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Original Paper

Inhibition of Growth of MDA-MB-231 Human Breast Cancer Xenografts in Nude Mice by Bombesin/Gastrin-releasing Peptide (GRP) Antagonists RC-3940-II and RC-3095

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Bombesin or gastrin-releasing peptide (GRP) may act as autocrine growth factors and play a role in the initiation and progression of breast cancer. We investigated the effect of bombesin/GRP antagonists RC-3095 and RC-3940-II on the growth of the MDA-MB-231 oestrogen-independent human breast cancer cell line xenografted into female nude mice. Bombesin/GRP antagonists, RC-3095 and RC-3940-II, were administered subcutaneously twice daily at a dose of 10 μg for 5 weeks. The growth of MDA-MB-231 tumours was inhibited during the treatment, as shown by a reduction in tumour volume. RC-3940-II and RC-3095 significantly decreased the final tumour volume by 72.4% and 57.7%, respectively, and greatly reduced tumour weights. RC-3940-II also significantly increased tumour doubling time and appeared to be more effective than RC-3095 in inhibiting the growth of MDA-MB-231 breast cancers. Serum gastrin and insulin-like growth factor-I (IGF-I) levels in animals treated with RC-3095 or RC-3940-II showed no significant changes as compared with controls. There was a significant decrease in the number of binding sites for epidermal growth factor (EGF), as well as bombesin, in tumour cells after chronic treatment with RC-3095 or RC-3940-II, which might be related to inhibition of tumour growth. Reverse transcription polymerase chain reaction, followed by Southern blot analysis, also showed a reduction in the expression of mRNA for EGF receptors in the group treated with RC-3940-II. Our findings suggest that bombesin/GRP antagonists such as RC-3095 or RC-3940-II could be considered for endocrine therapy for oestrogen-independent breast cancers, but further investigations are necessary. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: bombesin/GRP antagonists, oestrogen-independent breast cancer, tumour inhibition
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INTRODUCTION

BREAST CANCER is one of the most common malignancies among women in the Western world and a leading cause of mortality from cancer, resulting in approximately 50 000 deaths annually in the U.S.A. and 16 000 in the U.K. [1]. For breast cancers that are oestrogen-dependent, various hormonal interventions such as tamoxifen, bilateral oophorectomy, progestins and luteinising hormone releasing hormone (LH-RH) analogues have established efficacy for palliative therapy [2–5]. Other trials have provided evidence of the benefit of cytotoxic therapy, which can also be used as an adjuvant to surgery and radiotherapy [2–5]. However, some patients with breast cancer show poor responses to these therapies or become unresponsive following treatment, so new therapeutic approaches must be developed, particularly for oestrogen-independent breast cancer.

Much evidence suggests that tetradecapeptide bombesin, originally isolated from amphibia, and its mammalian counterpart, gastrin-releasing peptide (GRP), can function as growth factors [4, 6–9]. Bombesin-like peptides have been implicated as autocrine growth factors in the pathogenesis and progression of some human small cell lung carcinomas (SCLC) [4, 6–8]. Various studies have also demonstrated that bombesin/GRP may be involved in the function and growth of human breast cancer [4, 10–14]. Specific receptors

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for bombesin/GRP have been demonstrated in human breast tumour cell lines, MDA-MB-231, MCF-7 MIII, and T47D [10, 11, 14]. Recently, we found that bombesin/GRP receptors were present in approximately 33% of human breast cancer specimens [13]. The findings that bombesin and GRP promote the growth of some cancers, and function as autocrine growth factors, raises the possibility that their antagonists might inhibit the growth of certain tumours [4]. Several series of bombesin/GRP antagonists have been developed in various laboratories [4, 7, 15–23]. We have previously shown that the nonapeptide antagonist, D-Tpi^6, Leu^{13} ψ[CH_2NH]-Leu^{14} bombesin (6-14) (RC-3095), inhibited the growth of nitrosamine-induced pancreatic cancers in hamsters [24], MXT mammary cancers in mice [12], and MCF-7 MIII breast cancers [10, 11], HT-29 human colon cancer [25], PC-82 and DU-145 human prostate cancers [26, 27], and Hs746T human gastric cancer [28] in nude mice. The MDA-MB-231 human breast cancer cell line is a well accepted model of oestrogen-independent breast cancer [14]. In this study, we investigated the antitumour effects of RC-3095 and newly synthesized powerful antagonist RC-3940-II, on growth of MDA-MB-231 breast cancer xenografted into nude mice. Since in previous studies, tumour growth inhibition after treatment with bombesin/GRP antagonists was linked to a reduction in epidermal growth factor (EGF) receptors, we also carried out determination of binding sites for bombesin and EGF and molecular biology analyses of EGF receptors.

