Effective Treatment of Metastatic MDA-MB-435 Human Estrogen-independent Breast Carcinomas with a Targeted Cytotoxic Analogue of Luteinizing Hormone-releasing Hormone AN-207

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ABSTRACT

A highly potent derivative of doxorubicin, 2-pyrrolinodoxorubicin (AN-201), was linked to [d-Lys6]luteinizing hormone-releasing hormone (LH-RH) to form a cytotoxic analogue, AN-207, that can be targeted to LH-RH receptors. The effects of AN-207 were investigated in MDA-MB-435 human estrogen-independent breast carcinomas, which express LH-RH receptors. In experiment 1, nude mice bearing orthotopically implanted tumors received a single i.v. injection of AN-207, AN-201, or the carrier at 250 nmol/kg. Five weeks after administration of AN-207, tumor volume was significantly decreased by 66% (P < 0.001) and tumor burden by 71% (P < 0.05) as compared with controls, but no significant effects occurred in other groups. Six of eight (75%) control animals and 37.5% of mice treated with AN-201 developed metastases in the lymph nodes, whereas no lymphatic spread was found in any of the mice that received injections of AN-207. The antitumor effect of AN-207 could be partially blocked by pretreatment of the tumor-bearing mice with high doses of agonist [d-Trp6]LH-RH, which suggests that AN-207 acts on LH-RH receptors on tumors. The mortality due to toxicity was 25% in the group receiving AN-201 and 12.5% in the AN-207-treated group. Radioligand binding assays revealed the presence of high-affinity binding sites for LH-RH on tumor membranes, and mRNA for LH-RH receptors was demonstrated by reverse transcription-PCR. In experiment 2, two i.v. injections of AN-207 or AN-201 at 150 nmol/kg were given on days 0 and 28 to mice bearing orthotopic xenografts of MDA-MB-435. The outcome of the treatment was similar to that observed in experiment 1, but without any toxicity-related deaths. Tumor growth inhibition and prevention of metastatic disease suggest that cytotoxic LH-RH analogue AN-207 could be considered for the treatment of human estrogen-independent breast cancers expressing receptors for LH-RH.

INTRODUCTION

Breast cancer is the most common malignancy in women, accounting for 31% of all female cancers (1). The annual mortality due to this malignancy is about 45,000 in the United States (1). Although the death rate related to the disease has been reduced by the introduction of breast screening mammography and adjuvant therapies, more efficacious treatment modalities are needed. Chemo-therapy is used extensively for the treatment of breast cancer, but a dose escalation is always limited by treatment-related toxicity (2, 3).

Targeting of cytotoxic agents to cancer cells represents a modern approach to the treatment of human breast carcinomas and other cancers, which could increase inhibitory effects on tumors and reduce peripheral toxicity (4). Targeted chemotherapy is based on the concept that by linking cytotoxic radicals to a carrier, which is able to recognize cancer cells, a selective accumulation of the chemotherapeutic agents can be achieved in the tumor while sparing healthy tissues from exposure (reviewed in Ref. 4).

The presence of LH-RH receptors on various human tumors, including breast, ovarian, endometrial, and prostate cancers, indicated that LH-RH analogues might be suitable carrier molecules for cytotoxic agents (3–5). Receptors for LH-RH have been demonstrated in estrogen-independent MXT mouse mammary tumors and in several human breast cancer cell lines, including MCF-7, MX-1, and MDA-MB-231 (6–11). More importantly, receptor analyses of 500 primary human breast cancer specimens showed that 260 (52%) expressed binding sites for LH-RH (12). During the past decade, several cytotoxic LH-RH analogues have been developed and tested on various tumors in our laboratory (4, 7–10). Such hybrid molecules consisted of agonists or antagonists of LH-RH attached to various cytotoxic agents (4, 13). Recently, we synthesized AN-201,3 a highly active derivative of doxorubicin that is 500-1000 times more potent in vitro than the parent compound (14). This accounted for 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: AN-201, 2-pyrrolinodoxorubicin; LH-RH, luteinizing hormone-releasing hormone; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; m.f.p., mammary fat pad; NOR, nucleolar organizer region.
powerful cytotoxic agent was linked covalently to agonist [D-Lys°]LH-RH to form a cytotoxic LH-RH analogue designated AN-207 (13). We then demonstrated a high-binding affinity of AN-207 to human breast cancer specimens (8). A high antitumor efficacy of AN-207 and a lower toxicity than its radical AN-201 was shown in studies on LH-RH receptor-positive MX-1 and MDA-MB-231 estrogen-independent human breast cancers (9, 10).

