Gastrin-releasing peptide receptor as a molecular target in experimental anticancer therapy

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Received 18 September 2006; revised 18 January 2007; accepted 19 January 2007

Over the last two decades, several lines of experimental evidence have suggested that the gastrin-releasing peptide (GRP) may act as a growth factor in many types of cancer. For that reason, gastrin-releasing peptide receptor (GRPR) antagonists have been developed as anticancer candidate compounds, exhibiting impressive antitumoral activity both in vitro and in vivo in various murine and human tumors. In this article, the GRPR cell surface expression profile in human malignancies is reviewed aiming at the identification of potential tumor types for future clinical trials with GRP analogues and antagonists. In this review, we summarize the current literature regarding the GRPR status in human malignancies. Source data were obtained by searching all published material available through Medline, PubMed and relevant articles from 1971 to 2006. The data available demonstrated a high expression of GRPRs in a large spectrum of human cancers, demonstrating the potential relevance of this intracellular signaling pathway in various human tumor models. The GRPR may be an interesting target for therapeutic intervention in human malignancies, as carriers for cytotoxins, immunotoxins or radioactive compounds, being also a potential tool for tumor detection.

Key words: bombesin-like peptides, gastrin-releasing peptide, gastrin-releasing peptide receptor

introduction

Growth factor receptors are involved in all steps of tumor progression, enhancing angiogenesis, local invasion and distant metastases. Furthermore, the overexpression of growth factor receptors on the cell surface of malignant cells might be associated with a more aggressive behavior and a poor prognosis [1]. For these reasons, tumor-related growth factor receptors can be considered potential targets for therapeutic interventions [2]. The interference with epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF)-dependent cell surface signaling are examples of experimental therapeutic approaches that showed evidence of antitumour activity in patients with various types of malignancies, such as breast, colorectal and renal cancer [3, 4].

Over the last two decades, several lines of experimental evidence have suggested that the gastrin-releasing peptide (GRP) and other bombesin-like peptides (BLPs) may act as growth factors in many types of cancer [5–7]. For that reason, GRP-prefering receptor [gastrin-releasing peptide receptor (GRPR)] antagonists have been developed as anticancer candidate compounds, exhibiting impressive antitumor activity both in vitro and in vivo in various murine and human tumors [8–10]. Notably, the inhibition of GRPR was demonstrated to interfere with other relevant growth factor pathways such as the EGF- and VEGF-dependent signaling pathways [11, 12]. RC-3095 is a GRPR antagonist of this series, which showed a very favorable toxicity profile and antitumor activity in preclinical models. RC-3095 is currently undergoing clinical trials in our institution. Recently, a phase I trial with this compound was carried out in patients with advanced malignancies, confirming its favorable toxicity [13]. In this context, a review of GRPR cell surface profile in human malignancies is an issue of critical relevance, recognizing tumor types expressing sufficient amounts of these receptors for potential clinical applications of GRP analogues and antagonists.

bombesin-like peptides

Bombesin is a14-amino acid peptide first isolated from frog skin [14]. BLPs were identified in mammalian and the highest levels were observed in pulmonary neuroendocrine cells [15]. One major pulmonary BLP was determined to be GRP, a 27-amino acid homolog of frog bombesin. GRP and bombesin share a highly conserved 7-amino-acid COOH-terminal sequence, which is required for immunogenicity and for high-affinity binding to the GRP-prefering receptor [16]. Consequently, GRP and bombesin have essentially identical physiologic actions.
Three mammalian BLP receptors have been cloned [17, 18]: GRPR [19, 20], neurexin B receptors (NMBR) [21] and the orphan bombesin receptor subtype 3 (BRS-3) [22, 23]. These receptors are coupled to G-protein via their intracellular domain and, thus, belong to the G-protein receptor superfamily. Through the binding of GRPRs multiple cellular signal transduction pathways are activated, including mitogen-activated protein kinase, protein kinase C, tyrosine phosphorylation of focal adhesion kinase, paxillin, p130<sup>SH2</sup> and calcium mobilization, resulting in cell proliferation and growth [24, 25].

GRP plays an important role in regulating smooth muscle contraction, releasing hormones in the gastrointestinal (GI) tract, secreting pancreatic enzymes and serving as a neurotransmitter in the central nervous system [26]. These peptides are also thought to have pathogenic effects. Using animal models, Roessler et al. have conducted several preclinical studies investigating the role of BLPs and the GRPR in regulating brain function and discussing the implications of the GRPR for neurological and psychiatric disorders [27–36].

