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Preclinical report

Effective treatment of experimental ES-2 human ovarian cancers with a cytotoxic analog of luteinizing hormone-releasing hormone AN-207

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The receptors for luteinizing hormone-releasing hormone (LHRH) are found in 80% of human ovarian carcinomas. These receptors can be used for targeted chemotherapy with cytotoxic analogs of LHRH, such as AN-207, consisting of 2-pyrrolinodoxorubicin (AN-201) linked to [D-Lys^6]LHRH. We investigated the effects of AN-207 and AN-201 on the *in vivo* growth of LHRH receptor-positive ES-2 human ovarian cancers. The effects of the treatment on mRNA and protein levels of human epidermal growth factor (EGF) receptors (EGFR and HER-2) in ovarian tumors were determined by RT-PCR and immunoblotting. In Experiment 1, nude mice bearing ES-2 ovarian tumors were injected i.v. with 250 nmol/kg doses of AN-207, AN-201, the carrier [D-Lys^6]LHRH, an unconjugated mixture of AN-201 and [D-Lys^6]LHRH or vehicle. AN-207 caused a significant ($p < 0.01$) 59.5% inhibition in tumor growth while its components were ineffective. In Experiment 2, mice with large ES-2 tumors were treated with AN-207 or AN-201 at 250 nmol/kg. Again, AN-207, but not AN-201, inhibited tumor growth. In Experiment 3, the site of action of AN-207 was investigated. The blockade of LHRH receptors with Cetrorelix partially suppressed the antitumor effect of AN-207. Treatment with AN-207 significantly ($p < 0.01$) decreased the expression of mRNA for EGFR, and HER-2 by 27 and 34%, respectively, as compared to controls and reduced the receptor protein levels of EGFR and HER-2 by 35 and 36%, respectively ($p < 0.05$). The results indicate that cytotoxic LHRH analog AN-207 could be considered for chemotherapy of ovarian cancers expressing LHRH receptors.[© 2002 Lippincott Williams & Wilkins.]

Key words: Cytotoxic luteinizing hormone-releasing hormone analog, ovarian cancer, targeted chemotherapy.

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Introduction

Carcinoma of the ovary is the most frequent cause of death from gynecological cancer among women in the Western world.[1] Advanced epithelial ovarian cancer is currently treated by cytoreductive surgery and adjuvant chemotherapy, but after an initial response[2] most of the patients eventually relapse.[3] Thus, it is essential to develop new treatment modalities. Targeted chemotherapy represents a modern strategy for the treatment of various cancers, and it has been demonstrated that it improves the efficacy of cytotoxic drugs and decreases the peripheral toxicity.

Receptors for luteinizing hormone-releasing hormone (LHRH) are present in about 80% of epithelial ovarian cancers[4] and cytotoxic LHRH conjugates may be used for targeted therapy of this malignancy.[5,6] Recently, we synthesized a highly active derivative of doxorubicin (DOX), 2-pyrrolino-DOX (AN-201), that is 500–1000 times more potent *in vitro* than the parent compound.[7] AN-201 was linked to [D-Lys^6]LHRH through a glutaric acid spacer to form cytotoxic LHRH conjugate AN-207.[8] This hybrid exhibits high binding affinity to LHRH receptors on rat pituitary membranes and retains the antiproliferative activity of AN-201 *in vitro*.[8] We also demonstrated that AN-207 binds with high affinity to LHRH receptors on various tumors including ES-2 human ovarian cancers.[5,9]

The biological factors that regulate the growth of ovarian carcinomas are not completely understood. However, epidermal growth factor (EGF) and its structural homolog, transforming growth factor

(TGF)-α that bind with similar high affinity to EGF receptors (EGFR), are implicated in the regulation of cell proliferation in ovarian tumors.[10–12] EGFR belongs to the HER/ErbB family of tyrosine kinase receptors which are major mediators of growth and differentiation signals. Upon ligand binding, the EGFR forms homo- and heterodimeric complexes with HER-2/*neu* (c-erbB-2), as well as with other members of the family, to generate its intracellular signaling, leading to the phosphorylation of transcriptional factors, which in turn control several genes involved in cellular proliferation.[13] In various cancers including ovarian carcinoma, EGFR and HER-2 status is clinically associated with aggressive phenotype and poor survival.[14–18]

In this study, we evaluated the outcome of the treatment with cytotoxic LHRH analog AN-207 on the growth of ES-2 human epithelial ovarian cancer cell line xenografted into nude mice. The site of action of AN-207 was investigated by the blockade of the receptors with a high dose of LHRH antagonist Cetrorelix. The effect of AN-207 on the expression of EGFR and HER-2 was assessed by RT-PCR and immunoblotting.