In the present investigation, we evaluated the effect of AN-207 on the growth and the metastatic potential of the orthotopically implanted MDA-MB-435 human estrogen-independent breast carcinoma cell line, which expresses specific high-affinity receptors for LH-RH. The mechanism of action of AN-207 on MDA-MB-435 tumors was investigated by blocking LH-RH receptors with a high dose of agonist [D-Trp°]LH-RH. The effect of treatment on the receptors for LH-RH and the expression of their mRNA in MDA-MB-435 tumors were also studied.

MATERIALS AND METHODS

Peptides and Cytotoxic Agents. The agonistic analogue [D-Lys°]LHRH (pGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH₂), used as a carrier, was obtained from California Peptide Research Inc. (Napa, CA). Cytotoxic radical AN-201 and its 14-O-hemigluturate linked to [D-Lys°]LH-RH (AN-207) were prepared in our laboratory as described (14, 15). The cytotoxic compounds were purified by high-performance liquid chromatography, and their purity was 98%. Before the i.v. injection, the compounds were dissolved in 20 μl of 0.1 N acetic acid and diluted with 5% (w/v) aqueous D-mannitol solution (Sigma Chemical Co., St. Louis, MO) to the final volume.

Animals. Female athymic nude mice (Ncr nu/nu), 5–6 weeks of age, were obtained from the Frederick Cancer Research Facility of the National Cancer Institute (Frederick, MD), housed in sterile cages under laminar airflow hoods in a temperature-controlled room with a 12-h light and 12-h dark schedule, and fed autoclaved chow and water ad libitum. All experiments were performed according to institutional ethical guidelines on animal care.

Cell Line and Cytotoxicity in Vitro. The MDA-MB-435 estrogen-independent cell line, isolated from the pleural effusions of a patient with breast carcinoma, was obtained from American Type Culture Collection (Manassas, VA). It was maintained in MEM (Life Technologies Inc., Grand Island, NY) supplemented with 5% fetal bovine serum, 2 mM l-glutamine, nonessential amino acids, 1:50 MEM vitamins, 1 mM pyruvate, 100 units/l penicillin sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B (Life Technologies Inc.). Cells were cultured in Costar T-75 flasks (Corning Incorporated Science Products Division, Acton, MA) in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C. The cells were passaged weekly and routinely monitored for the presence of Mycoplasma using a test kit (Boehringer Mannheim, Indianapolis, IN). The cytotoxic activity of the analogues was determined in vitro using colorimetric assay based on quantitation of biomass by staining cells with crystal violet (16). IC₅₀ values, defined as the drug concentration that inhibited cell growth by 50% compared with untreated control cultures, were calculated from two independent experiments, consisting of seven replicate tests each, after exposure of MDA-MB-435 cells to cytotoxic agents for 91 h.

Experimental Protocol. Female athymic nude mice, 8–10 weeks of age, received injections orthotopically into the m.f.p. with MDA-MB-435 human breast carcinoma cells. Mice were anesthetized with methoxyflurane (Metofane; Pittman-Moore, Mundelein, IL), and a 5-mm incision was made in the skin over the lateral thorax as described previously (17). The m.f.p. was exposed, and an inoculum of 2 × 10⁶ cells/30 μl was implanted into the tissue through a 27-gauge needle. The skin incisions were closed with wound clips that were removed 1 week later.

In experiment 1, when tumors reached a volume of ~40–50 mm³, nude mice were randomly divided into seven experimental groups of seven to eight animals each and received the following treatment as a single i.v. injection, all of the drugs being injected at a dose of 250 nmol/kg: group 1, control, vehicle solution (5% mannitol); group 2, cytotoxic radical AN-201; group 3, cytotoxic analogue of LH-RH AN-207; group 4, unconjugated mixture of cytotoxic radical AN-201 and carrier analogue [D-Lys°]LH-RH; group 5, carrier, [D-Lys°]LH-RH; group 6, pretreatment with 200 μg agonist [D-Trp°]LH-RH 1 h before the injection of AN-207; and group 7, AN-207, the same as in group 3. The animals in groups 1–6 were sacrificed on day 33. In group 7, four animals were killed on day 48 and three mice on day 55, to study LH-RH receptor expression at different time points after the treatment with AN-207.

