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INHIBITORY EFFECTS OF ANTAGONISTS OF BOMBESIN/GASTRIN RELEASING PEPTIDE (GRP) AND SOMATOSTATIN ANALOG (RC-160) ON GROWTH OF HT-29 HUMAN COLON CANCERS IN NUDE MICE

SINISA RADULOVIC, ANDREW V. SCHALLY, HERTA REILE, GABOR HALMOS, KAROLY SZEPESHAZI, KATE GROOT, SLOBODAN MILOVANOVIC, GLENN MILLER and TETSU YANO

Nude mice bearing xenografts of HT-29 human colon cancer cell line were treated for 4 weeks with somatostatin analog (RC-160), bombesin/gastrin releasing peptide (GRP) antagonists (RC-3095 and RC-3440). In three separate experiments somatostatin analog RC-160 (50 μg/day) released from microgranules significantly reduced tumor growth. Bombesin/GRP antagonists, RC-3095 and RC-3440 injected subcutaneously (s.c.) twice daily at a dose of 10 μg had the greatest and consistently significant inhibitory effect on tumor growth. RC-3095 given once daily s.c. at a dose of 20 μg was less effective. RC-3095 also inhibited metastatic tumor growth after intrasplenic injection of HT-29 cells in nude mice. Specific binding sites of somatostatin, bombesin and epidermal growth factor (EGF) were detected on intact HT-29 cells or on the membranes from HT-29 tumor xenografts. The inhibitory effects of bombesin antagonists on tumor growth were consistently linked with a significant down-regulation of EGF receptors. Bombesin/GRP antagonists and somatostatin analogs could be considered for the development of new hormonal therapies for colon cancer.

Although the mortality from colon cancer has declined over the past two decades, the 5-year survival rate remains at 50% (1). Surgical excision is an effective treatment only in patients diagnosed at an early stage of cancer, and new treatment modalities are needed for patients with advanced metastatic disease.

Considerable evidence supports the belief that human colon cancer is under hormonal control. Gender and parity have been shown to influence the incidence of colorectal cancer (2, 3). Androgen receptors are present on human colorectal tumors (4) and castration in rats can diminish the risk of carcinogen-induced bowel cancer (5). Harrison et al. (6) reported significant trophic effects of physiologic concentrations of estradiol on in vitro growth of HT-29 and LoVo human colon cancer lines. LH-RH agonist [D-Trp^6]LH-RH has been shown to inhibit the growth of nitrosamine induced pancreatic cancers in hamsters (7) possibly by elimination of sex steroids.

The tetradecapeptide somatostatin has a wide spectrum of biological actions, and appears to be an endogenous antiproliferative agent (8). Somatostatin inhibits the release and action of growth hormone (GH) as well as some gastrointestinal (GI) hormones including gastrin and also influences the secretion or action of growth factors like IGF-I and EGF (8, 9). These hormones and growth factors have been shown to stimulate growth of experimental colon cancer in vivo as well as proliferation of colon cancer cells in vitro (8, 10–13). Smith & Solomon (14) reported that somatostatin-14 could significantly inhibit tumor growth in vivo and gastrin-induced proliferation in vitro of three human colon cancer cell lines. RC-160, a

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From the Endocrine, Polypeptide and Cancer Institute, Veterans Affairs Medical Center, and Department of Medicine, Tulane University Medical School, New Orleans, Louisiana, USA (All Authors).
Correspondence to: Dr. Andrew V. Schally, VA Medical Center, 1601 Perdido Street, New Orleans, LA 70146, USA, phone (504) 589-5230; fax (504) 566-1624.
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superactive octapeptide analog of somatostatin, has been shown to inhibit the growth of experimental pancreatic tumors (7), prostatic cancers (15), mammary carcinoma (16) and other tumors. These effects of somatostatin analogs may be direct and mediated by somatostatin receptors present on tumor cells or induced by inhibition of the release or action of GI hormones and growth factors like IGF-I and EGF (8).

Gastrin releasing peptide (GRP), the mammalian counterpart of the amphibian neuropeptide bombesin, is a GI hormone, stimulating gastrointestinal enteropancreatic hormone release and pancreatic and intestinal epithelial growth (10, 12, 17). Specific GRP receptors are found throughout the mammalian GI tract. That bombesin/GRP can function as autocrine growth factors in human small cell lung carcinoma (SCLC) is widely accepted (18). However, the involvement of bombesin/GRP in the promotion of proliferation of GI neoplasms is still unclear. We have synthesized various short chain pseudononapeptide antagonists of bombesin/GRP, including [D-Tpi^6, Leu$^{13}\Psi$(CH_2NH)Leu^{14}] bombesin (6–14) (RC-3095) and [Tpi^6, Leu$^{13}\Psi$(CH_2N)Tpi^{14}] bombesin (6–14) (RC-3440) (19, 20). These potent antagonists in nanomolar concentrations block GRP-stimulated amylase release from rat pancreatic acini, show strong binding affinity to Swiss 3T3 and H-346 SCLC cells and inhibit GRP-stimulated growth of these cells in vitro (19, 20). Their in vivo activity was also demonstrated in various systems (17, 19).

