Preclinical Evaluation of Targeted Cytotoxic Luteinizing Hormone-Releasing Hormone Analogue AN-152 in Androgen-Sensitive and Insensitive Prostate Cancers

Markus Letsch, Andrew V. Schally, Karoly Szepeshazi, Gabor Halmos, and Attila Nagy

Endocrine, Polypeptide, and Cancer Institute, Veterans Affairs Medical Center and Section of Experimental Medicine, Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana 70112 [M. L., A. V. S., K. S., G. H., A. N.]

ABSTRACT

Purpose and Experimental Design: To improve conventional chemotherapy, we developed cytotoxic analogues of luteinizing hormone-releasing hormone (LH-RH), which can be targeted to prostate cancers expressing LH-RH receptors. In view of pending clinical trials on cytotoxic LH-RH analogue AN-152, containing doxorubicin (DOX) linked to [D-Lys6]-LH-RH, we investigated the effects of AN-152 on tumor growth of s.c. implanted androgen-sensitive LNCaP and MDA-PCA-2b prostate cancers, as well as androgen-independent C4-2 prostate cancers xenografted into the tibiae of nude mice. In the C4-2 study, serum prostate-specific antigen (PSA) levels were also measured. LH-RH receptors were studied by reverse transcription-PCR and ligand competition assay. We also evaluated whether AN-152 can affect mRNA expression of human epidermal growth factor receptor and HER-2 and -3 oncogenes.

Results: After 32 days of treatment with AN-152, the growth of LNCaP cancers in castrated nude mice was strongly inhibited by 83% versus intact controls (P < 0.01) and 62% versus castrated controls (P < 0.05). In animals bearing MDA-PCA-2b prostate cancers, therapy with AN-152 for 25 days resulted in a 69% inhibition of tumor growth (P < 0.01 versus controls) and was more effective (P < 0.05) than equimolar doses of DOX or microcapsules of LH-RH agonist Decapeptyl. In nude mice bearing intraosseous C4-2 prostate cancers, treatment with AN-152 decreased serum PSA levels (P < 0.01) to 10.3 ± 3.4 ng/ml from 24.8 ± 4 ng/ml in controls, whereas DOX had no effect on PSA. The inhibitory effects of AN-152 on C4-2 tumors was accompanied by an increase in apoptosis and a decrease in tumor proliferation. Binding sites for LH-RH and the expression of mRNA for LH-RH receptors were found on s.c. C4-2 and MDA-PCA-2b tumors. The inhibition of MDA-PCA-2b tumors by AN-152 was associated with a significant decrease in mRNA expression for epidermal growth factor receptor, HER-2, and 3.

Conclusions: Our findings suggest that cytotoxic analogue AN-152 could be considered for therapeutic trials in patients with advanced prostate carcinoma.

INTRODUCTION

Despite advancements in methods for early detection, improved insights into the molecular mechanisms, and better treatment modalities, advanced prostate cancer is still responsible for 11% of all estimated male cancer deaths in the United States and therefore remains a major health problem for the aging man (1–4). Although curative treatment modalities are available for localized prostate cancer, only palliative therapies exist for patients with advanced disease (2–4). Our laboratory introduced LH-RH agonists as the primary treatment modality for advanced prostate cancer (3–6). However, surgical orchiectomy or androgen deprivation based on the use of LH-RH agonists cannot prevent an eventual relapse. Osteoblastic bone metastases, which occur in ~75% of patients with advanced disease, cause severe bone pain, pathological fractures, and nerve compression syndromes and impair the quality of life (7, 8). Early attempts to treat metastatic prostate cancer with systemic chemotherapy produced only marginal benefits (cited in Ref. 9). Recent multiple Phase II trials using a combination of agents, such as estramustine phosphate, Taxol, DOX, or suramin, showed clear improvements in palliative end points (9–11), such as pain relief and a decline in PSA. Although survival benefits have not yet been demonstrated in randomized trials, the increasing rates of response to chemotherapy are promising. However, continued research is necessary to improve the effectiveness and overcome the problems of toxicity of systemic chemotherapy (10–14).