In experiment 2, when tumors measured 60–65 mm³, mice were divided into three groups of five animals each and were given i.v. injections on days 1 and 28: group 1, control, vehicle solution (5% mannitol); group 2, cytotoxic radical AN-201 at a dose of 150 nmol/kg; and group 3, cytotoxic analogue of LH-RH AN-207 at a dose of 150 nmol/kg. The experiment was terminated 50 days after the initiation of therapy.

All injections were administered through the tail vein under light methoxyflurane anesthesia. The tumors were measured once or twice a week with microcalipers, and tumor volume was calculated as length × width × height × 0.5236. Tumor growth delay was defined and calculated as T-C, where T and C are the median times (in days) of the first tumor volume doubling from the beginning of therapy in the treated and the control groups, respectively. Body weights of the animals were measured on the days of the injections and weekly thereafter. Fifteen and 22 days after treatment in experiment 1, blood samples were collected from the tail vein of the control animals and those that received cytotoxic compounds using Unopette microcollection kit (Becton Dickinson, Franklin Lakes, NJ). Total leukocyte and platelet counts were determined manually using a hemacytometer. At the end of the experiments, an autopsy was performed and the tumor weights were measured. Samples of each tumor were either fixed in formalin for histological examination or snap-frozen at −70°C for preparation of membranes for receptor assay and for extraction of RNA for RT-PCR.

Histological Procedure. Tumor samples were processed as described previously (7). Sections (6 μm thick) were cut and stained with H&E. Mitotic and apoptotic cells were counted in 10 standard high-power microscopic fields, and their number per 1000 cells was accepted as the mitotic and apoptotic indices.
Table 1  Effect of cytotoxic radical AN-201, cytotoxic LH-RH analogue AN-207, carrier peptide [D-Lys6]-LH-RH, and the unconjugated mixture of AN-201 and the carrier peptide on tumor volume, growth delay, tumor burden, and animal mortality in athymic nude mice bearing MDA-MB-435 human estrogen-independent breast carcinomas implanted orthotopically into the m.f.p.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial</th>
<th>Final</th>
<th>Growth delay (days)</th>
<th>Tumor burdena</th>
<th>Mortalityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>48.0 ± 5.1</td>
<td>418.6 ± 82.8</td>
<td>0</td>
<td>18.8 ± 3.7</td>
<td>0/8</td>
</tr>
<tr>
<td>AN-201</td>
<td>45.3 ± 6.1</td>
<td>351.8 ± 51.5</td>
<td>0</td>
<td>15.1 ± 1.9</td>
<td>2/8</td>
</tr>
<tr>
<td>AN-207</td>
<td>45.3 ± 4.7</td>
<td>142.4 ± 26.1c</td>
<td>11h</td>
<td>7.1 ± 1.5f</td>
<td>1/8</td>
</tr>
<tr>
<td>[D-Lys6]-LH-RH</td>
<td>42.2 ± 4.4</td>
<td>314.1 ± 40.1</td>
<td>2</td>
<td>11.4 ± 1.1</td>
<td>0/8</td>
</tr>
<tr>
<td>AN-201 + [D-Lys6]-LH-RH</td>
<td>45.3 ± 6.1</td>
<td>266.3 ± 55.3</td>
<td>1</td>
<td>11.3 ± 2.3</td>
<td>2/8</td>
</tr>
<tr>
<td>[D-Trp6]LH-RH + AN-207f</td>
<td>43.1 ± 3.7</td>
<td>205.2 ± 48.4</td>
<td>1</td>
<td>9.7 ± 2.4</td>
<td>2/8</td>
</tr>
<tr>
<td>Experiment 2b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>62.3 ± 8.5</td>
<td>570.2 ± 144.1</td>
<td>0</td>
<td>16.9 ± 2.6</td>
<td>0/5</td>
</tr>
<tr>
<td>AN-201</td>
<td>59.4 ± 13.8</td>
<td>540.6 ± 148.3</td>
<td>−2</td>
<td>16.6 ± 3.0</td>
<td>1/5f</td>
</tr>
<tr>
<td>AN-207</td>
<td>64.6 ± 10.3</td>
<td>180.6 ± 32.3</td>
<td>13</td>
<td>8.8 ± 1.8</td>
<td>0/5</td>
</tr>
</tbody>
</table>

