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Preclinical report

In vitro targeting of a cytotoxic analog of luteinizing hormone-releasing hormone AN-207 to ES-2 human ovarian cancer cells as demonstrated by microsatellite analyses

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Targeting of cytotoxic agents represents a modern approach to the treatment of various cancers, that improves the efficacy and reduces peripheral toxicity. Recently we developed a powerful cytotoxic analog of luteinizing hormone-releasing hormone (LHRH), AN-207, designed to be targeted to tumors that express LHRH receptors. This analog consists of the superactive derivative of doxorubicin (DOX), 2-pyrrolino-DOX (AN-201), linked to [D-Lys^6]LHRH carrier. In the present study we investigated the cytocidal effects of AN-207 and AN-201 on the LHRH receptor-positive ES-2 ovarian cancer cells. The targeting of AN-207 to ES-2 cells in the presence of LHRH receptor-negative UCI-107 ovarian cancer cells was also evaluated by semi-quantitative polymerase chain reaction (PCR) amplification of microsatellite markers. Ligand competition assays showed a single class of high-affinity and low-capacity binding sites in ES-2 cells with a mean dissociation constant (K_D) of 3.93 ± 0.1 nM and a mean maximal binding capacity (B_{max}) of 271 ± 26.1 fmol/mg membrane protein. Kinetic assays indicated that AN-207 caused cell death in a concentration- and time-dependent manner in ES-2 cells, but not in UCI-107 cells, while the kinetics of cytotoxic effects of AN-201 were similar in both cell lines. To investigate targeting, ES-2 cells were co-cultured with UCI-107 cells, treated with 10 nM AN-207 or AN-201 for different times and then cultured for 48 h in the absence of cytotoxic agents. Genomic DNA was extracted for microsatellite analyses using different markers. Semi-quantitative analyses of the intensity of the alleles that correspond to each cell line indicated that AN-207 was selectively targeted to ES-2 cells, while AN-201 showed no selectivity for either cell line. These results extend our previous findings that AN-207 can be targeted to ovarian cancers and other tumors that express receptors for LHRH. Cytotoxic analogs of LHRH, such as AN-207, should be considered for treatment of LHRH receptor-positive tumors. [© 2001 Lippincott Williams & Wilkins.]

Key words: Cytotoxic LHRH analog, LHRH receptor, ovarian cancer, targeted cancer therapy.

Introduction

Ovarian cancer is one of the least curable malignancies and ranks as the fourth most frequent cause of cancer-related deaths among women in the western world.[1] This low survival is in part due to the advanced stage of the ovarian cancer at the time of presentation.[2] Treatment of epithelial ovarian cancer is currently based on debulking surgery combined with chemotherapy or radiation. Chemotherapy is usually accompanied by severe toxicity which restricts the systemic administration of an effective dose of a wide variety of cytotoxic drugs. A new strategy to decrease the toxicity and improve the effectiveness of cytotoxic drugs is based on Paul Ehrlich's concept of a selective agent, a so-called 'magic bullet', that will target tumor cells while sparing healthy tissues.[3] Targeting of cytotoxic agents represents a modern approach to the treatment of various cancers that improves the efficacy and reduces peripheral toxicity.[4] Several methods have been proposed including immunotoxins and hormonotoxins (for a review, see Schally and Nagy[4]). The presence of specific receptors for peptide hormones on various tumor cells makes the analogs of these peptides suitable for use as carriers that can be

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linked to cytotoxic radicals.[4] Luteinizing hormone-releasing hormone (LHRH) receptors have been found in approximately 80% of human epithelial ovarian tumors,[5] in various human ovarian cancer cell lines,[6,7] as well as in endometrial, prostatic and breast cancers.[8] Thus, in the past decade, several prototypes of cytotoxic conjugates of LHRH have been synthesized by our group and tested on experimental tumor models.[4] Recently we developed a new cytotoxic analog of LHRH (AN-207),[9] in which the potent derivative of doxorubicin (DOX) 2-pyrrolino-DOX (AN-201) is covalently linked to [D-Lys^6]LHRH, and showed that this conjugate effectively inhibits growth of human prostatic, mammary and ovarian cancer lines xenografted into nude mice.[4]