Previously, we reported briefly that somatostatin analog RC-160 and bombesin antagonist RC-3095 could inhibit the growth of HT-29 colon cancers in nude mice, while agonist [D-Trp^6]LH-RH was ineffective (21). The present report describes additional studies on the effects of somatostatin analog RC-160 and two bombesin-GRP antagonists RC-3095 and RC-3440 on the growth of xenografts of human colon cancer cell line HT-29 in nude mice and the evaluation of the levels of epidermal growth factor (EGF) receptors in treated tumors. The influence of RC-3095 administration on the hepatic metastases of HT-29 line was also investigated.

Material and Methods

Animals and tumors

Athymic male nude (nu/nu) mice, approximately 6 weeks old on arrival, were obtained from the National Cancer Institute (Bethesda, MD, USA). They were housed in sterile plastic cages and placed in continuous laminar air flow hoods in a temperature-controlled room with an automatic light/dark schedule (12 h light: 12 h darkness). Commercially available pelleted diet, bedding, water bottles and water were sterilized. All manipulations of mice were carried out using sterile gloves under a horizontal laminar air flow hood.

The human colonic adenocarcinoma cell line HT-29 was routinely grown as a monolayer in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% colostrum-free bovine serum and antibiotics and antimycotics at 37°C in a humidified 5% CO_2 atmosphere. Tumor cells growing exponentially were harvested by brief incubation with 0.25% Trypsin-EDTA solution (Gibco). Xenografts were initiated by subcutaneous (s.c.) injection of 1×10^7 cells into the right flank of nude mice. The resultant tumors were aseptically dissected, mechanically minced and 2 mm^3 pieces of tumor tissue implanted s.c. with a trocar needle into animals. Two weeks after transplantation, tumors had grown to a volume of approximately 20 mm^3 and the animals were divided into experimental groups with approximately equal average tumor volumes.

Peptides and sustained delivery systems

Microgranules of pamoate salt of somatostatin analog RC-160 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2), in poly(DL-lactide-co-glycolide) (22) were prepared by P. Orsolini at Cytotech (Martigny, Switzerland) and supplied by Debiopharm (Lausanne, Switzerland) and designed to release about 50 μg/day RC-160 for two weeks from an aliquot of 16 mg (22). Sterile suspensions of microgranules of RC-160 were injected every 15 days s.c. through an 18-gauge needle translateral to tumor. For the injection, microgranules were suspended in 0.7 ml of injection vehicle solution containing 2% carboxymethylcellulose and 1% Tween 80 in water. Bombesin antagonist RC-3095, originally synthesized in our laboratory by solid phase methods (20) was made by Asta Medica (Frankfurt/M, Germany). Bombesin antagonist RC-3440 was synthesized by standard solid phase methodology after formation of Boc-LeuΨ(CH_2N)Tpi-BHA resin from LeuΨ(CH_2NH) Trp-BHA resin by reaction with formaldehyde (19). Tpi is Trp analog 2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indol-3-carboxylic acid. Crude product was purified by reverse-phase HPLC on a Vydac C_{18} support and amino acid analyses performed with a Beckman 6300 amino acid analyzer. Bombesin was also synthesized in our laboratory by solid phase methods.

Experimental protocol

Experiment 1. The mice were divided into 5 groups (10–13 animals per group). Group 1, the control, was injected with the vehicle only, group 2 with [D-Trp^6]LH-RH microcapsules and group 3 with RC-160 microgranules. Group 4 was treated with RC-3095 dissolved in 0.1% dimethyl sulfoxide (DMSO) in saline and injected s.c. at a dose of 10 μg twice daily. Group 5 consisting of 13 animals received a continuous s.c. infusion of RC-3095 by osmotic minipumps (Alzet Corp., Palo Alto, CA, USA, model

2002), delivering 0.5 μl/h and corresponding to 20 μg/day of peptide. RC-3095 was dissolved in 50% (v/v) propylene glycol in water, and the osmotic minipumps were filled and implanted as described previously (21).

Experiment 2. Xenografts were obtained from the tumor of a control animal from experiment 1. Tumor transplantation and treatment were performed in the same way as in experiment 1. The mice were divided into 3 groups (8–10 per group). Group 1, the control, was injected with vehicle only, group 2 with RC-3440 dissolved in 0.1% DMSO in 0.9% saline at a dose of 10 μg twice daily s.c., and group 3 with bombesin, 5 μg s.c. twice daily.