a Tumor weight (mg)/body weight (g).
b Number of dead animals/number of total animals.
c A single i.v. injection of 250 nmol/kg body weight of each compound was given on day 1. The experiment lasted for 33 days.
d Values are mean ± SE; P < 0.01 versus control and P < 0.05 versus groups 2, 4, and 5.
e Mean doubling time was significantly different from other groups.
f Values are mean ± SE; P < 0.05 versus control.
g Mice received [D-Trp6]LH-RH at 200 µg s.c. 1 h before the administration of AN-207 at 250 nmol/kg.
h Two i.v. injections of 150 nmol/kg body weight of each compound were given on days 1 and 28. The experiment lasted for 50 days.
i The death of one mouse was accidental.

RESULTS

Tumor Inhibition and Toxicity. In the first experiment, we compared the antitumor effects and toxicity of single i.v. injections of cytotoxic radical AN-201, targeted cytotoxic LH-RH analogue AN-207, carrier peptide [D-Lys6]-LH-RH, and the unconjugated mixture of [D-Lys6]-LH-RH and AN-201 all at 250 nmol/kg in nude mice bearing xenografts of MDA-MB-435 human breast carcinoma grown in the m.f.p. The results are shown in Table 1. Administration of AN-207 produced a sig-
significant growth inhibition of the MDA-MB-435 tumors. Thirty-three days after the injection with AN-207, tumor volume was significantly reduced (P < 0.001) to 142.4 ± 26.1 mm³ compared with controls, which measured 418.6 ± 82.8 mm³, corresponding to a growth inhibition of 66% (Fig. 1a). In contrast, in groups that received injections of equimolar doses of AN-201, the unconjugated mixture of AN-201 and carrier [d-Lys⁶]LH-RH, or the carrier peptide alone, all tumors grew steadily (Fig. 1a). The growth of cancers treated with AN-207 was significantly delayed compared with all other groups (P < 0.01–0.012; Table 1). The weight of AN-207-treated tumors (200.0 ± 40.0 mg) was also reduced (P = 0.051) compared with

Fig. 1 Effect of treatment with cytotoxic LH-RH analogue AN-207 on the growth of orthotopically implanted MDA-MB-435 human estrogen-independent breast carcinomas in female athymic nude mice. a, changes of the tumor volume after treatment with a single i.v. injection of cytotoxic LH-RH analogue AN-207, cytotoxic radical AN-201, unconjugated mixture of AN-201, and the carrier peptide or the carrier peptide [d-Lys⁶]LH-RH alone, at 250 nmol/kg. Vertical bars show SEs. *, P < 0.01 versus control; **, P < 0.01 versus control and P < 0.01 versus AN-201 group; ***, P < 0.001 control, AN-201, and carrier groups. b, changes of the tumor volume after treatment with two i.v. injections of AN-207 or AN-201 at 150 nmol/kg given 28 days apart. Arrows indicate the days of treatment. Vertical bars show SEs. **, P < 0.01 versus control and P < 0.05 versus the group receiving AN-201; *, P < 0.05 versus control. c, effect of pretreatment with [d-Trp⁶]LH-RH administered at 200 μg/animal 1 h before the injection of AN-207 at 250 nmol/kg. The control and the AN-207 group are the same as in a. Vertical bars show SEs. *, P < 0.01 versus control; **, P < 0.001 versus control.
controls (490.0 ± 90.0 mg). Tumor burden in this group was significantly (P < 0.05) decreased as compared with controls (Table 1). After treatment with AN-207 only one of eight mice (12.5%) died due to toxicity, whereas AN-201 alone or together with [D-Trp^6]LH-RH resulted in the deaths of two of eight animals (25%; Table 1). The carrier peptide [D-Lys^6]LH-RH was not toxic but had no significant tumor inhibitory effects. On day 4, the average WBC counts in the AN-207-treated group and the AN-201-treated group were reduced markedly by 72.7% and 78%, respectively. In both groups the leukocyte count returned to normal values within 2 weeks (data not shown). Significant loss of body weight occurred in all groups treated with cytotoxic agents, but body weights of the surviving animals returned to normal by the end of the experiment (Fig. 2).

