Growth Inhibition of Human Ovarian Cancers by Cytotoxic Analogues of Luteinizing Hormone-Releasing Hormone

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Background: Receptors for luteinizing hormone-releasing hormone (LH-RH) are found in nearly 80% of human ovarian cancers. The chemotherapeutic agent doxorubicin can be linked to [D-lysine6]LH-RH to form a cytotoxic analogue (AN-152) that may have greater specificity for tumor cells. This study was conducted to investigate the effects of AN-152 on the growth of LH-RH receptor-positive OV-1063 human epithelial ovarian cancers.

Methods: Nude mice bearing human ovarian tumors, OV-1063 or UCI-107 (LH-RH receptor negative), were injected intraperitoneally with saline (control) or with equimolar doses of AN-152 or doxorubicin; experiments involving mice with OV-1063 tumors also included groups that were administered [D-lysine6]LH-RH either alone or in combination with doxorubicin. Tumor volume, weight, doubling time, and burden (i.e., tumor weight/body weight) as well as tumor apoptotic and mitotic indices were determined. The levels of receptors for LH-RH and epidermal growth factor (EGF) and their messenger RNAs were measured by use of radioreceptor and reverse transcription–polymerase chain reaction assays, respectively.

Results: The growth of OV-1063 ovarian tumors in nude mice, as based on reduction in tumor volume, was inhibited significantly (all P<0.05, two-sided) 4 weeks after treatment with AN-152, even at the lowest dose tested (413 nmol/20 g weight); the toxic effects of an equivalent dose of doxorubicin caused substantial mortality. High-affinity receptors for LH-RH and EGF were found on cell membranes of OV-1063 cancers; however, after in vivo treatment with AN-152, LH-RH receptor-binding sites were not detectable and EGF receptors were reduced in number. The growth of UCI-107 ovarian cancers was not inhibited by AN-152.

Conclusions: In nude mice bearing LH-RH receptor positive OV-1063 epithelial ovarian cancers, systemic administration of AN-152 is less toxic and inhibits tumor growth better than equimolar doses of doxorubicin. [J Natl Cancer Inst 1997;89:1803–9]

Ovarian cancer is the most common cause of death from gynecologic cancers in the Western world. It is estimated that in 1996 about 27,000 new cases of ovarian cancer were diagnosed and nearly 15,000 deaths occurred in the United States due to this disease (1,2). The current treatment of ovarian cancer is based on debulking surgery combined with chemotherapy (3–7). Various antineoplastic agents, such as cisplatin, cyclophosphamide, paclitaxel (Taxol), or doxorubicin are being used for chemotherapy, mostly in combination regimens (3–7). However, long-term outcome of such therapies is disappointing and prolonged use of cytotoxic agents is associated with toxic effects.

A modern approach to reducing the toxicity is the targeting of cytotoxic agents directly to cancerous cells. The presence of specific binding sites for peptide hormones on tumor cells renders these peptides appropriate for use as carriers that can be conjugated with cytotoxic radicals. Receptors for luteinizing hormone-releasing hormone (LH-RH) have been found on ovarian, endometrial, breast, and prostate cancers. Approximately 80% of human epithelial ovarian cancer specimens have been found to be LH-RH receptor positive (3,4). In the past decade, several prototypes of cytotoxic analogues of LH-RH have been synthesized in our laboratory (8–10). These hybrids consisted of LH-RH agonists or antagonists linked to various cytotoxic agents (8–10). Recently, we developed a cytotoxic LH-RH analogue containing doxorubicin (AN-152), which fully retains cytotoxic activity of the radical and receptor binding affinity of the peptide carrier (11). In this study, we have evaluated the effects of AN-152 on the growth of OV-1063 human epithelial ovarian cancers (12) that have high-affinity receptors for LH-RH (13). We also tested AN-152 on UCI-107 human epithelial ovarian cancers (14,15), which have been found to be negative for the presence of the LH-RH receptor.

Materials and Methods

Chemicals

Agonistic analogue [D-lysine6]LH-RH (pyroGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH2) used as a carrier was synthesized in our laboratory by solid-phase method (8). Cytotoxic LH-RH conjugate (AN-152) was made in our laboratory by coupling one molecule of doxorubicin-14-O-hemiglutlate to the ε-amino group of the D-lysine side chain of the carrier and purified by high-performance liquid chromatography (HPLC) as described previously (11). The approximate half-life of AN-152 in the serum of nude mice at 37 °C was 10 minutes as based on HPLC measurements. The compounds were dissolved in 0.9% sodium chloride and injected intraperitoneally.