The evaluation of the selectivity of targeted cytotoxic analogs on tumor cells *in vitro* can be very complicated depending on the chemical characteristics of the drug. The performance of costly and time-consuming *in vivo* experiments in animal model systems is often required. Lately, we have used a new technique based on microsatellite markers to demonstrate the targeting of cytotoxic peptide analogs *in vitro*.[10] This assay is based on the evaluation of genetic heterogeneity in biological specimens by means of quantitation of the allelic imbalance using microsatellite markers.[11]

In this study we first demonstrated the presence of LHRH receptors in the ES-2 human ovarian cancer cell line by a binding assay. Next, we evaluated the *in vitro* effects of the cytotoxic LHRH analog AN-207 on ES-2 cells and the LHRH receptor-negative human ovarian cancer cell line UCI-107. The targeting of AN-207 to ES-2 cells co-cultured with UCI-107 cells was then investigated by microsatellite analysis.

Materials and methods

Cytotoxic agents

Cytotoxic radical 2-pyrrolino-DOX (AN-201) and the cytotoxic LHRH analog AN-207 consisting of 2-pyrrolino-DOX-14-*O*-hemiglutarate linked to the ε-amino side chain of [D-Lys^6]LHRH were synthesized in our laboratory as described.[9] For cytotoxicity assays the compounds were dissolved in serum-free medium (SFM). Stability of AN-207 in SFM was tested as previously described.[12]

Cell lines

Human ovarian cancer cell line ES-2, a poorly differentiated ovarian clear cell carcinoma derived from a tumor of a 47-year-old African-American female,[13] was obtained from ATCC (Manassas, VA). The LHRH receptor-negative[14] UCI-107 epithelial ovarian cancer cell line, which originated from a patient with papillary adenocarcinoma of the ovary,[15] was kindly provided by Dr A Manetta (University of California, Irvine Medical Center, Orange, CA). Both cell lines were grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (100 U/ml) at 37°C in an humidified atmosphere of 95% air/5% CO_2. Cells were passaged weekly and routinely monitored for mycoplasma contamination using a detection kit (Boehringer, Mannheim, Germany). All culture media components were purchased from Gibco (Grand Island, NY).

Receptor assay

For binding studies ES-2 cells were detached by non-enzymatic methods and homogenized in 50 mM Tris–HCl (pH 7.4) supplemented with 0.25 mM phenylmethylsulphonyl fluoride, 0.4% (v/v) aprotinin and 2 μg/ml pepstatin A as described.[16] The homogenate was then centrifuged at 500 *g* to remove nuclear debris and lipid layer, and the supernatant containing the crude membrane fraction was ultracentrifuged at 70 000 *g* and washed with fresh buffer. Protein concentration was determined by the Bradford method using a BioRad protein assay kit (BioRad, Hercules, CA). Radioiodinated [D-Trp^6]LHRH was prepared by the chloramine-T method and purified by reversed-phase HPLC in our laboratory.[17] LHRH receptor binding assays were carried out as previously described[16–18] using *in vitro* ligand competition assays based on binding of [^{125}I][D-Trp^6]LHRH to cell membrane fractions and the results were analyzed by the Ligand-PC computerized curve fitting program of Munson and Rodbard.[19]

Cytotoxicity assays

ES-2 and UCI-107 cells were seeded at a density of 2000 cells/well in a 96-well plate and allowed to attach for 24 h after which the medium was removed and cells were washed twice with SFM to remove traces of serum enzymes. Cytotoxic LHRH analog AN-207 and its cytotoxic radical AN-201 were dissolved in SFM at 100, 10, 1 and 0.1 nM in experiment I and at 8, 6, 4 and 2 nM concentrations in experiment II, and added to the cells. Control cells received SFM only. After 30, 60, 120 and 240 min, the medium containing the peptides, as well as the medium from control cells, was removed, cells were washed twice with SFM and

cultured for an additional 48 h with fresh medium that contained FBS, but not the cytotoxic agents. Cell proliferation after exposure to AN-201 or AN-207 was measured by the crystal violet assay as previously described.[20] The OD values obtained after a 48 h incubation for the control untreated cells were considered as 100%. The experiments were performed in duplicates and repeated at least twice in different passages of the cells.

