Targeted Doxorubicin-containing Luteinizing Hormone-releasing Hormone Analogue AN-152 Inhibits the Growth of Doxorubicin-resistant MX-1 Human Breast Cancers

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ABSTRACT

Purpose: The receptors for luteinizing hormone-releasing hormone receptor (LHRH-R) are found in >50% of human breast cancers. Doxorubicin (DOX) was linked to [D-Lys6]LHRH to form a cytotoxic conjugate, AN-152, which can be targeted to tumor cells expressing LHRH-R.

We evaluated the effects of AN-152 on the estrogen-independent, DOX-resistant human mammary carcinoma line MX-1, xenografted into nude mice.

Experimental Design: Nude mice bearing MX-1 tumors were administered five i.v. injections of AN-152 or DOX at doses equivalent to 3 mg/kg DOX. Tumor growth was followed, and changes in the expression of LHRH-R on tumors were evaluated by radioreceptor assays, reverse transcription-PCR, and Western blotting. The effects of AN-152 on the expression of human epidermal growth factor receptor (HER)-2 were investigated. Because LHRH-R is coupled to various G proteins, which are involved in mitogenic signaling, we determined the outcome of treatment with AN-152 on the levels of mRNA for different G proteins.

Results: Treatment with AN-152 significantly (P < 0.05) decreased the final tumor volume to 978.56 ± 176.85 mm3, compared with the control tumors, which measured 2837.38 ± 515.38 mm3. Tumor doubling time was likewise significantly (P < 0.05) extended by AN-152 to 12.01 ± 1.99 days from 6.45 ± 0.36 days for the controls. Therapy with AN-152, but not with DOX, resulted in a significant decrease of LHRH-R levels on MX-1 tumors. The expression of mRNAs for HER-2, HER-3, Gαq,i2, and Gαq,i1, and the levels of HER-2 and HER-3 proteins were also significantly reduced by AN-152.

Conclusions: Cytotoxic LHRH analogue AN-152 could be considered for targeted chemotherapy of DOX-resistant breast cancers expressing LHRH-R.

INTRODUCTION

Breast cancer is the most common malignancy in women and the second leading cause of cancer-related deaths (1). Nearly two-thirds of all breast cancers are estrogen independent, and the main treatment modality for such cases is cytoreductive surgery followed by adjuvant chemotherapy and/or radiotherapy. However, in many cases, the intrinsic or acquired resistance of the cancer cells to chemotherapy would require the use of elevated doses, which are not feasible because of the toxic side effects. The overexpression of HER-2, a member of the EGF ErbB/HER type I tyrosine kinase receptor family, has been associated with resistance to chemotherapy in breast cancers (2). The overexpression of HER-2, which is found in 20–30% of breast cancers, is also associated with poor prognosis (3). A modern approach to reduce the toxicity and improve the efficacy of chemotherapy is based on the targeting of chemotherapeutic agents to peptide hormone receptors, such as the LHRH-R, which is found on >50% of breast carcinomas (4–6). In recent years, we developed a series of cytotoxic LHRH conjugates, including AN-152, which consists of LHRH agonist carrier [D-Lys6]LHRH linked through a glutaric acid spacer to one molecule of DOX (7). We showed a high affinity binding of AN-152 to LHRH-R on human breast cancer cells and specimens and demonstrated its LHRH-R-mediated internalization by MCF-7 human estrogen-dependent breast cancer cells (8, 9).

The LHRH-R is a G-protein-coupled receptor, which after ligand binding, relies primarily on the G proteins for downstream signaling. The activation of the receptor by an LHRH agonist leads to the dissociation of the heterotrimeric GTP-binding G proteins into their i1,i2,q, and i11 subunits, both of which can activate or inhibit various downstream effector molecules. Ten of the 17 α-subunits, which have been cloned to date, including Gα1, Gα11, Gα22, Gαq,i1, and Gαq,i11, were shown to be involved in the regulation of cell growth (10). The mechanisms, by which multiple G proteins interact with the LHRH-R, are still unknown. However, the second and third intracellular loops of the receptor appear to be involved in signal transduction, suggesting that multiple sites on the receptor may interact with G proteins (11). It is also known that the ability of the LHRH-R to

1 The abbreviations used are: LHRH, luteinizing hormone-releasing hormone; LHRH-R, luteinizing hormone-releasing hormone receptor; DOX, doxorubicin; RT-PCR, reverse transcription-PCR; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; HER, human epidermal growth factor receptor.
couple to multiple G proteins is not specific to the gonadotroph, because it was also observed in another cell type expressing LHRH-R (11). The ability of the LHRH-R to couple to multiple G proteins enables it to activate multiple signal transduction pathways.

