Bombesin Receptor Subtypes in Human Cancers: Detection with the Universal Radioligand ^{125}I-[D-TYR^{6}, β-ALA^{11}, PHE^{13}, NLE^{14}] Bombesin(6–14)

Jean Claude Reubi,[1] Sandra Wenger, Jacqueline Schmuckli-Maurer, Jean-Claude Schaer, and Mathias Gugger

Division of Cell Biology and Experimental Cancer Research, Institute of Pathology, University of Berne, CH-3010 Berne, Switzerland

ABSTRACT

Purpose: **Bombesin and bombesin receptors have been shown to play a role in cancer. Whereas the gastrin-releasing peptide (GRP) receptor is a bombesin receptor subtype frequently expressed by tumors, the other three subtypes, the neuromedin B (NMB), BB3, and BB4 receptors, have been poorly investigated in human tissues.**

Experimental Design: **We investigated 161 human tumors for their bombesin receptor subtype expression using *in vitro* receptor autoradiography with the universal bombesin radioligand ^{125}I-[D-Tyr^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) in displacement experiments with unlabeled GRP, bombesin, NMB, and [D-Tyr^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14). The distinct rank order of potencies of these analogues for each receptor subtype allows us to identify the predominant subtype expressed by each tumor.**

Results: **Twelve of 12 prostate cancers, 41 of 57 breast cancers, and 5 of 5 gastrinomas expressed predominantly GRP receptors; 11 of 24 intestinal, 1 of 26 bronchial, and 1 of 1 thymic carcinoids had preferentially NMB receptors; 9 of 26 bronchial carcinoids, 1 large cell neuroendocrine lung carcinoma, and 4 of 9 small cell lung carcinomas had preferentially BB3 receptors, whereas 3 of 9 small cell lung carcinomas had GRP receptors. Renal cell carcinomas had GRP receptors in 6 of 16 cases and BB3 receptors in 4 of 16 cases. Finally, 2 of 10 Ewing sarcomas had BB3 receptors. *In situ* hybridization detected BB3 receptor mRNA in neuroendocrine tumors expressing the BB3 protein.**

Conclusions: **This is the first study detecting the proteins of BB3, NMB, and GRP receptors in a group of human tumors using differential binding techniques. Particularly relevant is the BB3 expression in lung carcinoids and other neuroendocrine lung tumors, whereas gastrointestinal carcinoids preferably express NMB receptors. These tumors may be targets for diagnostic and radiotherapeutic applications of subtype-selective bombesin analogues.**

INTRODUCTION

The overexpression of peptide receptors in human tumors is of considerable clinical interest (1). The past 10 years have shown that the overexpressed somatostatin receptors in human neuroendocrine tumors can be targeted successfully. On one hand, the long-term octreotide treatment of patients with somatostatin receptor-expressing neuroendocrine tumors has been successful in relieving the symptoms related to excessive hormone production by the tumors (2). On the other hand, the use of radiolabeled somatostatin analogues has permitted us to visualize *in vivo* neuroendocrine tumors and their metastases in patients (3). More recently, it has been shown that somatostatin receptor-positive neuroendocrine tumors could be targeted with ^{90}Y-labeled somatostatin analogues as radiotherapy (4). Similar targeting strategies have been applied for the overexpressed cholecystokinin-B receptors in medullary thyroid carcinomas (5) and for the overexpressed vasoactive intestinal peptide receptors in gastrointestinal tumors (6).

Bombesin and its human counterpart GRP[2] belong to a family of brain-gut peptides, shown, in addition to physiological effects, to play also an important role in cancer (7, 8). It has been observed several years ago that cancer cell lines as well as primary human tumors can synthesize bombesin and GRP (9). These peptides probably act in an autocrine fashion to stimulate the growth of the tumor cells they originate from, through bombesin receptors expressed on the membranes of these cells (7, 10). More recently, it has been shown that the GRP receptor proteins can be overexpressed in a large variety of human tumors, including prostate cancers, breast carcinomas, SCLCs, and non-SCLCs, as well as renal cell carcinomas (11–15). Most of the studies up to now have been able to primarily identify the GRP receptor subtype, although it is known that the bombesin receptor family includes at least four different subtypes, namely the GRP receptor subtype (BB2), the NMB receptor subtype (BB1), and the BB3 and BB4 subtypes (16–19). Except for the GRP receptor, the other three subtypes have been poorly characterized, in particular in regard to their distribution and function in human tissues. Recently, a very potent ligand, the [D-Tyr^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14), shown to bind to all four of the bombesin receptors, has been developed by Mantey *et al.* (20) and Pradhan *et al.* (21). This compound,

Received 9/21/01; revised 12/28/01; accepted 1/7/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Division of Cell Biology and Experimental Cancer Research, Institute of Pathology, University of Berne, Murtenstrasse 31, CH-3010 Berne, Switzerland. Phone: 41-31-63-23-24-2; Fax: 41-31-63-28-99-9; E-mail: reubi@patho.unibe.ch.

[2] The abbreviations used are: GRP, gastrin-releasing peptide; SCLC, small cell lung carcinoma; NMB, neuromedin B.

Table 1 Incidence of bombesin receptor subtype expression in various human cancers

Tumor type	*n* of cases	Receptor incidence		
		GRP-R	NMB-R	BB_3-R
Prostate carcinomas	12	12/12	0/12	0/12
Breast carcinomas	57	41/57	0/57	0/57
NE[a] GEP tumors				
Gastrinomas	5	5/5	0/5	0/5
Intestinal carcinoids	24	0/24	11/24	0/24
Thymic carcinoid	1	0/1	1/1	0/1
NE lung tumors				
Bronchial carcinoids	26	0/26	1/26	9/26
Small cell lung cancers	9	3/9	0/9	4/9
LCNEC	1	0/1	0/1	1/1
Renal cell carcinomas	16	6/16	0/16	4/16
Ewing sarcomas	10	0/10	0/10	2/10

[a] NE, neuroendocrine; GEP, gastroenteropancreatic; LCNEC, large cell neuroendocrine carcinoma.

iodinated at the D-Tyr^6 residue, is a useful radioligand able to distinguish the various bombesin receptor subtypes on the basis of the rank order of their affinity for GRP, NMB, [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14), or bombesin. Using this approach, we could recently detect specific BB3 receptor expression in human pancreatic islets (22).

