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Original Paper

Inhibition of Growth of Androgen-independent DU-145 Prostate Cancer *In Vivo* by Luteinising Hormone-releasing Hormone Antagonist Cetrorelix and Bombesin Antagonists RC-3940-II and RC-3950-II*

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The aim of this study was to test the antagonist of LH-RH (Cetrorelix), agonist [D-Trp^6]LH-RH (triptorelin) and new bombesin antagonists RC-3940-II and RC-3950-II for their effect on the growth of an androgen-independent prostate cancer cell line, DU-145, xenografted into nude mice. Xenografts were grown in male nude mice, and after 4 weeks, the animals were treated either with saline (control) or with one of the analogues. One group of mice was given a combination of Cetrorelix and RC-3950-II. Treatment was given for 4 weeks. Tumour and body weights, and tumour volumes were measured. At sacrifice, tumours were dissected for histological examination and receptor studies. Serum was collected for measurement of hormone levels. The final tumour volume in control animals injected with saline was 577 ± 155 mm^3 and that of animals treated with Cetrorelix only 121.4 ± 45 mm^3 ($P < 0.01$). Bombesin antagonists RC-3940-II and RC-3950-II also significantly reduced DU-145 tumour volume in nude mice to 84.9 ± 19.9 and 96.8 ± 28 mm^3, respectively. Agonist [D-Trp^6]LH-RH did not significantly inhibit tumour growth. Serum levels of LH were decreased to 0.08 ± 0.02 ng/ml ($P < 0.05$) in the Cetrorelix treated group as compared to 1.02 ± 0.1 ng/ml for the controls, and testosterone levels were reduced to castration levels (0.01 ± 0.01 ng/ml). Specific receptors for EGF and LH-RH in DU-145 tumours were significantly downregulated after treatment with Cetrorelix, RC-3940-II and RC-3950-II. Although LH-RH could be a local regulator of growth of prostate cancer, the fall in LH-RH receptors is not fully understood and the inhibitory effects of Cetrorelix and bombesin antagonists on DU-145 tumour growth might be attributed at least in part to a downregulation of EHF receptors. Since Cetrorelix and bombesin antagonists inhibit growth of androgen-independent DU-145 prostate cancers, these compounds could be considered for the therapy of advanced prostate cancer in men, especially after relapse. © **1997 Elsevier Science Ltd.**

Key words: bombesin/GRP antagonists, LH-RH antagonist, androgen-independent prostate cancer

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INTRODUCTION

CARCINOMA OF the prostate is the most common malignant tumour in men [1]. In spite of screening programmes for prostate cancer, approximately 40% of all prostate cancer patients are diagnosed at initial presentation with an advanced stage of the disease [1]. For the advanced stages,

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surgical or medical castration is the only established therapy with an effective first-line response rate of 70–80% [2], but the majority of these patients eventually relapse within 18–36 months when androgen suppression fails to control tumour growth. The mean survival of these patients is less than 1 year after the relapse [3].

The prostate is a highly hormone-sensitive organ, which is primarily under the control of the pituitary–gonadal axis. However, there is also good evidence that growth factors, such as EGF (epidermal growth factor) and IGF-I (insulin-like growth factor) and some neurohormones such as LH-RH (luteinising hormone-releasing hormone), growth hormone-releasing hormone (GH-RH), somatostatin and bombesin/gastrin releasing peptide (GRP) can affect the function and growth of this gland [4, 5]. Some of these growth factors or hormones might be responsible for the relapse of prostate cancer under androgen ablation therapy. Inhibitors of these factors or their receptors may be of use in the treatment of androgen-independent prostate cancer [4].

DU-145 is an androgen-independent cell line derived from a human prostate adenocarcinoma metastatic to the brain [6]. It has been shown that DU-145 cells possess receptors for LH-RH and EGF, produce both EGF and transforming growth factor-α (TGF-α) and respond to IGF-I [4, 7, 8]. DU-145 can be xenografted into nude mice and provides a valuable model for investigating the effects of new compounds on androgen-independent prostate cancer *in vivo*.

LH-RH agonists such as Decapeptyl (triptorelin), Leuprolide and Zoladex produce androgen deprivation and are widely used for therapy of prostate cancer patients [4]. Recently, a powerful LH-RH antagonist, Cetrorelix (SB-75), has been synthesised in our laboratory [4]. This compound suppresses the proliferation of prostatic, mammary and ovarian cancers in experimental models [9–11]. High-affinity receptors for bombesin/GRP are also present on the membranes of DU-145 cells [12]. Bombesin and its mammalian counterpart GRP have been shown to act as autocrine growth factors by stimulating the growth of various tumours such as small cell lung carcinoma, pancreatic, gastric and breast cancers *in vivo* or *in vitro* [13–15]. In a previous study we demonstrated that the somatostatin analogue, RC-160, could inhibit the growth of DU-145 prostate cancer, but the bombesin/GRP antagonist, RC-3095, was less effective [7]. In this study, we tested the effect of the LH-RH antagonist Cetrorelix and two new potent bombesin/GRP receptor antagonists, RC-3940-II and RC-3950-II [16, 17], on the growth of DU-145 xenografts in nude mice.