It has been suggested recently that there is a “cross-talk” between G-protein-coupled receptors, such as the LHRH-R, and other signaling pathways (12, 13). Thus, it was shown that ligand-induced activation of LHRH-R can result in the activation of the ErbB/HER receptor family without the involvement of EGF-like ligands (13). Although the ErbB/HER family of receptors can form homodimers, the preferred dimerization partner for all of these receptors is HER-2, and the most commonly found heterodimer is the HER-2/HER-3. Thus, HER-2 provides an active tyrosine kinase for the catalytically impaired HER-3 to produce a heterodimer with a higher signaling potency than those not containing HER-2 (14).

In this study, we evaluated whether targeting DOX to LHRH-R on the estrogen-independent, DOX-resistant MX-1 human breast tumors xenografted into nude mice can overcome the chemoresistance of these cancers. The effects of the treatment on the expression of LHRH-R, various G-proteins, and members of the ErbB/HER family were also determined.

MATERIALS AND METHODS

Chemicals. Cytotoxic LHRH conjugate AN-152 was synthesized in our laboratory by coupling one molecule of DOX-14-O-hemiglutarate to the e-amino group of the D-Lys side chain of the carrier peptide [D-Lys6]LHRH and purified by high-performance liquid chromatography as described (7). DOX hydrochloride was obtained from Chemex Export-Import GmbH (Vienna, Austria). The compounds were dissolved in 5% (w/v) aqueous D-mannitol (Sigma, St. Louis, MO) solution.

Animals and Tumors. Six-week-old female athymic NCR/c (nu/nu) nude mice were purchased from the National Cancer Institute (Frederick Cancer and Development Center, Frederick, MD) and housed in a laminar air-flow cabinet under pathogen-free conditions with a 12-h light/dark schedule. Hormone-independent, DOX-resistant human mammary carcinoma xenograft MX-1, originating from a surgical explant, was obtained from the National Cancer Institute. Mice maintained in donor animals were aseptically dissected and mechanically minced, and 1-mm3 tumor pieces were transplanted s.c. by trocar needle into the right flank of the experimental animals. When the tumors measured ~160 mm3, the mice were assigned to experimental groups. All studies were conducted in accordance with institutional guidelines of animal care and the welfare of animals in experimental neoplasia.

Experimental Protocol. Mice with MX-1 tumors were divided into three groups of 10 animals each, which received the following treatments as five i.v. injections on days 1, 5, 9, 13, and 21: (a) group 1, vehicle solution (5% mannitol, control); (b) group 2, AN-152 at doses of 103.5 nmol/20 grams, which is equivalent to 3 mg/kg DOX hydrochloride; and (c) group 3, DOX at doses of 103.5 nmol/20 grams (3 mg/kg).

All injections were administered through the jugular vein under methoxyflurane (Metofane; Pittman-Moore, Mundelein, IL) anesthesia. Tumors were measured once or twice a week with microcalipers, and tumor volume was calculated using the following formula: length × width × height × 0.5236 (15). The measurement of tumor growth was continued for 4 weeks. Body weights of the animals were measured just before the injections and on the day the experiment was terminated. Five days after treating nude mice with the last injection of cytotoxic compound or the vehicle, blood samples were collected from the tail vein using Unopette microcollection kit (Becton-Dickinson, Franklin Lakes, NJ). WBC count was determined manually using a hemacytometer. The treatment-related toxicity was evaluated based on differences in mortality, body weights, and WBC count between treatment groups and controls. The experiment was terminated 28 days after the first injection. The mice were sacrificed under light methoxyflurane anesthesia according to institutional standards. Trunk blood was collected and centrifuged at 1000 × g for 30 min at 4°C, and serum was stored at −20°C until assayed. Tumors were dissected and snap frozen for receptor studies and molecular biology analyses. The liver, kidneys, heart, uterus, and ovaries were carefully removed, cleaned, and weighed. Tumor doubling time was calculated between the start of the treatment and end of the experiment. Tumor burden was calculated as tumor weight (milligrams)/body weight (grams).