Animals

Female athymic (nude) mice (Ncr nu/nu), 5 or 6 weeks old on arrival, were obtained from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The mice were housed in sterile cages under laminar air 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.

All experiments were performed according to institutional ethical guidelines. Tumor cells were transplanted into the mice in our laboratory.

Cells and Tumors

Human epithelial ovarian cancer cell line OV-1063 was obtained from American Type Culture Collection (Rockville, MD). This cell line originated from a metastatic papillary cystadenocarcinoma of the ovary in a 57-year-old woman and is positive for carcinoembryonic antigen (12). It was maintained in RPMI-1640 medium, supplemented with 10% fetal bovine serum (13), 1 mM pyruvate, and 1:100 minimal essential medium vitamins, 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) (all from Life Technologies, Inc. [GIBCO BRL]; Grand Island, NY). Cells were cultured in T-75 flasks (Corning Costar Corp., Cambridge, MA) in a humidified atmosphere of 5% CO2 and 95% air at 37°C and 5% CO2.
tumor tissue were fixed in 10% buffered neutral formalin for histologic examination. Tumor pieces were also stored at −18 °C for receptor studies and molecular biology analysis. **Pathologic Procedure**

**In Vivo Studies**

Xenografts were initiated by subcutaneous injection of 1 × 10^7 OV-1063 cells or UCI-107 cells into the right flank of four nude mice (13). Tumors resulting after 4 weeks were aseptically dissected and mechanically minced; 1-mm^3 tumor pieces were transplanted subcutaneously by trocar needle into the right flank of the mice. The tumor take rate was nearly 90%. One week after tumor transplantation, when the tumors measured about 15–25 mm^3, the mice were assigned to experimental groups of eight to 10 animals each and the therapy was initiated. The concentration of LH-RH receptors on OV-1063 tumors grown in nude mice was similar to that found on human epithelial ovarian cancer specimens. In experiment 1, mice with OV-1063 tumors were divided into six groups of nine animals each, which received the following treatments as single intraperitoneal injections: group 1, saline (control); group 2, AN-152 at a dose of 700 nmol/20 g; group 3, AN-152 at a dose of 413 nmol/20 g; group 4, doxorubicin at a dose of 413 nmol/20 g (12 mg/kg), which corresponds to maximum tolerated dose (MTD) in mice; group 5, the unconjugated mixture of 700 nmol/20 g of carrier agonist [D-lysine]LH-RH and doxorubicin; and group 6, agonist [D-lysine]LH-RH at a dose of 700 nmol/20 g.

In experiment 2, mice with OV-1063 tumors were divided into four groups and were given intraperitoneal injections as follows: group 1 (control), 10 animals received saline; group 2 (nine animals) received AN-152 at a dose of 413 nmol/20 g twice (days 1 and 10); group 3 (nine animals) received AN-152 at a dose of 600 nmol/20 g as a single injection; and group 4 (eight animals) received doxorubicin at a dose of 413 nmol/20 g twice (days 1 and 10).

In the third experiment, UCI-107 tumors were used and the mice were divided into three groups that received the following treatment as a single intraperitoneal injection: group 1 (control, nine animals) received saline; group 2 (nine animals) received AN-152 at a dose of 413 nmol/20 g; and group 3 (eight animals) received doxorubicin at a dose of 413 nmol/20 g.

Tumors were measured once or twice a week with microlipers, and tumor volume was calculated using the following formula: length × width × height × 0.5236 (16). Measurement of tumor growth was performed as previously described (19,20). [3H]LH-RH was radioiodinated by the chloramine-T method in our laboratory (19) and 125I-labeled EGF was purchased from Amershams Life Science Inc. (Arlington Heights, IL). The ligands were quantified spectrophotometrically. The optical density values (260 nm/280 nm) of the RNA preparations were greater than 1.8.