Materials and methods

Peptides and cytotoxic agents

LHRH antagonist Cetrorelix (SB-75), originally synthesized in our laboratory by solid-phase methods,[19] was kindly provided by Zentaris (Frankfurt/Main, Germany). 2-Pyrrolino-DOX (AN-201) was prepared as described.[7] Cytotoxic LHRH conjugate AN-207 was formed by linking one molecule of AN-201-14-*O*-hemiglutarate to the ε-amino group of the D-lysine side chain of the carrier peptide [D-Lys^6]LHRH and purified by high-performance liquid chromatography.[8] [D-Lys^6]LHRH was purchased from California Peptide Research (Napa, CA).

Cell line and animals

Human epithelial ovarian cancer cell line ES-2 was obtained from ATCC (Manassas, VA) and maintained as described.[9] Four- to 6-week-old female athymic NCR/c (*nu/nu*) nude mice were purchased from the Frederick Cancer Research Facility of the National Cancer Institute (Frederick, MD) 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*. Their care was in accordance with institutional guidelines.

In vivo studies

ES-2 cells (10^7) were injected into the right flank of three female nude mice. Tumors resulting after 6 weeks were aseptically removed and mechanically minced. Pieces (3 mm^3) were transplanted by s.c. injection with a trocar needle.

Experiment 1 was started when tumors had grown to a volume of approximately 200 mm^3. The animals were randomly divided into five treatment groups of six mice each, and received i.v. injections of AN-207, AN-201, unconjugated mixture of AN-201 and [D-Lys^6]LHRH, [D-Lys^6]LHRH or vehicle. For the i.v. injection, the compounds were dissolved in 20 μl of 0.01 N acetic acid and diluted with 5.5% aqueous D-mannitol solution (Sigma, St Louis, MO) to a volume of 0.2 ml/20 g body weight. All compounds were administered through the jugular vein under methoxyflurane anesthesia (Metofane; Pittman-Moore, Mundelein, IL) at a dose of 250 nmol/kg body weight. The experiment was terminated 28 days after the administration of the drugs.

Experiment 2 was designed to evaluate the effect of AN-207 and AN-201 on relatively large tumors. When tumors had grown to a volume of approximately 400 mm^3, mice were randomly divided into three treatment groups of five animals each and received i.v. injections of AN-201, AN-207 or vehicle at a dose of 250 nmol/kg body weight as described above. The experiment was terminated 19 days after treatment.

In Experiment 3, we studied the mechanism of action of cytotoxic LHRH analog AN-207 by a blockade of receptors for LHRH on tumors. When tumors had grown to a volume of approximately 150 mm^3 the mice were randomly divided into three treatment groups of eight mice each. Group 1 received two i.v. injections of AN-207 at a dose of 250 nmol/kg body weight. Group 2 was given Cetrorelix at a dose of 200 μg/mouse s.c. 4 h before the i.v. injection of AN-207 at a dose of 250 nmol/kg body weight. Group 3 received the vehicle only. All the injections were given on days 0 and 21. The experiment was terminated 27 days after the initiation of therapy.

In all experiments, animal weights and tumor volumes (length × width × height × 0.5236) were recorded twice a week.[20] At the end of each

experiment, the mice were anesthetized with metoxyflurane and sacrificed by decapitation. Tumors were removed, rapidly frozen in liquid nitrogen and stored at −70°C.