Determination of Serum LH Levels. Blood samples taken from mice at the end of the experiment were tested for serum murine LH levels by RIA, using mouse LH-RP (AFP-5366A), rat LH-I-9/AFP-10250C, and antirat LH RIA S-11/AFP C697071P. All RIA materials were provided by Dr. A F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program (Torrance, CA).

RNA Extraction and RT-PCR. Total RNA was extracted from frozen tissue samples and cells by RNAzolB (Test, Friendswood, TX) according to the manufacturer’s instructions. Three µg of total RNA were reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase according to the manufacturer’s instructions (Perkin-Elmer Corp., Norwalk, CT). For the amplification of the cDNA transcripts, gene-specific primers for human β-actin, EGF-R, HER-2 and -3, LHRH-R, Gαs, Gα11, Gα12, Gαq, and Gα11 were used as described in detail (16–22). The number of cycles was determined in preliminary experiments to be within the exponential range of PCR amplification. Negative controls were run in parallel to exclude genomic DNA contamination. PCR products were subjected to electrophoresis on a 2% agarose gel, then stained with ethidium bromide and visualized under UV light. Bands of PCR products were then analyzed semiquantitatively by using a zoom digital camera (DC 290) with EDAS 290 imaging system (Kodak, Rochester, NY). All experiments were repeated at least twice, and mRNA levels of each gene were normalized versus the corresponding levels of β-actin.

LHRH-R Receptor Binding Studies. Receptors for LHRH on MX-1 tumors from the experimental groups were characterized by the ligand competition assay. Preparation of tumor membrane fractions and receptor binding studies of LHRH were performed as described (8). The LIGAND-PC computerized curve-fitting program of Munson and Rodbard (23) was used to determine the type of receptor binding, dissociation constant (Kd), and maximal binding capacity of the
receptors (B\textsubscript{max}). Receptor binding affinity of cytotoxic LHRH analogue AN-152 to tumor membranes from animals that received AN-152 treatment was measured in displacement experiments based on competitive inhibition of \textsuperscript{125}I-[D-Trp\textsuperscript{6}] LHRH binding using various concentrations of AN-152 (10\textsuperscript{-6}–10\textsuperscript{-12} M; Ref. 8). IC\textsubscript{50}\textsuperscript{8} were calculated with a computerized curve fitting program and is defined as a dose of AN-152 causing 50% inhibition of \textsuperscript{125}I-[D-Trp\textsuperscript{6}]LHRH binding (23).

**Preparation of Samples for Immunodetection Studies.**

Tumor membranes for the immunodetection of HER family members were prepared as follows: tumor tissue was homogenized using homogenization buffer [50 mm Tris-HCl (pH 7.6), 0.25% sucrose, 0.5 mm EDTA, 0.1 mm phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 0.5 mg/ml bacitracin]. The homogenate was centrifuged at 500 × g for 5 min at 4°C. The supernatant was centrifuged at 25,000 × g for 60 min at 4°C. The membranes were diluted in washing buffer (homogenization buffer without sucrose) and then frozen at ~70°C until use. For immunodetection of LHRH-R, the tumors were homogenized in ice cold extraction buffer [50 mm Tris-HCl (pH 7.6), containing 1% Triton X-100, 200 mm NaCl, and 10 mm CaCl\textsubscript{2}]. Chilled samples were stirred for 30 min, and the supernatant was centrifuged at 12,000 × g, for 20 min, and then frozen at ~70°C until use. The protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) using BSA as a standard.

