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## International Union of Pharmacology. LXVIII. Mammalian Bombesin Receptors: Nomenclature, distribution, pharmacology, signaling and functions in normal and disease states.

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### Introduction

The unusual name of this family of receptors, bombesin (Bn), comes from the original terminology used by V. Erspamer and his colleagues to name the first natural ligand described, bombesin, which was an amidated tetradecapeptide isolated from the skin of the European frog *Bombina orientalis* (Erspamer *et al.*, 1970; Erspamer *et al.*, 1972) (Fig.1). They isolated many related peptides from other frog skins and most were named after the genus of frog from which it was isolated (Erspamer and Melchiorri, 1973; Erspamer, 1988). In terms of their structural similarities they were originally divided into three general groups (Fig.1): the bombesin group which all had a carboxyl terminus of Gly-His-Leu-Met-NH<sub>2</sub> [bombesin, altytesin, [pGlu<sup>1</sup>] bombesin<sub>6–14</sub>]; the ranatensin group which had a carboxyl terminus of Gly-His-Phe-Met-NH<sub>2</sub> (ranatensin, ranatensin R and C, litorin, rodhei-litorin, [Glu (Ote)<sup>2</sup> or (Ome)<sup>2</sup>] litorin) and the phyllolitorin group which had a carboxyl terminal Gly-Ser-Phe/Leu-Met-NH<sub>2</sub> [phyllolitorin, [Leu<sup>8</sup>]phyllolitorin, [Thr<sup>5</sup>, Leu<sup>8</sup>]Phyllolitorin (Falconieri Erspamer *et al.*, 1988; Erspamer, 1988)] (Fig.1). Recent molecular studies show the occurrence of these peptides in amphibian's skins is more complicated than originally thought with both Leu and Phe penultimate forms present in the same frog species in many cases (Nagalla *et al.*, 1996; Spindel, 2006). For example in the skin of the frog, *Bombina orientalis* [Leu<sup>13</sup>] bombesin, [Phe<sup>13</sup>] bombesin and [Ser<sup>3</sup>, Arg<sup>10</sup>, Phe<sup>13</sup>] bombesin (SAP bombesin) are found and each of these three forms are derived from separate genes (Nagalla *et al.*, 1996; Spindel, 2006).

Subsequently, in mammals two Bn-like peptides were isolated, gastrin-releasing peptide (GRP) (McDonald *et al.*, 1979) and neuromedin B (NMB) (Minamino *et al.*, 1983). GRP, a 27 amino acid peptide was originally isolated from porcine stomach and shares the same seven carboxyl terminal amino acids with bombesin (McDonald *et al.*, 1979) accounting for similar biological activity (Fig.1). The decapeptide of GRP was later isolated from porcine spinal cord and originally called neuromedin C (Minamino *et al.*, 1984b), although it is recommended a more appropriate name is either GRP-10 or GRP<sub>18–27</sub> (Anonymous, 1988). The mammalian equivalent of ranatensin, NMB, was isolated from porcine spinal cord and shown to be a decapeptide (McDonald *et al.*, 1979), which also occurs in precursor forms of 30 and 32 amino acids (Minamino *et al.*, 1985). The carboxyl terminal seven amino acids are identical in

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ranatensin, except for the replacement of threonine in NMB for valine in ranatensin at the penultimate position from the carboxyl terminus (Fig.1).

Studies of GRP and NMB immunoreactivity as well as mRNA studies have demonstrated these peptides and their mRNA are widely distributed in mammals in both the nervous system and peripheral tissues, especially the gastrointestinal tract (Penman *et al.*, 1983; Wada *et al.*, 1990; Battey and Wada, 1991; Spindel *et al.*, 1993; Moody and Merali, 2004). In the alimentary tract GRP-like IR is found primarily in neurons as well as the submucosal and myenteric plexuses and not in endocrine cells (Penman *et al.*, 1983). Using Northern blots the highest levels of mRNA occur in colon with lower amounts in the stomach and small intestine (Sunday *et al.*, 1988). In the spinal cord GRP-IR was found in both the posterior and anterior horn and in the CNS, GRP-IR and mRNA is widely distributed in neurons with high levels in the hypothamic nuclei, forebrain and medullary nuclei that participate in autonomic functions, as well as in sensory nuclei (Panula *et al.*, 1982; Panula *et al.*, 1988; Wada *et al.*, 1990; Battey and Wada, 1991; Spindel *et al.*, 1993). NMB-IR and mRNA is found throughout the GI tract, but generally at lower levels than GRP except in the esophagus (Spindel *et al.*, 1993). In general in the brain and spinal cord, NMB-IR is greater than GRP-IR (Minamino *et al.*, 1984a), NMB mRNA is most abundant in the olfactory bulb, dentate gyrus and dorsal root ganglia, whereas GRP mRNA is highest in the forebrain and some hypothamic nuclei (Wada *et al.*, 1990; Battey and Wada, 1991). In most brain regions the NMB mRNA distribution does not overlap with GRP (Wada *et al.*, 1990; Battey and Wada, 1991; Moody and Merali, 2004; Ohki-Hamazaki *et al.*, 2005).

The mammalian bombesin peptides, GRP and NMB demonstrate a broad spectrum of pharmacological and biological responses. GRP stimulates smooth muscle contraction in both the gastrointestinal tract and urogenital system and has profound effects on GI motility; stimulates release of numerous gastrointestinal hormones/neurotransmitters: stimulates secretion and/or hormone release from the pancreas, stomach, colon, and numerous endocrine organs; has potent effects on immune cells (macrophages, dendritic cells, lymphocytes, leukocytes) (Ruff *et al.*, 1985; De la Fuente *et al.*, 1991; van Tol *et al.*, 1993; De la Fuente *et al.*, 1993b; Del Rio and De la Fuente, 1994; Del Rio *et al.*, 1994; Plaisancie *et al.*, 1998; Makarenkova *et al.*, 2003); has potent growth effects on both normal tissues and tumors; has potent CNS effects, including regulation of circadian rhythm, thermoregulation; regulation of anxiety and fear response, food intake, behavioral effects as well as involved in mediating numerous CNS effects on the GI tract (Tache *et al.*, 1988; Bunnnett, 1994; Martinez and Tache, 2000; Jensen *et al.*, 2001; Jensen, 2003; Grider, 2004; Jensen and Moody, 2006). In many tissues the effects of NMB overlap with GRP, however NMB has specific effects in some tissues such as: contraction of smooth muscle; growth effects in various tissues (Moody *et al.*, 2000; Matusiak *et al.*, 2005); CNS effects including on feeding, thermoregulation; regulation of TSH release, stimulation of various CNS neurons, behavioral effects; and effects on spinal sensory transmission (von Schrenck *et al.*, 1989; Rettori *et al.*, 1992; Ladenheim *et al.*, 1997b; Ohki-Hamazaki, 2000; Merali *et al.*, 2006; Oliveira *et al.*, 2006). GRP and to a lesser extent NMB affects the growth and/or differentiation of a number of important human tumors including colon, prostate, lung and some gynecologic cancers (Cuttitta *et al.*, 1985; Schally *et al.*, 2000; Jensen *et al.*, 2001; Glover *et al.*, 2003; Jensen and Moody, 2006).

Early studies on the biologic effects of the different bombesin peptides isolated from frog skins, primarily examining their effects on contraction of isolated smooth muscle preparations from various tissues, demonstrated markedly varying potencies, suggesting more than one subtype of bombesin receptor might exist (Falconieri Erspamer *et al.*, 1988; Regoli *et al.*, 1988; Severi *et al.*, 1991). Binding studies and the development of highly selective antagonists established unequivocally the existence of two different classes of receptors in mammalian tissues mediating the actions of these peptides (Moody *et al.*, 1978; Jensen *et al.*, 1978; Jensen and

Gardner, 1981; Coy *et al.*, 1988; von Schrenck *et al.*, 1989; Ladenheim *et al.*, 1990; von Schrenck *et al.*, 1990; Jensen and Coy, 1991; Metz *et al.*, 1992). One class had a high affinity for GRP and a lower affinity for NMB (termed GRP-R, GRP receptor or GRP-preferring receptor) and the other class had a higher affinity for NMB than GRP (termed NMB-R, NMB receptor or NMB-preferring receptor) (Jensen and Gardner, 1981; Moody *et al.*, 1988; von Schrenck *et al.*, 1989; Ladenheim *et al.*, 1990; von Schrenck *et al.*, 1990; Moody *et al.*, 1992; Wang *et al.*, 1992; Ladenheim *et al.*, 1992). Subsequently, two mammalian receptors with high affinity for GPR (Spindel *et al.*, 1990; Battey *et al.*, 1991) or NMB (Wada *et al.*, 1991) have been cloned in addition to a closely related orphan receptor (Gorbulev *et al.*, 1992; Fathi *et al.*, 1993b) and one related receptor from amphibians (Nagalla *et al.*, 1995) which will be discussed in more detail below (Table 1).

### I. Molecular basis for nomenclature

Once the receptors were defined using binding studies, cross-linking studies and studies of biological activity (Kris *et al.*, 1987; Tache *et al.*, 1988; Sinnott-Smith *et al.*, 1988; von Schrenck *et al.*, 1989; Huang *et al.*, 1990; Ladenheim *et al.*, 1990; Lebacqz-Verheyden *et al.*, 1990), an active effort to clone the GRP-preferring receptor (GRP-R) was undertaken by Dr Eliot Spindel, Oregon Regional Primate Center and Dr James Battey, National Institutes of Health. In 1990 using electrophysiological and luminometric *Xenopus* oocyte expression assays, Spindel (Spindel *et al.*, 1990) succeeded in cloning the GRP-R from murine Swiss 3T3 cells, which express high levels of this receptor (Rozenfurt, 1988). The cDNA for the same receptor was isolated and described by Battey *et al.* in 1991 (Battey *et al.*, 1991) by using an enriched library from Swiss 3T3 cells and specific oligonucleotide probes based on information from a partial sequence of the GRP-R in these cells obtained after solubilization and purification using wheat germ agglutinin-agarose and a ligand affinity chromatography (Feldman *et al.*, 1990). Pharmacology studies demonstrated the cloned receptor preferred GRP to NMB and its activation was blocked by specific GRP-preferring receptor antagonists (Rozenfurt, 1988; Battey *et al.*, 1991). Subsequently, using low stringency conditions with a mouse GRP-R cDNA probe (Wada *et al.*, 1991); the NMB preferring receptor (NMB-R) was cloned from a cDNA library made from the rat esophagus, a tissue that had been reported to have a high density of NMB-R (von Schrenck *et al.*, 1989; von Schrenck *et al.*, 1990). The structure of the cDNA of the human GRP-R and NMB-R were described from a small cell lung cancer cell line in 1991 (Corjay *et al.*, 1991).

In 1992 a novel receptor was cloned from guinea-pig uterus (Gorbulev *et al.*, 1992) which showed the highest amino acid identity to the GRP-R (52%) and the NMB-R (47%). This receptor bound GRP and NMB, but only with relatively low affinities ( $IC_{50}$ -290 nM and 20,000 nM, respectively). The human analogue of this novel receptor was cloned in 1993 (Fathi *et al.*, 1993b) and expression studies showed it was specifically activated by bombesin-related peptides, but only with low affinity and thus was classified as an orphan receptor. It was termed BRS-3 for bombesin receptor subtype 3 (Fathi *et al.*, 1993b). Subsequent binding studies and signaling studies using synthetic ligands of bombesin with high affinity for hBRS-3 (Mantey *et al.*, 1997), demonstrated that it not only had low affinity for GRP and NMB, but all known naturally occurring bombesin related peptides (Wu *et al.*, 1996; Mantey *et al.*, 1997; Pradhan *et al.*, 1998; Ryan *et al.*, 1998a; Ryan *et al.*, 1998b) and therefore it remains an orphan receptor. Subsequently it was cloned from mouse (Ohki-Hamazaki *et al.*, 1997a), rat (Liu *et al.*, 2002) and sheep (Whitley *et al.*, 1999).

Searching for receptors for bombesin-related peptides in amphibians (Nagalla *et al.*, 1995), clones were isolated which had a similar sequence to the mammalian GRP-R and NMBR. A clone was isolated which encoded for a novel bombesin receptor, which had 61%, 56% and 70% amino acid identities to the human GRP-R, NMB-R and BRS-3 (Nagalla *et al.*, 1995).

This receptor had the highest affinity for [Phe<sup>13</sup>]bombesin, the form most prevalent in frog brain, and had lower affinity for GRP and NMB. This receptor was called BB4 for bombesin receptor subtype 4 (Nagalla *et al.*, 1995). Subsequent detailed binding studies and studies of cell signaling confirmed these findings and showed this receptor had greater affinity for [Phe<sup>13</sup>] bombesin than any other naturally occurring bombesin-related peptide (Katsuno *et al.*, 1999). At present no mammalian equivalent of this receptor has been described and therefore it is not included in the classification discussed below. Recently in chickens a receptor was cloned that had high amino acid identity to frog BB4 [fBB4](70%) as well as human BRS-3 (69%) and lower for human GRP-R (58%) and human NBR-R (52%) (Iwabuchi *et al.*, 2003). When expressed this receptor had low affinity for GRP and NMB, but it retained high affinity for [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> (Iwabuchi *et al.*, 2003), a synthetic analogue which has high affinity for hBRS-3, GRPR, NMBR and fBB4 (Mantey *et al.*, 1997; Pradhan *et al.*, 1998). It was proposed this receptor be termed chBRS-3.5 because of its resemble to both fBB4 and BRS-3. No mammalian equivalent of this receptor has been described and therefore it is also not included in the classification below.

Based on the above molecular studies, three classes of mammalian bombesin receptors are proposed for which the nomenclature and a few features are summarized in Table 1. Although the usual NC-IUPHAR nomenclature uses the endogenous mammalian ligand, the substantial historical use of the frog peptide bombesin in the field to describe this system was retained. The BB<sub>1</sub>-BB<sub>3</sub> receptors will each be dealt with in more detail in the following sections, but a few important points will be briefly covered here. The BB<sub>1</sub> receptor was previously referred to as the NMB receptor, NMB-R or NMB-preferring receptor. This terminology is the same used for this bombesin receptor subclass in the Sigma-RBI Handbook of Receptor Classification and Signal Transduction (Watling, 2007) and is the same as the BB1 in the BJP Guide to Receptors and Channels (Alexander *et al.*, 2006). The BB<sub>2</sub> receptor was previously referred to as the GRPR, GRP receptor or GRP-preferring receptor (Table 1). This terminology is the same used for this bombesin receptor subclass in the Sigma-RBI Handbook of Receptor Classification and Signal Transduction (Watling, 2007) and is the same as the BB2 subclass in the BJP Guide to Receptors and Channels (Alexander *et al.*, 2006). The BB<sub>3</sub> receptor was previously referred to as the BRS-3 receptor, BRS-3, and bombesin receptor subtype 3 (Table 1). This terminology is the same used for this bombesin receptor classes in the Sigma-RBI Handbook of Receptor Classification and Signal Transduction (Watling, 2007) and is the same as the bb3 receptor in the BJP Guide to Receptors and Channels (Alexander *et al.*, 2006). Finally, the amphibian BB<sub>4</sub> receptor does not have a mammalian equivalent so is not included in this classification. This receptor was also not classified in the Sigma-RBI Handbook of Receptor Classification and Signal Transduction (Watling, 2007) or the BJP Guide to Receptors and Channels (Alexander *et al.*, 2006).

## II. BB<sub>1</sub> Receptor

**III.1. Early studies of the BB<sub>1</sub> receptor**—Prior to the identification of the BB<sub>1</sub> in 1989 in rat esophageal muscle tissue sections by direct binding studies using <sup>125</sup>I-Bolton-Hunter-labeled NMB and subsequent esophageal muscle strip contraction studies (von Schrenck *et al.*, 1989), there were no early studies that unequivocally established the existence of BB<sub>1</sub>. Numerous previous studies had demonstrated the frog peptides ranatensin and litorin, which closely resembled NMB (Minamino *et al.*, 1983), had potent effects on various tissues and especially on smooth muscle contraction, which in some classes had differences from bombesin (Falconieri Erspamer *et al.*, 1988; Regoli *et al.*, 1988). However, these differences were not significant enough to clearly establish the existence of a separate class of BB<sub>1</sub> receptors (Minamino *et al.*, 1983; Falconieri Erspamer *et al.*, 1988; Regoli *et al.*, 1988). Although there had been many binding studies to numerous tissues from the late 1970's, in almost all cases <sup>125</sup>I-[Tyr<sup>4</sup>] bombesin or another radiolabeled bombesin analogue was used (Moody *et*

*et al.*, 1978; Shapira *et al.*, 1993; Ladenheim *et al.*, 1993b). Unfortunately, bombesin has high affinity for both BB<sub>1</sub> and BB<sub>2</sub> making it more difficult to distinguish subtypes. Numerous classes of selective BB<sub>2</sub> receptor antagonist were developed prior to the cloning of the BB<sub>1</sub> and these also confirmed the presence of the BB<sub>1</sub> on esophageal smooth muscle (von Schrenck *et al.*, 1990). After the pharmacologic description of BB<sub>1</sub> on esophageal muscle and prior to its cloning in 1991, using selective BB<sub>2</sub> receptor antagonists or binding studies using radiolabeled NMB and selective agonists or BB<sub>2</sub> receptor antagonists, BB<sub>1</sub> receptors were demonstrated in the CNS (Ladenheim *et al.*, 1990) and on gastric smooth muscle cells (Severi *et al.*, 1991).

**III.2. Cloned BB<sub>1</sub> receptor and receptor structure**—The human BB<sub>1</sub> receptor is a 390 amino acid protein and it shows an 89% amino acid identity with the rat BB<sub>1</sub> (Corjay *et al.*, 1991). The human BB<sub>1</sub> receptor has 55% amino acid identities with the human BB<sub>2</sub> (Corjay *et al.*, 1991) and 47% with the human BB<sub>3</sub> receptor (Fathi *et al.*, 1993b). The human BB<sub>1</sub> receptor has two consensus sites for potential PKC phosphorylation and three potential N-linked glycosylation sites (Corjay *et al.*, 1991). Hydropathy plots yielded results consistent with a seven transmembrane structure typical for a G-protein coupled receptor (Corjay *et al.*, 1991). The BB<sub>1</sub> receptor has been cloned from rat (Wada *et al.*, 1991) (Fig. 2), mouse (Ohki-Hamazaki *et al.*, 1997a) and the frog, *bombina orientalis* (Nagalla *et al.*, 1995). Cross-linking studies demonstrate the mature human BB<sub>1</sub> receptor had a molecular weight of 72 ± 1 kDa and when deglycosylated 43 ± 1 kDa (Benya *et al.*, 1995b). Detailed cross-linking and serial deglycosylation studies using enzymatic digestion in the rat BB<sub>1</sub> receptor demonstrated a molecular weight of 63 kDa in the membrane and showed there were no O-linked carbohydrates but that the mature BB<sub>1</sub> receptor was a sialoprotein (Kusui *et al.*, 1994). However, each of the potential N-linked glycosylation sites was, in fact, glycosylated, with tri-antennary and/or tetra-antennary complex oligosaccharide chains (Kusui *et al.*, 1994).

**III.3. BB<sub>1</sub> receptor genomic organization**—The human BB<sub>1</sub> receptor gene is localized at human chromosome 6p21-qter and in the mouse on chromosome 10 (Table 1). Both the human, rat and mouse genes contained three exons with two introns (Corjay *et al.*, 1991; Wada *et al.*, 1991; Ohki-Hamazaki *et al.*, 1997a; Ohki-Hamazaki, 2000). In the mouse the gene for BB<sub>1</sub> receptor spanned more than 10 kb with exon 1 of the BB<sub>1</sub> gene separated from exon 2 by 6 kb and this in turn separated from exon 3 by 3 kb (Ohki-Hamazaki *et al.*, 1997a). In human and mouse the first intron of the BB<sub>1</sub> gene was located between transmembrane domains 3 and 4 and the second between transmembrane domains 5 and 6 (Corjay *et al.*, 1991; Ohki-Hamazaki *et al.*, 1997a). The first intron interrupted a codon for arginine located immediately COOH terminal to the transmembrane domain 3, and the second intron was located between glutamine and methionine codons in both the mouse and human BB<sub>1</sub> gene (Corjay *et al.*, 1991; Ohki-Hamazaki *et al.*, 1997a). The positions of the first and second introns were identical in the mouse and human BB<sub>1</sub> receptor gene (Corjay *et al.*, 1991; Ohki-Hamazaki *et al.*, 1997a).

**III.4. BB<sub>1</sub> receptor expression**—Expression levels of BB<sub>1</sub> receptor mRNA has been reported in human, mouse, rat, and monkey (Corjay *et al.*, 1991; Wada *et al.*, 1991; Ohki-Hamazaki *et al.*, 1997a; Sano *et al.*, 2004). In the monkey where it was studied in detail, highest levels of BB<sub>1</sub> mRNA are found in the CNS and in the testis (Sano *et al.*, 2004). In the CNS the BB<sub>1</sub> receptor was expressed widely in different brain regions including the amygdala, caudate nucleus, hippocampus, hypothalamus, thalamus, brain stem, spinal cord and in peripheral tissues in addition to the testis and the stomach, which is a similar distribution to that found in rats and mice (Wada *et al.*, 1991; Ohki-Hamazaki *et al.*, 1997a; Ohki-Hamazaki, 2000; Sano *et al.*, 2004). In the rat and mouse, BB<sub>1</sub> mRNA is present in high amounts in the olfactory region and esophagus (Wada *et al.*, 1991; Ohki-Hamazaki *et al.*, 1997a). Binding studies and studies of biological activity provide evidence for BB<sub>1</sub> on both gastrointestinal and urogenital



smooth muscle cells (von Schrenck *et al.*, 1989; Severi *et al.*, 1991; Bitar and Coy, 1992; Kim *et al.*, 1993). Binding studies have confirmed the widespread distribution of BB<sub>1</sub> in the brain showing especially high levels in the olfactory tract of the rat (Ladenheim *et al.*, 1990; Ladenheim *et al.*, 1992; Ladenheim *et al.*, 1993a).

Using binding studies and/or assessment of BB<sub>1</sub> mRNA, BB<sub>1</sub> receptors have been shown to exist on a large number of different tumors (Reubi *et al.*, 2002; Jensen and Moody, 2006) including CNS tumors (glioblastomas) (Wada *et al.*, 1991; Wang *et al.*, 1992), small cell and nonsmall cell lung cancers (Corjay *et al.*, 1991; Moody *et al.*, 1992; Toi-Scott *et al.*, 1996; Siegfried *et al.*, 1997; Moody *et al.*, 2000; Jensen and Moody, 2006), carcinoids (intestinal, thymic, bronchial) (Reubi *et al.*, 2002), human ovarian epithelial cancers (Sun *et al.*, 2000b) and pancreatic cancer cell lines (Jensen and Moody, 2006).

### III.5. BB<sub>1</sub> receptor pharmacology

**III.5.a. BB<sub>1</sub> receptor agonists:** The human BB<sub>1</sub> receptor (Moody *et al.*, 1992; Benya *et al.*, 1995b; Reubi *et al.*, 2002) as well as the rat BB<sub>1</sub> receptor (von Schrenck *et al.*, 1989; von Schrenck *et al.*, 1990; Wang *et al.*, 1992; Ladenheim *et al.*, 1992; Ladenheim *et al.*, 1993a) has a greater than a 100-fold higher affinity for NMB than GRP (Fig. 1,2). Bombesin and the frog peptides, ranatensin and litorin also had relatively high affinity for the BB<sub>1</sub> receptor (affinities 1–10 fold less than NMB) (Wang *et al.*, 1992; Mantey *et al.*, 1997; Katsuno *et al.*, 1999) (Table 1 and 2). The synthetic bombesin analogue [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6–14</sub> (Mantey *et al.*, 1997), which has high affinity for the human BB<sub>3</sub> receptor also has a high affinity for the human BB<sub>1</sub> receptor as well as the human BB<sub>2</sub> receptor and fBB4 (Mantey *et al.*, 1997; Pradhan *et al.*, 1998) (Table 2).

