Inhibition of the Growth of Caki-I Human Renal Adenocarcinoma In Vivo by Luteinizing Hormone–Releasing Hormone Antagonist Cetrorelix, Somatostatin Analog RC-160, and Bombesin Antagonist RC-3940-II

Andreas Jungwirth, M.D.[1,2]
Andrew V. Schally, Ph.D., M.D.h.c.[1,2]
Gabor Halmos, Ph.D.[1,2]
Kate Groot, Ph.D.[1]
Karoly Szepeshazi, M.D.[1,2]
Jacek Pinski, M.D.[1,2]
Patricia Armatis, M.S.[1]

[1] Endocrine, Polypeptide and Cancer Institute, Veterans Affairs Medical Center, New Orleans, Louisiana.

[2] Section of Experimental Medicine, Tulane University School of Medicine, New Orleans, Louisiana.

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At the time this manuscript was prepared, Dr. Jungwirth was on leave from the Department of Urology, Salzburg General Hospital, Salzburg, Austria.

Address for reprints: Andrew V. Schally, Ph.D., M.D.h.c., Veterans Affairs Medical Center, 1601 Perdido Street, New Orleans, LA 70146.

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BACKGROUND. Metastatic or recurrent renal cell carcinoma (RCC) is a therapeutic challenge because it is resistant to chemotherapy and external radiotherapy. No uniformly effective therapeutic agents are available for the management of patients with RCC. Hormones and growth factors may play a role in promoting the transformation and/or proliferation of kidney neoplasms.

METHODS. Luteinizing hormone–releasing hormone (LH-RH) antagonist Cetrorelix (SB-75), somatostatin analog RC-160, and bombesin antagonist RC-3940-II were tested for their effects on the growth of the Caki-I renal adenocarcinoma cell line xenografted into nude mice.

RESULTS. After 4 weeks of treatment, tumor volume was significantly ($P < 0.01$) decreased in animals receiving RC-160, to 167.5 ± 34.2 mm^3, compared with the control group (485.7 ± 77.2 mm^3). LH-RH antagonist SB-75 and bombesin antagonist RC-3940-II also significantly reduced the volume of Caki-I tumors, to 159.9 ± 18.1 and 234.7 ± 81.8 mm^3, respectively. Somatostatin analog RC-160 decreased serum levels for growth hormone (GH) and insulin-like growth factor-I compared with controls. Treatment with RC-160, Cetrorelix, and RC-3940-II significantly reduced the number of high-affinity receptors for epidermal growth factor on Caki-I tumors.

CONCLUSIONS. LH-RH antagonist Cetrorelix, somatostatin analog RC-160, and bombesin antagonist RC-3940-II effectively inhibit the growth of human Caki-I renal adenocarcinomas in nude mice. These peptide analogs should be considered for the therapy of patients with metastatic or recurrent RCC. ***Cancer*** **1998;82:909–17.** © *1998 American Cancer Society.*

KEYWORDS: renal cell carcinoma, somatostatin analog, luteinizing hormone–releasing hormone antagonist, bombesin antagonist, anticancer therapy.

Renal cell carcinoma (RCC) is the most common malignant neoplasm of the kidney.[1,2] RCC occurs more frequently in men than in women, with a ratio of nearly 2:1, and its incidence seems to increase slowly.[1,2] It is expected that in 1996, approximately 30,000 patients in the U.S. were diagnosed with kidney cancer[1–2] and that about 12,000 deaths occurred from this malignancy. It is also estimated that annual deaths from kidney cancer around the world may exceed 100,000.[1,2] The unusual natural history of RCC, including spontaneous regression, delayed growth of metastatic lesions, and varying tumor doubling times, makes the prediction of the clinical course for

the individual patient difficult. About 30% of patients with RCC have evidence of metastatic disease at initial presentation, and the 2-year survival rate in this group is poor, varying from 10% to 20%.[2] For the advanced stages of RCC, no uniformly effective systemic therapy is available. Cytotoxic chemotherapy shows marginal efficacy at best and fails to show statistically significant survival advantages for responding patients.[3] Radiotherapy has proven ineffective for the treatment of local recurrent or metastatic disease.[4] Immunotherapy involving the administration of interferons and interleukins has shown some promising results but is still in experimental phases.[4]

The regulatory mechanisms responsible for malignant transformation, tumor progression, and metastasis in RCC are still unclear, but there is some evidence that hormones such as growth hormone (GH) and somatostatin and growth factors such as EGF, transforming growth factor (TGF)-α and -β, angiogenin, and tumor necrosis factor-α might have direct or indirect regulatory effects on the behavior and growth of this malignancy.[4–6] Somatostatin receptors were detected in 72% of the samples of surgically removed human RCCs.[5] Mydlo et al. have shown that TGF-α and EGF receptors are both overexpressed in kidney tumor tissue, pointing to a possible autocrine control of tumor cell proliferation.[7] All together, these findings suggest that hormonal treatment with agents that interfere with the stimulatory action or receptors of these growth factors might be of some benefit in the treatment of RCC.