**Immunodetection of HER Family Members and LHRH-R.** Proteins (10–30 μg) were solubilized in 20 mm Tris-HCl buffer (pH 8.0) containing 10% (v/v) glycerol, 1% (w/v) SDS, 1 mm EDTA, and 1 mm DTT and heated for 15 min. Proteins were resolved on a 6% (for EGF-R and HER-2 and -3) or 10% (for actin and LHRH-R) SDS-PAGE and then transferred to nitrocellulose sheets (Hoefer, San Francisco, CA). The nitrocellulose sheets were soaked in Tris-buffered saline [10 mm Tris-HCl (pH 7.5) and 50 mm NaCl] containing 0.1% Tween 20 (TBST buffer). Excess protein-binding sites were saturated with TBST buffer containing 5% nonfat dried milk. The blotted membranes were incubated for 1 h at room temperature with rabbit polyclonal antihuman EGF-R, HER-2 and HER-3, goat polyclonal antihuman LHRH-R, and antia cetin antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA) at dilutions of 1:1000–1:3000 in TBST buffer. The immunoreactive proteins were developed with peroxidase-conjugated antirabbit and antigoat IgG antibodies (Santa Cruz Biotechnology) and visualized by a chemiluminescence detection system (Pierce, Rockford, IL). The bands were analyzed with the imaging densitometer as specified above, and the relative protein levels were normalized versus the corresponding levels of actin.

**Statistical Methods.** Data are expressed as mean ± SE, and statistical analyses of the tumor data were performed using Student’s t test. All Ps are based on two-sided hypothesis testing, P < 0.05 being considered significant.

**RESULTS**

**Antitumor Effects and Toxicity.** Fourteen days after the initiation of the therapy, after the mice had already received four injections of AN-152 at doses equivalent to 3 mg/kg DOX, the volume of the MX-1 breast cancers was significantly (P < 0.05) reduced to 357.74 ± 72.92 mm\textsuperscript{3}, compared with the control group, in which the tumors measured 848.6 ± 112.9 mm\textsuperscript{3}. This represented a 57.8% decrease in tumor volume. On day 21, the mice were given another injection of AN-152 at the same dose. On day 28, when the experiment was terminated, the tumor volume in the AN-152 group was significantly (P < 0.05) reduced to 978.56 ± 176.85 mm\textsuperscript{3}, whereas the control tumors measured 2837.38 ± 515.38 mm\textsuperscript{3}. This corresponded to a 68.9% growth inhibition (Fig. 1; Table 1). Tumor doubling time in mice receiving AN-152 was significantly (P < 0.05) extended to 12.01 ± 1.99 days from 6.45 ± 0.36 days in the control group (Table 1). The tumor burden was also significantly (P < 0.05) decreased by AN-152 to 50.78 ± 11.51 mg/gram body weight compared with 113.4 ± 24.2 mg/gram body weight in the control animals (Table 1); DOX administered according to the same dose regimen had no effect on any of the tumor growth parameters (Table 1). None of the animals died during the therapy, and there was no significant difference in body weights in the treated groups compared with the controls (Table 1). Five days after the nude mice received the last injection, their WBC counts were performed. No significant differences were found in the WBC counts between the treated groups and controls, but the actual WBC values were ~20% lower in the group treated with DOX compared with the other two groups (Table 1). Routine examination of the organs at autopsy revealed that the animals treated with DOX or AN-152 showed a significant (P < 0.01) decrease in ovarian weights, the ovaries weighing 3.56 ± 0.56 and 3.6 ± 0.7 mg, respectively. In the control group, the ovaries weighed 7 ± 0.43 mg. The examination of liver, lungs, kidneys, heart, and uterus showed no significant changes.

**Fig. 1** Tumor volume in athymic nude mice bearing s.c. transplanted xenografts of MX-1, hormone-independent DOX-resistant human breast carcinoma after five i.v. injections of DOX or the cytotoxic LHRH analogue AN-152 at doses of 103.5 nmol/20 grams, corresponding to 3 mg/kg DOX hydrochloride. Arrows, days of injections. Vertical bars, SE. *, P < 0.05 versus control by Student’s two-tailed t test.
after treatment with AN-152 to 184.4/11006 194, 102, 260, and 340 bp, respectively (Figs. 2 A treated with AN-152, the decrease in the expression of mRNA genomic DNA contamination. In MX-1 tumors from animals give rise to any detectable product, ruling out the possibility of Negative control samples without reverse transcription did not RT-PCR with specific primers for LHRH-R, Gs, G1, Gq, and Go subunits yielded single products of 319, 203, 194, 102, 260, and 340 bp, respectively (Figs. 2A and 3). Negative control samples without reverse transcription did not give rise to any detectable product, ruling out the possibility of genomic DNA contamination. In MX-1 tumors from animals treated with AN-152, the decrease in the expression of mRNA for LHRH-R was 24% (P < 0.01). The reduction in the mRNA for Go11 and Go12 was 10% (P < 0.01) and 16% (P < 0.05), respectively (Tables 2 and 3). No significant differences in the expression of mRNA were found between samples from the control group and the animals treated with DOX (Fig. 2; Table 4).

