Receptors for Luteinizing Hormone Releasing Hormone Expressed on Human Renal Cell Carcinomas Can Be Used for Targeted Chemotherapy with Cytotoxic Luteinizing Hormone Releasing Hormone Analogues

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Abstract

Purpose: To determine the expression of luteinizing hormone releasing hormone (LHRH) receptors in specimens and cell lines of human renal cell carcinoma (RCC) and to evaluate the antitumor efficacy of targeted therapy with a cytotoxic analogue of LHRH, AN-207, in vivo. AN-207, consisting of [D-Lys6] LHRH linked to a cytotoxic radical, 2-pyrrolinodoxorubicin (AN-201), binds with high affinity to LHRH receptors and can be targeted to tumors expressing these receptors.

Experimental Design: The expression of LHRH receptors was investigated in 28 surgically removed specimens of human renal cell carcinoma (RCC) by immunohistochemistry and in three human RCC cell lines A-498, ACHN, and 786-0 by radioreceptor assays, Western immunoblotting, and reverse transcription-PCR analysis. Antitumor efficacy of AN-207 was examined in experimental models of these cell lines.

Results: Positive staining for LHRH receptors was found in all (28 of 28) of the examined human RCC specimens. mRNA for LHRH receptor, receptor protein, and LHRH binding sites were detected in all three cell lines. AN-207 significantly (P < 0.05) inhibited the growth of A-498, ACHN, and 786-0 xenografts in vivo producing a 67.8% to 73.8% decrease in tumor volume and a 62.2% to 77.3% reduction in tumor weight. Nontargeted cytotoxic radical AN-201 had no significant antitumor effects. Blockade of LHRH receptors by an excess of LHRH agonist Decapeptyl suppressed tumor inhibitory effects of AN-207.

Conclusions: Our findings indicate that LHRH receptors are expressed in human RCC specimens and can be used for targeted chemotherapy with cytotoxic LHRH analogues.

In 2004, about 36,000 patients in the United States will be diagnosed with renal cancer, with an estimated 12,000 to 13,000 deaths (1). Renal cell carcinoma (RCC) accounts for ~85% of all kidney tumors and its incidence has been increasing by 2% to 4% per year since the 1970s (2, 3). The standard treatment for patients diagnosed with localized RCC is surgical removal (4). However, 30% to 50% of all patients will show recurrence of disease and one third of the cases initially present with evidence of systemic spread (5). With a 5-year survival rate of <10%, these patients face a dismal prognosis and currently no standard treatment modality is effective in the therapy of metastatic RCC (3). Systemic chemotherapy results in partial and complete response rates only in 4.7% and 1.3% of the cases, respectively (6). Immunotherapy with IFNs, interleukin-2, and lymphokine-activated killer cells achieves response rates of 10% to 20%, some of which were lasting (7, 8). However, these therapeutic approaches are accompanied by severe side effects and consequently by a low quality of life. These clinical observations underline the importance of developing a new, more effective treatment for RCC.

The elucidation of molecular characteristics of cancer cells prompted the development of a new class of drugs known as targeted therapeutics. The targets for antibodies or conjugates of ligands linked to toxins, radionuclides, or chemotherapeutic agents include surface antigens, growth factor signaling pathways, and peptide hormone receptors (9–16). Targeted chemotherapy can be based on peptide hormones, such as luteinizing hormone releasing hormone (LHRH), somatostatin, or bombesin (13, 15). A direct delivery of the cytotoxic agent to the tumor cells is expected to increase therapeutic efficacy and reduce the systemic toxicity. Recently, we developed a targeted cytotoxic conjugate AN-207, which consists of the LHRH

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agonist [D-Lys\(^6\)] LHRH linked to a highly potent derivative of doxorubicin 2-pyrrolino-DOX (AN-201; refs. 17–19). AN-207 effectively inhibits the growth of experimental breast, ovarian, endometrial, and prostate cancers, which are known to express specific LHRH receptors (13, 15). In nonmalignant human tissue, receptors for LHRH seem expressed mainly in organs regulated by the pituitary-gonadal axis but not in tissues such as lymph nodes or hematopoietic stem cells (20). However, binding sites for LHRH were also detected in human tumor specimens and cancer cell lines originating from liver, larynx, pancreas, colon, and skin (21–27). In addition, specific binding for a LHRH agonist, \([^{125}\text{I}]\) buserelin, was found in four of six human RCC specimens (28).