RNA extraction and RT-PCR

Total RNA was extracted from ES-2 tumors from Experiments 1 and 2 using the RNAzol B kit (Tel-Test, Friendswood, TX) following the manufacturer's instructions and quantified spectrophotometrically at 260 nm.RT-PCR. was performed using the Gene Amp RNA PCR kit (Perkin-Elmer, Norwalk, CT). Total RNA (1 μg) was reverse transcribed into cDNA and subsequently subjected to multiplex PCR for the semiquantitative evaluation of the mRNA expression of EGFR, using GAPDH as internal control.[21] Amplification for HER-2 was performed by AmpliTaq Gold Polymerase (Perkin-Elmer) and the expression of β-actin was used as internal control.[21] Aliquots of amplified PCR products were resolved by electrophoresis on 1.8% agarose gel and stained with ethidium bromide. Gels were directly scanned (Alpha Imager 2200; Alpha Innotech, San Leandro, CA) and quantified densitometrically using Bio-Rad PC Analysis software (Bio-Rad, Richmond, CA).[9]

Immunoblotting

Tumor tissue obtained in Experiments 1 and 2 was homogenized in 0.05 M. Tris–HCl (pH 7.5) containing 0.25 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/μl leupeptin, 0.5 mg/ml bacitracin and 0.5 mM EDTA (homogenation buffer). The homogenate was centrifuged at 500 *g* for 5 min at 4°C. The pellet was discarded and the supernatant centrifuged at 45 000 *g* for 45 min at 4°C. The pellet containing the membrane protein fraction was washed once, resuspended in homogenation buffer without sucrose and immediately frozen at −70°C. Protein concentration was measured by the Bradford method[22] using bovine serum albumin as a standard. Membrane proteins (20 μg) were solubilized in loading solution (60 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1 M DTT and 0.01% bromophenol blue), heated for 15 min at 70°C, resolved on a 5% polyacrylamide gel and transferred onto nitrocellulose membrane.

Immunodetection was carried out at room temperature using antiserum 1005 for EGFR and antiserum C-18 for HER-2 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:3000 dilution in Tris-buffered saline, pH 7.4 (TBS). Briefly, membranes were incubated for 1 h in blocking solution [TBS, 0.1% Tween 20 and 5% (w/v) non-fat dry milk] and then with the specific polyclonal antibody for 1 h. After several washes in blocking buffer, the membranes were incubated for 1 h with the secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG; Santa-Cruz Biotechnologies) diluted 1:2000 in TBS, washed with blocking buffer and visualized by enhanced chemiluminescence (Pierce, Rockford, IL). Quantification of the bands obtained after exposure of the blots to X-ray films was performed with a densitometer (model GS-7000; Bio-Rad) coupled to the Bio-Rad PC Analysis software.[21]

Statistical analysis

All data are expressed as the mean ± SE. Tumor volume changes were analyzed by two-way ANOVA and analysis of mRNA expression was performed by Student's two-sided *t*-test. Differences were considered statistically significant at $p < 0.05$.

Results

Effect of treatment with AN-207 on the growth of ES-2 tumors in nude mice

In Experiment 1, we compared the antitumor effects of targeted cytotoxic analog AN-207 and its constituents, radical AN-201 and carrier [D-Lys^6]LHRH, at a dose of 250 nmol/kg body weight. Twenty-eight days after a single i.v. injection of AN-207, the volume of ES-2 tumors was reduced significantly ($p < 0.01$) to 856 ± 52 mm^3 compared with controls which measured 2051 ± 478 mm^3, corresponding to a 59.5% inhibition. An equimolar dose of cytotoxic radical AN-201, the unconjugated mixture of AN-201 and [D-Lys^6]LHRH, and [D-Lys^6]LHRH, caused no significant differences in tumor volume compared with the control group (Figure 1A). The body weights of mice decreased in the groups treated with cytotoxic agents, but returned to normal by the end of the experiment (Figure 1B). No animals died from toxicity in this experiment.

The effect of AN-207 and its cytotoxic moiety AN-201 on relatively large tumors (about 400 mm^3) was evaluated in Experiment 2. AN-207 at a single dose of 250 nmol/kg body weight significantly diminished

