

Regression of rat Dunning R-3327-H prostate carcinoma by treatment with targeted cytotoxic analog of luteinizing hormone-releasing hormone AN-207 containing 2-pyrrolinodoxorubicin

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Abstract. The effects of AN-207, a new targeted cytotoxic analog of LH-RH, were evaluated in rats bearing hormone-dependent Dunning R-3327-H prostate carcinomas. AN-207 consists of the agonist [D-Lys⁶]LH-RH linked to 2-pyrrolinodoxorubicin, an intensely potent derivative of doxorubicin. In the first experiment, 2-pyrrolinodoxorubicin was administered at a concentration of 50 nmol/kg, as a single drug (AN-201) and as an unconjugated mixture with [D-Lys⁶]LH-RH or conjugated to the carrier [D-Lys⁶]LH-RH (AN-207). Following the second administration of radical AN-201 alone or mixed with the carrier, all rats died with signs of general toxicity, but all animals treated with the conjugate AN-207, survived. After 5 weeks of treatment with a total dose of 150 nmol/kg AN-207, the tumors regressed from an initial volume of $8.35 \pm 1.7 \text{ cm}^3$ to $4.47 \pm 0.8 \text{ cm}^3$, while tumors in the control group measured $17.84 \pm 2.2 \text{ cm}^3$. The therapy with AN-207 also significantly reduced tumor weight and tumor burden. In the second experiment, we compared the efficacy and toxicity of 3 injections of 25 nmol/kg AN-201 or 25 nmol/kg and 50 nmol/kg AN-207. The initial tumor volume in all groups was between 3.9 and 4.5 cm³. After 5 weeks of therapy, the tumors of rats treated with 50 nmol/kg AN-207 regressed to $2.3 \pm 0.51 \text{ cm}^3$, whereas 25 nmol/kg AN-201 was still toxic in contrast to 25 nmol/kg AN-207, while the reduction in final tumor volume was similar ($6.76 \pm 1.4 \text{ cm}^3$ and $6.74 \pm 1 \text{ cm}^3$, respectively), as compared to $15.6 \pm 2.2 \text{ cm}^3$ for untreated

animals. High capacity LH-RH receptors were found in the membranes of untreated Dunning tumor specimens, but after treatment with AN-207, they could no longer be detected. This is the first demonstration that the new targeted cytotoxic LH-RH analog AN-207 is an effective antitumor agent. Our work indicates that the cytotoxic analog AN-207 is much less toxic than the antineoplastic radical (AN-201) incorporated, and significantly more active in inhibiting tumor growth. Further development of approaches based on targeted cytotoxic analog AN-207 may lead to major improvements in current palliative therapy of prostate cancer.

Introduction

Carcinoma of the prostate is the most common malignant tumor in men. It is expected that in 1996 approximately 317,000 new cases of prostate cancer will be diagnosed in the USA, and about 41,000 deaths will occur from this disease (1). In spite of screening programs, about 40% of all prostate cancer patients are only diagnosed at an advanced stage of the disease, with no possibility of cure by radical prostatectomy (2). Conventional chemotherapy shows low response rates and high toxicity (3). Thus, a weekly administration of doxorubicin, an anticancer antibiotic, which is widely used in the therapy of advanced, androgen-independent prostate cancer, shows only marginal response accompanied with significant toxic side effects (4). A local delivery of chemotherapeutic agents to tumor cells would greatly reduce their toxicity. A modern approach, which is being developed to overcome the problem of non-selective toxic effect on normal cells is targeting, based upon the selectivity of carrier molecules for specific binding sites on tumor tissues (5). Thus, highly active cytotoxic agents or toxins can be conjugated to various carriers for a more specific delivery to malignant cells, sparing healthy tissues (5). Specific high affinity binding sites for LH-RH are present in about 50% of human breast cancer specimens (5), nearly 80% of ovarian and endometrial cancers (6,7) as well as 86% of human prostate cancers (8,9). Consequently, targeted chemotherapy based on cytotoxic LH-RH analogs might be more efficient and less toxic in these malignancies than conventional regimens of antineoplastic agents (5). Previously, prototypes of various cytotoxic analogs of LH-RH containing antineo-

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Abbreviations: LH, luteinizing hormone; LH-RH, luteinizing hormone-releasing hormone; EGF, epidermal growth factor; NS, not significant

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plastic radicals such as cisplatin, D-melphalan, anthraquinone derivatives and methotrexate were synthesized and tested in our laboratory (5,10,11). Preliminary work indicated that some of these early hybrids in large doses could partially suppress the growth of experimental prostatic, mammary, endometrial and ovarian cancers, *in vitro* and/or *in vivo* (5,10,11).

