Alterations of EGFR/HER, angiogenesis and apoptosis pathways after therapy with antagonists of growth hormone releasing hormone and bombesin in non-small cell lung cancer

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Abstract. New therapeutic strategies are necessary to improve the treatment of lung cancer. We investigated the effects of bombesin/gastrin-releasing peptide (GRP) antagonist, RC-3940-II, and growth hormone-releasing hormone (GHRH) antagonists, MZ-J-7-114 and MZ-J-7-118, on the expression of epidermal growth factor receptor (EGFR)/HER (-2, -3, and -4) family, angiogenic factors, VEGF-A and VEGF receptors (VEGF-R1 and VEGF-R2), and the apoptotic molecules Bax and Bcl-2, in H-460 and A-549 non-small cell lung carcinomas (NSCLC). Nude mice bearing xenografts of H-460 and A-549 NSCLC were treated daily with these peptide analogues for 4 weeks. The treatment resulted in growth inhibition of H-460 by 22-77% and A-549 NSCLCs by 64-84%. The inhibition of tumor growth was associated with a down-regulation of members of EGFR/HER family. A significant reduction of the levels of expression of EGFR/HER family on both tumors varied from 29-96%: the greatest inhibition being induced by RC-3940-II. Similarly, a significant decrease in the levels of VEGF-A in tumors by 19-60% and VEGF receptors (VEGF-R1, 24-74% and VEGF-R2, 25-50%) was detected after therapy. An up-regulation of Bax by 21-63% and a down-regulation of Bcl-2 by 23-39% was observed only for H-460 NSCLC. Our study demonstrates that human H-460 and A-549 NSCLC, express receptors for GHRH and bombesin/GRP, and respond to the respective antagonists. The antagonists of bombesin/GRP and GHRH could provide a new strategy for treatment of NSCLC through down-regulation of EGFR/HER family and an interference with the angiogenic and apoptotic pathways.

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Abbreviations: GRP, gastrin-releasing peptide; GRP-R, gastrin-releasing peptide receptor; NMB-R, neuromedin receptor; BRS-3, bombesin receptor subtype 3; EGFR, EGF receptor; GHRH, growth hormone releasing hormone; NSCLC, non-small cell lung carcinoma; SV, splice variant

Key words: GHRH antagonists, bombesin/GRP antagonist, EGFR/HER, VEGF/VEGF-R, Bax/Bcl-2

Introduction

Lung cancer is the leading cause of cancer related death in the USA (1). Non-small cell lung carcinoma (NSCLC) accounts for approximately 87% of all lung cancers based on an analysis of data from Surveillance Epidemiology and Research (Page *et al,* Proc ASCO, abs. 1216, 2002). Despite advances in the treatment of lung cancer combining chemotherapy and radiotherapy, new therapeutic strategies are necessary. A better knowledge of lung carcinogenesis based on identification of molecular pathways could lead to clinical studies with new agents targeting tyrosine kinase receptors, angiogenic factors, and apoptotic molecules.

The bombesin receptor family comprises at least 4 subtypes, namely the gastrin-releasing peptide (GRP) receptor, the neuromedin B (NMB) receptor, and 2 receptor subtypes, BRS-3 and BRS-4 (2,3). Except for the GRP receptor, the other 3 subtypes have been poorly characterized in regard to their distribution and function in human tissues (2). The bombesin/GRP receptors are members of the G-protein coupled receptor superfamily and the signal transduction pathways involve the activation of phospholipase C, generation of inositol trisphosphate, the release of intracellular calcium, and the activation of protein kinase C (3). An up-regulation of these receptors has been reported in various cancers including carcinomas of the lung (both SCLC and NSCLC), prostate, colon, kidney, breast and ovary, as well as glioblastoma (4). Anti-proliferative effects of bombesin/GRP antagonists are attributed to both the blockade of mitogenic

stimuli of bombesin/GRP and interference with the action of epidermal growth factor (EGF) through the dephosphorylation of epidermal growth factor receptors (EGFR) and down-regulation of their expression in tumors (5,6). We showed previously that bombesin/GRP antagonist RC-3095 inhibits growth of H-69 SCLC, but not of H-157 NSCLC xenografted into nude mice (7). Subsequently, we demonstrated that antagonist RC-3940-II possesses greater anti-tumor activity on H-69 SCLC than RC-3095 (5). The inhibition of SCLC growth by RC-3095 and RC-3940-II was associated with a decrease in the levels and mRNA expression of EGFR (5). It has also been shown that growth hormone-releasing hormone (GHRH) antagonists inhibit the growth of experimental human cancer cell lines xenografted into nude mice or cultured *in vitro* (8-11). Recently we demonstrated in H-69 SCLC, that RC-3940-II and GHRH antagonists, JV-1-65 and MZ-J-7-110, decreased the tumor volume, reduced the protein levels of PKC isoforms, MAPK, as well as the mRNA levels of *c-fos* and *c-jun* oncogenes, and caused down-regulation of the protein expression of EGF/HER-2 and -3 receptor family (8,9). The expression of 4 splice variants (SV1-SV4) of the full length human GHRH receptor (GHRH-R) was reported previously in normal tissues and in certain cancer cell lines (12,13). The inhibitory effect of GHRH antagonists is exerted in part by endocrine mechanisms through the inhibition of GHRH release from the pituitary, which in turn, results in the reduction of the hepatic production of insulin-like growth factor I (IGF-I) (11). However, much evidence suggests that the anti-tumor effects are also mediated by a direct action independent of hepatic IGF axis (12,14-17). These investigations demonstrated that the direct effects of GHRH and its antagonists on tumors are mediated by SVs of GHRH receptors (12,14-17). mRNAs for 4 of the these SVs of the GHRH-R were found in many cancers, SV1 being more common than the other forms (12,14). SV1 has been detected in many human cancers including gastroenteropancreatic, renal, lung and prostatic tumor cell lines and surgical specimens of human prostate cancer (13,16,18,19). Unlike the other three SVs, the SV1 has all 7 transmembrane domains and the whole third intracellular loop, the latter being critical for the interaction with G-proteins (12,20-22).