The aim of the present study was to evaluate in a selection of human tumors of different origins the expression of the various bombesin receptor subtypes using *in vitro* receptor autoradiography with ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) as a radioligand in displacement experiments with unlabeled GRP, bombesin, NMB, and [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14). In all of the cases, we have compared the results obtained with ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) with those using [^{125}I-Tyr^4]bombesin, a ligand that preferentially identifies GRP receptors. In selected tumors, the corresponding receptor mRNA was measured with *in situ* hybridization techniques.

MATERIALS AND METHODS

Tissues. Aliquots of surgically resected tumors or of biopsy specimens submitted for diagnostic histological analysis were frozen immediately after surgical resection and stored at −70°C. The following tumors were investigated: 12 prostate carcinomas; 57 ductal breast carcinomas; 29 neuroendocrine gastroenteropancreatic tumors consisting of 5 gastrinomas and 24 intestinal carcinoids; 36 neuroendocrine lung tumors consisting of 26 bronchial carcinoids, 9 SCLCs, and 1 large cell neuroendocrine carcinoma; 1 thymic carcinoid; 16 renal cell carcinomas; and 10 Ewing sarcomas.

Receptor Autoradiography. Cryostat sections (20-μ thick) of the tissue samples were processed for receptor autoradiography as described in detail previously for other peptide receptors (23). The radioligands used were [^{125}I-Tyr^4]bombesin, known to preferentially label GRP receptors (24), and the newly developed radioligand ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14), which has been reported to be an outstanding ligand identifying all four of the bombesin receptor subtypes (21, 22). For autoradiography, tissue sections were mounted on precleaned microscope slides and stored at −20°C for at least 3

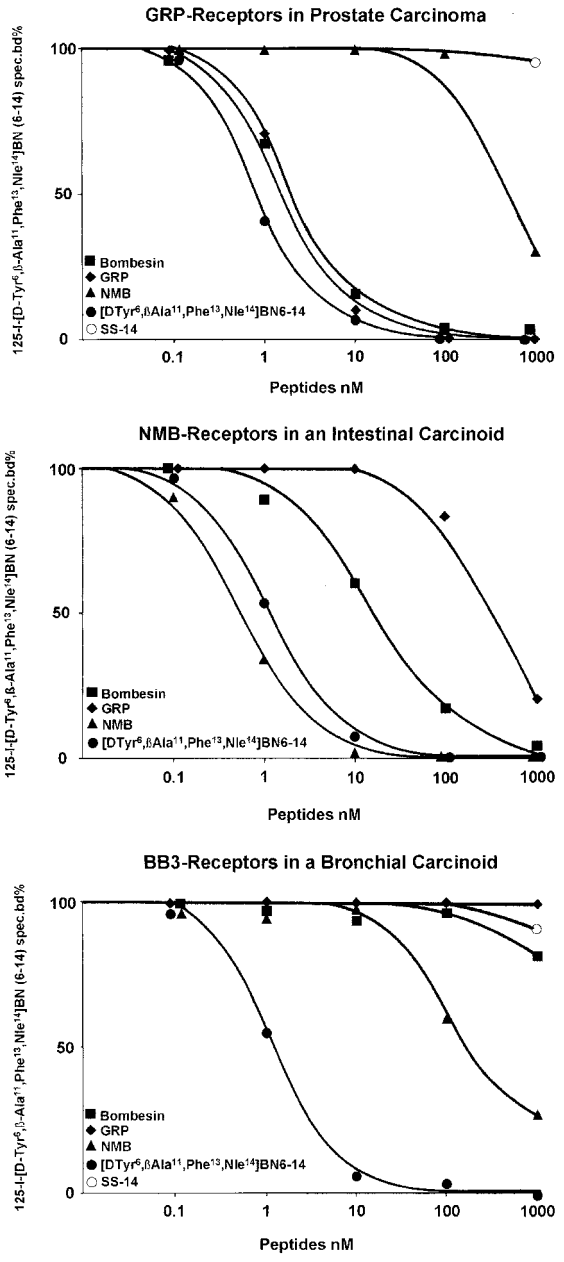

Fig. 1 Displacement curves in three different human tumors, using ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) as radioligand. *Top graph*, GRP receptor-expressing prostate carcinoma. *Middle graph*, NMB receptor-expressing intestinal carcinoid. *Bottom graph*, BB3 receptor-expressing bronchial carcinoid. Each graph is a representative example of one receptor-positive tumor. Successive tumor sections were incubated with 20 pM of the radioligand and increasing concentrations of unlabeled bombesin (■), GRP (◆), NMB (▲),[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14; ●), and 1000 nM of somatostatin (○). The GRP receptors in the tumor in the *top graph* are characterized by a high affinity for GRP, bombesin, and [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) but a very low affinity for NMB and no affinity for somatostatin. The NMB receptors in the tumor in the *middle graph* are characterized by a high affinity for NMB and [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14), moderate affinity for bombesin, and very low affinity for GRP. The BB3 receptors in the tumor in the *bottom graph* are characterized by high affinity for [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) but a low affinity for NMB and an even lower affinity for bombesin, GRP, and somatostatin.

