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Selective induction of apoptosis by the cytotoxic analog AN-207 in cells expressing recombinant receptor for luteinizing hormone-releasing hormone

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ABSTRACT **The selectivity of ligands specific for certain cells can be used to preferentially target chemotherapeutic compounds to neoplastic cells. Human breast, ovarian, endometrial, and prostatic cancers express receptors that can mediate the delivery of targeted cytotoxic compounds to neoplastic cells. Recently, a potent derivative of 2-pyrrolinodoxorubicin (AN-201) conjugated to [D-Lys[6]] luteinizing hormone-releasing hormone (LH-RH) (AN-207), was demonstrated to be less toxic than the nonconjugated chemotherapeutic radical and significantly more active in slowing neoplastic cellular growth. In this study we investigate the molecular mechanisms underlying the cytotoxic action of AN-207. We stably transfected COS cells with a LH-RH receptor (LH-RH-Rc) mammalian expression vector and examined the effect of AN-207 on known markers of cellular apoptosis. Apoptotic induction by AN-207, as measured by Bax and Bcl-2 protein levels, was increased in stable cells that express LH-RH-Rc compared with parental cells. DNA fragmentation also was increased by AN-207 treatment when compared with AN-201. Clinically used LH-RH antagonists partially inhibited apoptotic Bax expression and DNA fragmentation induced by AN-207, and blocked AN-207 induced down-regulation of Bcl-2 steady-state protein levels. In cell proliferation studies, after 72 h AN-207 exhibited greater cytotoxicity than AN-201 at equivalent concentrations, in COS cells expressing LH-RH-Rc but not in parental COS cells. In addition, survival of LH-RH-Rc positive cells treated with AN-207 was partially restored by LH-RH antagonist. This study demonstrates the receptor-specific cytotoxic effect of 2-pyrrolinodoxorubicin conjugated to [D-Lys[6]] LH-RH, exerted through induction of apoptosis and modulation of Bax, Bcl-2, and DNA fragmentation.**

Chemotherapeutic analogs conjugated to cell- and tumor-specific ligands have been designed because of their potential to reduce the nonspecific toxic side-effects and to increase their efficacy on targeted tissues (1, 2). The presence of luteinizing hormone-releasing hormone (LH-RH) receptors in breast, ovarian, and endometrial cancers, as well as in most prostate cancers, has led to synthesis of novel cytotoxic LH-RH analogs as potential targeted therapeutic agents (1–6). A potent derivative of the chemotherapeutic agent doxorubicin, 2-pyrrolinodoxorubicin (AN-201) (7), conjugated to [D-Lys[6]] LH-RH (8), was demonstrated to be less toxic than nonconjugated chemotherapeutic radicals and significantly more active in slowing neoplastic cellular growth (6, 9).

Recently, intracellular signaling mechanisms that induce apoptosis have been increasingly well characterized (10–12). Although there are multiple points of apoptosis induction and downstream effectors, cytotoxicity and inducible cellular death ultimately rely on a central apoptotic pathway that involves the caspases proteolytic cascade in both nuclear DNA and cellular structural and regulatory proteins. Caspases are dynamically regulated by a net equilibrium of proapoptotic Bax and antiapoptotic Bcl-2 regulatory proteins. Bax may heterodimerize with Bcl-2 to competitively inhibit its action, and Bax homodimers can induce proapoptotic intracellular signaling pathways (12). Therefore, stoichiometric ratios of these two effectors are critical in determining cell viability and apoptotic induction.

Thus we studied the effects of cytotoxic conjugate consisting of 2-pyrrolinodoxorubicin linked to [D-Lys[6]] LH-RH (AN-207) by using mammalian COS cells that stably express recombinant human LH-RH receptor (LH-RH-Rc), and compared its effects to parental COS cells that do not express endogenous LH-RH-Rc. We examined nucleosome cleavage patterns in transfected and parental cell lines exposed to either unconjugated 2-pyrrolinodoxorubicin (AN-201) or AN-207. Steady-state levels of both Bax and Bcl-2, as well as overall cell proliferative rates, also were assessed for the two compounds in both cell lines. Our results indicate that stable cell lines expressing LH-RH-Rc are more sensitive to the cytotoxic effects of LH-RH conjugate AN-207 and that its apoptotic action is induced through the Bax/Bcl-2 regulatory pathway.