Thus, receptors of bombesin/GRP and SV1 are members of the G-protein coupled receptors. Recent studies on the signaling pathways activated by mitogenic neuropeptides through this mechanism revealed that these receptors not only stimulate the synthesis of conventional second messengers, but also induce tyrosine phosphorylation cascades (23). In this signaling network, an increase in the tyrosine kinase activity of the EGFR has been identified as an important element, but little is known about the contribution by which G-protein coupled receptor agonists induce EGFR transactivation leading to carcinogenesis and tumor growth (4).

The EGFR/HER family of receptors plays a key role in tumorigenesis and disease progression. The HER molecules are cell membrane-bound proteins comprising four distinct receptors: EGFR/HER-1, HER-2, HER-3, and HER-4 (24). Members of the HER family and their ligands are in general overexpressed in many tumor types, including lung, pancreatic, ovarian, renal cell, gastric, hepatocellular, and breast cancers (25). Consequently, the blockade of EGFR activation has been proposed as a target for anticancer therapy since the overexpression of EGFR has been associated with a more aggressive disease and a poor prognosis (26). Activation of EGFR leads to receptor tyrosine kinase activation and a series of downstream signaling events which mediate increases in cellular proliferation, motility, adhesion, invasion, blocking of apoptosis and resistance to chemotherapy (27). In addition, VEGF is considered one of the most potent mitogen promoting the vascularization and growth of the primary tumor (28). Thus, inhibition of VEGF and its receptors VEGF-R1 (Flt-1) and VEGF-R2 (Flk-1/KDR) offers an attractive target for anti-angiogenic therapy in cancer. Besides, parameters concerning apoptosis such as Bax and Bcl-2 are of great importance since the imbalance of these apoptotic members, including overexpression of Bcl-2 and a decreased Bax/Bcl-2 ratio is responsible, in part, for the tumor growth (29).

Therefore, we investigated the effects of bombesin/GRP antagonist, RC-3940-II and GHRH antagonists, MZ-J-7-114 and MZ-J-7-118 on the growth of xenografts of H-460 and A-549 NSCLC cell lines in athymic nude mice, and whether these antagonists would affect the expression of EGF/HER receptor family as well as the angiogenic and apoptotic signaling molecules. This is the first study in NSCLC tumors xenografted into nude mice reporting the effects of bombesin/GRP antagonist and GHRH antagonists on the levels of protein expression for EGFR/HER family, the angiogenic proteins VEGF-A and VEGF receptors, in addition to the apoptotic molecules, Bax and Bcl-2.

Materials and methods

Peptides. The bombesin/GRP antagonist [Hca^{6}, Leu$^{13}\Psi$ (CH_2N)-Tac^{14}]BN(6-14) (RC-3940-II) originally synthesized in our laboratory (11,30) was made and provided by Zentaris AG (Frankfurt on Main, Germany). The GHRH antagonists MZ-J-7-114 and MZ-J-7-118 were synthesized by solid-phase as described (10,31) and the chemical structures were described previously (8,32). For injections, all the peptides were dissolved in 0.1% dimethyl sulfoxide (DMSO) in sterile aqueous 10% propylene-glycol and administered s.c.

Cell lines and animals. Human NSCLC cell lines H-460 and A-549 were obtained from American Type Culture Collection (Manassas, VA, USA). H-460 NSCLC cell line was routinely grown in RPMI supplemented with 1 μM sodium pyruvate and A-549 NSCLC was grown in F-12K medium. The culture media for both cell lines were supplemented with 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin. Athymic male (Ncr *nu/nu*) nude mice, approximately 6-week-old on arrival, were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD), and housed in laminar air-flow cabinets under pathogen-free conditions with a 12-h light/12-h dark schedule, and fed autoclaved standard chow and water *ad libitum*. All experiments were carried out in accordance with institutional guidelines for animal care.

In vivo studies. Xenografts were initiated by s.c. injection of 1x10^{6} H-460 NSCLC and 1x10^{8} A-549 NSCLC cells into the

right flanks of male nude mice. The developed tumors were dissected and minced, and 3-mm^3 pieces of tumor tissue were transplanted s.c. to both flank areas of the experimental animals. When the tumors became measurable, control and experimental groups were formed with animals bearing tumors about the same size. The mice were randomly divided into 6 groups of 7-9 animals each and received the following s.c. daily treatment for 4 weeks: group 1 (control), vehicle solution; group 2, GHRH antagonist MZ-J-7-114 (10 μg); group 3, MZ-J-7-114 (40 μg); group 4, GHRH antagonist MZ-J-7-118 (10 μg); group 5, MZ-J-7-118 (40 μg); and group 6, bombesin/GRP antagonist RC-3940-II (10 μg). Tumor volumes and body weights were measured every week. The volume of tumors was calculated as length x width x height x 0.5236. Antitumor activity of the compounds was evaluated by calculation of tumor growth reduction (TGR) as described previously (8). Tumors were excised, weighed, snap-frozen and stored at -70˚C until further analyses. All animal experiments were reviewed by the institutional animal care and use committee and were performed in accordance with institutional guidelines for animal care.