MATERIALS AND METHODS

Peptides

The bombesin/GRP antagonists D-Tpi^6, Leu^{13}ψ[CH_2NH]-Leu^{14} bombesin (6-14) (RC-3095), originally synthesised in our laboratory [7], was made by Asta Medica (Frankfurt am Main, Germany). RC-3095 acetate (D22213) was used for treating nude mice. The new bombesin antagonist Hca^6, Leu^{13}ψ[CH_2N]Tac^{14}-BN(6-14) (RC-3940-II) was synthesised in our laboratory by solid phase methods [15, 17, 23]). Hca is a desaminophenylalanine and Tac is thiazolidine-4-carboxylic acid. RC-3095 and RC-3940-II were dissolved in dimethyl sulphoxide (DMSO) and diluted with 0.9% saline. The final concentration of DMSO was 0.1%.

Animals

Female athymic nude mice (Ncr nu/nu), 5–6 weeks old on arrival, were obtained from the Frederic Cancer Research Facility of the National Cancer Institute (Frederick, Maryland, U.S.A.). The mice were housed in sterile cages under laminar flow hoods in a temperature-controlled room with a 12 h light/12 h dark schedule and were fed autoclaved food and water *ad libitum*. The tumours were regularly transplanted in our laboratory. All animal studies were conducted in accordance with institutional guidelines for the care and use of experimental animals.

Cells and tumours

The human breast cancer cell line MDA-MB-231 was obtained from American Type Culture Collection (Rockville, Maryland, U.S.A.). MDA-MB-231 cells were grown in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/l D-glucose plus 10% newborn calf serum (NCS), 2 mM glutamine penicillin (100 unit/ml), streptomycin (100 μg/ml) and amphotericin B (100 units/ml). Cells were cultured in Costar T-75 flasks in a humidified atmosphere of 5% CO_2 and 95% air at 37°C and passed every 7–10 days. Tumour cells growing exponentially were harvested by a brief incubation with 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) solution (Gibco/Life Technologies, Grand Island, New York, U.S.A.).

Experimental protocol

Xenografts were initiated by subcutaneous injection of 1×10^7 MDA-MB-231 cells into four nude mice. Tumours resulting after 4 weeks were aseptically dissected and mechanically minced; 1 mm^3 MDA-MB-231 tumour pieces were transplanted subcutaneously by trocar needle into the right flank of 30 mice. Ten days after tumour transplantation, when the tumours measured approximately 30 mm^3, the mice were assigned to experimental groups of eight to nine animals each and the therapy was initiated as follows: group 1 (control), 0. 9% saline; group 2, RC-3095 at a dose of 10 μg twice daily; group 3, RC-3940-II at a dose of 10 μg twice daily. All compounds were administered by subcutaneous injection into the left lower back area of mice. The treatment was continued for 35 days.

The tumours were measured weekly with microcalipers, and the tumour volume calculated using the following formula: length×width×height×0.5236 [29]. The measurement of tumour growth was continued for 5 weeks. The percentage change in tumour volume from the start of the treatment was used as a parameter of growth rate. Tumour doubling time was calculated between the start of the treatment and the end of the experiment. After the treatment period, the mice were sacrificed under light methoxyflurane anaesthesia according to institutional standards. Trunk blood was collected and centrifuged at 2000 rpm for 30 min at 4°C and the serum stored at −20°C until assayed. Tumours were carefully removed, cleaned and weighed. Tumour burden at the end of the experiment was calculated as tumour weight (mg)/body weight (g). Samples of tumour tissue were fixed in 10% buffered neutral formalin for histological examination. Tumour samples were also stored at −80°C for receptor assay and molecular biology analysis.

Pathological procedures

Samples of tumour tissue were fixed in 10% buffered formalin. Specimens were embedded in Paraplast (Oxford Labware, St Louis, Missouri, U.S.A.). Six micrometre thick sections were cut and stained with haematoxylin–eosin. Mitotic and apoptotic cells were counted in 10 standard high power microscopic fields containing, on average, 300 cells, and their numbers per 1000 cells were accepted as the mitotic and apoptotic indices, respectively. For demonstration of the nucleolar organiser region (NOR) in tumour cell nuclei, the argyrophilic NOR (AgNOR) method of Chiu and colleagues [30] was used with small modification [31]. NORs are sites of genes that encode for ribosomal RNA. They are associated with argyrophilic non-histone acidic proteins that can be detected by the AgNOR method. AgNOR numbers correlate with cell proliferation rate. The silver-stained black grains in 50 cells of each tumour were counted and the AgNOR number per cell was calculated.