**III.5.b. BB<sub>1</sub> receptor antagonists:** Whereas the search for high affinity receptor antagonists for the BB<sub>2</sub> receptor has been very successful (see section below) (Jensen and Coy, 1991; Jensen *et al.*, 1993; de Castiglione and Gozzini, 1996), the results with BB<sub>1</sub> receptor have been much less successful and only a few high affinity receptor antagonists are available. None of the strategies used for making high affinity BB<sub>2</sub> antagonists were successful with the BB<sub>1</sub> receptor including the synthesis of bombesin or NMB COOH terminal pseudopeptide analogues, COOH terminal truncated analogues or [des-Met<sup>10</sup>] NMB amides, alkylamides or esters (Lin *et al.*, 1995). Subsequently, it was discovered that certain substituted somatostatin (SS) analogues selectively antagonized the BB<sub>1</sub> receptor compared to the BB<sub>2</sub> receptor (Orbuch *et al.*, 1993). The most potent analogue was cyclo-SS-octa [D-Nal-Cys-Tyr- D-Trp-Lys-Val-Cys-Nal-NH<sub>2</sub>], which had a 100-fold higher affinity for the BB<sub>1</sub> receptor than the BB<sub>2</sub> receptor (K<sub>i</sub> –230 vs. 3000 nM) (Orbuch *et al.*, 1993; Ryan *et al.*, 1999) (Table 2). Unfortunately this analogue also interacted with high affinity with somatostatin receptors (IC<sub>50</sub> 0.80 nM) and mu opioid receptors (IC<sub>50</sub> 430 nM) (Orbuch *et al.*, 1993). Substitution of an ornithine for Lys greatly reduced the affinity for somatostatin receptors, and a related analogue, (BIM-23127), [D-Nal-Cys-Tyr- D-Trp-Orn-Val-Cys-Nal-NH<sub>2</sub>] inhibited NMB cell signaling in rat BB<sub>1</sub> receptor transfected Rat-1 cells (Lach *et al.*, 1995) and selectively reversed NMB feeding suppression, but had no effect on the action of GRP (Ladenheim *et al.*, 1997b). However, a recent study reports BIM-23127 also functions as a receptor antagonist of both human and rat urotensin-II receptors (Herold *et al.*, 2003) limiting its utility. Peptoid antagonists of BB<sub>1</sub> have been described including PD 165929 (Eden *et al.*, 1996) and PD 168368 (Ryan *et al.*, 1999), which have high affinity and selectivity for BB<sub>1</sub>. In a detailed comparison of bombesin receptors from different species, PD 168368 was found to have a similar high affinity (K<sub>i</sub>-15 –45 nM) for BB<sub>1</sub> receptors from each species, a 30- to 60-fold lower affinity for the BB<sub>2</sub> receptor from different species and a >300 fold lower affinity for the BB<sub>3</sub> receptor or fBB4 (Ryan *et al.*, 1999) (Table 2). It also inhibited NMB-stimulated cellular signaling in a competitive manner (Ryan *et al.*, 1999) as well as inhibiting NMB-induced proliferation of rat

C6 glioblastoma cells (Moody *et al.*, 2000) and NMB stimulation of NCI-H1299 lung cancer cell proliferation (Moody *et al.*, 2000).

### III.6. BB<sub>1</sub> receptor structural basis of receptor binding/activation

**III.6.a. BB<sub>1</sub> receptor agonist binding/activation:** Structure-function studies of NMB demonstrate the COOH terminal octapeptide is the minimal peptide length required for BB<sub>1</sub> receptor activation and the full decapeptide was required for full affinity for the BB<sub>1</sub> receptor (Lin *et al.*, 1996). NMB differs from GRP in the COOH octapeptide, which is the biologically active end (Broccardo *et al.*, 1976; Lin *et al.*, 1996), at 3 residues; substitution of a leucine in NMB for a histidine in GRP at position 3, a threonine for valine at position 6 and a phenylalanine for leucine at position 9 of NMB from the amino terminus (Minamino *et al.*, 1983; Lin *et al.*, 1996) (Fig.1). Structure-function studies of all naturally occurring bombesin-related peptides for BB<sub>1</sub> and BB<sub>2</sub> receptors suggested the presence of the phenylalanine instead of leucine, as the penultimate amino acid from the COOH terminus in NMB was not important for selectivity for the BB<sub>1</sub> receptor. Single amino acid substitutions in NMB demonstrated the Leu for His substitution in position 3 was the most important for determining high affinity and selectivity for the BB<sub>1</sub> receptor (Lin *et al.*, 1996) (Fig.1).

A chimeric receptor approach (Fathi *et al.*, 1993a) and homology screening after computer alignment of bombesin receptor family members (Sainz *et al.*, 1998), followed by site-directed mutagenesis studies have been used to explore the molecular basis of NMB high affinity and selectivity for the BB<sub>1</sub> receptor over the BB<sub>2</sub> receptor (Fig. 3). A study of BB<sub>1</sub>/BB<sub>2</sub> chimeric receptors (Fathi *et al.*, 1993a) demonstrated that differences in the amino terminus of the two receptors were of minimal importance for high affinity NMB interaction. High affinity and selectivity for the BB<sub>1</sub> receptor was primarily determined by differences in transmembrane domain 5 (TM5)(Fathi *et al.*, 1993a)(Fig. 3). Site-directed mutagenesis of the amino acid differences between the BB<sub>1</sub> receptor and the BB<sub>2</sub> receptor in this region demonstrated the substitution of an Ile<sup>216</sup> instead of Ser in the comparable position of the TM5 of the BB<sub>2</sub> receptor was the critical difference accounting for high affinity NMB interaction with the BB<sub>1</sub> and not the BB<sub>2</sub> receptor (Fathi *et al.*, 1993a). A second study (Sainz *et al.*, 1998) used a different approach to select potentially important amino acids for NMB selectivity for the BB<sub>1</sub> receptor and further study. Using amino acid sequence alignment of bombesin receptor family members and identifying conserved amino acids in members with similar peptide affinities (Akeson *et al.*, 1997), four amino acids were identified that could be important for high affinity bombesin binding to either the BB<sub>1</sub> or BB<sub>2</sub> receptor (Akeson *et al.*, 1997)(i.e. in the BB<sub>1</sub> receptor: Gln<sup>123</sup>, Pro<sup>200</sup>, Arg<sup>290</sup>, Ala<sup>310</sup> and in the BB<sub>2</sub> receptor: Gln<sup>121</sup>,Pro<sup>199</sup>,Arg<sup>288</sup>,Ala<sup>308</sup>). Possible gain of affinity mutants were made in the BB<sub>3</sub> receptor, which has a low affinity for NMB (Mantey *et al.*, 1997; Ryan *et al.*, 1998a; Ryan *et al.*, 1998b), by substituting alone or in combination each of these four BB<sub>1</sub> receptor amino acids for the comparable amino acid(s) of the BB<sub>3</sub> receptor (Arg<sup>127</sup>, Ser<sup>205</sup>, His<sup>294</sup>, Ser<sup>315</sup>) (Fig. 3). It was found that each of these four amino acids are important for determining NMB affinity because the affinity for NMB of the BB<sub>3</sub> mutants with these BB<sub>1</sub> receptor amino acids substituted one at a time were increased (Sainz *et al.*, 1998). The substitution of all 4 amino acids for the comparable amino acid in the BB<sub>3</sub> receptor, which has a low very affinity for NMB (i.e. K<sub>i</sub>-3450 nM) increased the affinity and the potency for NMB, almost up to that seen with the native BB<sub>1</sub> receptor (Sainz *et al.*, 1998). This study helps to define the binding pocket for NMB by identifying four amino acids needed for high affinity NMB interaction in markedly different BB<sub>1</sub> regions [transmembrane domain 2 (Gln<sup>123</sup>), extracellular domain 2 (Pro<sup>200</sup>), extracellular domain 3 (Arg<sup>290</sup>) and transmembrane region 7 (Ala<sup>310</sup>) (Fig. 3) (Sainz *et al.*, 1998).

**III.6.b. BB<sub>1</sub> receptor antagonist binding:** Using a chimeric receptor approach combined with site-directed mutagenesis and receptor modeling the molecular basis of selectivity of the BB<sub>1</sub> receptor antagonist, PD 168,368 was studied (Tokita *et al.*, 2001a)(Fig. 3). PD 168,368 is a new class of antagonists described as a peptoid, because this group of antagonists is nonpeptide ligands, which were designed using the chemical structure of the mammalian neuropeptide of interest as a starting point (Horwell *et al.*, 1994; Horwell, 1995). This approach has yielded antagonists for cholecystokinin, somatostatin, tachykinins and bombesin receptors (Boden *et al.*, 1993; Horwell *et al.*, 1994; Boyle *et al.*, 1994; Horwell, 1995; Eden *et al.*, 1996; Tran *et al.*, 1998; Tokita *et al.*, 2001a). However, little is known about the molecular basis of their affinity and whether they resemble peptide or other nonpeptide ligands in the basis of their selectivity and affinity (Tokita *et al.*, 2001a). The receptor extracellular domains were shown not to be important for the selectivity of PD 168,368 by studying both loss-of-affinity BB<sub>1</sub> receptor chimeras in which the extracellular domains of the BB<sub>1</sub> were replaced by those from BB<sub>2</sub>, one at a time or the reverse study performed by making PD 168,368 gain-of-affinity chimeras in the BB<sub>2</sub> receptor (Tokita *et al.*, 2001a). Additional PD 168,368 loss and gain-of-affinity chimeric studies made by exchanging the upper transmembrane regions of BB<sub>1</sub> and BB<sub>2</sub> receptors showed that differences in the upper transmembrane 5 (TM5) were the key determinants of selectivity of PD 168,368 (Tokita *et al.*, 2001a). Site-directed mutagenesis studies of the different amino acids between the BB<sub>1</sub> receptor and the BB<sub>2</sub> receptor in the upper TM5 region demonstrated the substitution of Tyr at position 220 of BB<sub>1</sub> for Phe in the comparable position in BB<sub>2</sub> was the critical difference (Tokita *et al.*, 2001a)(Fig. 3). Three-dimensional modeling studies showed the critical Tyr<sup>220</sup> was facing the interior of a large binding pocket formed primarily by transmembrane domains 3–7 and minimum energy conformation of the ligand showed that it was dominated by a large hydrogen bond accepting region around the nitrophenyl group (Tokita *et al.*, 2001a). It was concluded that the Tyr<sup>220</sup> hydroxyl of the BB<sub>1</sub> receptor was critical for interacting with the nitrophenyl group of PD 168,368, likely primarily by hydrogen bonding. This result showed the binding of this peptoid antagonist was similar to that reported with other non-peptide antagonists of GRPR's, in that it was primarily dependent on interaction with transmembrane regions (Tokita *et al.*, 2001a).

### **III.7. BB<sub>1</sub> receptor signaling, activation, and modulatory processes (internalization, down-regulation, desensitization)**

—The human BB<sub>1</sub> receptor (Moody *et al.*, 1986; Corjay *et al.*, 1991; Moody *et al.*, 1992; Moody *et al.*, 1995a; Benya *et al.*, 1995b), as well as the rat BB<sub>1</sub> receptor (Wada *et al.*, 1991; Wang *et al.*, 1992; Jones *et al.*, 1992; Dobrzanski *et al.*, 1993; Lach *et al.*, 1995; Akeson *et al.*, 1997; Vigne *et al.*, 1997; Tsuda *et al.*, 1997b; Hou *et al.*, 1998) is coupled to phospholipase C resulting in breakdown of phosphoinositides, mobilization of cellular calcium and activation of protein kinase C. BB<sub>1</sub> receptor activation also result in the stimulation phospholipase A<sub>2</sub> (Moody *et al.*, 1995a) and phospholipase D by a PKC-dependent and independent mechanism (Tsuda *et al.*, 1997b), but does not activate adenylate cyclase (Benya *et al.*, 1992). BB<sub>1</sub> receptor stimulation also results in activation of tyrosine kinases (Lach *et al.*, 1995; Tsuda *et al.*, 1997b) stimulating tyrosine phosphorylation of p125<sup>FAK</sup> by a phospholipase C independent mechanism which requires p21<sup>rho</sup> and the integrity of the actin cytoskeleton (Tsuda *et al.*, 1997b). BB<sub>1</sub> receptor activation also stimulated tyrosine phosphorylation of paxillin and MAP kinase activation (Lach *et al.*, 1995). The native as well as transfected rat BB<sub>1</sub> receptor in BALB 3T3 cells has been shown to behave in a similar manner in their binding and signaling cascades (Benya *et al.*, 1992) demonstrating the usefulness of this cell line for studying BB<sub>1</sub> receptor interaction and signaling.

The BB<sub>1</sub> receptor is coupled to heterotrimeric guanine-nucleotide binding proteins in both native and BALB 3T3 transfected cells (Benya *et al.*, 1992; Wang *et al.*, 1993). In a *Xenopus* oocyte assay with the injection of antisense oligonucleotides, G<sub>oq</sub> was identified as a mediator of the BB<sub>1</sub> receptor response (Shapira *et al.*, 1994). With an *in situ* reconstitution



assay with purified G protein  $\alpha$  subunits, it was found that cells expressing the BB<sub>1</sub> receptor activated G<sub>αq</sub>, but not G<sub>αt</sub> or G<sub>αi/o</sub> (Jian *et al.*, 1999). This activation was enhanced by  $\beta\gamma$  dimers with a relative potency of:  $\beta\gamma > \beta 1\gamma 2 >> \beta 1\gamma 1$ . In this study (Jian *et al.*, 1999) these results were contrasted with the BB<sub>2</sub> receptor and differences were found in their kinetics of activation, preference for G<sub>αq</sub> proteins from different sources and for  $\beta\gamma$  dimers demonstrating distinct coupling mechanisms for these two closely related receptors (Jian *et al.*, 1999).

In contrast to the BB<sub>2</sub> receptor there have been few studies of BB<sub>1</sub> receptor modulatory processes (internalization, down-regulation, or desensitization). Both the human (Benya *et al.*, 1995b) and rat BB<sub>1</sub> receptors (Benya *et al.*, 1992; Wang *et al.*, 1993; Benya *et al.*, 1994c) are rapidly internalized with receptor activation of the rat BB<sub>1</sub> receptor. The rat BB<sub>1</sub> receptor internalized 60–80% of the bound ligand and human BB<sub>1</sub> receptors 70% of the bound ligand. In addition to being rapidly internalized by BB<sub>1</sub> receptor bearing cells, the ligand is rapidly degraded by these cells (Benya *et al.*, 1992; Wang *et al.*, 1993). Protease inhibitors markedly decreased ligand degradation by either rat native or rat BB<sub>1</sub> receptor transfected BALB 3T3 cells (Benya *et al.*, 1992; Wang *et al.*, 1993) with the acid proteinase inhibitor, leupeptin being the most potent followed by bacitracin > chymostatin > phosphoramidon >> bestatin, amastatin. The BB<sub>1</sub> receptor also undergoes desensitization, which is mediated by receptor down-regulation and internalization (Benya *et al.*, 1994c). Pre-incubation for 3 hours with 3 nM NMB markedly attenuated the ability of a maximally effective concentration of NMB (1  $\mu$ M) to subsequently stimulate either native or BB<sub>1</sub> transfected BALB 3T3 cells, but did not alter the response to other stimulants (Benya *et al.*, 1994c). This desensitization was associated with a rapid decrease in BB<sub>1</sub> receptors due to internalization of the receptors. Restoration of receptor number and response recovered over a 6-hour period and it was not dependent on new protein synthesis, but was due to receptor recycling, because it was inhibited by the recycling inhibitor, monesin, a monocarboxylic acid cation ionophore (Benya *et al.*, 1994c).

**III.8. BB<sub>1</sub> receptor function in various tissues and *in vivo***—One of the main difficulties in assessing the effects of BB<sub>1</sub> receptor activation in the CNS as well as peripheral tissues, especially in older studies, is that bombesin was frequently used as the agonist, and it interacts with both BB<sub>1</sub> and BB<sub>2</sub> receptor with relatively high affinity. Furthermore, many tissues possess both BB<sub>1</sub> and BB<sub>2</sub> receptors and therefore it was difficult to assess whether a particular response was due to activation of the BB<sub>1</sub> or BB<sub>2</sub> receptors present.

Numerous effects of NMB in both *in vivo* and *in vitro* studies have been reported, but it is not clear in many cases which are physiological and which are pharmacological. Studies comparing the potencies of NMB to GRP as well as binding studies or antagonist studies provide evidence that BB<sub>1</sub> receptor can stimulate contraction of urogenital and gastrointestinal smooth muscle (esophageal, gastric, colonic, gallbladder) (Regoli *et al.*, 1988; von Schrenck *et al.*, 1989; von Schrenck *et al.*, 1990; Severi *et al.*, 1991; Kilgore *et al.*, 1993; Parkman *et al.*, 1994; Milusheva *et al.*, 1998); potentially inhibit thyrotropin release from the pituitary acting as an autocrine and paracrine regulator (Rettori *et al.*, 1992; Pazos-Moura *et al.*, 1996; Ortega-Carvalho *et al.*, 2003); and have potent CNS effects including inhibiting food intake independent of BB<sub>2</sub> stimulation (Ladenheim *et al.*, 1994; Ladenheim *et al.*, 1996b; Ladenheim *et al.*, 1997b; Merali *et al.*, 1999; Ladenheim and Knipp, 2007), mediating aspects of the stress and fear responses as well as various behaviors such as spontaneous activity (Merali *et al.*, 2002; Merali *et al.*, 2006).

BB<sub>1</sub> receptor knockout mice are now available and have undergone a limited number of investigations for actions of NMB (Ohki-Hamazaki *et al.*, 1999; Oeffner *et al.*, 2000; Yamano *et al.*, 2002; Yamada *et al.*, 2002b; Yamada *et al.*, 2003) (Table 1). In these mice the hypothermic effect of NMB was reduced by 50% without a change in the GRP response

supporting a possible BB<sub>1</sub> receptor mediated role in thermoregulation: NMB-mediated gastric smooth muscle contraction was not effected suggesting this is mediated not through BB<sub>1</sub> receptors; and no effect on feeding could be confirmed, although NMB did not have an effect in the control animals (Ohki-Hamazaki *et al.*, 1999). BB<sub>1</sub> receptor's satiety effects are mediated through different peripheral neural pathways than BB<sub>2</sub> receptor's satiety effects, because only BB<sub>1</sub> receptors satiety effect is inhibited by capsaicin treatment suggesting the involvement of primary sensory afferent neurons (Ladenheim and Knipp, 2007). Recently, NMB has found to be expressed in human and rodent adipose tissue and to be regulated by changes in energy balance. It was proposed that because of the known anorectic effects of NMB centrally, it may form part of a new adipose tissue-hypothalamic regulating system for food intake (Hoggard *et al.*, 2007). In the BB<sub>1</sub> receptor knockout mice dysregulation of the thyroid occurs suggesting BB<sub>1</sub> receptor pathways are importantly involved in both TSH gene regulation and function (Oliveira *et al.*, 2006); dysfunction in response to stress was seen (Yamano *et al.*, 2002; Yamada *et al.*, 2002b), impairment in the modulation of the CNS 5-HT system in response to stress occurred (Yamano *et al.*, 2002) and an impairment of learning and memory was seen (Yamada *et al.*, 2003). The alterations in the CNS 5-HT and stress in these animals is particularly interesting, because the dorsal raphe nucleus is one of the brain regions that has a preponderance of BB<sub>1</sub> receptors (Wada *et al.*, 1990; Ladenheim *et al.*, 1992; Pinnock *et al.*, 1994; Merali *et al.*, 2006), which are located on 5-HT neurons, and stimulation of this nucleus by NMB stimulates release of 5-HT resulting in angiogenesis (Merali *et al.*, 2006). In a study in rats using BB<sub>1</sub> and BB<sub>2</sub> receptor agonists and antagonists (Bedard *et al.*, 2007) data was provided that both GRP and NMB affect the stress response. NMB affected both anxiety and fear responses, whereas GRP affected only fear responses (Bedard *et al.*, 2007).

Whereas the growth effects of the BB<sub>2</sub> receptor on normal and especially in neoplastic tissues have received the most attention, stimulation of the BB<sub>1</sub> receptor and/or administration of NMB has been shown to have growth promoting effects in a number of neoplastic tissues. NMB is an autocrine growth factor for non-small cell lung cancer (NSCLC) with 14/14 such cell lines possessing BB<sub>1</sub> receptors in one study (Siegfried *et al.*, 1997) and in 4 NSCLC cell lines examined in detail NMB was synthesized and released into the media by the tumor cell in 7–15 times greater amounts than GRP (Siegfried *et al.*, 1997). Blockade of the BB<sub>2</sub> receptor only partially blocked the proliferative effect of NMB on these cells demonstrating the importance of BB<sub>1</sub> receptor activation for the proliferative effects in these tumor cells (Siegfried *et al.*, 1997). Furthermore, in human colon cancers NMB and the BB<sub>1</sub> receptor are co-expressed and they act in an autocrine growth fashion (Matusiak *et al.*, 2005). Activation of BB<sub>1</sub> receptors causes proliferation of rat C6 glioblastoma cells (Moody *et al.*, 1995a), BB<sub>1</sub> receptor transfected RAT-1 cells (Lach *et al.*, 1995), small cell lung cancers (Moody *et al.*, 1992) and adrenal zona fasciculata cells (Malendowicz *et al.*, 1996).

**III.9. BB<sub>1</sub> receptor in diseases**—At present no disease has been shown to be caused specifically by alterations in the BB<sub>1</sub> receptor. Activation of the BB<sub>1</sub> receptor in various human cancers due to an autocrine growth pathway may have an important effect on their growth particularly human small cell lung cancers, nonsmall cell lung cancers, colonic cancer, and various carcinoid tumors (Moody *et al.*, 1992; Moody and Jensen, 1996; Siegfried *et al.*, 1999; Matusiak *et al.*, 2005; Jensen and Moody, 2006). In various studies BB<sub>1</sub> receptors are over-expressed by 55% of small cell lung cancers, 67% of non-small cell lung cancers, 46% of intestinal carcinoids, and a proportion of colon cancers, prostate cancers and CNS tumors such as glioblastomas (Moody *et al.*, 1995a; Reubi *et al.*, 2002; Matusiak *et al.*, 2005; Jensen and Moody, 2006).

Numerous studies (Rettori *et al.*, 1992; Pazos-Moura *et al.*, 1996; Ortega-Carvalho *et al.*, 2003) including BB<sub>1</sub> receptor knockout studies (Oliveira *et al.*, 2006) support the conclusion that NMB plays an important physiological role in the regulation of thyrotropin release

primarily having an inhibitory effect. NMB is produced in the pituitary (Jones *et al.*, 1992) and it is proposed that NMB functions as a tonic inhibitor of TSH secretion, acting as an autocrine/paracrine regulator (Rettori *et al.*, 1992; Oliveira *et al.*, 2006) (Table 1). Conditions with increased TSH release such as hypothyroidism are associated with decreased pituitary NMB levels (Jones *et al.*, 1992; Ortiga-Carvalho *et al.*, 2003), while in hyperthyroidism where the TSH levels are suppressed; there is an increased pituitary NMB level (Jones *et al.*, 1992; Ortiga-Carvalho *et al.*, 1997). These results suggest NMB could play an important role in human thyroid disorders causing hyper- or hypo-function.

The role of NMB in human feeding disorders is unclear at present. Two genetic studies have suggested that the NMB gene is a possible candidate for eating disorders and predisposition to obesity (Oeffner *et al.*, 2000; Bouchard *et al.*, 2004).

### III. BB<sub>2</sub> Receptor

**IV.1. Early studies of the BB<sub>2</sub>**—Many of the early studies provide limited information on the BB<sub>2</sub> receptor, as discussed in III.1 above for the BB<sub>1</sub> receptor. This occurred because many of the tissues studied are now known to possess both BB<sub>2</sub> and BB<sub>1</sub> receptor and in most studies bombesin analogues were used which have high affinity for both subclasses of receptors. This continued to occur after the isolation of GRP in 1978 (McDonald *et al.*, 1979), even though it had greater selectivity than bombesin analogues for the BB<sub>2</sub> over the BB<sub>1</sub> (von Schrenck *et al.*, 1989; Lin *et al.*, 1995; Benya *et al.*, 1995b; Reubi *et al.*, 2002) because of its limited availability. *In vivo* studies were even more difficult to interpret because numerous studies demonstrated that GRP-related peptides can both have a direct action on tissues as well as indirect action because they are potent at stimulating the release of many hormones (gastrin, insulin, somatostatin, CCK, pancreatic polypeptide, enteroglucagon, pancreatic glucagon, gastric inhibitory peptide) (McDonald *et al.*, 1979; Modlin *et al.*, 1981; Ghatei *et al.*, 1982; McDonald *et al.*, 1983; Greeley, Jr. *et al.*, 1986; Pettersson and Ahren, 1987; Knuhtsen *et al.*, 1987; Kawai *et al.*, 1988; Hermansen and Ahren, 1990). With the development of selective BB<sub>2</sub> receptor antagonists (von Schrenck *et al.*, 1990; Jensen and Coy, 1991; Benya *et al.*, 1995b) and the increased use of BB<sub>2</sub> selective ligands such as GRP, it became clear that a separate GRP-preferring receptor existed, even before the cloning of the mouse and human BB<sub>2</sub> receptor in the early 1990's (Spindel *et al.*, 1990; Corjay *et al.*, 1991; Battey *et al.*, 1991) (Table 2). It subsequently became clear that a number of the tissues that had been extensively used to characterize bombesin receptors/responses such as pancreatic acinar cells (Jensen *et al.*, 1978; Jensen, 1994) and Swiss 3T3 cells (Rozengurt, 1988) possessed only BB<sub>2</sub> receptors, whereas others such as the CNS (Battey and Wada, 1991; Ladenheim *et al.*, 1992) and smooth muscle preparations possessed both BB<sub>1</sub> and BB<sub>2</sub> receptors (Severi *et al.*, 1991).