A modern approach to improve conventional chemotherapy is the direct targeting of chemotherapeutic agents to cancer cells (15, 16). This local delivery to tumors should enhance the...
tumoricidal effect and reduce general toxicity (15, 16). High affinity binding sites for LH-RH are expressed in >80% of human prostatic adenocarcinoma specimens as well as in breast, endometrial, and ovarian cancers (15–21). This provided a rationale for the development of new anticancer agents, consisting of potent cytotoxic radicals linked to a LH-RH analogue carrier, which can be targeted to tumoral LH-RH receptors (3, 4, 15, 16). Diverse studies with cytotoxic analogues of LH-RH, carried out in various models of prostate, mammary, and ovarian cancers, clearly demonstrated that targeted chemotherapy with LH-RH analogues can inhibit growth and even cause a regression of these tumors (3, 4, 15, 22–30). One of the cytotoxic analogues, AN-152, synthesized in our laboratory, consists of [D-Lys6]-LH-RH linked to DOX, a DNA-intercalating antibiotic, which is widely used as an anticancer agent (15, 30). Although we have also developed another analogue containing 2-pyrrolino-DOX, which is much more potent in vivo, we selected AN-152 for initial clinical trials because of the extensive clinical experience with DOX and its liposomal or other targeted derivatives (16). In an early in vivo study, Miyazaki et al. (24) demonstrated that AN-152 given i.p. was more effective and less toxic than equimolar doses of DOX in LH-RH receptor-positive OV-1063 human ovarian cancers in nude mice. Subsequent studies in ovarian, endometrial, and mammary cancers confirmed the inhibitory action of AN-152 on tumor growth (25–27, 29–31) and showed that these effects are associated with the suppression of members of the EGFR type I tyrosine kinase family: EGFR, human EGFR (HER)-2 and -3, also known as ErbB-2 and -3 (24, 28, 29). These oncogenes are linked to tumor progression in various malignancies, including prostate cancer (32). However, the effects of AN-152 on prostate cancers have not yet been evaluated.

In this study, we tested the antitumor effects of AN-152 in androgen-sensitive LNCaP and MDA-PCa-2b prostate cancers xenografted s.c. into nude mice. We also evaluated AN-152 in a bone metastasis model of prostate cancer based on androgen-independent C4-2 tumors implanted into the tibiae of nude mice. Binding characteristics of LH-RH receptors and the expression of mRNA for LH-RH receptors in MDA-PCa-2b and C4-2 tumors were also estimated. In addition, we determined histological changes and the expression of mRNA for LH-RH receptors in MDA-PCa-2b and C4-2 tumors implanted into the tibiae of nude mice.

**Materials and Methods**

**Peptides and Cytotoxic Agents**

Cytotoxic LH-RH conjugate AN-152 was synthesized by coupling one molecule of DOX-14-0-hemiglutarate to the ε-amino group of the D-Lysine side chain of the carrier peptide [D-Lys6]-LH-RH and purified by high-performance liquid chromatography (30). DOX hydrochloride was obtained from Chemex Export-Import GmbH (Vienna, Austria). Before i.v. injection, the compounds were dissolved in 20 μl of 0.01 N acetic acid and diluted with 5.5% w/v aqueous D-mannitol solution (Sigma Chemical Co., St. Louis, MO). Microcapsules of the LH-RH agonist [D-Trp8]-LH-RH (Decapeptyl), dispersed in a polymeric matrix of poly(DL-lactide-co-glycolide; Ipsen Pharma Biotech, Toulon, France), were suspended in injection vehicle and 375 μg/0.2 ml doses, releasing 12.5 μg of Decapeptyl/day for 30 days were injected s.c. into each mouse (33).

**Animals**

Male athymic (Ncr nu/nu) nude mice ~6 weeks old were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD) and housed in laminar airflow cabinets under pathogen-free conditions with 12-h light/12-h dark schedule. They were fed autoclaved standard chow and water ad libitum. All experiments were performed in accordance with institutional guidelines of animal care.

**Cell Lines and Tumors**

Human androgen-sensitive LNCaP prostatic cancer cell line was obtained from American Type Culture Collection (Mannassas, VA). MDA-PCa-2b human androgen-sensitive prostate cancer cell line was obtained from Dr. N. Navone, the University of Texas, M.D. Anderson Cancer Center (Houston, TX). C4-2 human androgen-independent prostate cancer cell line was acquired from Urocor, Inc. (Oklahoma City, OK). The three cell lines were maintained in cultures as described (23, 34–38).

**In Vivo Studies**

**Experiment I.** s.c. xenografts of LNCaP cancers were induced as described (34, 38) when tumors had grown to a mean volume of ~180 mm³. The animals were randomly assigned to four groups containing 7 mice/group: (1) sham operation; (2) castration and vehicle solution; and group 3, castration and AN-152. AN-152 was injected through the jugular vein under isoflurane anesthesia (Abbott Laboratories, North Chicago, IL) on days 1 and 8 at doses of 103.5 nmol/20 grams BW and on days 14 and 21 at doses of 138 nmol/20 grams equivalent to 3 and 4 mg/kg DOX hydrochloride, respectively. Orchietomy or sham operation (control) was performed 1 week before the first injection, as described (38). Tumor volumes (length × width × height × 0.5236) and body weights were recorded weekly. On day 33, the mice were euthanized in all groups under isoflurane anesthesia.