Table 2 IC_{50} values for [D-Phe^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) (univ.BN), bombesin, GRP, and NMB measured in three groups of tumors expressing preferentially GRP-, NMB-, or BB3-receptors

Tumor group	IC_{50} (nM) mean ± SEM			
	univ.BN	Bombesin	GRP	NMB
Group 1: GRP receptor-expressing tumors (4 prostate and 3 breast carcinomas)	1.4 ± 0.2	5 ± 1.1	2.6 ± 0.5	366 ± 75
Group 2: BB3 receptor-expressing tumors (6 bronchial carcinoids)	2.1 ± 0.7	>10.000	>10.000	269 ± 45
Group 3: NMB receptor-expressing tumors (4 gut and 1 thymic carcinoids)	1.9 ± 0.3	19 ± 3.2	304 ± 43	0.67 ± 0.2

days to improve adhesion of tissue to the slide. The sections were then processed as described previously (13, 24). They were first preincubated in 10 mM HEPES (pH 7.4) for 5 min at room temperature. They were then incubated in 10 mM HEPES, 130 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 1 mM ethylenglycol-bis (β-aminoethylether)-*N*-*N*′-tetraacetic acid, 0.1% BSA, 100 μg/ml bacitracin (pH 7.4), and ~100 pM [^{125}I-Tyr^4]-bombesin (2000 Ci/mmol; Anawa, Wangen, Switzerland) or 20 pM ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14; 2000 Ci/mmol; Anawa) in the presence or absence of 1 μM bombesin or [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) for 1 h at room temperature. Because each of the bombesin receptor subtypes is characterized by a specific rank order of potencies of GRP, bombesin, NMB, and [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14), the use of these four peptides as unlabeled competitors in displacement experiments allows us to identify the receptor subtypes predominantly expressed in tissues (20, 21, 25). Therefore, sections from selected tumors, labeled with ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14), were incubated in the presence of increasing amounts of nonradioactive bombesin, GRP, NMB (Bachem, Bubendorf, Switzerland), and [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) to generate competitive inhibition curves. These displacement experiments showed that a 50 nM concentration of each of the four competitors {bombesin, GRP, NMB, and [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14)} given in successive tumor sections labeled with ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) was adequate to discriminate between the four receptor subtypes. This procedure was performed in all of the tumors in duplicate. After incubation, the sections were washed four times for 2 min each in 10 mM HEPES with 0.1% BSA (pH 7.4) at 4°C. Finally, the slides were rinsed twice for 5 s each at 4°C in distilled water. The slides were then dried at 4°C under a stream of cold air. The slides were placed in apposition to [^{3}H]hyperfilms (Amersham, Aylesbury, United Kingdom) and exposed for 7 days in X-ray cassettes. The autoradiograms were quantified using a computer-assisted image processing system, as described previously (13, 14, 23).

***In Situ* Hybridization Histochemistry.** BB3 receptor mRNA was identified in selected tumors with *in situ* hybridization histochemistry on cryostat sections as described in detail previously (26). BB3-specific oligonucleotides that do not span the very conserved transmembrane domains of the human BB3 receptor gene (27) were selected. The following oligonucleotide probes were used for *in situ* hybridization: BRS3-S5 acc aat tct tcc gaa cag cca tcc ttc tgc aag gta gtg agt tgc (nucleotides 313–357 from atg) and BRS3-S9 ggt cac act aat ttc aga cat ctg tat gct ccc agt gcc cgg gac (nucleotides 1111–1155 from atg). Sequence similarity searches were performed using all four of the oligonucleotide sequences against the GenBank database at National Center for Biotechnology Information, Bethesda, MD,[3] with the BlastN program to detect possible homologies to other sequences (28). No strong homologies to human sequences were detected. The probes were synthesized and purified on a 20% polyacrylamide-8 M urea sequencing gel (Microsynth, Balgach, Switzerland). They were labeled at the 3′-end by using [α-^{32}P]dATP (>3000 Ci/mmol; NEN, Life Science Products, Boston, MA) and terminal deoxynucleotidyltransferase (Roche Diagnostics, Mannheim, Germany) to specific activities of 0.9–2.0 × 10^4 Ci/mmol. Control experiments were carried out as reported previously (26) to determine the specificity of the hybridization signal obtained.

RESULTS

Table 1 summarizes the results of the bombesin receptor subtype expression in 161 human tumors. From the 12 prostatic carcinomas tested, all expressed GRP receptors, whereas they were devoid of the other bombesin receptor subtypes. Similarly, 41 of 57 ductal breast carcinomas expressed GRP receptors, whereas no other subtype was found in significant amount. The results of the neuroendocrine gastroenteropancreatic tumors could be divided as follows: all 5 gastrinomas had preferentially GRP receptors, whereas 11 of 24 intestinal carcinoids preferentially expressed NMB receptors. Also 1 thymic and 1 bronchial carcinoid had preferentially NMB receptors. Interestingly, the neuroendocrine lung tumors much more frequently expressed BB3 receptors: 9 of 26 bronchial carcinoids and 1 of 1 large cell neuroendocrine carcinoma had preferentially BB3 receptors; furthermore, 7 of 9 SCLCs expressed bombesin receptors, 4 of them having BB3 receptors and 3 others having GRP receptors. Also renal cell carcinomas expressed bombesin receptors (8 of 16), either BB3 receptors alone (2 of 16), GRP receptors alone (4 of 16), or both subtypes together (2 of 16). Finally, 2 of the 10 tested Ewing sarcomas expressed BB3 receptors (Table 1).

The tumors expressing predominantly GRP receptors were all characterized in the experiments using ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) as a radioligand by a high affinity for GRP, bombesin, and [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14), but a very low affinity for NMB. An example of a displacement experiment with such a pattern of rank order of potency is shown in Fig. 1 in a GRP

[3] Internet address: http://www.ncbi.nlm.nih.gov/.