**IV.2. Cloned BB<sub>2</sub> and receptor structure**—The human BB<sub>2</sub> receptor has 384 amino acids and shows high homology (90% amino acid identities) with the mouse BB<sub>2</sub> receptor (Corjay *et al.*, 1991) (Fig. 4). The human BB<sub>2</sub> receptor has 55% amino acid identities with the human BB<sub>1</sub> receptor (Corjay *et al.*, 1991) and 51% with human BB<sub>3</sub> receptor (Fathi *et al.*, 1993b). Hydrophathy analysis of the predicted human BB<sub>2</sub> structure revealed seven regions of hydrophobic amino acids consistent with a seven transmembrane structure typical for G-protein coupled receptors (Corjay *et al.*, 1991). There were two consensus sites of potential PKC phosphorylation and two potential sites for N-linked glycosylation in the human BB<sub>2</sub> receptor (Corjay *et al.*, 1991). The BB<sub>2</sub> receptor has been completely or partially cloned from 21 species (Baldwin *et al.*, 2007) and the most highly conserved regions are in the transmembrane domains and the third intracellular domain (Baldwin *et al.*, 2007). The presence of a likely disulphide bond between cysteines at the end of the extracellular domain I and middle of extracellular domain 2 (Cys<sup>113</sup> and Cys<sup>196</sup> in human BB<sub>2</sub>) is preserved in all non-insect species (Baldwin *et al.*, 2007) (Fig. 4). Solubilization studies as well as cross-linking studies demonstrate the

mature human BB<sub>2</sub> receptor has a molecular weight greater than that predicted from the structure (Kris *et al.*, 1987; Rozengurt, 1988; Feldman *et al.*, 1990; Huang *et al.*, 1990; Staley *et al.*, 1993; Williams and Schonbrunn, 1994; Kusui *et al.*, 1994; Benya *et al.*, 1994b; Benya *et al.*, 1995b). Cross-linking studies demonstrate the mature human BB<sub>2</sub> receptor has a molecular weight of 60 ± 1 kDa, the mouse BB<sub>2</sub> receptor 82 ± 2 kDa and when each is deglycosylated 43 ± 1 kDa (Kris *et al.*, 1987; Rozengurt, 1988; Huang *et al.*, 1990; Williams and Schonbrunn, 1994; Kusui *et al.*, 1994; Benya *et al.*, 1994b; Benya *et al.*, 1995b). These results demonstrate that 35% of the mature human BB<sub>2</sub> receptor's molecular weight is due to glycosylation, whereas in the mouse BB<sub>2</sub> receptor it is 47%. This difference is likely due to the existence of 2 potential sites of N-linked glycosylation in the human BB<sub>2</sub> receptor compared to four potential sites in the mouse BB<sub>2</sub> receptor (Spindel *et al.*, 1990; Corjay *et al.*, 1991; Battey *et al.*, 1991; Benya *et al.*, 1995b)(Fig. 4). Using cross-linking studies with serial deglycosylation by enzymatic digestion (Kusui *et al.*, 1994; Kusui *et al.*, 1995), as well as a molecular approach involving mutating the four potential N-linked glycosylation sites either alone or in combination in the murine BB<sub>2</sub> receptor followed by receptor expression and cross-linking analysis (Benya *et al.*, 1994d), the murine BB<sub>2</sub> receptor was shown to be glycosylated at all 4 potential N-linked sites (Asn<sup>5</sup>, Asn<sup>20</sup>, Asn<sup>24</sup> and Asn<sup>191</sup>) (Kusui *et al.*, 1994; Benya *et al.*, 1994d; Kusui *et al.*, 1995). The extent of glycosylation varied however, with carbohydrate residues of 12 kDa on Asn<sup>5</sup>, 10 kDa on Asn<sup>20</sup>, 5 kDa on Asn<sup>24</sup> and 9 kDa on Asn<sup>191</sup> (Benya *et al.*, 1994d). The presence of the glycosylation on Asn<sup>24</sup> and Asn<sup>191</sup> were especially important for sorting and expression of the murine BB<sub>2</sub> receptor on the plasma membrane (Benya *et al.*, 1994d). Digestion of the cross-linked receptor with different enzymes demonstrated the murine BB<sub>2</sub> receptor was not a sialoprotein, contained no O-linked glycosylation, and had four tri-antennary and or tetra-antennary complex oligosaccharide chains (Kusui *et al.*, 1994). Studies using baculovirus expression of the BB<sub>2</sub> receptor (Kusui *et al.*, 1995) demonstrated that neither full glycosylation was needed for receptor expression on the cell surface nor did the glycosylation have to be tri- or tetra-antennary for expression, because in the baculovirus only 11 kDa of glycosylation was seen on different sites and the glycosylation was entirely biantennary complex oligosaccharide chains (Kusui *et al.*, 1995).

**IV.3. BB<sub>2</sub> genomic organization**—The human BB<sub>2</sub> receptor gene was localized to Xp22 (Maslen and Boyd, 1993; Xiao *et al.*, 2001) and the murine BB<sub>2</sub> receptor gene to X chromosome between the Pdha-1 and Amg loci (Maslen and Boyd, 1993). Both the human (Xiao *et al.*, 2001) and murine (Weber *et al.*, 2000) BB<sub>2</sub> receptor gene organization have been studied in detail. The human BB<sub>2</sub> receptor gene has three exons (Corjay *et al.*, 1991; Xiao *et al.*, 2001) spanning more than 27 kb with intron 1 and intron 2 being 23 and 1.6 kb (Xiao *et al.*, 2001). Exon one encodes the first three membrane-spanning domains of the BB<sub>2</sub> receptor and the splice site is located in the proximal second intracellular loop (residue 137). Exon 2 encodes for the transmembrane regions 4 and 5 and most of the 3<sup>rd</sup> intracellular loop with the splice site located at residue 254. Exon 3 encodes for the transmembrane domains 5 as well as the cytoplasmic carboxyl terminus of the BB<sub>2</sub> receptor (Xiao *et al.*, 2001). Two major transcription start sites for the human BB<sub>2</sub> receptor gene were found in gastrointestinal and breast cancer cells located 43 and 36 bp downstream of a TTTAAA motif which is identified 407 to 402 bp upstream of the ATG start codon (Xiao *et al.*, 2001). Truncation studies of the transfected promoter region suggested a cyclic AMP response element (CRE) motif located 112 bp upstream of the major transcription start site is required to confer basal BB<sub>2</sub> receptor promoter activity in duodenal cancer cells (Xiao *et al.*, 2001).

**IV.4. BB<sub>2</sub> receptor expression**—Expression levels of BB<sub>2</sub> receptor mRNA has been reported in human, mouse and monkey (Spindel *et al.*, 1990; Corjay *et al.*, 1991; Battey *et al.*, 1991; Ohki-Hamazaki *et al.*, 1997a; Sano *et al.*, 2004). BB<sub>2</sub> receptor mRNA distribution was studied in detail in the monkey where it is found in the greatest amount in the pancreas

and lesser amounts in the stomach, prostate, skeletal muscle and in the CNS (Sano *et al.*, 2004). This result generally agrees with studies of location of the human BB<sub>2</sub> receptor gene where a very strong signal was found in the normal pancreas with 4 specific transcripts of 9, 4.6, 3.1 and 2.1 kb sizes, a weaker signal in the stomach with 2 transcripts of 9 and 3.1 kb and very weak 9 kb transcript signal in whole brain and adrenal (Xiao *et al.*, 2001). In the monkey CNS the BB<sub>2</sub> receptor mRNA was widely expressed with the highest amounts in hippocampus, hypothalamus, amygdala and pons (Sano *et al.*, 2004). In the mouse BB<sub>2</sub> receptor mRNA was present in high amounts in the digestive tract in the colon, but not in the stomach or small intestine (Battey *et al.*, 1991). Detailing mapping in the rat brain was reported which showed BB<sub>2</sub> receptor expression in all brain regions, with the highest amounts of BB<sub>2</sub> receptor mRNA in the hypothalamus, particularly the suprachiasmatic and supraoptic nuclei as well in the magnocellular preoptic nucleus in the basal ganglia and the nucleus of the lateral olfactory tract (Battey and Wada, 1991).

Detailed CNS location of the murine BB<sub>2</sub> receptor has been reported using a specific BB<sub>2</sub> receptor antibody (Kamichi *et al.*, 2005). The BB<sub>2</sub> receptor was widely distributed in the mouse brain in the isocortex, hippocampal formation, pyriform cortex, amygdala, hypothalamus and brain stem (Kamichi *et al.*, 2005). Strong BB<sub>2</sub> immunoreactivity was observed in many nuclei of the amygdala and in the nucleus tractus solitarius (Kamichi *et al.*, 2005). Double labeling studies in the amygdala demonstrated subpopulations of BB<sub>2</sub> receptors present in the GABAergic neurons providing support for a possible role of BB<sub>2</sub> receptors mediating memory by modulating neurotransmitter release in the local GABAergic network (Kamichi *et al.*, 2005).

Binding studies have confirmed the widespread distribution of BB<sub>2</sub> receptors in the brain showing high levels in the cortex as well as the suprachiasmatic and supraoptic nuclei of the rat (Ladenheim *et al.*, 1990; Ladenheim *et al.*, 1992; Ladenheim *et al.*, 1993a; Moody and Merali, 2004). Binding studies and studies of biological activity provide evidence for BB<sub>2</sub> on both gastrointestinal and urogenital smooth muscle cells (Severi *et al.*, 1991; Kilgore *et al.*, 1993; Ladenheim *et al.*, 1997a; Milusheva *et al.*, 1998; ter Beek *et al.*, 2004; Fleischmann *et al.*, 2005). BB<sub>2</sub> receptors in the gastrointestinal tract are also found in gastric antral G cells (Giraud *et al.*, 1987), other gastric mucosa cells (D cell, mucus cell, parietal cell) (Nakamura *et al.*, 1988) and pancreatic acinar cells (Jensen *et al.*, 1978; Jensen *et al.*, 1988a; Jensen, 1994). In the epithelial cells lining the normal human gastrointestinal tract, BB<sub>2</sub> receptor mRNA was only found in the antrum with the esophagus, jejunum, ileum and not in the descending colon (Ferris *et al.*, 1997).

BB<sub>2</sub> receptors are present on a large number of different tumors using binding studies, immunohistochemical localization using specific receptor antibodies and/or assessment of BB<sub>2</sub> receptor mRNA. BB<sub>2</sub> receptors have been widely studied in prostate cancer (Reubi *et al.*, 2002; Jensen and Moody, 2006; Patel *et al.*, 2006), small cell lung cancer (Corjay *et al.*, 1991; Toi-Scott *et al.*, 1996; Jensen and Moody, 2006; Patel *et al.*, 2006), non small cell lung cancer (Corjay *et al.*, 1991; Toi-Scott *et al.*, 1996; Siegfried *et al.*, 1997; Jensen and Moody, 2006), breast cancer (Gugger and Reubi, 1999; Reubi *et al.*, 2002; Jensen and Moody, 2006; Patel *et al.*, 2006), head and neck squamous cell cancer (Lango *et al.*, 2002; Jensen and Moody, 2006), colon cancer (Carroll *et al.*, 1999b; Carroll *et al.*, 2000a; Jensen *et al.*, 2001; Glover *et al.*, 2003; Patel *et al.*, 2006), uterine cancer (Fleischmann *et al.*, 2005), various CNS/neural tumors [glioblastomas, neuroblastomas] (Jensen and Moody, 2006), ovarian cancer (Sun *et al.*, 2000b), gastrointestinal carcinoid tumors (Reubi *et al.*, 2002; Scott *et al.*, 2004) and renal cell cancers (Reubi *et al.*, 2002; Heuser *et al.*, 2005).



## IV.5. BB<sub>2</sub> receptors pharmacology

**IV.5.a. BB<sub>2</sub> receptors agonists:** The human BB<sub>2</sub> receptor (Frucht *et al.*, 1992; Benya *et al.*, 1995b; Reubi *et al.*, 2002) as well as in the rat (von Schrenck *et al.*, 1990; Ladenheim *et al.*, 1992; Ladenheim *et al.*, 1993a; Lin *et al.*, 1996; Katsuno *et al.*, 1999; Ryan *et al.*, 1999), mouse (Huang *et al.*, 1990; Ryan *et al.*, 1999) and guinea pig BB<sub>2</sub> receptors (Jensen and Gardner, 1981; Mantey *et al.*, 1993) have greater than 50-fold higher affinity for GRP than NMB (Fig. 2). Bombesin and various frog peptides, including ranatensin litorin, PG-L and [Phe<sup>13</sup>] bombesin also have high affinities for the BB<sub>2</sub> receptor, where as other frog peptides such as phyllolitorin, [Leu<sup>8</sup>] phyllolitorin, SAP bombesin and *Xenopus* NMB have low affinity for this receptor (Jensen and Gardner, 1981; Frucht *et al.*, 1992; Mantey *et al.*, 1997; Katsuno *et al.*, 1999) (Fig.1) (Table 2). The synthetic bombesin analogue, [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> (Iwabuchi *et al.*, 2003), which has high affinity for human BB<sub>3</sub> receptor, also has high affinity for the BB<sub>2</sub> receptor as well as the BB<sub>1</sub> receptor and fBB4 (Mantey *et al.*, 1997; Pradhan *et al.*, 1998; Ryan *et al.*, 1998b).

**IV.5.b. BB<sub>2</sub> receptor antagonists, partial agonists and biased agonists: IV.5.b.1. BB<sub>2</sub> receptor antagonists:** There have been a large number of different compounds reported to function as BB<sub>2</sub> receptor antagonists (Jensen and Coy, 1991; Jensen *et al.*, 1993; de Castiglione and Gozzini, 1996). They can be divided into six general classes of BB<sub>2</sub> receptor antagonists (Jensen and Coy, 1991; Jensen *et al.*, 1993; de Castiglione and Gozzini, 1996)(Table 2). All classes are peptides or peptoid antagonists, except for Class 6, which are flavone derivatives, isolated from extracts of the mulberry tree *Morus bombycis* (Mihara *et al.*, 1995). These six classes include substituted substance P analogues (Class 1); [D-Phe<sup>12</sup>] bombesin analogues (Class 2); modified position 13–14 bombesin or position 26–27 GRP analogues (Class 3); desMet<sup>14</sup> or GRP<sup>27</sup> analogues (Class 4); peptoids (Class 5) and finally the nonpeptide analogues, kuwanon G and H (Class 6) (Fig.1).

Jensen and co-workers described in 1984 that the D-amino acid substituted substance P (SP) analogue, [D-Arg<sup>1</sup>, D-Pro<sup>2</sup>, DTrp<sup>7,9</sup>, Leu<sup>13</sup>] SP, not only functioned as a substance P receptor antagonist, but also inhibited both radiolabeled bombesin binding and bombesin-stimulated amylase release from guinea pig pancreatic acini, which are now known to possess only BB<sub>2</sub> receptors. Later, they showed various D-amino acid substituted substance P analogues had broad inhibitory activity against a number of GPCR (Zhang *et al.*, 1988; Jensen *et al.*, 1988b). The inhibition of the action of bombesin by [D-Arg<sup>1</sup>, D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>, Leu<sup>13</sup>] SP was competitive in nature with a Schild plot having a slope of 0.996 and the inhibition was specific for the substance P and BB<sub>2</sub> receptor, because it did not inhibit VIP, secretin or carbamylcholine-stimulated secretion (Jensen *et al.*, 1984). Subsequent studies demonstrated numerous D-amino acid substance P and SP<sub>4-11</sub> analogues including [D-Arg<sup>1</sup>, D-Phe<sup>5</sup>, D-Trp<sup>7,9</sup>, Leu<sup>13</sup>] SP functioned as BB<sub>2</sub> receptor antagonists (Woll and Rozengurt, 1988b; Jensen *et al.*, 1988b; de Castiglione and Gozzini, 1996). These analogues were reported to inhibit bombesin-stimulated growth of lung cancer cells and Swiss 3T3 cells (Woll and Rozengurt, 1988a; Woll and Rozengurt, 1988b) as well as a number of other bombesin-stimulated changes in the CNS and peripheral tissues (Jensen and Coy, 1991). This class of BB<sub>2</sub> receptor antagonists is now rarely used, not only because of their relatively low affinities for the BB<sub>2</sub> receptor (1–40 μM), but also because of their lack of selectivity for the BB<sub>2</sub> over the BB<sub>1</sub> receptor. In addition, some show agonist activity in various tissues (von Schrenck *et al.*, 1990; Jensen and Coy, 1991; Patel and Schrey, 1991; Lin *et al.*, 1995; Mantey *et al.*, 1997; Katsuno *et al.*, 1999) (Table 2). These various D-amino acid substituted SP analogues were reported not only to inhibit the action of bombesin, but also to function as antagonists of substance P, cholecystokinin, vasopressin and endothelin (Zhang *et al.*, 1988; Langdon *et al.*, 1992; Jarpe *et al.*, 1998). Subsequent detailed studies of the mechanism of action of these

substance P analogues provided evidence that they were functioning as biased agonists rather than antagonists. This will be discussed below in the next section dealing with biased agonists.

Early bombesin structure-function studies demonstrated that Trp<sup>8</sup> and His<sup>12</sup> in the COOH terminus of bombesin were essential for biologic activity (Broccardo *et al.*, 1976; Rivier and Brown, 1978; Marki *et al.*, 1981). The substitution of a number of D-amino acids (DPhe, D-chloro-phenylalanine, D-Tyr) for His<sup>12</sup> in bombesin analogues produced antagonists (Class 2) (Heinz-Erian *et al.*, 1987; Saeed *et al.*, 1989) (Fig.1). These antagonists inhibited bombesin-stimulated amylase release from pancreatic acini (Heinz-Erian *et al.*, 1987; Saeed *et al.*, 1989) and the satiety effect of bombesin in rats (Flynn, 1997) which were both due to BB<sub>2</sub> receptor activation. The use of these antagonists is limited by their relatively low affinities for the BB<sub>2</sub> receptor (0.4–10 μM), their low aqueous solubility and their low selectivity for BB<sub>2</sub> over BB<sub>1</sub> receptors (Lin *et al.*, 1995; Mantey *et al.*, 1997; Katsuno *et al.*, 1999).

Numerous studies have demonstrated that the biologically active portion of GRP or bombesin is the COOH terminus (Broccardo *et al.*, 1976; Rivier and Brown, 1978; Heimbrook *et al.*, 1988; Lin *et al.*, 1996). In 1988 Coy and coworkers reported a new class of BB<sub>2</sub> receptor antagonists by substituting into the COOH terminus of bombesin, pseudopeptide bonds ( $\psi$  bonds) (i.e. each CONH group one at a time replaced by CH<sub>2</sub>NH), a strategy that had been used successfully to make antagonists for gastrin, secretin and substance P (Martinez *et al.*, 1985; Rodriguez *et al.*, 1986; Coy *et al.*, 1988; Qian *et al.*, 1989; Haffar *et al.*, 1991) (Table 2) (Fig.1). Two of the pseudopeptides were antagonists with the  $\psi$  13–14 analogues having a higher affinity than the  $\psi$  9–10 bond analogue. This  $\psi$ 13–14 bombesin analogue was the first bombesin receptor antagonist described with an affinity <0.1 μM (Coy *et al.*, 1988). Subsequent studies demonstrated this analogue had 50–100-fold selectivity for the BB<sub>2</sub> receptor in human or rat than the BB<sub>1</sub> receptor (Benya *et al.*, 1995b; Ryan *et al.*, 1999). This antagonist was shown to inhibit a number of BB<sub>2</sub> receptor-stimulated processes including bombesin stimulated enzyme secretion from isolated acini and growth of Swiss 3T3 cells as well as of various small cell lung cancer cell lines (Trepel *et al.*, 1988; Coy *et al.*, 1988; Coy *et al.*, 1989; Liu *et al.*, 2002). A subsequent study described short chain pseudopeptide bombesin receptor antagonists ([such as [D-Phe<sup>6</sup>, Cpa<sup>14</sup>,  $\psi$ 13–14]Bn<sub>6–14</sub>]) that had fewer proteolytic sites, and could be more easily synthesized (Coy *et al.*, 1989; Coy *et al.*, 1990; Jensen and Coy, 1991; Coy *et al.*, 1992a)(Table 2). Furthermore, some of the  $\psi$ 13–14 analogues had partial agonist activity in some species (particularly the rat) which was not seen in a number of the newer, shortened substituted pseudopeptide analogues such as [D-Phe<sup>6</sup>, Cpa<sup>14</sup>,  $\psi$ 13–14]Bn<sub>6–14</sub> (Dickinson *et al.*, 1988; Coy *et al.*, 1990; Houben and Deneff, 1991; Coy *et al.*, 1992a) (Fig.1). A number of the shortened D-Phe substituted [ $\psi$ 13–14] Bn<sub>6–14</sub> analogues are > 100 fold selective for the BB<sub>2</sub> over the BB<sub>1</sub> receptor (von Schrenck *et al.*, 1990; Mantey *et al.*, 1997; Katsuno *et al.*, 1999). Subsequently, a particularly potent group of pseudopeptide antagonists were described having a D-Pro- $\psi$  (CH<sub>2</sub>NH)-Phe-NH<sub>2</sub> moiety at the COOH terminus of GRP (Leban *et al.*, 1993). One of the most potent and widely used analogue in this series is: (3-PhPr)-His, Trp, Ala, Val, D-Ala, His, D-Pro- $\psi$  (CH<sub>2</sub>NH)-Phe-NH<sub>2</sub> [K<sub>i</sub>-0.001 nM murine BB<sub>2</sub> (Leban *et al.*, 1993); 0.7 nM rat BB<sub>2</sub> (Mantey *et al.*, 1997), 10 nM human BB<sub>2</sub> (Moody *et al.*, 1996a)] (BW2258U89). BW2258U89 has >10,000 fold selectivity for the rat BB<sub>2</sub> over the rat BB<sub>1</sub> receptor (Mantey *et al.*, 1997; Katsuno *et al.*, 1999)(Table 2). BW2258U89 is reported to inhibit small cell lung cancer growth (Moody *et al.*, 1995b), inhibit bombesin-stimulated gastrin release *in vivo* in dogs and rats (Singh *et al.*, 1992) and blocked the satiety effect of bombesin in rats (Kirkham *et al.*, 1994). An additional series of substituted pseudopeptide analogues with position 14 substitutions in addition to the  $\psi$ 13–14 bond have been described and widely used by Schally's group for inhibition of various tumor cell growth (Radulovic *et al.*, 1991a; Cai *et al.*, 1992; Qin *et al.*, 1994; Cai *et al.*, 1994; Qin *et al.*, 1995; Jungwirth *et al.*, 1998; Bajo *et al.*, 2004). Two analogues with high potency in this group include [D-Phe<sup>6</sup>,  $\psi$ 13–14, Tac<sup>14</sup>] Bn<sub>6–14</sub> (tac=thiazolidine-4-carboxylic acid)[RC-3950-II] (Cai *et al.*, 1994) (K<sub>i</sub>-0.078 nM,

murine BB<sub>2</sub> receptor and [D-Tpi<sup>6</sup>, ψ13–14] Bombesin<sub>6–14</sub> (RC-3095) (K<sub>i</sub>-0.92 nM, murine BB<sub>2</sub> receptor (Reile *et al.*, 1994; Qin *et al.*, 1994; Qin *et al.*, 1995)). A final group of potent antagonists in this class were synthesized by J. Martinez's group, with the most potent being JMV641 and JMV594 (Azay *et al.*, 1996; Lamharzi *et al.*, 1998). JMV641 [H-D-Phe, Gln, Trp, Ala, Val, Gly, His-NH-\*CH [CH<sub>2</sub>-CH (CH<sub>3</sub>)<sub>2</sub>]-\*\*CHOH-(CH<sub>2</sub>)<sub>3</sub>-CH<sub>3</sub> [where \*(S) and \*\*92% of (S isomer)] contains a pseudopeptide bond that mimics the transition state analogue [K<sub>i</sub>-murine BB<sub>2</sub> 0.85 nM](Azay *et al.*, 1996) and has a >3000 fold selectivity for the BB<sub>2</sub> over the BB<sub>1</sub> receptor (Tokita *et al.*, 2001b). JMV594 [DPhe<sup>6</sup>, statine<sup>13</sup>]Bn<sub>6–14</sub>(where statine=4-amino-3-hydroxy-6-methylheptanoic acid) also has a high affinity for the murine BB<sub>2</sub> receptor (K<sub>i</sub>-0.60 nM) (Azay *et al.*, 1998; Linares *et al.*, 1999) and has >5000 fold selectivity for the BB<sub>2</sub> over the BB<sub>1</sub> receptor (Tokita *et al.*, 2001b)(Table 2).