Radioimmunoassay of gastrin, insulin-like growth factor-I (IGF-I) and EGF

Serum gastrin levels were measured by double-antibody radioimmunoassay (RIA) with a kit provided by Becton

Dickinson (Orangeburg, New York, U.S.A.). All serum samples for IGF-I and EGF determination were extracted by a modified acid–ethanol cryoprecipitation method described previously [32, 33]. This method eliminates most of the IGF binding proteins which can interfere in the RIA. The extracted IGF-I was measured by RIA using IGF-I (88-G4; Genentech, San Francisco, California, U.S.A.) as a standard in the range of 2–500 pg/tube and for iodination using the standard chloramine-T method. Antibodies UB3-189 and UB2-495 (a gift from Dr Underwood and J. van Wyk) obtained from NIDDK were used at the final dilution of 1:10 000 and 1:14 000, respectively, in the RIA. The extracted EGF was measured using mouse EGF standard (receptor grade, UBI, Lake Placid, New York, U.S.A.) in the range of 0.006–12.2 ng/tube and mouse EGF antiserum provided by Collaborative Research (Bedford, Massachusetts, U.S.A.) in the final dilution of 1:167 000.

Receptor assay

Receptors for bombesin/GRP and EGF on the membrane of MDA-MB-231 tumours were measured. Preparations of tumour membrane fractions and receptor binding studies of bombesin/GRP and EGF were performed as previously described [34, 35]. The LIGAND-PC computerised curve fitting program of Munson and Rodbard [36] was used to determine the types of receptor binding, dissociation constant (K_d), and the maximal binding capacity of receptors (B_{max}).

Isolation of mRNA

Total RNA was extracted from frozen tissue samples by using RNA zol B (TEL-TEST Inc., Friendswood, Texas, U.S.A.) according to the manufacturer's instructions. The RNA pellets were suspended in 100 µl Tris (10 mM)–EDTA (1 mM) buffer (pH 8.0) and quantified spectrophotometrically. The optical density ratios (260 nm/280 nm) of the RNA preparations were greater than 1.8.

Reverse transcription (RT)

One microgram of total RNA was used in a test tube containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM each of deoxynucleoside triphosphate (dNTP), 1 U RNase inhibitor, 2.5 µM random hexamer primers in a final volume of 19 µl of RNase-free deionised distilled water. The mixture was heated for 10 min at 65°C and quenched on ice and 2.5 U of Moloney murine leukaemia virus reverse transcriptase (Perkin Elmer, Norwalk Connecticut, U.S.A.) in 1 µl were added for a total reaction volume of 20 µl. The mixture was incubated at room temperature for 10 min and then at 42°C for 1 h. The reaction was stopped by heating at 95°C for 5 min and quenching on ice.

Polymerase chain reaction (PCR) amplification

Twenty microlitres of solution from the RT reaction from each sample was diluted to a final volume of 100 µl using a mixture of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$. Fifty picomoles of each primer and 2.5 U of Amplitaq DNA polymerase (Perkin Elmer) were also added. The primers used for the human EGF receptor were sense 5′ACGCAGATAGTCGCCCAAAGTTCC-3′ (base pair 3047–3070) (Clontech, Palo Alto, California, U.S.A.) and antisense 5′-AGGAAGGTGTCGTCTATGCTGTCC-3′ (base pairs 3423–3446). The primers for human glyceraldehyde, 3-phosphate dehydrogenase (hGAPDH) used for internal control were sense 5′-TCCTCTGACTTCAACAGCGACACC-3′ (base pairs 907–930) and antisense 5′-TCTCTCTTCCTCTTGTGCTCTTGG-3′ (1091–1114). These PCR oligonucleotide primers were synthesised using 394 DNA/RNA synthesiser (Applied Biosystem, Foster City, California, U.S.A.). PCR was started after an initial denaturation step at 95°C for 3 min by the addition of 2.5 U Ampli-taq (Perkin Elmer), followed by 30 cycles of replication (1 min at 94°C, 1 min at 54°C, 1 min at 72°C), and the final extension was carried out for 7 min at 72°C using a Stratagene Robocycler 40 system (Stratagene, La Jolla, California, U.S.A.). The number of cycles was previously determined to be within the exponential range of PCR product amplification necessary for quantitative densitometry. Negative controls were run in parallel to check for DNA contamination of samples. Ten microlitres of the final PCR product was electrophoresed using a 1.8% agarose gel. Bands were visualised by ethidium bromide staining on an ultraviolet transilluminator.