Recently, we developed highly active derivatives of doxorubicin, such as 2-pyrrolinodoxorubicin (AN-201), which is 500-1,000 times more potent *in vitro* than its parent compound (12). This powerful cytotoxic agent was attached covalently to agonist [D-Lys⁶]LH-RH to form a cytotoxic LH-RH analog, AN-207 (13). This hybrid molecule fully retains cytotoxic activity of AN-201, as well as hormonal and binding properties of the peptide carrier *in vitro* (13). Hormone dependent rat prostate carcinoma Dunning R-3327-H (14,15) has been shown to be a suitable experimental model for early stages of human prostate cancer (16,17). Previously we have also shown that LH-RH receptors are present in the membranes of this tumor (18). In this study, we evaluated the effects of cytotoxic LH-RH analog AN-207 and compared it with its antineoplastic radical AN-201 on the growth of Dunning R-3327-H prostate carcinoma in rats.

Materials and methods

Peptide and cytotoxic agent. The agonistic analog [D-Lys⁶]LH-RH (pyro-Glu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH₂) was synthesized in our laboratory by solid phase methods (19). 2-pyrrolinodoxorubicin (AN-201) and its conjugate with [D-Lys⁶]LH-RH (AN-207) were synthesized in our laboratory as described by Nagy *et al* (12,13).

Animals and tumors. Male Copenhagen rats were obtained from Charles River Laboratories (Frederick, MD). They were housed four/cage in a temperature-controlled room with a 12-h light/12-h dark schedule and fed water and standard rat chow *ad libitum*. All experiments were performed according to institutional ethical guidelines. Dunning R-3327-H tumors were a generous gift from Dr John T. Isaacs (The Johns Hopkins Oncology Center, Baltimore, MD) and were transplanted in our laboratory.

Experimental protocol. Experiment I: rats bearing Dunning R-3327-H tumors were sacrificed and the tumors were aseptically dissected and mechanically minced; 3-mm³ pieces of tumor tissue were then transplanted s.c. by trocar needle into 40 male animals. The tumor take rate was 85%. Six months after transplantation, Dunning tumors had grown to a volume of approximately 8.5 cm³. Tumor-bearing rats were divided into 5 groups of 7 animals each, which received the following treatments: group 1, saline only intraperitoneally (i.p.); group 2, agonist [D-Lys⁶]LH-RH at a dose of 50 nmol/kg, i.p.; group 3, AN-201 at a dose of 50 nmol/kg, i.p.; group 4, AN-207, 50 nmol/kg i.p.; group 5, the unconjugated mixture of 50 nmol/kg of carrier agonist [D-Lys⁶]LH-RH and AN-201, i.p. The treatment consisted of 3 applications, which were scheduled as follows: in the initial loading phase 50 nmol/kg of the respective compounds were administered once in the first week (day 1) and second week (day 8). In the maintenance phase a third dose was given in the fifth week (day 29). Tumor

volumes were measured with microcalipers once a week and the tumor volume was calculated as length x width x height x 0.5236 (10). After 5 weeks, rats were anesthetized with methoxyflurane (Metofane, Pitman-Moore, Mundelein, IL), killed by decapitation and trunk blood was collected. The serum was separated and frozen for further analyses. 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: forty male Copenhagen rats bearing Dunning R-3327-H tumors were divided into 4 groups of 10 animals each, which received the following treatments: group 1, (control) saline only, i.p.; group 2, AN-201: 25 nmol/kg, i.p.; group 3, AN-207: 25 nmol/kg, i.p.; group 4, 50 nmol/kg AN-207, i.p. The treatment was repeated 3 times and scheduled as in experiment I, on days 1, 8 and 29. After 5 weeks, the experiment was terminated as described above.

Radioimmunoassays. LH was determined by RIA using material provided by the National Hormone and Pituitary Program (NHPP, Rockville, MD) (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 by Coat-A-Count RIA kit from Diagnostic Products Corp. (Los Angeles, CA, USA). 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 the NHPP (GH-RP-2/AFP-3190B, rat GH-I-6/AFP-5676B, and anti-rat GH-RIA-5/AFP-411S).

Receptor assay. The characteristics of receptors for EGF and LH-RH in the membranes of Dunning R-3327-H tumors were evaluated as previously described (20,21). 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}) (22).