The GRPR system seems to have a functional interaction with other neurotransmitter and receptor systems (GABA, dopamine and glucocorticoid receptors) implicated in the pathogenesis of Parkinson’s disease, schizophrenia and in mediating anxiety and stress responses. In addition, recent evidence indicates that drugs acting at the GRPR might be potential tools for the treatment of memory dysfunction associated with Alzheimer’s disease [27]. GRP has also been implicated in inflammatory processes. Dal Pizzol et al. [37] have recently reported that a selective GRPR antagonist attenuates the release of proinflammatory cytokines in vitro and in vivo and improves survival in ‘established’ sepsis. These findings are consistent with the involvement of a new inflammatory pathway regulated by BLPs and the GRPR relevant to the development of inflammation and sepsis.

BLPs in cancer

BLPs have been studied in several tumor models as well as in human cancer. They were shown to be mitogenic, causing growth of Swiss 3T3 murine fibroblasts [38] as well as normal bronchial epithelial cells in culture [39], and have been implicated in the development of diseases of the lung [16]. Small-cell lung carcinoma cells produce and secrete GRP and express high-affinity receptors for BLPs, thus establishing an autocrine growth loop, involved in the abnormal growth of these tumors [5]. Bombesin stimulates early signal transduction mechanisms in human breast cancer cell line MCF-7 [40]. The trophic effect on cellular growth has been established in the normal intestinal mucosa [6] and in pancreas [41], as well as in cancers of the lung [42], stomach [43, 44], colon [7], breast [45] and prostate [46, 47].

GRPR expression in human tumors

GRPR expression has been evaluated in a variety of human malignancies. Through competitive RT-PCR experiments, some authors demonstrated the widespread but variable expression of GRPR in messenger RNA (mRNA) in fresh-frozen specimens of prostatic carcinoma and benign prostatic hypertrophy [48]. To address which cells in the prostate express the GRPR, they used in situ hybridization methods to stain selectively GRPR mRNA. The highest levels were found in basal and luminal epithelial cells in both histologically normal and cancerous glands within sections of normal and diseased tissue. GRPR mRNA staining in cancerous tissue ranged widely from very intense to not detectable (~30% of the cases), while normal tissue consistently displayed a low message staining.

Other researchers have also found overexpression of GRPR in invasive prostatic carcinomas and in the earliest phase of neoplastic transformation of the prostate [49]. The receptors were localized in 100% of carcinomas, in 100% of intraepithelial lesions and in 57% of bone metastases, with higher density in well-differentiated tumors. Non-neoplastic prostatic tissue only rarely expressed a measurable amount of GRPRs, so the authors concluded that these receptors may be markers for early molecular events in prostate carcinogenesis and useful in differentiating hyperplasia from neoplasia.

Another study evaluated 80 specimens of primary prostate cancer by receptor binding assays, and 68% showed high affinity for GRPR [50]. When the samples were analyzed by RT-PCR, 91% expressed GRPR mRNA. No correlation was observed between receptor expression and patient’s age or pathological data. Reubi et al. [51] investigated 161 human malignancies using in vitro receptor autoradiography with radioligand, and there was a high incidence of GRPR expression in all of the 12 samples of prostate cancer. This study also evaluated other tumors. GRP receptors were found in five of five gastrinomas, three of nine small-cell lung carcinomas, six of 16 renal cell carcinomas (RCCs) and in 41 of 57 breast cancers.

The first report of GRPR expression in breast cancer tissues showed 33 positive cases in 100 carcinomas [52]. Subsequently, Gugger and Reubi [53] evaluated GRPR expression in human non-neoplastic and neoplastic breast tissues and in axillary lymph node metastases through binding assays. GRPRs were detected, often in high density, in non-neoplastic epithelial mammary cells in 29 of 46 invasive ductal carcinomas, in 11 of 17 ductal carcinomas in situ, in one of four invasive lobular carcinomas, in one of two lobular carcinomas in situ and in one mucinous and one tubular carcinoma. A heterogeneous GRPR distribution was found in 61% of the invasive carcinomas and 63% of the carcinomas in situ. The 33 lymph node metastases from those primary carcinomas expressing GRPR were all positive, whereas surrounding lymphoreticular tissue was negative. GRPRs were also present in high density but with heterogeneous distribution in ducts and lobules from all available breast tissue samples. These data describe a ubiquitous GRPR expression in non-neoplastic human breast tissue, indicating a role of GRP in breast physiology, and a high percentage of GRPR-positive non-neoplastic breast tissues.