**Experiment II.** MDA-PCa-2b androgen-sensitive tumors were initiated and implanted s.c. as described previously (23, 37, 38). Treatment was started when tumors reached an average size of ~80 mm³. Nude mice were randomly assigned to four groups containing 7 mice/group: (1) Control; (2) Decapeptyl; (3) DOX; and (4) AN-152. On Day 1, group 2 received a single s.c. injection of Decapeptyl microcapsules, which released ~12.5 μg/mouse/day for 30 days as described (33). At this time, the other groups received vehicle injection only. AN-152 and DOX were administered i.v. at a dose of 138 nmol/20 grams BW as described in experiment I on days 7, 11, 15, 20, and 25. This dosage corresponds to 4 mg/kg DOX hydrochloride. Total leukocyte count (WBC) was determined on day 15. The experiment was terminated after 29 days by euthanizing the animals. Liver, heart, lungs, kidneys, spleen, testicles, prostate, and seminal vesicles were carefully removed under a dissecting microscope and weighed. The tumors were excised, weighed, snap frozen, and stored at −70°C until further analyses.
Experiment III. To investigate the LH-RH receptor characteristics in s.c. C4-2 prostate cancers, tumors were initiated as described previously (35, 38). When the tumors had reached an average size of 200 mm³, the mice were sacrificed, and the tumors were excised, weighed, snap frozen, and stored at −70°C until further analyses.

Subsequently, intraosseous implantation of C4-2 cells in nude mice was performed as described by Wu et al. (36). Briefly, nude mice were anesthetized, and a midline incision was made on the left and right knee under a dissecting microscope. A TB Syringe with an attached 27-gauge needle was used to drill a hole in the tibial tuberosity, until the cortex of the tibial bone head was traversed. One million C4-2 cells diluted in 20 μl of PBS were slowly injected into the bone marrow cavity of the tibia. The needle was removed, and the wound was sutured. After 5 weeks, when the PSA had risen >5 ng/ml, the treatment was initiated. The mice were randomly divided into three groups and received weekly injections of cytotoxic LH-RH analogue AN-152, DOX, and vehicle solution as control. Cytotoxic LH-RH analogue AN-152 and DOX were administered i.v. according to the dosage regimen of experiment II on days 1, 7, 14, and 21. Blood samples (~50 μl) were collected at weekly intervals from the tail vein. Quantitative measurement of total PSA in serum was performed as described (34, 38). WBC count was determined on day 16 as described (22). The mice were euthanized on day 27. Necropsy was performed as described above. All intraosseous injected areas were removed, fixed in 10% formalin, and decalified in Cal Ex (Fisher Chemicals) solution before histological processing.

PSA Measurement

Quantitative measurement of total PSA in serum was performed using the reagents and protocol of DSL-9700 Active PSA Coated-Tube IRMA kit provided by Diagnostic Systems Laboratories (Webster, TX) as reported (34, 38).

LH-RH-Receptor Binding Studies

Receptors for LH-RH on MDA-PCa-2b control tumors in experiment II and from s.c. grown C4-2 tumors in experiment III were characterized by ligand competition assay. Preparation of tumor membrane fractions and receptor binding studies were performed as described (17).

mRNA Isolation and RT-PCR

The methods of isolation of mRNA and RT-PCR for the analysis of mRNA expression for LH-RH receptor, EGFR, HER-2 and-3, PSA, and β-actin were performed as described previously (29, 34, 39, 40).

Histological Procedure

Fixed tumor specimens were embedded in Paraplast (Oxford Labware, St. Louis, MO), and 6-μm-thick sections were cut and stained with H&E. The number of mitotic and apoptotic cells per 1000 cells was accepted as mitotic and apoptotic indices, respectively. For the demonstration of the NOR in tumor cells, the AgNOR method was used, and their number per cell was calculated as described (24, 25, 28).

Statistical Analysis

Data are expressed as mean ± SE. Differences between the values were evaluated with two-tailed Student’s t test and Tukey’s multiple comparison test, *P* < 0.05 being considered significant.

RESULTS

Inhibition of the Growth of s.c. Xenografts of Androgen-sensitive LNCaP Tumors in Castrated Nude Mice by Treatment with AN-152. In experiment I, we evaluated the antitumor activity resulting from four i.v. injections of targeted cytotoxic analogue AN-152 into castrated nude mice bearing s.c. xenografts of LNCaP human prostate cancers. The effects of AN-152 were compared with those produced by the vehicle solution in castrated controls and noncastrated controls as shown in Fig. 1A. After 32 days of treatment with AN-152, the volume of LNCaP tumors (406.1 ± 31.1 mm³) was significantly smaller than that of control tumors (2336.7 ± 482 mm³), corresponding to an 83% inhibition (*P* < 0.01; Fig. 1A). AN-152 also reduced tumor growth by 62% compared with tumors in castrated animals (1080.8 ± 263.8 mm³; *P* < 0.05). Tumor doubling time was similarly extended by treatment with AN-152 to 28.2 ± 4.7 days versus 11.2 ± 1.4 days for the control group (*P* < 0.01) and 16.2 ± 2.1 days (*P* < 0.05) for the castration group.