DNA extraction and microsatellite analysis

Co-cultured ES-2 and UCI-107 cells, seeded at a 1:1 ratio, were exposed to 10 nM of AN-201 or AN-207 for 30 min as described above. Untreated co-cultured cells were used as controls. Genomic DNA was extracted by standard phenol–chloroform extraction. Microsatellite markers were obtained from Research Genetics (Huntsville, AL). DNA (50 ng) was amplified in 20 μl of an aqueous solution containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 nM each dNTP, 0.4 mM of each primer and 2.5 U Taq DNA polymerase (Perkin-Elmer, Norwalk, CT). PCR consisted of 1 cycle of denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, with a final extension step of 3 min at 72°C using a Stratagene Robocycler 40 system (Stratagene, La Jolla, CA). The number of cycles was previously determined in preliminary experiments to be within the exponential range of PCR amplification. Aliquots of 5 μl of each PCR product were electrophoresed in a 12% polyacrylamide gel, stained with silver and quantified densitometrically using a scanning densitometer (model GS-700; BioRad) coupled with the BioRad PC Analysis software.

Statistical analyses

Statistical analyses were performed by using Student's two-sided *t*-test. $p < 0.05$ was considered significant.

Results

Receptor binding assay

The presence of specific LHRH binding sites and characteristics of [^{125}I][D-Trp^6]LHRH binding to membrane receptors on ES-2 cells were determined by ligand competition assays. The results showed that radiolabeled [D-Trp^6]LHRH was bound to a single class of high-affinity and low-capacity binding sites with a mean dissociation constant (K_D) of 3.93 ± 0.1 nM and a mean maximal binding capacity (B_{max}) of 271 ± 26.1 fmol/mg membrane protein (Figure 1). Other experiments showed that the concentration of AN-207 required to inhibit 50% of the specific [^{125}I][D-Trp^6]LHRH binding (IC_{50}) was 9.11 nM.

Cytotoxicity assay

The effects of cytotoxic LHRH analog AN-207 and its cytotoxic radical AN-201 on the proliferation of ES-2 and UCI-107 ovarian cancer cell lines are presented in Figure 2 and 3.

In experiment I, the treatment of ES-2 cells with AN-207 resulted in a cytocidal effect when the cytotoxic peptide was used at a concentration of 10 or 100 nM, while doses of 1 nM and lower did not affect cell proliferation (Figure 2A). At 100 nM concentration, significant ($p < 0.01$) cell death occurred after 30 min of exposure to the analog and approximately only 25% of the cell population survived as compared to controls (100%). This survival rate did not decrease with longer periods of exposure to the cytotoxic agent (Figure 2A). Significant cell death compared to controls also occurred at 10 nM, being 35% ($p < 0.01$) after 30 min, 53% ($p < 0.01$) after 60 min, 59% ($p < 0.01$) after 2 h and 69% ($p < 0.01$) after 4 h of exposure (Figure 2A). Similar results were obtained after treatment of UCI-107 cells at 100 nM, whereas at 10 nM only 35% of the initial

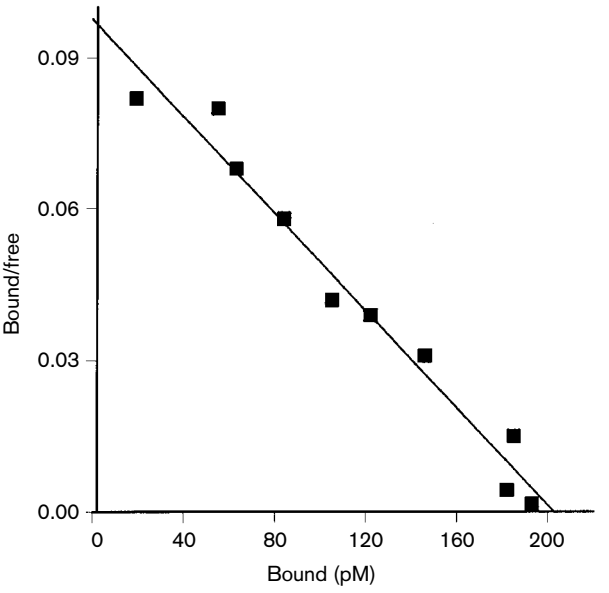

Figure 1. Representative example of Scatchard plots of [^{125}I][D-Trp^6]LHRH binding to the membrane fraction isolated from ES-2 human ovarian cancer cells (60 min incubation at 4°C). Increasing concentrations (10^{-12} to 10^{-6} M) of [D-Trp^6]LHRH were added in the presence of approximately 60 000 c.p.m. radioligand. Each point represents the mean of triplicate determinations.