**Isolation of Messenger RNA (mRNA)**

Total RNA was extracted from frozen tissue samples by using RNAzol B (TEL-TEST Inc., Friendswood, TX) according to the manufacturer’s instructions. The RNA pellets were suspended in 100 μl of 10 mM Tris, 1 mM EDTA buffer (pH 8.0), and quantified spectrophotometrically. The optical density values (260 nm/280 nm) of the RNA preparations were greater than 1.8.

**Reverse Transcription (RT)**

One microgram of total RNA was used in a test tube containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.5 mM dNTPs, 1 U RNase inhibitor, and 2.5 μM random hexamer primers in a final volume of 19 μL of RNase-free deionized distilled water. The mixture was heated for 10 minutes at 65 °C, quenched on ice, then 2.5 U of Moloney murine leukemia virus reverse transcriptase (The Perkin-Elmer Corp., Norwalk, CT) in 1 μl was added, for a total reaction volume of 20 μL. The mixture was incubated at room temperature for 10 minutes and then at 42 °C for 1 hour. The reaction was ended by heating at 95 °C for 5 minutes and quenching on ice.

**Polymerase Chain Reaction Amplification**

The 20-μl solutions of the RT reaction from each sample were diluted into a final volume of 100 μL by a mixture of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, and 2 mM MgCl₂. Fifty picomoles of each primer and 2.5 U AmpliTaq DNA polymerase (The Perkin-Elmer Corp.) were also added. In the first experiment using reverse transcription–polymerase chain reaction (RT–PCR), the sense and antisense primers for LH-RH receptors were 5′-GACCTTGTGGTGGAAAGATCC-3′ and 5′-ACGCTGTGAATCACCACATCA-3′, respectively. These primers yield a 319-base-pair (bp) fragment that is complementary to nucleotide positions 93–411 in human LH-RH receptor complementary DNA (cDNA). The primers for EGF receptor were 5′-CTACACACACCTTTTGAACGACAAAGG-3′ (sense) and 5′-CTATGATCCCTCAGGTCCGACAGTATCG-3′ (antisense). These primers yield a 675-bp fragment that is complementary to nucleotides at positions 1488–2162 in human EGF receptor cDNA. The sense and antisense primers for β-actin used for internal control were 5′-ATCTGGGACACACCTTCTCAATCTGAGCTGCG-3′ and 5′-CCTGTTTAATCTCTGTATTCAACATGTCG-3′, respectively. These primers yield a 838-bp fragment (nucleotides 294–1131). These PCR oligonucleotide primers were synthesized by use of the model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). PCR was started after an additional denaturation step at 95 °C for 3 minutes by the addition of 2.5 U AmpliTaq (The Perkin-Elmer Corp.), followed by 40 cycles of replication (1 minute at 94 °C, 1 minute at 54 °C, and 1 minute at 72 °C), and the final extension was done for 7 minutes at 72 °C using a Stratagene Robocycler 40 system (Stratagene, La Jolla, CA).

The second experiment was done to investigate the expression of mRNA for EGF receptors. The primers used for human EGF (hEGF) receptor were sense 5′-AACGAGATAGTGGCCCAAGATTGCC-3′ (nucleotides 3047–3070) and antisense 5′-AGGAAATGATGCTGCTATGCTAACC-3′ (nucleotides 3423–3446). The primers for human glycerolaldehyde-3-phosphate dehydrogenase (bGAPDH) used for another internal control were sense 5′-TCTCTGACTTCAACACGCGACACC-3′ (nucleotides 907–930) and antisense 5′-TCTCTCTTTCCTGATCTTGG-3′ (nucleotides 1091–1114). Twenty-three cycles of PCR for hEGFR receptor and for bGAPDH were carried out under the same conditions as in the first experiment. The number of cycles was previously determined to be within the exponential range of PCR product amplification necessary for quantitative densitometry. Negative controls were run in parallel to check for DNA contamination of samples. Ten microliters of the final PCR product was electrophoresed in a 1.8% agarose gel.
gel. Bands were visualized on an ultraviolet transilluminator following ethidium bromide staining.