Schally et al. evaluated the presence of the three bombesin receptor subtypes in human ovarian epithelial cancers, through RT-PCR [54]. Of the 22 ovarian cancer specimens analyzed, 17 tumors expressed mRNA for GRPR, 19 showed NMBR mRNA and six revealed BRS-3 mRNA. Thus, ~64% of the specimens expressed mRNA for both GRPR and NMBC, and ~23% expressed all three subtypes. The expression of GRPR appeared to be greater in poorly differentiated carcinomas, and
a higher incidence of BRS-3 expression was found in samples with tumor stage IV.

GRP has been shown to stimulate the growth of normal pancreas in vivo and to modulate growth of pancreatic cancer in experimental models; therefore, Ehlers et al. [55] analyzed the expression of GRP and other GI peptide receptors in 26 human pancreatic adenocarcinomas. Only 8% of the pancreatic cancers expressed GRPR, similar to the results of 12% of expression found by Tang et al. [56] some years before. The authors postulate that GRP can stimulate the release of all GI hormones, except for secretin, as well as stimulating pancreatic and intestinal secretions. For this reason, the well-characterized in vivo effects of GRP on pancreatic growth may be secondary to indirect effects as a result of stimulation of other hormones, which then act directly on the pancreas.

Another study compared GRP expression in chronic pancreatitis (n = 23) and pancreatic carcinoma (n = 29) using in vitro receptor autoradiography on tissue sections [57]. GRPRs were identified in the pancreatic exocrine parenchyma in 17 of 20 cases of chronic pancreatitis. No measurable bombesin receptors were found in the tumor tissue of ductal pancreatic carcinomas, however, GRPRs were detected in a subset of peritumoral vessels in 65% of the neoplastic samples. Moreover, residual pancreatic islets in these tissues were shown to express the BRS-3 receptor subtype. These data demonstrate the presence of bombesin receptors in three distinct tissue compartments of the pancreas. The authors point that such a selective expression of bombesin receptor subtypes in pancreatic tissues may be of pathophysiological significance and represent the basis for potential diagnostic and therapeutic clinical applications for bombesin analogues, including GRPR scintigraphy to differentiate chronic pancreatitis from ductal pancreatic carcinoma.

Human colorectal cancer tissue and matched uninvolved mucosa from 21 patients were examined by radioligand displacement for the presence of GRP binding sites. There was no involvement of normal mucosa, whereas five cancers showed high affinity for GRPR [58]. Chave et al. [59] also evaluated the expression of BLPs and their receptor subtypes in normal and neoplastic colorectal tissues. The analysis was made by RT-PCR and in situ hybridization in cancer tissue and matched normal mucosa from 23 patients. NMBR expression was not detectable, as well as BRS-3. GRPR was present in all samples but with overall overexpression in the tumoral ones supporting the possibility that GRP may act as an autocrine growth factor in colorectal cancer.

Other researchers aimed to test whether GRPR expression was correlated with characteristics and usual prognostic factors in colorectal adenocarcinomas [60]. Again, GRPR mRNA was not detected in normal colonic epithelium, but a distinct signal was observed after PCR amplification in 93% of the tumors. Moreover, mRNA levels were significantly higher in poorly/moderately differentiated tumors and in those with lymphatic vessel invasion. Studies with immunohistochemistry also confirmed aberrant expression of GRPR in human colon cancers, which was absent in adjacent normal mucosa [61].

Pansky et al. [62] demonstrated the expression of GRPR in human RCC, but not in normal kidney tissue, using RT-PCR and autoradiography with radioligand. GRPR expression was also demonstrated in four human kidney carcinoma cell lines, especially in CAKI-2 cells. The effect of GRP agonists and/or antagonists on growth was investigated in vitro on these cells. Bombesin alone significantly stimulated growth of CAKI-2 cells, whereas a GRPR antagonist completely reversed this effect. These results indicated that malignant transformation of human kidney tissue into RCC is accompanied by novel expression of GRP receptors, and that BLPs might act as mitogens in these carcinomas.

Other researchers also evaluated the presence of GRPR in RCC and corresponding normal tissue using RT-PCR, immunohistochemistry and confocal laser scanning microscopy [63]. They aimed to analyze the effects of GRPR blockade in angiogenesis in RCC. Therefore, multicellular spheroids of the A498 RCC line were implanted into dorsal skin fold chambers of athymic nude mice, and neoangiogenesis was measured after blockade of GRPRs by the antagonist RC-3095. The results showed that GRPR expression was immunolocalized in tumor cells and microvessels. Implanted tumor cell spheroids and spheroid microvessels of the chamber also expressed GRPR, and spheroid neoangiogenesis was significantly inhibited by RC-3095 when given immediately after its implantation. VEGF secretion of A498 cells was not affected by GRP. The authors concluded that RCC angiogenesis is sensitive to GRPR blockade, so that GRPR may not only stimulate tumor cell proliferation, but also affect tumor microcirculation.