These findings prompted us to investigate the expression of LHRH receptors in human RCC specimens as well as in three human RCC cell lines and to evaluate the efficacy of cytotoxic LHRH analogue AN-207 in models of human RCC in vivo.

## Materials and Methods

### Immunohistochemistry.

Tissues of 37 human RCC specimens derived from primary tumors and metastases as well as three normal human kidneys were fixed for 16 to 20 hours in 4% neutral buffered formalin and then embedded in paraffin. Two- to 4-μm-thin sections of selected tissue blocks were cut, mounted on siliconized glass slides (Sigma Chemical Co., St. Louis, MO), and dried at 60°C for 30 minutes and at 38°C overnight. Sections were deparaffinized by incubation in a xylene bath for 5 minutes, twice, in acetone for 5 minutes twice, and finally in distilled water for 5 minutes. Heat pretreatment of the sections was done with 120°C PBS buffer for 7 minutes. The slides were then washed in PBS/0.05% Tween (pH 7.4, PBS buffer) for 7 minutes and incubated with an antibody for LHRH receptors (clone AE94, Novocasta, New Castle, United Kingdom) for 4 hours (1:10 dilution in antibody diluent; DAKO). The reaction was stopped with 100 μL PBS buffer per slide. After washing in 1:400 PBS buffer for 7 minutes, the slides were incubated with 120 μL EnVision horseradish peroxidase antimouse antibody (DAKO) for 30 minutes. After washing as before, the staining reaction was done with 120 μL per slide 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 in distilled water. Endogenous peroxidase was blocked by incubation in a freshly prepared solution of 0.3% H\(_2\)O\(_2\) in methanol for 20 minutes at room temperature followed by washing in distilled water for 5 minutes. Except for counterstaining with hematoxylin and mounting, the following steps were done overnight using the Tecan-Immunostainer Genesis RSP 200 (Software: Gemini 3.40), according to the manufacturer’s EnVision Plus–staining procedure (DAKO, Carpinteria, CA): The slides were rinsed twice in PBS/0.05% Tween (pH 7.4, PBS buffer) for 7 minutes and incubated with an antibody for LHHRH receptors (clone AE94, Novocasta, New Castle, United Kingdom) for 4 hours (1:10 dilution in antibody diluent; DAKO). The reaction was stopped with 100 μL PBS buffer per slide. After washing in 1:400 PBS buffer for 7 minutes, the slides were incubated with 120 μL EnVision horseradish peroxidase antimouse antibody (DAKO) for 30 minutes. After washing as before, the staining reaction was done with 120 μL per slide 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 in 1400 μL PBS buffer for 7 minutes. The slides were then washed thrice with PBS buffer every 2 hours. 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 expression of LHRH receptors was 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-pyrrolino-DOX-14-O-hemiglutarate to the ε-amino-group of carrier peptide [D-Lys\(^6\)] LHRH (19). Cytotoxic radical AN-201 was prepared as described (18). 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 Chemical). Human RCC cell lines A-498, ACHN, and 786-0 were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (Sigma Chemical), 50 μL penicillin G, 50 μL gentamicin, and 100 μL streptomycin (all from Life Technologies, Grand Island, NY).

### Animals.

Five- to 6-week-old male 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 6 to 10 weeks after s.c. cell injection in donor animals were aseptically dissected and mechanically minced. Pieces of tumor tissue, about 3 mm\(^3\), were transferred 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 and necropsy was done. The right kidneys were fixed in 4% neutral buffered formalin. Tumors were excised, weighed, snap frozen, and stored at −70°C.

All experiments were in accordance with the institutional guidelines for the welfare of experimental animals and the NIH Guide for Care and Use of Laboratory animals.

### Experiment 1.

Four weeks after transplantation of A-498 tumors, mice were assigned to three groups of nine animals each, with an average tumor size of 50 to 60 mm\(^3\). Mice received the following treatment as single i.v. injections into the jugular vein on day 1: group 1, control, vehicle solution (5% mannitol); group 2, cytotoxic analogue AN-207 at a dose of 200 nmol/kg; group 3, cytotoxic radical AN-201 at a dose of 200 nmol/kg. The experiment was terminated on day 29.

### Experiment 2.

Nude mice bearing ACHN tumors were divided into three groups of nine animals each, with an average tumor volume of 50 mm\(^3\). Mice received the following treatment on days 1 and 29 of the experiment: 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 57.