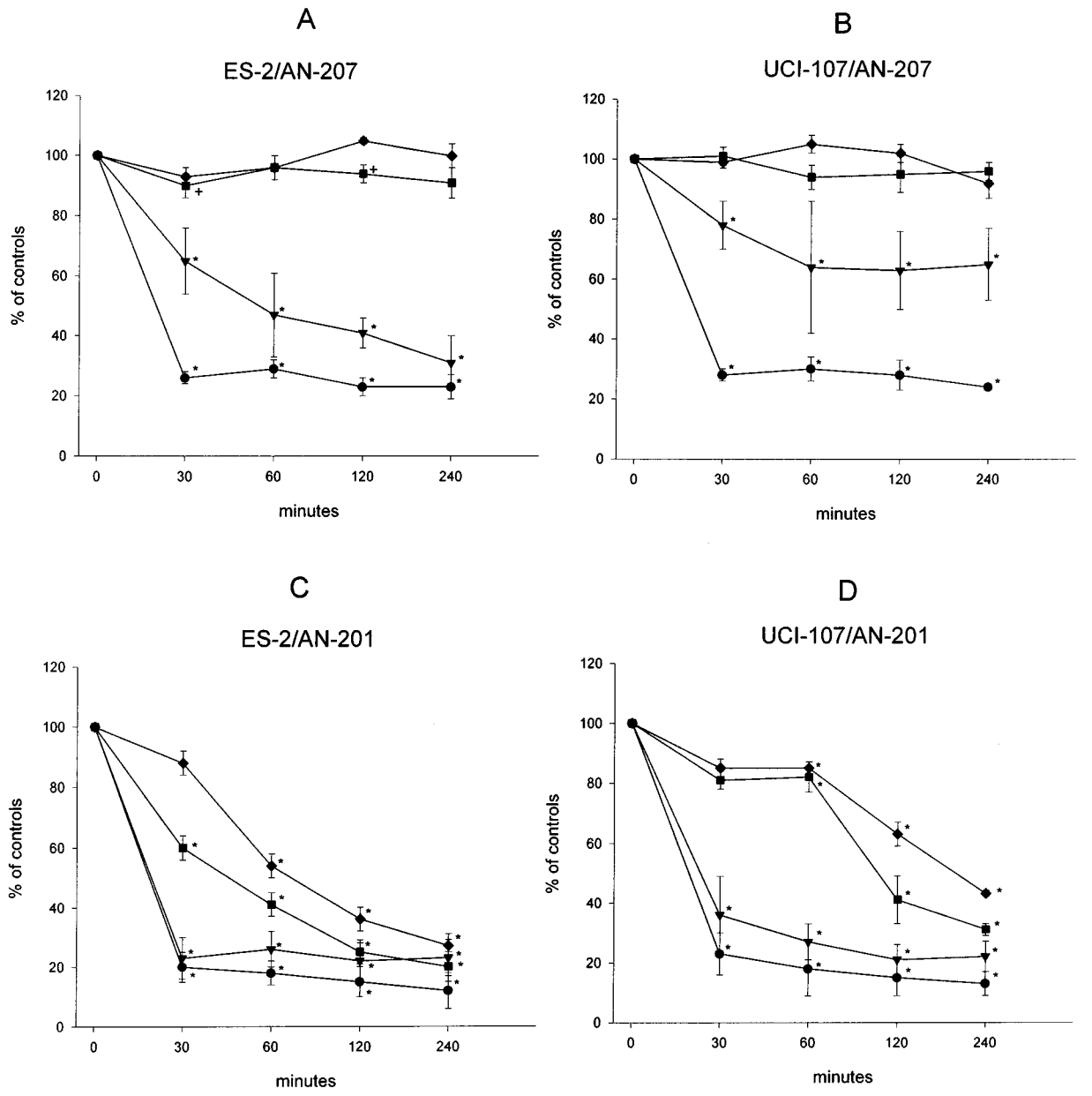

Figure 2. Experiment I: effect of cytotoxic LHRH analog AN-207 and its cytotoxic moiety AN-201 on the proliferation of human ovarian cancer cell lines ES-2 and UCI-107. Cells were seeded at a density of 2000 cells/well and incubated for different times in the presence of the cytotoxic agents dissolved in SFM at 100 (●), 10 (▼), 1 (■) and 0.1 (◆) nM. After incubation, the cells were washed and allowed to grow for 2 days in culture medium containing FBS. Vertical bars represent SE. $^{+}p<0.05$; $^{*}p<0.01$.

population died after 60 min and longer exposure did not affect cell survival (Figure 2B). When ES-2 and UCI-107 cells were treated with cytotoxic radical AN-201, inhibition of cell proliferation was achieved at all the doses tested, and at 10 and 100 nM approximately 80% cell death was reached after 30 min (Figure 2C and D). In experiment II, the effects of AN-207 and AN-201 in the concentration range of 2–8 nM were also determined on both cell lines (Figure 3). As shown in Figure 3(A), the effect of AN-207 in ES-2 cells is time and dose dependent, and the effects are apparent at 30 min. In UCI-107 cells, however, there is no significant cell death within the first hour of treatment, but significant ($p<0.01$) effects can be seen after 2 h for concentrations above 2 nM (Figure 3B). In contrast, cytotoxic radical AN-201 was effective in both cell lines at concentrations of 2–8 nM (Figure 3C and D).