Approximately two-thirds of small-cell lung cancer (SCLC) tumors produce pro-gastrin-releasing peptide (pro-GRP), and it can be used as a specific marker for SCLC. Given the limited information available concerning expression of pro-GRP mRNA and protein, Uchida et al. [64] measured the levels of serum pro-GRP in individuals with SCLC and the expression of GRPR and pro-GRP in their neoplastic tissues. GRPR was detected in 30% of the samples. The results showed that pro-GRP mRNA transcripts and protein production could only be detected in tumor tissues recovered from individuals with high serum pro-GRP levels, evidencing a close relationship between the pro-GRP expression and the synthesis of pro-GRP protein which is eventually released into the blood.

GRPR and its ligand GRP expression was investigated in normal and neoplastic specimens of patients with squamous cell carcinoma of the head and neck (SCCHN), in control noncancer patients and in 14 SCCHN cell lines [65]. Tumoral and mucosal tissues, respectively, from SCCHN patients expressed six-fold and four-fold higher levels of GRPR than normal mucosa from noncancer patients (P < 0.001). The levels of GRPR expression in the tumor and normal adjacent epithelium of patients with SCCHN were correlated, indicating that increased GRPR expression is an early event in SCCHN formation. SCCHN cells expressed five-fold higher levels of GRPR mRNA than cultured normal mucosal epithelial cells. These results showed that GRP and GRPR appear to take part in an autocrine regulatory pathway in SCCHN.

Some authors evaluated GRP and GRPR expression in 33 samples of neuroblastomas and in four neuroblastoma cell lines (SK-N-SH, IMR-32, SH-SY5Y, LAN-1) [66]. Immunohistochemical analysis showed an increased GRPR expression in a higher percentage of more aggressive and undifferentiated tumors compared with those that were benign.
GRPR mRNA was confirmed in neuroblastoma cell lines and GRP treatment stimulated growth of all four cell lines. This effect was inhibited in SK-N-SH cells by pretreatment with GRP antibody. Sebesta et al. [67] analyzed 19 human resected neuroblastomas, intending to correlate GRP expression with other known predictors of poor prognosis. GRP and GRPR were qualitatively present in all of the specimens, regardless of the age of the patient or the stage of the disease, failing to assist in the biological interpretation of the tumors.

GRP expression was observed in human esophageal squamous cell carcinoma (ESCC) samples and in cell lines [68]. Using a RT-PCR method, significant overexpression of GRP was observed in 83% of the tumors and in all four ESCC cell lines tested. With in situ hybridization, GRP mRNA expression was detected in nine out of 21 (42.8%) samples with basal cell hyperplasia, five out of seven (71.4%) samples with dysplasia and 17 out of 24 (70.9%) ESCC samples. The authors supposed that GRP overexpression may play a role in ESCC development and growth.

To establish whether GRP and GRPR are expressed together in GI carcinoid tumors, Scott et al. [69] investigated 26 tumors from the stomach, small intestine, appendix, colon and rectum using immunohistochemistry. GRP was detected in 47% of the tumors and GRPR in 84%. GRPR but not GRP was strongly expressed in appendix and colonic tumors. Coexpression of both the ligand and receptor was seen in six of 19 cases. The results indicate a possible autocrine/paracrine pathway for GRP-stimulated cell proliferation in some of these neoplasms.

Epithelial cells lining the GI tract except in the gastric antrum do not normally express GRPR. Preston et al. first described binding sites for GRP in 50% of fresh resected gastric cancer. Only one of the 23 matched mucosal samples specifically bound bombesin and was the sample from a patient with Menetrier’s disease, a disorder of mucosal growth known to be premalignant [70]. Another study evaluated the incidence and quality of GRP expression in nonantral gastric adenocarcinomas and its impact on patient survival [71]. Among 20 tumors, 40% aberrantly expressed GRPR. Of these, 75% were found to be mutated. Contrary to expectations, expression of functional GRPR did not alter patient survival. Watson et al. [72] reviewed the emerging role of GRP, somatostatin and mainly gastrin in gastric carcinogenesis and reinforced the complex network between these three hormones.