MATERIAL AND METHODS

Peptides

Bombesin antagonists RC-3940-II (Hca^6, Leu^{13} Ψ[CH_2N]Tac^{14}BN (6-14)) and RC-3950-II (D-Phe^6, Leu^{13}Ψ[CH_2N]Tac^{14}BN (6-14)) were synthesised by solid-phase methods and purified in our laboratory [16, 17]. 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 synthesised in this laboratory [4], was obtained from ASTA Medica (Frankfurt/Main, Germany). Agonist [D-Trp^6]LH-RH was provided by Debiopharm (Lausanne, Switzerland). Microcapsules of [D-Trp^6]LH-RH in poly(DL-lactide-co-glycolide) were prepared by a phase-separation process by Dr P. Orsolini at Cytotech (Martigny, Switzerland) and supplied by Debiopharm. RC-3940-II, RC-3950-II and [D-Trp^6]LH-RH were dissolved in 0.1% dimethyl sulphoxide (DMSO) in 0.9% saline. Cetrorelix was dissolved in distilled water containing 5% mannitol.

Animals

Male athymic NCR/c (nu/nu) 6-week-old mice were obtained from the National Cancer Institute (Bethesda, Maryland, U.S.A.) and housed in a laminar airflow cabinet under pathogen-free conditions with a 12 h light/12 h dark schedule and fed autoclaved standard chow and water *ad libitum* throughout the experiment. All animal studies were conducted in accordance with institutional guidelines for the care and use of experimental animals.

Cell culture

The human androgen-independent prostatic carcinoma line DU-145 was obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, U.S.A. It was grown as a monolayer in RPMI 1640 medium (Gibco, Grand Island, New York, U.S.A.) supplemented with 5% colostrum-free bovine serum, antibiotics and antimycotics, at 37°C in a humidified 95% air/5% CO_2 atmosphere. Tumour cells growing exponentially were harvested by a brief incubation with 0.25% trypsin-EDTA solution (Gibco). Xenografts were initiated by s.c. injection of 1×10^7 cells into the right flanks of 5 male nude mice.

Experimental protocol

Tumours resulting after 8 weeks were aseptically dissected and mechanically minced; 3 mm^3 pieces of tumour tissue were transplanted s.c. by trocar needle into 60 male animals. The tumour take rate was around 70%. Four weeks after transplantation, tumours had grown to a volume of approximately 70–90 mm^3. The tumour-bearing mice were then divided into six experimental groups of 8 animals each, which received the following treatments: group 1, (control) saline only; group 2, agonist [D-Trp^6]LH-RH at a dose of 100 μg/day/animal s.c.; group 3, Cetrorelix, 100 μg/day/animal s.c.; group 4, RC-3950-II at a dose of 10 μg/twice daily s.c.; group 5, the combination of 100 μg/day Cetrorelix and RC-3950-II, 10 μg/twice daily s.c.; group 6, bombesin antagonist RC-3940-II at a dose of 10 μg/twice daily s.c. The treatment was continued for 4 weeks. The tumours were measured once a week with microcalipers and the tumour volume was calculated as length × width × height × 0.5236 [18]. Tumour volume doubling time was calculated as described [19]. At the end of the experiment, mice were anaesthetised with methoxyflurane (Metofane; Pitman–Moore, Mundelein, Illinois, U.S.A.), killed 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. Tumours were cleaned and weighed, and samples were taken for histology and receptor studies.

To confirm our findings on sex hormones in animals with DU-145 prostate cancers, non-tumour bearing nu/nu male nude mice were divided into four experimental groups of 7 animals each. They received the following treatment: group 1 (control), saline only; group 2, [D-Trp^6]LH-RH agonist at a dose of 100 μg/day/animal s.c; group 3, [D-Trp^6]LH-RH microcapsules releasing 25 μg/day injected once s.c.;

group 4, RC-3940-II at a dose of 10 μg/twice daily s.c. After 15 days mice were killed as described above, and blood was collected for further analysis.

Radioimmunoassay

LH was determined by RIA (radio-immuno-assay) using materials provided by the National Hormone and Pituitary Program (NHPP, Rockville, Maryland, U.S.A.) (rat LH-RP-3/AFP-71871B/, rat LH-I-9/AFP-10250C/, anti-rat LH-RIA-11/AFP C 697071P). Serum testosterone levels were determined using a Coat-A-Count RIA kit from Diagnostic Products Corp. (Los Angeles, California, U.S.A.). Interassay and intra-assay coefficients of variation were less than 15% and 10%, respectively, for the assays. Serum gastrin levels were measured by double antibody RIA using a kit provided by Becton Dickinson (Orangeburg, New York, U.S.A.). The interassay variation was less than 7.3%, and the intra-assay variation was less than 4.0%.

Receptor assay

Measurements of receptors for bombesin, EGF and LH-RH in the membranes of DU-145 tumours were performed as previously described [19–21]. The LIGAND-PC computerised 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}) [22].

Histological procedure

Sex organs and a part of each tumour were fixed in 10% buffered formalin. Specimens were embedded in Paraplast (Oxford Labware, St. Louis, Missouri, U.S.A.). 6 μm 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, 200 cells and their number per 1000 cells were accepted as the mitotic and apoptotic indices, respectively. Areas of necrosis or inflammation were avoided. As criteria for mitotic figures, the absence of nuclear membrane and a condensation of chromosomes with arrangements characteristic of meta-, ana- and telophase of mitosis were accepted. Apoptotic alterations affecting usually single cells or small groups of cells included the shrinkage of isolated tumour cells, karyopyknosis, karyorrhexis and the appearance of small liberated cell fragments between the epithelial cells or in vacuoles within the cells (phagocytosed apoptotic bodies). For the demonstration of the nucleolar organiser region (NOR) in tumour cell nuclei, the AgNOR method was used [23]. The silver-stained black dots in 50 cells of each tumour were counted and the AgNOR number per cell was calculated. Alteration of testicles were classified according to the percentage rate of tubules containing the most advanced germ cell type, i.e. elongated and round spermatids, spermatocytes, spermatogonia. A total number of 100 tubules were analysed on one cross-section of each testicle.