### Experiment 3.

Animals with 786-0 tumors were assigned to six groups when tumors had reached a size of 40 to 50 mm\(^3\). The mice received the following treatment on days 1 and 15: group 1, control, vehicle solution (n = 10 mice); group 2, AN-207 at 150 nmol/kg (n = 10 mice); group 3, AN-201 at 150 nmol/kg (n = 10 mice); group 4, the carrier [D-Lys\(^6\)] LHRH at 150 nmol/kg (n = 5 mice); group 5, unconjugated mixture of the cytotoxic radical AN-201 and the carrier [D-Lys\(^6\)] LHRH at 150 nmol/kg (n = 5 mice); group 6, 200 μg of the LHRH agonist Decapeptyl ([D-Trop\(^6\)] LHRH) i.v. 15 minutes before the i.v. injection of cytotoxic analogue AN-207 at 150 nmol/kg (n = 6 mice). The experiment was terminated on day 29.

### Evaluation of systemic and local renal toxicity.

General toxicity was evaluated on the bases of total leukocyte count (WBC) and bodyweight. WBC was determined with the Unopette microcollection kit (Becton Dickinson, Franklin Lakes, NJ) before, and 7 days after the drug administration as well as at the end of the experiments. Bodyweight was measured weekly.

Toxic effects to renal tissue were examined by serum creatinine, urine analyses, and light microscopy of kidney tissue. For serum creatinine analyses, nude mice were assigned to three groups and received the following treatment: group 1, control, vehicle solution (n = 4 mice); group 2, AN-207 at a dose of 200 nmol/kg (n = 5 mice); group 3, AN-201 at a dose of 200 nmol/kg (n = 5 mice). Eight days after the injection blood was collected during necropsy and serum creatinine levels were measured by Antech Diagnostics (Lake Success, NY). Urine was collected from mice 7 days after the application of AN-207 and AN-201 as well as from control animals. Urine analyses were carried out by Antech Diagnostics.

Kidneys from mice treated with single or repeated injections of AN-207 and AN-201 and from control animals were excised and fixed in 10% inactivated FCS (Sigma Chemical), 50 μL penicillin G, 50 μL gentamicin, and 100 μL streptomycin (all from Life Technologies, Grand Island, NY).

### RNA extraction and reverse transcription-PCR.

Tumor tissue from human RCC cell lines. 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 reverse transcription 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 (Norwalk, CT). For the amplification of the cDNA, gene-specific primers for the LHRH receptor were used as described (29). Ten microliters of each PCR product were loaded on a 1.8% agarose gel and subjected to electrophoresis, staining with ethidium bromide, and analysis using Kodak 1D 3.6 (Rochester, NY) imaging analysis software. A total RNA negative control, which received only water in the reverse transcription reaction, was used in each PCR to rule out genomic DNA contamination. DU-145 human prostate cancer cell line was used as a positive control for LHRH receptors in the PCR reaction.

**Mouse kidney.** For the detection of the LHRH receptor in mouse kidney tissue, normal mouse kidney was homogenized and RNA isolated in Tri reagent according to the manufacturer’s protocol. Five micrograms of total RNA were used in the Superscript III cDNA synthesis system (Invitrogen, Carlsbad, CA) in a volume of 20 μL. The cDNA was amplified using the AmpliTaq system (Applied Biosystems) in a reaction volume of 25 μL for 40 cycles using the primer sequence specific for mouse LHRH receptor: sense 5'-TGATGGTGGTGATTAGCC-3', antisense 5'-CACATTGCGAGAAGACTG-3'.

**Western immunoblot analysis.** For the immunodetection of LHRH receptors, an extraction of membrane protein from A-498, ACHN, and

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### Table 1. LHRH receptor expression in surgically removed human RCC determined by immunohistochemistry