Histological procedure. Parts of each tumor were fixed in 10% buffered formalin. Specimens were embedded in Paraplast (Oxford Labware, St. Louis, MO). Six μ m thick sections were cut and stained with hematoxylin-eosin. Mitotic and apoptotic cells were counted in 10 standard high-power microscopic fields containing on the average 200 cells and their numbers per 1,000 cells were accepted as the mitotic and apoptotic indices, respectively. For the demonstration of the nucleolar organizer region (NOR) in tumor cell nuclei, the AgNOR method was used (23). The silver-stained black dots in 50 cells of each tumor were counted and the AgNOR number per cell was calculated.

Statistical methods. All data are expressed as the mean \pm SEM, and statistical analyses of the tumor data were performed using Duncan's new multiple range test (24).

Results

Effects on tumor volume and weight, body weight and survival. Experiment I: all animals treated with the doxorubicin derivative AN-201, given at a dose of 50 nmol/kg either as a

Table I. Effect of treatment with [D-Lys⁶]LH-RH, 2-pyrrolinodoxorubicin (AN-201) and cytotoxic LH-RH analog AN-207 on tumor volume, body and tumor weight, and tumor burden in rats bearing transplanted Dunning R-3327-H tumors.

Treatment group	Tumor volume (cm ³)		Body weight (g)	Tumor weight (g)	Tumor burden (mg/g body wt)
	Initial	Final			
Experiment I					
Control	8.56±1.4	17.8±2.2	394±16	10.1±1.1	26.0±3.2
[D-Lys ⁶]LH-RH	8.30±1.3	16.0±2.8	392±8	8.53±1.5	22.8±4.1
AN-207 50 nmol/kg	8.35±1.7	4.47±0.8 ^b	316±10 ^a	4.27±0.9 ^b	13.2±2.9 ^a
Experiment II					
Control	4.35±0.8	15.6±2.2	394±11	7.2±0.9	19.3±2.2
AN-201 25 nmol/kg	4.35±0.6	6.76±1.4 ^b	273±10 ^b	4.6±1.6 ^b	16.8±4.2
AN-207 25 nmol/kg	4.15±0.6	6.74±1.0 ^b	365±7	4.9±0.2 ^b	13.4±0.8 ^a
AN-207 50 nmol/kg	4.28±0.5	2.30±0.5 ^b	323±18 ^b	2.1±0.2 ^b	6.5±0.8 ^b

Values are mean ± SE; ^ap<0.05 vs. control; ^bp<0.01 vs. control.

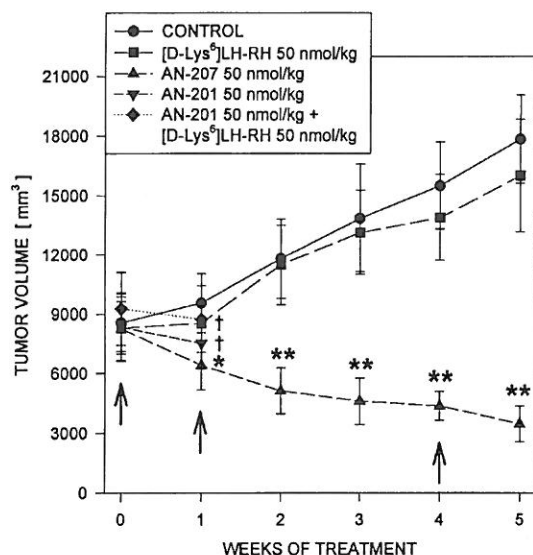


Figure 1. Experiment I: tumor volume in rats bearing Dunning R-3327-H prostate cancers during the treatment consisting of 3 applications of 50 nmol/kg agonist [D-Lys⁶]LH-RH and 50 nmol/kg of cytotoxic LH-RH analog AN-207. Vertical lines indicate the SEM. *p<0.05; **p<0.01 versus control by Duncan's new multiple range test. The treatment indicated by arrows was applied on days 1, 8 and 29. †The animals treated with AN-201 as a single drug or an unconjugated mixture with [D-Lys⁶]LH-RH died in the second week. In these two groups the volume of tumors recorded on day 8 is shown.