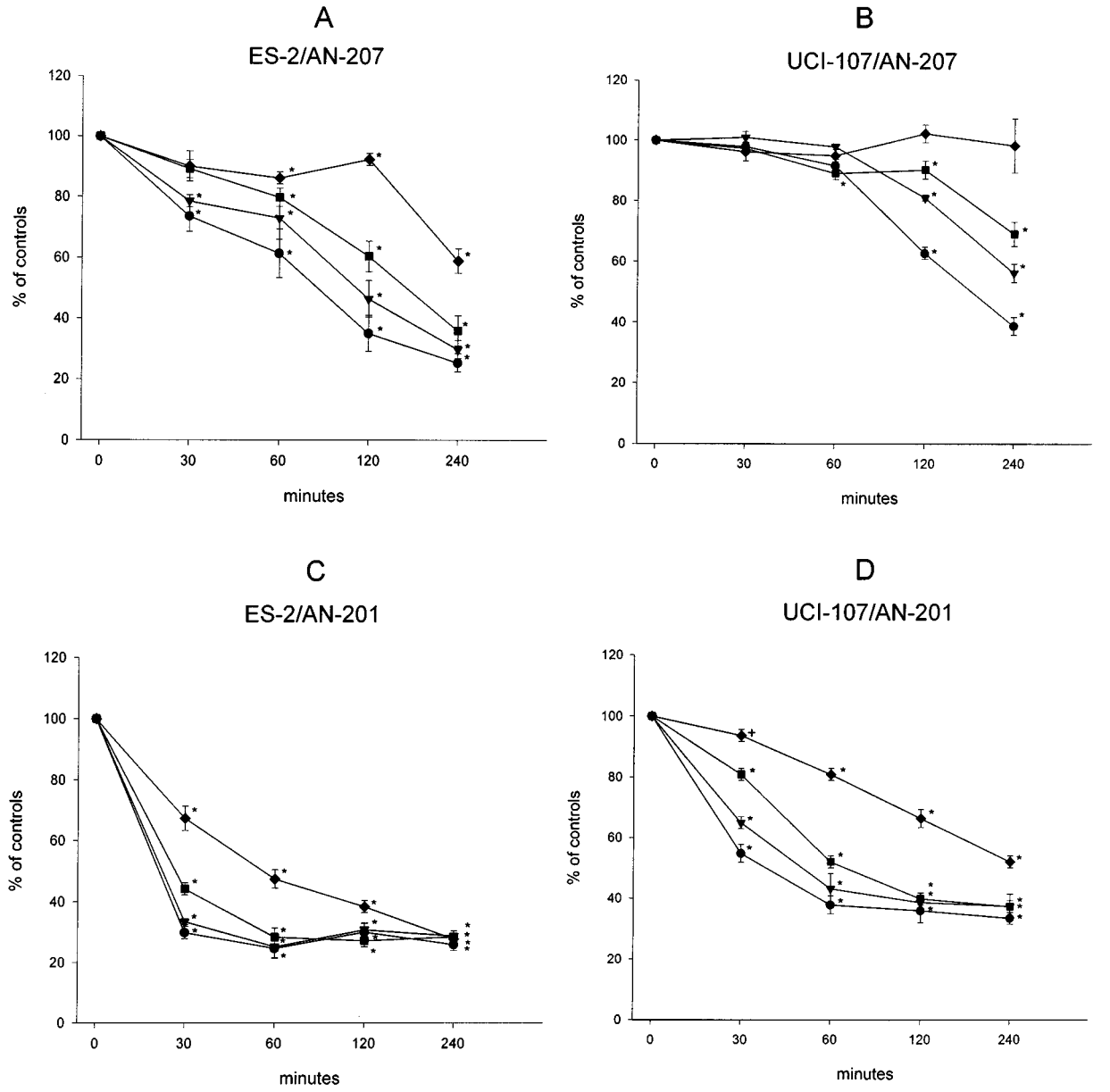

Figure 3. Experiment II: effect of cytotoxic LHRH analog AN-207 and its cytotoxic moiety AN-201 on the proliferation of human ovarian cancer cell lines ES-2 and UCI-107. Cells were seeded at a density of 2000 cells/well and incubated for different times in the presence of the cytotoxic agents dissolved in SFM at 8 (●), 6 (▼), 4 (■) and 2 (◆) nM. After incubation, the cells were washed and allowed to grow for 2 days in culture medium containing FBS. Vertical bars represent SE. $^{+}p<0.05$; $^{*}p<0.01$.

Microsatellite analysis

Microsatellite markers D3S2409 and D13S317 were chosen from a variety of available markers because the bands associated with each cell line were easily identifiable. DNA from co-cultured cells was amplified by PCR with D3S2409 primers and the product electrophoresed in 12% polyacrylamide gel. The results are shown in Figure 4(A). The gel was directly scanned, the bands specific for each cell line were quantified by densitometrical analysis, and the ratio of the intensity of the bands specific for ES-2 and UCI-107 was calculated. The ratio of their intensities reflects the proportion of living cells present in the co-cultured wells. Untreated cells and cells treated with AN-201 showed an ES-2 : UCI-107 ratio of 1.30 ± 0.03 and 1.31 ± 0.03, respectively, while in AN-207-treated cells this ratio was significantly decreased to 1.18 ± 0.04