The fourth class of BB<sub>2</sub> receptor antagonists are all [desMet<sup>14</sup>] Bn or [desMet<sup>27</sup>] GRP analogues (Jensen and Coy, 1991; Jensen *et al.*, 1993; de Castiglione and Gozzini, 1996), but vary widely in chemical groups attached and they including desMet amides (Heimbrook *et al.*, 1989; Wang *et al.*, 1990a; Wang *et al.*, 1990b), alkylamides (Camble *et al.*, 1989; Heimbrook *et al.*, 1989; Wang *et al.*, 1990a; Wang *et al.*, 1990b), esters (Heimbrook *et al.*, 1989; Wang *et al.*, 1990b; Coy *et al.*, 1992b), hydrazides (Wang *et al.*, 1990b) and with other COOH terminal groups attached (Heimbrook *et al.*, 1989; Heimbrook *et al.*, 1991)(Table 2) (Fig. 1). A number of these analogues have high potency for the BB<sub>2</sub> receptor in all species studied and have high selectivity for the BB<sub>2</sub> over the BB<sub>1</sub> receptor (Heimbrook *et al.*, 1989; Jensen and Coy, 1991; Jensen *et al.*, 1993; Benya *et al.*, 1995b; de Castiglione and Gozzini, 1996; Mantey *et al.*, 1997; Katsuno *et al.*, 1999). Two widely used antagonists in this class are [D-Phe<sup>6</sup>]Bn<sub>6–13</sub>methylester or its analogues (Wang *et al.*, 1990b; Coy *et al.*, 1992b) and Ac-[N-GRP<sub>20–26</sub>.ethylester (Heimbrook *et al.*, 1989) with each having high affinity for the BB<sub>2</sub> receptor [K<sub>i</sub>-2–5 nM](Heimbrook *et al.*, 1989; Wang *et al.*, 1990b; Coy *et al.*, 1992b; Benya *et al.*, 1995b; Mantey *et al.*, 1997; Katsuno *et al.*, 1999) and having > 1000 fold selectivity for the BB<sub>2</sub> over the BB<sub>1</sub> receptor (von Schrenck *et al.*, 1990; Katsuno *et al.*, 1999). [D-Phe<sup>6</sup>] Bn<sub>6–13</sub>methylester and/or Ac- [N-GRP<sub>20–26</sub>.ethylester are reported to inhibit GRP-stimulated mitogenesis in 3T3 cells (Heimbrook *et al.*, 1989) (Fig. 1). GRP-dependent acid secretion (Heimbrook *et al.*, 1989), GRP-induced signaling in small cell lung cancer cells, GRP/Bn-induced smooth muscle contraction (Maggi *et al.*, 1992), BB<sub>2</sub> receptor mediated pancreatic enzyme secretion (Wang *et al.*, 1990b) and *in vivo* to inhibit bombesin/GRP stimulated pancreatic enzyme secretion (Varga *et al.*, 1991; Coy *et al.*, 1992b), satiety (Stratford *et al.*, 1995; Ladenheim *et al.*, 1996a), hypothermia (Cai *et al.*, 1994) and acid secretion (Weigert *et al.*, 1997). *In vivo* a number of these antagonist were found to have a short duration of action (Alptekin *et al.*, 1991; Coy *et al.*, 1992b) and it was found that by adding a D-Ala<sup>11</sup> in place of Gly<sup>11</sup> in bombesin, as well as lipophilic moieties to the amino terminus, the *in vivo* stability was improved and analogues with long duration of action were obtained. [D-pentafluoro-Phe<sup>6</sup>, D-Ala<sup>11</sup>]Bn<sub>6–13</sub>methylester not only retained high affinity for the BB<sub>2</sub> receptor (human BB<sub>2</sub> 0.9 nM; rat BB<sub>2</sub> K<sub>i</sub>-5 nM) it had >400 to 10,000 fold selectivity for the BB<sub>2</sub> over the BB<sub>1</sub> receptor in rat and human (Coy *et al.*, 1992b; Benya *et al.*, 1995b) it had a 15-fold longer duration of action *in vivo* (Coy *et al.*, 1992b) (Fig. 1). This analogue was subsequently used in a number of human studies (Guex and Peitsch, 1997; Hildebrand *et al.*, 2001) which will be reviewed in Section IV.8. below.

In contrast to the BB<sub>1</sub> receptor (Eden *et al.*, 1996; Moody *et al.*, 2000; Tokita *et al.*, 2001a), there are no selective peptoid BB<sub>2</sub> receptor antagonists (Class 5). However, PD 176252 is a peptoid antagonist that has nanomolar affinity for both the BB<sub>2</sub> [K<sub>i</sub> 1 nM] and BB<sub>1</sub> receptor [K<sub>i</sub> 0.1 nM](Ashwood *et al.*, 1998; Moody *et al.*, 2003b). Subsequent studies demonstrated PD 176252 inhibited the growth of lung cancer cells; potentiated the growth inhibitory effects of histone deacetylase inhibitors (Moody *et al.*, 2006a); inhibited GRP/Bn-stimulated signaling in lung cancer cells [Ca<sup>2+</sup> and tyrosine phosphorylation of p125<sup>FAK</sup>] as well as the stimulation

of increases in c-fos mRNA (Moody *et al.*, 2000) and growth (Moody *et al.*, 2000); and in rats had an anxiolytic effect *in vivo* in rats (Merali *et al.*, 2006).

The only nonpeptide, nonpeptoid antagonists of BB<sub>2</sub> receptors reported are kuwanon G and kuwanon H which are two closely related flavone compounds that were isolated from the Mulberry tree, *Morus bombycis* (Mihara *et al.*, 1995). Only one study (Mihara *et al.*, 1995) has examined their ability to interact with BB<sub>2</sub> receptors on Swiss 3T3 cells. Kuwanon G and kuwanon H had affinities of 290 and 470 nM, respectively for the murine BB<sub>2</sub> receptor and Kuwanon H had a 22 fold higher affinity for the murine BB<sub>2</sub> receptor than the rat BB<sub>1</sub> receptor (Mihara *et al.*, 1995). Kuwanon H inhibited both bombesin-stimulated changes in cytosolic calcium and growth in Swiss 3T3 cells, which are both, mediated by BB<sub>2</sub> receptors (Mihara *et al.*, 1995).

**IV.5.b.2. BB<sub>2</sub> receptor partial agonists:** None of the naturally occurring mammalian or frog bombesin related peptides is a partial agonist for the BB<sub>2</sub> receptor (Jensen *et al.*, 1978; Jensen *et al.*, 1988a; von Schrenck *et al.*, 1989; Lin *et al.*, 1996). However, one of the main difficulties found with the various classes of peptide antagonists is that, in some species or some cellular systems they demonstrated partial agonist activity or even full agonist activity, whereas they are antagonists in other species or cell systems (Jensen and Coy, 1991; Coy *et al.*, 1991b; Coy *et al.*, 1992a). This was reported for both class 3 pseudopeptide analogues as well as for class 4 potent desMet<sup>14</sup> bombesin analogues in a number of studies (Dickinson *et al.*, 1988; Coy *et al.*, 1990; Wang *et al.*, 1990b; Houben and Deneff, 1991; Coy *et al.*, 1992a; Wu *et al.*, 1995). Furthermore, some BB<sub>2</sub> receptor antagonists functioned as partial agonists for BB<sub>1</sub> receptors (Ryan *et al.*, 1996). Detailed studies with both bombesin pseudopeptide and desMet<sup>14</sup> analogues, which functioned as pure BB<sub>2</sub> receptor antagonists in the guinea pig or mouse, demonstrated that many showed partial agonist activity in the rat BB<sub>2</sub> receptor (Coy *et al.*, 1990; Wang *et al.*, 1990b; Jensen and Coy, 1991; Coy *et al.*, 1991b). The conclusion from these studies was that there exist important differences in the ability of the same ligand to activate the BB<sub>2</sub> receptor from different species with the rat having less stringent peptide structural requirements for BB<sub>2</sub> receptor activation than the guinea pig or mouse. The expression level of the BB<sub>2</sub> receptor can have a marked effect on the magnitude of various agonist responses such as phospholipase C activation with stimulation of phosphoinositide breakdown (Tsuda *et al.*, 1997a) and calcium mobilization (Wu *et al.*, 1995) or stimulation of mitogenesis (Wu *et al.*, 1995). This may contribute to the presence or magnitude of the partial agonist activity of some of these compounds in different tissues.

**IV.5.b.3. BB<sub>2</sub> receptor biased agonists:** As discussed above after the initial description (Jensen *et al.*, 1984) in 1984 of the ability of D-amino acid substituted analogues of substance P to function as bombesin receptor antagonists, the same group reported that some of these analogues could function as broad-spectrum antagonists inhibiting the activation of a number of peptide hormone GPCR's (Zhang *et al.*, 1988; Jensen *et al.*, 1988b). It is now clear these compounds can inhibit activation of a wide range of different G-protein coupled receptors [i.e. substance P, cholecystokinin, vasopressin and endothelin] (Zhang *et al.*, 1988; Langdon *et al.*, 1992; Jarpe *et al.*, 1998). A number of subsequent studies have proposed different mechanisms for the ability for the substituted SP analogues to function as broad spectrum GPCR antagonists, with some studies, but not others, suggesting they function as biased agonists at the BB<sub>2</sub> receptor (Jarpe *et al.*, 1998; Sinnott-Smith *et al.*, 2000; MacKinnon *et al.*, 2001; Djanani *et al.*, 2003). Initially it was shown (Jarpe *et al.*, 1998) that the substance P analogue [D-Arg<sup>1</sup>, D-Phe<sup>5</sup>, D-Trp<sup>7,9</sup>, Leu<sup>11</sup>] SP at concentrations that inhibited bombesin-stimulated calcium mobilization at the BB<sub>2</sub> receptor, stimulated c-jun kinase activation and cytoskeletal changes. To explain this unexpected result it was proposed (Jarpe *et al.*, 1998) that the substance P analogue functions as a biased agonist in that it causes the BB<sub>2</sub> receptor to preferentially activate Gα<sub>12</sub> over Gα<sub>q</sub> and this results in activation of the Gα<sub>12</sub> stimulated events



(c-Jun kinase activation, changes in cytoskeletal events) and inhibition of the  $G\alpha_q$  stimulated events (i.e. calcium mobilization). A latter study (Sinnott-Smith *et al.*, 2000) challenged this hypothesis by providing evidence that D-amino acid substituted SP analogues prevented  $BB_2$ , bradykinin and vasopressin receptor activation of both  $G\alpha_{12}$  and  $G\alpha_q$ . A more recent study (MacKinnon *et al.*, 2001) provides evidence that [D-Arg<sup>1</sup>, D-Phe<sup>5</sup>, D-Trp<sup>7,9</sup>, Leu<sup>11</sup>] SP differentially modulates the activation of the G proteins  $G\alpha_{12}$ ,  $G\alpha_i$  and  $G\alpha_q$ . This unique ability allows  $BB_2$  receptor activation to couple to  $G\alpha_i$  and at the same time block  $G\alpha_q$  supporting the proposal that [D-Arg<sup>1</sup>, D-Phe<sup>5</sup>, D-Trp<sup>7,9</sup>, Leu<sup>11</sup>] SP is functioning as a biased agonist at the  $BB_2$  receptor.

#### IV.6. $BB_2$ receptors structural basis of receptor binding/activation

**IV.6.a.  $BB_2$  receptors agonist binding/activation:** Structure-function studies of GRP or bombesin demonstrate the COOH terminal heptapeptide is the minimal peptide length required for  $BB_2$  receptor activation and the COOH terminal nonapeptide is the minimal fragment required for full affinity for  $BB_2$  (Mazzanti *et al.*, 1982; Heimbrook *et al.*, 1988; Lin *et al.*, 1996). GRP differs from NMB in 3 residues in the biologically active COOH decapeptide: a histidine in GRP 8 amino acids from the COOH terminus instead of Leu in NMB, at 5 amino acids from the COOH terminus a valine in GRP instead of a threonine and a leucine at the penultimate position of GRP instead of phenylalanine in NMB (Minamino *et al.*, 1983; Lin *et al.*, 1996). Structure-function studies of all natural occurring bombesin-related peptides for the  $BB_2$  and  $BB_1$  receptors suggested primarily the presence His for Leu and to a lesser extent the presence of Leu for Phe were the most important differences in GRP from NMB determining high affinity and selectivity for the  $BB_1$  receptor (Lin *et al.*, 1996). Correlating biological activity with binding affinity, especially of antagonists, demonstrated the presence of a COOH terminal amino acid in position 14 of bombesin, is not essential for high affinity for the  $BB_2$  receptor, but it is essential for biologic activity (Coy *et al.*, 1988; Wang *et al.*, 1990a; Wang *et al.*, 1992).

From studies correlating binding results with biological activity, especially for COOH terminal pseudopeptides, a model was proposed for the biologically active conformation of GRP/Bn at the  $BB_2$  receptor (Coy *et al.*, 1988; Wang *et al.*, 1990a; Coy *et al.*, 1991b). In a study (Coy *et al.*, 1988) of the effects on bombesin's affinity and potency for the  $BB_2$  receptor of substitution of a pseudopeptide bond ( $\psi$  bond) (i.e.  $CH_2NH_2$  instead of  $CONH$ ) between each amino acid pair at the COOH terminus, it was found only a  $\psi_{13-14}$  and  $\psi_{8-9}$  substitution resulted in peptides that retained affinity for the  $BB_2$  receptor but did not activate it and thus functioned as antagonists. Because previous studies of somatostatin analogues had shown hydrogen bonding was the prime factor in stabilizing the peptide's conformation (Sasaki *et al.*, 1987), the loss of efficacy with retention of affinity in these two bombesin pseudopeptides suggested the elimination of these CO groups were likely having an effect on the conformation of the peptide due to both loss of a potential intramolecular hydrogen-bonding point and increased rotation about the C-N bond (Coy *et al.*, 1988). The model proposed (Coy *et al.*, 1988) was based on the known solution conformation of somatostatin in which the COOH terminus of bombesin had a  $\beta$ -bend beginning at Val<sup>10</sup> and the rest of the amino acid chains arranged in an antiparallel  $\beta$ -pleated sheet. In this model the hydrogen bonding between Leu<sup>13</sup>-Leu<sup>14</sup> CO groups and Ala<sup>9</sup>-Val<sup>10</sup>-CO groups is important and their destruction by a pseudopeptide bond would lead to a conformational shift and loss of efficacy. Support for this conformation has come from studies of both agonists and antagonists (Wang *et al.*, 1990a; Coy *et al.*, 1991a; Kull, Jr. *et al.*, 1992). Only the agonist results will be discussed here with the antagonist result in the next section. The proposed folded conformation of the COOH terminus of GRP/bombesin was supported by findings from a study of various covalently cyclized analogues of the COOH terminus of bombesin (Coy *et al.*, 1991a). Using such an approach both agonists and antagonists were identified supporting the proposal that both  $BB_2$  receptor



agonists and antagonist likely adopted a folded conformation. A subsequent study (Lin *et al.*, 1996) demonstrated one cyclized analogue, [D-Cys<sup>6</sup>, D-Ala<sup>11</sup>, Cys<sup>14</sup>]Bn<sub>6-14</sub> had >400 fold greater potency at activation the BB<sub>2</sub> receptor than the BB<sub>1</sub> receptor, suggesting the constrained conformation induced by cyclization resembled more the active conformation for the BB<sub>2</sub> than the BB<sub>1</sub> receptor. It also suggested the active conformation for BB<sub>2</sub> and BB<sub>1</sub> receptor are significantly different (Lin *et al.*, 1996). The substitution of D-Ala in position 11 of bombesin for glycine would be expected to stabilize the folding in the above proposed model and therefore not lead to a decrease in affinity/potency (Lin *et al.*, 1996). The finding that [D-Ala<sup>11</sup>] bombesin was equipotent to native bombesin for the BB<sub>2</sub> receptor, but resulted in a marked decrease in affinity for the BB<sub>1</sub> receptor, supports both the folded conformation model proposed for the GRP/Bn COOH terminus (Coy *et al.*, 1988) and also suggests the active conformation of bombesin for these two receptors is very different (Lin *et al.*, 1996).

To elucidate the molecular basis of BB<sub>2</sub> receptor agonist selectivity, high affinity and receptor activation both a chimeric receptor approach (Tseng *et al.*, 1995a; Tseng *et al.*, 1995b; Maughfling *et al.*, 1997; Tokita *et al.*, 2002) either alone or followed by site-directed mutagenesis (Tokita *et al.*, 2002), a comparison of receptor selectivity for agonists combined with homology screening after computer alignment of bombesin receptor family members (Akeson *et al.*, 1997; Nakagawa *et al.*, 2005) and site-directed mutagenesis of specific residues (Benya *et al.*, 1993; Slice *et al.*, 1994; Benya *et al.*, 1994d; Donohue *et al.*, 1999; Lin *et al.*, 2000; Schumann *et al.*, 2003) has been used. A study (Maughfling *et al.*, 1997) of chimeric BB<sub>2</sub>/BB<sub>1</sub> receptors demonstrated receptor regions between the end of transmembrane (TM) domain 3 (TM3) and TM6 were responsible for the high affinity and selectivity of NMC (GRP<sub>18-27</sub>) for the BB<sub>2</sub> receptor. A subsequent detailed study (Tokita *et al.*, 2002) examined both GRP loss and gain of affinity chimeric BB<sub>2</sub>/BB<sub>1</sub> receptors followed by site-directed mutagenesis, demonstrated differences in the extracellular (EC) domain 3 (EC3)[where the N terminus is EC1] was the specific critical region for determining GRP high affinity and selectivity (Fig. 4). Site-directed mutagenesis (Tokita *et al.*, 2002) of each of the 20 amino acid differences between the BB<sub>2</sub> and BB<sub>1</sub> receptor in the EC3 demonstrated that two amino acid differences were the most important; the substitution of Phe<sup>185</sup> in the BB<sub>2</sub> receptor for Ile in the comparable position in the BB<sub>1</sub> receptor and Ala<sup>198</sup> in the BB<sub>2</sub> for Ile in the comparable position of the BB<sub>1</sub> receptor (Fig. 4). Additional point mutations in these positions (Tokita *et al.*, 2002) demonstrated that an amino acid with an aromatic ring in position 185 of the BB<sub>2</sub> receptor was the most important of these two changes, whereas the size of the backbone substitution in position 198 was the difference from the BB<sub>2</sub> receptor at this position but it was less important than the position 185 difference for determining high affinity for GRP. The mechanism (Tokita *et al.*, 2002) of the aromatic substitution's effect in position was not studied in detail, but it was proposed it might be due to cation- $\pi$  or  $\pi$ -receptor interaction.

Important amino acids for GRP selectivity/high affinity were also identified using a different approach of comparison of receptor selectivity for agonists combined with homology screening after computer alignment of bombesin receptor family members (Akeson *et al.*, 1997; Nakagawa *et al.*, 2005). This approach made use of the fact that the BB<sub>2</sub>, BB<sub>1</sub> and frog BB<sub>4</sub> receptors all have relatively high affinity for bombesin, whereas the BB<sub>3</sub> receptor has a very low affinity. In the first study (Akeson *et al.*, 1997) nine amino acids were identified which were the same in BB<sub>1</sub>, BB<sub>2</sub>, and frog BB<sub>4</sub> receptor, but differed in the BB<sub>3</sub> receptor. Site-directed mutagenesis (Akeson *et al.*, 1997) demonstrated the occurrence of Arg<sup>288</sup> in the BB<sub>2</sub> receptor or comparable position of the other receptors with high affinity for bombesin, instead of a histidine in the comparable position of the BB<sub>3</sub> receptor (i.e. R<sup>288</sup>H change), a glutamine in position 121 instead of arginine (Q<sup>121</sup>R), a proline in position 199 instead of a serine (P<sup>199</sup>S change) and an alanine in position 308 instead of a serine (A<sup>308</sup>S change) were the critical differences accounting for high affinity for bombesin (Fig. 4). Of these four critical differences the Q<sup>121</sup>R and R<sup>288</sup>H change had the most profound effect on determining both

GRP and bombesin's affinity for the BB<sub>2</sub> receptor (Akeson *et al.*, 1997). Molecular modeling (Akeson *et al.*, 1997) demonstrated the Q<sup>121</sup>, R<sup>288</sup> and A<sup>308</sup> all lie in a plane pointing inward toward the binding pocket. Furthermore, the critical Q<sup>121</sup> lies in the same position in the BB<sub>2</sub> receptor in TM3 as the highly conserved aspartate in biogenic amine receptors, which has been shown to be critical for their high affinity interaction, suggesting a similar interaction is critical for GRP high affinity. In a second study (Nakagawa *et al.*, 2005) a modification of the above approach was used where amino acid differences from receptors with high affinity for GRP (BB<sub>2</sub> receptor and frog BB<sub>4</sub> receptor) were identified compared to the BB<sub>3</sub>, which has low affinity for GRP. Fourteen amino acid differences (Nakagawa *et al.*, 2005) were found and each analyzed by site-directed mutagenesis with the results compared to the effects of the Q<sup>121</sup>R, P<sup>199</sup>S, R<sup>288</sup>H and A<sup>308</sup>S point mutations described above (Akeson *et al.*, 1997). This study (Nakagawa *et al.*, 2005) demonstrated that GRP's selectivity for the BB<sub>2</sub> receptor was primarily determined by K<sup>101</sup>, Q<sup>121</sup>, A<sup>198</sup>, P<sup>199</sup>, S<sup>293</sup>, R<sup>288</sup> and T<sup>297</sup> of the BB<sub>2</sub> receptor (Fig. 4). Molecular modeling of the BB<sub>2</sub> receptor (Nakagawa *et al.*, 2005) demonstrated the backbone substitution of eight of the 14 amino acids identified using this approach were facing inward to the binding pocket and were within 6 Å including the Q<sup>121</sup>, A<sup>193</sup>, S<sup>293</sup> and R<sup>288</sup> which were especially important for a GRP affinity. A phylogenetic analysis of the structures of the BB<sub>2</sub> receptor from 21 species was performed and compared to other bombesin receptor members as well as other GPCR's (Baldwin *et al.*, 2007). This analysis (Baldwin *et al.*, 2007) demonstrated the sequence GVSVFTLTALS (125–136 in murine BB<sub>2</sub> receptor) in the cytoplasmic side of TM3 is unique to the bombesin receptor family and retained by all members; the cysteines residues in positions C94, C114, C197, C277 and C317 in the murine BB<sub>2</sub> are highly conserved in all BB<sub>2</sub> receptors; and the important amino acids described for determining GRP affinity are generally well-conserved in all BB<sub>2</sub> receptors.

BB<sub>2</sub> receptor mutations are reported to occur in human colon and gastric cancer and a number of these have identified and characterized (Carroll *et al.*, 1999a; Carroll *et al.*, 2000b; Glover *et al.*, 2003). In the human BB<sub>2</sub> receptor P<sup>145</sup>Y, P<sup>198</sup>L, P<sup>200</sup>S and V<sup>316</sup>E mutations (equivalent to positions 146,199,210,317 in murine BB<sub>2</sub> receptor, (Fig. 4) are found in colon and/or gastric cancers (Carroll *et al.*, 1999a; Carroll *et al.*, 2000b; Glover *et al.*, 2003) and each resulted in no ligand binding of the expressed BB<sub>2</sub> receptor, demonstrating these amino acids in the BB<sub>2</sub> receptor are either essential for receptor expression and/ or binding.

A number of studies have attempted to examine the important amino acids in BB<sub>2</sub> receptor mediating activation as well as the stimulation of various receptor modulatory processes (internalization, down-regulation and or desensitization) (Benya *et al.*, 1994a; Tseng *et al.*, 1995a; Donohue *et al.*, 1999; Schumann *et al.*, 2003). Because the BB<sub>2</sub> receptor as well as the BB<sub>1</sub> and BB<sub>3</sub> receptors have a conserved aspartate residue at position 98 (D<sup>98</sup>) just at the extracellular border of TM 2 and a arginine residue (R<sup>309</sup>) at the top of TM7 (Fig. 4), the effect of these on receptor binding and activation was explored using site-directed mutagenesis, binding studies and an *in situ* reconstitution assay. The results (Donohue *et al.*, 1999) demonstrated that these residues are not only important for high affinity binding, but they are critical for efficient coupling of the BB<sub>2</sub> receptor to G<sub>αq</sub>. The authors (Donohue *et al.*, 1999) suggested that these results are consistent with the existence of a salt bridge interaction between these two polar and oppositely charge amino acids that maintains the proper BB<sub>2</sub> receptor conformation necessary to interact with G proteins. The importance of the second and third intracellular domains (IC2, IC3) of the BB<sub>2</sub> receptor for affinity, activation and internalization were examined by making BB<sub>2</sub> receptor-/m3 muscarinic cholinergic receptor chimeras (Tseng *et al.*, 1995a). Replacement of IC2 and/or IC3 domain alone or together in the BB<sub>2</sub> receptor had minimal or no effect on receptor affinity or the occurrence of the high affinity receptor binding state, however the replacement of IC3, but not IC2, dramatically decreased the ability of the BB<sub>2</sub> receptor to internalize bombesin or to activate the receptor and stimulate phospholipase A<sub>2</sub> or C (Tseng *et al.*, 1995a). It was proposed from these results that agonist

activation of a similar conformational state is required for BB<sub>2</sub> receptor G protein-coupling and internalization, but is not needed for generation of a high affinity binding state (Acs *et al.*, 2000). The BB<sub>2</sub> receptor, as well as other bombesin receptors and many GPCR's, have a retained DRY sequence at the beginning of the second intracellular domain and a conserved alanine in the distal third intracellular domain (Benya *et al.*, 1994a) which have been shown in a number of GPCR's to be important for G-protein coupling and cell signaling (Benya *et al.*, 1994a). Site-directed mutagenesis (Benya *et al.*, 1994a; Benya *et al.*, 1995a) was used to make a R<sup>139</sup>G and A<sup>263</sup>E mutant (Fig. 4) to explore the importance of these conserved residues for BB<sub>2</sub> receptor affinity, cell signaling and activation of receptor modulatory processes (internalization, down-regulation, desensitization) (Benya *et al.*, 1994a; Benya *et al.*, 1995a). Both of these mutations decreased BB<sub>2</sub> receptor affinity for bombesin by 9-fold, neither receptor could activate phospholipase C and the R<sup>139</sup>G, but not the A263E mutant, was uncoupled from G-proteins. Both mutant receptors demonstrated impaired internalization, however the impairment was much greater with the R<sup>139</sup>G mutant. These results demonstrated that BB<sub>2</sub> receptor internalization occurs by both phospholipase-dependent and phospholipase-independent mechanisms and that both are dependent on G-protein coupling of the activated BB<sub>2</sub> receptor. In contrast (Benya *et al.*, 1995a), each of these mutant BB<sub>2</sub> receptors demonstrated no bombesin-stimulated receptor down-regulation, whereas the wild type receptor underwent a > 75% decrease in receptor number when exposed to agonist. These results demonstrated that BB<sub>2</sub> receptor internalization and down-regulation are at least partially mediated by different signaling mechanisms. In studies of the muscarinic cholinergic M3 receptor the central portion of the second intracellular domain (IC2) is important for G protein coupling and internalization (Moro *et al.*, 1993; Moro *et al.*, 1994). Results of a systematic analysis of this region of the BB<sub>2</sub> receptor (amino acids 142–148, Fig. 4) are reported (Schumann *et al.*, 2003). In this study (Schumann *et al.*, 2003) each amino acid was mutated to an alanine either alone or in combination. The mutations had a minimal (<2 fold) to no effect on agonist receptor affinity, however 5 mutants showed decreased efficacy for activation of phospholipase C (Schumann *et al.*, 2003). Two mutations the IM<sup>143,147</sup>AA and VM<sup>144,147</sup>AA showed markedly decreased abilities to activate phospholipase C. The IM double mutant had defective internalization, whereas the R<sup>145</sup>A mutant had enhanced internalization (Schumann *et al.*, 2003). Both double mutants and 3 single mutants also had decreased down-regulation. Maximal changes in phospholipase C were significantly correlated with maximal down-regulation, but not with internalization. Therefore, amino acids within the IC2 of the BB<sub>2</sub> receptor are important for activation of phospholipase C and support the proposal that internalization and down-regulation have a different dependence on phospholipase C activation and are largely independent processes (Schumann *et al.*, 2003). Kinetic analysis of the effect of the R<sup>145</sup>A mutation on BB<sub>2</sub> receptor binding and internalization support the conclusion that the R<sup>145</sup> in the native receptor is having a restraining effect on internalization and its mutation decreased receptor recycling without altering the endocytotic rate (Schumann *et al.*, 2003).