MATERIALS AND METHODS

Generation of Stable COS Cell Lines with Inducible Human LH-RH-Rc Expression. COS-7 cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin (GIBCO/BRL) in 95% air/5% CO_2 at 37°C. Recombinant human LH-RH-Rc cDNA (provided by Thomas Gudermann, Freie Univerisität, Berlin, Germany; ref. 13) with an N-terminal hemagglutinin (HA) tag was inserted into the *Hin*dIII/*Nco*I site of pIND inducible expression vector (Invitrogen) to generate LH-RH-Rc/pIND. Cells (2×10^5/well) then were cotransfected with LH-RH-Rc/pIND and pVgRXR plasmids (ecdysone inducible expression system, Invitrogen), using Lipofectamine (GIBCO/BRL) according to manufacturer's optimized protocol for COS cells. Stable COS cell clones were selected with zeocin (200 μg/ml) (Invitrogen) and neomycin (400 μg/ml) (GIBCO/BRL). Several clonal cell lines were expanded from single foci and subsequently were screened by Western blotting for expression of HA-tagged LH-RH-Rc in

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Abbreviations: LH-RH, luteinizing hormone-releasing hormone; LH-RH-Rc, LH-RH receptor; FCS, fetal calf serum.

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the presence of the ecdysone analogs at indicated concentration for 24 h (Invitrogen).

Transient Transfection/Functional Assays of Recombinant LH-RH-Rc. A human glycoprotein hormone α-subunit responsive reporter (α-846 Luc/paLuc, courtesy of J. L. Jameson, Northwestern University, Chicago) was transiently transfected (1 μg/well) in stable COS cell lines by using Lipofectamine according to manufacturer's protocol (GIBCO/BRL). Ecdysone analog (10 μM; Invitrogen) was added for 24 h to culture medium. [D-Lys[6]] LH-RH and/or LH-RH antagonist (Cetrorelix, ASTA Medica, Frankfurt Am-Main, Germany) then were added at the indicated concentrations to triplicate wells of transiently transfected cells. After 24 h, cells were lysed in 150 μl of lysis buffer containing 1% Triton X-100, 10% glycerol, 2 mM EDTA, 2 mM DTT, and 25 mM Tris-phosphate, pH 7.8. Luciferase activity was assayed in 100 μl of cell lysate by using a luminometer (EG & G Berthold, Gaithersburg, MD) in the presence of D-luciferin (Molecular Probes) and ATP (Sigma).

DNA Fragmentation Assay. Stable COS cell lines were seeded in 6-well plates at 10^4 cells/well and incubated for 24 h in the presence of 10 μM ecdysone analog in 10% FCS/DMEM. AN-201, AN-207, both synthesized at Tulane University as described (7, 8), and the LH-RH antagonist antide (Bachem) then were added to the medium at the indicated concentrations. After 72 h, cells were harvested and incubated with lysis buffer [20 mM EDTA/10 mM Tris·HCl, pH 8.0/0.5% SDS (Sigma)/100 μg/ml Proteinase K (Boehringer Mannheim)] at 37°C for 1 h, followed by ribonuclease A (Promega) (100 μg/ml) digestion. Genomic DNA was extracted by phenol-chloroform protocol (Amresco, Solon, OH) and precipitated with 60% (vol/vol) isoproterenol. DNA (2 μg) was labeled with α[P^{32}]dCTP by using 5 units of terminal deoxynucleotidyl transferase (Amersham Pharmacia) per sample for 90 min at 37°C as described (14). Labeled DNA fragments were reprecipitated with 60% isoproterenol and resolved by using 10% PAGE, followed by autoradiography.

Immunoblotting Assay. Stable LH-RH-Rc COS cells were incubated for 36 h in the presence of 10 μM ecdysone analog in 10% FCS/DMEM, followed by administration of AN-201, AN-207, and/or Cetrorelix to culture medium at the indicated concentrations for 1 h. Then cells were rinsed once with PBS and solubilized with buffer containing 25 mM Tris·HCl, 250 mM sodium chloride, 2 mM EDTA, 1% Triton X-100, and 10 μg/ml aprotinin (Sigma) on ice. The homogenates were centrifuged at 10,000 × *g* for 15 min. Protein concentration in each sample was determined by Bradford assay (Sigma), and equal amounts of protein (70 μg/lane) were separated by 10% SDS/PAGE. The resolved proteins were electroblotted onto poly(vinylidene difluoride) filters (Micron Separations) in transfer buffer (192 mM glycine/20% methanol/25 mM Tris·HCl). To reduce nonspecific binding, the filters were incubated in a blocking buffer (20 mM Tris, pH 7.6/137 mM sodium chloride/10% nonfat milk/0.1% Tween-20) at 5°C overnight. Filters then were incubated with specific primary antibodies at indicated dilution for 1 h at room temperature, rinsed in wash buffer (Tris-buffered saline, pH 7.6, and 0.1% Tween-20) four times for 10 min each. Primary antibodies used were anti-Bax (1:2,000) and anti-Bcl-2 (1:1,000) rabbit polyclonal antibodies (Santa Cruz Biotechnology), as well as antiactin (1:2,000) and antihemagglutinin (1:500) mAb (Boehringer Mannheim). Secondary antibodies used were anti-rabbit (1:4,000) or anti-mouse (1:4,000) IgG conjugated with horseradish peroxidase (Boehringer Mannheim). Western blots were visualized by enhanced chemiluminescence (Boehringer Mannheim).