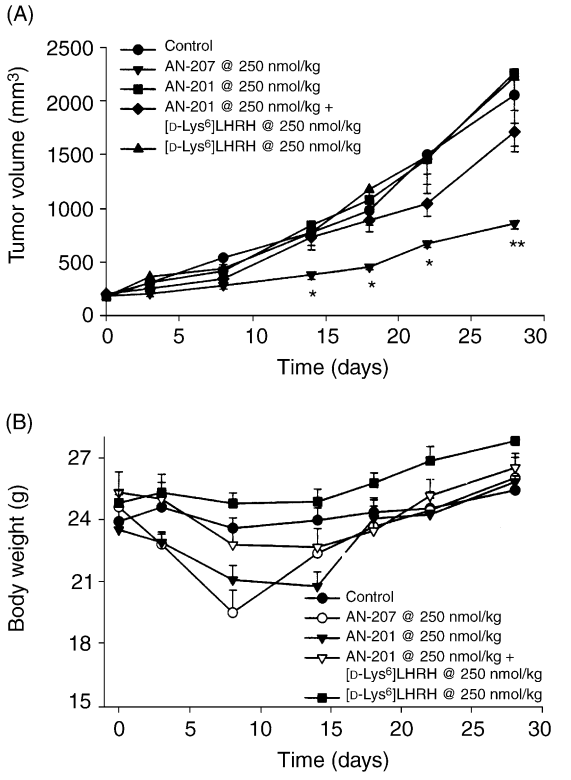

Figure 1. Effect of treatment with the cytotoxic LHRH analog AN-207 and its components on the growth of ES-2 human ovarian cancers in nude mice. (A) Changes in tumor volume after a single i.v. injection on day 0 at a dose of 250 nmol/kg of AN-207, the cytotoxic radical AN-201, an unconjugated mixture of [D-Lys^6]LHRH and AN-201, and the carrier [D-Lys^6]LHRH. Controls received i.v. injections of the vehicle solution (5% mannitol). (B) Changes in body weights in athymic nude mice bearing ES-2 human ovarian tumors after treatment with a single dose of cytotoxic LHRH analog AN-207 or its components at 250 nmol/kg body weight. Vertical bars show SEM. *$p<0.05$ versus controls; **$p<0.01$ versus controls.

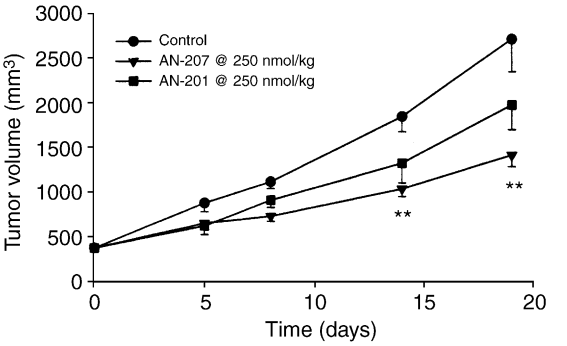

Figure 2. Changes in tumor volume after a single i.v. injection of the cytotoxic LHRH analog AN-207 or its cytotoxic moiety AN-201 at 250 nmol/kg body weight in nude mice with relatively large ES-2 tumors. Controls received i.v. injection of the vehicle solution (5% mannitol).

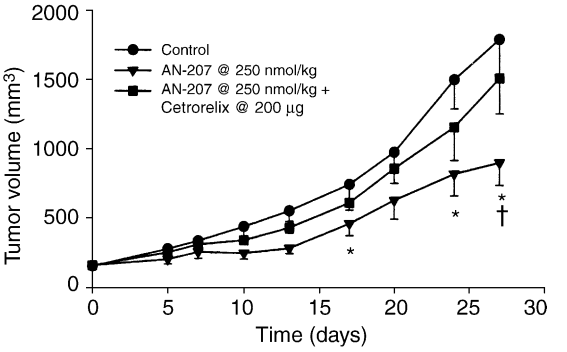

Figure 3. Effect of targeted therapy based on AN-207 with or without blockade of LHRH receptors. Mice received AN-207 at a dose of 250 nmol/Kg, LHRH antagonist Cetrorelix was administered s.c. at a dose of 200 μg 4 h before the injection of AN-207 or vehicle. Injections were given on days 0 and 21. Vertical bars show SEM. *$p<0.05$ versus controls; †$p<0.05$ versus the group pretreated with Cetrorelix.

tumor volume to 1415 ± 129 ($p<0.01$) corresponding to a 47.9% inhibition compared with controls, which measured 2716 ± 366. An equimolar dose of AN-201 reduced tumor volume to $1977\pm276\,mm^3$, but this (27.2%) inhibition was not significant (Figure 2). No animals died from toxicity in this experiment, although administration of AN-207 and AN-201 caused 7 and 10% loss in body weights, respectively, after 5 days. Because of the large volume of the control tumors, the experiment was terminated after 19 days.