RNA extraction and real-time (RT)-PCR. Total RNA was isolated from approximately 10 mg of tumor tissue according to the TRI-Reagent® protocol (Sigma-Aldrich, St. Louis, MO). Tumor samples from H-460 and A-549 NSCLCs were homogenized, the RNA extracted and re-suspended in nuclease free water. Real-time PCR was then performed for the 3 human bombesin receptor subtypes (GRP-R, NMB-R and BRS-3). The RT reaction was performed with the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA) according to manufacturer's protocol. RNA (2 μg) was transcribed into cDNA with a final volume of 40 μl. The RT reaction was performed in an Applied Biosystem PCR system 2700. Bio-Rad's iCycler was used for all real-time PCR reactions and the gene-specific primers for the bombesin receptor subtypes and ß-actin were: GRP-R (113 bp): sense 5'-TAC CTC TGT TGG GGT GTC TGT-3', antisense 5'-GGC AGA TCT TCA TCA GGG CAT-3', probe 5'-hex CAC ACT CAC GGC GCT CTC GGC A-3' BHQ1; NMBR (141 bp): sense 5'-GAC AGG TAC AGA GCC ATC GTT A-3', antisense 5'-GCG AGC CAC TTC TGA AAA CAC-3', probe 5'-Texas Red ACT GCCAGC AAC ACG GAG ACC ACC-3' BHQ-2; BRS-3 (104 bp): sense 5'-CGC TGA CAG ATA CAA GGC AGT TG-3', antisense 5'-TCA TAG ACA CGA TCC AGA CGC AG-3', probe 5'-Cy5™ AGC CAC TTG AGC GAC AGC CCT CCA-3' BHQ-3; ß-actin (140 bp): sense 5-CTG GAA CGG TGA AGG TGA CA-3', anti-sense 5'-AAG GGA CTT CCT GTA ACA ATG-3', probe 5'-FAM CAG TCG GTT GGA GCG AGC ATC CCC-3' BHQ-1. All reactions followed the same thermal cycling protocol, comprising an initial denaturation step at 95˚C for 3 min, followed by 40 cycles of two-step PCR including 95˚C for 20 sec and 60˚C for 45 sec. Bio-Rad's iQ supermix was used for all the PCR reactions. Total volume in each well was 25 μl in which 2 μl corresponded to cDNA. All probes found to be efficient in the range of 95-105%. Efficiency in this instance is the amount of PCR product doubled after each cycle. The C_T (threshold cycle) was compared between the control and the samples treated with RC-3940-II, and compared to the C_T value of their respective housekeeping gene (ß-actin). Four tumor samples of each group were run in triplicate for each primer. Negative controls included PCR amplification without cDNA and reverse transcriptase. PC3 prostate cancer cell line was used as a positive control for GRP-R and H-69 SCLC cell line was used as a positive control for NMB-R and BRS-3. Relative expression ratio (R) was calculated using the mathematical model described by Pfaffl *et al* (33). Ratio = $2^{-\Delta\Delta C_T}$ = delta-delta method for comparing relative expression results between treated and control groups.

Western blot assays. The whole tissue homogenate (10-20 μg/lane) were separated by SDS-PAGE Tris-HCl Criterion Precasted Gels (Bio-Rad, Hercules, CA), varying from 7.5% to 15%, depending on the molecular weight of the protein. The membranes were incubated for 3-5 h at room temperature in 5% non-fat dry milk in TBS-Tween, followed by incubation with the specific polyclonal antibodies to VEGF-A (1:1000), VEGF-R1/Flt-1 (1:500), VEGF-R2/Flk-1/KDR (1:500), Bax (1:1000), and monoclonal antibody to Bcl-2 (1:1000); all from Santa Cruz Biotechnology, and to SV1 (1:2000) antiserum purified in our laboratory. The specificity and optimal dilutions of the SV1 was determined in preliminary study (34) and the batch utilized in our assays was 2317/7. The blots were probed at 4˚C overnight with the specific antisera and the signal for the immunoreactive proteins was developed with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and visualized by exposure to the chemiluminescence substrate (Amersham Bioscience Corp., Piscataway, NJ). The protein bands were quantified by normalizing the signals of different proteins to ß-actin signal (1:2000, Santa Cruz Biotechnology) using the Kodak EDAS 290 imaging system with Kodak 1D Image Analysis Software (Kodak, Rochester NY).

Statistical analyses. The SigmaStat Software was used for the statistical analysis of data. Results are presented as means ± SE. Results of the '*in vivo*' studies were evaluated by one way ANOVA and the Fisher test.

Results

Effect of antagonists of GHRH and bombesin/GRP on the growth of H-460 and A-549 NSCLC tumors xenografted into nude mice. Nude mice bearing subcutaneous xenografts of H-460 and A-549 NSCLCs were treated daily with the GHRH antagonists MZ-J-7-114 and MZ-J-7-118 at two different doses (10 and 40 μg) and with 10 μg of bombesin/GRP antagonist RC-3940-II. No significant difference was observed between 10 and 40 μg doses of both GHRH antagonists. A significant inhibition in the tumor growth became evident following 3 weeks of treatment with either GHRH antagonists or bombesin/GRP antagonist, in both NSCLC tumors (Fig. 1). In H-460 NSCLC, the tumor volumes measured in the third week following treatment with MZ-J-7-118 at 10 and 40 μg, were significantly smaller ($P<0.05$), 344±55 and 341±52 mm^3, respectively, compared to the control group (475±50) (Fig. 1A), but at the end of the therapy this reduction was no longer statistically significant, although an inhibitory tendency was still observed (Fig. 1A).