Effect of AN-152, DOX, and [D-Trp⁶]LH-RH (Decapeptyl) on the Growth of s.c. Implanted MDA-PCa-2b Tumors. In experiment II, we compared the effects of AN-152 given at 138 nmol/20 grams on days 7, 11, 15, 20, and 25 with those of equimolar doses of DOX, corresponding to 4 mg/kg and a depot injection of Decapeptyl microcapsules, which released 12.5 μg/mouse/day, on the growth of MDA-PCa-2b tumors in nude mice. As shown in Fig. 1B and Table 1, after 3 weeks of therapy, AN-152 significantly (*P* < 0.01) inhibited the growth of MDA-PCa-2b tumors by 69% compared with controls. Treatment with AN-152 also decreased significantly the tumor volume by 54 and 59% compared with an equimolar dose of DOX and microcapsules of LH-RH agonist Decapeptyl, respectively (both *P* < 0.05). Tumor doubling time in animals treated with AN-152 was extended by 85% to 12.4 ± 1.1 days, compared with 6.7 ± 0.4 days for the controls (*P* < 0.01) and was also extended by 49 and 47% compared with DOX and Decapeptyl, respectively (both *P* < 0.05). Therapy with AN-152 reduced tumor weight by 64% (*P* < 0.01) compared with controls (Table 1), this decrease being much greater than that obtained with DOX or Decapeptyl (*P* < 0.05). Decapeptyl had only a small inhibitory effect on tumor growth (NS versus control). DOX also inhibited tumor growth only nonsignificantly by 32%, extended slightly tumor doubling time to 8.3 ± 1.1 days (NS), and reduced tumor weight by 17% compared with controls (NS; Table 1; Fig. 1B). On day 15, the average WBC count in animals treated with DOX or AN-152 was not significantly different compared with the controls and the Decapeptyl group (Table 1). At the end of the observation period, body weights in the treatment groups did not differ significantly from the control group (Table 1). The necropsy revealed significant differences in the weights of the reproductive organs in nude mice after treatment with AN-152, DOX,
injections of AN-152 and DOX at doses of 138 nmol/kg in the intraosseous osteoblastic C4-2 prostate cancer model. Because tumor volume or weight could not be measured in this study, we used serum PSA as one of the parameters of tumor growth (Fig. 2). Serum PSA levels in the control group of intraosseous C4-2 tumors rose from an average of 7.4 ± 2 ng/ml to 24.8 ± 4 ng/ml by day 28. Treatment with AN-152 reduced final serum PSA levels to 10.3 ± 3.4 ng/ml, representing a 58% decrease compared with control values (P < 0.01; Fig. 2). Serum PSA levels after therapy with DOX (24.9 ± 4.2 ng/ml) were not different from controls (Fig. 2). There were no significant changes in the WBC count after 16 days of treatment with AN-152 or DOX (5912 ± 853.7 and 5362 ± 1092 leukocytes/mm³, respectively) compared with 5280 ± 1359 leukocytes/mm³ in the control group. At the end of the experiment, the BW in the treatment groups with AN-152 (26 ± 0.4 gram) and DOX (24.5 ± 1.3 grams) were not significantly different compared with controls (27.5 ± 1.4 grams). The necropsy revealed similar effects on the reproductive organs as in experiment II. AN-152 and DOX reduced significantly the weights of the testicles from 200 ± 10.4 mg in the controls to 57.5 ± 2.5 mg and 88 ± 1.1 mg, respectively (P < 0.01). The weights of all other measured organs did not differ from the control group.

**Histological Analysis.** Histological examination of MDA-PCa-2b tumors in experiment II showed highly cellular tumors consisting of undifferentiated cells with a minimal amount of stroma. Treatment with AN-152 and Decapetyl caused a significant (P < 0.05) reduction in the number of AgNORs but did not decrease mitotic and apoptotic indices (Table 2). AgNOR counts and mitotic and apoptotic indices remained unchanged after therapy with DOX.

In experiment III, growth of intraosseous C4-2 prostate tumors occurred in >80% of the tibiae of the nude mice. Representative tumors are shown in Fig. 3. Treatment with AN-152 produced a highly significant (P < 0.01) increase in apoptotic indices and a decrease in mitotic indices (Table 2). The cytotoxic LH-RH analogue caused also a significant reduction (P < 0.05) in the number of AgNORs per cell compared with control (Table 2). Equimolar doses of DOX caused no changes in morphological indices.

**LH-RH Receptor Studies in s.c. Implanted MDA-PCa-2b and C4-2 Prostate Cancers.** RT-PCR analyses demonstrated the presence of mRNA for LH-RH-receptors in representative samples of MDA-PCa-2b and C4-2 tumors (Fig. 4). PCR products of 319 bp for human LH-RH receptors were detected in all samples investigated (Fig. 4). Using ligand competition assays, a single class of high affinity binding sites for LH-RH was found in MDA-PCa-2b control tumors with a mean Kd value of 6.2 ± 0.7 nM and a mean Bmax value of 657.8 ± 24.6 fmol/mg membrane protein (Table 3). Treatment with DOX and Decapetyl did not significantly affect the affinity or capacity of receptors for LH-RH. However, AN-152 significantly (P < 0.01) decreased LH-RH receptor levels to 419.5 ± 9.3 fmol/mg membrane protein (Table 3). The s.c. grown C4-2 prostate cancers also expressed high affinity binding sites for LH-RH with a mean Kd value of 7.1 ± 1 nM and a mean Bmax value of 500.4 ± 4.7 fmol/mg membrane protein.
Effects of Treatment with AN-152 and DOX on the Expression of mRNA for EGFR, HER-2, and HER-3 in MDA-PCa-2b Tumors. In experiment II, we also evaluated whether treatment with AN-152, Decapetyl, or DOX had an effect on the expression of mRNA for EGFR, HER-2, and HER-3 in androgen-sensitive MDA-PCa-2b cancers grown in nude mice (Fig. 5; Table 3).