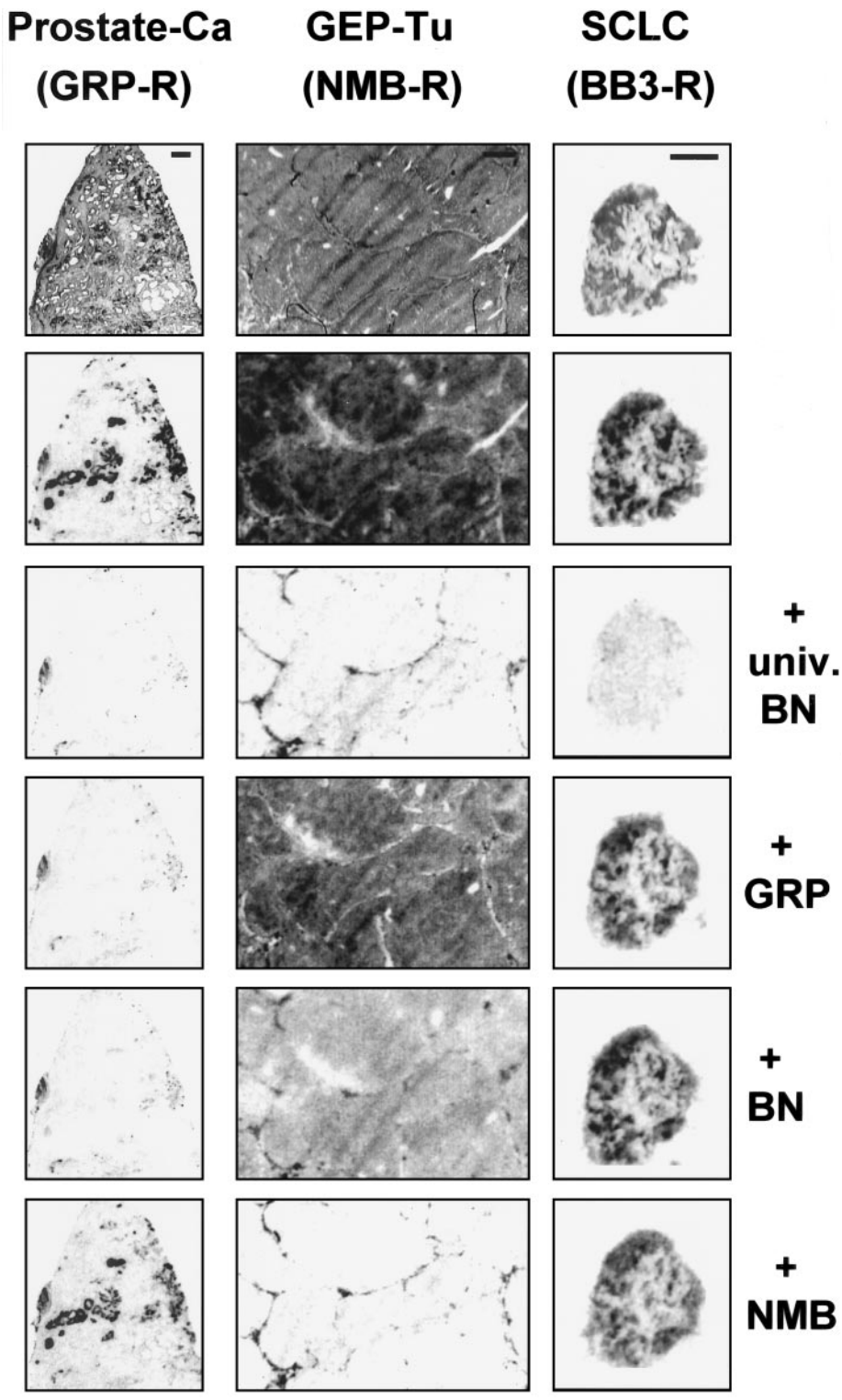

Fig. 2 Autoradiographic evidence of bombesin receptor subtypes in three different cancers (*left*, GRP receptor-expressing prostate carcinoma; *middle*, NMB receptor-expressing intestinal carcinoid; *right*, BB3-expressing SCLC) using ^{125}I-[D-Tyr^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]-bombesin(6–14) as radioligand with selective competitors. *Row 1*, H&E-stained sections; *bars*, 1 mm. *Row 2*, autoradiograms showing total binding of ^{125}I-[D-Tyr^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14). All three tumors are strongly labeled. *Row 3*, autoradiograms showing nonspecific binding of ^{125}I-[D-Tyr^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) {in presence of 50 nM [D-Tyr^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) = universal bombesin (*univ. BN*)}. The radioligand is displaced in all three tumors. *Row 4*, autoradiograms showing ^{125}I-[D-Tyr^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) binding in presence of 50 nM GRP. The radioligand is displaced in the prostate carcinoma but not in the carcinoid or SCLC. *Row 5*, autoradiograms showing ^{125}I-[D-Tyr^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) binding in presence of 50 nM of bombesin (*BN*). The radioligand is displaced in the prostate carcinoma but not in the SCLC and only partly in the carcinoid. *Row 6*, autoradiograms showing ^{125}I-[D-Tyr^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) binding in presence of 50 nM NMB. The ligand is completely displaced in the carcinoid but not in the prostate carcinoma or in the SCLC.

receptor-expressing prostate carcinoma. Table 2 shows the IC_{50}s for the different ligands measured in a series of GRP receptor-expressing tumors. Conversely, the tumors with a predominant expression of NMB receptors were characterized by a very high affinity for NMB and for [D-Tyr^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14), a moderate affinity for bombesin, but a very low affinity for GRP. An example of a displacement curve using a NMB receptor-expressing ileal

carcinoid is shown in Fig. 1. Table 2 summarizes the IC_{50} values for the four peptides, as measured in several NMB receptor-expressing tumors. Furthermore, all of the tumors expressing predominantly BB3 receptors were characterized by a very high affinity for [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) only, whereas NMB had a low affinity, and GRP and bombesin an even lower affinity. An example of a displacement experiment in a BB3 receptor-expressing bronchial carcinoid is shown in Fig. 1. IC_{50} values calculated in six bronchial carcinoids are shown in Table 2 for the corresponding analogue peptides. In this study, we have not seen tumors having a pattern of rank order of potencies for peptides corresponding to BB4 receptors. Such a BB4 profile would retain a very high affinity for [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) but also a high affinity for NMB and bombesin, and a high to moderate affinity for GRP (25); a BB4-selective analogue is presently lacking that would allow us to assess more specifically the presence of BB4 receptors.

When comparing tumor labeling by [^{125}I-Tyr^4]-bombesin and by ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14), we observed that all of the tumors expressing predominantly GRP receptors were labeled with both tracers, whereas the tumors expressing predominantly BB3 receptors were not labeled by ^{125}I-[Tyr^4]-bombesin but were labeled by ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14). The NMB receptor-expressing tumors were labeled with both ligands, although the labeling with ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) was more intense, as expected from the rank order of potencies of the two ligands.