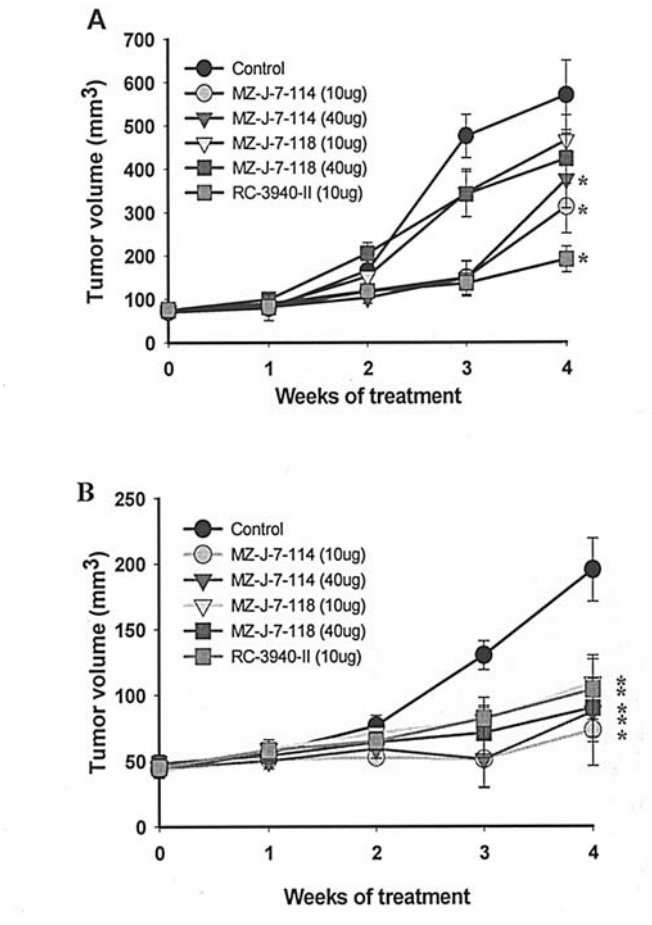

Figure 1. Tumor volumes in nude mice bearing H-460 (A) and A-549 (B) human NSCLC during treatment with GHRH antagonists MZ-J-7-114 and MZ-J-7-118 and bombesin/GRP antagonist RC-3940-II. Vertical bars indicate SE. *P<0.05 vs. control.

On the other hand, the other GHRH antagonist, MZ-J-7-114, caused a significant tumor suppression from the third week of treatment and when the experiment was concluded, an inhibition of tumor growth by 40% and 54% (P<0.05) was observed after doses at 10 and 40 μg, respectively (Fig. 1A, Table I). The bombesin/GRP antagonist, RC-3940-II, caused the greatest inhibition of 77% in the growth of H-460 NSCLC (Fig. 1A, Table I). The same therapy with both antagonistic peptide analogues was applied to the A-549 NSCLC tumors. In this cell line, both GHRH antagonists as well as bombesin/GRP antagonist, caused a significant decrease in the tumor growth from the third week of treatment (Fig. 1B). When the therapy was completed, we observed a decline of 64-84% (P<0.05) in the tumor volumes as compared with the control group (Fig. 1B, Table I). The weights of H-460 and A-549 NSCLC tumors were significantly (P<0.05) decreased by treatment with the antagonists of GHRH and bombesin/GRP analogues (Table I). No significant differences in body weights and the weights of various organs were observed in treated and control animals, indicating that these analogues had no toxic side effects (data not shown).

Bombesin receptors assay on the H-460 and A-549 tumor tissues. Total RNA from tumor tissues of mice treated with 10 μg of RC-3940-II and untreated controls was isolated and subjected to real-time RT-PCR analysis. PCR efficiencies were in the range of 95-105% for the bombesin subtype receptors or ß-actin. We observed significant changes in the expression level of the mRNA for GRP-R, NMB-R and BRS-3 receptors in the treated animals compared to controls (Table II), as calculated by the relative expression ratio (see Materials and methods). The ratio (R) determined by using delta-delta method for comparing relative expression results between treated and control groups, displayed values by 0.63-2.05 (Table II). In H-460 NSCLC, we detected a significant (P<0.05) down-regulation (R=0.63) of NMB-R, an up-regulation (R=2.05) of BRS-3 and no alteration (R=0.94) in the GRP-R mRNA expression (Table II). On the contrary, treatment of A-549 showed an up-regulation of ~1.8 for all 3 bombesin receptor subtypes (Table II). No significant amounts of PCR products were detected in negative controls without cDNA or reverse transcriptase.

Western blot assays for the expression of SV1 receptor in H-460 and A-549 NSCLC tumors. Preliminary studies based on RIA and Western blot assays have shown that the anti-

Table I. Effect of treatment with antagonistic analogues of bombesin/GRP and GHRH on tumor volume and weight in nude mice xenografted with H-460 and A-549 human NSCLC cell lines.

Groups (μg)	H-460 NSCLC		A-549 NSCLC	
	Tumor weight (g)	Final tumor volume in mm^3 (% inhibition)	Tumor weight (mg)	Final tumor volume in mm^3 (% inhibition)
Control	4.0±0.6	569±8	25±0.03	195±24
MZ-J-7-118 (10)	2.1±0.5[a]	465±58 (22)	17±0.02	109±21[a] (64)
MZ-J-7-118 (40)	2.5±0.4[a]	423±58 (34)	16±0.02	90±22[a] (74)
MZ-J-7-114 (10)	1.8±0.3[a]	375±58[a] (40)	14±0.02	73±27[a] (84)
MZ-J-7-114 (40)	1.4±0.2[a]	312±71[a] (54)	13±0.02	87±23[a] (73)
RC-3940-II (10)	1.3±0.2[a]	191±51[a] (77)	12±0.02	104±23[a] (67)

[a]P<0.05 vs. control. Values are expressed as the mean ± SE.

Table II. Relative expression and ratio of GRP-R, NMB-R, and BRS-3 receptors in the H-460 and A-549 NSCLC tumor tissues.