**Southern Blot Analysis**

The gel was soaked in denaturation buffer containing 50 mM NaOH and 1.5 M NaCl and then in neutralization buffer containing Tris–HCl (pH 8.0) and 1.5 M NaCl. The gel was then blotted onto a nylon membrane (Hybond N+, Amersham Life Science Inc.) by capillary transfer, and the DNA was linked to the membrane by heating for 2 hours at 80 °C. Sample blots were prehybridized at 60 °C for 16 hours in a buffer containing 4× standard saline citrate (SSC), 2× Denhardt’s solution, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, and 100 μg/mL denatured salmon sperm DNA. After prehybridization, the sample blots were hybridized at 60 °C for 20 hours in hybridization buffer containing 5× SSC, 0.5× Denhardt’s solution, 0.02 M Tris–HCl, 100 μg/mL sonicated salmon DNA, and 150 ng of 5′(32P)-end-labeled oligonucleotide probe. The oligonucleotide probe used for hEGF receptor was 5′- TGGATGAGAAGACATGACCAGCTG-GTGG-3′ (nucleotides 3191–3220) and for hGAPDH was 5′-TGTCAAGGCTTTTCCTGTC-GACACCA-3′ (nucleotides 981–1010). Probes were labeled in a reaction mixture containing 70 μCi [32P]adenosine triphosphate 3000 Ci/mmol; 10 mCi/mL. (Amersham Life Science, Inc.), 150 ng oligonucleotide probe, and 10 U of T4 polynucleotide kinase (Amersham Life Science, Inc.). After incubation at 37 °C for 45 minutes, reactions were stopped by adding 1 μL of 0.5 M EDTA and unincorporated radionucleotides were removed using NucTrap columns (Stratagene). The blots were washed under stringent conditions (i.e., twice for 30 minutes in 2× SSC/0.1% SDS at room temperature and once for 1 hour in 0.2× SSC/0.1% SDS at 65 °C) and the signals from samples were scanned and quantified by use of an imaging densitometer (Model GS-700, Bio-Rad Laboratories, Richmond, CA).

**In Vitro Cytotoxicity Assay**

The crystal violet assay was performed to determine inhibitory effects in vitro of AN-152 and doxorubicin on proliferation of OV-1063 and UCI-107 ovarian cancer cell lines. This assay is based on quantification of biomass, as described by Bernhardt et al. (22). Relative cell number in treated and control wells was determined by crystal violet staining and expressed as T/C values where T/C = (T – C0)/C0 × 100 [T = absorbance of treated cultures, C0 = absorbance of control cultures, and C0 = absorbance of cultures at the start of incubation (t = 0)]. The measured absorbance is proportional to the cell number. T/C values obtained at three different concentrations were used to calculate IC50 values, the drug concentration that inhibited cell growth by 50% compared with untreated control cultures.

**Statistical Methods**

All data are expressed as the mean ± standard error of the mean, and statistical analyses of the tumor data were performed with use of Student’s t test (two-tailed). Differences were considered statistically significant when P < 0.05.

**Results**

**Effect of AN-152 on the Growth of Human Epithelial Ovarian Cancers OV-1063 and UCI-107 in Nude Mice**

In experiment 1, after 2 weeks, the volume of the OV-1063 ovarian cancers in the two groups receiving AN-152 was significantly reduced to 3.9 ± 1.2 mm3 for the high-dose group (700 nmol/20 g) (P = .0024) and to 58.2 ± 23.5 mm3 for the low-dose group (413 nmol/20 g) (P = .0027) compared with the control group (892.7 ± 224.4 mm3), corresponding to 99% and 93% decreases in tumor volume, respectively (Fig. 1, A). Treatment with AN-152 for 4 weeks significantly decreased the final tumor volume and tumor weight in the group treated with a high dose to 149.0 ± 49.2 mg (97% reduction; P = .0049) and 94.8 ± 49.2 mg (97% reduction; P = .0056), respectively, and in the low-dose group to 623.4 ± 215.1 mm3 (84% reduction; P = .0106), respectively. In the control group, tumors measured 3984.8 ± 1053.6 mm3 and weighed 3016.8 ± 820.2 mg. Tumor doubling time in mice receiving 413 nmol/20 g of AN-152 was significantly (P = .044) extended to 11.1 ± 3.3 days from 3.8 ± 0.5 days in the control groups. At the end of the experiment, there was no significant difference in body weight in the group treated with the low dose of AN-152 (413 nmol/20 g) compared with the control group and tumor burden was decreased even more to 6.2 ± 3.3 mg/g body weight (P = .0099), although body weights were decreased to 16.2 ± 0.5 g compared with the control animals (22.8 ± 0.8 g) and two of nine animals died. Mice treated with a mixture of high-dose doxorubicin (700 nmol/20 g) and [α-labeled]LH-RH were all dead within 5 days after treatment. In the group treated with 413 nmol/20 g doxorubicin, six of nine animals died because of the toxic effects of the drug. Three days after treating nude mice with high-dose AN-152, their white blood cell (WBC) counts were 2640 ± 303/mm3 of peripheral blood compared with 4820 ± 638/mm3 in control animals (P = .015); if the mice were treated with AN-152 at a dose of 413 nmol/20 g, 3 days later their WBC counts were reduced to 4040 ± 856/mm3 (P = .41). After 7 days, WBC counts were normal in both groups treated with AN-152. In the group that received the high-dose AN-152, weights of the ovary (P = .0407), heart (P = .026), and liver (P = .0029) were significantly decreased.