The Caki-I cell line was established in 1971 and derived from a human renal adenocarcinoma that was metastatic to the skin.[8] This cell line can be xenografted into nude mice, forming poorly differentiated tumors.[9] It has been demonstrated that monoclonal antibodies against the Caki-I cell line react with antigens present in most human RCCs but not with normal adult kidney tissue.[10] Caki-I has been shown to express the c-*myc* oncogene, which is known to stimulate EGF receptor expression and the multidrug resistant phenotype (gp170).[11] This cell line is susceptible to cytokines and interferons and shows many similarities to native human RCC.[11]

Somatostatin inhibits cell proliferation and the secretion of various hormones.[5,12–17] A potent somatostatin analog RC-160, synthesized in our laboratory,[12] has been shown to inhibit the growth of pancreatic, colorectal, gastric, mammary, and prostate carcinomas, small cell and nonsmall cell lung carcinomas, and brain tumors in experimental animals.[13–17] The antitumor activity of RC-160 was associated with a significant down-regulation of EGF receptors in membranes of these tumors.[13–17]

Cetrorelix (SB-75) is a powerful luteinizing hormone–releasing hormone (LH-RH) antagonist that causes an immediate inhibition of secretion of LH, FSH, and sex steroids.[17,18] Cetrorelix suppresses the proliferation of prostate, mammary, and ovarian carcinomas in experimental models[14,17–19] and is now used clinically in the treatment of patients with prostate carcinoma, benign prostatic hyperplasia (BPH), and other conditions.[17,18]

Bombesin and its mammalian homologue, gastrin-releasing peptide (GRP), can function as autocrine growth factors and stimulate various tumors, such as small cell lung carcinomas[20] and pancreatic, gastric, and breast carcinomas in vivo or in vitro.[17,21,22] Various potent bombesin/GRP antagonists have been synthesized[17,23] in an attempt to develop new methods for cancer treatment. Bombesin/GRP antagonists RC-3095 and RC-3940-II have been shown to suppress the proliferation of pancreatic, mammary, gastric, and prostate carcinomas in vivo.[13,15,17,21–25]

In the current study, we evaluated the effects of LH-RH antagonist Cetrorelix, somatostatin analog RC-160, and the new bombesin/GRP antagonist RC-3940-II on the growth of the renal adenocarcinoma cell line Caki-I xenografted into nude mice or cultured in vitro.

MATERIALS AND METHODS

Peptides

Somatostatin analog RC-160 [D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2], first synthesized in our laboratory,[12] was made by classical synthesis by Novabiochem (Laufingen, Switzerland) and supplied by Debiopharm S.A. (Lausanne, Switzerland). LH-RH antagonist Cetrorelix (SB-75), [Ac-D-Nal(2)^1, D-Phe(4Cl)^2, D-Pal(3)^3, D-Cit^6, D-Ala^{10}] LH-RH, originally synthesized in this laboratory,[17] was obtained from Asta Medica (Frankfurt/Main, Germany).[18] Bombesin antagonist RC-3940-II, [Hca^6, Leu^{13} ψ[CH_2N]Tac^{14}-BN(6-14)], was synthesized by solid phase methods and purified in our laboratory.[23] For administration, RC-3940-II was dissolved in 0.1% dimethyl sulfoxide (DMSO)/0.9% saline. RC-160 was dissolved in 0.1 M acetic acid and diluted with 0.9% saline. Cetrorelix was dissolved in distilled water containing 5% mannitol.

Animals

Male athymic (NCr nu/nu) nude mice, age approximately 6 weeks on arrival, were obtained from the National Cancer Institute (Bethesda, MD) and housed in a laminar air flow cabinet under pathogen free conditions on a 12-hour light/12-hour dark schedule and fed autoclaved standard chow and water ad libitum. Their care was in accord with institutional guidelines.

Cell Culture

The human renal adenocarcinoma cell line Caki-I was obtained from the American Type Culture Collection (Rockville, MD) and cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin-G sodium, 100 mg/mL streptomycin sulfate, and 0.25 mg/mL amphotericin B (all from GIBCO, Grand Island, NY), at 37 °C in a humidified 95% air/5% CO_2 atmosphere. Tumor cells growing exponentially were harvested by brief incubation with 0.25% trypsin-ethylenediamine-tetraacetic acid solution (GIBCO). Xenografts were initiated by subcutaneous (s.c.) injection of 1×10^7 cells into the left flanks of 5 male nude mice.