	H-460 NSCLC			A-549 NSCLC		
	Cycle threshold (C_T)		Ratio	Cycle threshold (C_T)		Ratio
	Control	RC-3940-II		Control	RC-3940-II	
GRP-R	15.42±0.16	15.51±0.18	0.94	15.68±0.56	14.83±0.58[a]	1.8
NMB-R	19.74±0.04	20.40±0.18[a]	0.63	12.87±0.07	11.94±0.38[a]	1.9
BRS-3	9.94±0.06	8.90±0.39[a]	2.05	12.92±0.25	12.01±0.36[a]	1.8

All data represent means ± SE of triplicates, presented as cycle threshold (C_T). Ratio = $2^{-\Delta\Delta C_T}$ = delta-delta method for comparing relative expression results between treatments in real-time PCR [developed by PE Applied Biosystems (Perkin-Elmer)].

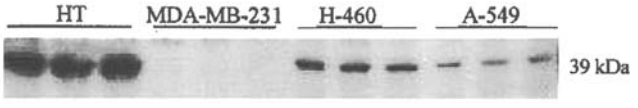

Figure 2. Western blot analysis of expression of protein for GHRH receptor splice variant I (SV1). Bands of the expected size of 39 kDa were found in RL lymphoma, H-460 and A-549 NSCLC tumors.

serum batch 2317/7 for the purified-SV1 was more specific (34). The purified SV1-antiserum recognized protein bands at 39 kDa obtained from H-460 and A-549 NSCLC cell lines (Fig. 2). HT lymphoma and MDA-MB-231 breast carcinoma were used as positive and negative control, respectively. The specificity of the bands was confirmed by the considerable decrease of the specific bands after re-incubation of the membranes with the specific blocking peptide (data not shown).

Effect of antagonists of GHRH and bombesin/GRP on the expression of EGFR/HER family receptor proteins in H-460 and A-549 NSCLC cell lines. Members of the EGF/HER receptor family (EGFR, HER-2, -3, and -4) were investigated by immunoblot assays with specific antisera, in H-460 and A-549 tumors treated with GHRH antagonists, MZ-J-7-114 and MZ-J-7-118, and bombesin/GRP antagonist, RC-3940-II. We detected bands at 172, 187, 162, and 181 kDa corresponding to EGFR, and HER-2, -3, and -4 proteins, respectively (Fig. 3). Densitometric analyses of the bands representing members of the EGFR/HER family after normalization to ß-actin signals are shown on Table III. A significant decrease ($P<0.05$) in the protein levels of EGF/ HER (-2, -3, and -4) receptors was observed on both NSCLC cell lines. In the H-460 NSCLC, a significant reduction in the levels of EGFR/HER protein expression varied from 29 to 65% and in the A-549 tumors, the variation was in the range of 34-96% (Table III). However, MZ-J-7-114 had no significant effect on the protein expression of EGFR/HER (-2, -3, and -4) in H-460 tumors and EGFR expression in A-549 NSCLC (Fig. 3, Table III).

Figure 3. Effect of antagonists of GHRH and bombesin/GRP on protein expression of EGFR and HER-2, -3, and -4 in H-460 (A) and A-549 (B) NSCLC tumors. Protein matched samples of tumors treated with MZ-J-7-114, MZ-J-7-118, and RC-3940-II were submitted to Western blot assays. Tissue homogenate was examined for EGFR and HER-2, -3, and -4, using specific antisera, and the molecular masses were 172, 187, 162, and 181 kDa, respectively. Three to five representative tumors from each group are shown. Protein levels were normalized to ß-actin protein (42 kDa) and are expressed as percentages of control values shown in Table III.

Expression of VEGF-A and VEGF receptors in NSCLC human lung cancers after therapy with peptide antagonistic analogues of GHRH and bombesin/GRP. VEGF-A, VEGF-R1 and VEGF-R2 expressions in H-460 and A-549 NSCLC tumors were investigated by Western blot analysis. The levels of expression of VEGF-A protein were decreased by 13-33% after treatment of H-460 tumors with MZ-J-7-114, MZ-J-7-118 and RC-3940-II (Fig. 4). We also detected significant reduction ($P<0.05$) in the expression of VEGF-R1 varying from 24-56% and by 25-50% of VEGF-R2 in all H-460 NSCLC-treated groups (Fig. 4). Interestingly, a greater inhibition in the expression of VEGF-A (19-60%) and VEGF-R1 (62-74%) was detected in the A-549-treated animals (Fig. 4). Similarly, VEGF-R2 was inhibited by 8-46% in A-549-treated tumors (Fig. 4).

Modulation of the expression of Bax and Bcl-2 proteins in tumors treated with GHRH antagonists and bombesin/GRP antagonist. The levels of Bax and Bcl-2 proteins in both

Table III. Expression of protein for EGFR/HER receptors family in H-460 and A-549 NSCLC after treatment with 10 μg/day of bombesin/GRP antagonist RC-3940-II and 40 μg/day of GHRH antagonists MZ-J-7-114, and MZ-J-7-118.

	Protein (% of control)							
	H-460 NSCLC				A-549 NSCLC			
	Control	MZ-J-7-114	MZ-J-7-118	RC-3940-II	Control	MZ-J-7-114	MZ-J-7-118	RC-3940-II
EGFR	100.0±19.4	96.0±2.2	63.0±1.6[a]	35.0±6.9[a]	100.0±7	111.0±19.0	65.0±15.0[a]	36.0± 8.0[b]
HER-2	100.0±4.0	95.0±9.0	71.0±18.0[a]	68.0±12.0[a]	100.0±6	27.0±1.1[a]	64.0±7.0[a]	4.0±2.5[c]
HER-3	100.0±34.6	86.0±7.6	41.0±8.2[a]	42.0±14.0[a]	100.0±33.0	22.0±1.4[a]	18.0±5.0[a]	12.0±0.4[b]
HER-4	100.0±6.0	80.0±18.0	45.0±23.0[a]	61.0±7.0[a]	100.0±10.0	66.0±9.0[a]	62.0±8.0[a]	30.0±5.0[b]

Values are means ± SE. Analyses of 5-8 tumors in each group were quantified by densitometric analysis and the data were normalized to ß-actin values. [a]P<0.05, [b]P<0.01, and [c]P<0.001 vs. control.

