Human Malignant Melanomas Express Receptors for Luteinizing Hormone Releasing Hormone Allowing Targeted Therapy with Cytotoxic Luteinizing Hormone Releasing Hormone Analogue

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Abstract

Cytotoxic analogue of luteinizing hormone releasing hormone (LHRH), AN-207, binds with high affinity to LHRH receptors and can be targeted to tumors expressing these receptors. We investigated the expression of LHRH receptors in surgical specimens of human malignant melanoma and evaluated the effects of AN-207 in models of human melanoma. Human melanoma specimens derived from primary tumors or metastases were examined for LHRH receptor expression by immunohistochemistry. Binding assays, Western immunoblotting, and reverse transcription-PCR analyses were used to investigate LHRH receptors in MRI-H255 and MRI-H187 transplantable human melanoma tumor lines. Antitumor effects of AN-207 and its components were evaluated in vivo in nude mice bearing xenografts of either melanoma tumor line. All 19 human melanoma specimens examined showed positive staining for LHRH receptors. The mRNA for LHRH receptors, receptor protein and binding sites for LHRH were detected in both transplantable melanoma tumor lines. AN-207 significantly inhibited the growth of MRI-H255 and MRI-H187 xenografts in vivo, reducing tumor volume by 59.9% to 79.2% and tumor weight by 61.0% to 76.9% (all P < 0.05). The components of AN-207 (LHRH analogue carrier and cytotoxic radical AN-201 as single drugs or as an unconjugated mixture) had no significant effects. Blockade of LHRH receptors by an excess of LHRH agonist Decapeptyl suppressed the effects of AN-207. LHRH receptors are expressed in a very high percentage of human malignant melanoma specimens and can be used for targeted chemotherapy with cytotoxic LHRH analogue AN-207. (Cancer Res 2005; 65(13): 5857-63)

Introduction

In the past four decades, the incidence of malignant cutaneous melanoma has steadily increased (1–4). It is estimated that 55,100 new cases of melanoma will occur in the United States in 2004 (5). When detected early, cutaneous melanoma can be cured by a wide surgical excision. However, once melanoma metastasizes to distant sites, patients face a dismal prognosis with a median survival of 7.5 months and an estimated 5-year survival rate of 5.5% (6). Various treatment modalities are available for therapy of disseminated melanoma, including surgical resection of metastatic lesions (7, 8), systemic chemotherapy with dacarbazine (DTIC; refs. 9, 10), and immunotherapy with vaccines (8, 11, 12) or biological response modifiers (13, 14). Although some promising clinical results were reported, no improvement in overall survival was achieved in the past 22 years (6), emphasizing the importance of developing new effective therapies.

The discovery of specific molecular characteristics of cancer cells prompted the development of a new class of drugs known as targeted therapeutics. These include inactivators of genes or gene products involved in oncogenesis, antibodies against tumor surface structures, and conjugates consisting of tumor-specific ligands linked to toxins, radionuclides, or chemotherapeutic agents. (15–18). Targeted chemotherapy can be based on peptide hormones, for which receptors are expressed on cancerous cells (19–22). Because of direct delivery of the cytotoxic agent to tumor cells, these targeted peptide conjugates are expected to have a higher antitumor efficacy with a reduced systemic toxicity. Recently, we developed a series of targeted cytotoxic conjugates consisting of luteinizing hormone releasing hormone (LHRH), somatostatin, or bombesin linked to the cytotoxic radicals doxorubicin or its highly potent derivative 2-pyrrolino-doxorubicin (AN-201; refs. 19, 20). One of these conjugates, AN-207, contains the agonist [D-Lys6] LHRH linked to AN-201 (23). AN-207 effectively inhibits the growth of breast, ovarian, endometrial and prostate cancers, 52% to 86% of which are known to express specific LHRH receptors (21, 22). Although LHRH receptors were found on cells of healthy reproductive organs, they are absent or only marginally expressed in other normal tissues (24). Interestingly, binding sites for LHRH were also detected in human tumor specimens and cancer cell lines originating from the liver, larynx, kidney, pancreas, colon, and brain (25–29). In addition, the presence of LHRH receptors was reported recently in two human melanoma cell lines (30).