Measurement of cell growth in vitro was done by the [^{3}H]-thymidine incorporation assay. Incorporation of [methyl-^{3}H] thymidine into DNA was used to determine the effects of RC-160, SB-75, RC-3940-II, and insulin-like growth factor (IGF)-I (Gibco BRL, Grand Island, NY) on the Caki-I cell line in vitro.[21,22,25] Cells were seeded into 96-well microplates (Falcon, Lincoln Park, NJ) in culture medium cited above. After 3–4 days, the confluent cultures were washed with McCoy's 5A medium and maintained in McCoy's 5A medium containing 2% fetal bovine serum (FBS) for 24–30 hours. Several concentrations of each compound diluted with media were then added to the quiescent cultures. Control cultures received the media without addition.[21,22,25] After a 20-hour incubation, all cultures were pulsed with 0.25 μCi/well of [methyl-^{3}H] thymidine (specific activity 25 Ci/mmol; Amersham Corp., Arlington Heights, IL) in a total volume of 175 μL/well for 4–6 hours. The cells were fixed with ice-cold 10% trichloroacetic acid (TCA), washed twice with TCA at 4 °C, and solubilized overnight in 0.2N NaOH at 37 °C. Radioactivity was determined by liquid scintillation counting (Searle Analytic Model 6880, Des Plaines, IL). Index of growth (inhibition or stimulation of DNA synthesis as compared with controls) was expressed using the formula

$$\frac{T - C}{C} \times 100$$

where T = the average dpm of test cultures and C = the average dpm of control cultures.

Experimental Protocol

Experiment I

Caki-I tumors resulting after 5 weeks were aseptically dissected and mechanically minced; 3-mm^3 pieces of tumor tissue were transplanted s.c. with a trocar into 45 male animals. The tumor take rate was 95%. Two weeks after transplantation, the tumor had grown to a volume between 37 and 51 mm^3. The tumor-bearing mice were then divided into 4 groups of 10 animals each, which received the following treatments: Group 1 (control), saline only, s.c.; Group 2, somatostatin analog RC-160 at a dose of 20μg/animal, twice daily, s.c.; Group 3, Cetrorelix (SB-75), 100μg/day/animal, s.c.; Group 4, RC-3940-II at a dose of 10μg/ twice daily, s.c. The treatment was continued for 4 weeks. The tumors were measured once a week with microcalipers and the tumor volume was calculated as length $\times$ width $\times$ height $\times$ 0.5236. Tumor volume doubling time was calculated as described.[13,15] At the end of the experiment, mice were anesthetized with methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL) and sacrificed by decapitation, and trunk blood was collected. The serum was separated for hormone analyses. Further, body weights were recorded and various organs were removed and weighed. Tumors were cleaned and weighed, and samples were taken for histology and receptor studies.

Experiment II

This experiment was designed to investigate the combination of Cetrorelix with bombesin antagonist using the conditions similar to those in Experiment I. Forty Caki-I tumor-bearing mice were divided into 4 experimental groups of 10 animals each, which received the following treatments: Group 1, (control) saline only, s.c.; Group 2, Cetrorelix (SB-75), 100μg/day/animal, s.c.; Group 3, RC-3940-II at a dose of 10μg/ twice daily, s.c..; Group 4, the combination of 100μg/day Cetrorelix and RC-3940-II, 10μg twice daily. The treatment period was 4 weeks, and the experiment was terminated as described above.

Radioimmunoassay

Luteinizing hormone (LH) was determined by radioimmunoassay (RIA) using materials provided by the National Hormone and Pituitary Program (NHPP, Rockville, MD) (rat LH-RP-3/AFP-7187/B/, rat LH-I-9/AFP-10250C/, anti-rat LH-RIA-11/AFP C 697071P). Serum testosterone levels were determined with a Coat-A-Count RIA kit from Diagnostic Products Corporation (Los Angeles, CA). Interassay and intra-assay coefficients of variation were less than 15% and 10%, respectively, for both assays. GH was determined by using materials provided by Dr. A. F. Parlow (Pituitary Hormones and Antisera Center, Torrance,CA, mouse GH reference preparation AFP10783B, mouse GH antigen AFP10783B, and anti-rat GH-RIA-5/AFP-411S). All serum samples for IGF-I and IGF-II determination were extracted by a modified acid-ethanol cryoprecipitation method as described previously.[26,27] This method eliminates most of the IGF binding proteins,

TABLE 1
Effect of Treatment with Peptide Analogs RC-160, Cetrorelix (SB-75), RC-3940-II, or the Combination of Cetrorelix and RC-3940-II on Body and Tumor Weight, Tumor Volume, and Tumor Doubling Time in Nude Mice Bearing Xenografts of the Caki-I Human Renal Adenocarcinoma[a]