[^{3}H] Thymidine assay

The ability of peptide analogues to inhibit the incorporation of [methyl-^{3}H] thymidine into the DNA of the human DU-145 cell line in culture was assayed as described earlier [14, 15].

Statistical methods

All data are expressed as the mean ± SEM, and statistical analyses of the tumour data were performed using Duncan's new multiple range test [24].

RESULTS

Effects of [D-Trp^6]LH-RH, Cetrorelix, RC-3940-II and RC-3950-II on DU-145 tumours in nude mice

The effects of treatment with peptide analogues on final tumour volume, body, tumour, sex-organ weights and tumour doubling time are shown in Tables 1 and 2. After 4 weeks of therapy, when the experiment was terminated, there was no significant difference in the body weights between the groups. Figure 1 shows the tumour volume as measured at weekly intervals. Agonist [D-Trp^6]LH-RH was ineffective in suppressing the growth of DU-145 androgen-independent tumours, whereas Cetrorelix, RC-3940-II, RC-3950-II and the combination of Cetrorelix and RC-3950-II powerfully inhibited tumour growth (Table 1, Figure 1). A significant inhibition could be achieved within 21 days from the start of the therapy ($P < 0.05$). After 4 weeks of treatment, the tumour volume was significantly reduced ($P < 0.01$) in groups receiving Cetrorelix, RC-3940-II or RC-3950-II to $121.4 \pm 45.8\ mm^3$, $84.9 \pm 19.9\ mm^3$ and $96.8 \pm 28\ mm^3$, respectively, compared with the control group ($577 \pm 155.1\ mm^3$) (Table 1). The control group had a tumour doubling time of 10.5 ± 1.5 days (Table 1). Tumour doubling time was prolonged by Cetrorelix, RC-3940-II, and RC-3950-II to 40.2 ± 16, 28.9 ± 3.9 and 37.7 ± 4.4 days, respectively. Therapy with the combination of Cetrorelix and RC-3950-II resulted in a decrease in tumour growth similar to that obtained with single drugs (Figure 1, Table 1). The final tumour weights were significantly reduced in the groups treated with Cetrorelix, RC-3940-II and RC-3950-II compared with the controls (Table 1). The combination of Cetrorelix and RC-3950-II did not lead to a further decrease in tumour weights. Agonist [D-Trp^6]LH-RH had no significant effect on tumour doubling time or tumour weights.

Effects of peptide analogues on sex organs and sex hormone levels

There was a significant decrease in the weights of testes, seminal vesicles and prostate in the two groups that received Cetrorelix alone or in combination with RC-3950-II (Table 2). LH-RH agonist Decapeptyl led only to a 25% reduction in the total weight of the sex organs, while the bombesin antagonists had no significant effect. Serum levels of LH, testosterone and gastrin in animals with DU-145 tumours treated with peptide analogues or saline are shown in Table 3. Serum LH levels were significantly reduced in groups treated with Cetrorelix given singly to 0.08 ± 0.02 ng/ml or in combination to 0.04 ± 0.02 ng/ml as compared to control animals (1.02 ± 0.1 ng/ml). [D-Trp^6]LH-RH agonist had no effect on the LH levels (0.99 ± 0.08 ng/ml), but interestingly RC-3940-II reduced LH levels by 64% to 0.37 ± 0.2 ng/ml. Serum testosterone was at castration levels in groups treated with Cetrorelix or the combination (0.01 ± 0.01 ng/ml and 0.02 ± 0.01 ng/ml, respectively, versus 8.1 ± 1.3 ng/ml for controls). The LH-RH agonist did not affect the testosterone levels, but bombesin antagonists RC-3940-II and RC-3950-II significantly lowered testosterone levels by 92% and 85%, respectively ($P < 0.05$) (Table 3). Serum gastrin levels were reduced by 20 to 23%

Table 1. Effect of treatment with [D-Trp^6]LH-RH, Cetrorelix, RC- 3950-II, the combination of Cetrorelix and RC-3950-II or RC-3940-II on tumour volume, body and tumour weight, and tumour doubling time in nude mice bearing xenografts of the human prostate cancer cell line DU-145

Treatment group	Tumour volume (mm^3) Initial	Final	Body weight (g)	Tumour weight (mg)	Tumour doubling time (days)
Control	89.5 ± 17.1	577 ± 155	30.2 ± 1.2	265 ± 70	10.5 ± 1.5
[D-Trp^6]LHRH	74.3 ± 21.2	518 ± 146	28.1 ± 2.4	224 ± 52	12.0 ± 3.9
Cetrorelix	78.3 ± 25.1	121.4 ± 45.8*	29.5 ± 0.8	61.7 ± 13†	40.2 ± 16†
RC-3950-II	88.4 ± 17.2	96.8 ± 28*	27.9 ± 1.6	57.1 ± 10†	37.7 ± 4.4*
Cetrorelix + RC-3950-II	106 ± 29.2	126 ± 45*	28.7 ± 1.6	64.1 ± 23†	38.2 ± 8.3†
RC-3940-II	66.4 ± 9.4	84.9 ± 19.9*	28.2 ± 1.6	62.2 ± 18†	28.9 ± 3.9*

Values are mean ± SEM. *$P < 0.05$ versus control; †$P < 0.01$ versus control.