single drug or as an unconjugated mixture with the carrier agonist [D-Lys⁶]LH-RH, died after the second injection of AN-201 with signs of severe weight loss, diarrhea and general toxicity (Fig. 1). The effects of therapy with AN-207 and carrier [D-Lys⁶]LH-RH on final tumor volume, body, and tumor weights, and tumor burden are shown in Table I. The tumor volume, as measured at weekly intervals is recorded in Fig. 1. The initial tumor volume was approximately 8.5 cm³. [D-Lys⁶]LH-RH agonist was ineffective in inhibiting tumor growth of this androgen-dependent tumor, whereas treatment with AN-207 caused a major reduction of tumor volume

and weight (Fig. 1; Table I). A significant (p<0.05) tumor regression as shown by a decrease in volume was achieved within 7 days from the start of therapy. After 5 weeks of therapy, tumor volume was significantly (p<0.01) reduced to 4.47±0.89 cm³ in the group that received a total dose of 150 nmol/kg AN-207, while in untreated animals the final tumor volume was 17.84±2.2 cm³ (Table I). The final tumor weights were also significantly reduced in the groups treated with AN-207 compared with the controls (Table I). As expected, there was a significant difference in body weights between untreated and AN-207 treated animals. Nevertheless, the tumor burden was significantly (p<0.05) lower in the AN-207 treated group compared to controls (13.25±2.9 versus 26.03±3.2 mg tumor/g body weight).

In experiment II, we evaluated the effect of 3 applications of 25 nmol/kg or 50 nmol/kg of the cytotoxic LH-RH conjugate AN-207 and 25 nmol/kg 2-pyrrolinodoxorubicin (AN-201). This experiment was designed solely for the purpose of comparing the efficacy and toxicity of AN-207 and AN-201. In view of the shortage of experimental animals, groups treated with carrier agonist and the unconjugated mixture, previously tested in experiment I, were deemed not to be essential. By the end of the experiment, 6 of 10 animals in the group that received altogether 75 nmol/kg AN-201 were dead, whereas only 2 of 10 animals died in the group treated with a total dose of 150 nmol/kg of AN-207. All animals treated with a total of 75 nmol/kg AN-207 survived without signs of toxicity. The administration of AN-207 at a dose of 50 nmol/kg on days 1, 8 and 29 caused the greatest reduction in tumor volume, from 4.28±0.5 cm³ at the beginning of the experiment to 2.3±0.5 cm³ (Table I; Fig. 2A). In 4 animals that survived after 3 doses of 25 nmol/kg AN-201, the mean tumor volume and weight after 5 weeks were 6.76±1.4 cm³ and 4.6±1.6 g, respectively, compared to 15.6±2.2 cm³ and 7.2±0.9 g for untreated controls. A similar suppression of tumor growth, but without accompanying toxicity was achieved with 3 doses of 25 nmol/kg of cytotoxic analog AN-207, tumor volume and weight corresponding to 6.74±1 cm³ and 4.9±0.2 g, respectively. The body weights of the animals in various