This finding prompted us to investigate the expression of LHRH receptors in human melanoma specimens as well as in transplantable human melanoma tumor lines and to evaluate the efficacy of our cytotoxic LHRH analogue AN-207 in experimental models of human melanoma.

Materials and Methods

Human Specimens and Detection of Luteinizing Hormone Releasing Hormone Receptors by Immunohistochemistry

Tissues of 19 human melanoma specimens derived from primary tumors and metastases were prepared for immunohistochemical staining as described earlier (31). Slides were incubated with an antibody to LHRH receptors (clone A9E4, Novocastra, Newcastle upon Tyne, United Kingdom) for 4 hours (1:10 dilution in antibody diluent; DAKO, Carpinteria, CA). The
reaction was stopped with 100 µL PBS buffer per slide. After washing in 1,400 µL PBS buffer for 7 minutes, the slides were incubated with 120 µL EnVision horseradish peroxidase antimouse antibody (DAKO) for 30 minutes as above. After washing, the staining reaction was done with 120 µL/slide of 3,3'-diaminobenzidine solution (DAKO; 1:50 dilution in substrate buffer) for 10 minutes. The reaction was stopped with 100 µL PBS buffer for 20 minutes followed by washing with 1,400 µL PBS buffer for 7 minutes and slides were washed thrice every 2 hours with PBS buffer. Finally, the slides were rinsed in water, counterstained with Harris’ hematoxylin, and covered with a glass slide. The slides were examined by light microscopy and the LHRH receptors were estimated on a four-point scale as absent (−), weak expression (+), distinct expression (++), and strong expression (+++).

Peptides and Cytotoxic Agents

Cytotoxic LHRH analogue AN-207 was synthesized in our laboratory by coupling one molecule of 2-pyrroldino-doxorubicin-14-O-hemigluturate to the ε-amino-group of carrier peptide [D-Lys6] LHRH (23). Cytotoxic radical AN-201 was prepared as described (20). For the i.v. injection, the compounds were dissolved in 20 µL of 0.01 N aqueous acetic acid and diluted with 5% (w/v) aqueous D-mannitol solution (Sigma, St. Louis, MO).

Tumor Lines

The two transplantable human melanoma tumor lines MRI-H255 and MRI-H187 were obtained as frozen tumor tissues from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD). Thawing and transplantation procedures were carried out as recommended. Because these are not cell lines, in vitro studies could not be done.

Animals

Five- to 6-week-old female athymic nude mice (Ncr nu/nu) were obtained from the National Cancer Institute (Bethesda, MD). The animals were housed in sterile cages under laminar flow hoods in a temperature-controlled room with a 12-hour light/12-hour dark schedule and were fed autoclaved chow and water ad libitum.

Experimental Protocol

Tumors resulting 8 weeks after transplantation in donor animals were aseptically dissected and mechanically minced. Pieces of tumor tissue, about 3 mm3, were transplanted s.c. into the experimental animals by a trocar needle. Tumor volume (length × width × height × 0.5236) and body weight were measured weekly. At the end of each experiment, mice were sacrificed under anesthesia, tumors were excised and weighed and necropsy was done. Tumor specimens were snap frozen and stored at −70°C.

All experiments were in accordance with the institutional guidelines for the welfare of experimental animals.

Experiment 1. Three weeks after transplantation, animals bearing MRI-H255 tumors were divided into three groups of nine mice each with an average tumor volume of −100 mm3. The mice received the following treatment as a single injection into the jugular vein: group 1, control, vehicle solution; group 2, AN-207 at 200 nmol/kg; group 3, AN-201 at 200 nmol/kg. The experiment was terminated on day 22.

Experiment 2. Animals with MRI-H255 tumors were assigned to six groups with an average tumor size of about 70 mm3 and received the following treatment as single i.v. injections: group 1, control, vehicle solution; group 2, AN-207 at 200 nmol/kg; group 3, AN-201 at 200 nmol/kg. The experiment was terminated on day 22.