Residues in the cytoplasmic carboxyl terminus of the receptor are important for various receptor modulatory processes such as internalization or desensitization in numerous GPCR's (Benya *et al.*, 1993; Tseng *et al.*, 1995b). Two different approaches have been used with the BB<sub>2</sub> receptor to investigate the importance of this region. In one study (Benya *et al.*, 1993) serial truncation mutants of the BB<sub>2</sub> receptor COOH terminus were constructed as well as site-directed mutation of PKC consensus sites, a potential palmitoylation site and of Ser/Thr residues. None of these mutations altered receptor affinity or altered the ability of the expressed mutant to activate phospholipase C. Longer truncations (at residue 358 or more proximal) resulted in increasing impairment of internalization, whereas the mutation of the potential palmitoylation site had no effect. Mutation of the distal PKC consensus site moderately reduced internalization (approximately 50%) whereas mutation of all Ser/Thr residues in the COOH tail almost completely inhibited internalization (Benya *et al.*, 1993). These results (Benya *et al.*, 1993) show that BB<sub>2</sub> receptor internalization is dependent on residues in the COOH

terminus and suggests that it is partially PKC-dependent, but completely dependent on the presence of at least some Ser or Thr residues in this region. A second approach used to examine the importance of the COOH terminus in BB<sub>2</sub> receptor function was to make BB<sub>2</sub> receptor/m<sub>3</sub> muscarinic cholinergic receptor chimeras or BB<sub>2</sub> receptor/CCK<sub>A</sub> receptor chimeras by substituting the COOH terminus of these receptors for that of the BB<sub>2</sub> receptor (Tseng *et al.*, 1995b). Each of the chimeric receptors demonstrated similar affinities to the wild type BB<sub>2</sub> receptor for bombesin and similar potencies for activation by bombesin. Ligand internalization as well as receptor recycling by the chimeric BB<sub>2</sub> receptors generally assumed the characteristics of the donor receptor (Tseng *et al.*, 1995b). This study (Tseng *et al.*, 1995b) demonstrated that carboxyl-terminal structures determine both the internalization of the ligand-receptor complex and the subsequent recycling. The BB<sub>2</sub> receptor undergoes rapid down-regulation and desensitization in addition to internalization with agonist stimulation (Benya *et al.*, 1994b; Benya *et al.*, 1994d; Kroog *et al.*, 1995a; Benya *et al.*, 1995a). A number of studies have explored the receptor structural elements involved in stimulation of these receptor modulatory processes as well as the signaling cascades involved. The latter will be discussed in a later section on BB<sub>2</sub> cell signaling mechanisms. In a number of GPCR's a conserved NPX (n)Y motif in the seventh transmembrane receptor domain (TM7) is important for mediating receptor internalization and/or resensitization (Slice *et al.*, 1994). Mutation of T<sup>324</sup> within this motif in the rat BB<sub>2</sub> receptor did not effect receptor internalization or its resensitization (Slice *et al.*, 1994) demonstrating that this motif is not universally involved in receptor internalization.

The importance of the COOH terminus of the BB<sub>2</sub> receptor for mediating chronic desensitization or down-regulation was explored by using mutant BB<sub>2</sub> receptors with increasing COOH terminal truncations, a distal PKC consensus mutation, a deletion of all COOH terminal Ser/Thr residues, or mutations that either prevent BB<sub>2</sub> receptor activated phospholipase C activation (R139G, A263E) or G protein-coupling (R139G) (Benya *et al.*, 1995a). Receptor mutants that did not activate phospholipase C did not show down-regulation or desensitization and removal of the distal PKC consensus sequence markedly attenuated both processes (Benya *et al.*, 1995a). These results lead the authors to conclude that PKC activation was essential for chronic desensitization and down-regulation and provide no evidence for the involvement of second messenger-independent mechanisms driving these receptor modulatory processes.

**IV.6.b. BB<sub>2</sub> receptors antagonist binding:** Numerous structure-function studies of primarily peptide antagonists demonstrated that the COOH terminal amino acid of GRP or bombesin was not required for high affinity interaction with the BB<sub>2</sub> receptor, however it was required to activate the receptor (Coy *et al.*, 1988; Heimbroom *et al.*, 1989; Wang *et al.*, 1990a; Wang *et al.*, 1992). A number of results from these studies and molecular modeling studies supported the model proposed by Coy and collaborators in which the COOH terminus of GRP existed in a folded conformation, stabilized by hydrogen bonding, with the rest of the amino acid chains arranged as an antiparallel  $\beta$ -pleated sheet (Coy *et al.*, 1988). Computer generated molecular modeling (Kull, Jr. *et al.*, 1992) of the COOH terminus of various GRP/Bn pseudopeptides and correlation with whether they behaved as an antagonist or partial agonists for the BB<sub>2</sub> receptor, supported the Coy model (Coy *et al.*, 1988). In detailed studies of [desMet<sup>14</sup>] bombesin amides and alkylamides (Wang *et al.*, 1990a) the resultant antagonist activity could also be explained by the proposed model (Coy *et al.*, 1988) with the loss of the COOH terminal carbonyl group disrupting hydrogen bonding and modifying the conformation from the active form. The effect of this disruption is similar to the introduction of pseudopeptide bonds, which were proposed to result in a conformation shift of the position 14-carboxamide groups in the receptor-bound peptide promoted by the increased rotational freedom and flexibility introduced (Coy *et al.*, 1988; Wang *et al.*, 1990a).



In contrast to agonists, only two studies have examined the BB<sub>2</sub> receptor structural elements responsible for BB<sub>2</sub> receptor high affinity or selectivity for antagonists (Maughfling *et al.*, 1997; Tokita *et al.*, 2001b) (Fig. 4). A chimeric approach using BB<sub>2</sub>/BB<sub>1</sub> receptor combinations was used to examine the region of the BB<sub>2</sub> receptor responsible for the 500-fold selectivity of [D-Phe<sup>6</sup>]Bn<sub>6-13</sub>ethylamide for the human BB<sub>2</sub> receptor over the human BB<sub>1</sub> receptor (Maughfling *et al.*, 1997). The region from the NH<sub>2</sub> terminus to the end of TM2 and regions in the EC4 and TM7 were primarily responsible for this antagonist selectivity. Using BB<sub>2</sub>/BB<sub>1</sub> receptor chimeras, site-directed mutagenesis and molecular modeling, the molecular basis was examined for the >3000-fold and >5000-fold selectivity of the two class 3 BB<sub>2</sub> receptor antagonists JMV641 ([D-Phe<sup>6</sup>, statine<sup>13</sup>] Bn<sub>6-14</sub>) and JMV594, which contains a pseudopeptide bond that mimics the transition state analogue (Azay *et al.*, 1996; Lamharzi *et al.*, 1998). Both loss of affinity and gain of affinity chimera studies showed only differences in the fourth extracellular domain (EC4) contributed to the BB<sub>2</sub> selectivity of these antagonists. Each of the 11 amino acid differences between BB<sub>2</sub> and BB<sub>1</sub> in EC4 was mutated one at a time. The important differences for determining each antagonist's selectivity was the presence of Thr<sup>297</sup> in BB<sub>2</sub> instead of a proline in the comparable position in the BB<sub>1</sub> receptor, Phe<sup>302</sup> in BB<sub>2</sub> instead of a Met in the BB<sub>1</sub> receptor and the presence of Ser<sup>305</sup> instead of Thr in the BB<sub>1</sub> receptor (Fig. 4). Receptor modeling showed that each of these three amino acids faced inward toward the binding pocket and each was within 5 Å of the putative binding pocket (Tokita *et al.*, 2001b). These results suggest that both receptor-ligand cation- $\pi$  interactions and hydrogen bonding are important for the high selectivity of these antagonists.

#### **IV.7. BB<sub>2</sub> receptors signaling, activation, and modulatory processes (internalization, down-regulation, desensitization)**

The human BB<sub>2</sub> receptor (Moody *et al.*, 1986; Corjay *et al.*, 1991; Williams and Schonbrunn, 1994; Benya *et al.*, 1995b; Moody *et al.*, 1996b), as well as the rat BB<sub>2</sub> receptor (Deschodt-Lanckman *et al.*, 1976; Matozaki *et al.*, 1991; Garcia *et al.*, 1997; Tapia *et al.*, 2006), mouse (Huang *et al.*, 1990; Garcia *et al.*, 1997) guinea pig BB<sub>2</sub> receptor (Jensen *et al.*, 1978; Jensen *et al.*, 1988a; Jensen *et al.*, 1988b; Jensen, 1994; Garcia *et al.*, 1997) and canine BB<sub>2</sub> receptor (Seensalu *et al.*, 1997) are coupled to phospholipase C resulting in breakdown of phosphoinositides, generation of diacylglycerol (DAG), stimulation of the mobilization of cellular calcium and PKC activation (Klein *et al.*, 1979; Rozengurt, 1988; Jensen, 1994; Rozengurt, 1998a). BB<sub>2</sub> receptor stimulation activates both phospholipase  $\beta_1$  and  $\beta_3$  and this is dependent on G $\alpha_q$  (MacKinnon *et al.*, 2001). Activation of the BB<sub>2</sub> receptor also results in activation of phospholipase D (Cook *et al.*, 1991; Briscoe *et al.*, 1994), phospholipase A<sub>2</sub> (Currie *et al.*, 1992; Nishino *et al.*, 1998) and is reported to stimulate increased cyclic AMP in some tissues (Rozengurt and Sinnott-Smith, 1983; Bjoro *et al.*, 1987; Millar and Rozengurt, 1988; Garcia *et al.*, 1997). The increase in cyclic AMP in Swiss 3T3 cells was reduced by PKC down-regulation and inhibition of cyclooxygenase suggesting these pathways were involved (Rozengurt *et al.*, 1987). However, a systematic study demonstrated the activation of BB<sub>2</sub> receptors in normal pancreas from three species (rat, mouse, guinea pig) (Garcia *et al.*, 1997) and the transfected human or mouse BB<sub>2</sub> receptor did not stimulate an increase in cAMP (Benya *et al.*, 1994b; Benya *et al.*, 1995b). These results compared to those in a number of studies in the literature led the authors (Garcia *et al.*, 1997) to propose that the BB<sub>2</sub> receptor may be coupled differentially to different adenylate cyclases in different tissues in the same species. Down-stream DAG leads to the activation of both classic and novel PKC's, which catalyze the phosphorylation of a number of membrane-bound and cytosolic proteins. Furthermore, specific protein kinase cascades are triggered including the Raf/MEK/ERK kinase cascade, activation of protein kinase D and rapamycin-sensitive p70s6k, which lead to increased expression of immediate early response genes (i.e. c-myc, c-jun, c-fos) leading to the regulation of the cell cycle and cell proliferation (Rozengurt, 1998a).



BB<sub>2</sub> receptor stimulation also results in the activation of tyrosine kinases and tyrosine phosphorylation of a number of proteins including p125 focal adhesion kinase (FAK) and PYK2, paxillin, ERK kinase and P130<sup>CAS</sup> (Rozengurt, 1998a; Rozengurt, 1998b). Paxillin and P130<sup>CAS</sup> function as important adaptors with paxillin promoting protein-protein interactions and P130<sup>CAS</sup> interacting with Src, c-Crk, and with numerous proteins that have SH2 and SH3 binding domains (Turner, 1994; Harte *et al.*, 1996). BB<sub>2</sub> receptor stimulation of P125<sup>FAK</sup> tyrosine phosphorylation occurs largely independent of PKC activation (Sinnott-Smith *et al.*, 1993), but is dependent on the small GTP binding protein Rho and the integrity of the actin cytoskeleton and focal adhesion plaques (Rozengurt, 1998a). In addition to BB<sub>2</sub> receptor activation stimulating the formation of focal adhesion plaques via a rho dependent mechanism, it also stimulates actin proliferation resulting in membrane ruffling via rac proteins (Nobes *et al.*, 1995). In contrast to P125<sup>FAK</sup> and paxillin tyrosine phosphorylation due to BB<sub>2</sub> receptor stimulation, stimulation of ERK activation and tyrosine phosphorylation is not dependent on rho or the other factors listed above (Seufferlein *et al.*, 1996a). GRP induced activation of ERK is dependent on PKC (Rozengurt, 1998b) and transactivation of the EGF receptor (MacKinnon *et al.*, 2001; Lui *et al.*, 2003; Thomas *et al.*, 2005) which may be mediated by G<sub>i</sub> proteins (MacKinnon *et al.*, 2001). Recent studies provide evidence that BB<sub>2</sub> receptor stimulation of tyrosine phosphorylation of P125<sup>FAK</sup>, paxillin and P130<sup>CAS</sup> occurs via an interaction with Gα<sub>12/13</sub> and rho (Rozengurt, 1998a). BB<sub>2</sub> receptor stimulation also leads to coupling to Gα<sub>12</sub> to elicit c-Jun N-terminal kinase (JNK) activation (MacKinnon *et al.*, 2001; Chan and Wong, 2005).

BB<sub>2</sub> receptor stimulation results in a rapid activation of Src kinase family members (Rodriguez-Fernandez and Rozengurt, 1996; Vincent *et al.*, 1999; Pace *et al.*, 2006) which is not dependent on either PKC or mobilization of calcium, nor is it dependent on rho or the integrity of the cytoskeleton (Rodriguez-Fernandez and Rozengurt, 1996). Blockade of Src family kinases decreases BB<sub>2</sub> receptor stimulated transactivation of the EGFR as well as MAP kinase stimulation (Vincent *et al.*, 1999). The EGFR transactivation by BB<sub>2</sub> receptor activation in head and neck squamous cancers is dependent on Src mediated cleavage and release of TGF-α and amphiregulin and is essential for invasion and growth of these cancers (Vincent *et al.*, 1999).

Acute and chronic BB<sub>2</sub> receptor stimulation results in an activation of a number of receptor modulatory processes (internalization, down-regulation, or desensitization) (Lee *et al.*, 1980; Pandol *et al.*, 1982; Millar and Rozengurt, 1990; Walsh *et al.*, 1993; Briscoe *et al.*, 1994; Benya *et al.*, 1994b; Kroog *et al.*, 1995a), and a number of studies have investigated the cell signaling processes involved. In cells containing human (Benya *et al.*, 1995b), mouse (Zachary and Rozengurt, 1987; Brown *et al.*, 1988; Wang *et al.*, 1993; Benya *et al.*, 1993; Benya *et al.*, 1994d; Tsuda *et al.*, 1997a; Acs *et al.*, 2000) or rat BB<sub>2</sub> receptors (Zhu *et al.*, 1991), with agonist exposure the receptor-ligand complex is rapidly internalized (t<sub>0.5</sub>-5 min) with 80–85%, 70–90%, and 50% respectively of the bound ligand internalized. In epithelial cells transfected with the murine BB<sub>2</sub> receptor, agonist ligand and receptor were internalized by 5 minutes into early endosomes, after 10 min both were in perinuclear vesicles and after 60 min the BB<sub>2</sub> receptor had recycled back to the surface (Grady *et al.*, 1995). In this study (Grady *et al.*, 1995) and in others (Benya *et al.*, 1994b; Benya *et al.*, 1995a) there was a rapid down-regulation of cell surface receptors and the recovery was decreased by acidotropic agents, but not by inhibitors of new protein synthesis. The internalization of the BB<sub>2</sub> receptor is partially dependent on phospholipase C activation (Benya *et al.*, 1994a; Williams *et al.*, 1998; Schumann *et al.*, 2003) and requires clathrin-coated pits since it is inhibited by hyperosmolar sucrose as well as phenylarsine oxide (Grady *et al.*, 1995). Acute desensitization of the BB<sub>2</sub> receptor occurs within seconds to minutes of agonist exposure (Walsh *et al.*, 1993; Briscoe *et al.*, 1994) and is reported to occur with stimulated phospholipase D activity as well as stimulation of phosphoinositides breakdown (Briscoe *et al.*, 1994; Williams *et al.*, 1998) and for

stimulation of changes in cytosolic calcium, with the latter shown to be homologous in nature (Walsh *et al.*, 1993). In some tissues acute desensitization and down-regulation of the BB<sub>2</sub> receptor is caused by hormones/neurotransmitters activating phospholipase C such as carbachol and cholecystokinin (Younes *et al.*, 1989; Vinayek *et al.*, 1990). Chronic BB<sub>2</sub> receptor desensitization occurs after prolonged incubation with agonist (1–2 hours) and is homologous in nature (Lee *et al.*, 1980; Benya *et al.*, 1995a). Receptor structure-function studies reviewed above provide strong support for the conclusion that down-regulation and chronic desensitization are coupled processes being effected by similar receptor structural alterations and cellular signaling cascades and have a distinct mechanism from that causing internalization (Benya *et al.*, 1994d; Benya *et al.*, 1995a; Tsuda *et al.*, 1997a; Schumann *et al.*, 2003). The results of these studies provided no evidence for second messenger independent processes in mediating down-regulation or desensitization, whereas internalization is equally stimulated by second messenger-dependent and independent processes and the presence of the COOH terminal serines and threonines were essential for mediating these effects. In HIT-T15 cells BB<sub>2</sub> receptor mediated desensitization was closely coupled to down-regulation (Swope and Schonbrunn, 1990).

Studies in the  $\beta$ -adrenergic receptor and a number of GPCR's demonstrate receptor phosphorylation, primarily by G protein-coupled receptor kinases (GRK) and subsequent binding of arrestins are critical for receptor internalization and deactivation during acute desensitization (Krupnick and Benovic, 1998; Ferguson, 2001; Premont and Gainetdinov, 2007). Studies demonstrate BB<sub>2</sub> receptor activation results in rapid phosphorylation of the receptor (Kroog *et al.*, 1995b; Williams *et al.*, 1996; Kroog *et al.*, 1999; Ally *et al.*, 2003) as does stimulation of the BB<sub>2</sub> receptor containing cells by the phorbol ester, TPA (Kroog *et al.*, 1995b; Williams *et al.*, 1996; Ally *et al.*, 2003). However, agonist and TPA-induced BB<sub>2</sub> receptor phosphorylation occur at different receptor sites (Williams *et al.*, 1998). GRK's are serine-threonine kinases that preferentially phosphorylate agonist occupied, active conformation GPCR's and lead to uncoupling from G protein and endocytosis (Szekeres *et al.*, 1998; Premont and Gainetdinov, 2007). Bn/GRP stimulates BB<sub>2</sub> receptor phosphorylation at serine/threonine residues in the COOH terminus, but does not stimulate tyrosine phosphorylation in the BB<sub>2</sub> receptor (Williams *et al.*, 1996; Ally *et al.*, 2003). With BB<sub>2</sub> receptor activation arrestin translocation occurs to the plasma membrane (Ally *et al.*, 2003) and requires an intact DRY sequence in the second intracellular domain of the BB<sub>2</sub> receptor (Ally *et al.*, 2003). BB<sub>2</sub> receptor internalization has been proposed to play a key role in acute BB<sub>2</sub> receptor desensitization (Swope and Schonbrunn, 1990) because the kinetics of each is identical. Furthermore, the kinetics of BB<sub>2</sub> receptor phosphorylation correlate closely with both internalization and acute desensitization (Kroog *et al.*, 1995b; Williams *et al.*, 1996; Williams *et al.*, 1998). Phosphorylation of the BB<sub>2</sub> receptor after GRP/ Bn stimulation is reported in one study (Williams *et al.*, 1996), but not another (Kroog *et al.*, 1995b), to be mediated by both a PKC-dependent and PKC-independent process (likely a GRK family member).

Studies demonstrate that radiolabeled GRP/Bn is rapidly degraded by the BB<sub>2</sub> receptor (Swope and Schonbrunn, 1987; Zachary and Rozengurt, 1987; Brown *et al.*, 1988; Zhu *et al.*, 1991; Wang *et al.*, 1993; Williams *et al.*, 1998). This degradation is best inhibited by the general inhibitor bacitracin or the thermolysin-like metalloproteinase inhibitor, phosphoramidon and to a less degree by leupeptin, bestatin>chymostatin>amastatin (Wang *et al.*, 1993). The lysosomal proteinase inhibitor, choroquine, also inhibits degradation (Swope and Schonbrunn, 1987; Williams *et al.*, 1998).

Activation of the BB<sub>2</sub> receptor results in growth of both normal and neoplastic tissues (Moody *et al.*, 2003a; Jensen and Moody, 2006). The cell signaling cascades involved have been studied extensively in both Swiss 3T3 cells and in numerous tumors cells. In 3T3 cells and a number

of tumor cells (prostate, head and neck squamous cell cancer, nonsmall cell lung cancer cells) the activation of the BB<sub>2</sub> receptor results in stimulation of phosphorylation of Akt (Liu *et al.*, 2007) and extracellular regulated kinase (ERK) phosphorylation (Sakamoto *et al.*, 1988; Rozengurt, 1998b; Koh *et al.*, 1999; Vincent *et al.*, 1999; Lui *et al.*, 2003; Thomas *et al.*, 2005) which has been shown in some cells to be dependent on the transactivation of the EGF receptor which is in turn depends on Src and changes in cytosolic calcium in some cases. Mitogenesis in 3T3 cells is dependent on BB<sub>2</sub> receptor stimulated changes in cytosolic calcium; activation of PKC, PKD, ERK kinases, and release of arachidonic acid (Rozengurt, 1998b). BB<sub>2</sub> receptor stimulation of ERK phosphorylation is dependent on Ras but not Rap1 in prostate tumor cells (Sakamoto *et al.*, 1988). The transactivation of the EGF receptor by BB<sub>2</sub> receptor activation is dependent on PKC and PKD activation in some cells (Seufferlein *et al.*, 1996b; Rozengurt, 1998b; Sinnott-Smith *et al.*, 2004; Sinnott-Smith *et al.*, 2007). EGF receptor transactivation upon BB<sub>2</sub> receptor stimulation as well as by a number of other GPCRs occurs via metalloproteinase-dependent cleavage and release of EGF-related peptides that then activate the receptor (Sakamoto *et al.*, 1988; Vincent *et al.*, 1999; Lui *et al.*, 2003). The inhibition of either EGF receptor transactivation or ERK activation inhibited BB<sub>2</sub> receptor-stimulated DNA synthesis in these tumor cells (Sakamoto *et al.*, 1988). BB<sub>2</sub> receptor activation stimulates the invasion and cell migration of tumor cells (Vincent *et al.*, 1999; Thomas *et al.*, 2005; Zheng *et al.*, 2006). This stimulation occurs via G<sub>α13</sub> leading to activation of RhoA and Rho-associated coiled-coil forming protein kinase (ROCK) (Zheng *et al.*, 2006). BB<sub>2</sub> receptor activation promotes progression from the G<sub>1</sub> to the S phase of the cell cycle by increasing the expression of cyclin D<sub>1</sub> and E through the early growth response protein Egr-1, down-regulating the cyclin-dependent kinase inhibitor p27<sup>kip1</sup> and hyperphosphorylating the retinoblastoma protein (Rb) (Mann *et al.*, 1997; Rozengurt, 1998b; Xiao *et al.*, 2005).

**IV.8. BB<sub>2</sub> receptor function in various tissues and *in vivo***—A major difficulty in assessing the effects of BB<sub>2</sub> receptor activation *in vivo* and in a number of tissues *in vitro* is the fact they frequently possess both classes of bombesin receptors and bombesin, the agonist frequently used, has high affinity for both receptor subtypes. Recently a number of developments have contributed to solving this problem. Selective receptor antagonists for the BB<sub>2</sub> receptor are described, studies on BB<sub>2</sub> receptor knockout animals are being increasing performed, more selective BB<sub>2</sub> receptor agonists such as GRP are being used and with the cloning of the mammalian bombesin receptors, it has become clear that some widely studied tissues such as Swiss 3T3 cells and pancreatic acinar cells only possess BB<sub>2</sub> receptors.