Fig. 2 is an autoradiographic illustration of the receptor status in three different tumors predominantly expressing GRP receptors, NMB receptors, or BB3 receptors. The GRP receptor-expressing prostate carcinoma shows a very strong binding in the tumor area of ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14), which is completely displaced by 50 nM of bombesin, GRP, or [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14), whereas it is virtually not displaced by 50 nM of NMB. Conversely, the NMB receptor-expressing intestinal carcinoid is strongly labeled by ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14), and it is fully displaced by 50 nM NMB or 50 nM of the universal bombesin ligand [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14); moreover, it is displaced to a large extent by 50 nM bombesin but only poorly by 50 nM GRP. Finally, the BB3 receptor-expressing SCLC is also strongly labeled by ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) but is fully displaced by 50 nM of unlabeled [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}] bombesin(6–14) only and not by 50 nM bombesin, GRP, or NMB.

Table 3 reports the mean density for the various bombesin receptor subtypes identified in several types of tumors. Prostate carcinomas and gastrinomas are usually characterized by a very high density of GRP receptors, whereas SCLCs have only a low density. Ileal carcinoids express a high density of NMB receptors. Neuroendocrine lung tumors have a high density of BB3 receptors, especially the bronchial carcinoids, whereas renal cell cancers and Ewing sarcomas have a low density only.

We have identified the presence of BB3 mRNA by *in situ* hybridization in eight tumors shown to have BB3 proteins in binding experiments; those were five bronchial carcinoids, two SCLCs, and one large cell neuroendocrine lung cancer. Fig. 3 is an example of a bronchial carcinoid containing abundant BB3 mRNA detected with *in situ* hybridization using the BRS3-S5 probe. This tumor was also strongly BB3 receptor-positive on receptor autoradiography.

Table 3 Density of bombesin receptor subtypes in various cancers

Bombesin receptor subtype[a]	Tumor type	Receptor density (dmp/mg tissue)[b]	*n*
GRP-R	Prostate carcinomas	2726 ± 164	12
	Gastrinomas	4268 ± 833	5
	SCLC	403 ± 218	3
NMB-R	Intestinal carcinoids	1313 ± 57	11
	Thymic carcinoid	583	1
BB3-R	Bronchial carcinoids	2230 ± 385	9
	SCLC	1096 ± 581	3
	LCNEC	1405	1
	Renal cell cancers	353 ± 78	3
	Ewing sarcomas	555, 424	2

[a] For quantification purposes, tumors expressing preferentially one bombesin receptor subtype were chosen.
[b] Mean ± SE was calculated for those tumors with $n \geq 3$.

DISCUSSION

This study shows that selected human tumors can express bombesin receptor subtypes other than GRP receptors. In particular, it is the first time that BB3 and NMB receptor proteins are identified in primary human tumors. Of considerable interest is the preferential expression of BB3 receptors in neuroendocrine tumors of the lung, whereas NMB receptors are predominantly found in carcinoids of the gastrointestinal tract.

The mRNAs of the various bombesin receptor subtypes have been detected previously in several human tumors, in particular mRNA for GRP receptors in prostate cancers, ovarian cancers, renal cancers, and colorectal cancers (11, 15, 29, 30), whereas mRNAs for NMB or BB3 were found less frequently or were even absent in these tumors (11, 29, 30). The GRP receptor proteins have also been frequently found overexpressed in tumors such as prostate cancers, breast cancers, renal cell carcinomas, and lung cancers (12–15, 31). However, the BB3 receptor proteins have never been detected in primary human tumors before. The NMB receptor proteins have only been identified in tumor cell lines; Moody *et al.* (32) have reported recently that functional NMB receptor proteins were present on several SCLC cell lines. The present study identifies NMB receptors in primary human cancers, predominantly in carcinoids originating from the gastrointestinal tract. We also show that there is a preferential expression of BB3 receptors in primary lung tumors having a neuroendocrine origin, namely bronchial carcinoids, SCLCs, and a large cell neuroendocrine carcinoma. These data suggest that carcinoids of bronchial and gut origin may be distinguished on the basis of their bombesin receptor subtypes. Because various types of neuroendocrine lung tumors (carcinoids, SCLCs, and large cell neuroendocrine carcinoma) can express BB3, it is conceivable that the neuroendocrine cells from which these tumors originate may express BB3 receptors. Evidence that BB3 receptors can be expressed in certain human neuroendocrine cells has been given recently, with the finding of BB3 receptors in human pancreatic islets (22). Furthermore,