Experiment 4. Mice with MRI-H187 tumors measuring 310 to 340 mm3 were assigned to three groups containing seven mice each and received the following treatment as single i.v. injections: group 1, control, vehicle solution; group 2, AN-207 at 200 nmol/kg; group 3, AN-201 at 200 nmol/kg. The experiment was terminated on day 22.

Evaluation of Toxicity

General toxicity was evaluated on the bases of total leukocyte (WBC) count and body weight. WBC was determined with the Unopette microcollection kit (Becton Dickinson, Franklin Lakes, NJ) before and 7 days after administration of AN-207 and AN-201 as well as in controls. Body weights were measured weekly.

<table>
<thead>
<tr>
<th>Table 1. LHRH receptor expression in surgically removed human melanoma specimens as determined by immuno-histochemistry</th>
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</thead>
<tbody>
<tr>
<td><strong>Primary tumors</strong></td>
</tr>
<tr>
<td>Cutaneous melanomas</td>
</tr>
<tr>
<td>Ulcerated nodular malignant melanoma</td>
</tr>
<tr>
<td>Ulcerated superficial, spreading melanoma with nodular parts</td>
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<tr>
<td>Ulcerated superficial, spreading melanoma with nodular parts</td>
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<td>Ulcerated superficial, spreading melanoma</td>
</tr>
<tr>
<td>Ulcerated superficial, spreading melanoma</td>
</tr>
<tr>
<td>Superficial, spreading melanoma</td>
</tr>
<tr>
<td>Lentigo maligna melanoma</td>
</tr>
<tr>
<td>Ulcerated, acral-lentiginous melanoma with nodular parts and lymphangioses melanoblastosis</td>
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<tr>
<td>Ulcerated nodular malignant melanoma of the vagina</td>
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<tr>
<td>Uveal melanomas</td>
</tr>
<tr>
<td>Malignant melanoma of the choroida</td>
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<tr>
<td>Metastases of malignant melanomas</td>
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<tr>
<td>Pigment forming, large cell metastasis of a cutaneous melanoma in the lung</td>
</tr>
<tr>
<td>Skin/soft tissue metastasis of a known malignant melanoma with nodular infiltration of cutis and subcutis</td>
</tr>
<tr>
<td>Poorly differentiated soft tissue metastasis of a low differentiated cutaneous melanoma</td>
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<tr>
<td>Amelanotic metastasis in cervical lymph node of a known cutaneous melanoma</td>
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<tr>
<td>Pigment forming metastasis of a cutaneous melanoma</td>
</tr>
<tr>
<td>Amelanotic metastasis of a cutaneous melanoma</td>
</tr>
<tr>
<td>Poorly differentiated metastasis of a known cutaneous melanoma</td>
</tr>
<tr>
<td>Visceral metastasis of a known malignant melanoma in the adrenal gland</td>
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</tbody>
</table>

NOTE: No expression (−), low expression (+), distinct expression (++), high expression (+++).
RNA Extraction and Reverse Transcription-PCR

RNA was isolated from approximately 100 mg of tumor tissue, according to the protocol for TRI-Reagent (Sigma-Aldrich, St. Louis, MO), following the manufacturer’s instructions. The RT reaction was done with the iScript cDNA synthesis kit from Bio-Rad (Hercules, CA) according to the manufacturer's instructions. Five hundred nanograms of RNA were transcribed into cDNA in a final volume of 10 µL. All PCR reactions were carried out in an Applied Biosystems PCR system 2700 (Applied Biosystems, Norwalk, CT). For the amplification of the cDNA, gene-specific primers for the LHRH receptor were used as described (32). Ten microliters of each PCR product was loaded on a 1.8% agarose gel and subjected to electrophoresis, staining with ethidium bromide, and analysis using Kodak 1D 3.6 (Kodak, Norwalk, CT). For the amplification of the cDNA, gene-specific primers for the LHRH receptor were used as described (32). Ten microliters of each PCR product was loaded on a 1.8% agarose gel and subjected to electrophoresis, staining with ethidium bromide, and analysis using Kodak 1D 3.6 (Kodak, Norwalk, CT).