In experiment 2, performed to find an improved regimen, AN-152 was administered according to two different protocols (Fig. 1, B). Final tumor volume and tumor weight in the group treated twice with AN-152 at a dose of 413 nmol/20 g decreased to 555.2 ± 215.1 mm3 (62% reduction; P = .0488) and 237.3 ± 89.6 mg (71.5% reduction; P = .0978), respectively. In the group that received a single administration of AN-152 at a dose of 600 nmol/20 g, tumor volume and weight decreased to 382.9 ± 90.3 mm3 (74% reduction; P = .0433) and 194.3 ± 48.7 mg (77% reduction; P = .0731), respectively, and only one of nine animals died. In the control group, tumors measured 1472.1 ± 456.9 mm3 and weighed 832.8 ± 311.4 mg. In the group treated twice with doxorubicin at the dose of 413 nmol/20 g, five of eight animals died by day 17 and seven of eight animals were dead by day 35. In the groups treated twice with 413 nmol/20 g AN-152 or once with 600 nmol/20 g, tumor burden was decreased (P = .062 and P = .116, respectively) and tumor doubling time was significantly increased (P = .0377 and P = .045, respectively). In experiment 2, mice treated with 600 nmol/20 g of AN-152 showed only a 14.6% weight loss and in mice treated twice with 413 nmol/20 g of AN-152, the mean body weight increased by 7.1%.

In experiment 3, neither AN-152 nor doxorubicin at a dose of 413 nmol/20 g demonstrated a statistically significant inhibitory effect on the growth of UCI-107 human epithelial ovarian cancer (Fig. 1, C).

**Histologic Findings and Serum LH Levels**

Mitotic index was significantly (P = .048) decreased in tumors treated with AN-152 at a dose of 600 nmol/20 g (10.5 ± 2.1) compared with the control group (16.7 ± 1.9). Mitotic indices were not changed in the groups receiving 413 nmol
of doxorubicin (11.3 ± 4.3) or 600 nmol of [d-Lys\(^6\)]LH-RH (14.5 ± 1.6). The number of AgNORs per nucleus was significantly reduced in the groups that received AN-152 twice at a dose of 413 nmol/20 g or once at a dose of 600 nmol/20 g in experiment 2 (\(P = .039\) and \(P = .04\), respectively), but was not changed by doxorubicin or [d-Lys\(^6\)]LH-RH alone. Both the apoptotic index (10.3 ± 1.0) (\(P = .049\)) and the ratio of apoptotic to mitotic indices (1.11 ± 0.21) (\(P = .022\)) were significantly higher in the OV-1063 tumors treated twice with AN-152 at a dose of 413 nmol/20 g compared with tumors in the control group (7.0 ± 1.2 and 0.46 ± 0.09, respectively). Doxorubicin and [d-Lys\(^6\)]LH-RH caused no changes in apoptosis.

In experiment 1, no significant differences in serum LH levels were observed between the groups and therefore serum LH was not followed in subsequent experiments.