Table 2. Weight of different sex organs of nude mice bearing DU-145 human prostate cancer cell line xenografts after treatment with [D-Trp^6]LH-RH, Cetrorelix, RC-3950-II, the combination of Cetrorelix and RC-3950-II or RC-3940-II

Treatment group	Testis (mg)	Seminal vesicles (mg)	Prostate (mg)	Total (mg)
Control	240 ± 18	585 ± 122	95 ± 13	1066 ± 70
[D-Trp^6]LHRH	N.D.	N.D.	N.D.	801 ± 41†
Cetrorelix	85 ± 47†	202 ± 72*	24 ± 22*	302 ± 28†
RC-3950-II	254 ± 30	468 ± 86	64 ± 7	865 ± 103
Cetrorelix + RC-3950-II	93 ± 23†	243 ± 57*	20 ± 15†	353 ± 52†
RC-3940-II	262 ± 22	530 ± 72	85 ± 3	877 ± 58

Values are mean ± SEM. *$P < 0.05$ versus control; †$P < 0.01$ versus control.
N.D., not determined individually.

in the groups treated with the bombesin antagonists, but this decrease was not statistically significant.

In the experiment with nude mice not bearing tumours, serum LH levels were significantly lower in the groups receiving [D- Trp^6]LH-RH microcapsules and RC-3940-II (0.27 ± 0.06 ng/ml and 0.27 ± 0.03 ng/ml, respectively, versus 0.56 ± 0.15 ng/ml for the controls; $P < 0.05$), whereas daily administration of agonist [D-Trp^6]LH-RH had no significant effect on serum LH (0.31 ± 0.03 ng/ml) (Table 4). Serum testosterone was reduced in all treated groups, but castration levels were only achieved in groups receiving [D-Trp^6]LH-RH microcapsules and RC-3940-II (0.36 ± 0.05 ng/ml and 0.5 ± 0.24 ng/ml, respectively, versus 7.18 ± 2.67 ng/ml for control; $P < 0.01$) (Table 4).

Histological findings

Histologically, DU-145 prostate cancers were undifferentiated tumours consisting of large epithelial cells. The cells were arranged in solid nests surrounded by a very scanty stroma. Only a few tumours showed focal glandular differentiation. The cells had large, round or oval nuclei, containing prominent nucleoli. Extensive necrosis was found in most tumours. The number of mitotic cells and the AgNOR count was not notably altered by the treatments. However, a significant enhancement of apoptosis was found in the tumours after therapy with Cetrorelix (Table 5). Sex organs of control animals and of those treated with [D-Trp^6]LH-RH showed normal histological structure. Moderate atrophic signs were found hitstologically in the prostates and seminal vesicles of mice after treatment with Cetrorelix.

Effects of peptide analogues on DU-145 receptor status

The results of the receptor assays for EGF, bombesin/GRP and LH-RH following treatment with the peptide analogues are shown in Table 6. High-affinity binding sites for EGF were present in the membranes of DU-145 tumours. A major and highly significant ($P < 0.01$) reduction in maximal binding capacity (B_{max}) of EGF receptors and a moderate, but non-significant, increase in affinity were observed after treatment with all analogues tested. The decrease in EGF receptor concentration after [D-Trp^6]LH-RH treat-

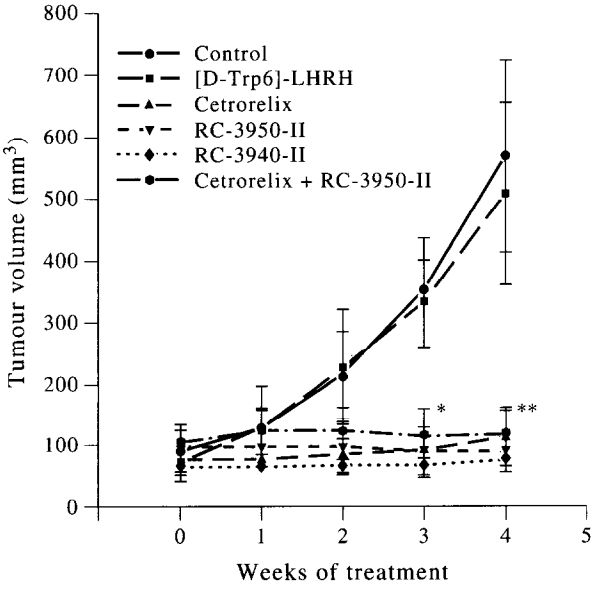

Figure 1. Tumour volume in nude mice with DU-145 human prostate carcinoma xenografts during treatment with agonist [D- Trp^6]LH-RH, LH-RH antagonist Cetrorelix, bombesin/GRP antagonists RC-3940-II and 3950-II, and the combination of Cetrorelix and RC-3950-II. Vertical lines indicate the SEM. *$P < 0.05$; **$P < 0.01$ versus control by Duncan's new multiple range test.