Experiment 3. The mice with HT-29 xenografts were divided into 3 groups (10 per group). Group 1, the control, was injected with vehicle, group 2 was treated with RC-160 microgranules releasing 50 μg/day, and group 3 with RC-3095 (dissolved in 0.1% DMSO in 0.9% saline) at a dose of 10 μg twice daily s.c.

Experiment 4. The same experimental protocol as in experiment 3 was used, with the exception that RC-3095 was administered once daily at a dose of 20 μg/day.

The tumors were measured twice weekly for 4 weeks. The tumor volume was calculated using the following formula: length × width × height × $\pi/6$ (23). Tumor volume doubling time was calculated during the logarithmic phase of tumor growth which occurred in experiments 1 to 3 between days 14 and 27 and for experiment 4 between day 11 and day 22. The inhibition of tumor growth after various treatments was represented by tumor growth delay time, i.e. the time difference in days required for the tumor to reach a volume of 300, 250, 250 and 150 mm^3 in all animal groups in experiments 1, 2, 3 and 4 respectively. Tumor burden at the end of the treatment period was calculated as mg of tumor weight per g of body weight.

After 27 days of treatment, mice were fasted overnight, anesthetized with Metofane (Pitman-Moore, Mundelein, IL, USA) and sacrificed by decapitation and trunk blood was collected. Serum was separated and stored at −20°C for further analyses. Body weights were recorded and various organs were removed, cleaned and weighed. Tumors were carefully cleaned and weighed, and then a sample was fixed in 10% buffered neutral formalin for histological examination and the rest was stored at −80°C for receptor studies.

Intrasplenic injection of HT-29 cells in nude mice

A piece of the growing tumor from a control animal in experiment 1 was cut into small fragments and incubated for 45 min in a Dubnoff incubator in 5 ml of enzyme solution [RPMI 1640, 1% collagenase (Type I, 162 units/mg), 0.25% bovine serum albumin (BSA, RIA grade), gentamicin and penicillin, and freshly added 0.03% (w/v) bacitracin, 0.01% (w/v) trypsin inhibitor and 15 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES)]. The suspension was incubated in 95% O_2 and 5% CO_2 and shaken at 120 oscillations per min at 37°C. A second 45 min digestion period followed in 5 ml incubation solution supplemented with 50 μM phenylmethylsulphonyl fluoride (PMSF). After this incubation the fragments of tissue were easily dispersed by 10–20 passes through 5 ml pipette with an automatic pipettor and filtered through 200 μm Nitex gauze. Tumor cells were washed 3 times in RPMI 1640. An aliquot was removed, cells were counted using a hemocytometer and viability was assessed by trypan blue exclusion. Mice were anesthetized, a left subcostal incision made to open the abdomen and one million viable HT-29 cells in total volume of 50 μl was injected into the exposed spleen. Peritoneum and skin were sutured and animals randomly divided into two groups of 6 animals. One group, the control, received vehicle only and animals in the second group were injected s.c. twice daily with 10 μg of RC-3095. Treatment started 3 days after surgery and lasted 45 days. The mice were sacrificed under light methoxyflurane anesthesia and spleen and liver were removed and weighed.

Histological procedures

Specimens were embedded in Paraplast (Oxford Labware, St. Louis, MO). Step-sections 6 μm thick were cut and stained with hematoxylin-eosin. For the determination of the number of mitotic or apoptotic cells (7, 16), 4 000 cells were considered in each tumor and the number of mitotic and apoptotic cells per 1 000 tumor cells was calculated. The extent of necrosis was measured on the slide containing the largest cross-section of tumors, and the percentage area of necrosis was calculated. The crossing points of an ocular net that hid or coincided with necrosis in tissue sections were counted. The ratio of these points to the number of all points on the slide represented the ratio of volume of necrosis to the volume of the tumor tissue. For demonstration of the nucleolar organizer region (NOR) in tumor cell nuclei the AgNOR method described earlier was used (24). NOR is a good indicator of tumor cell proliferation. All counts were carried out without knowledge of treatment.

Determination of serum gastrin, growth hormone and IGF-I levels

Serum levels of growth hormone were measured by radioimmunoassay (RIA) as described previously (22). Serum IGF-I levels were determined with the use of anti-IGF-I antiserum (UKB-487) provided by National Institute of Diabetes and Digestive and Kidney diseases. Serum gastrin levels were determined by specific RIA using Gastrin^{125}I RIA kit from Becton Dickinson (Orangeburg, NY).