Experiment 3 was designed to study the site of action of targeted cytotoxic agent AN-207 by blockade of LHRH receptors. Thus, mice bearing ES-2 tumors received an i.v. injection of vehicle (control), AN-207 at 250 nmol/kg body weight or a s.c. injection of LHRH antagonist Cetrorelix at a dose of 200 μg/mouse 4 h before an i.v. injection of AN-207 at 250 nmol/kg body weight. The treatment was repeated 21 days after the first injection. As shown in Figure 3, the inhibition of tumor growth after 27 days in the group that received AN-207 was 50.3% ($p<0.05$), the final tumor volume being $903\pm163\,mm^3$ compared with the control group which measured $1797\pm256\,mm^3$. All the animals survived the therapy. Blockade of the receptors with Cetrorelix suppressed the antitumor effect of the targeted cytotoxic agent, with the tumor volume corresponding to $1515\pm257\,mm^3$, a reduction of only 15.6% that was not significant. Blockade of LHRH receptors increased the toxic effects of AN-207 causing the deaths of three of eight mice after the administration of the first dose. Although the second

injection decreased the body weights (data not shown) there were no more deaths.

RT-PCR

The results of RT-PCR analysis of mRNA expression for EGFR and HER-2/*neu* in ES-2 human ovarian tumors from Experiment 1 are shown in Figure 4(A). Agarose gels were stained with ethidium bromide and the optical densities (OD) of the bands corresponding to EGFR and HER-2 were normalized by the OD obtained for GAPDH and β-actin, respectively. Treatment with AN-207 at a single dose of 250 nmol/kg body weight caused a significant 27% ($p<0.01$) decrease in EGFR mRNA expression as compared to the control group (accepted as 100%), while AN-201 at the same dose reduced the expression by 8% ($p<0.05$) (Figure 4B). Similarly, the expression of HER-2/*neu* mRNA was significantly decreased by AN-207 by 34% ($p<0.01$), compared to controls. In contrast, AN-201 reduced the level of expression by only 13% ($p<0.05$) (Figure 4B). Similar results were obtained in Experiment 2 (data not shown).

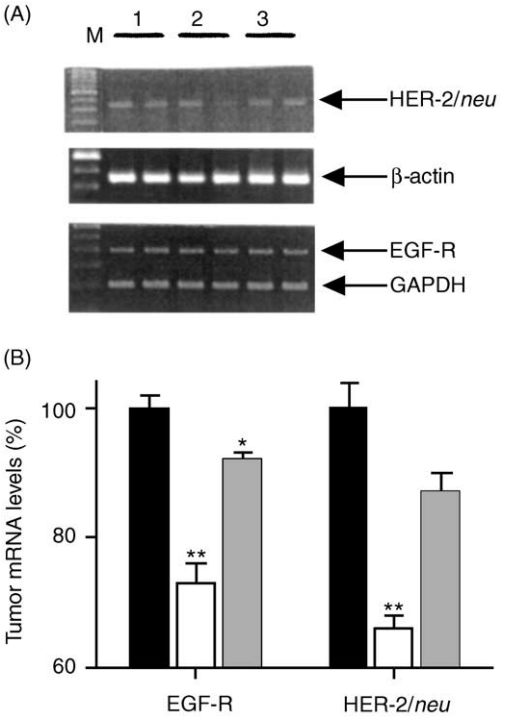

Figure 4. Effects of therapy with AN-207 or its cytotoxic radical AN-201 on EGFR and HER-2 mRNA expression. (A) PCR products obtained from tumors resulting after treatment with the cytotoxic LHRH analog AN-207 or its cytotoxic moiety AN-201 from Experiment 1 were analyzed by electrophoresis and visualized after ethidium bromide staining under UV light. Two representative samples from each group are shown. M, 100-bp DNA size marker; 1, controls; 2, AN-207-treated group; 3, AN-201-treated group. (B). The intensity of the bands for EGFR and HER-2 in the gel was analyzed by densitometry and normalized by those of GAPDH or β-actin. Filled columns, controls; open columns, AN-207-treated group; hatched columns, AN-201-treated group. Vertical bars show SEM. $*p<0.05$ versus controls; $**p<0.01$ versus controls.