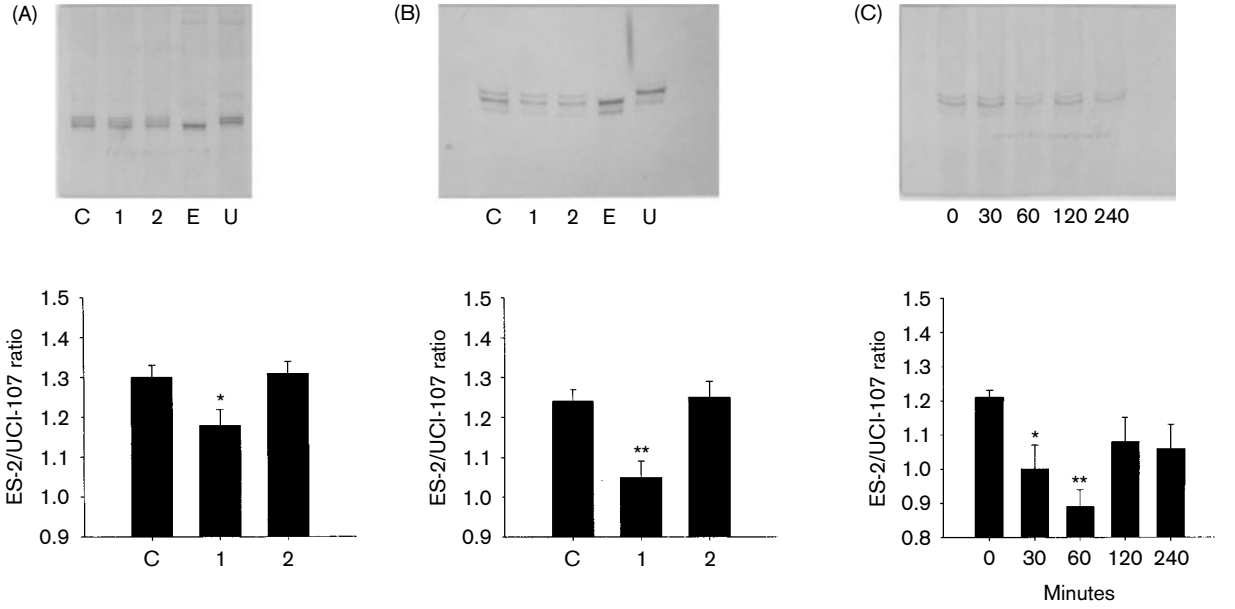

Figure 4. *In vitro* targeting of AN-207 to the LHRH receptor-positive ES-2 human ovarian cancer cell line co-cultured with LHRH receptor-negative UCI-107 as demonstrated by microsatellite alleles assay. DNA extracted from co-cultured cells treated for 30 min with 10 nM AN-207 or AN-201 was amplified by PCR with markers D3S2409 (A) and D13S317 (B), and the products were electrophoresed in polyacrylamide gels (upper panel). The bands specific for each cell line were densitometrically quantified and the ES-2 : UCI-107 ratio was calculated (lower panel). Similarly, DNA extracted from co-cultured cells treated with AN-207 for 0 (untreated controls), 30, 60, 120 and 240 min was amplified with marker D13S317(C). Lane C, untreated co-cultured cells (control); lane 1, AN-207-treated co-cultured cells; lane 2, AN-201-treated co-cultured cells; lane E, ES-2 cells; lane U, UCI-107 cells. $^{*}p<0.05$; $^{**}p<0.01$.

($p<0.05$) (Figure 4A). This decrease was significant, but represented less than 10% of the ratio in the untreated co-cultured cells. Consequently, microsatellite marker D13S317 was also used to verify whether this decrease was due to AN-207 treatment or was an artifact produced during PCR. The results with this new set of primers are shown in Figure 4(B). The ES-2: UCI-107 ratio was 1.24 ± 0.03 and 1.25 ± 0.04 for control and AN-201-treated cells, respectively, while in AN-207-treated cells it was 1.05 ± 0.05. In this case there was a significant ($p<0.05$) decrease