Recently, normal and neoplastic human uterine tissues were investigated for their bombesin receptor status [73]. Normal uterine tissues expressed GRPR in the myometrium, in subsets of secretory endometrial glands and in subsets of endometrial blood vessels of the late proliferative and the secretory phase. Most leiomyomas (20 of 26) expressed GRPR but not the leiomyosarcomas. GRPR were also detected in 10 of 28 adenocarcinomas, one of one carcinosarcoma and in blood vessels surrounding the adenocarcinomas. These findings may be of physiological and pathophysiological significance. The expression of GRPR in glands and vessels during specific phases of the cycle indicates a timely precise physiological action in these targets; in certain uterine neoplasms, the GRPR overexpression may contribute to tumor development. Furthermore, these findings may have diagnostic relevance, as the expression of GRPR in leiomyomas may allow distinguishing them from receptor-negative leiomyosarcomas, as well as therapeutic relevance, for GRPR may be candidates for receptor targeting in vivo.

Table 1 summarizes published studies of GRPR expression in primary malignancies and the different techniques for detection, showing a high expression of GRPR in a large spectrum of human cancers.

<table>
<thead>
<tr>
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<th>No. positive</th>
<th>% Method</th>
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<td>12</td>
<td>100</td>
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<tr>
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<tr>
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GRPR, gastrin-releasing peptide receptor; IH, Immunohistochemistry; SCLC, small-cell lung cancer; GI, gastrointestinal.
octreotide analogues that bind to cancer cells overexpressing somatostatin receptors [74, 75]. Octreotide conjugated with \( ^{90}Y \) was tested in a phase II clinical trial in patients with progressive neuroendocrine tumors and showed 24% of overall response rate, including one patient who achieved complete response (complete disappearance of all evidence of disease with no appearance of new lesions for a minimum period of 4 weeks) [76].

GRP and somatostatin receptor share some similarities. Several GRP conjugates have been developed in the last years. Technetium-99m continues to be at the forefront of nuclear medicinal applications due to its wide range availability, ideal nuclear characteristics and well-established labeling chemistries. Technetium conjugates were found to have high affinity for cells with GRPRs, also presenting satisfactory in vivo uptake in experimental tumor models [77]. Pilot clinical studies of a new bombesin-derived, \( ^{99m} \text{Tc} \)-labeled pentadecapeptide indicated significant uptake by breast cancer and invaded lymph nodes, as well as by prostate cancer, small-cell lung carcinoma, gastroenteropancreatic tumors and others [78].

A phase I study in patients with prostate cancer evaluated technetium [Leu 13] bombesin as cancer-seeking agent. There were no relevant side-effects and the tumors were well visualized on early 1–2 min images with planar as well as tomographic imaging. Due to the high tumoral uptake, the authors suggested that this radiopharmaceutical may also serve as a radiotracer for radioisotope-guided surgery [79]. Most recently Nock et al. [80] reported some remarkable results for the potent bombesin analogue \( ^{99m} \text{Tc}-\text{Demobesin} \) in mice bearing human PC-3 xenografts. Tumor uptake values were well above all radiolabeled bombesin conjugates that have been previously reported.

Another conjugated bombesin compound for imaging and systemic radiotherapy, \( ^{177} \text{Lu-AMBA} \), is now in phase I clinical trials. In preclinical studies, this compound showed nanomolar affinity for GRPR binding, low retention or radioactivity in kidney and significantly prolonged the life span of PC-3 tumor-bearing mice and decreased tumor growth rate over controls [81]. Many other radiolabeled GRP-like peptides are currently being investigated, including (Tyr(0),Bpa(4))bombesin [82], H-D-Phe-Gln-Trp-Ala-Val-betaAla-His-Phe-Nle-NH2 [83], \( ^{99m} \text{Tc}-\text{RP527} \) [84] and (111)In-DTPA-Pro(1),Tyr(4)]bombesin [85] and are reviewed by other authors [86, 87].

The antibody-based approach is also achieving interesting results. A murine monoclonal antibody (mAb), 2A11, which binds GRP with high affinity has already entered phase I and II clinical trials in patients with lung cancer [88, 89]. The phase I trial enrolled 13 patients who received 12 doses of 2A11 antibody three times a week for 4 weeks at one of four dose levels. There were no toxic reactions, and none of the patients developed detectable human anti-mouse antibody; however, no objective antitumoral responses were observed. This trial established the recommended dose of 250 mg/m\(^2\) for the phase II trial. A pilot imaging evaluation using 111In conjugated 2A11 mAb was also carried out in the same patients and tumor uptake was noted in 11 of the 12 patients who received the injection [87]. The phase II trial carried out in patients with previously treated SCLC used 2A11 at 250 mg/m\(^2\) >1 h three times per week for 4 weeks. One of 12 assessable patients had complete resolution of radiographically detectable tumor lasting 4 months. Four patients (33%) had stable disease less than a 50% reduction and less than a 25% increase in the sum of the products of two perpendicular diameters of all measured lesions and the appearance of no new lesions. Again, no toxic reactions were observed [88].