Receptor assays for bombesin, somatostatin and EGF

Radioiodination of Tyr^4-bombesin, Tyr^{11}-somatostatin-14 (Tyr^{11}-SS-14), was performed as previously described

(20, 25) and iodinated EGF was purchased from Amersham (Arlington Heights, IL). Samples of HT-29 xenografts were cleaned of connective tissue, cut into small slices and homogenized in 5 volumes of sucrose buffer (0.3 M sucrose, 25 mM Tris base, 0.25 mM PMSF, 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid), 10 mM monothioglycerol and Trasylol (aprotinin) at 10 000 kallikrein inactivator units/l, pH = 7.5) using an Ultra-Turrax homogenizer (Tissumizer, Tekmar, Cincinnati, OH) at maximal speed for 5 × 5-s strokes at 0°C. The homogenate was centrifuged at 500 × g for 10 min at 4°C. The supernatant containing the crude membrane fractions was then ultracentrifuged at 70 000 × g for 45 min at 4°C (Beckman Preparative Ultracentrifuge, Beckman Instruments, Inc., Palo Alto, CA). The resulting pellet was resuspended in wash buffer (25 mM Tris base, 1 mM dithiothreitol, 1 mM EDTA (ethylenediaminetetraacetic acid), 0.25 mM PMSF) and used for the receptor binding studies. The chemicals used for buffers were purchased from Sigma Chemical Co. (St. Louis, MO). Receptor assays on membrane preparation from tumor xenografts were performed as previously described (25).

The binding assays of Tyr^{11}-SS-14 were conducted on confluent HT-29 cells in 24-well plates. The cells were washed twice with RPMI 1640 and the binding reaction performed in Hanks Balanced Salt solution (HBSS) containing 0.1% BSA, 5 mM $MgCl_2$, 20 mM HEPES and 100 μg/ml bacitracin (pH 7.4). Displacement curves were obtained with 0.05 nM [^{125}I]Tyr^{11}-SS-14 in the presence of increasing amounts of unlabeled SS-14 (10^{-10}–10^{-5} M). The cells were incubated at 37°C for 60 min, as this was found to give optimal binding. At the end of the incubation period, binding reaction was stopped by adding 1 ml of ice-cold HBSS and cells were washed once more with the same amount of HBSS. The cells were detached by 0.6 ml of 0.25% trypsin solution and a sample of 0.5 ml was transferred in tubes for counting.

The LIGAND-PC computerized curve fitting program of Munson & Rodbard (26) was used to determine the types of receptor binding, dissociation constant (K_d) and the maximal binding capacity of receptors (B_{max}).

Statistical analysis. All data are expressed as the mean ± SEM. Statistical evaluation of data was performed by Student's t-test and Duncan's new multiple range test as previously described (22).

Results

Tumor growth and pathology

Xenografts were established in about 90%–95% of injected animals in all experiments. Fig. 1 shows the tumor volume in experiments 2, 3 and 4 as measured at 3–4 day intervals. The effects of various treatments on final tumor volume, percentage increase in tumor volume, tumor burden, tumor doubling or tumor growth delay time in these experiments are shown in Table 1. The results of experiment 1 were published earlier (21).

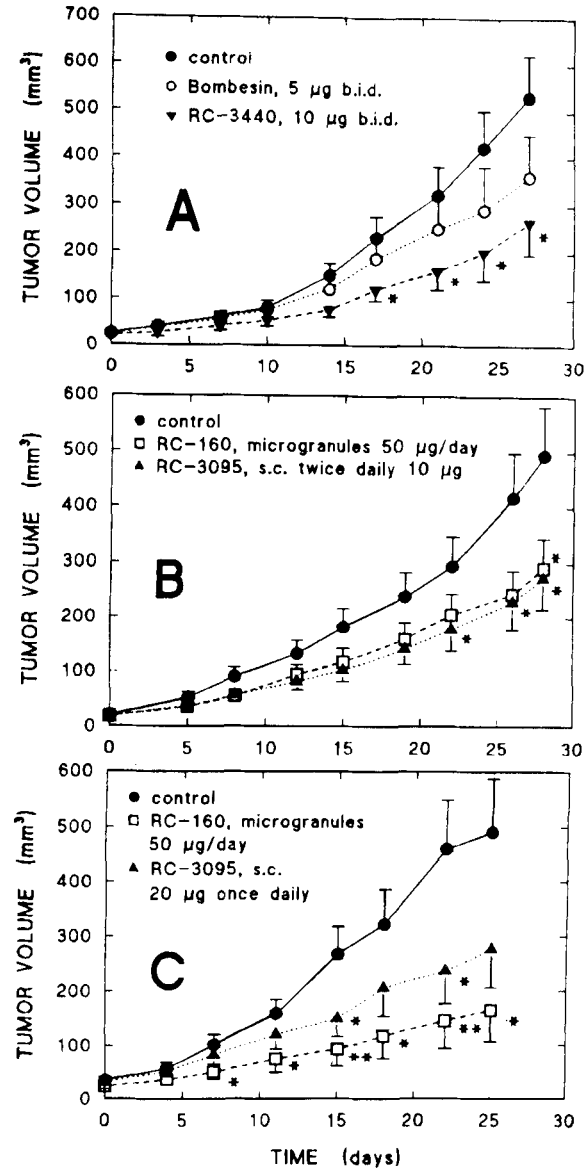

Fig. 1. Tumor volume in nude mice bearing HT-29 human colon carcinomas during treatment with bombesin/GRP antagonist RC-3440 given by s.c. injections and bombesin (s.c. injections) (A) and microgranules of RC-160 and RC-3095 s.c. injection (B and C). Vertical lines indicate the S.E.M.; *$p < 0.05$; **$p < 0.01$. Significance was determined by Student's t-test (A, B) and by Duncan's multiple range test (C).