<table>
<thead>
<tr>
<th>Expression level</th>
<th>Primary tumors</th>
<th>Metastases of renal cell carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Well-differentiated clear-cell RCC</td>
<td>Cervical lymph node metastasis of a papillary RCC</td>
</tr>
<tr>
<td></td>
<td>Well-differentiated clear-cell RCC with cystic parts</td>
<td>Bone metastasis in femur of a clear-cell RCC</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated clear-cell RCC</td>
<td>Bone metastasis in humerus of a clear-cell RCC</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated clear-cell RCC</td>
<td>Lung metastasis of a clear-cell RCC</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated clear-cell RCC</td>
<td>Liver metastasis of a clear-cell RCC</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated chromophilic papillary RCC</td>
<td>Bone metastasis of a clear-cell RCC</td>
</tr>
<tr>
<td></td>
<td>Infiltrating, moderately differentiated clear-cell RCC</td>
<td>Lymph node metastasis of a papillary RCC</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated clear-cell RCC</td>
<td>Lung metastasis of a clear-cell RCC</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated clear-cell RCC</td>
<td>Metastasis in the adrenal gland of a clear-cell RCC</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated clear-cell RCC</td>
<td>Metastasis in the opposite kidney of a clear-cell RCC</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated clear-cell RCC</td>
<td>Lung metastasis of a clear-cell RCC</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated clear-cell RCC</td>
<td>Metastasis in the retroperitoneum of a clear-cell RCC</td>
</tr>
</tbody>
</table>

**NOTE:** --, no expression; --/+ , tumor with parts of negative and low receptor expression; +, low expression; ++/+++ , tumor with parts of low and distinct receptor expression; ++, distinct receptor expression; +++, tumor with parts of distinct and high receptor expression; ++++, high receptor expression.
786-0 samples as well as from normal mouse kidneys was done as previously reported (30). The presence of LHRH receptor protein was then shown by Western blotting using a goat polyclonal antihuman LHRH receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA), as described (30).

**Luteinizing hormone releasing hormone receptor binding studies.** Receptors for LHRH on A-498, ACHN, and 786-0 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 (31, 32). The LIGAND-PC computerized curve-fitting program of Munson and Rodbard (33) 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. Differences between mean values were evaluated by two-tailed Student’s t test. $P < 0.05$ was considered significant.

**Results**

**Immunohistochemistry of human renal cell carcinoma specimens.** A total of 37 human RCC specimens were obtained from primary tumors and metastases and evaluated for LHRH receptor expression by immunohistochemistry. Positive staining for LHRH receptors was found in all samples examined, independently of the histologic subtype. In only two of the primary RCCs, areas with no LHRH receptor expression were detected. In six primary RCCs, LHRH receptor expression was low, whereas the majority of the primary RCC specimens (17 of 25) showed moderate or high LHRH receptor expression. Eleven of 12 samples of RCC metastases were moderately or strongly stained, whereas weak receptor expression was found in only one sample. In surrounding nonmalignant tissue, binding sites for LHRH were detected in cells of the renal tubular system (13 of 17 samples) with varying intensity. Other cells (e.g., cells of glomeruli, blood vessels, or connective tissue) showed no or only marginal LHRH receptor expression. Three normal, human kidneys examined showed a moderate LHRH receptor expression in the tubular system, which was comparable with the findings in the surrounding tissue of the tumors. (Table 1; Fig. 1).

**Inhibition of tumor growth in experimental renal cell carcinoma models.** In experiment 1, a single administration
of AN-207 at 200 nmol/kg significantly \( (P < 0.001) \) inhibited the growth of human A-498 RCC, resulting in a 71.1% reduction of tumor volume. In this group, tumor weight was significantly \( (P < 0.01) \) decreased by 77.3% and tumor-doubling time was significantly \( (P < 0.01) \) prolonged compared with controls. Equimolar doses of the cytotoxic radical AN-201 had no significant effects on any tumor growth characteristics (Fig. 2; Table 2).

In experiment 2, two injections of AN-207 at 200 nmol/kg on days 1 and 29 completely arrested the growth of human ACHN RCC tumors, until the end of the experiment on day 57 when final tumor volume was 73.8% \( (P < 0.001) \) smaller than in the control group. Tumor weights in the group treated with AN-207 were 75.5% lower than in the control animals \( (P < 0.01) \) and tumor-doubling time was also significantly extended \( (P < 0.05) \). The cytotoxic radical AN-201 at equimolar doses had no significant effects on tumor growth. (Fig. 3; Table 2).