Using a different approach, in which an anti-CD3 mAb, OKT3 was conjugated to a GRP antagonist (Antag2) and evaluated cytotoxicity against SCLC cells [90]. The bispecific molecule functioned as a cross-linker between T cells and SCLC cells. The results showed 40%–80% growth inhibition and apoptosis of SCLC cells by activated T cells in vitro and in vivo. The use of bispecific molecule-targeting GRPR and immune effector cells has been explored by Chen et al. [91] since 1995 when they reported a construction of an immunon conjugate of bombesin and a mAb directed to the high-affinity FcgammaRII (mAb 22). It was demonstrated that this bispecific molecule had capacity to redirect immune effector cells towards SCLC cells and elicit lysis of these target cells. The same group of investigators further added common chemotherapeutic agents (cisplatin, etoposide and paclitaxel) to the targeted immunotherapy, and the association significantly enhanced targeted SCLC cell killing [92].

Bombesin analogues have been linked to several cytotoxic agents for delivering drugs in cancer treatment. AN-215 is a combination of 2-pyrrolinodoxorubicin (AN-201) and bombesin that has been studied for almost a decade in many types of cancer. This compound was recently used as single and combined therapy in experimental ovarian cancers. Cytotoxic radical AN-201 was toxic and had no effect on tumor growth. In contrast, the toxicity of the conjugated peptide was low. Because ovarian cancers tend to acquire chemo-resistance, multidrug resistance was also tested and the authors found low or unmeasurable rates [93]. The same group of authors tested AN-215 in human breast cancer cell lines xenografted to nude mice. All five cell lines expressed GRPR and AN-215 significantly (\( P < 0.05 \)) inhibited tumor growth in all models. However, this effect was nullified by a blockade of GRPR with a bombesin antagonist [94]. Besides ovarian and breast cancers, a wide range of other malignancies were inhibited by AN-215, including RCCs [95], pancreatic [96], endometrial [97], prostate cancers [98] and glioblastomas [99].

Safavy et al. [100] reported a paclitaxel model conjugate using a bombesin receptor-recognizing peptide in which the drug cytotoxicity against H1299 human non-small-cell lung cancer (NSCLC) was enhanced compared with unconjugated taxol. They proposed recently a multiligand approach in a ‘scorpion’ model conjugate consisting of 2 bombesin ‘claws’ and one paclitaxel ‘tail’ to improve the targeting properties [101]. In a preliminary random screening, the conjugate showed superior cytotoxic activity in several GRPR-positive human cancer cell lines as compared with free paclitaxel and two single-drug single-ligand conjugates. In a receptor blocking experiment, addition of excess unconjugated bombesin ligand reduced the cytotoxicity of the conjugate, indicating the receptor-mediated mechanism of drug delivery. In order to verify the role of specific tumoral receptor interaction in its mediation, some authors synthesized camptothecin (CPT) bombesin analogues,
in which CPT was coupled via a novel carbamate linker, L2 [N-(N-methyl-amino-ethyl)-glycine carbamate], that were chemically similar but differed markedly in their potency/affinity for human bombesin receptors. Their ability to interact with bombesin receptors and cause \textit{in vitro} and \textit{in vivo} tumor cytotoxicity was tested. There was a significant difference between the compounds demonstrating that specific tumoral receptor interaction is important in mediating the ability of peptide ligand cytotoxic constructs to cause cytotoxicity [102].

**GRPR antagonists**

The synthetic antagonists are produced to bind with high affinity to the receptors, blocking the signaling pathways. Many GRP antagonists have been synthesized and tested. Schally et al. conducted numerous studies with RC-3095 e RC-3940-II. They demonstrated that GRP antagonists inhibit the growth of the androgen-independent prostate cancer cell line PC-3 \textit{in vivo}, possibly exerting a direct inhibitory effect on tumor growth through a down-regulation of EGF receptors [103]. The antagonists potentiated the inhibitory effects of growth hormone-releasing hormone (GHRH) in this cancer line [11] and showed efficacy for the treatment of advanced prostate cancer in preclinical metastatic models [8].

The combination of RC3094-II and GHRH antagonist inhibited orthotopic tumor growth by 77%, intratibially implanted tumors by 86% and subcutaneous tumors by 86%. The combined therapy also reduced the local tumor spread and distant metastases and was effective in reducing the incidence and severity of tibial osteolytic lesions and pathologic fractures in intraosseously implanted tumors. The use of RC-3940-II and RC-3095 in estrogen-independent human breast cancer cell line MDA-MB-231 xenografted in nude mice showed a decrease in final tumor volume by 72.4% and 57.7%, respectively, and greatly reduced tumor volume. There was a significant decrease in the number of binding sites for EGF, as well as bombesin, in tumor cells after chronic treatment with these antagonists [104].