Immunoblotting

EGFR and HER-2 receptor protein levels in tumors from Experiment 1 and 2 were investigated by Western blot analysis (Figure 5A and B). A single dose of AN-207 at 250 nmol/kg body weight significantly decreased the level of EGFR and HER-2 by 35% ($p<0.05$) and 36% ($p<0.05$), respectively, compared to controls (100%). An equimolar dose of AN-201 did not have a significant effect on EGFR protein levels, but it produced a significant 33% reduction in HER-2 receptor protein levels ($p<0.05$) of the controls. Similar results were obtained in Experiment 2 (data not shown).

Discussion

The modalities currently used for the treatment of patients with epithelial ovarian cancer are not satisfactory and new approaches must be developed. Targeting chemotherapeutic agents to hormone receptors on tumors is a modern strategy, intended to achieve a more efficient and selective delivery of drugs to malignant lesions, thereby reducing the peripheral toxicity. As about 80% of human ovarian cancer specimens express receptors for LHRH, cytotoxic conjugates of LHRH analogs may be suitable for the targeted chemotherapy of this malignancy.[4,5] Recently, we developed a series of cytotoxic LHRH conjugates, including AN-207 containing 2-pyrrolino-DOX (AN-201), a potent derivative of DOX. AN-207 has been shown to powerfully inhibit the growth of various experimental cancers, such as breast, prostate and ovarian cancers (reviewed in Schally and Nagy[5]). It was also demonstrated that while AN-207 very effectively inhibited the growth of LHRH receptor-positive OV-1063 human ovarian cancers in nude mice, it had no effect on LHRH receptor-negative UCI-107 human ovarian tumors.[23]

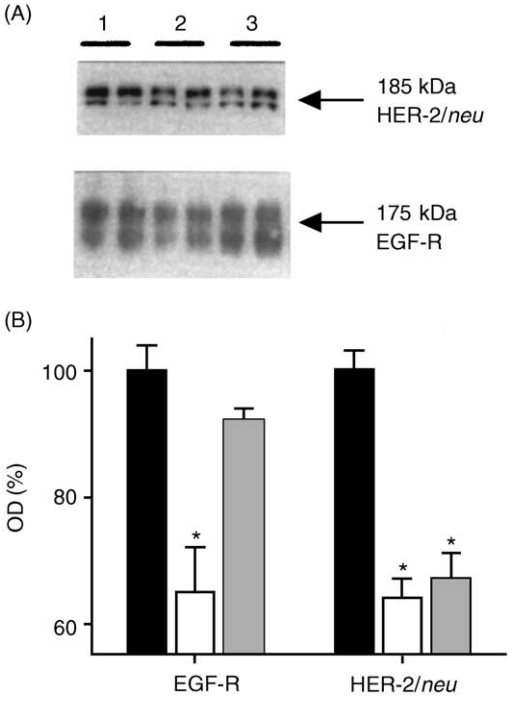

Figure 5. Effects of cytotoxic LHRH analog AN-207 or its cytotoxic moiety AN-201 on EGFR and HER-2 protein levels. (A) Proteins were extracted from control tumors and tumors treated with the cytotoxic LHRH analog AN-207 or its cytotoxic moiety AN-201 in Experiment 1 as described in Materials and methods, and were resolved by PAGE, transferred onto nitrocellulose membranes and revealed by enhanced chemiluminescence. 1, Controls; 2, AN-207-treated group; 3, AN-201-treated group. (B) The intensity of the bands for EGFR and HER-2 proteins was analyzed by densitometry. Filled columns, controls; open columns, AN-207-treated group; hatched columns, AN-201-treated group. Vertical bars show SEM. $*p < 0.05$ versus controls.

In this study, we tested the effects of targeted cytotoxic LHRH analog AN-207 on the growth of LHRH receptor-positive ES-2 human ovarian cancers xenografted into nude mice. In all three experiments, a single injection of AN-207 produced a significant inhibition of tumor growth. In the first experiment, we demonstrated that its components, radical AN-201, carrier [D-Lys^6]LHRH or their mixture were ineffective. The inhibitory effect of AN-207, even on relatively large initial tumors that measured about 400 mm^3 in Experiment 2, holds a promise for the treatment of patients whose tumors can only be partially removed by surgery.