Therapy with bombesin antagonist RC-3095, administered by osmotic minipumps in doses of 20 μg/day or by s.c. injections (10 μg b.i.d.), was the most effective among various treatments in experiment 1 and resulted in the greatest inhibition of the tumor weight, tumor volume and percentage increase in tumor volume. Chronic treatment

Table 1

Effect of treatment with various peptides on tumor volume, percentage change in tumor volume, tumor burden, tumor doubling time and tumor growth delay time in nude mice with xenografts of the human colon cancer cell line HT-29

Treatment group	Tumor volume, mm^3		Percentage increase in tumor volume from day 0	Tumor burden mg/g body weight	Tumor doubling time (days)	Growth delay days (days)
	Initial	Final				
Experiment 2						
Control	25.6 ± 4.5	523 ± 70	2043 ± 228	19.8 ± 3.5	6.5 ± 0.4	—
RC-3440[a]	24.7 ± 4.5	277 ± 87[e]	1121 ± 128[e]	11.9 ± 3.2[e]	9.5 ± 0.5[e]	7.5
Bombesin[b]	22.5 ± 5.1	357 ± 85	1764 ± 300	16.9 ± 5.8	8.1 ± 0.6	3
Exeriment 3						
Control	19.8 ± 4	494 ± 88	2386 ± 408	17.2 ± 3.1	7.0 ± 0.5	—
RC-160	18.7 ± 3	291 ± 53[e]	1717 ± 213	9.9 ± 3	9.0 ± 0.5	6.5
RC-3095[a]	18.3 ± 3	272 ± 57[e]	1276 ± 243[e]	8.9 ± 3.2	11.0 ± 0.6[e]	7.5
Experiment 4						
Control	34.9 ± 6.1	491 ± 97	1296 ± 91	19.4 ± 3.3	7.7 ± 0.6	—
RC-160	23.1 ± 5.0	165 ± 58	557 ± 168[d]	4.9 ± 1.6[e]	–N.D.	12.2
RC-3095[c]	30.9 ± 5.8	277 ± 71	765 ± 151[e]	20.3 ± 6.7	13.8 ± 2.0	4.2

[a] RC-3095 and RC-3440 were injected s.c. at dose of 10 μg twice daily.
[b] Bombesin was injected s.c. at dose of 5 μg twice daily.
[c] RC-3095 was injected s.c. once daily at a dose of 20 μg.
N.D. Since 3 animals of this group showed tumor regression, the determination of tumor doubling time could not be calculated.
[d] $p < 0.01$; [e] $p < 0.05$, significance calculated by Duncan's multiple range test.
Values are mean ± SE.

with somatostatin analog RC-160 inhibited tumor growth, but this suppression was significant as compared with controls only after 21 days and after 24 days. At the end of the experiment, the tumor volume, as well as final tumor weight, were still reduced, but not significantly different compared with controls (21).

In the second experiment, treatment with bombesin antagonist RC-3440 by twice daily s.c. injection significantly inhibited tumor growth (about 45% inhibition vs. control), prolonged tumor doubling time to 9.5 days and tumor growth delay time was 7.5 days. Interestingly, twice daily s.c. injections of 5 μg bombesin inhibited tumor growth by about 30% compared with control, but the effect was not statistically significant (Fig. 1A and Table 1).

In the third and fourth experiment, a significant inhibitory effect of RC-160 and RC-3095 on tumor growth was again obtained (Fig. 1B and C and Table 1). In experiment 4, treatment with a single daily injection of 20 μg RC-3095 was less effective in inhibiting tumor growth than twice daily injections of RC-3095 in experiments 1 and 3.

At the end of all experiments, there were no significant differences in body weights between various treated and control groups. Histologically, the tumors in the control and treated groups were moderately differentiated adenocarcinomas. The tumor cells were large, cylindrical or cuboidal, and arranged as solid nests or irregular glands with multilayer epithelial lining. Numerous mucin-containing cells were found in some areas and a mucinous content in glandular lumina. The tumorous glands were supported by a delicate and scant stroma. Smaller or larger foci of necrosis were found in the central area of most of the tumors. No difference could be detected in histological structure of tumors between treated and untreated groups. The quantitatively measured parameters: the extent of necrosis, the number of mitotic cells, the frequency of apoptosis or the number of AgNORs showed no significant changes after treatment with peptides compared with control values.