Treatment group	Tumor volume (mm^3)		Body weight (g)	Tumor weight (mg)	Tumor doubling time (days)
	Initial	Final			
Experiment I					
Control	37.6 ± 4.8	485.7 ± 77.2	29.4 ± 1.6	847 ± 108	7.7 ± 0.8
RC-160	39.9 ± 2.4	167.5 ± 34.2[c]	29.0 ± 1.5	295 ± 43[c]	16.3 ± 3.0[b]
SB-75	50.1 ± 6.3	159.9 ± 18.1[c]	28.3 ± 0.8	329 ± 69[c]	17.7 ± 2.7[c]
RC-3940-II	51.0 ± 8.5	234.7 ± 81.8[c]	27.0 ± 1.2	425 ± 99[c]	14.0 ± 1.5[b]
Experiment II					
Control	29.8 ± 1.9	519 ± 110	29.7 ± 0.8	734 ± 135	6.7 ± 0.4
SB-75	29.7 ± 4.7	160 ± 46[c]	27.4 ± 0.9	303 ± 66[c]	12.1 ± 1.4[c]
RC-3940-II	28.2 ± 3.9	187 ± 56[c]	28.9 ± 0.6	366 ± 83[c]	11.6 ± 1.2[c]
RC-3940-II + SB-75	34.7 ± 2.3	185 ± 31[c]	28.7 ± 0.5	345 ± 78[c]	11.8 ± 0.9[c]

[a] Values are mean ± standard error.
[b] $P < 0.05$ vs. control.
[c] $P < 0.01$ vs. control.

which can interfere in the RIA.[26] The extracted IGF-I was measured by RIA using IGF-I (88-G4, from Genentech, San Francisco, CA) for standard in the range of 2–500 pg/tube and also for iodination by the chloramine-T method. Antibody UB3-189 and UB2-495 (a gift from Dr. L.E. Underwood and J. van Wyk) obtained from NIDDK was used in the final dilution of 1:10,000 and 1:14,000 in the RIA.

IGF-II was measured using recombinant human insulin-like growth factor-II (Bachem Chemical, Torrance, CA) in the range of 2–500 pg/tube. IGF-II was iodinated by lactoperoxidase method and purified by reverse phase HPLC using Vydac C18 column. For the assay, Amano monoclonal antibody generated against rat IGF-II (10 mg/mL) was used at the final dilution of 1:14,285 (Amano Enzymes USA, Troy, VA). This antibody cross-reacts 100% with human IGF-II and rat IGF-II and 10% with hIGF-I.

Receptor Assay

Evaluation of receptors for EGF in the membranes of Caki-I tumors was performed as previously described.[13,15,28] The LIGAND-PC computerized curve-fitting program of Munson and Rodbard was used to determine the types of receptor binding, dissociation constant (K_d), and maximal binding capacity of receptors (B_{max}).[29]

Histologic Procedure

Sex organs and a part of each tumor were fixed in 10% buffered formalin. Specimens were embedded in Paraplast (Oxford Labware, St. Louis, MO). Sections 6 μm thick were cut and stained with hematoxylin and eosin. Mitotic and apoptotic cells were counted in 10 standard high-power microscopic fields containing, on the average, 200 cells; and their numbers per 1000 cells were accepted as the mitotic and apoptotic indices, respectively.[30]

Alteration of testicles was classified according to the percentage of tubules containing the most advanced germ cell type, i.e., elongated and round spermatids, spermatocytes, and spermatogonia. A total number of 100 tubules were analyzed on one cross section of each testicle.

Statistical Methods

All data are expressed as the mean ± standard error of the mean. Statistical analyses of the tumor data were performed using Duncan's new multiple range test.[31]

RESULTS

The effects of treatment with peptide analogs on final tumor volume, body, tumor, and sex-organ weights, and tumor doubling time are shown in Tables 1 and 2. Figures 1 and 2 show the tumor volumes in both experiments as measured at weekly intervals. After 4 weeks of therapy, when the experiment was terminated, there were no significant differences in the body weights between the groups. In the first experiment, all three analogs tested powerfully inhibited growth of Caki-I RCC tumors (Table 1, Fig. 1). A significant inhibition could be achieved within 7 days from the start of the therapy ($P < 0.05$). After 4 weeks of treatment, tumor volume was significantly reduced ($P < 0.01$) in groups receiving RC-160, Cetrorelix, or RC-

TABLE 2
Weight of Sex Organs of Nude Mice Bearing Caki-I Human Renal Adenocarcinoma Xenografts after Treatment with RC-160, Cetrorelix (SB-75), RC-3940-II, or the Combination of Cetrorelix and RC-3940-II[a]