Southern blot analysis

The gel was treated with denaturation buffer containing 50 mM NaOH, 1.5 M NaCl, then in neutralisation buffer containing Tris–HCl (pH 8.0), 1.5 M NaCl. The gel was blotted on to a nylon membrane (Hybond N+, Amersham, Arlington Heights, Illinois, U.S.A.) by capillary transfer, and the DNA was bonded to it by heating for 2 h at 80°C. Sample blots were prehybridised at 60°C for 16 h in a buffer containing 4XSSC, 2X Denhardt's solution, 0.1% sodium dodecyl sulphate (SDS), 5 mM EDTA and 100 µg/ml denatured salmon sperm DNA. After prehybridisation, the sample blots were hybridised at 60°C for 20 h in hybridisation buffer containing 5XSSC, 0.5X Denhardt's solution, 0.02 M Tris–HCl, 100 µg/ml sonicated salmon DNA and 150 ng of (^{32}P)5′-end labelled oligonucleotide probe [37]. The oligonucleotide probe used for the hEGF receptor was 5′-TGGATGAAGAAGACATGGACGACGTGGTGG-3′ (base pairs 3191–3220) and for hGAPDH 5′-TGTCAAGCTCATTTCCTGGTCTGACAACGA-3′ (base pairs 981–1010). Probes were labelled in a reaction mixture containing (^{32}P) ATP 3000 Ci/mmol, 10 mCi/ml, 150 ng oligonucleotide probe and 10 units of T4 polynucleotide kinase. After incubation at 37°C for 45 min, reactions were stopped by adding 1 µl of 0.5 M EDTA and unincorporated radionucleotides were removed using Nuctrap columns (Stratagene). The blots were washed under stringent conditions and the signals from samples were scanned and quantified using an imaging densitometer (Model GS-700; BioRad, California, U.S.A.).

Statistical methods

All data are expressed as the mean ± standard error of the mean (SEM) and statistical analyses of the tumour data were performed using Duncan's new multiple range test [38] or Student's *t*-test. All *P* values are based on two-sided hypothesis testing.

RESULTS

Effect of bombesin/GRP antagonists RC-3095 and RC-3940-II on the growth of human breast cancer MDA-MB-231 in nude mice

Both bombesin/GRP antagonists powerfully inhibited growth of MDA-MB-231 tumours. A significant inhibition could be achieved within 14 days from the start of the therapy ($P < 0.01$) (Figure 1). After 2 weeks, the volume of the

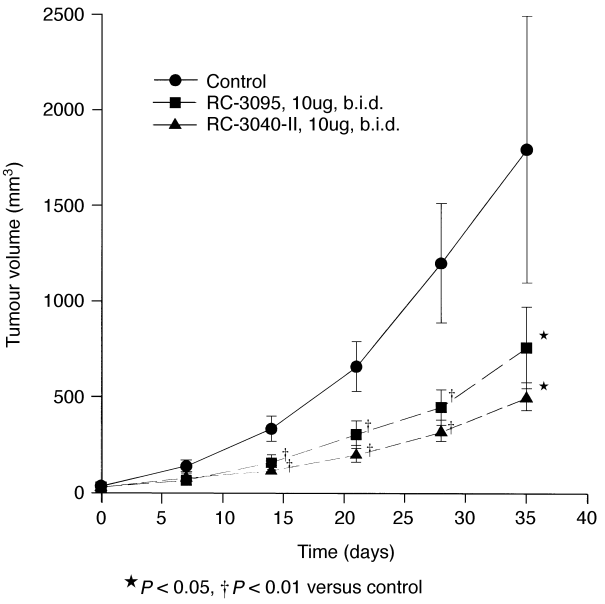

Figure 1. Tumour volume in athymic nude mice bearing subcutaneously transplanted MDA-MB-231 human breast cancer during treatment with the bombesin/gastrin-releasing peptide (GRP) antagonists RC-3095 and RC-3940-II administered subcutaneously. Bars represent standard error (SE). *$P < 0.05$, †$P < 0.01$ versus control by Duncan's new multiple range test.

MDA-MB-231 breast cancers in the groups receiving RC-3095 and RC-3940-II was significantly ($P < 0.01$) reduced to $159.5 \pm 40.0\,mm^3$ and $114.7 \pm 31.4\,mm^3$, respectively, as compared with the control group ($334.1 \pm 66.1\,mm^3$), corresponding to a 52.3% and a 65.7% decrease in tumour volume, respectively (Figure 1). After 5 weeks of therapy, when the experiment was terminated, there was no significant difference in body weights between the groups. Treatment with RC-3095 or RC-3940-II for 5 weeks significantly ($P < 0.05$) decreased the final tumour volume and tumour weight as compared with those for the controls (Table 1). Final tumour growth inhibition was 57.7% for RC-3095 and 72.4% for RC-3940-II. Tumour doubling time in mice receiving RC-3940-II was significantly ($P < 0.05$) extended to 9.35 ± 1.27 days from 6.52 ± 0.82 days in control groups, but no significant increase in tumour doubling time was found in the group treated with RC-3095. Tumour burden was significantly decreased ($P < 0.05$) in the groups treated with RC-3095 and RC-3940-II (Table 1).

Histological findings

MDA-MB-231 tumours were undifferentiated carcinomas consisting of large irregular polygonal cells without any specific arrangements. The cytoplasms were wide with homogenous or finely granular pale eosinophilic staining. The nuclei of tumour cells were large having different shapes and various chromatin contents. Several bizarre large cells and multinuclear giant cells were visible. There was very little connective tissue stroma in the tumours. All of the cancers contained necrotic areas which were very extensive in some tumours. The histological appearance of the tumours was the same in treated and control groups. Some quantitative histological data are shown in Table 2. There were no significant differences in mitotic and apoptotic indices and the numbers of AgNORs in cells.