representing about 16% of the ratio in untreated cells. Longer periods of exposure to the cytotoxic analog AN-207 decreased specifically the number of ES-2 cells by 26% after 60 min ($p<0.01$) (Figure 4C), but the specificity did not increase after 2 h. Different proportions of starting populations of ES-2 and UCI-107 were also tested, and similar results on specific cytotoxicity of AN-207 against ES-2 cells were obtained (data not shown).

Discussion

Targeted chemotherapy is a modern approach to the treatment of cancer. Because of the presence of specific receptors for LHRH in various tumors such as breast,[21] prostatic,[18,22,23] ovarian[24–26] and endometrial cancers,[27] we have developed targeted cytotoxic analogs of LHRH, including AN-207, which contains 2-pyrrolino-DOX, a superactive derivative of DOX, covalently linked to [D-Lys^6]LHRH. We have demonstrated that AN-207 fully preserves the high binding affinity of the carrier peptide [D-Lys^6]LHRH to LHRH receptors on rat pituitary membranes.[9] We have also shown that AN-207 has a high binding affinity to LHRH receptors on human breast cancer specimens and MCF-7 and MDA-MB-231 cell lines.[28] The expression of messenger RNA for LHRH receptor in human ovarian cancer cell line ES-2 was reported recently.[20] In this study we show for the first time the presence of a single class of receptors for LHRH, that can be classified as high-affinity receptors in ES-2 cells. The expression of such functional receptors is required for effective targeting of AN-207.