Receptor Analysis

In experiments 1 and 2, receptor assays on OV-1063 tumor membranes of control animals showed high-affinity binding sites for EGF (Table 1). The number of receptors for EGF was decreased after treatment with AN-152 compared with animals in the control group, although the reduction was not significant in the groups that were treated one time with 413 or 700 nmol/20 g in experiment 1 (Table 1). In both experiments, specific binding sites for LH-RH were found on OV-1063 tumors of control animals. However, in all groups treated with AN-152, receptors for LH-RH were no longer detectable (Table 1). In experiment 3, the receptors for LH-RH could not be found in UCI-107 ovarian tumors of control animals.

Molecular Biology Analysis

The mRNAs for LH-RH receptor and for EGF receptor were detected on OV-1063 human epithelial ovarian cancers by use of a RT–PCR assay (Fig. 2, A). However, the expression of mRNA for LH-RH receptor was not found in UCI-107 human ovarian cancers (Fig. 2, A). The PCR products of hEGF receptors from OV-1063 tumors in experiment 2, obtained after RT–PCR, were confirmed by Southern blot analysis (Fig. 2, B). Semiquantitative analysis of the developed bands by densitometry showed that in the group treated...
In Vitro Cytotoxicity Assay

The concentrations of AN-152 and doxorubicin that inhibited the proliferation of OV-1063 human ovarian cancer cell line in vitro at 115 hours by 50% (IC₅₀) were 1.4 × 10⁻⁸ M and 1.9 × 10⁻⁸ M, respectively. In the case of the UCI-107 ovarian cancer line, IC₅₀ values for AN-152 and doxorubicin at 68 hours were 1.7 × 10⁻⁸ M and 2.2 × 10⁻⁸ M, respectively.

Discussion

Chemotherapy provides effective treatment for many cancers, but in spite of the development of modern antineoplastic drugs, their peripheral toxicity remains a major problem (1,10). Targeted chemotherapy may be more selective and would greatly reduce the side effects of cytotoxic agents. On the basis of the presence of receptors for LH-RH on mammary, ovarian, endometrial, and prostatic tumors, a novel class of multitumor drugs was developed, based on LH-RH analogues linked covalently to various cytotoxic radicals (8–11). In one of these hybrid molecules (AN-152), doxorubicin, which can be cytotoxic by membrane action without entering the cells (23–25), is linked to [d-lysine⁶]LH-RH (11).

This study demonstrates that the cytotoxic LH-RH analogue AN-152 inhibits the growth of OV-1063 human epithelial ovarian cancers in vivo. [d-Lysine⁶]LH-RH showed no inhibitory effect at a dose of 700 nmol/20 g. This result is in agreement with the findings of a recent clinical trial (26) in which patients with advanced epithelial ovarian cancer were treated with agonist [d-tryptophan⁶]LH-RH. Doxorubicin showed no significant anticancer effect; in fact, at a dose of 413 nmol/20 g, which corresponds to the maximum tolerated dose (27), most mice died because of the toxic effects of the drug. In experiment 1, the unconjugated mixture of doxorubicin and [d-lysine⁶]LH-RH at doses of 700 nmol/20 g each, killed all animals within 5 days after treatment, whereas 700 nmol/20 g of AN-152 was much less toxic and produced a statistically significant (P = .0049) decrease in tumor volume. These results show that the cytotoxic LH-RH conjugate is more effective and less toxic than an equimolar amount of radical alone, carrier alone, or a mixture of radical and carrier. The final reductions in measured tumor volumes were 96% for 700 nmol/20 g and 84% for 413 nmol/20 g of AN-152. Although the dose of 700 nmol/20 g of AN-152 demonstrated an excellent tumor inhibitory effect, it caused a marked weight loss. Doses of 600 nmol/20 g of AN-152 or 413 nmol/20 g of AN-152, given twice, were less toxic and still able to effectively inhibit tumor growth. Among known side effects of doxorubicin is leukopenia (1,7,10,27), which was observed only in

Table 1. Characteristics of luteinizing hormone-releasing hormone (LH-RH) receptors and epidermal growth factor (EGF) receptors on membranes of OV-1063 human ovarian cancer xenografts in nude mice after 4 weeks of treatment with saline (control), AN-152, DOX, or the carrier [d-Lys⁶]LH-RH*