In the second experiment, mice bearing MDA-MB-435 tumors grown in the m.f.p. received injections of AN-207 or AN-201 at 150 nmol/kg on days 1 and 28. Therapy with AN-207 at 150 nmol/kg on days 1 and 28. The experiment lasted for 50 days. Pretreatment with [D-Trp^6]LH-RH at a high dose was intended to block the LH-RH receptors on the tumor cells. As shown in Table 1 and Fig. 1c, pretreatment with [D-Trp^6]LH-RH attenuated the suppressive effects of the cytotoxic agent AN-207 on the growth of MDA-MB-435 tumors. The inhibition of tumor growth produced by AN-207 alone was 66.0%, whereas the administration of [D-Trp^6]LH-RH before AN-207 decreased the tumor inhibition to 51.0% and increased toxicity-related deaths to 25% (two of eight mice), as compared with 12.5% (one of eight mice) with AN-207 alone.

**Effect of AN-201 and AN-207 on the Metastatic Potential of Orthotopically Implanted MDA-MB-435 Tumors in Nude Mice.** In the first experiment, six of eight mice (75%) in the control group and three of eight mice (37.5%) in the group given AN-201 developed metastases in the lymph nodes (Table 2). No metastatic lesions could be found in the animals treated with AN-207. Pretreatment of nude mice with [D-Trp^6]LH-RH 1 h before injection of AN-207 partially blocked the effect of AN-207 on the metastases of the MDA-MB-435 tumors, as shown by the development of lymphatic spread in two of eight mice (25%; Table 2). Distant hematogenous metastases were found in one of eight control animals (in the lungs), but not in the AN-201 or AN-207-treated groups.

In the second experiment, AN-201 and AN-207 were administered to mice twice at 150 nmol/kg body weight (Table 2). Four of five mice in the control group (80%) and three of five mice treated with AN-201 (60%) developed metastases in the lymph nodes. In contrast, no metastases were found in any of the five mice that received injections of AN-207. Lung metastasis was found in one of the control animals, but not in the AN-201- or AN-207-treated mice.

**Cytotoxicity in Vitro.** Antiproliferative activities of AN-207 and AN-201 were also evaluated in vitro. The concentration of AN-207 that inhibited growth of MDA-MB-435 cells by 50% as compared with control (IC_{50}) was 1.9 × 10^{-10} M, whereas the

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**Table 2** Effect of cytotoxic radical AN-201 and cytotoxic LH-RH analogue AN-207 on the metastatic incidence of MDA-MB-435 human estrogen-independent breast carcinomas implanted orthotopically into athymic female nude mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence of lymph node metastases</th>
<th>Incidence of distant metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Control 6/8 1 (lung)</td>
<td>AN-201 3/8 0/8</td>
</tr>
<tr>
<td></td>
<td>AN-207 0/8 0/8</td>
<td>[D-Trp^6]LH-RH + AN-207&lt;sup&gt;b&lt;/sup&gt; 2/8 0/8</td>
</tr>
<tr>
<td>Experiment 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Control 4/5 1 (lung)</td>
<td>AN-201 3/5 0/5</td>
</tr>
<tr>
<td></td>
<td>AN-207 0/5 0/5</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> A single i.v. injection of 250 nmol/kg body weight of each compound was given on day 1. The experiment lasted for 33 days.

<sup>b</sup> Two i.v. injections of 150 nmol/kg body weight of each compound were given on days 1 and 28. The experiment lasted for 50 days.
respectively, compared with the control group, in which the
controls (Table 3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Area of necrosis (%)</th>
<th>Mitotic index</th>
<th>Apoptotic index</th>
<th>Ratio of apoptotic: mitotic indices</th>
<th>No. of AgNORs/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24 ± 8.0</td>
<td>13.3 ± 1.8</td>
<td>8.2 ± 2.2</td>
<td>0.64 ± 0.16</td>
<td>5.05 ± 0.07</td>
</tr>
<tr>
<td>AN-201</td>
<td>23 ± 4.0</td>
<td>8.4 ± 1.7</td>
<td>6.4 ± 0.8</td>
<td>0.87 ± 0.15</td>
<td>5.04 ± 0.14</td>
</tr>
<tr>
<td>AN-207</td>
<td>46 ± 8.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.3 ± 1.3&lt;sup&gt;8&lt;/sup&gt;</td>
<td>10.3 ± 1.1</td>
<td>1.22 ± 0.19&lt;sup&gt;9&lt;/sup&gt;</td>
<td>4.43 ± 0.13&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>[D-Trp&lt;sub&gt;6&lt;/sub&gt;]LH-RH + AN-207&lt;sup&gt;c&lt;/sup&gt;</td>
<td>44 ± 9.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.4 ± 0.8&lt;sup&gt;8&lt;/sup&gt;</td>
<td>9.4 ± 0.8</td>
<td>1.55 ± 0.22&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4.88 ± 0.15</td>
</tr>
</tbody>
</table>