Expression of LHRH-R Protein. After treatment with AN-152 or DOX, LHRH-R was investigated by Western blotting with specific antibodies revealing a band at M, 60,000 (Fig. 2B). In MX-1 tumors of animals treated with AN-152, densitometric analysis of the bands representing the LHRH-R showed a significant (P < 0.01), 38% reduction in the protein levels compared with control values (Fig. 2B) after normalization to the values of actin protein (M, 43,000). In contrast, DOX caused only a slight, but not significant decrease in LHRH-R protein levels (Fig. 2B; Table 2).

LHRH-R Binding Studies. Radioligand binding studies were performed to evaluate the treatment effect of cytotoxic LHRH analogue AN-152 on LH-RH-R in membrane fractions of MX-1 human breast cancer. In membranes of MX-1 tumors from the control group, receptor analyses revealed a single class of high affinity (Kd of 5.1 ± 0.5 nm) binding sites for LHRH, with a mean Bmax value of 383.9 ± 2.6 fmol/mg membrane protein. The number of receptors for LHRH in tumors was significantly (P < 0.01) reduced to 213 to 24.2 fmol/mg membrane protein after treatment with AN-152, compared with tumors in the control group (Table 2). No significant changes were found in Kd or Bmax values in tumors from animals treated with DOX (Table 2). The concentration of cytotoxic LHRH analogue AN-152 required to inhibit the binding of 125I-[D-Trp6]LHRH by 50% (IC50) was 14.5 ± 1.3 nM, indicating a high affinity of AN-152 to LHRH receptors expressed on tumors from animals that received AN-152 treatment.

Expression of mRNA for the Members of the ErbB/HER Type I Tyrosine Kinase Receptor Family. To investigate whether the treatment with cytotoxic LHRH analogue

Serum LH Levels. There were no significant changes in serum LH levels after chronic treatment with DOX (110.1 ± 27.7 pg/ml) compared with controls (92.7 ± 6.5 pg/ml). In contrast, serum LH levels increased significantly (∑ 0.01) compared with controls (92.7 ± 6.5 pg/ml). The figure shows a representative assay of three independent experiments.

**Table 4** Effects of cytotoxic LHRH analogue AN-152 and DOX administered i. v. five times at doses of 103.5 nmol/20 grams, on the growth of MX-1 estrogen-independent, DOX-resistant human breast carcinoma in nude mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial tumor volume (mm³)</th>
<th>Final tumor volume (mm³)</th>
<th>Tumor burden (mg/gram)</th>
<th>Tumor doubling time (days)</th>
<th>Body weight (grams)</th>
<th>WBCs/mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>157.3 ± 25.9</td>
<td>2837.4 ± 515.8</td>
<td>113.4 ± 24.2</td>
<td>6.45 ± 0.36</td>
<td>10972.5 ± 1958.7</td>
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<tr>
<td>DOX</td>
<td>157.4 ± 19.4</td>
<td>3145.8 ± 844.3</td>
<td>102.91 ± 22.6</td>
<td>6.93 ± 0.69</td>
<td>8497.5 ± 2004.5</td>
<td></td>
</tr>
<tr>
<td>AN-152</td>
<td>161.9 ± 27.5</td>
<td>978.5 ± 176.8</td>
<td>50.78 ± 11.5</td>
<td>12.01 ± 1.99</td>
<td>10697.5 ± 2632.5</td>
<td></td>
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</table>

*a P < 0.05 vs. control.
AN-152 had an effect on the expression of mRNAs for the receptors of the ErbB/HER family. RT-PCR analyses were performed. PCR products of 400, 420, and 371 bp, corresponding to the mRNAs for EGF-R and HER-2 and -3, respectively, were detected after ethidium bromide staining under UV light (Fig. 4A). In MX-1 tumors from animals treated with AN-152, densitometric analysis revealed a 20% (P < 0.05) decrease in the expression of mRNA for both HER-2 and -3, compared with controls (Table 4). DOX had no effect on the mRNA levels for ErbB/HER family members, as shown in Fig. 4A.