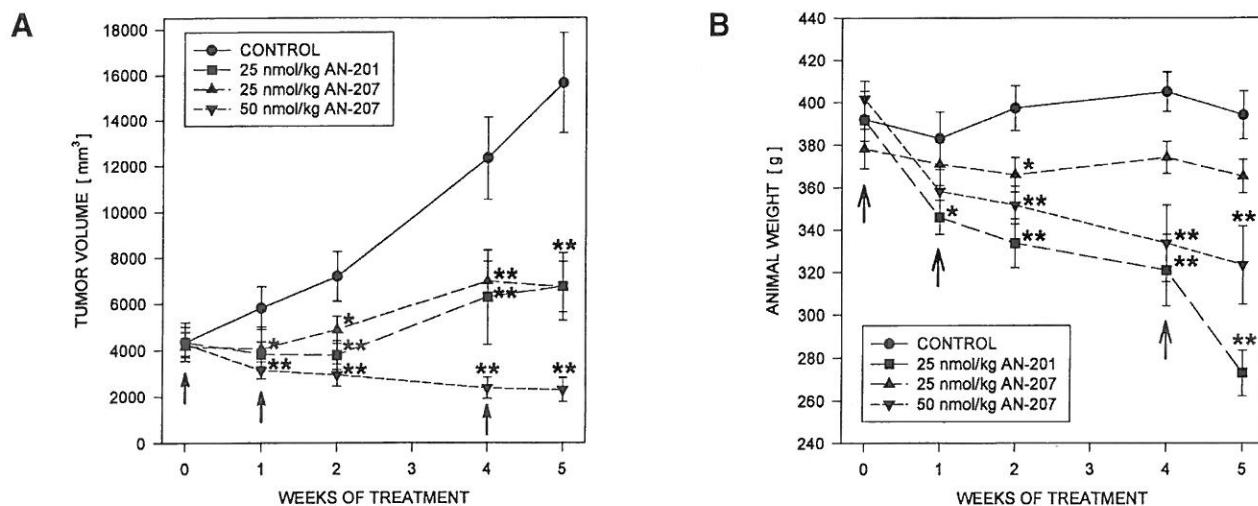


Figure 2. A, Experiment II: effect of treatment with 25 nmol/kg 2-pyrrolinodoxorubicin AN-201, 25 nmol/kg and 50 nmol/kg cytotoxic LH-RH analog AN-207 on the tumor volume in rats with Dunning R-3327-H prostate cancer. Vertical lines indicate the SEM. * $p < 0.05$; ** $p < 0.01$ versus control. The treatment indicated by arrows was applied 3 times, that is on days 1, 8 and 29. B, Experiment II: effect of treatment with 25 nmol/kg 2-pyrrolinodoxorubicin AN-201, 25 nmol/kg and 50 nmol/kg cytotoxic LH-RH analog AN-207 on the body weight of Copenhagen rats bearing Dunning R-3327-H prostate cancer. Vertical lines indicate the SEM. * $p < 0.05$; ** $p < 0.01$ versus control. The treatment indicated by arrows was applied 3 times, that is on days 1, 8 and 29.

groups are shown in Table I and Fig. 2B. Administration of a total dose of 75 nmol/kg AN-201 caused the greatest weight loss. A smaller decrease in final body weight resulted from a total of 150 nmol/kg of the cytotoxic analog AN-207. The low dose of AN-207 (25 nmol/kg given three times) did not significantly affect body weights. Tumor burden was calculated to be 16.8 ± 4.2 (NS) for animals that received 75 nmol/kg AN-201, and 13.4 ± 0.8 ($p < 0.05$) and 6.5 ± 0.8 mg tumor/g body weight ($p < 0.01$) for animals treated with 75 nmol/kg and 150 nmol/kg of cytotoxic analog AN-207, respectively, as compared to 19.3 ± 2.2 mg tumor/g body weight for untreated rats.

Side effects. In addition to loss of body weight, other toxic side effects of the therapy were diarrhea and a moderate anemia in the AN-201 treated group (3.77×10^6 erythrocytes versus 4.8×10^6 in the control animals). No leucopenia was observed during this experiment (data not shown).

Serum hormone levels. The levels of serum luteinizing hormone (LH), testosterone and growth hormone (GH) in controls, and in animals treated with carrier [D-Lys⁶]LH-RH, AN-201 and AN-207 are shown in Table II. Agonist [D-Lys⁶]LH-RH at the dose of 50 nmol/kg did not change serum LH or testosterone levels. Serum LH levels were significantly reduced in the groups receiving 25 nmol/kg AN-201, 25 nmol/kg and 50 nmol/kg AN-207 to 0.39 ± 0.17 ng/ml, 0.56 ± 0.06 ng/ml and 0.56 ± 0.11 ng/ml, respectively, compared to 0.84 ± 0.05 ng/ml for controls. Low serum levels of testosterone were recorded in groups treated with 25 nmol/kg AN-201 (0.04 ± 0.03 ng/ml) and 50 nmol/kg AN-207 (0.53 ± 0.2 ng/ml), as compared to 1.67 ± 0.18 ng/ml for control. Serum GH levels were unchanged and in the normal range for all different treatment groups (Table II).

Histology. Dunning R-3327-H tumors were well differentiated adenocarcinomas, consisting of small and tortuous tumorous

glands which were surrounded by a moderate amount of stroma. A flattening of the epithelial layer of glands together with a significant widening of connective tissue stroma was detected in the tumors treated with cytotoxic analog AN-207. The tumors contained small necrotic areas. The quantitative histological data are shown in Table III. In the groups of animals treated with 25 nmol/kg and 50 nmol/kg AN-207, a significant reduction of mitotic cells was observed. Although the frequency of apoptosis was not changed significantly by the treatments, a highly significant ($p < 0.01$) increase in the ratio of

Table II. Serum luteinizing hormone, testosterone and growth hormone levels in rats bearing Dunning R-3327-H tumors after treatment with [D-Lys⁶]LH-RH, 2-pyrrolinodoxorubicin (AN-201) and cytotoxic LH-RH analog AN-207.