Many effects of GRP are observed both *in vivo* and *in vitro*, but it remains unclear in many cases which are pharmacological or which are physiological. Studies support a role for BB<sub>2</sub> receptor in numerous gastrointestinal functions, including regulation of gastric acid secretion via both stimulation of gastrin release from antral G cells and somatostatin release from D cells and stimulation of acid secretion (Schubert *et al.*, 1991; Hildebrand *et al.*, 2001; Schubert, 2002); regulation of gastrointestinal motility, especially gastric emptying, small intestinal transit and gallbladder emptying (Degen *et al.*, 2001; Yegen, 2003); stimulation of pancreatic secretion (Niebergall-Roth and Singer, 2001; Nathan and Liddle, 2002), insulin release (Persson *et al.*, 2002), colonic ion transport (Traynor and O'Grady, 1996); and stimulation of the secretion of a variety of hormones (gastrin, somatostatin, CCK, pancreatic polypeptide, enteroglucagon, pancreatic glucagon and gastric inhibitory polypeptide) (Modlin *et al.*, 1981; Ghatei *et al.*, 1982; Pettersson and Ahren, 1987; Bunnett, 1994). Activation of BB<sub>2</sub> receptors have a number of immunologic effects including functioning as a chemoattractant in peritoneal macrophages, monocytes and lymphocytes (Ruff *et al.*, 1985; Del Rio and De la Fuente, 1994), stimulating lymphocyte proliferation (Del Rio *et al.*, 1994), and stimulating natural killer and antibody-dependent cellular cytotoxicity in leukocytes (De la Fuente *et al.*, 1993a). BB<sub>2</sub> receptors are reported to be important for fetal lung development including lung branching, cell proliferation and differentiation (Subramaniam *et al.*, 2003) as well as a number

of lung diseases, which will be discussed below. BB<sub>2</sub> receptors are widely expressed in the CNS and in the spinal cord and numerous central effects have been described with their activation including: effects on satiety, regulation of circadian rhythm, thermoregulatory effects, grooming behaviors, modulation of stress, fear and anxiety response, memory and effects on gastrointestinal function such as acid secretion (Martinez and Tache, 2000; Yegen, 2003; Moody and Merali, 2004; Karatsoreos *et al.*, 2006; Roesler *et al.*, 2006a; Roesler *et al.*, 2006b; Kallingal and Mintz, 2007; Presti-Torres *et al.*, 2007). The satiety effect of BB<sub>2</sub> receptors has been extensively studied (Gibbs *et al.*, 1979; Gibbs and Smith, 1988; Flynn, 1997; Ladenheim and Knipp, 2007; Fekete *et al.*, 2007). A recent study (Ladenheim and Knipp, 2007) shows the satiety effect of peripherally administered NMB, but not GRP, is inhibited by capsaicin pretreatment suggesting either the neural pathways involved in BB<sub>2</sub> receptor mediated satiety are either by capsaicin-insensitive neurons or involve direct activation of BB<sub>2</sub> receptors in the CNS (Ladenheim and Knipp, 2007). The importance of BB<sub>2</sub> receptors in mediating the satiety effects of GRP was demonstrated by the ability of a specific BB<sub>2</sub> receptor antagonist administered in the hindbrain of rats, to inhibit the satiety effects of peripherally administered GRP (Ladenheim *et al.*, 1996a).

BB<sub>2</sub> receptor knockout mice have been described and limited study results are available (Wada *et al.*, 1997; Hampton *et al.*, 1998). In the initial study performed with these mice (Hampton *et al.*, 1998), no developmental abnormalities were seen, however bombesin failed to suppress glucose intake, whereas it caused a dose-dependent decrease in normal mice (Hampton *et al.*, 1998). In a second study (Wada *et al.*, 1997) the intracerebroventricular administration of GRP failed to cause hypothermia in the BB<sub>2</sub> receptor knockout mice as observed in the wild type mice. Furthermore, the BB<sub>2</sub> receptor knockout mice demonstrated abnormal behaviors and altered spontaneous activity during darkness (Wada *et al.*, 1997). In a more detailed study of feeding behavior in these mice, neither GRP, NMB nor bombesin altered satiety in the knockout mice however the satiety response to cholecystokinin was present and in fact enhanced (Ladenheim *et al.*, 2002). In a long term study (Ladenheim *et al.*, 2002) BB<sub>2</sub> receptor knockout mice ate more food than normal mice due to a defect in terminating meals and had greater weight gain supporting the conclusion that the BB<sub>2</sub> receptor has important roles in satiety. These mice were used to study the effects of BB<sub>2</sub> receptor activation on islet function (Persson *et al.*, 2000; Persson *et al.*, 2002). BB<sub>2</sub> receptor knockout mice had impaired glucose tolerance, a defect in early insulin release (Persson *et al.*, 2000) and the plasma GLP-1 response to gastric glucose administration was significantly reduced in the knockout mice suggesting BB<sub>2</sub> receptor had an important role in normal GLP-1 release and insulin and glucose responses after glucose a glucose meal. In a second study (Persson *et al.*, 2002) GRP was found to potentiate glucose-stimulated insulin release in wild type but not BB<sub>2</sub> receptor knockout mice. This study (Persson *et al.*, 2002) demonstrated that BB<sub>2</sub> receptor activation contributes to insulin secretion induced by activation of autonomic nerves and that the deletion of the BB<sub>2</sub> receptor is compensated for by increased cholinergic sensitivity (Persson *et al.*, 2002). These results are consistent with earlier studies which demonstrated GRP potentiated glucose-induced insulin release by both a ganglionic and direct effect, but did not alter glucagon or pancreatic somatostatin release (Hermansen and Ahren, 1990; Gregersen and Ahren, 1996; Karlsson *et al.*, 1998). BB<sub>2</sub> receptor knockout mice were used to study possible behavioral effects of GRP (Shumyatsky *et al.*, 2002). In one study the BB<sub>2</sub> receptor was found in wild type, but not knockout mice, to be highly expressed in the lateral nucleus of the amygdala, which is important in mediating fear responses. BB<sub>2</sub> receptor knockout mice showed more persistent long-term fear responses (Shumyatsky *et al.*, 2002) supporting other study results, which suggest the BB<sub>2</sub> receptor, has an important role in memory and fear responses (Roesler *et al.*, 2006a). Other behavior changes seen in BB<sub>2</sub> receptor knockout mice include increased social investigatory behavior (Yamada *et al.*, 2000b), preference for conspecific odors (Yamada *et al.*, 2000b) and altered social preferences in females (Yamada *et al.*, 2001). BB<sub>2</sub> receptor knockout mice have also been used



to investigate the role of this receptor in specific diseases, which will be discussed in the next section.

BB<sub>2</sub> receptor activation has important growth effects on normal and neoplastic tissues (Moody *et al.*, 1992; Jensen and Moody, 2006). BB<sub>2</sub> receptor activation stimulates growth of normal endometrial stromal cells (Endo *et al.*, 1991), bronchial epithelial cells (Willey *et al.*, 1984; Siegfried *et al.*, 1993), melanocytes (Terashi *et al.*, 1998), chondrocytes (Hill and McDonald, 1992), normal enterocyte growth and turnover post small bowel resection (Chu *et al.*, 1995; Sukhotnik *et al.*, 2007) as well as normal development of the intestinal villus (Carroll *et al.*, 2002) and normal fetal lung development (Emanuel *et al.*, 1999; Shan *et al.*, 2004). The effects of BB<sub>2</sub> receptor activation on neoplastic growth have been extensively studied (Moody *et al.*, 1992; Jensen *et al.*, 2001; Patel *et al.*, 2006). This widespread interest occurred after human small cell lung cancers were shown to possess high affinity BB<sub>2</sub> receptors (Moody *et al.*, 1985) and bombesin was shown to have an autocrine growth effect on these cells (Cuttitta *et al.*, 1985). Subsequent studies demonstrated such an autocrine growth effect where the tumor cells not only possessed BB<sub>2</sub> receptors, but also secreted bombesin-like peptides resulted in a growth stimulatory effect (Moody *et al.*, 2003a; Jensen and Moody, 2006; Patel *et al.*, 2006) in a large number of tumor cells including neuroblastomas (Kim *et al.*, 2002), squamous head and neck tumors (Lango *et al.*, 2002; Lui *et al.*, 2003), pancreatic cancer cells (Wang *et al.*, 1996; Murphy *et al.*, 2001), colon cancer (Chave *et al.*, 2000), prostate cancer (Plonowski *et al.*, 2000), human glioblastoma cells (Sharif *et al.*, 1997) and non-small cell lung cancer (Siegfried *et al.*, 1999). Furthermore, many human cancers or the blood vessels in the cancers either overexpress or ectopically express BB<sub>2</sub> receptors and the stimulation or inhibition of these receptors is reported to effect growth/differentiation (Jensen *et al.*, 2001; Moody *et al.*, 2003a; Heuser *et al.*, 2005; Jensen and Moody, 2006; Patel *et al.*, 2006; Fleischmann *et al.*, 2007). The potential clinical importance of this will be discussed further in the next section. The role of the ectopic expression or overexpression in various cancers may be different with different tumors. Whereas, many of the studies referred to in the following paragraph emphasize the growth stimulatory effects of BB<sub>2</sub> receptor on tumor cells, other studies especially in colon cancer support the conclusion that the ectopic expressing of BB<sub>2</sub> receptor has a morphogenic effect rather than a mitogenic effect (Jensen *et al.*, 2001). Whereas in normal colonic mucosal epithelial cells, the BB<sub>2</sub> receptor is not found (Preston *et al.*, 1995; Ferris *et al.*, 1997; Carroll *et al.*, 1999b), in 40–100% of colonic cancers (Carroll *et al.*, 1999b) BB<sub>2</sub> receptor is aberrantly expressed. BB<sub>2</sub> receptor activation on some colonic cancer cells is reported to result in proliferation (Radulovic *et al.*, 1991b; Frucht *et al.*, 1992; Narayan *et al.*, 1992). However, in detailed studies, although 62% of the tumors expressed both GRP and the BB<sub>2</sub> receptor, their co-expression was equally frequent in early or late stage cancers and was rarely detected in metastases (Carroll *et al.*, 1999b). However, GRP/ BB<sub>2</sub> receptor expression was seen in all well-differentiated tumors, whereas poorly differentiated tumors never co-expressed GRP/ BB<sub>2</sub> receptors (Carroll *et al.*, 1999b). Furthermore, no difference in survival occurred in patients expressing or not expressing cancers with GRP/ BB<sub>2</sub> receptor (Carroll *et al.*, 1999b). In a study (Carroll *et al.*, 2000a) of BB<sub>2</sub> receptor knockout mice with colonic tumors induced by azoxymethane, larger tumors were better differentiated in wild type mice than BB<sub>2</sub> receptor knockout mice. From these studies and others it was proposed that BB<sub>2</sub> receptor activation in these cells is functioning primarily as a morphogenic or differentiating factor (Carroll *et al.*, 1999b; Jensen *et al.*, 2001). More recent studies show this morphogenic effect is mediated by activation of p125<sup>FAK</sup>, which inhibits invasion/metastases by enhancing cell attachment (Glover *et al.*, 2004), most likely by up-regulating the expression of intracellular adhesion protein-1 (ICAM-1) (Taglia *et al.*, 2007). Subsequent studies showed that BB<sub>2</sub> receptor mutations occurred frequently in poorly differentiated colonic tumor, resulting in the formation of inactive receptors, and the generation of these mutations correlated inversely with the differentiation of the tumor suggesting their production represents a new



mechanism allowing for the differentiation of tumors (Carroll *et al.*, 2000b; Glover *et al.*, 2003).

A recent study (Ruginis *et al.*, 2006) used a proteomic approach to identify proteins selectively up regulated in human colorectal cancer cells subsequent to BB<sub>2</sub> receptor activation. This study took advantage of the fact that pre-confluent human colorectal cancer cells such as Caco-2 and HT-29 only secrete GRP and express BB<sub>2</sub> receptors when they are pre-confluent and not when they are post-confluent (Glover *et al.*, 2005). Total cellular proteins were isolated from pre-confluent, GRP and BB<sub>2</sub> receptor expressing cells in the presence and absence of specific BB<sub>2</sub> receptor antagonist [D-Phe<sup>6</sup>]bombesin<sub>6-13</sub>methyl ester, and from post-confluent cells not expressing GRP or BB<sub>2</sub> receptors. Using 2D gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Ruginis *et al.*, 2006), at least six separate proteins are up-regulated subsequent to BB<sub>2</sub> signaling when this receptor is aberrantly expressed in human colorectal cancer: gephyrin, heat shock protein 70, heterochromatin associated protein 1, ICAM-1, and THIL/acetyl co-A acetyltransferase (ACAT). These findings promise to further define the mechanism whereby aberrantly expressed BB<sub>2</sub> receptors in human colorectal cancer promote tumor cell differentiation and improve patient outcome.

**IV.9. BB<sub>2</sub> receptor in diseases**—BB<sub>2</sub> receptor activation has been proposed to be important in the mediation of a number of human disorders including disorders of lung development, various pulmonary diseases, CNS disorders, and the growth/differentiation of human cancers. The tumor differentiation effects of BB<sub>2</sub> receptor activation were discussed in the previous section and the growth effects and effects of BB<sub>2</sub> receptor overexpression will be considered here. BB<sub>2</sub> receptors are ectopically or over-expressed in a large number of tumors including 85–100% of small cell lung cancers, 74–78% of non-small cell lung cancer cells, 38–72% of breast cancer, 75% of pancreatic cancer cell lines and 10% of pancreatic cancers, 62–100% of prostate cancers, 100% of head/neck squamous cell cancers and 72–85% of neuroblastomas/glioblastomas (Jensen and Moody, 2006; Patel *et al.*, 2006). Bn-like peptides are critical to the growth of some, but not all small cell lung cancers (Cuttitta *et al.*, 1985), and as discussed above have been shown to have an autocrine growth effect on a large number of tumors as well as stimulating growth in a large number of these tumors (Jensen and Moody, 2006; Patel *et al.*, 2006). In some tumors such as prostate cancer, BB<sub>2</sub> receptor overexpression correlates with neoplastic transformation (Markwalder and Reubi, 1999). Bn peptides also function as proangiogenic factors in various tumors (Levine *et al.*, 2003; Kanashiro *et al.*, 2005; Martinez, 2005). The production of GRP-related peptides and/or overexpression of BB<sub>2</sub> receptors by tumors are playing a potential role in a number of aspects of the treatment and management of these tumors. These aspects include functioning as targets for anti-tumor treatment, as prognostic factors, as targets to image the tumors and as targets to deliver cytotoxic treatment selectively to the tumor (Smith *et al.*, 2003; Schally and Nagy, 2004; Jensen and Moody, 2006). Attempts to inhibit the autocrine growth effect of GRP-like peptides on tumor growth are reported in human and/or animal studies using monoclonal antibodies to GRP, antisense constructs, BB<sub>2</sub> receptor antagonists or other inhibitors (Zhou *et al.*, 2004). Infusion of the monoclonal antibody 2A11 directed against the biologically active COOH terminus of GRP is safe in humans (Chaudhry *et al.*, 1999) and was given to 13 patients with small cell lung cancer (Kelley *et al.*, 1997). One patient had complete remission and four patients had radiologically stable disease and further evaluation was recommended (Kelley *et al.*, 1997). In a recent study (Schwartzmann *et al.*, 2006) the BB<sub>2</sub> receptor antagonist, RC-3095 was administered to 25 patients with different advanced malignancies. No side effects occurred and there were no tumor responses, however, maximal doses could not be reached by the methods used despite dose-escalation (Schwartzmann *et al.*, 2006). EGF receptor transactivation upon BB<sub>2</sub> receptor stimulation may also be a therapeutic site for intervention. Experimental studies demonstrate that activation of the BB<sub>2</sub> receptor rescues tumor cells from

the growth inhibiting effect of the EGF receptor inhibitor, gefitinib, by stimulating the release of amphiregulin and activation of the Akt pathway (Liu *et al.*, 2007). When a BB<sub>2</sub> receptor antagonist is combined with an EGF receptor inhibitor (erlotinib) there is marked enhanced antitumor activity (Zhang *et al.*, 2007b), suggesting such an approach may be useful in some cancers such as head/neck tumors or lung tumors. In a number of studies plasma levels of GRP precursors such as pro-GRP or assessment of GRP expression in tumors provides prognostic information (Hamid *et al.*, 1990; Okusaka *et al.*, 1997; Sunaga *et al.*, 1999; Shibayama *et al.*, 2001; Yonemori *et al.*, 2005).

Recent clinical and laboratory studies with somatostatin receptors demonstrate that many endocrine tumors over-express or ectopically express these receptors and that radiolabeled analogues of somatostatin can be used to both localize these tumors as well as for somatostatin receptor mediated cytotoxicity (Van Essen *et al.*, 2007; Breeman *et al.*, 2007). Somatostatin analogues coupled to <sup>111</sup>Indium are now widely used to image neuroendocrine tumors and numerous studies demonstrate that they have greater sensitivity than conventional imaging modalities (computed tomography, magnetic resonance imaging, angiography, ultrasound) and that its routine use changes patient management in 20–47% of cases (Gibril *et al.*, 1996; Gibril and Jensen, 2004; Breeman *et al.*, 2007). Recent studies with somatostatin analogues coupled to <sup>111</sup>Indium, <sup>90</sup>Yttrium and <sup>177</sup>Lutetium show promising results for somatostatin receptor-mediated cytotoxicity in patients with advanced neuroendocrine tumors and have entered Phase 3 studies (Van Essen *et al.*, 2007; Breeman *et al.*, 2007; Forrer *et al.*, 2007). Unfortunately many common tumors (colon, pancreas, head/neck, prostate, lung) may not overexpress somatostatin receptor, however they frequently overexpress Bn receptors, especially the BB<sub>2</sub> receptor (Jensen *et al.*, 2001; Reubi *et al.*, 2002; Moody *et al.*, 2003a; Heuser *et al.*, 2005; Jensen and Moody, 2006; Patel *et al.*, 2006; Fleischmann *et al.*, 2007). This observation has led to considerable interest in the possibility of developing radiolabeled analogues of Bn that could be use for localization of the tumors containing Bn receptors or the development of radiolabeled Bn analogues or Bn analogues coupled to cytotoxic agents that could be used to treat tumors overexpressing Bn receptors through bombesin receptor-mediated cytotoxicity (Breeman *et al.*, 2002; de Jong *et al.*, 2003; Cornelio *et al.*, 2007; de Visser *et al.*, 2007a). Numerous radiolabeled [<sup>111</sup>Indium, <sup>68</sup>Gallium, <sup>177</sup>Lutetium, <sup>64</sup>Copper, <sup>86</sup>Yttrium, <sup>18</sup>F, <sup>99m</sup>Tc] GRP analogues with enhanced stability that bind with high affinity to BB<sub>2</sub>-receptors are reported, as well as there ability to image various human tumors *in vivo* using gamma detectors or PET imaging (Breeman *et al.*, 2002; Smith *et al.*, 2003; Nock *et al.*, 2003; Smith *et al.*, 2005; Zhang *et al.*, 2006; Lantry *et al.*, 2006; Johnson *et al.*, 2006; Waser *et al.*, 2007; Parry *et al.*, 2007; Dimitrakopoulou-Strauss *et al.*, 2007; Garrison *et al.*, 2007; Prasanphanich *et al.*, 2007; de Visser *et al.*, 2007a; Zhang *et al.*, 2007a). In some preliminary studies in humans, tumors were imaged in the majority of patients, and in some cases, tumors were detected using radiolabeled Bn analogues that were not seen on other commonly used imaging modalities (Scopinaro *et al.*, 2004; De Vincentis *et al.*, 2004; Scopinaro *et al.*, 2005; Dimitrakopoulou-Strauss *et al.*, 2007). At present no study has established the value of imaging using radiolabeled Bn analogues.

A number of Bn analogues coupled to radiolabeled compounds [<sup>177</sup>Lutetium] (Smith *et al.*, 2005; Lantry *et al.*, 2006; Johnson *et al.*, 2006; Zhang *et al.*, 2007a) as well as to cytotoxic agents (camptothecin-a topoisomerase inhibitor, doxorubicin analogues, paclitaxel) (Schally and Nagy, 1999; Breeman *et al.*, 2002; Moody *et al.*, 2004; Schally and Nagy, 2004; Engel *et al.*, 2005; Buchholz *et al.*, 2006; Safavy *et al.*, 2006; Panigone and Nunn, 2006; Nanni *et al.*, 2006; Moody *et al.*, 2006b; Engel *et al.*, 2007) have been described which retain high affinity for Bn receptors and are internalized by Bn-receptor-bearing tissues, for the possibility of delivering Bn-receptor mediated tumoral cytotoxicity. Many of these compounds have been shown to cause tumor cytotoxicity in animal studies and one study has provided evidence that it is due to specific interaction with the BB<sub>2</sub>-receptors overexpressed on the tumor (Moody *et*

*et al.*, 2006b). At present it is unclear whether this approach will be effective *in vivo* in human tumors whether alone or in combination with other anti-tumor treatments. A recent study using a chemically identical active and inactive cytotoxic GRP analogue (i.e. camptothecin-L2-[D-Tyr<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> or its D-Phe<sup>13</sup> inactive form, where L2=[N-(N-methyl-amino ethyl)glycine carbamate]), demonstrated that specific tumor receptor interaction is important in mediating the tumor cytotoxicity of these compounds (Moody *et al.*, 2006b). Various studies demonstrate that such an approach can inhibit the growth of pancreatic lung, prostate and gastric cancers (Schally and Nagy, 1999; Breeman *et al.*, 2002; Moody *et al.*, 2004; Schally and Nagy, 2004). At present the usefulness of GRP or BB<sub>2</sub> receptor in management of human tumors in each of the areas discussed above is not established (Jensen and Moody, 2006).

BB<sub>2</sub> receptor activation, GRP secretion, or abnormalities of either have been proposed to be important in a number of other diseases. In a recent study (Sun and Chen, 2007) evidence was presented that activation of the BB<sub>2</sub>-receptor in the dorsal spinal cord is important for mediating pruritus. GRPR knockout mice showed significantly decreased scratching behavior in response to pruritogenic stimuli, while other responses were normal. Furthermore, administration of a BB<sub>2</sub> receptor antagonist into the spinal fluid inhibited scratching behavior in three different models of itching (Sun and Chen, 2007). The authors (Sun and Chen, 2007) point out the BB<sub>2</sub> receptor may represent the first molecule identified that is dedicated to mediating the itch response in the spinal cord and may provide an important therapeutic target for the treatment of chronic pruritic conditions. Abnormalities of GRP, BB<sub>2</sub> receptors and other bombesin-like peptides and/or their receptors are proposed to be important in normal lung development and mediating the lung injury in premature infants with bronchopulmonary dysplasia (Li *et al.*, 1994; Sunday *et al.*, 1998; Emanuel *et al.*, 1999; Cullen *et al.*, 2000; Ashour *et al.*, 2006; Ganter and Pittet, 2006; Subramaniam *et al.*, 2007). In one recent study (Ashour *et al.*, 2006) GRP given to newborn mice induced features of human BPD including interstitial pulmonary fibrosis and alveolarization. In a hyperoxic baboon model of BPD (Subramaniam *et al.*, 2007) the early overproduction of Bn-like peptides correlated with the development of BPD-like histological features and the blockage of GRP partially reversed these effects, leading the authors to suggest such an approach could have important implications for preventing BPD in premature infants. GRP has been shown to be protective to the small intestine in various injury models (Assimakopoulos *et al.*, 2004; Kinoshita *et al.*, 2005; Assimakopoulos *et al.*, 2005a; Assimakopoulos *et al.*, 2005b; Kimura *et al.*, 2006b), enhance gut barrier function, prevent the atrophy of enteric ganglia caused by FK506 in small bowel (Assimakopoulos *et al.*, 2005a; Higuchi *et al.*, 2006; Kimura *et al.*, 2006a; Kimura *et al.*, 2006b) and in a recent study (Fujimura *et al.*, 2007) to prevent the atrophy of Peyer's patches and dysfunction of M cell in rabbits receiving long-term parenteral nutrition. These studies suggest GRP agonists may have a potential therapeutic role in diseases causing this type of injury. Numerous studies in rodents provide evidence that GRP/ BB<sub>2</sub>-receptor activation is important for memory as well as a number of social behaviors (learning, grooming, stereotypy) (Roesler *et al.*, 2006a; Roesler *et al.*, 2006b). These results were supported by a recent study (Presti-Torres *et al.*, 2007) in which the administration of BB<sub>2</sub>-receptor antagonists in neonatal rats resulted in marked impairment of memory, and social interaction. These changes have led one group (Roesler *et al.*, 2006a) to propose that the BB<sub>2</sub>-receptor should be considered a therapeutic target in a subset of human CNS diseases, especially those involving memory, learning and fear. Specifically, in the CNS it has been proposed that alterations in either the GRP and/or BB<sub>2</sub> receptor may be important in schizophrenia, Parkinson's disease, anxiety disorders, anorexia, bulimia, and mood disorders (Merali *et al.*, 1999; Frank *et al.*, 2001; Yegen, 2003; Moody and Merali, 2004; Merali *et al.*, 2006; Roesler *et al.*, 2006a).

## V. BB<sub>3</sub> receptor

**V.1. Early studies of the BB<sub>3</sub> receptor**—Prior to the identification of the BB<sub>3</sub> receptor when it was cloned in 1992 from guinea pig uterus (Gorbulev *et al.*, 1992) no pharmacological or functional studies suggested its existence.