Radioimmunoassay

Serum gastrin, EGF and IGF-I levels in controls and in animals treated with RC-3095 or RC-3940-II were measured (Table 3). There were no significant changes in gastrin serum levels after chronic treatment with RC-3095 or RC-3940-II compared with controls. Serum EGF levels were decreased in groups treated with RC-3095 and RC-3940-II compared with the controls, but the changes were not significant. Serum IGF-I levels also showed no significant differences after treatment with RC-3095 or RC-3940-II compared with those of controls.

Receptor analysis

The binding characteristics of receptors for bombesin/GRP and EGF on MDA-MB-231 tumours were analysed

Table 1. *Effect of treatment with bombesin/gastrin-releasing peptide antagonists RC-*3095 *and RC-*3940*-II on tumour volume, fold increase in tumour volume, tumour doubling time, tumour weight and tumour burden in nude mice bearing MDA-MB-*231 *human breast cancer xenografts*

Treatment	Initial tumour volume (mm^3)	Final tumour volume (mm^3)	Fold increase in tumour volume	Tumour doubling time (days)†	Final tumour weight (mg)	Tumour burden (mg/g bw)
Control	36.93 ± 3.81	1793.24 ± 697.26	48.6	6.52 ± 0.82	1263.86 ± 469.24	47.48 ± 14.57
RC-3095 (10 µg, bid)	30.36 ± 5.16	758.81 ± 212.82★	25.0	7.95 ± 0.84	669.43 ± 173.90	29.03 ± 7.45★
RC-3940-II (10 µg, bid)	28.69 ± 3.83	495.18 ± 82.39★	17.3★	9.35 ± 1.27★	423.56 ± 100.82★	16.90 ± 3.63★

Values are mean ± standard error of the mean (SEM). *$P < 0.05$ versus control. †Calculated on the basis of individual tumours.
bid, twice daily; bw, body weight.

Table 2. *Effect of treatment with bombesin/gastrin-releasing peptide antagonists RC-*3095 *and RC-*3940*-II on some histological characteristics of MDA-MB-*231 *human breast cancers growing in nude mice*

Treatment	Mitotic index	Apoptotic index	Ratio of apoptotic to mitotic indices	Number of AgNORs per cell
Control	11.2 ± 0.6	5.9 ± 1.4	0.56 ± 0.15	5.43 ± 0.31
RC-3095	11.3 ± 1.3	8.4 ± 1.6	0.79 ± 0.17	5.33 ± 0.25
RC-3940-II	10.9 ± 1.9	5.9 ± 0.4	0.65 ± 0.13	5.39 ± 0.14

Values are mean ± SEM.

Table 3. *Serum gastrin, epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) levels in nude mice bearing MDA-MB-231 human breast cancer xenografts after treatment with RC-3095 and RC-3940-II*

Treatment	Gastrin (pg/ml)	EGF (ng/ml)	IGF-I (ng/ml)
Control	108.86 ± 7.57	26.02 ± 10.27	80.55 ± 6.72
RC-3095 (10 μg, bid)	125.80 ± 9.71	5.18 ± 0.74	98.93 ± 6.86
RC-3940-II (10 μg, bid)	129.77 ± 13.14	16.38 ± 5.90	116.7 ± 8.97

Values are mean ± SEM.
bid, twice daily.

(Table 4). Receptor assays on MDA-NM-231 tumour membranes showed high-affinity binding sites for bombesin/GRP (K_d = 1.40 ± 0.01 nM) and EGF (K_d = 1.06 ± 0.09 nM). Treatment with RC-3095 or RC-3940-II significantly ($P < 0.01$) decreased the concentration of receptors for bombesin/GRP to 330.4 ± 23.3 and 320.6 ± 7.06 fmol/mg of membrane protein, respectively, as compared with the control group (576.4 ± 8.79 fmol/mg of membrane protein). The concentration of receptors for EGF was also significantly ($P < 0.01$) reduced by therapy with RC-3095 or RC-3940-II (Table 4).

Molecular biology analysis

mRNA for EGF receptors was detected on MDA-MB-231 human breast cancers using RT-PCR (Figure 2a). Since RC-3940-II exerted a more powerful tumour growth inhibition, the tumours from this group and the control were subjected to additional analyses. The PCR products of hEGF receptors (Figure 2a) and hGAPDH (Figure 2b) obtained after RT-PCR were confirmed by Southern blot analysis (Figure 2c,d). Semiquantitative analysis of the developed bands by densitometry showed that in the group treated with

Table 4. *Characteristics of bombesin/gastrin-releasing peptide (GRP) receptors and epidermal growth factor (EGF) receptors of MDA-MB-231 human breast cancer xenografts in nude mice after treatment with RC-3095 and RC-3940-II*

	Bombesin/GRP		EGF	
Treatment	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)
Control	1.40 ± 0.01	576.4 ± 8.79	1.06 ± 0.09	234.1 ± 5.95
RC-3095 (10 μg, bid)	0.97 ± 0.05*	330.4 ± 23.3†	0.85 ± 0.18	93.6 ± 6.65†
RC-3940-II (10 μg, bid)	1.06 ± 0.12	320.6 ± 7.06†	0.53 ± 0.21	75.1 ± 6.95†

Values given are the mean ± SEM of three to four independent experiments, each performed in duplicate. bid, twice daily. *$P < 0.05$, †$P < 0.01$ versus control.