<sup>a</sup> A single i.v. injection of 250 nmol/kg body weight of each compound was given on day 1. The experiment lasted for 33 days.
<sup>b</sup> Values are mean ± SE; P < 0.01 versus control.
<sup>c</sup> Mice received [D-Trp<sub>6</sub>]LH-RH at 200 μg s.c. 1 h before the administration of AN-207 at 250 nmol/kg.
<sup>d</sup> Values are mean ± SE; P < 0.05 versus control.

**Histological Findings.** MDA-MB-435 mammary cancers were undifferentiated tumors consisting of polygonal epithelial cells without any special arrangement. The nuclei of cells were round or oval, relatively pale, and contained prominent nucleoli. The amount of stroma was minimal. The group treated with AN-207 had a more extensive area of necrosis than the controls (P < 0.01; Table 3). Large necrosis was also found in tumors from mice given AN-207 after pretreatment with [D-Trp<sub>6</sub>]LH-RH (P < 0.05). The area of necrosis in tumors from mice treated with AN-207 was similar to that of the controls. Therapy with AN-207 alone or after [D-Trp<sub>6</sub>]LH-RH pretreatment significantly increased the ratio of apoptotic to mitotic indices to 1.55 ± 0.22 (P < 0.01) and 1.22 ± 0.19 (P < 0.01) respectively, compared with the control group, in which the ratio was 0.64 ± 0.16. A marked reduction of the mitotic cells (P < 0.01) was seen in all groups treated with cytotoxic agents, whereas the number of AgNORs/cell was significantly (P < 0.01) decreased only in the group receiving AN-207 compared with controls (Table 3).

**Effect of Treatment with AN-207 on the Expression of mRNA for LH-RH Receptor.** RT-PCR analysis of mRNA expression for LH-RH receptors yielded a product of the expected size of 319 bp for LH-RH receptor in samples from the control group as well as the AN-207-treated group at various time points after therapy (Fig. 3). Amplification with primers specific for human GAPDH produced a single PCR product of 207 bp (Fig. 3). Densitometric analysis of the RT-PCR products, after normalization of the expression of LH-RH receptor mRNA versus the corresponding levels of mRNA for human GAPDH, showed a 26% (P < 0.05) decrease in the expression of mRNA for LH-RH receptor in tumors 33 days after injection of AN-207 as compared with the controls. However, 48 days after treatment mRNA expression returned to the level of controls.

**LH-RH Receptor Binding Analyses.** Using ligand competition assays, a single class of high-affinity binding sites for LH-RH receptors was found in membranes of MDA-MB-435 mammary tumors with a mean K<sub>d</sub> of 7.21 ± 1.6 nM and a mean B<sub>max</sub> of 577 ± 125.8 fmol/mg membrane protein (Table 4). Receptors for LH-RH were also detected in tumors from animals treated with AN-201 (Table 4). High-affinity receptors for LH-RH with a K<sub>d</sub> of 6.85 nm and a B<sub>max</sub> of 273 fmol/mg were detected only in one of the three tumors from the AN-207-treated group, 33 days after the administration of AN-207 at 250 nmol/kg. Forty-eight days after the injection of AN-207 no specific binding for LH-RH was detected in any of the three tumors tested, whereas after 55 days only one of two tumors investigated showed high-affinity receptors for LH-RH with a K<sub>d</sub> of 8.16 nm and a B<sub>max</sub> of 484.0 fmol/mg membrane protein. In the group of animals pretreated with [D-Trp<sub>6</sub>]LH-RH 1 h before to the injection with AN-207, receptors for LH-RH at a concentration of 589.0 fmol/kg membrane protein (Table 4) were detected only in one of the three tumors tested.