Cell Proliferation Assay. The Cell Titer 96AQueous One solution cell proliferation assay (Promega) was used to assess the number of viable cells after AN-201 and AN-207 administration. Cells were plated in 6-well plates at 5,000 cells/well in 10% FCS/DMEM and treated with 10 μM ecdysone analog for 36 h. AN-201, AN-207, and LH-RH antagonist Cetrorelix were added in the indicated dosages to triplicate wells for 1 h in the presence of ecdysone analog at 10 μM concentration. The cells were washed twice with PBS and were cultured further in 10% FCS/DMEM medium. Cell Titer 96AQueous One solution was added to each well for 30 min, and the light absorbance at 490 nm was recorded by using a spectrophotometer as a direct measurement of cell viability. Cell viability data at 72 h are presented as the mean ± SD of triplicate wells. Statistical significance was tested by Student's *t* test by using SIGMAPLOT, version 3.06, Jandel Software.

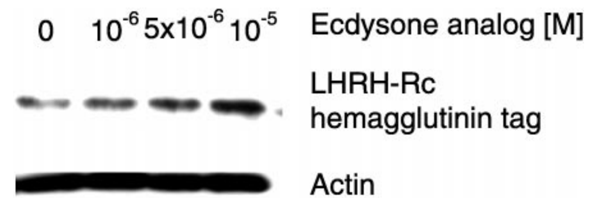

FIG. 1. Human recombinant LH-RH-Rc protein expression in response to ecdysone analogs. After 24-h induction with ecdysone analog at the indicated concentration, 100 μg of cell lysate was fractionated on 6% PAGE and transferred onto poly(vinylidene difluoride) membrane for Western analysis. Western blotting was performed with monoclonal primary antibody to hemagglutinin tag of LH-RH-Rc. Actin blots show no statistical difference between amounts of protein loaded in any two groups.

RESULTS

Characterization of Stable COS Cell Lines Expressing Human Recombinant LH-RH-Rc. Stable COS cell lines harboring recombinant LH-RH-Rc first were functionally tested for inducible expression by the ability of the receptor to up-regulate a human glycoprotein hormone α-subunit promoter/luciferase (αSU/Luc) reporter fusion gene. Fig. 1 shows Western blots demonstrating inducibility of recombinant LH-RH-Rc protein expression in response to ecdysone analog, with maximal stimulation at a concentration of 10 μM. Fig. 2 demonstrates that inducible receptor expression leads to significant ($P < 0.0001$) transcriptional up-regulation of αSU/Luc reporter that can be blocked by coadministration of the LH-RH antagonist Cetrorelix. No transcriptional up-

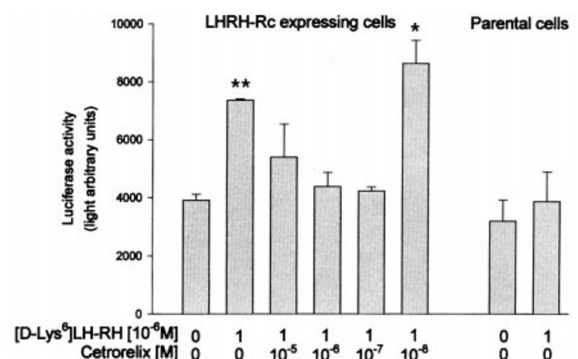

FIG. 2. Luciferase activity in LH-RH-Rc stable expressing cells induced by [D-Lys[6]] LH-RH (mean ± SEM). Stable expressing LH-RH-Rc cells were transiently transfected with 1 μg α-846 Luc/paLuc, and the receptor was induced with 10 μM ecdysone analog for 24 h. [D-Lys[6]] LH-RH agonist and/or LH-RH antagonist (Cetrorelix) then were added at the indicated concentrations to triplicate wells of transiently transfected cells. After 24 h, cells were lysed in 150 μl of lysis buffer, and luciferase activity was assayed in 100 μl of cell lysate according to vendor. *, $P < 0.005$; **, $P < 0.0001$, significant difference from the value of control group, by Student's *t* test.

regulation of the αSU/Luc reporter was observed in parental COS cells that are LH-RH-Rc negative.