Western Immunoblot Analysis

For immunodetection of LHRH receptors, an extraction of membrane protein from MRI-H255 and MRI-H187 samples was done as reported (33). The presence of LHRH receptor protein was then shown by Western blotting using a goat polyclonal human LHRH receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA), as described (33). ES-2 human ovarian cancer served as a positive control, PC-3 human prostate cancer as a negative control for LHRH receptors in the PCR reaction.

Luteinizing Hormone Releasing Hormone Receptor Binding Studies

Receptors for LHRH on MRI-H255 and MRI-H187 tumors from the experimental groups were characterized by the ligand competition assay. Preparation of tumor membrane fractions and receptor binding studies of LHRH were done as described (34, 35). The LIGAND-PC computerized curve-fitting program of Munson and Rodbard (36) was used to determine the type of receptor binding, dissociation constant ($K_d$) and maximal binding capacity of the receptors ($B_{max}$).

Statistical Analysis

Data are expressed as means ± SE. Results were compared by one-way ANOVA and Bonferroni test was carried out for post hoc comparisons. $P < 0.05$ was considered significant.

Results

Immunohistochemistry of human melanoma specimens.

Altogether, 19 human melanoma samples of different histologic subtypes were obtained from primary tumors and metastases and examined by immunohistochemistry. Positive staining for LHRH receptors could be observed in all melanoma specimens. (Table 1; Fig. 1) Four samples stained strongly positive for LHRH receptors, 14 samples expressed the receptor at a moderate level, whereas only one showed a weak expression. LHRH receptor expression was independent of histologic subtype and stage of the primary tumors. Similar expression levels were observed in primary melanomas and the metastatic specimens indicating that no loss of LHRH receptors took place during the metastatic process. In the surrounding nonmalignant tissue, no or only marginal expression of LHRH receptors was found.

Inhibition of tumor growth in experimental models.

In experiment 1, a single administration of AN-207 at 250 nmol/kg significantly inhibited the growth of MRI-H255 human melanomas compared with the controls, resulting in a 70.9% reduction in tumor volume ($P = 0.018$) and a 67.4% decrease in tumor weight ($P = 0.036$). In the group treated with AN-207, tumor doubling time was significantly prolonged as compared with control animals ($P < 0.001$) or the AN-201 group ($P = 0.001$). Equimolar doses of the cytotoxic radical AN-201 had no significant effects on any tumor growth characteristics compared with controls (Fig. 2A; Table 2).

In experiment 2, administration of AN-207 at 200 nmol/kg on day 1 and at 150 nmol/kg on day 15 significantly reduced the volume of MRI-H255 melanomas from day 8 ($P = 0.024$) until the end of the experiment on day 29 ($P = 0.003$), with the final tumor volume being 79.2% smaller than in the controls. Mean tumor weight was also significantly ($P = 0.002$) decreased by 76.9% compared with the controls. The mean tumor volume and weight of the group treated with AN-207 were also significantly ($P = 0.005$ and $P = 0.007$, respectively) lower than in animals that received...
cytotoxic radical AN-201. Equimolar doses of the carrier [D-Lys6] LHRH and the mixture of AN-201 and [D-Lys6] LHRH had no significant effects on the variables of tumor growth compared with the controls. The effect of AN-207 could be blocked by pretreatment with 200 µg of the LHRH agonist Decapeptyl, 15 minutes before the administration of AN-207. Tumor doubling time of the AN-207 group was significantly increased when compared with the control group ($P < 0.001$) or any other treatment regimen ($P < 0.001$; Fig. 2B; Table 2).

In experiment 3, a single injection of AN-207 at a dose of 200 nmol/kg significantly inhibited the growth of MRI-H187 human melanoma xenografts as compared with the control group (59.9% reduction in tumor volume; $P < 0.001$) or the animals treated with AN-201 ($P = 0.028$). AN-207 also significantly decreased the tumor weight by 61.0% versus controls ($P < 0.001$) and by 51.9% versus AN-201 ($P = 0.005$). Tumor doubling time in animals that received AN-207 was significantly prolonged compared with the controls ($P < 0.001$) and the AN-201 group ($P = 0.002$). Equimolar doses of AN-201 did not significantly effect tumor growth when compared with the controls (Fig. 2C; Table 2).

