ATP binding site (Lynch *et al.,* 2004; Paez *et al.*, 2004) and are used to treat NSCLC patients who fail chemotherapy. Lapatinib, a TKI for the EGFR and HER2, is used to treat breast cancer patients while panitumumab (EGFR mAb) is used to treat metastatic colorectal cancer patients. Cetuximab (EGFR mAb) is used to treat patients with advanced squamous cell carcinoma of the head and neck. Thus TKI and mAbs are available to treat cancer patients whose tumors contain EGFR.

The wild type EGFR can be activated by several growth factors including amphiregulin, EGF, HB-EGF and transformingα(HanandLo,growth2012)facto r. (TGF) The EGFR can form homodimers with itself or heterodimers with HER2. The EGFR can phosphorylate itself or protein substrates such as PI3K. PI3K activates PDK-1 leading to tyrosine phosphorylation of Akt and mTor resulting in increased survival of cancer cells. The EGFR can interact with adapter proteins such as Grb2 which results in Ras and Raf activation leading to tyrosine phosphorylation of MEK and ERK. Phosphorylated ERK can enter the nucleus and increase nuclear oncogene expression leading to increased proliferation. The EGFR can activate JAK2 leading to STAT3 tyrosine phosphorylation. STAT3 forms homo- or heterodimers and enters the nucleus and alters gene transcription. While the EGFR is important in regulating cancer proliferation, resistance to EGFR TKI can develop (Nguyen *et al*. 2009). This acquired resistance is often due to secondary EGFR T790M mutations (Bell *et al.,* 2005). There is a need to develop new drugs to overcome lung cancer TKI resistance. GPCR antagonists can increase the potency of EGFR TKI in cancer (Moody *et al.,* 2015). In this communication, the mechanism by which the BBR regulates EGFR transactivation is reviewed.

2. BB_2R

The BB_2R is active in the normal central nervous system (CNS) and periphery. GRP is released from brain neurons and in a paracrine manner activates the BB_2R present in adjacent cells. BB or GRP administration into animals causes a variety of effects such as anxiety/fear responses, altered circadian rhythm, pruritis, satiety and hypothermia (Gonzalez *et al.,* 2008). GRP administration into the periphery stimulates hormone release especially gastrin from the stomach, stimulates smooth muscle contraction and increases gastrointestinal (GI) motility (Jensen *et al.,* 2008).

High levels of immunoreactive GRP are detected in extracts from SCLC cell lines (Moody et al., 1981; Wood *et al.,* 1981). The secretion of BB-like peptides from SCLC cells is increased in vitro and in vivo by vasoactive intestinal peptide (VIP) or secretin, which causes elevation of intracellular cAMP (Korman et al., 1987). A mAb named 2A11, which neutralizes

GRP, was developed which inhibits the

growth of SCLC in vitro and in vivo (Cuttitta *et al.*, 1985). 2A11was nontoxic in clinical trials but unfortunately only 1 SCLC patient out of 12 had a clinical response (Kelley *et al.*, 1997). SCLC, which kills approximately 30,000 U.S. citizens annually, is traditionally treated with radiation and chemotherapy. While patients initially respond to treatment, relapse occurs and the mean survival time is less than 1 year.

Subsequently BB_2R antagonists were developed. The 10 amino acid BW2258U89 inhibited the growth of SCLC xenografts in nude mice, however, it was cytostatic but not cytotoxic in that when its administration was discontinued the tumors resumed rapid growth (Moody *et al.*, 1995). Additional BB_2R antagonists include RC-3940-II and its analog RC-3095 which inhibits the growth of breast, colon, gastric, pancreatic, prostate and liver cancer cells (Szepeshazi *et al.*, 2012). In a phase I clinical trial, RC-3095 had little toxicity but was of no clinical benefit to patients with advanced solid cancers (Schwartsmann *et al.*, 2006).