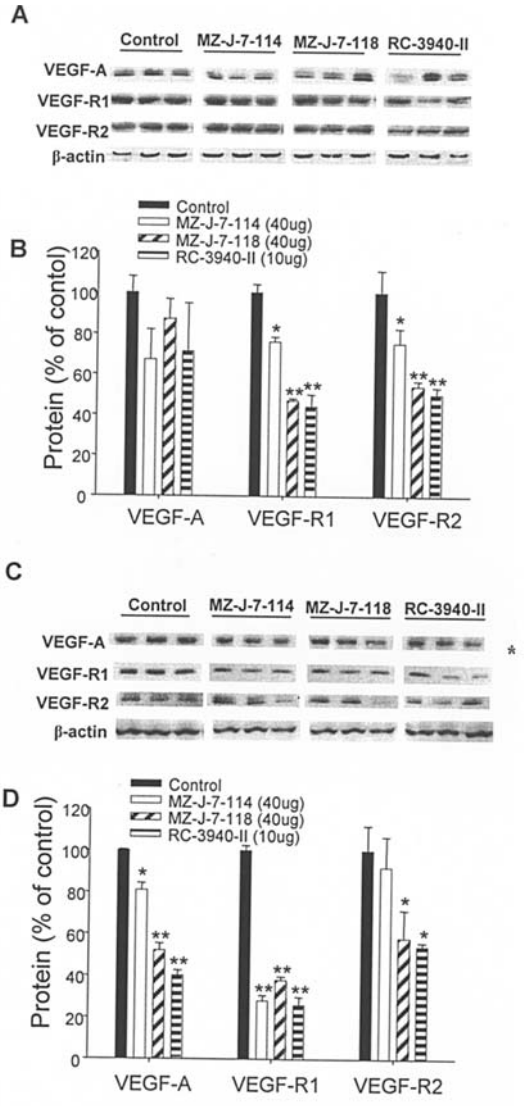

Figure 4. Representative Western blotting for VEGF-A, VEGF-R1 (Flt-1), and VEGF-R2 (Flk-1/KDR) in H-460 NSCLC (A) and A-549 NSCLC (C). Levels of these proteins expressed as a percentage of control after standardization to the expression of ß-actin levels in H-460 NSCLC (B) and A-549 NSCLC (D). The specific antisera for VEGF-A, VEGF-R1, and VEGF-R2, recognized bands at 22, 210, and 207 kDa, respectively. Three representative tumors from each group are shown and the experiments were repeated at least three times. The data are expressed as means ± SE. *P<0.05 vs. control; **P<0.01.

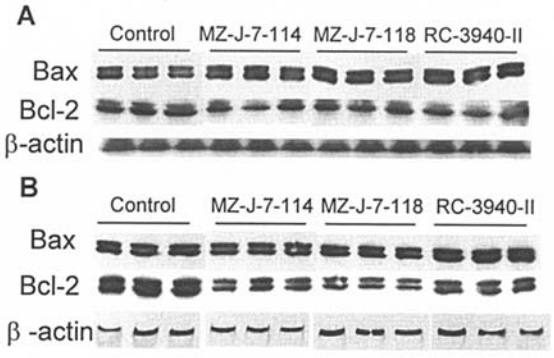

Figure 5. Expression of Bax and Bcl-2 in H-460 NSCLC (A) and A-549 NSCLC (B) tumors. Protein matched samples of tumors from mice untreated or treated with MZ-J-7-114, MZ-J-7-118 and RC-3940-II were submitted to Western blot assays. Tissue homogenates were examined for Bax and Bcl-2 using specific antibodies (1:1000) and the molecular weights were 22 and 26 kDa, respectively. Three representative tumors from each group are shown. ß-actin (43 kDa) was used as an internal control.

NSCLC tumors, H-460 and A-549, were determined by immunoblot assays. As shown in Fig. 5A and Table IV, in H-460 NSCLC, the levels of Bax protein were significantly (P<0.05) increased by antagonists of GHRH and bombesin/GRP while the levels of Bcl-2 were significantly (P<0.05) decreased by treatment with all three analogs, MZ-J-7-114, MZ-J-7-118 and RC-3940-II. Bax and Bcl-2 are thought to have opposite effects in the apoptotic pathway, therefore a relative ratio of Bax/Bcl-2 was calculated. Given that this ratio has pro-apoptotic protein in the numerator and the anti-apoptotic protein in the denominator, higher ratio values are observed with higher levels of the pro-apoptotic proteins and/or lower levels of the anti-apoptotic proteins. In the treated groups of H-460 NSCLC, the ratio of the Bax to Bcl-2 proteins, considered as a parameter of a net apoptotic gain, was 57-167% higher than the control values, indicating the effectiveness of treatment (Table IV). No significant effects on the levels of Bax and Bcl-2 were observed after treatment with the same compounds on the A-549 NSCLC (Fig. 5B, Table IV).

Table IV. Expression of protein for Bax and Bcl-2 in H-460 and A-549 NSCLC after treatment with 10 μg/day of bombesin/GRP antagonist RC-3940-II and 40 μg/day of GHRH antagonists MZ-J-7-114, and MZ-J-7-118.