RC-3095 ad 3094-II also inhibited the growth of human breast cancer cells MDA-MB-468 [9] and MDA-MB-435 [105] xenografted in nude mice, and the authors found a substantial reduction in the expression of mRNA and protein levels of the ErbB/HER receptor family, as well as a decrease in the expression of c-jun and c-fos oncoproteins. A single \textit{in vivo} administration of RC-3095 reduced the levels and mRNA expression of EGF receptors in MXT mouse mammary cancers [12]. The overexpression of angiogenic factors such as VEGF, fibroblast growth factor (FGF) and insulin-like growth factors (IGFs) plays a role in the migration and proliferation of endothelial cells in many cancers. Using breast cancer tumors implanted in nude mice, the authors evaluated the effect of bombesin antagonists on the expression of these factors, on vessel density and matrix metalloproteinases (MMPs) [10]. Both RC-3095 and RC-3940-II significantly decreased the vessel density and MMPs activity. The inhibition of tumor growth was associated with a substantial reduction in the expression of mRNA and protein levels of FGF, IGF-II and VEGF-A in the tumors.

Investigating the effect of the synthet GRP antagonists in H-69 SCLC, Koppan et al. found that the inhibition of growth of these cells was accompanied by a marked decrease in the levels of mRNA expression of EGF-R [106]. Hamsters with nitroamine-induced pancreatic cancers were treated for 8 weeks with RC-3095, somatostatin analogue RC-160 or the luteinizing hormone-releasing hormone antagonist cetrorelix to establish the pattern of changes in the number and affinity of EGF in tumors [107]. Chronic treatment with RC-3095 and cetrorelix resulted in an early (day 10) and sustained reduction (71% and 69%, respectively) in EGF receptors. In contrast, RC-160 decreased receptor concentration by 60% only after 20 days. The concentration of the receptors returned to the control level 4 days after cessation of chronic treatment with RC-3095. The authors concluded that the pattern of down-regulation of EGF receptors in pancreatic cancers appears to depend on the peptide used for the therapy. RC-3095 and RC-3940-II inhibited the growth of U-87MG human malignant glioblastomas implanted in nude mice by a mechanism that may involve the down-regulation of c-fos oncogene [108]. The same antagonists tested on ovarian carcinoma cell line OV-1063 also showed inhibition on tumor growth, associated to decreased levels of c-jun and c-fos proto-oncogenes [109].

A phase I trial was recently conducted in our institution to determine the safety and feasibility of RC-3095 subcutaneous administration to 25 patients with advanced solid malignancies [13]. The doses were given once or twice daily ranging from 8 to 96 ug/kg. The only toxicity observed was local discomfort in the injection site at the highest doses. There were no objective tumor responses in patients included in the study. A short-lasting minor tumor response was observed in a patient with a GRP-expressing progressive medullary carcinoma of the thyroid. A single-dose administration of RC-3095 at the highest dose level (96 ug/kg) was tested in a clearly hypergastrinemic individual with the Zollinger–Ellison syndrome and produced a decrease in plasma gastrin down to 50% of basal levels in 6 h.

Other bombesin/GRP antagonists were developed in the last decade. Compounds like BW2258U89 [110, 111] and BW1023U90 [112] antagonized the growth of SCLC cells, as well as PD176252, the first nonpeptide GRPR antagonist [113, 114]. Another class of peptide antagonists, a statine analogue, JM594, and a pseudopeptide analogue, JMV641, demonstrated high affinity for GRPR [115]. Azay et al. [116] proved that JMV641 was highly potent to dose dependently inhibit Swiss 3T3 cells proliferation, while bombesin had the opposite effect. Kuwanon G and H, nonpeptide bombesin receptor antagonists isolated from the methanol extract of \textit{Morus bombycis}, antagonized bombesin-induced increases in the cytosolic free calcium concentration and GRP-induced DNA synthesis in Swiss 3T3 cells [117]. In addition to the antagonist described above, several other compounds have shown efficacy blocking GRPR [118–121].