In experiment 3, the administration of AN-207 at a dose of 150 nmol/kg on days 1 and 15 significantly inhibited the growth of human 786-0 RCC xenografts by 67.8% compared with the control group \( (P < 0.001) \). AN-207 significantly decreased tumor weight by 62.2% \( (P < 0.001) \) and significantly \( (P < 0.001) \) prolonged tumor-doubling time. Equimolar doses of AN-201, the carrier \([\text{D-Lys}^6]\) LHRH or the mixture of AN-201 and \([\text{D-Lys}^6]\) LHRH had no significant effects on any variables of tumor growth. The effect of AN-207 could be blocked by injecting 200 \( \mu \)g of the LHRH agonist Decapeptyl, 15 minutes before the administration of AN-207 (Fig. 4; Table 2).

**Toxicity.** In all treatment groups, a slight loss of body weight was observed 8 days after treatment, which ranged from 0.3% to 6.9% in the AN-207 groups and from 1.9% to 11.6% in the AN-201 groups (Table 3). In experiment 1, body weights in the AN-201 group were significantly \( (P < 0.05) \) reduced from day 8 until the end of the experiment. No significant loss of body weight was observed in any group in experiments 2 and 3 (Table 3).

AN-207 did not significantly decrease the WBC in any of the three experiments. AN-201 significantly \( (P < 0.001) \) suppressed WBC on day 8 in all three experiments compared with controls. In experiments 2 and 3, the number of leucocytes in the blood of mice treated with AN-201 was within reference ranges before the second injection. However, the second administration of

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**Table 2. Effects of therapy with cytotoxic LHRH analogue (AN-207), the cytotoxic radical AN-201, the carrier \([\text{D-Lys}^6]\) LHRH, an unconjugated mixture of AN-201 and \([\text{D-Lys}^6]\) LHRH, and AN-207 after blockade of the receptors with Decapeptyl on the growth of A-498, ACHN, and 786-0 human RCC xenografted in nude mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor volume (mm³)</th>
<th>Tumor-doubling time (d)</th>
<th>Tumor weight (mg), % inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final (% inhibition)</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 1 (A-498)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>59.7 ± 12.1</td>
<td>361.6 ± 54.0</td>
<td>12.7 ± 1.1</td>
</tr>
<tr>
<td>AN-207</td>
<td>53.4 ± 5.2</td>
<td>104.6 ± 15.1* (711)</td>
<td>34.3 ± 2.9*</td>
</tr>
<tr>
<td>AN-201</td>
<td>58.3 ± 10.9</td>
<td>338.5 ± 60.9 (–6.5)</td>
<td>14.5 ± 2.4</td>
</tr>
<tr>
<td><strong>Experiment 2 (ACHN)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>50.8 ± 6.7</td>
<td>207.0 ± 45.7</td>
<td>47.3 ± 10.1</td>
</tr>
<tr>
<td>AN-207</td>
<td>47.7 ± 6.9</td>
<td>54.3 ± 12.8* (73.8)</td>
<td>1,055.7 ± 315.2*</td>
</tr>
<tr>
<td>AN-201</td>
<td>53.3 ± 6.7</td>
<td>153.5 ± 31.3 (25.8)</td>
<td>73.4 ± 20.3</td>
</tr>
<tr>
<td><strong>Experiment 3 (786-0)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>46.1 ± 6.1</td>
<td>342.4 ± 44.5</td>
<td>10.5 ± 0.6</td>
</tr>
<tr>
<td>AN-207</td>
<td>46.5 ± 7.7</td>
<td>110.6 ± 10.6* (67.8)</td>
<td>21.3 ± 2.4*</td>
</tr>
<tr>
<td>AN-201</td>
<td>45.5 ± 6.2</td>
<td>318.8 ± 51.6 (6.9)</td>
<td>10.9 ± 0.7</td>
</tr>
<tr>
<td>Carrier</td>
<td>49.6 ± 7.7</td>
<td>388.7 ± 63.8 (–13.5)</td>
<td>10.1 ± 1.2</td>
</tr>
<tr>
<td>Mixture</td>
<td>45.9 ± 6.9</td>
<td>298.6 ± 56.2 (12.8)</td>
<td>13.0 ± 2.6</td>
</tr>
<tr>
<td>Blockade</td>
<td>50.0 ± 11.6</td>
<td>244.4 ± 61.8 (28.6)</td>
<td>15.5 ± 2.9</td>
</tr>
</tbody>
</table>

\* \( P < 0.001 \) versus controls.  
1 \( P < 0.05 \) versus controls.  
2 \( P < 0.01 \) versus controls.
the cytotoxic radical significantly ($P < 0.05$) decreased the WBC in both studies until the experiments were terminated.