Table 3. Serum levels of LH, testosterone and gastrin in nude mice bearing DU-145 human prostate cancer cell line xenografts after treatment with [D-Trp^6]LH-RH, Cetrorelix, RC-3950-II, the combination of Cetrorelix and RC-3950-II or RC-3940-II

Treatment group	LH (ng/ml)	Testosterone (ng/ml)	Gastrin (pg/ml)
Control	1.02 ± 0.1	8.1 ± 1.3	116 ± 18
[D-Trp^6]LH-RH	0.99 ± 0.08	10.1 ± 1.5	90.8 ± 15
RC-3950-II	N.D.	1.2 ± 0.8*	92.3 ± 9
Cetrorelix	0.08 ± 0.02*	0.01 ± 0.01*	143 + 69
Cetrorelix + RC-3950-II	0.04 ± 0.02*	0.02 ± 0.01*	79.2 ± 8
RC-3940-II	0.37 ± 0.2	0.62 ± 0.3*	89.3 ± 7.8

Values are mean ± SEM. *$P < 0.05$ versus control.
N.D., not determined.

ment was only 36%, whereas all other compounds produced a decrease of 61–73%. The greatest decrease in EGF receptor levels was achieved with cetrorelix (Table 6).

Specific, high-affinity binding sites for ^{125}I-[Tyr^4]-bombesin were also found in membranes from untreated control DU-145 prostate tumours (Table 6). Binding characteristics were $K_d = 9.6 \pm 0.93$ nM and $B_{max} = 1101 \pm 68.4$ fmol/mg membrane protein. After treatment with bombesin antagonists RC-3940-II and RC-3950-II (alone or in combination with Cetrorelix), the number of receptors for [Tyr^4]-bombesin showed a statistically significant decrease. Cetrorelix alone and [D-Trp^6]LH-RH agonist had no significant effect on bombesin receptor characteristics. Specific, moderate high-affinity binding sites for ^{125}I-[D-Trp^6] LH-RH were similarly detected in the membranes of DU-145 cells. The calculation showed that $K_d = 11.5 \pm 0.05$ nM and $B_{max} = 1191 \pm 34.6$ fmol/mg membrane protein. After treatment with Cetrorelix, alone or in combination with RC-3950-II, the concentration of receptors for LH-RH was significantly decreased (by 80% and 73%, respectively, versus controls). Agonist [D-Trp^6]LH- RH also reduced the number of LH-RH binding sites, but to a lesser extent than Cetrorelix. After the administration of RC-3940-II and RC-3950-II, LH-RH receptors were also downregulated by 57 and 63%, respectively (Table 6).

Effects of Cetrorelix and RC-3940-II on DU-145 cells in vitro

In cell culture experiments, inhibition of DU-145 cell proliferation could be achieved only at high concentrations of Cetrorelix or RC-3940-II. In the [methyl-^{3}H] thymidine incorporation assay, 10^{-5} M concentrations of either analogue were needed to obtain an approximate 20% reduction

Table 4. Serum LH and testosterone levels in nude mice without tumours after treatment with [D-Trp^6]LH-RH, [D-Trp^6]LH-RH microcapsules and RC-3940-II

Treatment group	LH (ng/ml)	Testosterone (ng/ml)
Control	0.56 ± 0.15	7.18 ± 2.67
[D-Trp^6]LH-RH	0.31 ± 0.03	1.58 ± 0.39*
[D-Trp^6]LH-RH microcapsules	0.27 ± 0.06*	0.36 ± 0.06†
RC-3940-II	0.27 ± 0.03*	0.50 ± 0.24†

Values are mean ± SEM. *$P < 0.05$ versus control; †$P < 0.01$ versus control.

Table 5. Evaluation of treatment with [D-Trp^6]LH-RH and Cetrorelix on some histological characteristics of DU-145 hormone-independent human prostate cancers growing in nude mice

Treatment group	Tumours Number of mitoses per 1000 cells	Number of apoptotic cells/1000 cells	AgNOR number/cell
Control	18.0 ± 2.9	10.7 ± 1.6	7.98 ± 0.33
[D-Trp^6]LH-RH	15.0 ± 2.5	13.2 ± 1.8	8.37 ± 0.37
Cetrorelix	15.7 ± 3.2	20.9 ± 1.6*	7.34 ± 0.26

Values are mean ±SEM. *$P < 0.05$ versus control.

in the [^{3}H] thymidine uptake into DNA of DU-145 cells during 48 h of incubation (data not shown).

DISCUSSION

The DU-145 cell line can be used to study the effect of different compounds on androgen-independent prostate cancer cells. It has been shown that bombesin/GRP [25] and growth factors such as EGF, TGF-α [8, 26] and IGF-II [27] may stimulate cell growth in different prostate cancer models by autocrine and paracrine mechanisms. These factors might be involved in the autonomous growth of androgen-independent cells. The role of EGF in the growth of DU-145 prostate cancers is suggested by the downregulation of EGF receptors in membranes of tumours inhibited by treatment with bombesin antagonists. This is in accordance with our previous studies in which the inhibition of growth of pancreatic, gastric, colorectal, prostatic, and mammary cancers and other tumours by bombesin/GRP antagonists was invariably accompanied by a major downregulation of EGF receptors [4, 7, 19, 23, 28]. Receptors for bombesin/GRP are present on membranes of DU-145 cells [12]. Various studies have shown that bombesin-like peptides act as autocrine growth factors in stimulating the growth of various tumours, such as small cell lung cancer and pancreatic and mammary cancers [13–15]. The bombesin-like peptides can bind to high-affinity bombesin–GRP receptors on the cell surface of these cells. This elicits a complex cascade of biological responses, starting with the activation of G-proteins, enhanced inositol phospholipid breakdown and mobilisation of Ca^{2+} from intracellular stores and activation of protein kinase C [29]. Activation of protein kinase C causes phosphorylation of EGF receptors on threonine residues. Bombesin and GRP have also been shown to enhance the phosphorylation of EGF receptors, and antagonist RC-3095 inhibits this effect in various cancer lines and cancer specimens [30]. Bombesin/GRP antagonists might also block the initial phases of the G-protein-mediated messenger mechanisms.