Treatment group	Testes (mg)	Seminal vesicles (mg)	Prostate (mg)
Experiment I			
Control	252 ± 7	816 ± 115	68 ± 11
RC-160	245 ± 16	945 ± 138	58 ± 14
SB-75	70 ± 17[c]	205 ± 32[c]	10 ± 5[c]
RC-3940-II	195 ± 22[b]	560 ± 26	57 ± 5
Experiment II			
Control	272 ± 10	685 ± 74	90 ± 20
SB-75	100 ± 17[c]	220 ± 10[c]	10 ± 6[b]
RC-3940-II	223 ± 17[c]	520 ± 17[b]	65 ± 10
RC-3940-II + SB-75	102 ± 19[c]	243 ± 13[c]	17 ± 5[c]

[a] Values are mean ± standard error.
[b] $P < 0.05$ vs. control.
[c] $P < 0.01$ vs. control.

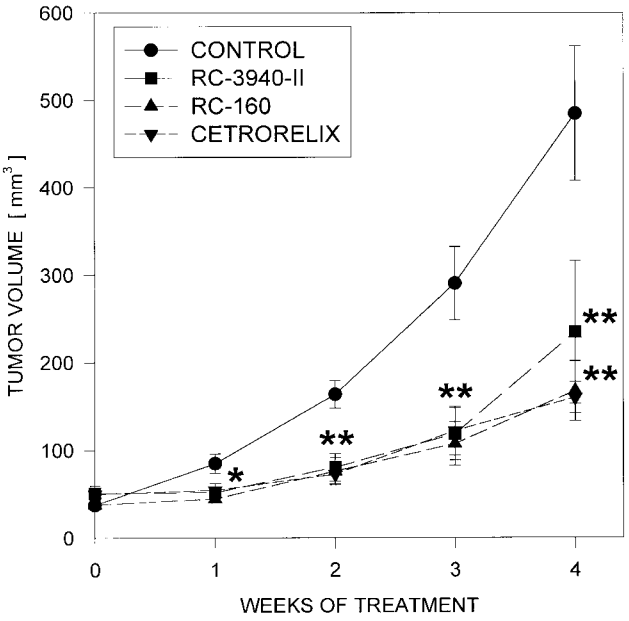

FIGURE 1. Tumor volume is shown in male nude mice bearing xenografts of Caki-I renal adenocarcinoma during treatment with somatostatin analog RC-160, luteinizing hormone–releasing hormone antagonist Cetrorelix, and bombesin antagonist RC-3940-II. Vertical lines indicate the standard error of the mean. $*P < 0.05$; $**P < 0.01$ versus control by Duncan's new multiple range test.

3940-II to 167.5 ± 34.2 mm^3, 159.9 ± 18.1 mm^3, and 234.7 ± 81.8 mm^3, respectively, compared with the control group (485.7 ± 77.2 mm^3) (Table 1). The calculations indicated a tumor doubling time of 7.7 ± 0.8 days for the control group. The tumor doubling time

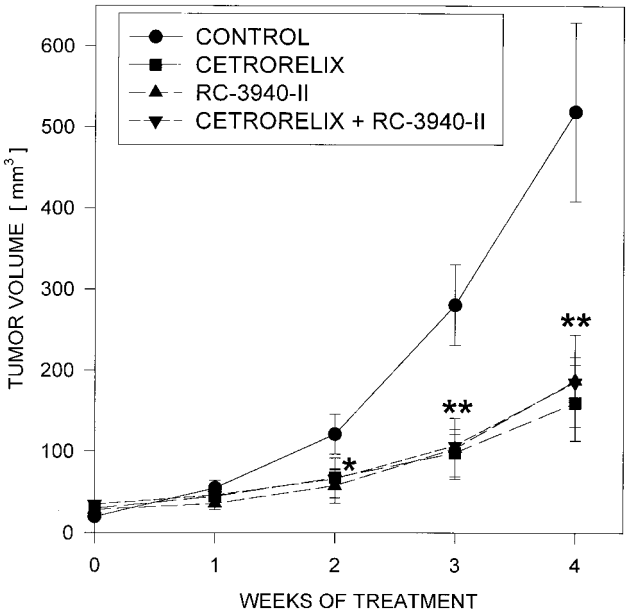

FIGURE 2. The effect of treatment with luteinizing hormone–releasing hormone antagonist Cetrorelix, bombesin antagonist RC-3940-II, or the combination of Cetrorelix and RC-3940-II on tumor volume in nude mice bearing xenografts of Caki-I renal cell carcinoma is shown. Vertical lines indicate the standard error of the mean. $*P < 0.05$ versus control; $**P < 0.01$ versus control.

was prolonged by RC-160, Cetrorelix, or RC-3940-II to 16.3 ± 3.0, 17.7 ± 2.7, and 14.0 ± 1.5 days, respectively (Table 1). In the second experiment, the combination of Cetrorelix and RC-3940-II resulted in a decrease in tumor growth similar to that obtained with single drugs (Fig. 2, Table 1). The final tumor weights were significantly reduced in the groups treated with RC-160, Cetrorelix, or RC-3940-II compared with the controls (Table 1). The combination of Cetrorelix and RC-3940-II did not lead to a further decrease in tumor weights.