	Protein (% of control)							
	H-460 NSCLC				A-549 NSCLC			
	Control	MZ-J-7-114	MZ-J-7-118	RC-3940-II	Control	MZ-J-7-114	MZ-J-7-118	RC-3940-II
Bax	100.0±9.8	121.0±1.2[a]	157.0±2.0[a]	163.0±1.1[a]	100.0±26.0	85.0±2.1	92.0±1.0	104.0±3.6
Bcl-2	100.0±4.0	77.0±4.9[a]	75.0±9.6[a]	61.0±7.5[a]	100.0±27.0	73.0±5.3	83.0±1.1	93.0±2.6
Bax/Bcl-2 ratio	1.00±0.07	1.57±0.06[a]	2.09±0.15[a]	2.67±0.17[a]	1.00±0.2	1.16±0.05	1.11±0.01	1.12±0.03

Values are means ± SE. Analyses of 5-8 tumors in each group were quantified by densitometric analysis and the data were normalized to ß-actin values. [a]P<0.05 vs. control.

Discussion

The incidence of lung cancer in the world is increasing (1). When lung cancer is detected at the early stage, followed by surgery, it presents a curative potential, but unfortunately 30-50% of patients experience relapses (35). Despite improvements in the diagnosis and treatment, the prognosis for lung cancer remains poor, the overall 5-year survival being 4-14% (36).

SCLC and some NSCLC have neuroendocrine features including production of neuropeptides, expression of its receptors in the membrane, and autocrine stimulation by the peptides (37). Thus, peptide receptors represent excellent targets for *in vivo* cancer diagnosis and therapy, and investigation of their expression in the tumors is critical for the successful therapeutic uses of peptide antagonists. Previously we showed in various experimental cancer models, that antagonists of bombesin/GRP and GHRH developed in our laboratory can be used for therapy. The treatment with our bombesin/GRP antagonists, RC-3095 and RC-3940-II, resulted in a significant reduction in the growth of several cancers, such as H-69 SCLC, breast, ovarian, prostate, glioblastomas, pancreatic and renal cell carcinomas, among others reported previously (11). Bombesin/GRP is a recognized growth factor for SCLC and the effectiveness of their antagonists for the *in vivo* treatment of experimental human SCLC has been well documented (2,5-9,11). In contrast, much less is known about the involvement of bombesin/GRP antagonistic analogues in the regulation of NSCLC (38,39), and the possible therapeutic benefits of bombesin/GRP antagonists for the therapy of NSCLC have not been evaluated in studies *in vivo*. Similarly, our GHRH antagonists strongly suppress the growth of various experimental cancers such as lung, osteosarcomas, glioblastomas, pancreatic, colorectal, breast and ovarian tumors (11).

The present study demonstrates the inhibitory effects of GHRH antagonists, MZ-J-7-114 and MZ-J-7-118, and that of the bombesin/GRP antagonist, RC-3940-II, on the growth of the H-460 and A-549 human NSCLC tumors xenografted into nude mice. The A-549 tumors treated for 4 weeks with our peptide analogues, exhibited a major inhibition of the tumor volumes. Interestingly therapy of H-460 tumors for 3 weeks with MZ-J-7-118 caused a significant inhibition of the tumor growth, but after the treatment for one additional week, tumor volume reduction was no longer statistically significant, although an inhibitory tendency was observed. One possible explanation could be that H-460 probably has much more aggressive characteristics than A-549, allowing these cells to escape from some inhibitory signaling pathways.

Our findings from Western blot and real-time PCR assays show that both cell lines, H-460 and A-549, express SV1 receptors besides bombesin/GRP receptor subtypes, such as GRP-R, NMB-R, and BRS-3. Based on these data, part of the inhibitory effect of the tumor growth observed on both cell lines, could be attributed to the direct effect of our antagonists on their specific receptors in the cell membrane. It is interesting that although we found inhibition of the growth of both H-460 and A-549 NSCLC after therapy with antagonists of GHRH and bombesin/GRP (NSCLC), it seems that these tumors make use of distinct molecular mechanisms for cellular proliferation in spite of the fact that both cell lines belong to the same NSCLC category of lung cancer. For example, after therapy with RC-3940-II, A-549 NSCLC showed an up-regulation of the three bombesin subtype receptors while H-460 showed a heterogeneous effect. The observed up-regulation of the bombesin/GRP receptors in A-549 tumors, could be due to tolerance developed by the cells, perhaps as a protective mechanism induced by preconditioning stimulus such as the exposure to the bombesin/GRP antagonist. In the case of H-460-treated tumors, because of the diversity of effects observed in the levels of mRNA expression of bombesin receptors, there could be a specific modulation for each subtype receptor by RC-3940-II and the final biological response, e.g. tumor volume, would be reflected by the summation of their activation. It was demonstrated previously that GRP and NMB play an important role in the pathophysiology of the lung by stimulating cell proliferation (40). Little information is available about the role of BRS-3 receptors in cancer, however, a recent report indicates that activation of BRS-3 may cause cancer cell proliferation, since the use of a bombesin analogue caused increased nuclear oncogene expression, MAPK phospho-

rylation and ELK-1 activation in lung cancer cells (41). The presence of bombesin receptors in human tumors may allow us to investigate the function of these receptors in more detail and consequently to identify new clinical applications of bombesin analogues. In this regard, van de Wiele and coworkers showed the successful visualization of GRP receptor with a 99Tc-labeled GRP analog in prostate and breast cancers (41).

It is described that bombesin/GRP receptors and the splice variant (SV1) of the GHRH receptor exert their direct anti-proliferative effects, at least in part, through G-protein coupled receptors (3,8,34). It is also reported that >95% of NSCLC tumors present EGFR overexpression (4). However, the mechanism of EGFR transactivation by G-protein coupled receptors leading to carcinogenesis and tumor growth is still unclear (4).