Serum gastrin, growth hormone and IGF-I levels in nude mice were measured after termination of experiments 1, 2 and 3. In the first experiment, the levels of serum gastrin in animals treated with RC-3095 given by minipumps or daily s.c. injections were decreased by about 30% compared to control levels (21). Gastrin levels were also decreased in the second experiment in animals which were treated with antagonist RC-3440 (67.8 ± 11 pg/ml vs. 97 ± 15 pg/ml for controls; $p < 0.05$), but bombesin did not cause significant changes in serum gastrin. In experiment 1, in animals treated with RC-160, serum gastrin levels were higher, but did not differ significantly from control values (21). In the third experiment treatment with RC-3095 did not change serum gastrin levels, whereas in animals treated with RC-160 an increase in serum gastrin

Table 2

Effects of treatment with bombesin antagonist RC-3095 (10 μg twice daily s.c.) on tumorous liver and tumorous spleen weights and on number of liver metastatic nodules in nude mice after intrasplenic injection of HT-29 cells

	Control	RC-3095	p[a)]
Liver (mg)	1948 ± 207	1475 ± 69	0.05
Spleen (mg)	456.8 ± 74	273.7 ± 93	0.05
No. of tumor nodules in livers	9.1 ± 1.1	3.6 ± 0.8	0.01

[a)] Significance determined by Duncan's new multiple range test. Treatment started 3 days after surgery and lasted 45 days. Values are mean ± SE.

levels was observed. There were no changes in serum growth hormone levels after chronic treatment with the peptide analogs tested. Only bombesin significantly increased serum growth hormone levels in experiment 2. Serum IGF-I levels were decreased in all treatment groups in the first experiment, and maximal reduction occurred in animals receiving RC-3095 by s.c. injection ($p < 0.05$) (21). There was no change in IGF-I in animals treated with RC-160 and RC-3095 in experiment 3.

Intrasplenic injection of HT-29 cells

Forty-five days after the injection of HT-29 cells into the spleen of the nude mice, local tumor growth was found in all animals. Liver metastases were found in all animals except in one treated with RC-3095. The number of metastatic liver nodules in control mice was 9.1 ± 1.1 and in the group treated with RC-3095 3.6 ± 0.8 ($p < 0.01$). Chronic treatment with bombesin antagonist RC-3095 significantly reduced the weights of tumorous liver and spleen in nude mice as compared to untreated animals (Table 2).

Receptor assays

The binding of ^{125}I-labelled Tyr^{11}-SS-14 to intact HT-29 cells was found to be temperature and time dependent, being optimal at 37°C and stable between 60 and 180 min. The total binding was found to be 8.79% with 88.3% specific binding. There was no displacement with any of the following: insulin, Substance P, [D-Trp^6]LH-RH or GRP (14–27). The displacement of labelled SS-14 by native SS-14 (Fig. 2) and Scatchard analysis of these data indicate that in HT-29 cells, labelled peptide was bound to a single class of binding sites. SS-14 displaced half of the specific ^{125}I-Tyr^{11}-SS-14 binding at concentration of 298 nM.

Very low (less than 1% of total binding) of labelled Tyr^4-bombesin has been detected only on the membranes from control HT-29 tumor xenografts, but not on RC-3095 treated tumors. However, this binding was 78% specific and analysis of binding data revealed the presence of a single class of high affinity ($K_d = 0.07$ nM) binding sites with very low maximal binding capacity (B_{max} about 2 fmol per mg protein).

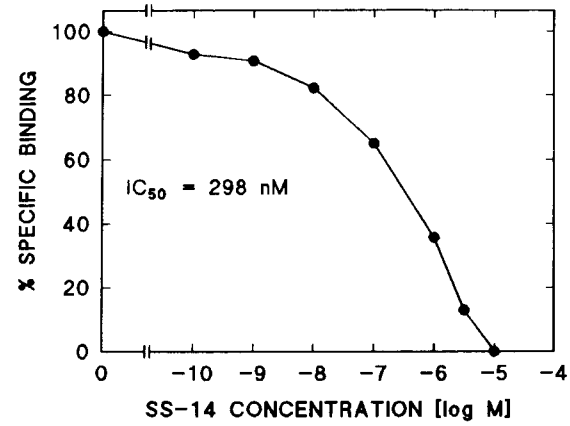

Fig. 2. Displacement of ^{125}I-SS-14 by unlabelled SS-14 from HT-29 cells. The cells were incubated in 24-well plates for 60 min at 37°C with 0.05 nM iodinated SS-14 in the presence of increasing concentrations of unlabelled SS-14 (10^{-10}–10^{-5} M). The individual data points are means of triplicate determinations.