In experiment 2, one mouse died on day 32 in the AN-201 group and one on day 45 in the AN-207 group. In experiment 3, one mouse died in the AN-201 group on day 11 (Table 3).

Eight days after the injection of AN-207 and AN-201 at 200 nmol/kg serum creatinine levels did not differ from the values in the control animals. Mean serum creatinine levels were 0.20 mg/d in the control and the AN-201 group and 0.22 mg/d in mice treated with AN-207 (Table 3).

Urine analyses did not show any differences between control animals and mice after treatment with AN-207 or AN-201. No leucocytes, RBC, or casts were detectable. Total protein was within the reference range for nude mice (Table 3).

Microscopic examinations of mouse kidneys showed minor changes after treatment with AN-207 or AN-201. Few apoptotic and necrotic cells were detected in the tubular system of kidneys of both groups. No mononuclear cell infiltration was found in periglomerular or peritubular areas (Table 3).

Expression of mRNA for luteinizing hormone releasing hormone receptors in tumors of human renal cell carcinoma cell lines. mRNA for the human LHRH receptor was found in human A-498, ACHN, and 786-O RCC xenografts by reverse transcription-PCR analyses. 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. 5A).

Expression of luteinizing hormone releasing hormone receptor protein in tumors of human renal cell carcinoma cell lines. The presence of LHRH receptor protein in untreated A-498, ACHN, and 786-O tumor tissues was evaluated by Western blotting using specific antibodies. A specific band at $M_r = 64,000$ was found in all investigated tumor samples (Fig. 5B).

Expression of mRNA for luteinizing hormone releasing hormone receptors and luteinizing hormone releasing hormone receptor protein in nonmalignant renal tissue of nude mice. mRNA for the LHRH receptors and the LHRH receptor protein in mouse kidneys were detectable at low levels in two of the three samples (Fig. 5C-D).

Luteinizing hormone releasing hormone receptor binding studies. In membranes of A-498, ACHN, and 786-O RCC tumors from the control group, receptor analyses revealed a single class of specific, high-affinity binding sites for LHRH (Table 4). Mean $K_d$ values were 8.51 nmol/L for A-498 tumors, 4.35 nmol/L for ACHN RCC, and 10.6 nmol/L for 786-0 tumors. Mean $B_{\text{max}}$ values were 439.8 fmol/mg membrane protein in A498 tumors, 654.2 fmol/mg membrane protein for ACHN tumors, and 437.2 fmol/mg membrane protein for 786-0 RCC. 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 compared with LHRH receptor characteristics in the controls in all three cell lines (Table 4).

Discussion

Patients with advanced RCC face a dismal prognosis and no effective therapy currently exists for the management of metastatic disease (3). A new class of drugs known as targeted therapeutics, directed against specific proteins in cancer cells, such as surface antigens, growth factors, or hormone receptors, is being developed (9–15). Recently, Bevacizumab, an antibody targeted to vascular endothelial growth factor, showed some promising results in patients with advanced RCC in a phase II clinical study (34).

Chemotherapy targeted to hormone receptors is a novel approach designed to deliver chemotherapeutic agents directly to cancer cells to increase antitumor activity and decrease the side effects. Because the receptors for peptide hormones were found on a wide variety of cancers, several cytotoxic hormone analogues were synthesized in our laboratory including cytotoxic LHRH analogue AN-207. AN-207 consists of [D-Lys$^6$] LHRH covalently linked to superactive derivative of doxorubicin, 2-pyrrolino-DOX (AN-201) and it shows enhanced antitumor activity in various LHRH receptor-positive experimental models of human cancers (13, 15). Previously, specific binding for LHRH agonist buserelin was detected in four of six human RCC (28). This finding prompted us to evaluate LHRH receptors in samples of surgically removed human RCC specimens derived from primary tumors or metastatic lesions.
Immunohistochemical analysis showed positive staining for LHRH receptors in all 25 tested specimens of primary RCC independently of histologic subtype and in all 12 samples of RCC metastases. These findings indicate that a very high percentage of primary RCC express receptors for LHRH and that there is no loss of LHRH receptor expression in metastatic disease. This suggests that treatment with targeted LHRH analogue AN-207 might be a promising therapy for advanced stages of RCC. In surrounding, nonmalignant tissue, LHRH receptor expression was only detected in cells of the renal tubular system. In line with our findings in human RCC specimens, specific, high-affinity LHRH receptors and the corresponding mRNA were also found in three human RCC cell lines, A-498, ACHN, and 786-0, by reverse transcription-PCR, Western blot analysis, and radioligand binding assays. Xenografts of these three RCC cell lines in nude mice were used to evaluate the efficacy and toxicity of AN-207 in vivo. These studies were designed to provide a rationale for a possible use of AN-207 in targeted chemotherapy of RCC.