The HER family of 4 naturally occurring receptors (EGFR/HER-1, -2, -3, and -4) and one tumor-specific mutant can activate signaling transduction through a complex range of mechanisms. The downstream signaling can lead to tumor growth and development by several processes, including enhanced cellular proliferation, survival, and metastasis (24). In fact, many EGFR inhibitors are presently undergoing evaluation clinical trials, including compounds acting on the extracellular domain of EGFR, such as IMC-C225, ABX-EGF, and EMD 72000 and small molecules inhibiting EGFR phosphorylation, such as ZD 1839, OSI-774, EW 2016 and C1-1033 (27). Therefore, we investigated whether our peptide analogues would affect the expression of EGFR/HER family in H-460 and A-549 tumors. We observed that MZ-J-7-118 at 40 μg and RC-3940-II at 10 μg caused a significant decrease in the levels of expression of EGFR/HER-2, -3, and -4 proteins. A significant decrease in the levels of expression of EGF/HER protein receptors on both NSCLC cell lines varied from 29 to 96%, but no effect was detected in H-460 NSCLC treated with MZ-J-7-114. Although distinct effects were again observed after these cell lines received similar therapy, these are important finding, because this is the first study reporting the inhibitory effect of bombesin antagonists and GHRH antagonists on the experimental NSCLC tumors and its association with a down-regulation of members of EGFR/HER family. Consequently, our GHRH and bombesin/GRP antagonists could be useful for therapy of NSCLC, since the members of the EGFR/HER family are viewed as promising therapeutic targets for the lung cancer.

Likewise, VEGF and VEGF receptor kinases are also good candidates for anti-angiogenic treatment strategy. In fact, it was shown that Gefitinib (ZD 1839), a potent and selective inhibitor of EGFR tyrosine kinase, caused inhibition of VEGF production in tumor cells through the inhibition of EGFR signaling, leading to a suppression of angiogenesis (42). Angiogenesis is a complex process regulated by a great variety of stimulators and inhibitors and of these, VEGF-A is considered as the most potent pro-angiogenic factor. VEGF-A through binding and activation of the tyrosine kinase receptors VEGF-R1 and VEGF-R2 (also called Flt-1 and FLk-1/KDR, respectively) can induce cell proliferation, migration and expression of tissue factor (43). A significant relationship between VEGF and VEGF-R1 expression and drug response was reported, showing that the expression of VEGF and VEGF-R1 was lower in resistant than in sensitive tumors (43). We observed that H-460 and A-549 NSCLC tumors after daily administration of MZ-J-7-114, MZ-J-7-118 and RC-3940-II for 4 weeks, showed a significant decrease in the levels of protein expression of the angiogenic-related factors, VEGF-A and VEGF receptors. Apparently, the A-549 NSCLC seems to be more sensitive in terms of reduction of the angiogenic factors as compared to the H-460 NSCLC. Nonetheless, this information is of great interest because agents targeting the angiogenic pathway, which plays a key role in the regulation of angiogenesis, may have an important role in enhancing the efficacy of anti-EGFR agents. Furthermore, the present study demonstrates for the first time, the alterations on the expression levels of VEGF-A, VEGF-R1 and VEGF-R2 proteins, in NSCLC cell lines after therapy with antagonists of GHRH and bombesin/GRP.

To elucidate the molecular alterations leading to the inhibition of H-460 and A-549 tumor growth after therapy with antagonists of GHRH and bombesin/GRP, we analyzed the apoptotic pathway, by measurements of the pro-apoptotic and anti-apoptotic molecules, Bax and Bcl-2, respectively. A recent study based on 94 NSCLC specimens, demonstrated that the expression of Bcl-2 was correlated with a shorter patient survival time and that the over-expression of Bax was of no prognostic significance (44). We found that treatment of H-460 tumors with either GHRH or bombesin/GRP antagonists induced apoptosis that was accompanied by the up-regulation of Bax and down-regulation of Bcl-2. The ratio of the Bax to Bcl-2 levels was increased >100% in the groups treated with MZ-J-7-118 and RC-3940-II and by 57% in the MZ-J-7-114 treated animals. On the other hand, in A-549 NSCLC treated groups no significant variations of Bax or Bcl-2 were observed, although an inhibitory tendency in the Bcl-2 levels was detected. Once again, this provides evidence that both NSCLC cell lines, A-549 and H-460, respond through distinct molecular mechanisms after being submitted to the same therapy. Moreover, this study confirms a relevant role for apoptosis-regulatory proteins in the pathogenesis of lung cancer, suggesting the possible role of Bcl-2 as a prognostic factor in some type of NSCLC lung cancers.

This study presents strong evidence that tumors classified in the same NSCLC category and receiving identical therapy, can eventually end-up with the same final biological response, such as inhibition of tumor volume, although the molecular pathways triggered in those cell lines were not the same. The current study points to possible variation in the mechanism/pathway intrinsic to the tumor, which could explain the contradictory findings in the literature and also in the clinical trials.

The present work in H-460 and A-549 NSCLC models of lung cancer xenografted into nude mice extends the findings on the effectiveness of GHRH antagonists, MZ-J-7-114 and MZ-J-7-118, and bombesin/GRP antagonist, RC-3940-II, for the therapy of NSCLC lung cancers. The results of this study show that the inhibition of NSCLC growth by antagonists of GHRH and bombesin, is associated with an inhibition of EGFR and HER-2, -3, and -4 receptor family, a decrease in the levels of angiogenic related-factors, such as VEGF-A, VEGF-R1, and VEGF-R2, and a reduction in the levels of the anti-apoptotic Bcl-2 protein.

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