High-affinity receptors for bombesin/GRP on DU-145 cell line have been characterised in our laboratory and it has also been established that bombesin antagonist RC-3095 powerfully blocks the specific binding sites [12]. In a previous study, Pinski and associates [7] showed that bombesin antagonist RC-3095 inhibited the growth of DU-145 prostate cancer and prolonged tumour doubling time by approximately 3.1 days, but the present work demonstrates that the new antagonists RC-3940-II and RC-3950-II are much more effective.

Table 6. Binding characteristics of EGF, bombesin/GRP and LH-RH receptors in membranes of DU-145 human prostate cancer cell line xenografts after in vivo *treatment with [D-Lys^6]LH-RH, Cetrorelix, RC-3950-II or the combination of Cetrorelix and RC-3950-II or RC-3940-II*

Treatment group	Epidermal growth factor receptor (EGF-R)		Bombesin/GRP receptor		LH-RH receptor	
	K_d (nM)	B_{max} (fmol/mg)*	K_d (nM)	B_{max} (fmol/mg)*	K_d (nM)	B_{max} (fmol/mg)*
Control	0.79 ± 0.0	330.7 ± 16.4	9.6 ± 0.93	1101 ± 68.4	11.5 ± 0.05	1191 ± 34.6
[D-Trp^6]LH-RH	0.70 ± 0.15	210.2 ± 24.6†	7.76 ± 0.82	1043 ± 59.9	9.63 ± 3.37	592 ± 30.9†
Cetrorclix	0.62 ± 0.08	88.7 ± 10.7†	9.22 ± 0.01	1022 ± 204	6.31 ± 0.55	242 ± 6.85†
RC-3950-II	0.34 ± 0.16	89.5 ± 8.7†	6.42 ± 0.85	346.8 ± 36.6†	10.7 ± 0.4	439 ± 74.8†
Cetrorelix + RC-3950-II	0.49 ± 0.11	104.7 ± 13.9†	8.37 ± 1.83	446.7 ± 52.4†	6.49 ± 2.11	322 ± 77.2†
RC-3940-II	0.59 ± 0.32	128.1 ± 1.0†	5.39 ± 3.08	407.2 ± 16.6†	11.7 ± 2.71	515 ± 124†

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 ± SEM.
*Per mg protein; †$P < 0.01$ versus control.

LH-RH agonists exert their effects by pituitary desensitisation and inhibition of sex steroid production. Furthermore, Dondi and associates [31] have shown that LH-RH agonists inhibit the proliferation of DU-145 prostate cancer cell line *in vitro*. This was attributed to a direct effect on LH-RH receptors, expressed in the membranes of this tumour [31]. LH-RH antagonists like Cetrorelix also inhibit the pituitary gonadal axis and lead to a dramatic fall in serum testosterone levels [4, 9, 32]. Clinical trials with Cetrorelix are now in progress in men with prostate cancer or benign prostate hyperplasia (BPH) [4, 32]. LH-RH antagonists may also exert a direct inhibitory effect on different tumours [4, 1, 32]. It is possible that the growth of DU-145 prostatic cancer cells is regulated at least in part by an autocrine loop based on LH-RH [31] and an inhibitory effect of LH-RH antagonists may be mediated through LH-RH binding sites on the tumour cell membranes [4, 9, 11]. Our histological studies show that Cetrorelix affects apoptosis rather than the proliferation rate of DU-145 cells. Enhancement of apoptotic cell death may be an important factor in tumour inhibition, although the exact mechanism by which Cetrorelix increases apoptosis in this hormone-independent cancer remains to be clarified.

In addition, our *in vivo* data point to another mechanism of action of LH-RH analogues, which could involve EGF receptors. However, the agonist [D-Trp^6]LH-RH decreased EGF receptor concentration much less than the antagonist Cetrorelix and, also in contrast to Cetrorelix, had no effect on tumour growth. Similarly, Cetrorelix but not [D-Trp^6] LH-RH inhibited growth of OV-1063 human epithelial ovarian cancers in nude mice and produced a reduction in levels of EGF receptor and its mRNA [11, 33]. The exact molecular mechanism of action of LH-RH antagonists on tumour growth still remains to be elucidated, but it is possible that antagonists like Cetrorelix affect EGF receptors [11].

In nude mice bearing DU-145 tumours, serum LH levels and testosterone levels were significantly lower in the Cetrorelix treated groups, as compared to the [D-Trp^6] LH-RH treated group. We have shown previously that to obtain the suppression of the pituitary–gonadal axis in athymic mice, sustained release formulations of agonist [D-Trp^6]LH-RH such as microcapsules must be used [9, 34]. These findings were confirmed in an experiment with nude mice without implanted tumours. In contrast to [D-Trp^6]LH-RH microcapsules, which significantly reduced LH levels and produced castration values of serum testosterone, [D-Trp^6]LH-RH administered at a dose of 100 µg once a day caused a non-significant fall in LH, which was not sufficient to achieve medical castration. It is also interesting that our powerful bombesin antagonist RC-3940-II lowered serum testosterone levels, most likely by suppressing pituitary LH release. The findings on serum hormone levels suggest that bombesin antagonists might cross the blood–brain barrier and inhibit LH release through an effect on the hypothalamus, or exert a direct suppressive action on the pituitary. In our previous work with a weaker bombesin/GRP antagonist RC-3095, an inhibition of LH release was found only after intracerebral, but not intravenous, administration in rats [35].