Induction of DNA Fragmentation by Cytotoxic LH-RH Conjugate AN-207. To determine whether administration of AN-207 induces nucleosomal fragmentation of DNA in COS cells stably expressing LH-RH-Rc, 24-h receptor induction was followed by treatment for 24 h with 1 nM AN-201, AN-207, or AN-207 plus LH-RH antagonist antide (1 μM). Labeled DNA fragmentation patterns at 72 h are shown in Fig. 3. DNA fragmentation was notably increased in AN-207-treated stable cells, whereas cells exposed to either AN-201 or cotreated with AN-207 and antide showed DNA fragmentation pattern similar to untreated control wells. No DNA fragmentation was observed in COS cells. These data indicate that AN-207 specifically induces cellular apoptotic nuclear DNA cleavage. However, an identical concentration of cytotoxic analog AN-201 appears to exhibit less cytotoxicity by this assay.

Regulation of Bax and Bcl-2 During AN-207-Induced Cellular Apoptosis. To study the effects of AN-207 on cellular factors that regulate apoptosis, we treated parental and stable COS cell lines with either AN-201, AN-207, and/or LH-RH antagonist Cetrorelix. Treatment of LH-RH-Rc stable expressing cell line with AN-207 caused Bax levels to increase compared with control levels and levels obtained in cells treated with similar concentration of unconjugated 2-pyrrolinodoxorubicin (AN-201) (Fig. 4). Decreased Bcl-2 protein levels were observed in cells treated with AN-207 compared with controls and similar concentration of AN-201 (Fig. 5). The specific changes in protein levels could be decreased by coadministration of LH-RH antagonist Cetrorelix. Parental COS cells exhibited no changes in Bcl-2/Bax proteins with any treatments.

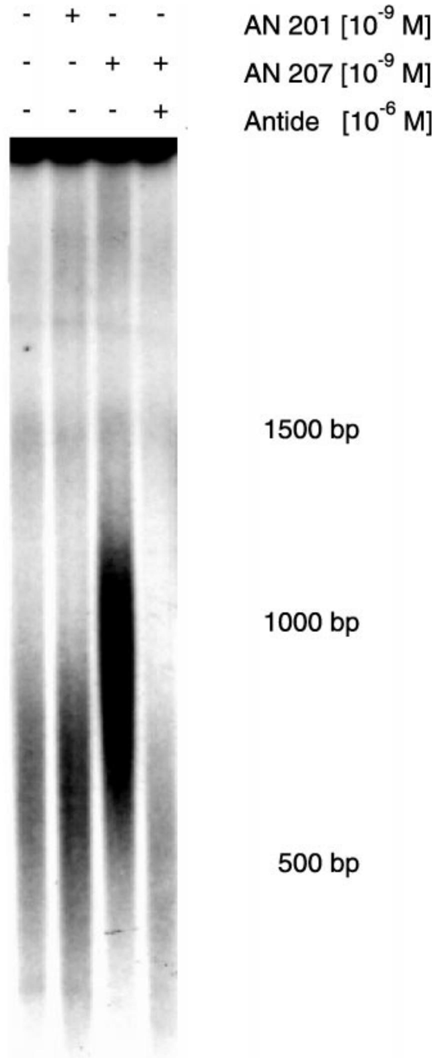

FIG. 3. Induction of DNA fragmentation by AN-207. Stable COS cell lines were seeded at 10^4 cells/well and incubated for 24 h with 10 μM ecdysone analog. AN-201, AN-207, and the LH-RH antagonist antide then were added to the medium at the indicated concentrations. After 72 h, cells were harvested and incubated with lysis buffer at 37°C for 1 h, followed by ribonuclease A digestion. [α-^{32}P]dCTP-labeled genomic DNA fragments were isoproterenol-reprecipitated and resolved by using 10% PAGE, followed by autoradiography.