Treatment group	Luteinizing hormone (LH) (ng/ml)	Testosterone (ng/ml)	Growth hormone (ng/ml)
Experiment I			
Control	0.61 ± 0.05	2.61 ± 0.6	nm
[D-Lys ⁶]LH-RH			
50 nmol/kg	0.62 ± 0.19	2.13 ± 0.2	nm
AN-207 50 nmol/kg	0.45 ± 0.08^a	0.83 ± 0.6^a	nm
Experiment II			
Control	0.84 ± 0.05	1.67 ± 0.18	11.1 ± 0.4
AN-201 25 nmol/kg	0.39 ± 0.17^b	0.04 ± 0.03^b	13.7 ± 1.5
AN-207 25 nmol/kg	0.56 ± 0.06^a	1.69 ± 0.19	10.7 ± 0.3
AN-207 50 nmol/kg	0.56 ± 0.11^a	0.53 ± 0.20^b	10.7 ± 0.8

Values are mean \pm SE; ^a $p < 0.05$ vs. control; ^b $p < 0.01$ vs. control; nm, not measured.

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Table III. Effect of treatment with carrier [D-Lys⁶]LH-RH, 2-pyrrolinodoxorubicin (AN-201) and cytotoxic LH-RH analog AN-207 on the histological characteristics of Dunning R-3327-H tumors.

Treatment group	Mitotic index	Apoptotic index	Ratio of apoptotic to mitotic indices	Number of AgNORs per cell
Experiment I				
Control	5.08±0.60	5.25±0.76	1.15±0.23	4.10±0.23
[D-Lys ⁶]LH-RH 50 nmol/kg	4.21±0.42	5.64±1.14	1.38±0.28	3.71±0.12
AN-207 50 nmol/kg	2.33±0.60 ^a	7.08±1.45	3.44±0.75 ^b	2.98±0.11 ^b
Experiment II				
Control	6.65±0.85	6.20±0.45	1.06±0.13	nm
AN-201 25 nmol/kg	5.75±0.52	3.75±0.78	0.69±0.16	nm
AN-207 25 nmol/kg	4.22±0.64 ^a	6.22±1.0	1.60±0.27	nm
AN-207 50 nmol/kg	3.93±0.66 ^a	4.86±1.06	1.43±0.42	nm

Values are mean ± SE; ^ap<0.05 vs. control; ^bp<0.01 vs. control; nm, not measured.

Table IV. Binding characteristics of receptors for LH-RH and EGF in membranes of Dunning R-3327-H prostate carcinomas after *in vivo* treatment with carrier [D-Lys⁶]LH-RH, 2-pyrrolinodoxorubicin (AN-201) and cytotoxic LH-RH analog AN-207.

Treatment group	LH-RH receptor		Epidermal growth factor receptor (EGF-R)	
	K _d (nM)	B _{max} (fmol/mg) ^a	K _d (nM)	B _{max} (fmol/mg) ^a
Experiment I				
Control	11.9±0.65	1,110±120	0.80±0.17	169.6±2.2
[D-Lys ⁶]LH-RH 50 nmol/kg	12.4±2.05	500±20 ^b	1.64±0.03 ^b	118.4±5.1 ^c
AN-207 50 nmol/kg	ND	ND	0.72±0.09	67.0±9.9 ^c
Experiment II				
Control	12.3±0.75	990±160	0.97±0.13	178.7±4.3
AN-201 25 nmol/kg	12.0±0.90	780±160	0.50±0.21	82.2±6.5 ^c
AN-207 25 nmol/kg	ND	ND	1.25±0.38	126.3±7.8 ^c
AN-207 50 nmol/kg	ND	ND	0.45±0.15	56.9±3.3 ^c

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 ± SE; ^aper mg protein; ^bp<0.05 vs. control; ^cp<0.01 vs. control; ND, not detectable.

apoptotic to mitotic cells could be detected in experiment I in tumors treated with 50 nmol/kg AN-207 (3.44±0.75), compared to 1.15±0.23 for controls. In the first experiment, the number of AgNORs per cell was significantly reduced in AN-207 treated group, to 2.98±0.11 (p<0.01), versus 4.1±0.23 for controls.