PCR products of 400, 420, and 371 bp, corresponding to the mRNA for EGFR, HER-2, and HER-3, were detected (Fig. 5). Amplification with primers for human β-actin yielded a single product of 459 bp. Densitometric analyses of the RT-PCR products indicated that treatment with AN-152 inhibited significantly the mRNA expression for EGFR, HER-2, and -3 (Fig. 5; Table 3) in comparison with control tumors. DOX administered at the same dose also caused a significant decrease in EGFR and HER-3 levels but did not significantly affect mRNA expression at the same dose also caused a significant decrease in EGFR and HER-3 levels. The rationale for using LH-RH for targeted chemotherapeutics is based on a concept by Paul Ehrlich, who postulated a century ago that antibodies could deliver toxic compounds to specific antigens on cancer cells (41). This targeting was conceived to eradicate mainly the tumors, thus sparing the normal tissue (41). In the search for cytotoxic hybrids with clinical potential, we developed a new class of targeted antitumor conjugates by linking LH-RH agonistic analogues of LH-RH, which have a high affinity for LH-RH receptors and their mRNA in breast, ovarian, endometrial, and prostatic carcinomas (4, 16, 30). The rationale for using LH-RH for targeted chemotherapy is based on the presence of specific high-affinity binding sites for this peptide hormone on various human tumors, including breast, ovarian, endometrial, and prostatic carcinomas (4, 15–17, 19–21). We found LH-RH receptors and their mRNA in 86% of clinical specimens of prostate cancer (17). The expression of mRNA for LH-RH receptors is significantly higher in

### Table 1: Effect of treatment with cytotoxic LH-RH analogue AN-152, DOX, or Decapetyl on the growth of MDA-PCa-2b human androgen-sensitive prostate cancer, bodyweights, and WBC counts in nude mice (Exp. II)

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Tumor volume (mm³)</th>
<th>Tumor doubling time (days)</th>
<th>Tumor weight (mg) (% inhibition)</th>
<th>Bodyweight (grams)</th>
<th>WBC (leukocytes/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final (% Inhibition)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>86.8 ± 26.2</td>
<td>1666.9 ± 292.9</td>
<td>6.7 ± 0.4</td>
<td>1551.5 ± 249.3</td>
<td>24.9 ± 0.9</td>
</tr>
<tr>
<td>Decapetyl</td>
<td>84.6 ± 9.9</td>
<td>1278.9 ± 335.4 (23%)</td>
<td>8.4 ± 1.2</td>
<td>1497.3 ± 371.1 (3%)</td>
<td>24.8 ± 0.8</td>
</tr>
<tr>
<td>DOX</td>
<td>84.4 ± 17.9</td>
<td>1134.8 ± 214.1 (32%)</td>
<td>8.3 ± 1.1</td>
<td>1291.8 ± 243.6 (17%)</td>
<td>23.3 ± 1.6</td>
</tr>
<tr>
<td>AN-152</td>
<td>84.2 ± 19.4</td>
<td>520.1 ± 65.1 (69%)³</td>
<td>12.4 ± 1.1³</td>
<td>564.4 ± 88.2 (64%)³</td>
<td>24.2 ± 1.1</td>
</tr>
</tbody>
</table>

¹ P < 0.01 vs. control.
² P < 0.05 vs. Decapetyl and DOX.
³ P < 0.01 vs. control.

### Table 2: The effect of treatment with cytotoxic LH-RH analogue AN-152 and DOX on histologic characteristics of s.c. implanted MDA-PCa-2b tumors (experiment II) and of intraosseously implanted C4-2 tumors (experiment III) in nude mice and on the weights of reproductive organs