The results of EGF receptor assays on membrane preparations from HT-29 xenografts from untreated animals and after treatment with different peptides are shown in Table 3. High affinity EGF binding occurred in mem-

Table 3

Binding characteristics of EGF receptors in membranes of HT-29 human colon cancer in nude mice after in vivo treatment with various peptides

Treatment	EGF	
	K_d(nM)	B_{max} (fmol/mg protein)
Experiment 1		
Control	2.1 ± 1.2	320 ± 69
RC-160	3.3 ± 1.4	94 ± 20
RC-3095	1.3 ± 0.7	26 ± 10[a)]
Experiment 2		
Control	1.7 ± 0.4	660 ± 176
RC-3440	0.3 ± 0.2	55 ± 19[a)]
Bombesin	0.4 ± 0.2	87 ± 25[a)]
Experiment 3		
Control	0.7 ± 0.2	195 ± 14
RC-160	1.4 ± 0.2	52 ± 6[b)]
RC-3095	1.4 ± 0.4	35 ± 3[b)]
Experiment 4		
Control	1.2 ± 0.3	260 ± 40
RC-160	2.2 ± 0.02[a)]	97 ± 4[b)]
RC-3095	4.4 ± 0.4[b)]	101 ± 28[b)]

Significance was calculated by Duncan's multiple range test, [a)] $p < 0.05$, [b)] $p < 0.01$
Values are the mean ± SEM

branes from HT-29 xenografts. Compared to EGF binding to intact HT-29 cells (27), maximal EGF binding capacity increased about 20 times (from 15 to 320 fmol/mg protein) after the cells were xenografted into nude mice. A highly significant reduction in EGF-binding capacity after treatment with bombesin antagonist RC-3095 was observed in experiments 1, 3 and 4. Similarly, in experiment 2, a significant reduction in EGF binding capacity was found after treatment with bombesin antagonist RC-3440 as well as with bombesin. A decrease in EGF-binding capacity also occurred after treatment with RC-160, but it was smaller than that induced by RC-3095 in experiments 1 and 3.

Discussion

HT-29, a human colon adenocarcinoma cell line, retains certain characteristics of the cells of origin. This cell line expresses receptors for gastrin and exhibits trophic responses to gastrin (13, 14). Gastrin stimulates growth of HT-29 cells in vitro and in vivo when the cells are xenografted into nude mice (13). This cell line also secretes the growth factors IGF-I and EGF (28). In our previous study, EGF binding sites were demonstrated on HT-29 cells (27). The present study showed somatostatin receptors and possibly specific binding sites for bombesin in this cell line. Consequently, HT-29 appears to be a suitable model for the study of the influence of hormonal manipulation on tumor growth. Results presented in this paper demonstrate that somatostatin analog RC-160 and bombesin antagonists RC-3095 and RC-3440 can inhibit the growth of this human colonic cancer line in nude mice. The modest reduction in growth of HT-29 tumor xenografts induced by chronic administration of the LH-RH agonist [D-Trp^6]LH-RH (21) may be due to sex steroid deprivation, considering the suggested dependence of colorectal tumors on sex steroids (5, 8).

A significant inhibitory effect of the potent somatostatin analog RC-160 on the growth of colonic tumor xenografts was shown in several experiments. As somatostatin and its analogs inhibit release and action of gastrin, the suppression of serum gastrin levels during therapy with RC-160 would be expected. Some elevation of serum gastrin observed in the RC-160 treated mice at the end of the experiments 1 and 3, possibly due to the rebound phenomena, suggests that by this time levels of circulating RC-160 released from microgranules were too low to suppress gastrin release (21). The same phenomenon i.e. low levels of RC-160 could be also used to explain poor suppression of GH and IGF-I levels at the end of experiment 3. Nevertheless, growth inhibition which occurred in response to RC-160 in experiment 1, 3 and 4 may be due to elimination of the stimulatory effect of gastrin on the HT-29 xenografts, directed effects upon somatostatin receptors or to inhibition of IGF-I secretion. We have demonstrated specific binding sites for somatostatin on HT-29 cells in culture, from which radiolabelled somatostatin can be displaced by unlabelled somatostatin. Somatostatin analogs may also act by inhibiting secretion of IGF-I through the suppression of GH or by interference with EGF receptors. Liebow et al. (29) found that RC-160 caused the dephosphorylation of EGF receptors. This phenomenon nullifies the EGF-induced growth of the human pancreatic cancer cell line MiaPaCa-2 (29). It is possible that similar phenomena occur with EGF receptors on HT-29 cells. RC-160 was recently found to inhibit the growth of DHD/K12 rat colon cancer in vivo (30), 320 DM and WidR human colon cancers in nude mice (31) and hepatic metastases of these cancers (31, 32).