<table>
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<tr>
<th>Treatment</th>
<th>Dissociation constant, nM</th>
<th>Binding capacity, fmol/mg protein</th>
<th>Dissociation constant, nM</th>
<th>Binding capacity, fmol/mg protein</th>
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<tr>
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<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>AN-152, 700 nmol/20 g</td>
<td>9.56 ± 1.45</td>
<td>488.5 ± 23.5</td>
<td>0.89 ± 0.23</td>
<td>176.1 ± 20.5</td>
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<td>AN-152, 413 nmol/20 g</td>
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<td>N.D.</td>
<td>1.32 ± 0.28</td>
<td>112.7 ± 0.75</td>
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<tr>
<td>Doxorubicin, 413 nmol/20 g</td>
<td>10.2 ± 4.12</td>
<td>469.0 ± 35.0</td>
<td>1.32 ± 0.33</td>
<td>186.0 ± 0.4</td>
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<tr>
<td>Doxorubicin, 600 nmol/20 g</td>
<td>8.65 ± 0.15</td>
<td>458.0 ± 17.0</td>
<td>1.28 ± 0.38</td>
<td>157.3 ± 11.8</td>
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*Statistically significant (P < .05).
†Values in columns = mean ± standard error.

An asterisk indicates not detectable.

Fig. 2. A) Reverse transcription–polymerase chain reaction (RT–PCR) analysis of messenger RNA for luteinizing hormone-releasing hormone (LH-RH) receptor and epidermal growth factor (EGF) receptors using 1 μg of total RNA from untreated xenografts of OV-1063 and UCI-107 human epithelial ovarian cancers. The PCR products were separated by 1.8% agarose gel electrophoresis, stained with ethidium bromide. The sizes of the expected PCR products were as follows: β-actin, 319 bp; EGF receptor, 475 bp; and LH-RH receptor, 319 bp. B) Southern blot analysis of human EGF (dEGF) receptor complementary DNA (400 bp) in OV-1063 ovarian cancers obtained after RT–PCR. Hybridization was performed with the oligonucleotide probe specific for dEGF receptors. Lane 1—negative control; lanes 2–7—tumor samples from treated animals; lanes 8–11—tumor samples from mice treated twice with AN-152 at a dose of 413 nmol/20 g; and lanes 12–15—tumor samples from mice treated with AN-152 at a dose of 600 nmol/20 g.
animals treated with AN-152 at a dose of 700 nmol/20 g.

Specific receptors for LH-RH and EGF were found on membranes of OV-1063 human epithelial ovarian cancers. After treatment with AN-152, the receptors for LH-RH vanished and the number of receptors for EGF was reduced. No receptors for LH-RH could be found on UCI-107 human ovarian cancers and AN-152 did not inhibit growth of this tumor. These findings indicate that this cytotoxic analogue might more selectively kill tumor cells expressing LH-RH receptors. The fact that UCI-107 ovarian cancers, which lack receptors for LH-RH, did not respond to AN-152, supports the view that the effects of AN-152 might be exerted, at least in part, directly on the tumors. The inhibition of the growth of OV-1063 ovarian cancer by analogue AN-152 was accompanied by a marked decrease in the number of EGF receptors and in the levels of their mRNA. This observation may be of special relevance. Various studies (28–31) suggest that EGF is involved in proliferation of human epithelial ovarian cancer. EGF receptor mRNA is expressed in primary human ovarian adenocarcinomas (30–32) and EGF receptor status has been associated with the biologic aggressiveness and poor survival in patients with ovarian cancer (29,32). Previously, we showed that LH-RH antagonist Cetrorelix suppressed the growth of OV-1063 cancers in vivo and decreased EGF receptor protein and mRNA expression (33). This down-regulation (i.e., decreased expression) of EGF receptors might be responsible for the inhibitory effects of Cetrorelix and cytotoxic analogue AN-152 on ovarian cancers.

Although cytotoxic LH-RH analogues could exert hormonal effects, the predominant mechanism of action in the case of AN-152 appeared to be based on the local cytotoxic and tumoricidal action. Our work supports the view that LH-RH analogues can serve as carriers for chemotherapy agents and that such conjugates might deliver cytotoxic radicals more selectively to tumor cells by binding to membrane LH-RH receptors. Our findings suggest the merit of further investigations to determine whether cytotoxic LH-RH analogues could be used for the treatment of advanced ovarian carcinomas that possess receptors for LH-RH.

References

18. Szepeshazi K, Korkut E, Schally AV. Decrease


Notes
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