**DISCUSSION**

Although some progress has been made in the treatment of estrogen-independent breast cancer, the overall results are not yet satisfactory and the development of novel therapeutic modalities is required (2, 3). Several investigators reported the presence of LH-RH receptors in various human mammary carcinoma cell lines and breast cancer specimens (7–10, 12, 19, 20). This provided a rationale for the development of targeted cytotoxic LH-RH analogues as potential candidates for the treatment of breast cancer (4).

In this study, we tested the effects of AN-207 on the growth of metastatic MDA-MB-435 estrogen-independent breast can-
Cytotoxic LH-RH Analogue in Metastatic Breast Cancer

Table 4  Binding characteristics of LH-RH receptors on membranes of orthotopic MDA-MB-435 human estrogen-independent breast carcinomas in athymic nude mice treated with cytotoxic radical AN-201 and cytotoxic LH-RH analogue AN-207

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Kd (nmol/L)</th>
<th>Bmax (fmol/mg protein)</th>
<th>No. of positive tumors for LH-RH receptors/no. of tumors tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.21 ± 1.6</td>
<td>577.7 ± 125.8</td>
<td>3/3</td>
</tr>
<tr>
<td>AN-201 on day 33</td>
<td>8.33 ± 3.1</td>
<td>505.0 ± 79.6</td>
<td>3/3</td>
</tr>
<tr>
<td>AN-207 on day 33</td>
<td>6.85</td>
<td>273.0</td>
<td>1/3</td>
</tr>
<tr>
<td>AN-207 on day 48</td>
<td>ND</td>
<td>ND</td>
<td>0/3</td>
</tr>
<tr>
<td>AN-207 on day 55</td>
<td>8.16</td>
<td>484.0</td>
<td>1/3</td>
</tr>
<tr>
<td>[D-Trp6]LH-RH + AN-207 on day 33</td>
<td>6.12</td>
<td>589.0</td>
<td>1/3</td>
</tr>
</tbody>
</table>

a A single i.v. injection of 250 nmol/kg body weight of each compound was given on day 1. The experiment lasted for 33 days.
b Kd, dissociation constant; Bmax, maximal binding capacity of receptors; ND, not detected.
c Mice received [D-Trp6]LH-RH at 200 μg s.c. 1 h before the administration of AN-207 at 250 nmol/kg.

cancers implanted in the m.f.p. of female athymic nude mice (17). Our results demonstrate that AN-207 inhibits significantly the growth of MDA-MB-435 tumors and totally suppresses their metastatic potential, whereas cytotoxic radical AN-201 given alone or as a mixture with the carrier peptide is more toxic and does not produce any significant tumor inhibition.

The antitumor efficacy of AN-207 was tested in two experiments differing in the dosage regimens used: in the first experiment, AN-207 was administered as a single i.v. injection at 250 nmol/kg; and in the second experiment, AN-207 was given twice at 150 nmol/kg. In both experiments, AN-207 produced a similar (65%) inhibition of tumor growth as compared with controls. However, a better tolerance to a lower dose of AN-207 is shown by an absence of toxicity-related deaths in mice treated twice with AN-207 at 150 nmol/kg, whereas one of eight mice died in the group that received one injection of AN-207 at 250 nmol/kg. Thus, repeated administration of low doses of AN-207 may represent a more efficient therapeutic option. In addition, while the administration of a single dose of AN-207 at 250 nmol/kg resulted in continuous, although suppressed, tumor growth, in contrast, when AN-207 was given twice at 150 nmol/kg, the second injection of AN-207 not only arrested the tumor growth for about 12 days but also produced a temporary tumor regression. The delay in tumor growth caused by AN-207 in the second experiment was not significantly different from controls because the calculation was based on the first doubling time. Growth inhibition by AN-207 in this experiment became significant only after the second injection (Fig. 1b).

Elimination of the lymphatic and hematogenous spread of MDA-MB-435 cells after treatment with the cytotoxic LH-RH analogue AN-207 was observed in both experimental protocols tested in this study. The most probable explanation for this finding is that the treatment with AN-207 prevented the dissemination of cancer by arresting the growth of primary tumors. It is possible, however, that AN-207 is also targeted directly to LH-RH receptor-positive metastatic lesions.