In experiment 4, the treatment was initiated at a mean starting tumor volume of >300 mm$^3$. AN-207 at a dose of 200 nmol/kg significantly decreased the volume and weight of MRI-H187 human melanoma xenografts by 66.3% ($P < 0.001$) and 61.7% ($P < 0.001$), respectively, compared with control animals. Tumor doubling time was also significantly ($P < 0.001$) extended by AN-207. Equimolar doses of AN-201 did not significantly inhibit tumor growth and AN-207 proved to be significantly ($P < 0.05$) more effective than AN-201 with respect to all growth variables (Fig. 2D; Table 2).

Toxicity. In all treatment groups, except for the carrier group, a slight loss of body weight was observed 8 days after treatment, which ranged from 1.3% to 7.0% in the AN-207 groups and from 3.6% to 11.4% in the AN-201 groups. In experiment 1, body weights in the AN-201 group were significantly reduced on day 8 ($P = 0.002$) and on day 15 ($P = 0.003$) compared with controls. No significant loss of body weight was observed in experiments 2 and 3.

WBC was measured before and 7 days after the injections. AN-207 did not significantly decrease the number of leucocytes in any of the three experiments and there was only a mild reduction of WBC ranging from 8.3% to 18.2% compared with control animals. AN-201 significantly suppressed WBC on day 8 in all three studies when compared with controls (in experiment 1: 67.8% reduction, $P = 0.02$; in experiment 2: 31.1% decrease, $P = 0.005$; and in experiment 3: 43.3% decline, $P < 0.001$). In experiments 1 and 3, WBC counts on day 8 were also significantly lower in animals treated with AN-201 than in those treated with AN-207 ($P = 0.029$ and $P = 0.003$). The measurement before the second injection in experiment 2 showed WBC numbers within reference ranges in all groups. However, 8 days

![Image](https://www.aacrjournals.org/upload/figures/2005_65_13_5860.png)

**Figure 2.** A, effects of targeted cytotoxic LHRH analogue AN-207 and its radical AN-201 on the growth of MRI-H255 human malignant melanoma xenografted into nude mice. *, $P < 0.05$ versus controls; **, $P < 0.01$ versus controls. B, effects of targeted cytotoxic LHRH analogue AN-207, the cytotoxic radical AN-201, the carrier [D-Lys$^6$] LHRH, an unconjugated mixture of AN-201 and [D-Lys$^6$] LHRH, and AN-207 after blockade of the receptors with Decapeptyl on the growth of MRI-H255 human melanomas in nude mice. *, $P < 0.05$ versus controls; **, $P < 0.01$ versus controls. C and D, effects of targeted cytotoxic LHRH analogue AN-207 and its radical AN-201 on the growth of MRI-H187 human malignant melanoma xenografts in nude mice. *, $P < 0.05$ versus controls; **, $P < 0.01$ versus controls.
after the second administration of AN-201, WBC significantly fell by 65.5% compared with the controls \( (P < 0.001) \) and the AN-207 group \( (P = 0.001) \) and remained significantly reduced until the end of the experiment on day 29 (43.5% versus control; \( P = 0.017 \)).

One mouse died in the control group on day 5 in experiment 1. In experiment 2, one animal died on day 15 in the AN-201 group and one on day 24 in the AN-207 group. In experiment 3, one mouse died in the AN-201 group on day 17. In experiment 2, one animal died on day 15 in the AN-201 group and one on day 24 in the AN-207 group. In experiment 3, one mouse died in the control group on day 5 in experiment 1.

Expression of mRNA for luteinizing hormone releasing hormone receptor. Reverse transcription-PCR (RT-PCR) analyses showed the expression of mRNA for the LHRH receptor in MRI-H255 and MRI-H187 melanoma xenografts of control animals. The PCR products were of the expected size of 319 bp. No PCR products were amplified from the negative controls, ruling out the possibility of genomic contamination (Fig. 3A).