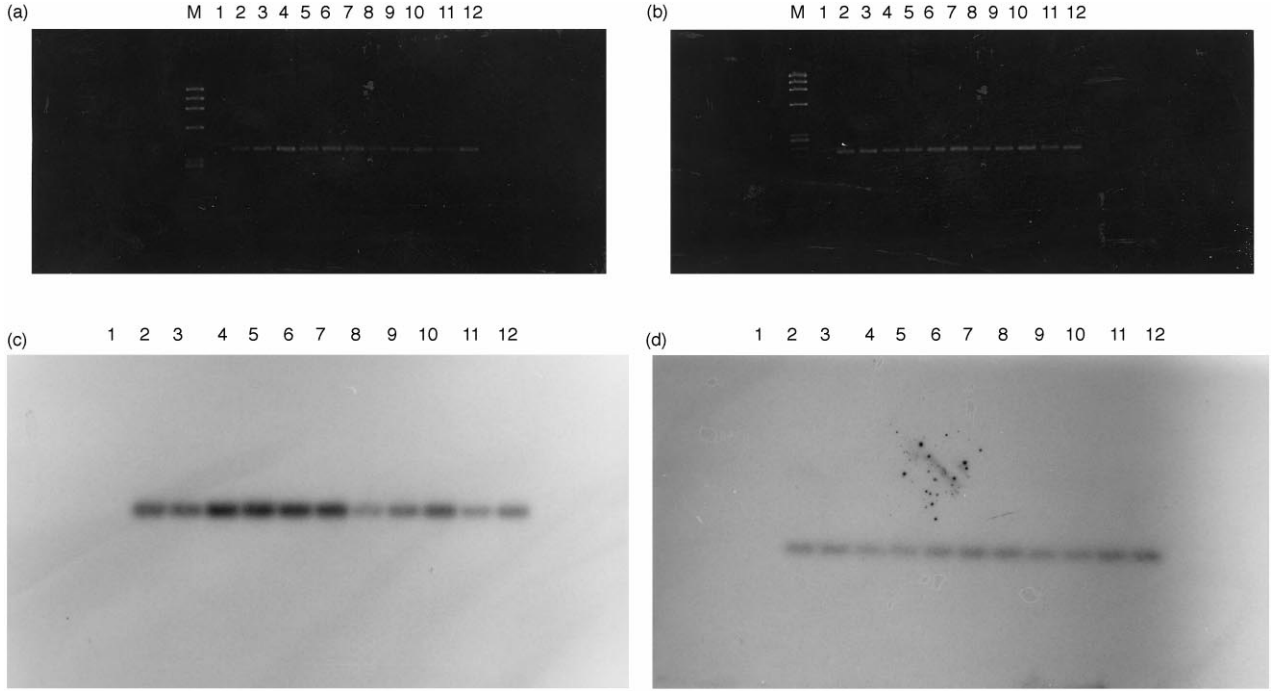

Figure 2. (a) Reverse transcription polymerase chain reaction (RT-PCR) analysis of human epidermal growth factor (EGF) receptors mRNA in MDA-MB-231 human breast cancers performed with specific primer. The expected length of the PCR product was 400 bp. M (molecular weight marker), ΦX174 Hae III digest; 1, negative control; 2–7, tumour samples from untreated animals; 8–12, tumour samples from mice treated with RC-3940-II at a dose of 10 μg twice daily. (b) RT-PCR analysis of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in MDA-MB-231 breast cancers performed with specific primers. The expected length of PCR product was 207 bp. (c) Southern blot analysis of human EGF receptors cDNA obtained after RT-PCR. Hybridisation was performed with the oligonucleotide probe specific for human EGF receptors. (d) Southern blot analysis of hGAPDH cDNA obtained after RT-PCR. Hybridisation was performed with the oligonucleotide probe specific for hGAPDH.