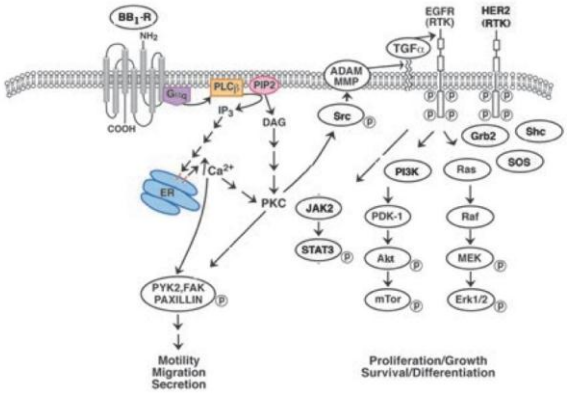
The BB_2R is detected using receptor binding techniques in gastrinomas, breast cancer, prostate cancer and SCLC (Reubi *et al.*, 2002). By immunocytochemistry, the BB_2R is present in SCLC and NSCLC biopsy specimens (Mattei *et al.*, 2014). In contrast, the EGFR is abundant in NSCLC and lung carcinoids but not SCLC cells (Moody *et al.*, 1990). Another tumor with high levels of BB_2R and EGFR is head and neck squamous cell carcinoma (HNSCC). GRP addition to HNSCC cells increased EGFR and ERK tyrosine phosphorylation after 2 min (Lui *et al.*, 2003). The EGFR and ERK tyrosine phosphorylation caused by GPR addition to HNSCC cells was inhibited by AG1478, an EGFR TKI, and GM6001, a matrix metalloprotease (MMP) inhibitor. Similarly, GM6001 and AG1478 blocked the ability of BB to increase EGFR transactivation and DNA synthesis (Santiskulvong and Rozengurt ; 2003). The HNSCC MMP increased production of transforming growth factor (TGF)αand amphiregulin (EGFR ligands) from inactive precursor proteins (Zhang *et al.*, 2004). The Src inhibitor A-419259 inhibited by ability of GRP to increase EGFR and ERK tyrosine phosphorylation in HNSCC cells. Subsequent studies showed that addition of GRP to HNSCC cells caused serine and threonine phosphorylation of TNF-α converting enzyme (TACE) in a Src-dependent manner (Zhang *et al.*, 2006). TACE is phosphorylated by PDK-1, leading to increased release of amphiregulin in a GRP-dependent manner. GRP addition to NSCLC cells increased phosphorylation of Akt, a PDK-1 substrate (Liu *et al.*, 2007). The phosphorylation of Akt was dependent upon c-Src and the EGFR. An important discovery was that PD176252, a BB_2R and BB_1 R antagonist, potentiated the growth inhibitory activity of erlotinib using HNSCC cells (Zhang *et al.*, 2007). PD176252 and erlotinib significantly inhibited HNSCC cellular proliferation relative to PD176252 or erlotinib alone by increasing apoptosis. As a result there were significantly less HNSCC cells in the S phase of growth when treated with PD176252 plus erlotinib relative to PD176252 or erlotinib alone. In addition PD176252 plus erlotinib significantly reduced phosphorylated EGFR, ERK and Akt in HNSCC cells relative to PD176252 or erlotinib alone. The results indicate that the BB_2R regulates EGFR transactivation and that GPCR antagonists potentiate the ability of TKI to reduce cancer cellular proliferation.

3. BB_1R

NMB administration into animals, similar to GRP, causes satiety, hypothermia and smooth muscle contraction (Gonzalez et al., 2008). NMB inhibits TSH release and hypothyroidism is associated with decreased pituitary NMB levels, whereas in hyperthyroidism there is increased pituitary NMB (Ortiga-Carvalho *et al.,* 2003). Immunoreactive NMB was present in 10/14 SCLC cell lines, 8/14 NSCLC cell lines and 5/5 lung carcinoids (Giaccone *et al.,* 1992). BB_1R receptor mRNA was detected in 14/14 NSCLC cell lines (Siegfried *et al.,* 1999). NMB is an autocrine growth factor in NSCLC.

Figure 1 shows that NMB addition to NSCLC cells increases cytosolic Ca^{2+} and PKC activity. This leads to phosphorylation of Src which tyrosine phosphorylates PYK2, FAK and paxillin altering cellular motility. Src activates ADAM and/or MMP increasing TGFαrelease, which binds to the EGFR. NMB addition to NSCLC cells increases EGFR tyrosine phosphorylation after 1 min (Moody et al., 2010). The increase in EGFR and ERK tyrosine phosphorylation caused by NMB addition to NSCLC cells is blocked by PD168368 or gefitinib. The increase in EGFR tyrosine phosphorylation is transient and after 30 min the phosphorylated EGFR levels returned to baseline due to protein tyrosine phosphatases. A surprising finding was that NMB addition to NSCLC cells increased reactive oxygen species (ROS), which may inhibit protein tyrosine phosphatases. Oxidation of cysteine in the catalytic Src homology 2-containing tyrosine phosphatase leads to reduced enzymatic activity resulting in a transient increase in EGFR tyrosine phosphorylation (Chen *et al.,* 2006). When tiron or N-acetylcysteine, which are antioxidants, are added to NSCLC cells, NMB did not increase EGFR tyrosine phosphorylation. The results indicate that the BB_1R regulates EGFR transactivation in a Src, MMP and ROS-dependent manner. Gefitinib or PD168368 inhibited the basal proliferation of NSCLC cells (Moody et al., 2010). Addition of PD168368 shifted the gefitinib dose-response curve to the left by approximately 1-order of magnitude. Thus GPCR antagonists increase the sensitivity of NSCLC cells with wild type EGFR to gefitinib. It remains to be determined if GPCR antagonists increase the sensitivity of NSCLC patients to gefitinib.