Receptor characteristics. The results of the receptor assays for LH-RH and EGF following treatment with compounds tested are shown in Table IV. Specific binding sites for [¹²⁵I]-[D-Trp⁶] LH-RH were detected in high concentrations in the membranes of Dunning R-3327-H cells. In experiment I, the calculation showed K_d=11.9±0.65 nM and B_{max}=1,100±120 fmol/mg membrane protein. After treatment with carrier agonist [D-Lys⁶]LH-RH, the concentration of receptors for LH-RH

was decreased to 500±20 fmol/mg protein. No receptors for LH-RH could be detected after treatment with 25 nmol/kg (experiment II) and 50 nmol/kg AN-207 (in experiments I and II). In untreated Dunning R-3327-H tumors, labeled EGF was bound to one class of binding sites with high affinity. In experiment II the mean binding capacity of EGF receptors (B_{max}) was 178.7±4.35 fmol/mg of membrane protein. A significant (p<0.01) reduction in maximal binding capacity (B_{max}) of EGF receptors was observed in both experiments after treatment with cytotoxic analog AN-207. In experiment II, the reduction in binding capacity of EGF receptors in Dunning tumor cell membranes was dose dependent and a greater decrease in EGF receptor levels was achieved with 50 nmol/kg AN-207 (B_{max}=56.9±3.35 fmol/mg) (Table IV).

Carrier [D-Lys⁶] LH-RH (experiment I) and 2-pyrrolinodoxorubicin also reduced the binding capacity of EGF receptors.

Discussion

Over the past two decades, many chemotherapeutic agents have been tested, alone or in combination, in patients with advanced hormonally unresponsive prostate carcinoma (3,4). However, the response rates that may be achieved at present with cytotoxic chemotherapy are generally low (3-5). In the experimental model of rat Dunning R-3327-H prostate cancer, the combination of chemotherapy and hormonal therapy can produce a better response than chemotherapy alone (17,25,26). Targeted chemotherapy may also yield better results than conventional cytotoxic therapy. In order to take advantage of the presence of LH-RH receptors on diverse tumors, including prostate carcinoma (5-9), we developed a new class of LH-RH analogs containing cytotoxic radicals. These analogs were designed for targeted chemotherapy and may represent a new class of antitumor drugs for the treatment of various malignancies (5,19,23). It has been established that high affinity binding sites for LH-RH are present in most surgically resected human prostate cancer samples (8,9). Qayum *et al*, (9) indicated that 86% of prostate cancer specimens exhibited LH-RH receptors and Fekete *et al*, reported similar findings (8). High affinity binding sites are also present in human androgen sensitive LNCaP prostate cancer line (9,27). However, only low affinity LH-RH receptors were reported in androgen independent DU-145 human prostate cancer cell line (9,28). In an earlier study, rats bearing Dunning R-3327-H prostate adenocarcinomas were treated with prototypes of hybrid cytotoxic LH-RH analogs containing anthraquinone or methotrexate (10). Large doses of these early cytotoxic analogs had moderate antitumor effects and caused a slightly greater tumor growth inhibition than the carrier peptide alone. Free anthraquinone or methotrexate given in equimolar doses were ineffective (10).

Recently, we developed an intensely potent derivative of doxorubicin, 2-pyrrolinodoxorubicin (AN-201), which is 500-1,000 times more active *in vitro* than its parent compound (12). This highly active antitumor agent was conjugated to agonist [D-Lys⁶]LH-RH and the resulting hybrid molecule (AN-207) fully preserved the hormonal and cytotoxic activity of its components *in vitro* (13).

This is the first report on the *in vivo* antitumor activities of AN-201 in comparison with its targeted LH-RH conjugate, AN-207, which was designed in an endeavor to produce a modern representative of the so called 'magic bullet' class of antitumor compounds. LH-RH carrier analog [D-Lys⁶]LH-RH was also tested alone and in an unconjugated mixture with AN-201 inasmuch as it has been shown in previous studies that cytotoxic LH-RH analogs retain their hormonal activities (23) and that LH-RH agonists administered in a proper concentration caused androgen deprivation and decreased tumor growth in Dunning R-3327-H prostate carcinoma (10). Because the carrier [D-Lys⁶]LH-RH was not administered at a therapeutic concentration, in this experiment it did not inhibit the growth of Dunning androgen-dependent prostate tumors.