In all three RCC models, targeted therapy with cytotoxic LHRH analogue AN-207 was highly efficacious and a complete arrest of tumor growth occurred in experiment 2 with ACHN RCC. To show that the growth-inhibiting effects of AN-207 are due to targeting to LHRH receptors, in experiment 3 we also included groups that received an unconjugated mixture of AN-201 and the carrier peptide, [D-Lys6] LHRH, or the carrier alone. Because a significant tumor inhibitory activity was observed only in the group injected with AN-207, the high efficacy of AN-207 cannot be attributed to any of its components but rather to the ability of [D-Lys6] LHRH to deliver AN-201 to malignant cells. The targeting concept is further supported by the fact that a blockade of LHRH receptors by Decapeptyl reduces the antitumor activity of the targeted cytotoxic analogue AN-207.

The binding characteristics and the concentration of LHRH receptors on tumor cell membranes were not changed by a single administration nor two consecutive injections of AN-207. This suggests that multiple dosing schedules of AN-207 are possible.

The toxicity of targeted cytotoxic LHRH analogue AN-207 and its cytotoxic radical AN-201 was compared with respect to systemic and local effects. The overall mortality in this study was very low with only one death after treatment with AN-207.

### Table 3. Systemic and local renal toxicity of cytotoxic LHRH analogue AN-207 and the cytotoxic radical AN-201 in mice bearing A-498, ACHN, and 786-0 human RCC tumors

<table>
<thead>
<tr>
<th>Variables of toxic side effects</th>
<th>AN-207</th>
<th>AN-201</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. deaths/treated animals</td>
<td>1/28</td>
<td>2/28</td>
</tr>
<tr>
<td>Decrease in WBC 8 days after first injection compared with controls</td>
<td>2.6-20.3%</td>
<td>39.7-69.6%</td>
</tr>
<tr>
<td>Decrease in WBC 8 days after second injection compared with controls</td>
<td>7.4-22.7%</td>
<td>48.3-64.8%</td>
</tr>
<tr>
<td>Decrease in WBC at the end of the experiments compared with controls</td>
<td>4.6-15.4%</td>
<td>44.6-50.1%</td>
</tr>
<tr>
<td>Loss of bodyweight on days 8 compared with controls</td>
<td>0.3-6.9%</td>
<td>1.9-11.6%</td>
</tr>
<tr>
<td>Microscopic examinations of mouse kidneys</td>
<td>Few apoptotic and necrotic cells</td>
<td>Few apoptotic and necrotic cells</td>
</tr>
<tr>
<td>Urine analyses</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>0.22 mg/dl</td>
<td>0.20 mg/dl*</td>
</tr>
</tbody>
</table>

*The same as control.