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Mitotic index</th>
<th>Apoptotic index</th>
<th>Ratio of apoptotic to mitotic indices</th>
<th>No. of AgNORs per cell</th>
<th>Testicles (mg)</th>
<th>Seminal vesicles (mg)</th>
<th>Prostate (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment II</strong> (s.c. MDA-PCa-2b tumors)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1. Control</td>
<td>72 ± 0.8</td>
<td>3.7 ± 0.5</td>
<td>0.55 ± 0.07</td>
<td>7.0 ± 0.1</td>
<td>118.0 ± 1.0</td>
<td>108.1 ± 15.8</td>
<td>65.0 ± 5.3</td>
</tr>
<tr>
<td>2. Decapetyl</td>
<td>4.7 ± 0.4</td>
<td>3.7 ± 0.5</td>
<td>0.86 ± 0.17</td>
<td>6.3 ± 0.2¹</td>
<td>141.4 ± 7.0b</td>
<td>64.3 ± 6.5b</td>
<td>50.0 ± 2.1b</td>
</tr>
<tr>
<td>3. DOX</td>
<td>5.9 ± 1.1</td>
<td>2.8 ± 0.4</td>
<td>0.60 ± 0.19</td>
<td>6.4 ± 0.2</td>
<td>71.4 ± 5.9b</td>
<td>80.0 ± 18.1</td>
<td>46.6 ± 8.9</td>
</tr>
<tr>
<td>4. AN-152</td>
<td>6.1 ± 0.3</td>
<td>2.1 ± 0.2</td>
<td>0.35 ± 0.05</td>
<td>6.3 ± 0.2²</td>
<td>84.0 ± 5.0b</td>
<td>86.0 ± 8.7</td>
<td>48.0 ± 3.7</td>
</tr>
<tr>
<td><strong>Experiment III</strong> (intraosseous C4-2 tumors)</td>
<td></td>
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</tr>
<tr>
<td>1. Control</td>
<td>22.6 ± 6.0</td>
<td>2.7 ± 0.9</td>
<td>0.19 ± 0.11</td>
<td>8.1 ± 0.1</td>
<td>200.0 ± 12.4</td>
<td>155.0 ± 31.2</td>
<td>111.8 ± 3.7</td>
</tr>
<tr>
<td>2. DOX</td>
<td>18.7 ± 3.5</td>
<td>2.6 ± 0.5</td>
<td>0.19 ± 0.06</td>
<td>7.5 ± 0.2</td>
<td>88.0 ± 11.1b</td>
<td>130.2 ± 7.4</td>
<td>98.0 ± 19.3</td>
</tr>
<tr>
<td>3. AN-152</td>
<td>6.4 ± 2.3</td>
<td>6.8 ± 0.9b</td>
<td>1.91 ± 0.70b</td>
<td>6.5 ± 0.2²</td>
<td>57.5 ± 2.5b</td>
<td>160.0 ± 15.3</td>
<td>97.5 ± 13.1</td>
</tr>
</tbody>
</table>

¹ P < 0.05 vs. control.
² P < 0.01 vs. control.

**DISCUSSION**

The principle of targeted chemotherapy is based on the concept of Paul Ehrlich, who postulated >100 years ago that antibodies could deliver toxic compounds to specific antigens on cancer cells (41). This targeting was conceived to eradicate mainly the tumors, thus sparing the normal tissue (41). In the search for cytotoxic hybrids with clinical potential, we developed a new class of targeted antitumor conjugates by linking agonistic analogues of LH-RH, which have a high affinity for LH-RH receptors, to a variety of chemotherapeutic agents (15, 16, 30). The rationale for using LH-RH for targeted chemotherapy is based on the presence of specific high-affinity binding sites for this peptide hormone on various human tumors, including breast, ovarian, endometrial, and prostatic carcinomas (4, 15–17, 19–21). We found LH-RH receptors and their mRNA in 86% of clinical specimens of prostate cancer (17). The expression of mRNA for LH-RH receptors is significantly higher in
prostate carcinoma (21). Thus, we developed cytotoxic analogue hormone-refractory disease compared with hormone-dependent prostate cancer, in the present study, we investigated if treatment with AN-152 would be effective in inhibiting the growth of human androgen-sensitive prostate carcinoma and its osteoblastic bone metastases in nude mice.

To estimate the antiproliferative potential of AN-152 in advanced prostate carcinoma, we chose the human androgen-sensitive prostate cancer cell lines LNCaP and MDA-PCa-2b. These two models of prostate cancer progression show common features of advanced clinical prostate cancer, such as PSA secretion and the missense mutation T877A in the androgen receptor, which renders these cells responsive to progesterone and corticosteroids (37, 42).

Because the LNCaP cell line is well established as an LH-RH receptor-positive model of prostate cancer and has retained androgen sensitivity (34, 36, 43), in a pilot study, we first evaluated in this cell line the effect of AN-152 in castrated animals and compared it with that in castrated mice and noncastrated controls injected with vehicle. Mice, which received treatment with AN-152, were castrated, to mimic the conditions existing in a clinical setting, because androgen ablation is the main palliative treatment modality for men with advanced prostate cancer (3, 4). Although LNCaP tumors in castrated animals eventually escaped androgen deprivation, castration combined with AN-152 had a strong and lasting growth inhibition throughout the study.