There was a significant decrease in the weights of testes, seminal vesicles, and prostate in the groups that received Cetrorelix alone or in combination with RC-3940-II. This shows that chronic treatment with Cetrorelix causes involution of the sex organs (Table 2). Somatostatin analog RC-160 had no effect on the weight of the different sex organs, but it is noteworthy that RC-3940-II significantly reduced the weights of the testes (in both Experiments I and II) and the seminal vesicles (in Experiment II).

Serum levels of LH, testosterone, GH, and IGF-I and -II in animals with Caki-I tumors treated with peptide analogs or saline are shown in Table 3. In Experiment I, Cetrorelix given singly significantly decreased serum LH and testosterone levels, as com-

TABLE 3
Serum Levels of Luteinizing Hormone, Testosterone, Growth Hormone, IGF-I, and IGF-II in Nude Mice Bearing Caki-I RCC Xenografts after Treatment with RC-160, Cetrorelix (SB-75), RC-3940-II, or the Combination of Cetrorelix and RC-3940-II[a]

Treatment group	LH (ng/mL)	Testosterone (ng/mL)	GH (ng/mL)	IGF-I (ng/mL)	IGF-II (ng/mL)
Experiment I					
Control	0.43 ± 0.19	1.0 ± 0.49	1.90 ± 0.15	127.7 ± 10.7	25.1 ± 2.0
RC-160	N.D.	N.D.	1.29 ± 0.06[b]	94.8 ± 7.9[b]	26.2 ± 3.4
SB-75	0.06 ± 0.03[b]	0.06 ± 0.01[b]	N.D.	N.D.	N.D.
RC-3940-II	0.30 ± 0.08	0.64 ± 0.07	N.D.	N.D.	N.D.
Experiment II					
Control	0.16 ± 0.04	0.56 ± 0.27	N.D.	N.D.	N.D.
SB-75	0.08 ± 0.04[b]	0.02 ± 0.01[b]	N.D.	N.D.	N.D.
RC-3940-II	0.15 ± 0.04	0.19 ± 0.07[b]	N.D.	N.D.	N.D.
RC-3940-II + SB-75	0.13 ± 0.07	0.03 ± 0.01[b]	N.D.	N.D.	N.D.

RCC: renal cell carcinoma; LH: luteinizing hormone; GH: growth hormone; IGF: insulin-like growth factor; N.D.: not determined.

[a] Values are mean ± standard error.

[b] $P < 0.05$ vs. control.

pared with control animals. In Experiment II, Cetrorelix administered either alone or in combination with RC-3940-II also significantly decreased LH levels as compared with controls. Although serum testosterone levels in the control group were low, Cetrorelix given singly or in combination reduced testosterone to castration values. Serum GH, IGF-I, and IGF-II levels were measured only in controls and in the group treated with the somatostatin analog RC-160. Serum GH was significantly decreased in the group given RC-160 to 1.29 ± 0.06 ng/mL as compared with controls (1.9 ± 0.15 ng/mL). Serum IGF-I levels were significantly reduced in the animals treated with RC-160, whereas serum IGF-II levels showed no changes after therapy with RC-160 (Table 3).

Histologically, the tumors consisted of large epithelial cells arranged in groups that were surrounded by a very scanty stroma. More extensive necrotic areas were found in the tumors treated with RC-3940-II than in those of other groups. The tumor cells were elongated and the stroma increased in three tumors after treatment with SB-75. An almost total arrest of spermatogenesis was seen in nude mice treated with Cetrorelix. The testicles of mice in other groups showed normal histologic structures. The quantitative histologic data are shown in Table 4. The mitotic index was significantly decreased in tumors treated with RC-160 and Cetrorelix, compared with that in control animals. The apoptotic index was not changed by the peptide analogs tested.

The results of the receptor assays for EGF after treatment with various peptide analogs are shown in Table 5. High-affinity binding sites for EGF were detected in the membranes of Caki-I RCC cells. A major and highly significant reduction in maximal binding capacity (B_{max}) of EGF receptors was observed after treatment with all analogs tested. The decrease in EGF receptor concentration after RC-160 and Cetrorelix treatment was 44% in Experiment I and 49% and 51% in Experiments I and II, respectively. The greatest decrease (61% and 71%) in EGF receptor levels was achieved after treatment with bombesin antagonist RC-3940-II. The combination of RC-3940-II and Cetrorelix caused a smaller reduction in EGF receptors. No receptors for LH-RH, somatostatin, or bombesin/GRP could be detected in the membranes of Caki-I cells.