The combination of Cetrorelix and RC-3950-II did not exert greater inhibitory effects on tumour growth and receptor levels than the administration of single analogues. Since these analogues are thought to work through different mechanisms [4, 7, 9–11, 14, 15, 19, 23, 29, 30], the expectation was that the combination might potentiate tumour growth suppression. It may be that virtually maximal tumour growth inhibition and possibly EGF receptor downregulation were already achieved by the single agent or that there was an interaction between these analogues, decreasing their individual effectiveness. This lack of potentiation with peptide combinations was observed in previous studies on prostatic, mammary and pancreatic cancer [19, 28, 36].

Collectively, our studies show that Cetrorelix (SB-75) as well as bombesin antagonists RC-3940-II and RC-3950-II effectively inhibit growth of DU-145 prostate carcinoma *in vivo*. The findings that tumour growth inhibition was accompanied by a marked downregulation of receptors for LH-RH and EGF in the Cetrorelix treated groups and those for bombesin and EGF in animals given RC-3940-II and RC-3950-II, suggests a possible involvement of LH-RH, bombesin and EGF in the autocrine stimulation of DU-145 tumour growth. Inhibition of these receptors might lead to a suppression of tumour growth. Since LH-RH an-

tagonist Cetrorelix and bombesin antagonists RC-3940-II and RC-3950-II decrease growth of this androgen-independent tumour, these analogues may be considered for the development of new approaches to the treatment of the androgen-independent prostate carcinoma in men, especially after relapse.