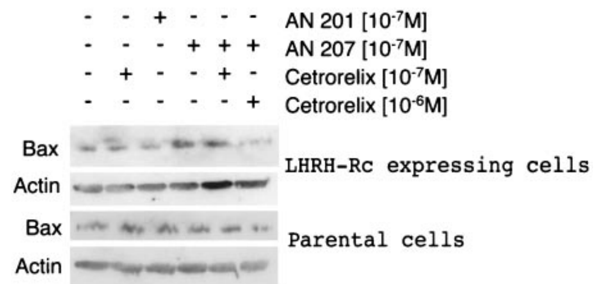

FIG. 4. Bax protein levels in parental and LH-RH-Rc-expressing cells. Stable LH-RH-Rc COS cells and parental COS cells were incubated for 36 h in the presence of 10 μM ecdysone analog, followed by administration of AN-201, AN-207, and/or Cetrorelix at doses indicated for 1 h. Equal amounts of protein (70 μg/lane) were separated by 10% SDS/PAGE and transferred onto poly(vinylidene difluoride) membranes. Membranes were incubated with anti-Bax and antiactin antibodies for 1 h at room temperature. Secondary antibodies used were anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase. Western blots were visualized by chemiluminescence.

Cell Proliferative Responses to AN-207. The cytotoxic effect of AN-207 as measured by cellular viability was compared with nonconjugated AN-201 in both parental and LH-RH-Rc-expressing COS cells. The results of this viability assay are shown in Fig. 6. The parental and LH-RH-Rc-induced stable COS cells showed no loss of cell viability at 24 h when treated with cytotoxic drugs for 1 h. At 72 h, treatment with 10^{-7} M AN-207 resulted in a significantly ($P < 0.0003$) lower viability of LH-RH-Rc-induced stable COS cells compared with AN-201. The specific effect of cytotoxic LH-RH conjugate AN-207 at 10^{-7} M was significantly inhibited by LH-RH antagonist Cetrorelix at concentrations of 10^{-7} M ($P < 0.002$) and 10^{-6} M ($P < 0.00006$). We observed significant cytotoxic effects at 72 h in both parental and stable cell lines after 1-h treatment.

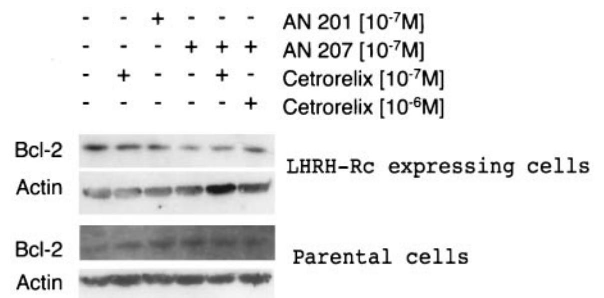

FIG. 5. Bcl-2 protein levels in parental and LH-RH-Rc-expressing cells. Stable LH-RH-Rc COS cells and parental COS cells were incubated for 36 h in 10 μM ecdysone analog, followed by administration of AN-201, AN-207, and/or Cetrorelix at doses indicated for 1 h. Equal amounts of protein (70 μg/lane) were separated by 10% SDS/PAGE and transferred onto poly(vinylidene difluoride) membranes. Membranes were incubated with anti-Bcl-2 and antiactin antibodies for 1 h at room temperature. Secondary antibodies used were anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase. Western blots were visualized by chemiluminescence.