In experiment I, among all the concentrations of analog AN-207 examined, only 1 nM or lower concentrations were ineffective in the ES-2 cell line, probably because the dissociation constant for LHRH receptors in this cell line is between 1 and 10 nM. In contrast, radical AN-201 was effective even at 0.1 nM. At high concentration (100 nM) AN-207 acts non-specifically, killing a

high percentage of the population of both receptor-positive and -negative cell lines. This non-selective action of analog AN-207 at 100 nM can be explained by its hydrolysis in SFM and the release of radical AN-201. HPLC analysis of AN-207 after incubation in SFM for 30 min at 37°C indicates that about 15% of AN-201 is released from the conjugate (data not shown). In line with these findings, our results show that AN-207 at 100 nM has the same cytotoxicity on LHRH receptor-negative UCI-107 cells as 10 nM concentration of AN-201. Consequently, concentrations higher than 10 nM were not used in further experiments.

Analog AN-207 at 2–10 nM concentrations specifically kills ES-2 cells and cell death increases with increasing times of treatment. In UCI-107 cells, AN-207 at 10 nM has an effect similar to that in ES-2 cells, although cell death does not increase after 60 min of treatment. It might thus be argued that the effect of AN-207 at 10 nM in ES-2 cells is not caused by the presence of receptors for LHRH that mediate the uptake of the cytotoxic drug into the cells because UCI-107 cells do not express receptors for LHRH and their growth is still inhibited. This effect of AN-207 on ES-2 cells can be explained by hydrolysis which occurs in the culture medium. After 30 min of incubation in SFM almost 15% of AN-207 was hydrolyzed, releasing AN-201, which can freely cross the cell membrane and inhibit cell growth. Because AN-201 at 10 nM kills about 80% of cells after 30 min, the observation that approximately 65% of the UCI-107 cells are still alive after 60 min of treatment with AN-207 at 10 nM is due to the fact that 'unhydrolyzed' AN-207 is not able to cross the cell membrane without specific receptors that can bind and transport it into the cytoplasm. In addition, AN-207 at concentrations ranging from 2 to 8 nM caused inhibition in UCI-107 only after 120 min of incubation when much AN-207 was hydrolyzed to AN-201 and the carrier.

The LHRH receptor-mediated internalization of a chromophore-labeled analog AN-152, the DOX-containing counterpart of AN-207, was recently demonstrated with a sophisticated optical tracking method in MCF-7 human breast cancer cells. In contrast, no uptake of the chromophore analog could be seen in UCI-107 cells even after 60 min.[29] These findings are in agreement with our results demonstrating specific targeting of AN-207 to LHRH receptor-positive ES-2 cells.

The quantification of the allelic imbalance between distinct microsatellite alleles can be used to provide evidence for the genetic heterogeneity that exists in biological specimens.[11] We have recently applied this methodology to quantify the selectivity of action of cytotoxic hormone analogs in mixtures of different cancer cell lines.[10,30]

Our microsatellite analyses on ES-2 cells co-cultured with the receptor-negative UCI-107 cells also demonstrate that AN-207 selectively targets ES-2 cells. When co-cultured cells were treated with AN-207, the ES-2: UCI-107 ratio of cell populations was 0.9:1 after 30 min and 0.75:1 after 60 min. Increasing times of incubation did not provide a clearer proof of targeting, most likely due to the deconjugation of radical AN-201 from analog AN-207. When different ratios of cell population were used at the seeding time, the proportion of untreated cells at the end of the experiment showed that both cell lines had similar kinetics because the ratio was maintained until the end of the experiment (not shown). Thus, the decrease in the number of ES-2 cells after treatment with AN-207 was not due to different kinetics of the cell lines tested, but to a selective effect of the cytotoxic LHRH analog AN-207.

Our findings *in vitro* explain and clarify the results of previous *in vivo* studies showing that cytotoxic analogs of LHRH, AN-152 and AN-207, powerfully inhibited the growth of LHRH receptor-positive human epithelial ovarian cancer cell line OV-1063 xenografted into nude mice, while the growth of UCI-107 was not affected.[14,31]

Collectively our results suggest that the use of targeted cytotoxic LHRH analogs such as AN-207 should permit a more selective delivery of the chemotherapeutic radicals to cancers such as ovarian that express LHRH receptors, leading to an improvement in the outcome of the treatment.

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