Expression of the ErbB/HER Type I Tyrosine Kinase Receptor Family Proteins. Protein levels of the ErbB/HER family were investigated by Western blotting with specific antibodies. Bands at M r 175,000; 185,000; and 160,000 corresponded to the EGF-R and HER-2 and -3 proteins, respectively (Fig. 4B). Densitometric analysis of the bands representing the ErbB/HER family members revealed that the HER-2 and -3 protein levels in MX-1 cancers from animals treated with AN-152 were reduced by 65% (P < 0.01) and 66% (P < 0.01), respectively, compared with controls (Fig. 4B; Table 4). In contrast, DOX caused no significant changes in the protein levels of the members of the ErbB/HER family (Fig. 4B; Table 4).

DISCUSSION

The overcoming of resistance of cancer cells to chemotherapy poses an enormous challenge to the oncologists, and new approaches need to be explored. About 20–30% of breast cancers express elevated levels of the HER-2 protein, which belongs to the ErbB/HER type I tyrosine kinase receptor family. These mammary cancers are resistant to chemotherapy and have very poor prognosis (3). In this study, we investigated whether targeting DOX to LHRH-R on MX-1 estrogen-independent DOX-resistant breast carcinomas could improve the efficacy of treatment. The presence of membrane receptors for LHRH on MX-1 tumors, and the efficacy of targeted therapy with superactive cytotoxic LHRH analogue AN-207, which contains 2-pyrrolino-DOX (AN-201) instead of DOX, has been demonstrated previously (18). In that study, AN-201, the cytotoxic radical itself, which is noncross-resistant with DOX, also showed a remarkable efficacy in MX-1 tumors. This efficacy was further improved by conjugation to the LHRH analogue carrier. In the present study, DOX showed no effect on tumor growth parameters or the levels of proteins we investigated. In contrast, a significant antitumor effect was observed after treatment with AN-152, in which DOX is linked to [D-Lys 6 ]LHRH, as demonstrated by significant decreases in tumor volume, tumor burden, and the extension of tumor doubling time. Although earlier experiments with AN-152 and DOX demonstrated a significantly lower toxicity for AN-152, in this study, we observed no toxic side effects of either compound, possibly attributable to the relatively low doses used. To evaluate the potential deleterious effects of AN-152 treatment on the pituitary, we measured the levels of serum LH in mice 1 week after the administration of the last dose of AN-152 and found a significant increase compared with controls. This result could be caused by the high dose of the hormone conjugate, which apparently caused a lasting stimulation of LH release. Previous results with cytotoxic LHRH analogue AN-207 also demonstrated that the LH-producing cells of the pituitary suffered no permanent damage after targeted therapy (24).

The crucial role of LHRH-R for the effectiveness of therapy with AN-152 was well demonstrated (9, 25–27). In one of these studies, we found that after treatment of OV-1063 ovarian cancers with AN-152, LHRH-R could not be detected by radioreceptor assay (25). In contrast, a recent study demonstrated no significant down-regulation of LHRH-R by AN-152 in OVCAR-3 human ovarian cancers and HEC-1B human endometrial cancers in nude mice (27). To further elucidate the effects of treatment with AN-152 on the expression of LHRH-R on tumor tissues, we investigated the changes in the levels of mRNA for LHRH-R and its protein product, as well as the binding characteristics. RT-PCR analysis demonstrated a significant down-regulation of mRNA for LHRH-R, and Western blot analysis showed a significantly lower protein expression. These data were confirmed by radioreceptor assay, revealing a significantly lower number of LHRH-binding sites on MX-1 tumors after treatment with AN-152. However, these reduced B max values were still >200 fmol/mg protein, and previous studies demonstrated that even a lower capacity of LHRH-R is sufficient for an effective targeting of AN-152 (25, 27). These findings suggest that repeated therapy with AN-152 is feasible. The results reported herein also showed high affinity binding of AN-152 to LHRH-R on tumors from animals that had been treated with AN-152.
The mechanism by which AN-152 can overcome the resistance of MX-1 tumors to DOX is not very clear, and there may be more than one mechanism involved. It is known that resistance to DOX in MX-1 tumors is not mediated by the transport system (28). Thus, the mechanism of overcoming DOX resistance by targeting may not be the result of evading the efflux pump system by internalization of DOX mediated by LHRH-R. It is more likely that a higher concentration of the cytotoxic agent delivered to target tumor tissue can overcome the mechanisms of chemoresistance, as it was shown in experimental human prostate cancers (29).