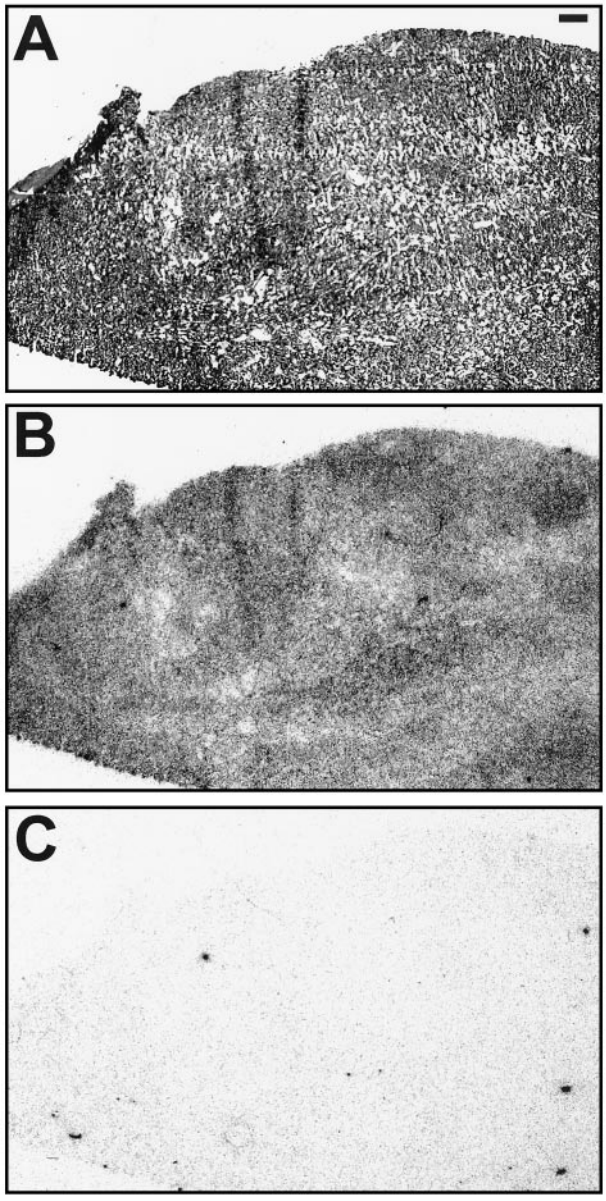

Fig. 3 *In situ* hybridization of BB3 receptor mRNA in a bronchial carcinoid. *A,* H&E stained section; *bar,* 1 mm. *B,* autoradiogram showing BB3 receptor mRNA. *C,* control: autoradiogram showing BB3 receptor mRNA hybridization blocked by 20-fold excess of unlabeled oligonucleotide.

BB3 receptors are also found in low density in two other distinct neoplastic entities, namely in some of the renal cell carcinomas and in a few Ewing sarcomas.

The method used in the present study, based on the binding of a universal radioligand to all bombesin receptor subtypes and its displacement by subtype-selective competitors, allows us to clearly identify the receptor subtypes that are predominantly expressed in a tumor; however, the method will not identify small amounts of additional bombesin receptor subtypes present in a low proportion in the same tumor, because those would be masked by the strong binding to the predominantly expressed receptor type (33). Moreover, one should be aware that measuring bombesin receptors with binding techniques is notoriously problematic in certain tumors (15) because of the presence of high amounts of membrane-bound endopeptidases that are able to rapidly degrade bombesin (34). This was reported in particular for gastrointestinal tumors (34). Therefore, it cannot be completely excluded that the reported incidence of GRP, NMB, and BB3 receptor-expressing tumors is somewhat underestimated because of a false-negative receptor status in some of the tested tumors (15). Nevertheless, the present data confirm the high incidence and high density of GRP receptors in prostate and breast carcinomas reported previously (13, 14, 31) and indicate that only a very low density, if any, of the other bombesin receptor subtypes may be present in theses tumors. This is in accordance with a recent mRNA study by Sun *et al.* (11) performed in prostate cancers showing that the majority of the tumors had GRP receptor mRNA, whereas only very few had mRNA for NMB receptors or for BB3 receptors. We can also confirm the presence of GRP receptors in renal cell carcinomas, reported recently by Pansky *et al.* (15) with binding assays and PCR methods. We could not only detect GRP receptors in some of the tested renal cell carcinomas but identify the presence of BB3 receptors in some of them as well.

Little information is available about the role and function of the NMB and BB3 receptors in cancer (35); however, a recent report by Weber *et al.* (36) gives first indications that the BB3 activation may cause cancer cell proliferation, since the analogue [D-Phe^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) caused increased nuclear oncogene expression, mitogen-activated protein kinase phosphorylation, and ELK.1 activation in lung cancer cells. The presence of these receptor subtypes in selected human tumors may allow us to investigate in more detail the function of these receptors in these tumors to identify new applications of bombesin analogues in the clinic.

The discovery of BB3 and NMB receptor expression in human tumors may suggest several clinical applications. For instance, the development of BB3- or NMB-selective analogues suitable for clinical applications may be of interest to visualize and treat selected tumors. A BB3-selective analogue has been recently developed by Mantey *et al.* (37); it should be possible to attach a chelator (diethylenetriaminepentaacetic acid or 1,4,7,10-tetra-azacylododecane *N,N′,N″,N‴*-tetraacetic acid) to this analogue to have a radiopharmaceutical suitable for *in vivo* clinical investigations. Clinical applications of such compounds may include tumor targeting for the visualization of receptor-positive tumors and their metastases as well as for the radiotherapy of such tumors (38–40). Linear bombesin analogues are known to be susceptible to degradation by proteases including the BB3-selective analogue [D-Phe^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) that has a half-life of $<$1 h. It is presently unclear to which extent this proteolytic effect may represent a problem for targeting. However, it has been shown that tumors are usually labeled within a few minutes after radiopeptide tracer injection (41), suggesting that most of the radioligand uptake occurs during the first passage, *i.e.,* before most of it is degraded. Highly encouraging in this regard is the successful visualization of GRP receptor-expressing prostate and breast cancers reported recently by Van de Wiele *et al.* (40) with a

^{99m}Tc-labeled linear GRP analogue. Moreover, the distinct bombesin receptor subtype expression in bronchial and gut carcinoids may permit us to distinguish the bronchial from the gastrointestinal origin of liver metastases of a carcinoid with unknown primary location, by identifying its BB3 or NMB receptor expression, respectively, *in vitro* in needle biopsies of liver metastases. An additional clinical application could be the use of subtype-selective analogues linked to cytotoxic drugs, in analogy to the bombesin analogues linked to doxorubicin developed previously by Nagy *et al.* (42).

ACKNOWLEDGMENTS

We thank Dr. A. Srinivasan (Mallinckrodt, Inc., St. Louis, MO) for providing [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14).

REFERENCES

1. Reubi, J. C. Neuropeptide receptors in health and disease: the molecular basis for *in vivo* imaging. J. Nucl. Med., *36:* 1825–1835, 1995.

2. Lamberts, S. W. J., Krenning, E. P., and Reubi, J. C. The role of somatostatin and its analogs in the diagnosis and treatment of tumors. Endocr. Rev., *12:* 450–482, 1991.

3. Krenning, E. P., Kwekkeboom, D. J., Pauwels, S., Kvols, L. K., and Reubi, J. C. Somatostatin Receptor Scintigraphy, pp. 1–50. New York: Raven Press, 1995.

4. Otte, A., Mueller-Brand, J., Dellas, S., Nitzsche, E. U., Herrmann, R., and Maecke, H. R. Yttrium-90-labelled somatostatin-analogue for cancer treatment. Lancet, *351:* 417–418, 1998.