**V.2. Cloned BB<sub>3</sub> and receptor structure**—The human BB<sub>3</sub> receptor is a 399 amino acid protein (Fathi *et al.*, 1993b) and it shows 95% amino acid identities with rhesus BB<sub>3</sub> receptor (Sano *et al.*, 2004), 80% amino acid identity with the rat BB<sub>3</sub> that shows 92% with the mouse, and 77% with the sheep BB<sub>3</sub> receptor (Liu *et al.*, 2002)(Table 2). The human BB<sub>3</sub> receptor has 51% amino acid identities with the human BB<sub>2</sub> and 47% with the human BB<sub>1</sub> receptor (Fathi *et al.*, 1993b). The human BB<sub>3</sub> receptor has a predicted molecular weight of 44.4 kDa (Fathi *et al.*, 1993b) and there are two potential N-linked glycosylation sites at Asn<sup>10</sup> and Asn<sup>18</sup> and a consensus site for potential PKC phosphorylation in the third cytoplasmic loop and carboxyl terminus (Fathi *et al.*, 1993b; Whitley *et al.*, 1999). A putative palmitoylation site existed at C<sup>347</sup> and C<sup>348</sup> (Fathi *et al.*, 1993b; Whitley *et al.*, 1999). Hydrophathy plots yielded results consistent with a seven transmembrane structure typical for a G-protein coupled receptor (Fathi *et al.*, 1993b). The BB<sub>3</sub> receptor has been cloned from rat (Liu *et al.*, 2002), mouse (Ohki-Hamazaki *et al.*, 1997a), sheep (Whitley *et al.*, 1999) and guinea pig (Gorbulev *et al.*, 1992). In the chicken a receptor was cloned that has similarities to both the mammalian BB<sub>3</sub> receptor and the frog BB<sub>4</sub> receptor and has been termed the chBRS-3.5 receptor (Iwabuchi *et al.*, 2003). No cross-linking studies have been performed on the mature BB<sub>3</sub> receptor so the extent of glycosylation or type is unknown at present.

**V.3. BB<sub>3</sub> genomic organization**—The human BB<sub>3</sub> receptor gene is localized at human chromosome Xq25 and in the mouse on chromosome XA7.1–7.2 (Fathi *et al.*, 1993b; Gorbulev *et al.*, 1994; Weber *et al.*, 1998). The human BB<sub>3</sub> receptor gene (Fathi *et al.*, 1993b; Gorbulev *et al.*, 1994; Weber *et al.*, 1998) contained two introns and three exons similar to the sheep (Whitley *et al.*, 1999), rhesus (Sano *et al.*, 2004), mouse (Ohki-Hamazaki *et al.*, 1997a), and rat BB<sub>3</sub> receptor gene (Liu *et al.*, 2002). In the mouse BB<sub>3</sub> receptor gene spanned more than 5kb with exon 1 of the BB<sub>3</sub> gene separated from exon 2 by 1.6 kb and this in turn separated from exon 3 by 1.6 kb (Weber *et al.*, 1998). In human, sheep, monkey, rat, mouse and guinea pig the exon/intron splice sites occurred at Arg<sup>145</sup> in the second intracellular loop and at Ile<sup>263</sup> in the third intracellular domain (Gorbulev *et al.*, 1994; Weber *et al.*, 1998; Sano *et al.*, 2004).

**V.4. BB<sub>3</sub> receptor expression**—Expression levels of the BB<sub>3</sub> receptor mRNA has been reported in the rat (Fathi *et al.*, 1993b; Liu *et al.*, 2002; Jennings *et al.*, 2003), sheep (Whitley *et al.*, 1999), mouse (Ohki-Hamazaki *et al.*, 1997a), monkey (Sano *et al.*, 2004) and guinea pig (Gorbulev *et al.*, 1992). In the monkey where it was studied in detail, BB<sub>3</sub> mRNA is found in the greatest amount in the CNS and in the testis (Sano *et al.*, 2004). This high expression in the testis is not seen in the sheep (Weber *et al.*, 2003) or mouse (Ohki-Hamazaki *et al.*, 1997a) but is similar to the rat (Fathi *et al.*, 1993b) where it was localized to the secondary spermatocytes and was not present in the sertoli cells or different maturation stages of the spermatogonia (Fathi *et al.*, 1993b). Detectable levels were also found in the monkey pancreas, thyroid and ovary in peripheral tissues and it was either undetectable or in very low amounts in other tissues showing a very different distribution than the BB<sub>1</sub> receptor or BB<sub>2</sub> receptor (Fathi *et al.*, 1993b; Sano *et al.*, 2004).

In the CNS the BB<sub>3</sub> receptor mRNA was expressed in a restricted distribution (Ohki-Hamazaki *et al.*, 1997a; Liu *et al.*, 2002; Jennings *et al.*, 2003; Sano *et al.*, 2004). In the rat and mouse (Ohki-Hamazaki *et al.*, 1997a; Liu *et al.*, 2002) the BB<sub>3</sub> receptor was present in the highest amounts in the hypothalamic area notable the paraventricular, arcuate, striohypothalamic,



dorsal hypothalamic and dorsomedial hypothalamic nuclei, a medial and lateral preoptic areas and lateral /posterior hypothalamic areas. In the rat expression was also detected in the medial habenula nucleus in one study (Liu *et al.*, 2002) and a second study (Jennings *et al.*, 2003) in the nucleus accumbens and the thalamus. In the monkey brain (Sano *et al.*, 2004) PCR quantitation showed the BB<sub>3</sub> receptor mRNA was present in highest amounts in the hypothalamus followed by the pituitary gland, amygdala, hippocampus and caudate nucleus.

Specific BB<sub>3</sub> receptor antibodies have been used to localize the receptor in the tunica muscularis of the rat gastrointestinal tract (Porcher *et al.*, 2005) and the rat CNS (Jennings *et al.*, 2003). In the gastrointestinal tract tunica muscularis BB<sub>3</sub> receptor immunoreactivity (IR) was observed in all regions studied (i.e. antrum, duodenum, ileum and colon) in nerves and nonneuronal cells but not in muscle cells (Porcher *et al.*, 2005). It was detected in both myenteric and submucosal ganglia as well as in nerve fibers interconnecting myenteric ganglia (Porcher *et al.*, 2005). BB<sub>3</sub> receptor IR was observed in cell bodies and processes of c-kit interstitial cells of Cajal leading the authors to propose that the BB<sub>3</sub> receptor was likely involved in the regulation of gastrointestinal motility through the enteric nervous system and possibly in the pacemaker function of the gastrointestinal smooth muscle (Porcher *et al.*, 2005). In the CNS, particularly strong BB<sub>3</sub> receptor IR was observed in the cerebral cortex, hippocampal formation, hypothalamus and thalamus (Jennings *et al.*, 2003).

Using assessment of BB<sub>3</sub> mRNA (Fathi *et al.*, 1993b) and/or binding studies (Reubi *et al.*, 2002), BB<sub>3</sub> receptors have been shown to exist on a number of different human tumors (Fathi *et al.*, 1993b; Reubi *et al.*, 2002) including small cell and non-small cell lung cancers (Fathi *et al.*, 1993b; Toi-Scott *et al.*, 1996; Ryan *et al.*, 1998b; Reubi *et al.*, 2002), carcinoids (lung) (Fathi *et al.*, 1993b; Reubi *et al.*, 2002), renal cell cancers (Reubi *et al.*, 2002), Ewing sarcomas (Reubi *et al.*, 2002), pancreatic cancer (Schulz *et al.*, 2006), pituitary tumors (Schulz *et al.*, 2006), ovarian cancer (Sun *et al.*, 2000b) and prostate cancer (Sun *et al.*, 2000a; Schulz *et al.*, 2006). BB<sub>3</sub> receptors have also been shown to exist on normal bronchial epithelial cells (DeMichele *et al.*, 1994; Tan *et al.*, 2006), human islets (Fleischmann *et al.*, 2000) and rat kidney cells (Dumesny *et al.*, 2004).

## V.5. BB<sub>3</sub> pharmacology

**V.5.a. BB<sub>3</sub> receptor agonists:** In the original studies describing the ability of GRP, neuromedin C or NMB to interact with the expressed cloned guinea pig BB<sub>3</sub> receptor (Gorbulev *et al.*, 1992) or GRP and NMB to activate the cloned human BB<sub>3</sub> receptor expressed in *Xenopus* oocytes (Fathi *et al.*, 1993b), it was clear that this receptor had low affinity for these peptides (Table 2). Similar results were later reported (Liu *et al.*, 2002) with the rat BB<sub>3</sub> receptor. A later study (Wu *et al.*, 1996) demonstrated that human BB<sub>3</sub> receptors expressed in Balb 3T3 cells had low affinity for all bombesin related peptides tested (i.e. ranatensin, litorin, NMB, GRP, bombesin and alytesin), but at concentrations >1  $\mu$ M, each could activate the BB<sub>3</sub> receptor and stimulate changes in cytosolic calcium. In 1997 Mantey and collaborators performed a detailed study of the ability of all naturally occurring bombesin related peptides and a number of novel synthetic analogues of bombesin to interact with the human BB<sub>3</sub> receptor (Mantey *et al.*, 1997). Because no cell lines existed with wild type BB<sub>3</sub> receptors, to check that the correct pharmacology and cell signaling were being obtained, in this study (Mantey *et al.*, 1997) human BB<sub>3</sub> receptors were expressed in Balb 3T3 cells, which have been shown with transfected BB<sub>1</sub> (Benya *et al.*, 1992) and BB<sub>2</sub> receptors (Benya *et al.*, 1994b) to have similar characteristics to the wild type receptors, as well as overexpressing BB<sub>3</sub> receptors in human nonsmall cell lung cancer cells, NCI-H1299. In this study (Mantey *et al.*, 1997) none of the 15 naturally occurring bombesin-related peptides had a affinity >1  $\mu$ M for the human BB<sub>3</sub> receptor. Furthermore, none of the 26 synthetic bombesin analogues which functioned as BB<sub>1</sub> or BB<sub>2</sub> receptor agonists or antagonists had high affinity for the BB<sub>3</sub> receptor including

[D-Phe<sup>6</sup> Bn<sub>6-13</sub> propylamide (K<sub>i</sub>-2 μM) which had been reported in another study (Wu *et al.*, 1996) assessing changes in cellular calcium to have a relatively high affinity of 84 nM for the human BB<sub>3</sub> receptor. In this study (Mantey *et al.*, 1997) one novel bombesin analogue, [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> was discovered which had high affinity (K<sub>i</sub>-4 nM) and potency for activating the BB<sub>3</sub> receptor and its Tyr<sup>6</sup> analogue retained high affinity and could be radiolabeled to study the pharmacology and ligand receptor interaction in detail. Using this radioligand it was demonstrated (Mantey *et al.*, 1997) that binding to the BB<sub>3</sub> receptor fit a single site-binding model; it was rapid and temperature-dependent, with slow dissociation, supporting ligand internalization; and the binding affinities of all agonists and antagonists for the BB<sub>3</sub> receptor could be determined for the first time and compared to BB<sub>1</sub> receptor and BB<sub>2</sub> receptor. These results demonstrated that the BB<sub>3</sub> receptor has a unique pharmacology, not interacting with high affinity with any known naturally occurring bombesin peptide, supporting the conclusion that the natural ligand is either an undiscovered member of the bombesin family with significant structural differences or an unrelated peptide (Mantey *et al.*, 1997). In a subsequent study (Ryan *et al.*, 1996) two human lung cancer cell lines, NCIN417 and NCI-H720, were found to possess sufficient wild type BB<sub>3</sub> receptors to allow assessment of the pharmacology of the native BB<sub>3</sub> receptor using the <sup>125</sup>I- [D-Tyr<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> ligand described above. Similar pharmacology for all agonists and antagonists was found for the native BB<sub>3</sub> receptor to that reported previously with the BB<sub>3</sub> receptor transfected cell lines (Mantey *et al.*, 1997) with only the agonist, [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> demonstrating high affinity (K<sub>i</sub>-7.4 nM).

Subsequent studies demonstrate that the synthetic bombesin analogue [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub>, in addition to having high affinity for the human BB<sub>3</sub> receptor, also has a high affinity for the human BB<sub>1</sub> receptor, the human BB<sub>2</sub> receptor, the BB<sub>1</sub> receptor and BB<sub>2</sub> receptor from all species studied and the fBB4 (Mantey *et al.*, 1997; Pradhan *et al.*, 1998; Katsuno *et al.*, 1999; Reubi *et al.*, 2002; Iwabuchi *et al.*, 2003) (Table 2). When the rat BB<sub>3</sub> receptor was cloned (Liu *et al.*, 2002) a surprising finding was that [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> had a low potency for this receptor (EC<sub>50</sub> 2 μM). In the chicken (Iwabuchi *et al.*, 2003) a receptor that had high affinity for [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub>, moderate affinity for bombesin and low affinity for GRP and NMB was found, which showed structural similarity to both mammalian BB<sub>3</sub> receptor and the amphibian BB4 receptor, and thus was called ChBRS-3.5. A subsequent study demonstrated the monkey BB<sub>3</sub> receptor (Sano *et al.*, 2004) had a high potency for [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> (EC<sub>50</sub>-5.6 nM) similar to the human receptor. The molecular basis for the difference in affinity of [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> between human/monkey and rat BB<sub>3</sub> receptors has been studied (Liu *et al.*, 2002) and will be discussed in the section III.6.BB<sub>3</sub> below.

Because of the lack of selectivity of the high affinity agonist, [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> for the human BB<sub>3</sub> receptor, there have been a number of groups that have attempted to develop more selective BB<sub>3</sub> receptor ligands. Each of the different groups used [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> as the starting point to identify BB<sub>3</sub> receptor selective agonists. In one study (Mantey *et al.*, 2001) rational peptide design was used by substituting conformationally restricted amino acids into the prototype peptide, [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> or its D-Tyr<sup>6</sup> analogue. A number of BB<sub>3</sub> receptor selective agonists were identified with two peptides with either an (R) or (S)-amino-3-phenylpropionic acid substitution for β-Ala<sup>11</sup> in the prototype ligand having the highest selectivity (i.e. 17–19-fold) (Mantey *et al.*, 2001). Molecular modeling demonstrated these two selective BB<sub>3</sub> receptor ligands had a unique conformation of the position of the 11 β-amino acids, which likely accounted for their selectivity (Mantey *et al.*, 2001). In a second study (Vincent *et al.*, 1999) two strategies were used to attempt to develop a more selective BB<sub>3</sub> receptor ligand: substitutions on the phenyl ring of Apa<sup>11</sup> and the substitution of additional conformationally

restricted amino acids into the position 11 of [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> or its D-Tyr<sup>6</sup> analogue. One analogue, [D-Tyr<sup>6</sup>, Apa-4Cl<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> retained high affinity for BB<sub>3</sub> receptor and was 227-fold selective for the BB<sub>3</sub> receptor over the human BB<sub>2</sub> receptor and 800-fold selective over the human BB<sub>1</sub> receptor (Mantey *et al.*, 2004). Using, [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-</sub> or its D-Tyr<sup>6</sup> analogue as the prototype, three studies (Weber *et al.*, 2002; Weber *et al.*, 2003; Boyle *et al.*, 2005) reported shortened analogues with selectivity for BB<sub>3</sub> receptor assessed by calcium or FLIPR calcium assays. A recent study has assessed the selectivity of four of the most selective of these shortened [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> analogues by binding assays as well as assessment of phospholipase C potencies (Mantey *et al.*, 2006). Three analogues which were reported selective in calcium assays for the BB<sub>3</sub> receptor; [H-D-Phe, Gln, D-Trp, NH (CH<sub>2</sub>)<sub>2</sub>C<sub>6</sub>H<sub>5</sub> and H-D-Phe, Gln, D-Trp, Phe-NH<sub>2</sub>, compounds 68 and 54 in (Weber *et al.*, 2002), and 3-phenylpropionyl-Ala, D-Trp, NH (CH<sub>2</sub>)<sub>2</sub>C<sub>6</sub>H<sub>5</sub> compound 17d in (Weber *et al.*, 2003)] were found (Mantey *et al.*, 2006) in binding studies as well as potency for activation of phospholipase C to have affinities >5 μM for all three human bombesin receptor subtypes and therefore not to be useful. The novel compound Ac-Phe, Trp, Ala, His (tBzl), Nip, Gly, Arg-NH<sub>2</sub> [compound 34 in (Boyle *et al.*, 2005)] had a 14-fold higher affinity for BB<sub>3</sub> receptor than BB<sub>1</sub> receptor and >20 fold for BB<sub>2</sub> receptor (Mantey *et al.*, 2006), however it was less BB<sub>3</sub> receptor selective than [D-Tyr<sup>6</sup>, Apa-4Cl<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> (i.e. >100 fold selectivity (Mantey *et al.*, 2006)(Table 2).

**V.5.b.BB<sub>3</sub> receptor antagonists:** No specific or potent antagonists of the BB<sub>3</sub> receptor exist. In four studies (Ryan *et al.*, 1996; Mantey *et al.*, 1997; Ryan *et al.*, 1998a; Ryan *et al.*, 1999) none of the members of the different classes of potent BB<sub>2</sub> receptor or BB<sub>1</sub> receptor antagonists had an affinity <3 μM for the human BB<sub>3</sub> receptor. In one study (Ryan *et al.*, 1996) the D-amino acid substituted somatostatin analogue, D-Nal, Cys, Tyr, D-Trp, Lys, Val, Cys, Nal-NH<sub>2</sub>, had an affinity of 1 μM for the human BB<sub>3</sub> receptor and was 30-fold more potent at inhibiting activation of the BB<sub>3</sub> receptor than any other compound (Table 2). Unfortunately, this compound also functions as a BB<sub>1</sub> receptor antagonist as well as a somatostatin and mu opioid receptor agonist (Orbuch *et al.*, 1993; Ryan *et al.*, 1999).

## V.6. BB<sub>3</sub> receptor structural basis of receptor binding/activation

**V.6.a. BB<sub>3</sub> receptor agonist binding/activation:** At present because the natural ligand of the BB<sub>3</sub> receptor is unknown there is minimal information available on the importance of amino acid residues in BB<sub>3</sub> receptor activation or determining high affinity interaction. For the only ligand known with high affinity for the BB<sub>3</sub> receptor, [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> (Ryan *et al.*, 1996; Mantey *et al.*, 1997; Ryan *et al.*, 1998a) limited structure-function studies suggest that it is unlikely the deletion of the first five amino acids in [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub>, the insertion of the D-Phe<sup>6</sup> or the presence of either Phe<sup>13</sup> or Nle<sup>14</sup> moieties are determining the high affinity for the BB<sub>3</sub> receptor, because other bombesin analogues with these substitutions do not have high affinity (Ryan *et al.*, 1996; Mantey *et al.*, 1997). These results suggest that the position 11 substitution (i.e. β-Ala<sup>11</sup>, Apa-4Cl<sup>11</sup>) in bombesin analogues is the key substitution for determining high affinity interaction with the BB<sub>3</sub> receptor. At present the basis for the high affinity with these substitutions is unknown.

One study (Liu *et al.*, 2002) investigated the molecular basis for the high affinity of [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> for the human BB<sub>3</sub> receptor, but low affinity for the rat BB<sub>3</sub> receptor. Using a chimeric receptor approach in which the individual extracellular loops of the rat BB<sub>3</sub> receptor were replaced with the corresponding human sequences the important residues were localized to the 4th extracellular domain (1<sup>st</sup>=N-terminus) (Liu *et al.*, 2002). Within this region using site-directed mutagenesis (Liu *et al.*, 2002) the mutation of

Y<sup>298</sup>E<sup>299</sup>S<sup>330</sup> (rat) to S<sup>298</sup>Q<sup>299</sup>T<sup>300</sup> (human) or of D<sup>306</sup>V<sup>307</sup>H<sup>308</sup> (rat) to A<sup>306</sup>M<sup>307</sup>H<sup>308</sup> (human) partially mimic the effect of switching the entire 4<sup>th</sup> extracellular domain. These results indicate that variations in the 4<sup>th</sup> extracellular domains of the rat and human BB<sub>3</sub> receptor are responsible for the differences in affinity for [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> (Liu *et al.*, 2002).

Whereas there is no information on the molecular basis of the selectivity of the various [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> analogues for the BB<sub>3</sub> receptor, a number of studies have assessed the molecular basis for the low affinity of the human BB<sub>3</sub> receptor for the natural occurring high affinity BB<sub>1</sub> receptor and BB<sub>2</sub> receptor agonists (GRP, bombesin, NMB). These studies have utilized an alignment of the receptor structures of the various bombesin receptors and identified key amino acid differences between the BB<sub>3</sub> receptor which has low affinity for GRP, bombesin or NMB and the BB<sub>2</sub> receptor, BB<sub>1</sub> receptor or fBB4 receptors that have high affinity for these ligands (Akeson *et al.*, 1997; Sainz *et al.*, 1998; Nakagawa *et al.*, 2005). The results of these studies are summarized above in either the BB<sub>1</sub> receptor or BB<sub>2</sub> receptor sections dealing with the structural basis of agonist binding.

No studies have investigated the structural basis for BB<sub>3</sub> receptor activation.

**V.6.b. BB<sub>3</sub> receptor antagonist binding:** No potent selective antagonists exist for the BB<sub>3</sub> receptor.

#### V.7. BB<sub>3</sub> receptor signaling, activation, and modulatory processes

**(internalization, down-regulation, desensitization)**—The human BB<sub>3</sub> receptor (Fathi *et al.*, 1993b; Ryan *et al.*, 1996; Wu *et al.*, 1996; Ryan *et al.*, 1998a), as well as the monkey (Sano *et al.*, 2004) and rat BB<sub>3</sub> receptor (Liu *et al.*, 2002) is coupled to phospholipase C resulting in breakdown of phosphoinositides, mobilization of cellular calcium and presumed activation of protein kinase C.

BB<sub>3</sub> receptor activation also results in the stimulation phospholipase D (Ryan *et al.*, 1996), but does not activate adenylate cyclase (Ryan *et al.*, 1996; Ryan *et al.*, 1998a). BB<sub>3</sub> receptor stimulation also results in activation of tyrosine kinases (Ryan *et al.*, 1998a; Weber *et al.*, 2001) stimulating tyrosine phosphorylation of p125<sup>FAK</sup> by a mechanism which is not dependent on either limb of the phospholipase C cascade (i.e. activation of PKC or mobilization of cellular calcium (Ryan *et al.*, 1998a)). Activation of the BB<sub>3</sub> receptor also stimulates MAP kinase activation resulting in rapid tyrosine phosphorylation of both a 42 and 44 kDa forms, which is inhibited by the MEK-1 inhibitor PD98059 (Weber *et al.*, 2001). In BB<sub>3</sub> receptor transfected NCI-1299 lung cancer cells, activation of the BB<sub>3</sub> receptor by [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> resulted in stimulation of Elk-1 in a MEK-1 dependent manner as well as a 47-fold increase in c-fos mRNA (Weber *et al.*, 2001). These results demonstrated that BB<sub>3</sub> receptor activation causes increased nuclear proto-oncogene expression and upstream events including activation of MAP kinase and Ek-1 activation (Weber *et al.*, 2001).

There have been no studies of BB<sub>3</sub> receptor modulatory processes (internalization, down-regulation, or desensitization).

**V.8. BB<sub>3</sub> receptor function in various tissues and *in vivo***—At present the function of the BB<sub>3</sub> receptor in normal physiology and pathologic conditions is largely unknown because the natural ligand is still not known. An important insight into possible BB<sub>3</sub> receptor function was provided by studies of BB<sub>3</sub> receptor knockout mice. In the initial study (Ohki-Hamazaki *et al.*, 1997b) mice lacking the BB<sub>3</sub> receptor developed mild obesity, associated with hypertension and impairment of glucose metabolism. These changes were associated with reduced metabolic rate, increased feeding behavior, a five-fold increase in serum leptin levels



and hyperphagia (Ohki-Hamazaki *et al.*, 1997b). These results suggested BB<sub>3</sub> receptor might play an important role in the mechanisms responsible for energy balance and control of body weight. A number of studies have been performed subsequently on BB<sub>3</sub> receptor knockout mice to attempt to establish the mechanism of these effects. BB<sub>3</sub> receptor knockout mice were shown to have altered taste preference (Yamada *et al.*, 1999) which was proposed to be due to the lack of BB<sub>3</sub> receptor expression in the medial and central nuclei of the amygdala and the hypothalamic nuclei which are known to be involved in taste perception (Yamada *et al.*, 1999) and to be possibly a contributory factor to the obesity. BB<sub>3</sub> receptors are present on pancreatic islets (Fleischmann *et al.*, 2000) and BB<sub>3</sub> receptor knockout mice have a 2.3 fold increase in plasma insulin levels (Matsumoto *et al.*, 2003) (Table 2). One study (Matsumoto *et al.*, 2003) concluded that the BB<sub>3</sub> receptor contributes to regulation of plasma insulin concentration/secretion and that dysregulation in this contribution in these mice contributes to obesity (Matsumoto *et al.*, 2003). In a second study (Nakamichi *et al.*, 2004) it was concluded that the impaired glucose metabolism in BB<sub>3</sub> receptor knockout mice is mainly due to impaired GLUT4 translocation in adipocytes.