**antisense oligonucleotide therapy**

Antisense oligonucleotide therapy is based on interference in mRNA translation to protein synthesis. This approach is showing promising results, especially in neutralizing BCL-2, which is a potent antiapoptosis protein overexpressed in many human cancers, and there are several ongoing clinical studies with hematological malignancies as well as solid tumors [122].
Langer et al. investigated the effect of antisense oligodeoxynucleotides (ODNs) directed against GRPR mRNA on proliferation of human SCLC NCI-H345 cells, which express the autocrine system for GRP. The cell lines were exposed to antisense ODNs or sense ODNs and their proliferation was measured by spectrophotometric assay or viable cell counts. The results showed that the single or combined antisense ODNs against GRPR inhibited proliferation significantly by 37% ($P < 0.01$). Specificity was also demonstrated by the observation that cells exposed to antisense ODNs had a decrease in GRPR expression as measured by specific binding of 34% ($P < 0.01$), and when all three antisense ODNs were used, binding was decreased by 60% ($P < 0.03$). Furthermore, antisense ODNs decreased by 75% the maximum percentage of cells responding to GRP in an intracellular calcium release assay [123].

**pro-GRP**

Pro-GRP is known as an immunohistochemical tissue marker and is closely associated with neuroendocrine differentiation in SCLC [64]. The usefulness of serum pro-GRP as a tumor marker for diagnosis, treatment and monitoring patients with SCLC was evaluated by Oremek and Sapoutzis [124]. This study comprised 80 patients with small cell carcinoma, 20 with chronic bronchitis, 30 with sarcoidosis, 20 with lung fibrosis and 80 healthy individuals. The levels of pro-GRP were significantly higher in the patients with benign diseases compared with the healthy group, and even more elevated in patients with SCLC, confirming the potential for pro-GRP as a tumor marker in this malignancy.

Other researchers indicated that pro-GRP should be considered before prophylactic cranial irradiation (PCI) is carried out in SCLC patients [125]. PCI reduces the incidence of brain metastasis with an effect on overall survival in patients with SCLC. In spite of multidisciplinary intensive treatment approaches, many patients still experience brain metastasis. The authors retrospectively analyzed the characteristics of the first failure event due to brain metastasis (FBM) in 71 patients treated with PCI. Elevation of pro-GRP level before PCI was found to be a significant predictive and prognostic factor for FBM, overall incidence of brain metastasis and survival on multivariate analysis ($P = 0.007, P = 0.025$ and $P = 0.009$, respectively).

Nisman et al. [126] aimed to assess the value of serum levels of pro-GRP and other markers in predicting response to chemotherapy and survival of 67 patients with unresectable NSCLC. Serum pro-GRP levels correlated with disease extent, being higher in patients with metastatic disease than in those with locoregional disease ($P = 0.02$), but were not significant to predict response to chemotherapy. The combined use of pro-GRP, chromogranin A and neuron-specific enolase allowed the definition of two sets of patients with significantly different median survival times (25.2 versus 8.8 months, $P = 0.0001$).

The prognostic value of pro-GRP was also examined in patients with prostatic diseases due to the high serum levels of this neuropeptide in patients with advanced cancer stages [127]. In a study that enrolled 460 men, the serum status steadily deteriorated performance status (PS), extent of bony disease, high serum alkaline phosphatase, serum pro-GRP and nadir prostate-specific antigen (PSA) levels were associated with a lower progression-free survival (PFS) rate ($P < 0.005$). Multivariate analysis demonstrated that PS, serum pro-GRP, and nadir PSA held an independent predictive value for PFS ($P < 0.05$) and all correlated with bone-related factors. Serum pro-GRP was the most significant predictor among pretreatment factors in this model ($P = 0.0094$).

**concluding remarks**

In this article, the potential relevance of GRP receptor-dependent intracellular pathways in human cancer is discussed. Its high expression in a large spectrum of human cancers, as well as the demonstration of its role as tumor growth factors in various tumor models gives support to the study of GRPR antagonists as experimental anticancer agents. Notably, the interference with GRPR signaling was shown to have indirect effects upon other intracellular signaling pathways already shown to have clinical importance in cancer therapy, such as the inhibition of VEGF-dependent tumor angiogenesis and EGF-dependent tumor growth.

Clinical trials with GRPR antagonists in cancer patients are in its initial phase, and, as anticipated by animal toxicology studies, preliminary evaluation in humans does not indicate the existence of major normal tissue limiting toxic effects. Presently, efforts at the identification of the most suitable candidates for clinical trials and at improving drug formulation for human use are considered priorities. It may also be anticipated that GRPRs may be exploited in the future as potential carriers for cytotoxins, immunotoxins or radioactive compounds. Furthermore, the visualization of these receptors through scintigraphy may become an interesting tool for tumor detection in future studies.

**acknowledgements**

RR and GS are funded by CNPq and the South American Office for Anticancer Drug Development.

**references**