Figure 1. Mechanism of EGFR transactivation in NSCLC cells.



4. BRS-3

BRS-3 is an orphan receptor whose natural ligand is unknown. BRS-3 knockout mice, however, become obese, have impaired glucose metabolism and reduced metabolic rates (Ohki-Hamazaki *et al*., 1997). Subsequently BA1, a universal agonist was synthesized, and used to characterize BRS-3 (Mantey *et al.,* 1997). Recently, MK-5046, a selective nonpeptide agonist, was developed (Moreno et al., 2013). In addition, the peptide antagonist Bantag-1

was synthesized which binds with high affinity to BRS-3 but not BB_1 R or BB_2R. In a clinical trial, MK-5046 achieved sufficient blood levels to activate BRS-3 and the half-life was 0.5-3.5 hours (Reitman *et al.,* 2012). MK-5046 had no effect on body weight, appetite or plasma glucose in this trial and side effects included increased blood pressure. It remains to be determined if BRS-3 agonists will be effective anti-obesity drugs in humans.

Human BRS-3 contains 399 amino acids and has 51% sequence homology with the 384 amino acid BB_2R and 47% sequence homology with the 390 amino acid BB_1R. BRS-3 mRNA is present on 5/6 SCLC, 2/2 lung carcinoid and 3/5 NSCLC cell lines examined (Fathi *et al.,* 1993). BRS-3 immunoreactivity is present in bronchial and intestinal carcinoids, prostate cancer, pancreatic cancer as well as pituitary adenomas (Schulz *et al.,* 2006). Using lung cancer cells containing BRS-3, addition of BA1 transiently increased EGFR and ERK tyrosine phosphorylation that was blocked by a BRS-3 antagonist or gefitinib but not BB_1R or BB_2R antagonists (Moody *et al.,* 2011). Because the ability of BA1 to increase lung cancer EGFR tyrosine phosphorylation was blocked by PP2 or GM6001, the EGFR transactivation occurs in a Src or MMP-dependent manner, respectively. Also, the BRS-3 regulation of EGFR transactivation was blocked by Tiron, N-acetylcysteine or diphenylene iodonium, a NADPH oxidase inhibitor. NADPH oxidase may release ROS after activation of BRS-3. The results indicate that BRS-3, BB_1R and BB_2R regulate EGFR transactivation in a similar manner. MK-5046 addition to NCI-H417 SCLC cells caused ERK, FAK and Akt phosphorylation which was inhibited by Bantag-1 (Moreno *et al.,* 2013). Currently we are investigating if MK-5046 causes EGFR transactivation in NSCLC cells.

CONCLUSION

The EGFR regulates epithelial cancer proliferation by interacting with endogenous EGFR ligands such as amphiregulin, epigen, epiregulin, HB-EGFα. andTheTGFEGFR forms homodimers when activated by endogenous growth factors, but can also form heterodimers with other RTK such as HER-2 or HER-3. Finally RTK transactivation is regulated by GPCR such as the BB_1R, BB_2R and BRS-3. The BBRs cause PI turnover leading to EGFR transactivation in a MMP, ROS and Src-dependent mannerα. causing release of EGFR ligands such as TGF

In NSCLC, high densities of EGFR are present. Approximately 13% of the NSCLC patients have EGFR mutations such as L858R, which makes them responsive to TKI such as erlotinib or gefitinib. For NSCLC cells with wild type EGFR, the cytotoxicity of gefitinib is increased by the BB_1R and BB_2R antagonists PD168368 and PD176252, respectively. Currently, we are investigating if the gefitinib sensitivity is increased by Bantag-1 using NSCLC cells with BRS-3 and wild type EGFR. It remains to be determined if GPCR antagonists will increase the potency of TKI in NSCLC patients whose tumors have wild type EGFR.

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