Our work shows that 50 nmol/kg of the cytotoxic LH-RH analog AN-207 effectively inhibited tumor growth even after the first administration. At the end of the experiment, in which 3 doses of AN-207 were given, the tumors of animals treated with a total dose of 150 nmol/kg AN-207 showed a significant regression. Lower doses of AN-207 were less effective and led only to a suppression of tumor growth. Unconjugated 2-pyrrolinodoxorubicin appeared to be toxic and after the second application of 50 nmol/kg AN-201 or the mixture of AN-201 and [D-Lys⁶]LH-RH, all animals died with signs of severe general toxicity. Lower concentrations of AN-201 (total of 75 nmol/kg) still caused a 60% death rate, and the surviving animals showed the highest weight loss of all groups. Interestingly, AN-207 in the high dose (total of 150 nmol/kg) was remarkably well tolerated, toxic side effects were minor and in the second experiment only 2 out of 10 animals died. The only measurable adverse effect was a weight loss of about 25% at the end of the experiment. Thus, our *in vivo* study shows that hybrid LH-RH analog AN-207 is much less toxic and significantly more potent in inhibiting the growth of Dunning prostatic cancers, than its radical 2-pyrrolinodoxorubicin.

Administration of a total dose of 150 nmol/kg AN-207 decreased significantly serum LH and testosterone levels. These effects on the pituitary-testicular axis might not be hormonally mediated, but due to a combined direct cytotoxic action of the drug (or its metabolite) on both LH-secreting cells and the Leydig cells (5,29). This is suggested by the fact that the administration of the carrier agonist [D-Lys⁶]LH-RH alone at equimolar concentrations had no influence on the LH and testosterone levels (experiment I), but a total dose of 75 nmol/kg unconjugated cytotoxic radical AN-201 caused even greater reduction of these hormones than AN-207. Growth hormone levels were not influenced either by the cytotoxic radical AN-201 or the LH-RH analog AN-207 (experiment II).

High capacity binding sites for LH-RH were present in membranes of Dunning R-3327-H prostate cancers. This is in agreement with previous studies (18). After administration of three doses of AN-207 corresponding to a total of 75 nmol/kg or 150 nmol/kg AN-207, no receptors for LH-RH were detected in the membranes of Dunning tumors. The exact mechanism of action of this cytotoxic peptide analog on the hormone-dependent Dunning prostate cancer model remains to be elucidated. The fall in LH-RH receptors could indicate that this hybrid compound might selectively kill tumor cells expressing these receptors, which is the goal of targeted chemotherapy. The fact that androgen-independent prostate cancers R-3327-AT-1, which do not have binding sites for LH-RH, did not respond to cytotoxic analogs of LH-RH (Pinski and Schally unpublished data), supports the concept that in our experiment with hormone dependent Dunning tumors, which possess LH-RH receptors, the effects of AN-207 might have been exerted at least in part directly on the tumors. A direct action is also suggested by the observation that radiolabeled cytotoxic LH-RH analog T-98 was shown to accumulate in MXT breast cancers 3 h after injection (23).

High concentrations of EGF receptors were found in Dunning tumors of untreated control animals. Numerous investigators have demonstrated the functional significance of a TGF- α /EGF-receptor mediated autocrine growth pathway

in cultured prostatic carcinoma cells (30). Agonist [D-Lys⁶] LH-RH decreased the binding capacity of EGF receptors in the membranes of this prostate cancer. This finding is in accord with previous studies with [D-Lys⁶] LH-RH and [D-Trp⁶] LH-RH (23,31,32). However, administration of a total dose of 150 nmol/kg of the conjugated cytotoxic analog AN-207 decreased the concentrations of EGF receptors to a much greater extent than the carrier alone. This points to another mechanism of action, linked to intercalating the tumor DNA.

Histological evaluation of the specimens showed a significant reduction of mitotic cells in the AN-207 treated groups, whereas in tumors treated with AN-201 alone, no significant change in the number of mitotic or apoptotic cells was observed. In the first experiment, a highly significant increase in the ratio of apoptotic to mitotic cells could be detected in the AN-207 treated Dunning R-3327-H tumors. These findings suggest that the cytotoxic doxorubicin derivative AN-201 becomes endowed with a higher tumor toxicity when linked covalently to an LH-RH analog.

The presence of receptors for LH-RH on various human tumors, including mammary, ovarian, endometrial and prostate cancers (5-9), makes modern highly active targeted cytotoxic analogs of LH-RH, such as AN-207, potential candidates for practical clinical use for the treatment of these malignancies (12,13). This approach based on cytotoxic LH-RH analogs requires much additional experimental work and still remains to be tested clinically, but it might possibly extend the oncological uses of LH-RH analogs from the current palliation of prostate cancer toward an eventual cure.

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