The presence of LHRH receptors is a prerequisite for targeted therapy with LHRH analogs. We have recently shown that AN-207 binds with high affinity to LHRH receptors present on membranes of ES-2 human ovarian cancer cell line.[9] In the same study, by means of a novel *in vitro* experiment, we have also shown that AN-207 selectively kills ES-2 cells cocultured with LHRH receptor-negative UCI-107 human ovarian cancer cell line.[9] To prove the targeting action of AN-207 *in vivo*, mice bearing ES-2 xenografts were pretreated with an excess of LHRH antagonist Cetrorelix before the administration of AN-207. Under these conditions, the antitumor activity of AN-207 was strongly attenuated, even after a second dose. In addition, the blockade of LHRH receptors by Cetrorelix increased the toxicity of AN-207, resulting in the deaths of three of eight mice. The high toxicity of AN-207 after pretreatment with Cetrorelix may be due to some unknown drug interaction. However, it is also possible that after blocking the LHRH receptors, the receptor-mediated uptake of the conjugate is inhibited and a longer exposure of the analog to non-specific carboxylesterase enzymes (EC.3.1.1.1) in the serum leads to hydrolytic release of the more toxic radical AN-201 into the circulation. A similar increase in toxicity was observed with our cytotoxic somatostatin analog AN-238, in which AN-201 is linked by the same 14-*O*-glutaryl ester bond to a somatostatin carrier peptide, when the receptors for somatostatin were blocked by a high dose of the carrier.[24,25] This indicates that the higher toxicity is less likely to be the result of nonspecific drug interactions, because different hormones were used in these experiments for the blockade of receptors. We have also shown that almost 15% of AN-207 was hydrolyzed to release AN-201 after 30 min of incubation in serum-free medium[9] and that AN-152, the DOX-containing counterpart of AN-207, is hydrolyzed by carboxylesterase enzymes in blood releasing DOX.[24] It has to be noted however, that while deconjugation of 50% of cytotoxic radical DOX in mouse serum takes about 20 min, in human serum it is approximately 2 h. Thus, much lower toxicity can be expected in human beings, as it has been recently shown in nude mice with suppressed serum esterase activity.[25]

Histological analysis by the AgNOR method[9] of representative tumors indicated that the rate of cell proliferation was significantly lower in the group treated with AN-207 than in the animals receiving AN-201 or in controls (data not shown).

In view of the finding that amplification of EGFR and overexpression of HER-2 are associated with the progression and chemoresistance of ovarian cancer and are indicators of poor prognosis,[15,17,18,26] we investigated the effect of the treatment with cytotoxic LHRH analog AN-207 on the expression of these genes in ES-2 tumors. AN-207 significantly decreased the expression of mRNA for EGFR and HER-2 in these

tumors, by 27 and 34% ($p<0.01$), respectively, compared to controls. In contrast, AN-201 at an equimolar dose produced a small reduction ($p<0.05$) only in the mRNA level for HER-2. The difference observed may be attributable to the targeting effect of AN-207, which can produce a higher intratumoral concentration of the cytotoxic moiety. After treatment with AN-207, the reduction of tumor mRNA for EGFR and HER-2, was accompanied by a significantly lower expression of EGFR and HER-2 receptor protein, as demonstrated by immunoblotting. Consequently, a suppression of stimulatory signaling associated with EGFR and HER-2 could account, at least in part, for the inhibition of tumor growth by AN-207 observed in the present study. However, it is also possible that AN-207 selectively kills cells that express elevated levels of LHRH receptors.[9] In view of the findings that EGF stimulates LHRH receptor expression,[27] these cells may also express higher EGFR and HER-2 levels. Thus, the death of these cells would lower EGFR and HER-2 expression. Although a similar decrease in HER-2 protein levels was found after treatment with AN-201, no significant reduction in EGFR protein was observed. HER-2, an orphan receptor, is thought to play an active role in cell proliferation through heterodimerization with EGFR and other members of the EGFR family,[28] after EGF stimulus. Thus, it is possible that therapy with AN-201 failed partially due to the fact that without targeting, EGFR levels were not affected. A decrease in HER-2 levels alone may not be sufficient to significantly influence the formation of heterodimers with EGFR and the ensuing mitogenic signaling.

In conclusion, our results indicate that AN-207 is a powerful targeted antitumor agent that could be considered for the treatment of LHRH receptor-positive ovarian cancers.

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