In a second, more comprehensive experiment, we tested the antiproliferative activity of AN-152 on another human androgen-sensitive prostate cancer model, MDA-PCa-2b, which was derived from a bone metastasis of a patient with advanced prostate cancer. In this study, we also included DOX and microcapsules of LH-RH-agonist Decapeptyl, representing the preferred method of androgen deprivation in the primary treatment of advanced prostate cancer (3, 4). The presence of LH-RH receptors was revealed by both RT-PCR and ligand binding assays in untreated MDA-PCa-2b tumors. AN-152 strongly reduced tumor growth and increased tumor doubling time compared with untreated controls, DOX, and Decapeptyl. Decapeptyl at 12.5 μg/day decreased tumor growth by only 23% in this model, which was not significant statistically, although there were significant reductions in weights of testes, seminal vesicles, and prostate. LH-RH agonists were not investigated previously in the MDA-PCa-2b model, and it is possible that for an effective inhibition of tumor growth, a release of 25 μg/day may be necessary. The absence of a marked down-regulation of tumoral LH-RH receptors (18) in the group treated with Decapeptyl supports this view. It should be noted that after 11 days of therapy with Decapeptyl, a 40% increase in tumor growth was observed, which could be interpreted as an initial stimulation ("flare-up") of the hormone-sensitive MDA-PCa-2b tumors. If AN-152 had a similar effect on these tumors, it could
Table 3 The effect of treatment with cytotoxic LH-RH analogue AN-152 and DOX on mRNA expression of EGFR, HER-2 and -3, and the binding characteristics of LH-RH-receptors on s.c. implanted MDA-PCa-2b tumors in nude mice

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>EGFR % of control</th>
<th>HER-2</th>
<th>HER-3</th>
<th>LH-RH-receptor-binding characteristicsb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>100 ± 5.8</td>
<td>100 ± 2.2</td>
<td>100 ± 3.4</td>
<td>Kd (nm) 6.2 ± 0.7, Bmax (fmol/mg protein) 657.8 ± 24.6</td>
</tr>
<tr>
<td>2. Decapeptyl</td>
<td>65.8 ± 2.5a</td>
<td>86.4 ± 6.5</td>
<td>87.2 ± 6.6</td>
<td>Kd (nm) 5.9 ± 0.4, Bmax (fmol/mg protein) 565.9 ± 41.6</td>
</tr>
<tr>
<td>3. DOX</td>
<td>85.6 ± 5.0b</td>
<td>86.3 ± 5.1</td>
<td>61.4 ± 4.5c</td>
<td>Kd (nm) 6.5 ± 0.4, Bmax (fmol/mg protein) 647.1 ± 13.5</td>
</tr>
<tr>
<td>4. AN-152</td>
<td>74.3 ± 2.3d</td>
<td>81.9 ± 3.5e</td>
<td>65.8 ± 10.3f</td>
<td>Kd (nm) 5.1 ± 0.6, Bmax (fmol/mg protein) 419.5 ± 9.3g</td>
</tr>
</tbody>
</table>

a Binding characteristics were obtained from 12-point displacement experiments. All values represent mean ± SE of three independent experiments, each done in duplicate or triplicate.
b P < 0.01 vs. control.

Fig. 5 The effect of AN-152, DOX, and Decapeptyl on the expression of mRNA for EGFR (400 bp), HER-2 (420 bp), and HER-3 (371 bp) in s.c. grown MDA-PCa-2b human androgen-sensitive prostate cancer in nude mice. mRNA for β-actin (459 bp) was used as an internal control. Lane M, the 100-bp molecular DNA marker.

have sensitized them to DOX, which, being a DNA intercalator, is more effective on more rapidly proliferating cancers. Although this mechanism of action cannot be ruled out, it must be pointed out that the initial increase in tumor growth during Decapeptyl therapy was not statistically significant. Even in the group treated with DOX, there was an initial enhancement of tumor growth, but again, it was not statistically significant compared with controls. In addition, in previous studies with targeted cytotoxic LH-RH analogues containing DOX or 2-pyrrolino-DOX, there was no improvement in the efficacy of the cytotoxic agent in mice receiving an unconjugated mixture of the cytotoxic radical and the carrier peptide, and often an even higher toxicity was observed (15, 16, 22, 24, 25, 28). AN-152 did not change the WBC count 7 days after the second of five injections, indicating that the low dose of 4 mg/kg does not affect the leukocytes. However, both DOX and AN-152 significantly reduced the weights of testes, which suggests that this effect is independent of targeting and may be attributable to partial hydrolysis of DOX by carboxylesterase enzymes (EC.3.1.1.1) in the circulation (16). The body weights were not affected by the therapies.