Expression of luteinizing hormone releasing hormone receptor protein. The presence of LHRH receptor protein in untreated MRI-H255 and MRI-H187 tumor tissues was evaluated by Western blotting using specific antibodies. A specific band at a \( K_d \) value of 6.85 nmol/L and a \( B_{\text{max}} \) of 623.6 fmol/mg membrane protein. High-affinity receptors for LHRH were also found in MRI-H187 tumors with a mean \( K_d \) value of 4.37 nmol/L and a \( B_{\text{max}} \) of 660.0 fmol/mg membrane protein. Treatment with AN-201 and AN-207 given as single or two consecutive injections did not affect the affinity or the concentration of LHRH receptors in either transplantable tumor line compared with the controls (Table 3).

### Discussion

Patients with advanced stages of malignant melanoma face a dismal prognosis and no improvement of overall survival has been achieved during the past two decades (2, 3). Systemic chemotherapy is one of the options for the therapy of disseminated melanoma. However, high intrinsic resistance of malignant melanoma cells to conventional chemotherapy and toxic side effects are responsible for the limitations of this treatment modality (3). Response rates to the traditionally used cytotoxic drug DTIC range between 11% and 28% and complete tumor regression occurs in only 4% of the patients (9). New treatment approaches including the combination of various chemotherapeutic agents or a recently introduced new cytotoxic drug, temozolomide, did not significantly exceed the efficacy of dacarbazine (12, 37, 38).

Targeted chemotherapy represents a modern approach to cancer therapy because of an anticipated more selective delivery of chemotherapeutic agents to malignant tissues. Therefore, it should be more effective and less toxic than conventional systemic chemotherapy (19, 21, 22). Receptors for peptide

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**Table 2. Effects of therapy with cytotoxic LHRH analogue AN-207, the cytotoxic radical AN-201, the carrier [D-Lys\(^6\)] LHRH, an unconjugated mixture of AN-201 and [D-Lys\(^6\)] LHRH, and AN-207 after blockade of the receptors with Decapeptyl on the growth of MRI-H255 and MRI-H187 human melanomas xenografted into nude mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor volume (mm(^3))</th>
<th>Tumor doubling time (d)</th>
<th>Tumor weight, mg (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>Final (% inhibition)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 1</strong> MRI-H255</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>107 ± 30</td>
<td>1,578 ± 377</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>AN-207</td>
<td>100 ± 25</td>
<td>460 ± 124(^a) (70.9)</td>
<td>8.4 ± 0.5 (^a)</td>
</tr>
<tr>
<td>AN-201</td>
<td>103 ± 21</td>
<td>1,180 ± 191 (25.2)</td>
<td>6.2 ± 0.4</td>
</tr>
<tr>
<td><strong>Experiment 2</strong> MRI-H255</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>70 ± 7</td>
<td>2,110 ± 338</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>AN-207</td>
<td>72 ± 7</td>
<td>439 ± 55(^a) (79.2)</td>
<td>12.3 ± 1.2(^a)</td>
</tr>
<tr>
<td>AN-201</td>
<td>69 ± 7</td>
<td>2,057 ± 289 (2.1)</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>Carrier</td>
<td>73 ± 9</td>
<td>2,097 ± 738 (0.6)</td>
<td>6.4 ± 0.4</td>
</tr>
<tr>
<td>Mixture</td>
<td>73 ± 9</td>
<td>2,457 ± 630 (–16.5)</td>
<td>5.9 ± 0.4</td>
</tr>
<tr>
<td>Blockade</td>
<td>73 ± 6</td>
<td>1,395 ± 160 (33.9)</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td><strong>Experiment 3</strong> MRI-H187</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>70 ± 8</td>
<td>1,059 ± 114</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>AN-207</td>
<td>68 ± 8</td>
<td>425 ± 47(^a) (59.9)</td>
<td>7.9 ± 0.5(^a)</td>
</tr>
<tr>
<td>AN-201</td>
<td>68 ± 8</td>
<td>805 ± 114 (24.0)</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td><strong>Experiment 4</strong> MRI-H187</td>
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</tr>
<tr>
<td>Control</td>
<td>340 ± 32</td>
<td>2,118 ± 307</td>
<td>9.0 ± 0.6</td>
</tr>
<tr>
<td>AN-207</td>
<td>334 ± 17</td>
<td>714 ± 47(^a) (66.3)</td>
<td>23.2 ± 3.2(^a)</td>
</tr>
<tr>
<td>AN-201</td>
<td>314 ± 40</td>
<td>1,679 ± 248 (20.7)</td>
<td>10.1 ± 0.9</td>
</tr>
</tbody>
</table>