5. Behr, T. M., Jenner, N., Radetzky, S., Béhé, M., Gratz, S., Yücekent, S., Raue, F., and Becker, W. Targeting of cholecystokinin-B/gastrin receptors *in vivo*: preclinical and initial clinical evaluation of the diagnostic and therapeutic potential of radiolabelled gastrin. Eur. J. Nucl. Med., *25:* 424–430, 1998.

6. Virgolini, I., Raderer, M., Kurtaran, A., Angelberger, P., Banyai, S., Yang, Q., Li, S., Banyai, M., Pidlich, J., Niederle, B., Scheithauer, W., and Valent, P. Vasoactive intestinal peptide-receptor imaging for the localization of intestinal adenocarcinomas and endocrine tumors. N. Engl. J. Med., *331:* 1116–1121, 1994.

7. Cuttitta, F., Carney, D. N., Mulshine, J., Moody, T. W., Fedorko, J., Fischler, A., and Minna, J. D. Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. Nature (Lond.), *316:* 823–825, 1985.

8. Kroog, G. S., Jensen, R. T., and Battey, J. F. Mammalian bombesin receptors. Med. Res. Rev., *15:* 389–417, 1995.

9. Moody, T. W., Pert, C. B., Gazdar, A. F., and Carney, D. N. High levels of intracellular bombesin characterize human small cell lung carcinoma. Science (Wash. DC), *214:* 1246–1248, 1981.

10. Moody, T. W., Carney, D. N., Cuttitta, F., Quattrocchi, K., and Minna, J. D. High affinity receptors for bombesin/GRP-like peptides on human small cell lung cancer. Life Sci., *37:* 105–113, 1985.

11. Sun, B., Halmos, G., Schally, A. V., Wang, X., and Martinez, M. Presence of receptors for bombesin/gastrin-releasing peptide and mRNA for three receptor subtypes in human prostate cancers. Prostate, *42:* 295–303, 2000.

12. Moody, T. W., Zia, F., Venugopal, R., Fagarasan, M., Oie, H., and Hu, V. GRP receptors are present in non-small cell lung cancer cells. J. Cell. Biochem., *24* (Suppl.)*:* 247–256, 1996.

13. Markwalder, R., and Reubi, J. C. Gastrin-releasing peptide receptors in the human prostate: relation to neoplastic transformation. Cancer Res., *59:* 1152–1159, 1999.

14. Gugger, M., and Reubi, J. C. GRP receptors in nonneoplastic and neoplastic human breast. Am. J. Pathol., *155:* 2067–2076, 1999.

15. Pansky, A., de Weerth, A., Fasler-Kan, E. V., Boulay, J. L., Schulz, M., Ketterer, S., Selck, C., Beglinger, C., von Schrenck, T., and Hildebrand, P. Gastrin releasing peptide-preferring bombesin receptors mediate growth of human renal cell carcinoma. J. Am. Soc. Nephrol., *11:* 1409–1418, 2000.

16. Spindel, E. R., Giladi, E., Brehm, P., Goodman, R. H., and Segerson, T. P. Cloning and functional characterization of a complementary DNA encoding the murine fibroblast bombesin/gastrin-releasing peptide receptor. Mol. Endocrinol., *4:* 1956–1963, 1990.

17. Wada, E., Way, J., Shapira, H., Kusano, K., Lebacq-Verheyden, A. M., Coy, D., Jensen, R., and Battey, J. cDNA cloning, characterization, and brain region-specific expression of a neuromedin-B preferring bombesin receptor. Neuron, *6:* 421–430, 1991.

18. Fathi, Z., Corjay, M. H., Shapira, H., Wada, E., Benya, R., Jensen, R., Viallet, J., Sausville, E. A., and Battey, J. F. BRS-3: novel bombesin receptor subtype selectively expressed in testis and lung carcinoma cells. J. Biol. Chem., *268:* 5979–5984, 1993.

19. Nagalla, S. R., Barry, B. J., Creswick, K. C., Eden, P., Taylor, J. T., and Spindel, E. R. Cloning of a receptor for amphibian [Phe13]bombesin distinct from the receptor for gastrin-releasing peptide: identification of a fourth bombesin receptor subtype (BB4). Proc. Natl. Acad. Sci. USA, *92:* 6205–6209, 1995.

20. Mantey, S. A., Weber, H. C., Sainz, E., Akeson, M., Ryan, R. R., Pradhan, T. K., Searles, R. P., Spindel, E. R., Battey, J. F., Coy, D. H., and Jensen, R. T. Discovery of a high affinity radioligand for the human orphan receptor, bombesin receptor subtype 3, which demonstrates that it has a unique pharmacology compared with other mammalian bombesin receptors. J. Biol. Chem., *272:* 26062–26071, 1997.

21. Pradhan, T. K., Katsuno, T., Taylor, J. E., Kim, S. H., Ryan, R. R., Mantey, S. A., Donohue, P. J., Weber, H. C., Sainz, E., Battey, J. F., Coy, D. H., and Jensen, R. T. Identification of a unique ligand which has high affinity for all four bombesin receptor subtypes. Eur. J. Pharmacol., *343:* 275–287, 1998.

22. Fleischmann, A., Läderach, U., Friess, H., Buechler, M., and Reubi, J. C. Bombesin receptors in distinct tissue compartments of human pancreatic diseases. Lab. Investig., *80:* 1807–1817, 2000.

23. Reubi, J. C., Kvols, L. K., Waser, B., Nagorney, D., Heitz, P. U., Charboneau, J. W., Reading, C. C., and Moertel, C. Detection of somatostatin receptors in surgical and percutaneous needle biopsy samples of carcinoids and islet cell carcinomas. Cancer Res., *50:* 5969–5977, 1990.

24. Vigna, S. R., Mantyh, C. R., Giraud, A. S., Soll, A. H., Walsh, J. H., and Mantyh, P. W. Localization of specific binding sites for bombesin in the canine gastrointestinal tract. Gastroenterology, *93:* 1287–1295, 1987.