1. Schroeder FH, Boyle P. Screening of prostate cancer—necessity or nonsense? *Eur J Cancer* 1993, **29A**, 656–661.
2. Sharifi R, Soloway M. The Leuprolide Study Group. Clinical study of leuprolide depot formulation in the treatment of advanced prostate cancer. *J Urol* 1990, **143**, 68–71.
3. Storlie JA, Buckner JC, Wiseman GA, Burch PA, Hartmann LC, Richardson RL. Prostate specific antigen levels and clinical response to low dose dexamethasone for hormone-refractory metastatic prostate carcinoma. *Cancer* 1995, **76**, 96–100.
4. Schally AV, Comaru-Schally AM, Gonzalez-Barcena D. Present status of agonistic and antagonistic analogs of LH-RH in the treatment of advanced prostate cancer. *Biomed Pharmacother* 1990, **46**, 465–471.
5. Reubi JC, Waser B, Schaer JC, Markwalder R. Somatostatin receptors in human prostate and prostate cancer. *J Clin Endocrin Metab* 1995, **80**, 2806–2814.
6. Stone KR, Mickey DD, Wunderli H, Mickey GH, Paulson DF. Isolation of a human prostate carcinoma cell line. *Int J Cancer* 1978, **21**, 274–281.
7. Pinski J, Halmos G, Schally AV. Somatostatin analog RC-160 and bombesin/gastrin-releasing peptide antagonist RC-3095 inhibit the growth of androgen-independent DU-145 human prostate cancer line in nude mice. *Cancer Lett* 1993, **71**, 189–196.
8. Connolly JM, Rose DP. Interaction between epidermal growth factor-mediated autocrine regulation and linoleic acid-stimulated growth of a human prostate cancer cell line. *Prostate* 1992, **20**, 151–158.
9. Redding TW, Schally AV, Radulovic S, Milovanovic S, Szepeshazi K, Isaacs JT. Sustained release formulations of luteinizing hormone-releasing hormone antagonist SB-75 inhibit proliferation and enhance apoptotic cell death of human prostate carcinoma (PC-82) in male nude mice. *Cancer Res* 1992, **52**, 2538–2544.
10. Szepeshazi K, Milovanovic S, Lapis K, Groot K, Schally AV. Growth inhibition of estrogen independent MXT mouse mammary carcinomas in mice treated with an agonist or antagonist of LH-RH, an analog of somatostatin, or a combination. *Breast Cancer Treat Res* 1992, **21**, 181–192.
11. Shirahige Y, Cook CB, Pinski J, Halmos G, Nair R, Schally AV. Treatment with luteinizing hormone-releasing hormone antagonist SB-75 decreases levels of epidermal growth factor receptor and its mRNA in OV-1063 human epithelial ovarian cancer xeongrafts in nude mice. *Int J Oncol* 1994, **5**, 1031–1035.
12. Reile H, Armatis P, Schally AV. Characterization of high-affinity receptors for bombesin/gastrin releasing peptide on the human prostate cancer cell lines PC-3 and DU-145: Internalization of receptor bound ^{125}I-(Tyr^4) bombesin by tumor cells. *Prostate* 1994, **25**, 29–38.
13. Cuttitta F, Carney DN, Mulshine J, *et al.* Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer cells. *Nature* 1986, **316**, 823–826.
14. Qin Y, Ertl T, Cai RZ, Halmos G, Schally AV. Inhibitory effect of bombesin receptor antagonist RC-3095 on the growth of human pancreatic cancer cells *in vivo* and *in vitro*. *Cancer Res* 1994, **54**, 1035–1041.
15. Yano T, Pinski J, Groot K, Schally AV. Stimulation by bombesin and inhibition by bombesin/gastrin-releasing peptide antagonist RC-3095 of growth of human breast cancer cell lines. *Cancer Res* 1992, **52**, 4545–4547.
16. Cai RZ, Qin Y, Ertl T, Schally AV. New pseudononapeptide bombesin antagonists with C-terminal LeuΨ(CH_2N)Tac-NH_2 showing high binding affinity to bombesin/GRP receptors in CFPAC-1 human pancreatic cancer cells. *Int J Oncol* 1995, **6**, 1165–1172.
17. Cai RZ, Reile H, Armatis P, Schally AV. Potent bombesin antagonists with C-terminal LeuΨ(CH_2 N)Tac-NH_2 or its derivatives. *Proc Natl Acad Sci USA* 1994, **91**, 12664–12668.
18. Janik P, Briand P, Hartmann NR. The effect of estrone–progesterone treatment on cell proliferation kinetics of hormone-dependent GR mouse mammary tumors. *Cancer Res* 1975, **35**, 3698–3704.
19. Pinski J, Schally AV, Halmos G, Szepeshazi K. Effect of somatostatin analog RC-160 and bombesin/gastrin releasing peptide antagonist RC-3095 on growth of PC-3 human prostate-cancer xenografts in nude mice. *Int J Cancer* 1993, **55**, 963–967.
20. Halmos G, Pinski J, Szoke B, Schally AV. Characterization of bombesin/gastrin-releasing peptide receptors in membranes of MKN45 human gastric cancer. *Cancer Lett* 1994, **85**, 111–119.
21. Szoke B, Horvath J, Halmos G, *et al.* LH-RH analogue carrying a cytotoxic radical is internalized by rat pituitary cells *in vitro*. *Peptides* 1994, **15**, 359–366.
22. Munson JP, Rodbard D. A versatile computerized approach for characterization of ligand binding systems. *Anal Biochem* 1980, **107**, 220–239.
23. Szepeshazi K, Cai RZ, Radulovic S, Milovanovic S, Szoke B, Schally AV. Inhibitory effect of bombesin/GRP antagonist RC-3095 and high dose of somatostatin analog RC-160 on nitrosamine-induced pancreatic cancers in hamsters. *Cancer Res* 1991, **51**, 5980–5986.
24. Steel RGD, Torrie J. *Principles and Procedures of Statistics*. New York, McGraw Hill, 1976, 114.
25. Bologna M, Festuccia C, Muzi P, Biordi L, Ciomei M. Bombesin stimulates growth of human prostatic cancer cells *in vitro*. *Cancer* 1989, **63**, 1714–1720.
26. Hofer DR, Sherwood ER, Bromberg WD, Mendelsohn J, Lee C, Kozlowski JM. Autonomous growth of androgen-independent human prostatic carcinoma cells: role of transforming growth factor α. *Cancer Res* 1991, **51**, 2780–2785.
27. Angelloz-Nicoud P, Binoux M. Autocrine regulation of cell proliferation by the insulin-like growth factor (IGF) and IGF binding protein-3 protease system in a human prostate carcinoma cell line (PC-3). *Endocrinology* 1995, **136**, 5485–5492.
28. Yano T, Pinski J, Szepeshazi K, *et al.* Inhibitory effect of bombesin/gastrin-releasing peptide antagonist RC-3095 and luteinizing hormone-releasing hormone antagonist SB-75 on the growth of MCF-7 MIII human breast cancer xenografts in athymic nude mice. *Cancer* 1994, **73**, 1229–1238.
29. Woll PJ, Rozengurt E. [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P, a potent bombesin antagonist in murine Swiss 3T3 cells, inhibits the growth of human small lung cancer cells *in vitro*. *Proc Natl Acad Sci USA* 1988, **81**, 1859–1863.
30. Liebow C, Crean DH, Lee MT, Kramer AR, Mang TS, Schally AV. Synergistic effects of bombesin and epidermal growth factor on cancers may stimulate growth through upregulation of EGF receptors. *Proc Natl Acad Sci USA* 1994, **91**, 3804–3808.
31. Dondi D, Limonta P, Moretti RM, Marelli MM, Garattini E, Motta M. Antiproliferative effects of luteinizing hormone-releasing hormone (LHRH) agonists on the human androgen-independent prostate cancer cell line DU-145: evidence for an autocrine-inhibitory LHRH loop. *Cancer Res* 1994, **54**, 4091–4095.
32. Gonzales-Barcena D, Vadillo-Buenfil M, Gomez-Orta F, *et al.* Responses to the antagonistic analog of LH-RH (SB-75, Cetrorelix) in patients with benign prostatic hyperplasia (BPH) and prostatic cancer. *Prostate* 1994, **24**, 84–92.
33. Yano T, Pinski J, Halmos G, Szepeshazi K, Groot K, Schally AV. Inhibition of growth of OV-1063 human epithelial ovarian cancer xenografts in nude mice by treatment with luteinizing hormone-releasing hormone antagonist SB-75. *Proc Natl Acad Sci USA* 1994, **91**, 7090–7094.
34. Redding TW, Schally AV. Inhibition of the pituitary–gonadal axis in nude male mice by continuous administration of LHRH agonists and antagonists. *J Endocrinol* 1990, **126**, 309–315.
35. Pinski J, Yano T, Schally AV. Inhibitory effects of the new bombesin receptor antagonist RC-3095 on the luteinizing hormone release in rats. *Neuroendocrinology* 1992, **56**, 831–837.
36. Szepeshazi K, Halmos G, Groot K, Schally AV. Combination treatment of nitrosamine-induced pancreatic cancers in ham-

sters with analogs of LH-RH and a bombesin/GRP antagonist. *Int J Pancreatol* 1994, **16**, 141–149.

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