\(^aP < 0.05\) versus control (one-way ANOVA and Bonferoni post hoc test).

\( ^{\dagger}P < 0.05\) versus AN-201 (one-way ANOVA and Bonferoni post hoc test).
hormones such as LHRH, somatostatin, or bombesin were found on a wide variety of cancers and to target these receptors, various cytotoxic hormone analogues have been designed in our laboratories. One of these agents, cytotoxic LHRH analogue AN-207 consists of [D-Lys⁶]LHRH linked covalently to 2-pyrrolidino-doxorubicin (AN-201), a superactive derivative of doxorubicin. AN-207 showed enhanced antitumor activity and a reduction of side effects compared with systemic therapy with the nontargeted cytotoxic radical, AN-201, in various LHRH receptor–positive experimental models of human cancers (21, 22). A high efficacy of AN-207 against chemoresistant tumors was shown in an experiment with nude mice bearing the doxorubicin-resistant MX-1 human mammary carcinoma. Sixty days after a single injection of AN-207, all mice were tumor free, whereas animals treated with AN-201 developed large tumors (32). The discovery of LHRH receptors in two human melanoma cell lines, BML and Me15392 (30), prompted us to evaluate the LHRH receptor status of human melanoma specimens. To provide a rationale for a possible use of AN-207 for the treatment of melanomas, we examined 19 samples of surgically removed human melanoma specimens derived either from primary tumors or metastatic lesions of various origins and with diverse histologic subtypes. Immunohistochemical analysis showed positive staining for LHRH receptors in all specimens tested, indicating that a very high percentage of melanomas express these receptors. In surrounding tissues, no expression or only marginal expression of LHRH receptors was found. Specific, high-affinity LHRH receptors and the corresponding mRNA were also detected in two transplantable human melanoma tumor lines, MRI-H255 and MRI-H187, by RT-PCR, Western blot analysis, and ligand binding assays.

In the first in vivo study, we evaluated the efficacy of AN-207 and its nontargeted cytotoxic radical in s.c. grown MRI-H255 cancers in nude mice. A single injection of AN-207 at 250 nmol/kg had highly significant antitumor effects, whereas the nontargeted cytotoxic agent AN-201 did not significantly influence tumor growth.

To show that the high efficacy of AN-207 is due to targeting of LHRH receptors on MRI-H255 tumors, a second experiment was carried out. Mice were treated with AN-207, AN-201, a nonconjugated mixture of AN-201 and the carrier peptide, [D-Lys⁶]LHRH, and the carrier alone. In addition, one group received a high dose of the LHRH agonist Decapeptyl, to block the LHRH receptors before the injection of AN-207. In this experiment, we also changed the dosing schedule compared with the first study. Instead of a single high dose, AN-207 and AN-201 were given at 200 nmol/kg on day 1 and at 150 nmol/kg on day 15. As a highly significant tumor inhibitory activity was observed only in the group treated with AN-207, this study clearly shows that the antitumor effects of AN-207 cannot be attributed to the cytostatic hormonal activity of the [D-Lys⁶]LHRH component of the conjugate but rather to the ability of this [D-Lys⁶]LHRH carrier to deliver AN-201 to malignant cells. The fact that a blockade of LHRH receptors by Decapeptyl led to the suppression of the antitumor effect of AN-207 provides additional evidence that this conjugate targets LHRH receptors. Because two consecutive injections of AN-207 did not change the characteristics of the LHRH receptors on MRI-H255 tumors compared with controls, a repeated administration of AN-207 is possible. In fact, a multiple injection regimen may be a favorable treatment schedule for AN-207, as two consecutive injections showed a somewhat more pronounced antitumor effects compared with a single dose.