The receptor-mediated mechanism of action of AN-207 was investigated by the administration of LH-RH agonist [D-Trp6]LH-RH at a high dose, before the injection of AN-207. This was expected to attenuate the antitumor activity of AN-207. Under these conditions, AN-207 had a weaker effect on the primary tumor and the capacity to suppress the metastatic potential of MDA-MB-435 cells was also reduced. It is likely that the excess of [D-Trp6]LH-RH occupied most LH-RH receptors on the tumors, whereas the unbound cytotoxic LH-RH analogue AN-207 was hydrolyzed by carboxylesterase enzymes (EC.3.1.1.1) in blood, releasing unconjugated AN-201 (21). This hypothesis is also in accordance with the finding that the mortality in this group was the same (25%) as in the AN-201-treated group. A very high esterase activity in mice, which is about 10 times higher than in humans (21), may be responsible for the similar mortality and losses in body weights in all groups treated with cytotoxic compounds (Fig. 2). The half-life of AN-207 in the serum of female nude mice incubated in vitro at 37°C in a humidified atmosphere of 95% air and 5% CO2 is ~20 min, whereas the deconjugation of 50% of cytotoxic radical AN-201 takes ~120 min in human serum under the same conditions (data not shown). Thus, in human beings a much lower toxicity of AN-207 can be expected, as it was shown recently in nude mice with suppressed serum esterase activity (21). Some toxicity to normal tissues expressing LH-RH receptors can be expected. However, in a recent study we found a full recovery of pituitary function in rats within 2 weeks after treatment with the maximum tolerated dose of AN-207 (22).

Histological analysis showed that treatment with AN-207 significantly increased the area of necrosis in MDA-MB-435 tumors and raised the ratio of apoptotic to mitotic indices. This suggests that tumor growth inhibition is not only caused by a decrease in the rate of cell proliferation, as indicated by the reduction in the AgNOR numbers and mitotic indices, but is also the result of cell death due to necrosis, which could be even more important (23).

The LH-RH receptor status of MDA-MB-435 tumors was studied by RT-PCR analysis and radioreceptor assay. Using RT-PCR, we detected mRNA for LH-RH receptor in all samples studied. The levels of mRNA for LH-RH receptor decreased significantly 33 days after the injection with AN-207, but after 48 days the levels returned to controls values. Considering that the treatment consisted of a single i.v. injection of the cytotoxic analogue 33 days before mRNA analysis, the decrease in the levels of mRNA for LH-RH receptor could be a consequence of inhibition of gene transcription due to tumor suppression rather than a direct result of the interaction between the cytotoxic LH-RH analogue and the LH-RH receptor. A down-regulation of LH-RH receptor due to endocrine effects is extremely unlikely because only a single low dose of about 5 nm of the cytotoxic LH-RH analogue AN-207 was used.

Radioligand binding assays showed that treatment with
AN-207 also affected the binding of LH-RH analogue to the membranes of MDA-MB-435 tumors. Although no significant difference was observed in the binding characteristics ($K_d$ and $B_{max}$) of MDA-MB-435 xenografts after the administration of AN-207, the number of tumors in which specific binding for LH-RH could be detected was decreased after the treatment. In contrast, RT-PCR analysis revealed the expression of mRNA for LH-RH receptor in all samples tested. A possible explanation for the results of radioligand binding studies as compared with RT-PCR is that mRNA for LH-RH receptors was not translated into functional receptor protein in some samples due to unknown mechanisms. However, a strong effect of a second injection of AN-207 on tumor growth in experiment 2 indicates that the receptors for LH-RH may still be available for targeting of AN-207 on previously treated tumors. In fact, in a recent study on the effects of AN-207 on MDA-MB-231 tumors, we demonstrated that although no receptors for LH-RH were detectable 21 days after AN-207 treatment, 39 days later high-affinity binding sites for LH-RH could be shown again on AN-207-treated tumors (10).

In conclusion, we showed that cytotoxic analogue AN-207 can be targeted to human estrogen-independent breast cancers that express receptors for LH-RH, producing a significant tumor growth inhibition and a suppression of the metastatic potential, whereas 2-pyrrolino-DOX itself is ineffective and toxic. Our results suggest that targeted chemotherapy with cytotoxic LH-RH analogues such as AN-207 could be a promising new modality for the management of estrogen-independent breast cancer.

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REFERENCES


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Effective Treatment of Metastatic MDA-MB-435 Human Estrogen-independent Breast Carcinomas with a Targeted Cytotoxic Analogue of Luteinizing Hormone-releasing Hormone AN-207

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