Previously, we observed that effective treatment of OV-1063 human ovarian cancers in nude mice with AN-152 was accompanied by a significant down-regulation of mRNA for EGF-R and the binding capacity of the receptor protein (25), whereas DOX had no effect on tumor growth and EGF-R expression. To find out whether treatment of MX-1 breast cancers with AN-152 would produce a similar outcome, we investigated the changes in the levels of EGF-R, HER-2, and HER-3. Our results show that although the levels of EGF-R were not affected by AN-152, both HER-2 and HER-3 levels were significantly reduced. In fact, Western blot analysis indicates that there was a substantial decrease of ~65% in the levels of protein expression for both HER-2 and HER-3. On the basis of the promising clinical results with the HER-2 antibody Herceptin (Trastuzumab) in the management of HER-2-overexpressing breast cancers (30, 31), this may be of crucial relevance from the clinical point of view. Because DOX had no significant effect on these receptors, it is possible that the mechanism by which AN-152 can overcome the chemoresistance of MX-1 tumors is related in part to the down-regulation of members of the ERbB/HER receptor family.

Another possible mechanism could involve the specific effect of AN-152 on the G-proteins that are coupled to the LHRH-R, including $G_s$, $G_i$, $G_q$, and $G_1$, which are known to play a role in the regulation of cell growth (10). To investigate whether AN-152 had an effect on the expression of these G proteins, we performed semiquantitative analyses of the RT-PCR products corresponding to their mRNA. Our results show that the levels of mRNAs for $G_i$ and $G_q$ were significantly decreased by treatment with AN-152, but none of the G proteins tested were affected by DOX. Thus, it is possible that a disruption in G protein signaling contributed to the antitumor effect of AN-152.

In conclusion, the enhanced efficacy of AN-152, as compared with DOX, can be attributed at least in part to receptor-mediated effects of AN-152, resulting in disruption of major signaling pathways. However, the exact mechanisms of action of AN-152 on cancers, which do not respond to DOX, require further investigation. This study provides additional evidence that the linking of

### Table 4  Effects of treatment with cytotoxic LHRH analogue AN-152 and DOX on the levels of mRNA and protein products of HER family from MX-1 breast cancers

Values are mean ± SE. mRNA was quantified by densitometric analysis, and the data were normalized to actin values.

<table>
<thead>
<tr>
<th></th>
<th>Control mRNA</th>
<th>Control Protein</th>
<th>DOX (% of control) mRNA</th>
<th>DOX (% of control) Protein</th>
<th>AN-152 (% of control) mRNA</th>
<th>AN-152 (% of control) Protein</th>
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</thead>
<tbody>
<tr>
<td>EGF-R</td>
<td>100.0 ± 2.7</td>
<td>100.0 ± 6.0</td>
<td>112.4 ± 8.4</td>
<td>80.0 ± 13.9</td>
<td>105.3 ± 5.9</td>
<td>100.0 ± 13.6</td>
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<tr>
<td>HER-2</td>
<td>100.0 ± 4.2</td>
<td>100.0 ± 10.6</td>
<td>92.5 ± 3.8</td>
<td>117.5 ± 25.3</td>
<td>80.4 ± 2.7</td>
<td>34.7 ± 5.1</td>
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<tr>
<td>HER-3</td>
<td>100.0 ± 3.3</td>
<td>100.0 ± 11.6</td>
<td>113.7 ± 7.5</td>
<td>76.9 ± 5.6</td>
<td>80.2 ± 6.5</td>
<td>33.2 ± 5.4</td>
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* P < 0.05 versus control.

* P < 0.01 versus control.
DOX to an LHRH analogue carrier can significantly improve the outcome of chemotherapy based on DOX, which is still one of the most widely used chemotherapeutic agents with a broad range of antitumor activity. Our findings that AN-152 can significantly inhibit the growth of estrogen-independent, DOX-resistant human mammary carcinoma in nude mice and reduce the expression of HER-2 protein suggest the merit of therapeutic clinical trials with AN-152 in breast cancer patients with LHRH-R positive and estrogen receptor-negative disease.

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