**IV.9. BB<sub>3</sub> receptor in diseases**—At present there are no diseases in which activation or alterations of the BB<sub>3</sub> receptor have been shown to be involved. BB<sub>3</sub> receptor activation has been proposed to be important in the mediation of a number of human disorders including disorders of lung development, various pulmonary diseases, CNS disorders, and the growth/differentiation of human cancers. The tumor differentiation effects of BB<sub>3</sub> receptor activation were discussed in the previous section; the growth effects and effects of BB<sub>3</sub> receptor overexpression will be considered here. In human cancer cells or cancers BB<sub>3</sub> receptors are not only ectopically expressed in a large number of tumors, as reviewed above (Fathi *et al.*, 1993b; Toi-Scott *et al.*, 1996; Fathi *et al.*, 1996; Sun *et al.*, 2000b; Reubi *et al.*, 2002; Schulz *et al.*, 2006), but their activation alters lung cancer behavior by increasing MAP kinase activation, nuclear oncogene expression (Weber *et al.*, 2001) and increasing adhesion of lung cancer tumor cells, which was proposed to contribute to increased tumor invasion and metastases by these tumors (Hou *et al.*, 2006). In BB<sub>3</sub> knockout mice (Maekawa *et al.*, 2004) the hyperphagic response to melanin-concentrating hormone (MCH) is impaired, but not in BB<sub>2</sub> receptor knockout mice. Furthermore, the levels of the MCH receptor and prepro-MCH mRNA's in the hypothalamus of BB<sub>3</sub> receptor knockout mice were higher than controls, suggesting that up-regulation of the MCH-R and MCH occurs in the knockout mice that triggers hyperphagia and likely upsets the mechanism by which leptin decrease MCH-R and feeding (Maekawa *et al.*, 2004). Studies of BB<sub>3</sub> receptor knockout mice suggest that this receptor is important in various behavioral effects including the neural mechanisms that regulate social isolation (Yamada *et al.*, 2000a) and are important in modulating emotion including forms of anxiety (Yamada *et al.*, 2002a).

BB<sub>3</sub> receptors as well as BB<sub>1</sub> receptor and BB<sub>2</sub> receptor are expressed in developing primate and murine fetal lungs (Emanuel *et al.*, 1999; Shan *et al.*, 2004). Studies (Tan *et al.*, 2006; Tan *et al.*, 2007) demonstrate that BB<sub>3</sub> receptors are expressed in the airway in response to ozone injury and that wound repair and proliferation of bronchial epithelial cells is accelerated by BB<sub>3</sub> receptor activation, suggesting it may mediate wound repair. The mechanism of lung ozone injury mediation of the up-regulation of BB<sub>3</sub> receptors has been studied by examining proteins interacting with the BB<sub>3</sub> receptor gene promoter region (Tan *et al.*, 2007). AP-2 alpha and PPARalpha increased the ozone-inducible DNA binding on the BB<sub>3</sub> receptor gene promoter suggesting they are specifically involved in the BB<sub>3</sub> receptor up-regulation (Tan *et al.*, 2007). BB<sub>3</sub> receptors are expressed on small cell and nonsmall cell lung cancers (Fathi *et al.*, 1993b; Toi-Scott *et al.*, 1996; Ryan *et al.*, 1998b; Reubi *et al.*, 2002) as well as lung carcinoids (Fathi *et al.*, 1993b; Reubi *et al.*, 2002). In the small cell lung cancer cell line, NCI-N417, which is known to possess functional BB<sub>3</sub> receptors (Ryan *et al.*, 1998b), [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> stimulated tumor cell adhesion, likely by stimulation of

focal adhesion formation (Hou *et al.*, 2006). It was proposed (Hou *et al.*, 2006) that BB<sub>3</sub> receptor activation in these cells may be important for their invasion and development of metastases.

Although the function of BB<sub>3</sub> receptors in the gastrointestinal tract is largely unknown, specific BB<sub>3</sub> receptor antibodies localized the receptor in the tunica muscularis of the rat gastrointestinal tract (Porcher *et al.*, 2005). BB<sub>3</sub> receptors were detected in both myenteric and submucosal ganglia as well as in nerve fibers interconnecting myenteric ganglia (Porcher *et al.*, 2005). BB<sub>3</sub> receptor IR was observed in cell bodies and processes of c-kit interstitial cells of Cajal leading the authors to propose that the BB<sub>3</sub> receptor was likely involved in the regulation of gastrointestinal motility.

The above studies and those reviewed in the previous section suggest that BB<sub>3</sub> receptor activation could be involved in human disorders of energy metabolism including obesity, glucose homeostasis, blood pressure control, lung injury, tumor growth, and possibility motility disorders, but all of these possibilities remain unproven at present.

One study screened 104 Japanese obese men for defects in the BB<sub>3</sub> receptor gene but no mutations or polymorphisms were found (Hotta *et al.*, 2000) suggesting BB<sub>3</sub> receptor gene mutations are unlikely to be a major cause of obesity in humans.

## VI. Therapeutic implications of Bn receptors

This area was partially covered under the sections dealing with disease for each of the three receptor classes, but a few important summary points will be made here. The principal therapeutic interests are in the BB<sub>2</sub> receptors, to a lesser extent in the BB<sub>3</sub> receptor and least in the BB<sub>1</sub> receptor. In the case of the BB<sub>2</sub> receptor the recent study (Sun and Chen, 2007) that provides evidence that activation of the BB<sub>2</sub> receptor in the spinal cord may be an important pathway in mediating pruritic signals has profound clinical implications. Chronic itching is a very common problem (Yosipovitch *et al.*, 2007): in a population survey of 18,770 adults in Norway (Dalgard *et al.*, 2007a; Dalgard *et al.*, 2007b) itching was the most common skin problem occurring in 7% and it is associated with poor general health. Often existing therapies provide limited relief and there are no general-purpose anti-pruritic drugs (Yosipovitch *et al.*, 2007) therefore, identification of the BB<sub>2</sub> receptor as possible central therapeutic target has significant therapeutic implications for this disorder. The tumoral growth effects and frequent overexpression or ectopic expression of all of the Bn receptors have important clinical implications. This is particularly true for the BB<sub>2</sub> receptor, which is the most frequently overexpressed, and has been the most extensively investigated for its growth effects on different human tumors (Schulz *et al.*, 2006; Lantry *et al.*, 2006; Jensen and Moody, 2006; Patel *et al.*, 2006; Engel *et al.*, 2007; Cornelio *et al.*, 2007). Studies demonstrating that GRP and NMB can have autocrine growth activity; in some tumors BB<sub>2</sub> receptor activation results in stimulation of the EGF receptor; that continued stimulation through the BB<sub>2</sub> receptor can counter the inhibitory effects of EGFR blockade on tumor growth and that the combination of a BB<sub>2</sub> receptor blockade and EGF receptor inhibition can have profound inhibitory effects, on tumor growth, all have important therapeutic implications (Santiskulvong *et al.*, 2001; Madarame *et al.*, 2003; Xiao *et al.*, 2003; Santiskulvong and Rozengurt, 2003; Santiskulvong *et al.*, 2004; Thomas *et al.*, 2005; Stangelberger *et al.*, 2005; Jensen and Moody, 2006; Patel *et al.*, 2006; Liu *et al.*, 2007; Zhang *et al.*, 2007b; de Visser *et al.*, 2007b). As discussed in detail in the BB receptor disease sections the over-expression of particularly BB<sub>2</sub> receptors, by many common tumors (breast, colon, head and neck squamous cancers, various CNS tumors, lung, prostate, ovary, and renal) has important therapeutic implications. This is particularly the case for the Bn family of receptors, because they are one of the classes of G protein-coupled receptors most frequently present on these tumors. Furthermore, in many cases existing therapies are inadequate with these tumors or the tumors frequently stop responding to current first-line

treatments, and therefore new approaches are needed. These therapeutic implications include not only for the development of labeled Bn analogues for enhanced tumor imaging and staging (Breeman *et al.*, 2002; Smith *et al.*, 2003; Nock *et al.*, 2003; Smith *et al.*, 2005; Zhang *et al.*, 2006; Lantry *et al.*, 2006; Johnson *et al.*, 2006; Waser *et al.*, 2007; Parry *et al.*, 2007; Dimitrakopoulou-Strauss *et al.*, 2007; Garrison *et al.*, 2007; Prasanphanich *et al.*, 2007; de Visser *et al.*, 2007a; Zhang *et al.*, 2007a), but also for use for bombesin receptor-mediated cytotoxicity, either with radiolabeled compounds, as is being widely evaluated with somatostatin analogues in Phase 3 studies (Van Essen *et al.*, 2007; Breeman *et al.*, 2007; Forrer *et al.*, 2007), or the use of Bn analogues coupled to other cytotoxic agents such as doxorubicin analogues, paclitaxel or camptothecin (Schally and Nagy, 1999; Breeman *et al.*, 2002; Moody *et al.*, 2004; Schally and Nagy, 2004; Engel *et al.*, 2005; Buchholz *et al.*, 2006; Safavy *et al.*, 2006; Panigone and Nunn, 2006; Nanni *et al.*, 2006; Moody *et al.*, 2006b; Engel *et al.*, 2007). The participation of BB<sub>3</sub> receptors in energy balance and in glucose homeostasis as manifested by the BB<sub>3</sub> receptor knockout animals developing obesity and diabetes (Ohki-Hamazaki *et al.*, 1997a) has potential important clinical implications. At present there has been increased understanding of the mechanisms of these effects (see BB<sub>3</sub> receptor diseases section: V.8 (Yamada *et al.*, 1999; Matsumoto *et al.*, 2003; Nakamichi *et al.*, 2004), but possible progress in extending this to a clinical application is limited by the lack of identification of the natural ligand for this receptor.

Numerous other actions of each of the three Bn receptors have potential importance for therapeutic interventions, but at present either the understanding of their participation in normal and pathological conditions is insufficient to specifically target these receptors or the drugs to do this are not available. In the case of the BB<sub>1</sub> receptor such areas include: involvement in thyroid function and alterations in thyroid disease (Ortiga-Carvalho *et al.*, 2003; Pazos-Moura *et al.*, 2003; Oliveira *et al.*, 2006); behavior effects in mediating aspects of fear, anxiety, stress responses (Ohki-Hamazaki *et al.*, 1999; Merali *et al.*, 2002; Yamada *et al.*, 2003; Merali *et al.*, 2006; Bedard *et al.*, 2007) and satiety effects (Merali *et al.*, 1999; Ladenheim and Knipp, 2007). For the BB<sub>2</sub> receptor such areas include: its role in motility with mediation of the descending peristaltic reflex (Grider, 2004); role in lung injury and development of lung diseases, particularly the neonatal lung disease, bronchopulmonary dysplasia, in which Bn-like peptides and the BB<sub>2</sub> receptor were shown to play an important role in various animal models (Li *et al.*, 1994; Sunday *et al.*, 1998; Emanuel *et al.*, 1999; Cullen *et al.*, 2000; Ashour *et al.*, 2006; Ganter and Pittet, 2006; Subramaniam *et al.*, 2007); its role in sepsis and in small intestinal mucosal protection and prevention from injury (Assimakopoulos *et al.*, 2004; Assimakopoulos *et al.*, 2005a; Assimakopoulos *et al.*, 2005b; Higuchi *et al.*, 2006; Dal-Pizzol *et al.*, 2006; Kimura *et al.*, 2006a; Kimura *et al.*, 2006b); satiety effects (Merali *et al.*, 1999; Ladenheim and Knipp, 2007) and its CNS effects on memory, learning, various behaviors and response to stress (Merali *et al.*, 1999; Yegen, 2003; Roesler *et al.*, 2004; Moody and Merali, 2004; Merali *et al.*, 2006; Luft *et al.*, 2006; dos Santos Dantas *et al.*, 2006; Roesler *et al.*, 2006a; Roesler *et al.*, 2006b; Presti-Torres *et al.*, 2007). For the BB<sub>3</sub> receptor such areas include: its possible role in lung development and responses to lung injury (Shan *et al.*, 2004; Hou *et al.*, 2006; Tan *et al.*, 2006; Tan *et al.*, 2007) and its possible role in regulation of aspects of gastrointestinal motility (Porcher *et al.*, 2005).

## VII. Unresolved nomenclature issues

The principal unresolved issue is the natural ligand of the BB<sub>3</sub> receptor remains unknown and therefore its pharmacology and roles in normal physiology or pathological processes is unknown. Another unresolved issue is whether an equivalent receptor to the frog BB<sub>4</sub> exists in human and mammals. Two studies have sought additional members of the bombesin receptor family and none were found in mammals (Fathi *et al.*, 1993b; Sano *et al.*, 2004). With human and mouse genome sequences now known, it is high unlikely that any other mammalian BB's

will be found besides BB<sub>1</sub>, BB<sub>2</sub>, and BB<sub>3</sub>. An additional key issue unresolved at present is whether COOH terminal extended or precursor form or fragments of GRP or NMB have physiological or pathological effects that are not mediated by the three classes of mammalian receptors described in the current nomenclature. A number of recent studies (Patel *et al.*, 2004; Dumesny *et al.*, 2004; Dumesny *et al.*, 2006; Patel *et al.*, 2007a; Patel *et al.*, 2007b) provide evidence that non-amidated precursor forms of GRP can stimulate proliferation of different tumors/tissues. COOH terminal precursor forms of GRP are reported to stimulate the proliferation and migration of the human colorectal carcinoma cell line DLD-1 (Patel *et al.*, 2007a; Patel *et al.*, 2007b) through a BB<sub>2</sub> receptor independent mechanism and the growth of the prostate cancer cell line DU145 (Patel *et al.*, 2007b). Furthermore, ProGRP immunoreactivity is reported in 90% of resected colorectal carcinomas and all endometrial, prostate and colon cancer cell lines tested, without any amidated forms present (Dumesny *et al.*, 2006). Recombinant ProGRP stimulated proliferation of the colon cancer cell line DLD-1, activating MAPK, but did not stimulate phospholipase C activity nor bind to known bombesin receptors, suggesting it was stimulating the tumor growth through a novel receptor (Dumesny *et al.*, 2006). At present no receptor has been isolated that mediates these actions, but they are not inhibited by BB<sub>2</sub> receptor antagonists, raising the possibility they could be mediated by a novel receptor. A final key problem area unresolved at present, are the roles of the three described mammalian bombesin receptors in normal physiology and pathological conditions which are still largely unknown. This is due in large part to lack of specific antagonists for all subclasses of bombesin receptors, especially high affinity, selective nonpeptide receptor antagonists.

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#### Abbreviations

Bn, bombesin  
 GRP, gastrin-releasing peptide  
 NMB, neuromedin B  
 CNS, central nervous system  
 IR, immunoreactivity  
 BRS-3, bombesin receptor subtype 3  
 fBB4, frog bombesin receptor subtype 4  
 COOH terminus, carboxyl terminus  
 PKC, protein kinase C  
 TM, transmembrane region  
 GPCR, G protein-coupled receptor  
 5-HT, serotonin, 5-hydroxytryptamine  
 NSCLC, nonsmall cell lung cancer cell  
 CCK, cholecystokinin  
 SP, substance P  
 Cpa, chlorophenylalanine  
 β—Ala, β—Alanine  
 ψ bonds, pseudopeptide bonds  
 DAG, diacylglycerol  
 p125<sup>FAK</sup>, p125 focal adhesion kinase  
 TPA, 12-O-tetradecanoyl-phorbol-13-acetate  
 EC1, extracellular domain 1



IC1, intracellular domain 1  
 PKD, protein kinase D  
 ERK, extracellular regulated kinase

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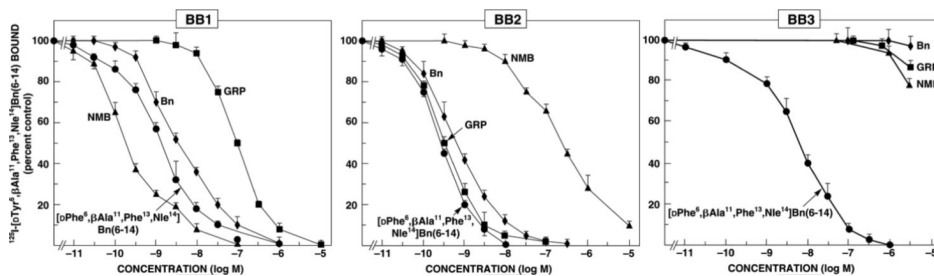
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Peptide	AMINO ACID POSITION (Reference to Bn)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Bombesin (Bn)	pGlu	Gln	Arg	Leu	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met
GRP (14-27)	Met	Tyr	Pro	Arg	-	-	His	-	-	-	-	-	-	-
NMC(GRP20-27)							His	-	-	-	-	-	-	-
Alytesin		Gly	-	-	-	Thr	-	-	-	-	-	-	-	-
Ranatensin				pGL	Val	Pro	-	-	-	-	-	-	Phe	-
NMB					-	-	Leu	-	-	Thr	-	-	Phe	-
PGL		pGlu	Gly	Gly	-	Pro	-	-	-	-	-	-	Phe	-
Litorin						pGlu	-	-	-	-	-	-	Phe	-
Rohdei-Litorin						pGlu	Leu	-	-	Thr	-	-	Phe	-
Phyllolitorin (PLL)						pGlu	Leu	-	-	-	-	Ser	Phe	-
[Leu <sup>8</sup> ]PLL						pGlu	Leu	-	-	-	-	Ser	Leu	-
[Thr <sup>5</sup> ,Leu <sup>8</sup> ]PLL						pGlu	Leu	-	-	Thr	-	Ser	Leu	-
[D-Phe <sup>6</sup> , (ψ <sup>13-14</sup> , Cpa <sup>14</sup> )Bn(6-14)						D-Phe								ψ Cpa
[D-Phe <sup>6</sup> ]Bn(6-13)propylamide(PA)						D-Phe								PA
[Tyr <sup>4</sup> ,D-Phe <sup>12</sup> ]Bn													D-Phe	-
[F <sub>5</sub> -D-Phe <sup>6</sup> , D-Ala <sup>11</sup> ]Bn(6-13)methyl ester(ME)						F <sub>5</sub> -D-Phe							D-Ala	ME
N-Ac-GRP(20-26)ethyl ester(EE)							N-Ac-							EE

**Figure 1.**

Structures of GRP, NMB and Bn-related agonists and antagonists. The entire structures of the different peptides are shown except for GRP which has 27 amino acids and only the COOH terminal 14 amino acids are shown, which is the biologically active end. Both natural occurring agonists and some of the antagonists referred to in the text are shown. Abbreviations. ψ, – CONH peptide bond changed to –CH<sub>2</sub>NH– ; pGlu, pyroglutamic acid; Cpa, chlorophenylalanine; NMC, neuromedin C; F<sub>5</sub>, pentafluoro-



**Figure 2.** Ability of the mammalian peptides, gastrin-releasing peptide (GRP) and neuromedin B NMB); the amphibian peptide, bombesin and the synthetic bombesin analogue, [D-Phe<sup>6</sup>, β-Ala<sup>11</sup>, Phe<sup>13</sup>, Nle<sup>14</sup>] bombesin<sub>6-14</sub> to interact with the three human classes of bombesin receptors. Data are partially from (Benya *et al.*, 1995b; Ryan *et al.*, 1998b).





**Table 1**

## Current bombesin receptor nomenclature and general characteristics

Variable	Mammalian bombesin receptor		
Receptor Code	BB <sub>1</sub>	BB <sub>2</sub>	BB <sub>3</sub>
Previous Names	NMB-R, NMB preferring receptor	GRP-R, GRP preferring receptor	BRS-3, bombesin receptor subtype 3
Cloned from mammals	Human, rat, mouse, monkey	Human, rat, mouse, monkey, chimpanzee, dog, sheep	Human, rat, mouse, monkey, sheep
Gene location	Chr 6p21 (human)	Chr Xp22 (human)	Chr Xq25 (human)
Structural information	390 aa (human)	384 aa (human)	399 aa (human)
Natural ligands	NMB>GRP	GRP>NMB	Unknown (low affinity NMB, GRP, all Bn natural related peptides)
Selective agonist	NMB, NMB30	GRP	[D-Tyr <sup>6</sup> , Apa-4Cl <sup>11</sup> , Phe <sup>13</sup> , Nle <sup>14</sup> ] bombesin <sub>6-14</sub> , Ac-Phe, Trp, Ala, His (tBz), Nip, Gly, Arg-NH <sub>2</sub>
Selective antagonists	PD 168368	[D-Phe <sup>6</sup> , Cpa <sup>14</sup> , Ψ13-14]Bn <sub>6-14</sub> , JMV641, JMV594, BW2258U89, Ac-GRP <sub>20-26</sub> methyl ester	None
Principal transduction	Gq/11	Gq/11	Gq/11
Preferred radioligand	<sup>125</sup> I-BH-[D-Tyr <sup>0</sup> ]-NMB, <sup>125</sup> I-[Tyr <sup>4</sup> ]-Bn	<sup>125</sup> I-[GRP], <sup>125</sup> I-[Tyr <sup>4</sup> ]-Bn, <sup>125</sup> I-[D-Tyr <sup>6</sup> ]-Bn <sub>6-13</sub> methyl ester	<sup>125</sup> I-[D-Phe <sup>6</sup> , β-Ala <sup>11</sup> , Phe <sup>13</sup> , Nle <sup>14</sup> ] Bn <sub>6-14</sub>
Tissue functions	CNS (regulate TSH release, satiety), GI tract (motility); regulate stress responses	CNS (thermoregulation, regulate circadian rhythm, satiety); GI tract (hormone release, motility, regulate secretions [pancreas, gastric acid, islets]); immunologic (chemoattractant, lymphocyte function); fetal development (lung), Tumor growth effects- morphogen, autocrine growth factor (lung/colon/prostate/breast/. head-neck tumors, others); lung diseases (bronchopulmonary dysplasia, tobacco injury)	Regulate energy homeostasis, glucose/insulin regulation; satiety; lung development and response to injury; present myenteric/submucosa ganglia, cells of Cajal proposed involved GI motility
Diseases	Altered hypo-, hyperthyroidism; autocrine tumor growth factor (lung/colon tumors, carcinoids, others)	Tumor growth effects- morphogen, autocrine growth factor (lung/colon/prostate/breast/. head-neck tumors, others); lung diseases (bronchopulmonary dysplasia, tobacco injury)	Tumor growth factor (lung, others)
Phenotype of knockout	Reduced hypothermic effect to NMB; abnormal behaviors; dysregulation of thyroid-pituitary axis; altered CNS 5-HT system with stress	Altered satiety, thermoregulation: abnormal behaviors, altered insulin release;	Mild obesity, hypertension, impaired glucose metabolism reduced metabolic rate, increased feeding behavior; altered lung response to injury

References are in text under the indicated receptor



**Table 2**

Affinity of bombesin receptor subtypes for various agonist/antagonists.

Variable	Affinity (nM) <sup>a</sup>		
	BB <sub>1</sub>	BB <sub>2</sub>	BB <sub>3</sub>
<b>I. Natural occurring Agonist</b>			
GRP	440	18	>10,000
NMB	4	248	>10,000
Bombesin (Bn)	34	4	>10,000
Litorin	7	6	>10,000
Ranatensin	13	2	>10,000
Alytesin	460	62	>10,000
Phyllolitorin	47	240	>10,000
Neuromedin C (GRP <sub>18-27</sub> )	140	20	>10,000
[Phe <sup>13</sup> ]Bombesin	350	0.77	>10,000
<b>II. Synthetic Agonists</b>			
[D-Phe <sup>6</sup> , β-Ala <sup>11</sup> , Phe <sup>13</sup> , Nle <sup>14</sup> ] Bn <sub>6-14</sub>	0.36	0.99	4.2
[D-Tyr <sup>6</sup> , (R)-Apa <sup>11</sup> , Phe <sup>13</sup> , Nle <sup>14</sup> ] Bn <sub>6-14</sub> <sup>b</sup>	7200	>1900	8.2
[D-Tyr <sup>6</sup> , Apa-4Cl <sup>11</sup> , Phe <sup>13</sup> , Nle <sup>14</sup> ] Bn <sub>6-14</sub> <sup>b</sup>	2400	151	2.8
Ac-Phe, Trp, Ala, His (tBzl), Nip, Gly, Arg-NH <sub>2</sub> <sup>b</sup>	3800	5000	259
[D-Phe <sup>6</sup> ] Bn <sub>6-14</sub>	14	2	>10,000
[D-Phe <sup>6</sup> , D-Ala <sup>11</sup> , Leu <sup>14</sup> ] Bn <sub>6-14</sub>	7600	13	>10,000
<b>II. Antagonists</b>			
[D-Phe <sup>6</sup> ] Bn <sub>6-13</sub> methyl ester	7500	1.1	>10,000
N-propionyl- [D-Ala <sup>11</sup> ] GRP <sub>20-26</sub> methyl ester	13,660	3.4	>10,000
PD 168368	39	1300	1010
D-Nal, Cys, Tyr, D-Trp, Lys, Val, Cys, Nal-NH <sub>2</sub>	59	2780	>10,000
[[Tyr <sup>4</sup> , D-Phe <sup>12</sup> ] Bn <sub>6-14</sub>	1900	>10,000	>10,000
[Leu <sup>13</sup> , ψ13-14, Leu <sup>14</sup> ] Bn <sub>6-14</sub>	>10,000	430	>10,000
[D-Phe <sup>6</sup> , Leu <sup>13</sup> , Cpa <sup>14</sup> , ψ13-14] Bn <sub>6-14</sub>	2700	42	6800
BW2258U89	>10,000	0.74	>10,000
[D-Arg <sup>1</sup> , D-Trp <sup>7,9</sup> , Leu <sup>11</sup> ] substance P	4,100	11,300	>10,000
JMV594	>10,000	2.2	>10,000
JMV641	1500	0.46	>10,000

<sup>a</sup> All data are for rat BB<sub>1</sub>, mouse BB<sub>2</sub> and human BB<sub>3</sub> except data indicated in footnote b. Data are from (Coy *et al.*, 1992b; Mantey *et al.*, 1997; Pradhan *et al.*, 1998; Ryan *et al.*, 1998b; Katsuno *et al.*, 1999; Ryan *et al.*, 1999; Tokita *et al.*, 2001b)

<sup>b</sup> Data are from human BB<sub>1</sub>, BB<sub>2</sub> and BB<sub>3</sub> and are from (Mantey *et al.*, 2001; Mantey *et al.*, 2004; Mantey *et al.*, 2006) Definition of compound structures are in text for each specific for receptor.