To evaluate the stimulatory effect of IGF-I and possible antiproliferative activity of RC-160, SB-75,

TABLE 4
Histologic Characteristics of Caki-I Renal Tumors and Testes in Nude Mice after Treatment with Analogs RC-160, Cetrorelix (SB-75), and RC-3940-II[a]

	Tumors		Testes
Treatment group	No. of mitoses per 1000 cells	No. of apoptotic cells per 1000 cells	% of seminiferous tubules containing mature spermatozoa
Experiment I			
Control	9.1 ± 1.4	3.8 ± 0.8	99.6
RC-160	4.0 ± 1.0[c]	3.5 ± 0.4	99.0
SB-75	4.7 ± 0.9[b]	2.9 ± 0.3	2.7[c]
RC-3940-II	6.7 ± 1.2	3.1 ± 0.6	100.0

[a] Values are mean ± standard error.

[b] $P < 0.05$ vs. control.

[c] $P < 0.01$ vs. control.

TABLE 5
Binding Characteristics of EGF Receptors in Membranes of Caki-I RCC Xenografts after In Vivo Treatment with RC-160, Cetrorelix (SB-75), RC-3940-II, or the Combination of Cetrorelix and RC-3940-II[a]

	EGF receptors	
Treatment group	K_d (nM)	B_{max} (fmol/mg protein)
Experiment I		
Control	0.97 ± 0.13	210 ± 27.0
RC-160	1.28 ± 0.32	117.5 ± 17.5[b]
SB-75	1.30 ± 0.40	106.5 ± 1.5[b]
RC-3940-II	1.16 ± 0.45	83.5 ± 9.5[c]
Experiment II		
Control	1.28 ± 0.11	215.0 ± 5.0
SB-75	0.82 ± 0.18[b]	105.5 ± 7.5[c]
RC-3940-II	0.72 ± 0.08[b]	62.0 ± 6.0[c]
RC-3940-II + SB-75	1.55 ± 0.05	124.0 ± 4.0[c]

RCC: renal cell carcinoma; EGF: epidermal growth factor.

[a] Binding characteristics were obtained from 10-point displacement experiments in duplicate tubes. Significance was calculated using Duncan's new multiple range test. All values represent mean ± standard error.

[b] $P < 0.05$ vs. control.

[c] $P < 0.01$ vs. control.

and RC-3940-II on Caki-I cell lines in vitro, the [^{3}H]-thymidine uptake assay was used. IGF-I at concentrations of 10 and 20 ng/mL significantly stimulated the thymidine incorporation into the Caki-I cell line by 66% and 101%, respectively. In cell culture experiments, only a slight inhibition of proliferation of Caki-I cells could be achieved even at high concentrations of RC-160, SB-75, or RC-3940-II. The extent of growth inhibition for these analogs at 10^{-5}M concentration ranged from 2% to 11% (not significant), compared with the controls (data not shown).

DISCUSSION

The Caki-I cell line was established in 1971 by Fogh et al. and is classified as clear cell renal adenocarcinoma.[8] It can be xenografted into nude mice and thus provides a valuable model for investigating new compounds for their effect on RCC in vivo. Our findings clearly demonstrate that LH-RH antagonist Cetrorelix (SB-75), somatostatin analog RC-160, and bombesin antagonist RC-3940-II significantly inhibit growth of xenografts of Caki-I cell line in nude mice. The antineoplastic actions of these three peptides, representing different classes of analogs, are thought to involve multiple mechanisms.

The human kidney secretes high amounts of EGF (urogastrone), and tissue levels of EGF are the highest in the kidney.[32] RCC synthesizes a number of growth factors, including TGF-α and -β, [33] and also expresses EGF receptors, implying an autocrine growth regulation.[7,34,35] A high level of EGF receptors in human RCC may contribute to the development and the maintenance of this malignant tumor. The presence of EGF receptors is a sign of aggressive tumor behavior and poor prognosis in many cancer types.[5] Our investigation characterized high-affinity receptors for EGF on the Caki-I cell line for the first time. The presence of specific somatostatin receptors has been demonstrated in a majority of human RCCs.[5] On the basis of the simultaneous presence of EGF and somatostatin receptors in kidney cancer, Reubi and Kvols[5] suggested that interaction between somatostatin and growth factors may affect tumor development.[5] EGF and somatostatin may be involved in the autonomous growth of Caki-I tumors, the former exerting stimulatory influences and the latter inhibitory. This view is supported by the down-regulation of EGF receptors in the tumors of animals treated with somatostatin analog RC-160, LH-RH antagonist Cetrorelix, and bombesin/GRP antagonist RC-3940-II. The reduction in EGF receptors appears to be a common event in the mechanism of action of these distinct analogs in the Caki-I RCC line.