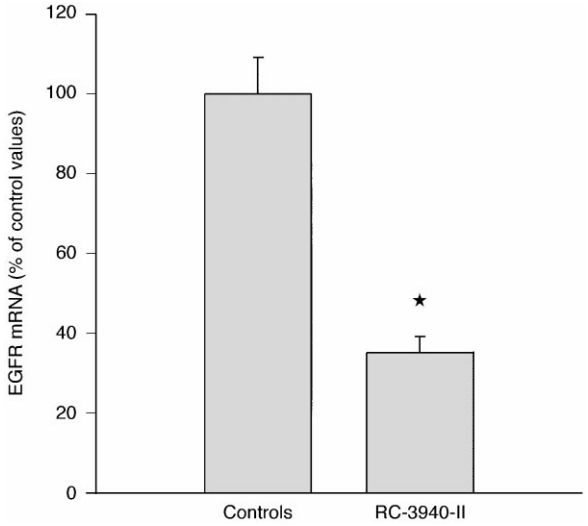

Figure 3. Densitometric analysis of epidermal growth factor (EGF) receptor mRNA from MDA-MB-231 breast tumours of control mice and animals treated with RC-3940-II. The levels of EGF receptor mRNA were standardised according to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and are expressed as a percentage of control value. The results are the mean ± SEM. The significance was calculated using Duncan's new multiple range test. *$P < 0.05$ versus control.

RC-3940-II, the level of EGF receptor mRNA expression was decreased to 35 ± 4% ($P < 0.05$) compared with that of controls (100 ± 9.2%) (Figure 3).

DISCUSSION

As part of a long-term project on the development of new hormonal approaches to treatment of various cancers, including oestrogen-independent breast cancers, a large number of bombesin/GRP antagonists has been synthesised and characterised in our laboratory [4, 7, 15, 19, 23]. Other laboratories have also pursued similar approaches [16, 18, 20–22] particularly with respect to synthesis of neuropeptide antagonists for the treatment of SCLC. Recently, we synthesised a series of new pseudononapeptide bombesin/GRP antagonists containing C-terminal Leuψ(CH_2N)Tac-NH_2 or its derivative [15, 17, 23]. Among them was the antagonist Hca^6, Leu$^{13}\psi$[CH_2N]Tac^{14}-BN(6-14) (RC-3940-II) containing structural modification of D-Tpi^6, Leu$^{13}\psi$[CH_2NH]-Leu^{14}-BN (6-14) (RC-3095), such as the replacement of N-terminal residue D-Tpi^6 by Hca and of C-terminal ψ(CH_2NH)Leu^{14} by ψ(CH_2N)Tac. Another new bombesin antagonist, D-Phe^6, Leu$^{13}\psi$[CH_2N]-Tac^{14}-BN (6-14) (RC-3950-II) was also synthesised as part of this series of analogues [15, 17, 23].

In the present study, we evaluated the *in vivo* effects of RC-3940-II and RC-3905 on growth of MDA-MB-231 oestrogen-independent human breast cancer. Both bombesin/GRP antagonists strongly inhibited the growth of MDA-MB-231 xenografts in nude mice producing significant reductions in the final tumour volume and tumour weight. A significant decrease in tumour volume could be detected 2 weeks after the start of treatment with either antagonist. RC-3940-II seemed to be somewhat more effective than RC-3095 in inhibiting tumour growth and significantly prolonged tumour doubling time. Final tumour growth inhibition was 72.4% ($P < 0.05$) for RC-3940-II and 57.7% ($P < 0.05$) for RC-3095. In groups treated with RC-3095 and RC-3940-II, serum EGF levels were reduced compared with the controls, but the decreases were not significant and serum IGF-I levels also showed no significant changes.

In a previous investigation [11], we evaluated the effectiveness of bombesin/GRP antagonists RC-3095 and RC-3950-II in MCF-7 MIII oestrogen-sensitive human breast cancers. The present study was carried out in MDA-MB-231 oestrogen-independent breast cancers. This line is representative of many human breast cancers and our work extends the findings on the therapeutic efficacy of bombesin/GRP antagonists to oestrogen-independent breast cancers.

Bombesin/GRP antagonist RC-3095, synthesised earlier in our laboratory [7], was shown to inhibit the growth of various tumours, including pancreatic, gastric, colorectal, prostatic and mammary cancers and SCLC [4, 10–12, 14, 24–28, 34, 39]. Among the analogues in the new series of bombesin/GRP antagonists, RC-3940-II and RC-3950-II appeared to be the most potent. RC-3940-II exhibited higher binding affinities to bombesin receptors on Swiss 3T3 cells than RC-3095 and RC-3950-II [17, 23]. Ligand competition assays in CFPAC-I human pancreatic cancer cells demonstrated that the binding affinity of RC-3940-II and RC-3950-II to bombesin/GRP receptors was 50 times and 5 times higher, respectively, than that of RC-3095. RC-3940-II had a higher binding affinity to the receptors on CFPAC-I human pancreatic cancer cells than any other bombesin antagonist tested in our laboratory [15]. The tumour growth inhibitory activity of RC-3940-II was greater than that of RC-3095 in hamsters with nitrosamine-induced pancreatic cancers and in nude mice bearing xenografts of SW-1990 human pancreatic adenocarcinoma [40, 41] and PC-3 or DU-145 human prostate cancers [27].