The present study confirms and extends our observations on significant inhibitory action of bombesin/GRP antagonist RC-3095 on human colon cancer xenografts and shows that a similar effect can be also obtained with antagonist RC-3440. After the treatment with antagonists RC-3440 or RC-3095, 45%–60% tumor growth inhibition was obtained. The mechanism of this effect is still not fully elucidated, although several concepts can be proposed. Tumor growth inhibition may be produced by blockade of the effects of endogenous GRP on gastrin production, and the consequent elimination of the stimulatory action of gastrin on HT-29 xenografts. However, gastrin suppression with RC-3095 and RC-3440 was inconsistent and when it was produced as in experiment 1, it was only equivalent to that caused by [D-Trp^6]LH-RH, while the tumor growth inhibitory effects of bombesin antagonists were vastly superior (21). Therefore, gastrin suppression may only partially explain the inhibitory effect induced by our bombesin antagonists. Another hypothesis is that bombesin/GRP antagonists RC-3095 and RC-3440 block the stimulatory effect of GRP on growth of colon cancer by an action on bombesin receptors. Recent studies have shown the presence of functional binding sites for bombesin/GRP in the human colon cancer cell line, NCI-H716, and murine colon cancer cell line MC-26 (33, 34). Bombesin stimulated tumor growth in MC-26 cell line in vitro, and its binding to the MC-26 cells was inhibited by bombesin antagonists (34). The present study demonstrates that very low concentrations of binding sites for bombesin are found on HT-29 cell line xenografts, but not on membranes from tumors treated with bombesin antagonists. This could be due to high binding affinity of bombesin/GRP antagonists to bombesin/GRP receptors (20) and a consequent down-regulation of these receptors. We reported previously that intact HT-29 cells did not appear to express bombesin receptors (27), but these receptors might have been down-regulated by long term culture or by growth factors present in serum. The presence of specific bombesin receptors on HT-29 cells or tumor xenografts needs to be confirmed by additional investigations.

The most likely mechanism of antitumor activity of bombesin antagonists may be based on the interference with the receptors for EGF. Reports of EGF and IGF-I production and elevated expression of IGF-I receptors by HT-29 cells (28), suggest that these growth factors may function as autocrine stimulants of cellular proliferation. Our previous work showed some suppression of circulating IGF-I levels in animals treated with bombesin antagonists (21). Above all, a significant down-regulation (60–90% reduction of B_{max} compared with control) of EGF receptors found in HT-29 xenografts after treatment with RC-3095 or RC-3440, might provide the best explanation for the tumor growth inhibition induced by bombesin/GRP antagonists. Our studies on EGF receptor concentration in nitrosamine induced pancreatic cancer in hamsters (7, 35), MXT mammary cancers in mice (36) and PC-82 prostate cancers in nude mice (15) have similarly demonstrated a major down-regulation of EGF-receptors induced by RC-3095 which would be in agreement with similar events in Swiss 3T3 cells after exposure to bombesin (37). Thus, bombesin/GRP antagonists may act locally by reducing available binding sites for EGF. Recently Liebow et al. (38) showed in several cancer lines that bombesin and GRP enhanced the phosphorylation induced by EGF and RC-3095 inhibited this phosphorylation. This suggests that GRP may upregulate EGF receptors and that RC-3095 prevents the upregulation of EGF receptors caused by bombesin/GRP.

Bombesin antagonist RC-3095 also reduced tumor growth after injection of HT-29 cells into the spleen of nude mice. From this site of injection, tumor cells gain access to the hepatic portal bloodstream and then reach the liver to proliferate into secondary tumor colonies. Liver is known to be the host for organ-specific metastatic growth of colorectal carcinoma, presumably because of a high content of EGF (39). The effect of bombesin antagonists on intrahepatic HT-29 cell proliferation might also indicate that there is interaction between bombesin and EGF. EGF binding to specific surface receptors in Swiss 3T3 cells was stated to be markedly inhibited by bombesin (40). In the present study bombesin caused some inhibition of growth of HT-29 colon cancer cell line xenografts in nude mice, although its effects were not significant. Previously, bombesin was reported to inhibit the growth of human pancreatic xenografts in nude mice (41) and nitrosamine-induced pancreatic cancers in hamsters (35). There is much evidence indicating that chronic administration of a hormone can down-regulate its receptors or other receptors inhibiting further response to the hormone. Down-regulation of receptors after chronic administration has been reported for bombesin itself (40, 42) and has been seen with other hormones such as LH-RH (43, 44). The inhibitory effects on the growth of pancreatic cancer and other cancers, produced by chronic administration of bombesin and GRP and possibly mediated by down-regulation of EGF receptors (35, 45 and this study), appear to resemble in some respects the actions of LH-RH agonists (43, 44). Interference with EGF-receptor pathways might be the common mechanism in the inhibitory effect of both agonists and antagonists of bombesin/GRP (35).

Our findings support the merit of further investigations of modern somatostatin analogs like RC-160 and new bombesin/GRP antagonists, such as RC-3095 or RC-3440, in experimental colon cancer models and suggest that a new therapy for this malignancy could be based on somatostatin analogs and bombesin antagonists.

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