The tumor inhibitory effects of AN-207 and AN-201 were also evaluated in MRI-H187 tumors. Again, AN-207 had a strong, significant effect, whereas AN-201 did not significantly influence tumor growth. The antitumor efficacy of AN-207 was independent of the tumor size and could also be shown in tumors with a starting volume greater than 300 mm³, suggesting that AN-207 is also effective in advanced malignant melanomas.

The toxicity of targeted cytotoxic LHRH analogue AN-207 and its cytotoxic radical AN-201 was compared with respect to weight loss, WBC suppression, and the incidence of death. The overall mortality was very low with only one death occurring after treatment with AN-207, two after AN-201 and one in the control group. In all treatment groups, except for the carrier group, a slight loss of body weight was observed 8 days after therapy. However, a significant decrease in body weights was only observed in experiment 1 in animals treated with AN-201. Myelotoxicity is

Table 3. Binding characteristics of LHRH receptors in MRI-H255 and MRI-H187 human melanoma xenografted into nude mice (experiments 2 and 3)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>(K_d) (nmol/L)</th>
<th>(B_{max}) (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRI-H255</td>
<td>Control</td>
<td>6.85 ± 0.65</td>
<td>623.6 ± 25.3</td>
</tr>
<tr>
<td>AN-207</td>
<td>6.01 ± 1.08</td>
<td>598.2 ± 33.7</td>
<td></td>
</tr>
<tr>
<td>AN-201</td>
<td>6.97 ± 0.88</td>
<td>612.3 ± 29.4</td>
<td></td>
</tr>
<tr>
<td>MRI-H187</td>
<td>Control</td>
<td>4.37 ± 0.33</td>
<td>660.0 ± 80.7</td>
</tr>
<tr>
<td>AN-207</td>
<td>5.13 ± 1.06</td>
<td>604.8 ± 47.2</td>
<td></td>
</tr>
<tr>
<td>AN-201</td>
<td>4.89 ± 0.64</td>
<td>688.5 ± 39.1</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Binding characteristics were obtained from ligand competition assays (each done in duplicate or triplicate tubes) based on binding of radiolaabeled [D-Trp⁶]LHRH to tumor membrane homogenates. All values represent mean ± SE.
usually the most serious side effect and the dose limiting factor of chemotherapy. In the present study, AN-207 did not significantly lower WBC at any time. However, in groups receiving AN-201, a significant decrease of WBC compared with controls was observed in all experiments 8 days after therapy. In experiments 1 and 3, the numbers of leucocytes in the AN-201 groups were also significantly lower than in animals of the AN-207-treated groups. In experiment 2, the second injection of AN-201 caused a highly significant decrease of WBC when compared with controls and AN-207 group and normal WBC ranges were not reached in this group until the end of the experiment. These results clearly show that targeting to tumoral LHRH receptors can significantly decrease the toxic side effects of chemotherapy because the receptors for LHRH are not expressed in most healthy tissues. A possible, specific toxicity of AN-207 to the LH-producing cells of the anterior pituitary has been investigated in previous studies in rats (39, 40). The results of these experiments indicated that AN-207 causes no permanent damage to the pituitary function at its maximum tolerated dose.

The current study shows for the first time, that LHRH receptors are expressed in a very high percentage (19 of 19, 100%) of human malignant melanoma specimens. Our work also shows that cytotoxic LHRH analogue AN-207 can be successfully targeted in vivo to human malignant melanomas, resulting in enhanced antitumor activity and a reduction of side effects compared with the cytotoxic radical AN-201. These results strongly suggest that targeted chemotherapy with cytotoxic LHRH analogues could be a promising new treatment option for the management of advanced malignant melanomas.

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Human Malignant Melanomas Express Receptors for Luteinizing Hormone Releasing Hormone Allowing Targeted Therapy with Cytotoxic Luteinizing Hormone Releasing Hormone Analogue

Gunhild Keller, Andrew V. Schally, Timo Gaiser, et al.


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