We then investigated whether AN-152 could exert antiproliferative effects in intraosseous xenografts of human androgen-independent C4-2 prostate cancers in nude mice. The LNCaP-derived C4-2 subline mimics clinically advanced prostate cancer by secreting PSA and its capability to produce spontaneous bone metastases (35, 36). Moreover, when injected intraosseously, C4-2 tumors also produce osteoblastic lesions, in contrast to the PC-3 human prostate carcinoma metastatic model, which generates exclusively osteolytic lesions without reliable means to evaluate the tumor progression by PSA measurement (35, 36, 44). We demonstrated the presence of high affinity receptors for LH-RH on C4-2 tumors by binding assays, which is a prerequisite for targeted chemotherapy with AN-152. The mRNA expression for LH-RH receptors was also shown. The effect of AN-152 and its cytotoxic radical DOX in the tibial prostate cancer xenografts was monitored by PSA measurement, which is a key clinical parameter in the evaluation of therapeutic responses in advanced prostate carcinoma (45). After 4 weeks of treatment, serum PSA levels in groups treated with AN-152 were significantly inhibited, compared with controls. DOX did not change serum PSA levels compared with the control group. The tumor growth inhibition by AN-152 was also demonstrated by histology, which showed a major decrease in mitosis and an increase in apoptosis (Fig. 3) and in the ratio of apoptotic to mitotic indices in C4-2 tumors. However, no such effects were observed on MDA-PCa-2b cancers, possibly because AN-152 may have different mechanisms of action on different types of tumors. This could be explained by different expression of various proteins, including p53 and Bcl-2, by the two tumor types, which we did not investigate in this study. In contrast, AN-152 significantly decreased the number of AgNORs per cell in both MDA-PCa-2b and C4-2 cancers. No changes could be observed in WBC count after three of four injections of AN-152 or DOX in experiment 3, indicating that these low doses do not have severe myelosuppressive side effects. The body weights were also not affected by the therapy.

Much evidence exists that the inhibitory effects of cytotoxic LH-RH analogues, such as AN-152, are mediated by the LH-RH receptors (15, 16, 24, 27, 29, 31, 46). Optical tracking methods in breast and ovarian cancers revealed that cytotoxic LH-RH analogue AN-152 exerts its effects through receptor-mediated endocytosis and a subsequent accumulation of the drug in the nucleus (26, 46). There are divergent results on the effects of treatment with AN-152 on the expression of LH-RH receptor. In an early study on OV-1063 ovarian cancers, no LH-RH receptors could be detected after therapy with AN-152 or DOX in experiment 3, indicating that these low doses do not have severe myelosuppressive side effects. The body weights were also not affected by the therapy.

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cantly, whereas DOX and Decapeptyl did not change the LH-RH receptor binding characteristics compared with control. However, despite the reduced binding capacity of LH-RH receptors on MDA-PCa-2b cancers after treatment with AN-152, the LH-RH receptor concentration characterized by a $B_{\text{max}}$ value of 419.5 ± 9.3 fmol/mg protein should still be adequate for further therapy with this cytotoxic LH-RH analogue, as shown in a previous study (31).

The activation of the LH-RH receptor has also been linked to effects on the EGFR family (24, 26, 27, 29), and this could constitute another mechanism, by which AN-152 may inhibit tumor growth. EGFR (HER-1) belongs to the type-1 family of receptor tyrosine kinases, which are important mediators of cell growth, differentiation, and survival and which are also associated with the invasiveness in prostate cancer (reviewed in Ref. 32). The EGFR family also comprises HER-2 and HER-3. Unlike the other members of the EGFR family, HER-2 has an intrinsic tyrosine kinase activity that activates signal transduction in the absence of ligand and thus can activate signaling through dimerization with other HER family members. Existing evidence suggests that an overexpression of HER-2 plays an important role in the progression of advanced prostate cancer (32, 47–49). The overexpression of HER-2 and HER-3 in experimental prostatic epithelial cells produces a phenotype with an increased rate of proliferation and the capacity for metastasis (49). Because previous work showed that therapy with cytotoxic LH-RH analogue AN-152 down-regulates the expression of EGFR and HER-2 (24, 29), in this study, we evaluated the treatment-related changes in the expression of the EGFR family. We found that therapy with AN-152 significantly decreased the expression of EGFR, HER-2, and HER-3 in MDA-PCa-2b tumors, which is in accordance with previous results in ES-2 human ovarian cancers (29). DOX decreased mRNA levels for EGFR and HER-3 but not the mRNA expression for HER-2. In contrast, treatment with Decapeptyl affected only mRNA expression for HER-2. Thus, AN-152 may affect growth-stimulatory signaling associated with the EGFR family, which could be an additional mechanism for growth inhibition by LH-RH analogues containing DOX moiety.

The experiments reported herein reveal for the first time the antitumor effects of cytotoxic LH-RH analogue AN-152 in androgen-sensitive prostate cancers. Our work supports the merit of forthcoming clinical Phase I trials with AN-152 in patients with advanced prostate carcinoma who have relapsed after androgen deprivation or other therapies. Because of the presence of receptors for LH-RH on a high percentage of prostate cancers, targeted chemotherapy based on AN-152 should be more efficacious and less toxic than the currently used systemic chemotherapeutic regimens and might permit an escalation of doses. In addition, it is possible that cytotoxic analogues of LH-RH might eventually be used also for primary therapy of patients with advanced prostate cancer.

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Preclinical Evaluation of Targeted Cytotoxic Luteinizing Hormone-Releasing Hormone Analogue AN-152 in Androgen-Sensitive and Insensitive Prostate Cancers

Markus Letsch, Andrew V. Schally, Karoly Szepeshazi, et al.


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