In this study, specific binding sites for bombesin/GRP and EGF were found on membranes of MDA-MB-231 human breast cancers. The suppressive effects of bombesin/GRP antagonists RC-3095 and RC-3940-II on the growth of MDA-MB-231 cells might be tentatively explained by the inhibition of the binding and action of endogenous bombesin-like peptides and interference with the complex cascade of intracellular events linked with the activation of EGF receptors [8, 9, 12, 16, 34, 42–44]. It is also possible that the effects of bombesin antagonists RC-3095 and RC-3940-II on the growth of MDA-MB-231 tumours may involve some other regulatory mechanisms as well. The mechanisms of action of bombesin or its analogues in the modulation of intracellular effector molecules are still unclear [34]. In the present study, a significant downregulation of EGF receptors occurred in MDA-MB-231 tumours after treatment with RC-3940-II or RC-3095. This major reduction in concentration of EGF receptors after treatment with RC-3095 was also previously demonstrated in other cancers including nitrosamine-induced pancreatic cancer in hamster [24], MXT mammary cancer in mice [12] and HT-29 colon cancer [25], DU-145 prostate cancer [27], MKN45 gastric cancer [39], H-128 SCLC [34] and MCF-7 MIII breast cancer in nude mice [10, 11]. Inhibition of growth of these cancers produced by bombesin antagonists was invariably linked to downregulation of EGF receptors [10–12, 24, 25, 27, 34]. The reduction in the number of EGF receptors on MDA-MB-231 tumours in nude mice produced by treatment with bombesin/GRP antagonist RC-3940-II was accompanied by a marked decrease in the levels of mRNA for EGF receptors. Since the reduction in

EGF receptor numbers on tumours after treatment with RC-3940-II coincided with the decrease in mRNA levels for the receptor, this suggests that loss of receptors is the result of downregulation of gene transcription or a change in the stability of mRNA for the receptor. These observations may be clinically relevant. Various studies suggest that EGF plays an important role in the regulation of proliferation of human breast cancer cells [4, 11, 45, 46]. EGF receptor positive status was shown to be correlated with the biological aggressiveness and poor survival in patients with breast cancer [45, 46]. The downregulation of EGF receptors might be responsible for the inhibitory effects of the bombesin/GRP antagonists RC-3095 and RC-3940-II on growth of mammary cancers. The antitumour effects of RC-3095 and RC-3940-II, mediated through bombesin/GRP receptors on tumours [6, 7, 16, 18, 20–23, 28, 42], might be produced by inhibition of the phosphorylation responses to EGF [34, 43].

It has been proposed that transmembrane signaling by bombesin and GRP involves a guanine-nucleotide-binding regulatory protein (G-protein) and the activation of adenyl cyclase in some cells [8, 16, 28]. The binding of bombesin to SCLC and Swiss 3T3 cells causes a rapid mobilisation of Ca^{2+} from internal stores which is mediated by inositol 1,4,5-triphosphate [16, 18]. Bombesin activates protein kinase C (PKC) by generating diacylglycerol as a second messenger [16, 18]. Bombesin also induces a release of arachidonic acid which contributes to mitogenesis [44, 47]. Benya and associates reported that bombesin and GRP induce an activation of adenyl cyclase which leads to an increase in intracellular cyclic adenosine monophosphate (cAMP) levels and results in the enhanced expression of proto-oncogene (*c-fos* and *c-myc*) and promotion of mitogenesis in Swiss 3T3 cells [48]. We have shown that RC-3095 inhibits cAMP production in SW-1990 cell cultures [41], but it is also possible that bombesin antagonists may exert their suppressive actions through other intracellular regulatory pathways independent of adenyl cyclase [43]. It has been shown that bombesin enhances phosphorylation of EGF receptors in various cancers and that the antagonist RC-3095 inhibited this effect [43]. These results suggest that bombesin and GRP may function by upregulating EGF receptors and RC-3095 prevents this up-regulation [43]. However, other mechanisms that still need to be elucidated might also be involved in the transmodulation of EGF receptors by bombesin antagonists. Further studies will be directed towards the identification of specific intracellular regulatory pathways involved in the inhibition of tumour cell growth induced by bombesin antagonists RC-3095 and RC-3940-II.

In conclusion, in nude mice with xenografted human breast cancer MDA-MB-231, the bombesin/GRP antagonist RC-3940-II showed a greater tumour growth inhibitory activity than RC-3095. The inhibition of tumour growth induced by these bombesin/GRP antagonists was accompanied by a major reduction in the number of membrane receptors for EGF and in the case of RC-3940-II, by a decrease in the mRNA levels for EGF receptors. Our findings suggest the merit of further investigations to determine whether bombesin/GRP antagonists could be used for the treatment of advanced oestrogen-independent breast carcinomas.

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