RC-160 could also act through the suppression of GH and IGF-1 levels, thereby removing a trophic stimulus to Caki-I xenografts. In our cell culture experiments, the addition of 10–20 ng/mL IGF-I stimulated proliferation of Caki-I cells, pointing to a direct involvement of IGF-I in Caki-I cell growth. Previously, we demonstrated high-affinity binding sites for IGF-1 in the cell membranes of Caki-I tumors.[36] However, our receptor binding studies showed no receptors for somatostatin on Caki-I tumors. The lack of an antiproliferative effect of RC-160 in vitro suggests that this cell line may have undergone changes in somatostatin receptor content during long term passages in the cell culture. Similar phenomena were observed for other cell lines[13,15,23] and might explain the differences in tumor growth inhibition in vivo and in vitro obtained with our somatostatin analog. Consequently, RC-160 might inhibit the growth of Caki-I RCC indirectly through the IGF-I or EGF pathway. It was shown in the MIA PACA-2 pancreatic carcinoma cell line that somatostatin and RC-160 may exert antitumor effects through inhibition of EGF functions at the level of EGF receptor phosphorylation.[37] Both somatostatin and RC-160 stimulated tyrosine phosphatase and promoted the dephosphorylation of EGF receptors.[37] Thus, RC-160 could reduce tumor growth also by down-regulation of EGF receptors, as observed in this study and in our previous investigations.[13–17, 30]

The current study likewise demonstrates that the new bombesin/GRP antagonist RC-3940-II effectively inhibits tumor growth and prolongs the doubling time

of Caki-I tumors. Various studies have shown that bombesin-like peptides act as autocrine growth factors in stimulating growth of various tumors, such as small cell lung carcinoma and pancreatic, gastric, and mammary carcinomas.[17,20–22] Bombesin and GRP enhance the phosphorylation of EGF receptors, and bombesin antagonist inhibits this effect in various cancer lines and cancer specimens.[38] This finding is in accord with our previous studies, in which the inhibition of growth of pancreatic, gastric, colorectal, prostatic, and mammary carcinomas and other tumors by bombesin/GRP antagonists was invariably accompanied by a major down-regulation of EGF receptors.[16,17,25,28,30]

The involvement of sex steroids in promoting the growth of RCC has been extensively discussed.[2–4] Sex steroids may induce DNA alterations and changes in growth factors in renal cells.[2–4] Medroxyprogesterone, tamoxifen, and flutamide have been tested in clinical trials with only marginal therapeutic benefit for the RCC patients. However, new findings concerning the involvement of sex hormones on tumor gene expression may explain the effects of these compounds in some patients.[3] LH-RH antagonists such as Cetrorelix inhibit the pituitary-gonadal axis and lead to a dramatic fall in sex hormone levels.[17–19,25] Thus, Cetrorelix could produce its inhibitory effect on RCC growth through sex steroid deprivation. LH-RH antagonists may also exert direct inhibitory effects on different tumors.[17–19,25] The inhibition of growth of Caki-I human RCC xenografts in nude mice by administration of LH-RH antagonist Cetrorelix is accompanied by a marked decrease in the number of EGF binding sites. A similar reduction in levels of EGF receptors after in vivo treatment with Cetrorelix was reported previously for PC-3 prostate carcinomas[25] and OV-1063 ovarian carcinomas.[39] In the case of OV-1063 tumors, a decrease in mRNA for EGF receptors was also found.[39] Thus, it appears that antagonists such as Cetrorelix affect EGF receptors.[19,25,39] The exact molecular mechanisms of action of LH-RH antagonists on EGF receptors remains to be elucidated.

The combination of SB-75 and RC-3940-II did not exert greater inhibitory effects on tumor growth and on the receptor levels than the administration of single analogs. The observed lack of potentiation could be explained by the possiblitity that virtually maximal tumor growth inhibition and EGF receptor down-regulation were already achieved by the single agents or that an interaction occurred between these analogs, decreasing their individual effectiveness. This lack of potentiation with combinations of some peptide analogs was observed in a previous study on prostatic, mammary, and pancreatic carcinomas.[25,28,40]

Our findings indicate that LH-RH antagonist Cetrorelix, somatostatin analog RC-160, and bombesin antagonist RC-3940-II inhibit growth of human Caki-I renal adenocarcinoma, but future studies will be required to elucidate the mechanisms involved. These peptide analogs could be considered for the clinical treatment of RCC, especially in the case of local recurrence or metastatic disease.

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