25. Katsuno, T., Pradhan, T. K., Ryan, R. R., Mantey, S. A., Hou, W., Donohue, P. J., Akeson, M. A., Spindel, E. R., Battey, J. F., Coy, D. H., and Jensen, R. T. Pharmacology and cell biology of the bombesin receptor subtype 4 (BB_4-R). Biochemistry, *38:* 7307–7320, 1999.

26. Reubi, J. C., Schaer, J. C., Waser, B., and Mengod, G. Expression and localization of somatostatin receptor SSTR1, SSTR2, and SSTR3 mRNAs in primary human tumors using *in situ* hybridization. Cancer Res., *54:* 3455–3459, 1994.

27. Gorbulev, V., Akhundova, A., Grzeschik, K. H., and Fahrenholz, F. Organization and chromosomal localization of the gene for the human bombesin receptor subtype expressed in pregnant uterus. FEBS Lett., *340:* 260–264, 1994.

28. Altschul, S. F., Madden, T. L., Schaumloffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. Gapped Blast and PSI Blast: a new generation of protein database search programs. Nucleic Acids Res., *25:* 3389–3402, 1997.

29. Sun, B., Schally, A. V., and Halmos, G. The presence of receptors for bombesin/GRP and mRNA for three receptor subtypes in human ovarian epithelial cancers. Regul. Pept., *90:* 77–84, 2000.

30. Chave, H. S., Gough, A. C., Palmer, K., Preston, S. R., and Primrose, J. N. Bombesin family receptor and *ligand* gene expression in human colorectal cancer and normal mucosa. Br. J. Cancer, *82:* 124–130, 2000.

31. Halmos, G., Wittliff, J. L., and Schally, A. V. Characterization of bombesin/gastrin-releasing peptide receptors in human breast cancer

and their relationship to steroid receptor expression. Cancer Res., *55:* 280–287, 1995.

32. Moody, T. W., Staley, J., Zia, F., Coy, D. H., and Jensen, R. T. Neuromedin B receptors are present on small cell lung cancer cells. J. Pharmacol. Exp. Ther., *263:* 311–317, 1992.

33. Reubi, J. C., Waser, B., Schaer, J. C., and Laissue, J. A. Somatostatin receptor sst1-sst5 expression in normal and neoplastic human tissues using receptor autoradiography with subtype-selective ligands. Eur. J. Nucl. Med., *28:* 836–846, 2001.

34. Bunnett, N. W., Wu, V., Sternini, C., Klinger, J., Shimomaya, J., Payan, D., Kobayashi, R., and Walsh, J. H. Distribution and abundance of neutral endopeptidase (EC 3.4.24.11) in the alimentary tract of the rat. Am. J. Physiol., *264:* G497–G508, 1993.

35. Ryan, R. R., Weber, H. C., Hou, W., Sainz, E., Mantey, S. A., Battey, J. F., Coy, D. H., and Jensen, R. T. Ability of various bombesin receptor agonists and antagonists to alter intracellular signaling of the human orphan receptor BRS-3. J. Biol. Chem., *273:* 13613–13624, 1998.

36. Weber, H. C., Walters, J., Leyton, J., Casibang, M., Purdom, S., Jensen, R. T., Coy, D. H., Ellis, C., Clark, G., and Moody, T. W. A bombesin receptor subtype-3 peptide increases nuclear oncogene expression in a MEK-1 dependent manner in human lung cancer cells. Eur. J. Pharmacol., *412:* 13–20, 2001.

37. Mantey, S. A., Coy, D. H., Pradhan, T. K., Igarashi, H., Rizo, I. M., Shen, L., Hou, W., Hocart, S. J., and Jensen, R. T. Rational design of a peptide agonist that interacts selectively with the orphan receptor, bombesin receptor subtype 3. J. Biol. Chem., *276:* 9219–9229, 2001.

38. Breeman, W. A. P., Hofland, L. J., de Jong, M., Bernard, B. F., Srinivasan, A., Kwekkeboom, D. J., Visser, T. J., and Krenning, E. P. Evaluation of radiolabelled bombesin analogues for receptor-targeted scintigraphy and radiotherapy. Int. J. Cancer, *81:* 658–665, 1999.

39. Safavy, A., Khazaeli, M. B., Qin, H., and Buchsbaum, D. J. Synthesis of bombesin analogues for radiolabeling with rhenium-188. Cancer (Phila.), *80* (Suppl.)*:* 2354–2359, 1997.

40. Van de Wiele, C., Dumont, F., Vanden Broecke, R., Oosterlinck, W., Cocquyt, V., Serreyn, R., Peers, S., Thornback, J., Slegers, G., and Dierckx, R. A. Technetium-99m RP527, a GRP analogue for visualisation of GRP receptor-expressing malignancies: a feasibility study. Eur. J. Nucl. Med., *27:* 1694–1699, 2000.

41. Krenning, E. P., Kwekkeboom, D. J., Bakker, W. H., Breeman, W. A. P., Kooij, P. P. M., Oei, H. Y., van Hagen, M., Postema, P. T. E., de Jong, M., Reubi, J. C., Visser, T. J., Reijs, A. E. M., Hofland, L. J., Koper, J. W., and Lamberts, S. W. J. Somatostatin receptor scintigraphy with [^{111}In-DTPA-D-Phe^{1}]- and [^{123}I-Tyr^{3}]-octreotide: the Rotterdam experience with more than 1000 patients. Eur. J. Nucl. Med., *20:* 716–731, 1993.

42. Nagy, A., Armatis, P., Cai, R., Szepeshazi, K., Halmos, G., and Schally, A. V. Design, synthesis, and *in vitro* evaluation of cytotoxic analogs of bombesin-like peptides containing doxorubicin or its intensely potent derivative, 2-pyrrolinodoxorubicin. Proc. Natl. Acad. Sci. USA, *94:* 652–656, 1997.

Clinical Cancer Research

Bombesin Receptor Subtypes in Human Cancers: Detection with the Universal Radioligand 125I-[d-TYR6, β-ALA11, PHE13, NLE14] Bombesin(6–14)

Jean Claude Reubi, Sandra Wenger, Jacqueline Schmuckli-Maurer, et al.

Clin Cancer Res 2002;8:1139-1146.