![Fig. 5. A, expression of mRNA for LHRH receptors in A-498, ACHN, and 786-0 human RCC xenografts as revealed by reverse transcription-PCR. Lane M, 100-bp ladder; lanes 1-3, 786-0; lanes 4-6, ACHN; lanes 7-9, A498; lane –, RNA negative control; lane +, positive control (DU-145 prostate cancer). B, expression of LHRH receptor protein in A-498, ACHN, and 786-0 human RCC xenografts as revealed by Western blot analysis. Lane M, molecular weight marker; lane 1, ES2 ovarian cancer (positive control); lanes 2-4, 786-0; lanes 5-7, A-498; lanes 8-10, ACHN. C, expression of mRNA for LHRH receptors in normal mouse kidneys as revealed by reverse transcription-PCR. Lane M, 100-bp ladder; lanes 1 and 2, normal mouse kidney; lane –, negative control; lane +, positive control. D, expression of LHRH receptor protein in normal mouse kidneys as revealed by Western blot analysis. Lane M, molecular weight marker; lane +, ES2 ovarian cancer (positive control); lanes 1 and 2, normal kidneys.](https://www.aacrjournals.org/content/11/15/5555)
and two after AN-201. In all treatment groups, except for the carrier group, a slight loss of body weight was observed 8 days after the therapy. However, a significant weight loss was observed only in experiment 1 in animals treated with AN-201. The myelotoxicity is usually the most serious side effect and the dose-limiting factor of chemotherapy. In the present study, AN-207 did not significantly lower the number of WBC was observed in all the experiments. This indicates that targeting of the cytotoxic radical AN-201 to LHRH receptors in the form of AN-207 can reduce its systemic toxicity. As normal kidneys were previously found to express low levels of mRNA (35) and 13 of 17 specimens in this study showed LHRH receptor expression in normal tubular cells, we also addressed the possibility of local toxicity by AN-207 in nonmalignant renal cells. Therefore, mouse kidneys were examined for LHRH receptor mRNA expression and nonmalignant renal tissue was histologically evaluated after therapy with AN-207 and AN-201. As in the case of human kidneys, some of the mouse kidneys were positive for LHRH receptors. Microscopic examination of kidneys after single or repeated injections of AN-207 and AN-201 showed only a few apoptotic and necrotic cells in both groups, indicating that the targeted therapy causes no specific toxicity to renal tissue. In addition, serum creatinine levels and urine analyses 8 days after the injection of AN-207 or AN-201 showed no differences compared with the control group, showing that renal function is not greatly affected by targeted therapy. A possible, specific toxicity of AN-207 to the LH-producing cells of the anterior pituitary has been investigated in previous studies in rats (36). The results of these experiments indicated that AN-207 causes no permanent damage to the pituitary function at its maximum tolerated dose. The low local toxicity of the cytotoxic radical AN-201 in AN-207 in LHRH receptor–positive nonmalignant tissue may be explained by the fact that AN-201 mainly kills rapidly proliferating cells.

The current study shows that LHRH receptors are expressed in a very high percentage (28 of 28) of human RCC specimens. In addition, our work shows that cytotoxic LHRH analogue AN-207 can be successfully targeted in vivo to human RCC, resulting in a high antitumor activity. Thus, a reduction of systemic side effects compared with the cytotoxic radical AN-201 can be achieved by targeting to tumoral LHRH receptors. In conclusion, our findings suggest that targeted therapy with cytotoxic LHRH analogues could be a promising new therapeutic modality for the treatment of advanced RCC.

Acknowledgments

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Table 4. Binding characteristics of LHRH receptors in A-498, ACHN, and 786-0 human RCC xenografted into nude mice

<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>A-498</td>
<td>Control</td>
<td>8.51 ± 0.38</td>
<td>439.8 ± 21.9</td>
</tr>
<tr>
<td>AN-207</td>
<td>AN-207</td>
<td>9.11 ± 0.83</td>
<td>398.4 ± 55.8</td>
</tr>
<tr>
<td>AN-201</td>
<td>AN-201</td>
<td>8.08 ± 0.77</td>
<td>414.2 ± 32.7</td>
</tr>
<tr>
<td>ACHN</td>
<td>Control</td>
<td>4.35 ± 0.33</td>
<td>654.2 ± 35.4</td>
</tr>
<tr>
<td>AN-207</td>
<td>AN-207</td>
<td>4.11 ± 0.74</td>
<td>599.1 ± 51.6</td>
</tr>
<tr>
<td>AN-201</td>
<td>AN-201</td>
<td>4.73 ± 0.58</td>
<td>670.2 ± 58.8</td>
</tr>
<tr>
<td>786-0</td>
<td>Control</td>
<td>10.6 ± 0.25</td>
<td>437.2 ± 39.9</td>
</tr>
<tr>
<td>AN-207</td>
<td>AN-207</td>
<td>11.4 ± 0.76</td>
<td>404.6 ± 47.1</td>
</tr>
<tr>
<td>AN-201</td>
<td>AN-201</td>
<td>9.93 ± 0.58</td>
<td>415.5 ± 28.3</td>
</tr>
</tbody>
</table>

NOTE: Binding characteristics were obtained from ligand competition assays (each done in duplicate or triplicate tubes) based on binding of radiolabeled [D-Trp$^6$]LHRH to tumor membrane homogenates. All values represent mean ± SE.

References

Receptors for Luteinizing Hormone Releasing Hormone Expressed on Human Renal Cell Carcinomas Can Be Used for Targeted Chemotherapy with Cytotoxic Luteinizing Hormone Releasing Hormone Analogues

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