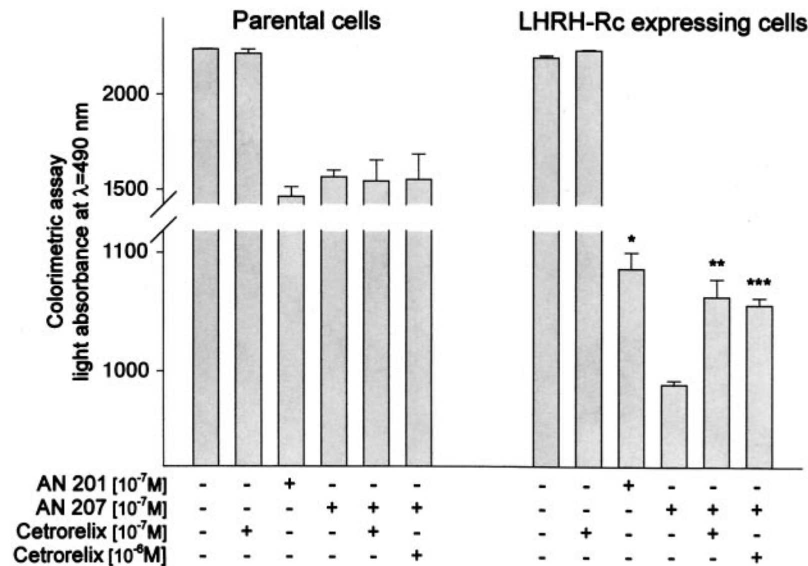

FIG. 6. Cytotoxicity of AN-207 as measured by cellular viability at 72 h, compared with AN-201 in both parental and LH-RH-Rc stable expressing cells. Cells were plated in 6-well plates at 5,000 cells per well in DMEM/10% FCS, and treated with 10 μM ecdysone analog for 36 h. AN-201, AN-207, and LH-RH antagonist Cetrorelix were added in the indicated doses to triplicate wells for 1 h. Cells were washed twice with PBS and were cultured further in 10% FCS/DMEM medium. Cell Titer 96AQueous One solution was added to each well for 30 min, and the light absorbance at 490 nm was recorded by using a spectrophotometer as a direct measurement of cell viability. Cell viability data at 72 h are presented as the mean ± SD of triplicate wells. Statistical significance was tested by using Student's *t* test. $*$, $P < 0.0003$; $**$, $P < 0.002$; $***$, $P < 0.00006$, significant difference from the value of AN-207-treated LH-RH-Rc-expressing cells.

In parental cells, AN-201 and AN-207 had the same cytotoxic potency measured by colorimetric titers.

DISCUSSION

The selectivity of ligands specific for certain cells can be used to preferentially target chemotherapeutic compounds to neoplastic cells (1–5). Human breast, ovarian, endometrial, and prostatic cancers express receptors that can mediate the delivery of targeted cytotoxic compounds to neoplastic cells. Antineoplastic compounds conjugated to hormone derivatives may provide a viable approach to targeted chemotherapy. In this study we investigated the molecular mechanisms underlying the cytotoxic action of 2-pyrrolinodoxorubicin conjugated to [D-Lys^6] LH-RH (AN-207) (8) on COS cells stably expressing LH-RH-Rc.

We studied apoptosis markers in parallel in COS cells and COS cells stably transfected with the LH-RH-Rc gene and found a cell-specific cytotoxicity of AN-207 as determined by changes in the expression of known apoptotic markers Bax and Bcl-2. Clinically used LH-RH antagonists, Cetrorelix (15–17) or antide, partially inhibited apoptotic Bax expression and DNA fragmentation induced by AN-207 and blocked AN-207 induced down-regulation of Bcl-2 steady-state protein levels. In cell proliferation studies at 72 h after a 1-h exposure to the analogs, AN-207 exhibited greater cytotoxicity than its cytotoxic radical AN-201 at equivalent concentrations in COS cells stably expressing LH-RH-Rc but not in parental COS cells. Although AN-207 was more effective than AN-201 for induction of nucleosome cleavage, Bax/Bcl-2 expression, and cellular death, we noted an overall nonspecific effect of unconjugated 2-pyrrolinodoxorubicin (AN-201) in stable COS cells expressing LH-RH-Rc. This effect was greater than that observed in parental cell lines. Survival of LH-RH-Rc positive cells treated with AN-207 was restored by LH-RH antagonist Cetrorelix to levels observed after AN-201 treatment. These data support the hypothesis that AN-207 exerts its effect through binding to LH-RH-Rc.

Apoptosis is an integral biologic mechanism of many developmental and pathophysiologic states and reflects multiple biochemical events that ultimately lead to cell death via common mechanisms of cellular membrane perforation, chromatin condensation, DNA fragmentation, cell shrinkage, and disassembly into membrane-enclosed vesicles (apoptotic bodies) (10). Apoptotic signaling varies from cell to cell, and different inducers may trigger distinct signaling pathways. Recent studies suggest that these pathways converge on self-destruction mechanisms that are in part modulated by a net equilibrium of proapoptotic Bax and antiapoptotic Bcl-2 regulatory proteins (12). Repression or down-regulation of steady-state protein levels of the antiapoptotic Bcl-2 with concurrent increases in Bax levels is a common mechanism for apoptotic induction. Bcl-2 homodimers prevent the apoptosis signaling cascade whereas Bax heterodimerizes with death antagonists such as Bcl-2 and Bcl-x_L. Bax homodimers trigger apoptotic signaling at multiple levels. This ratio of Bcl-2 family death agonists to antagonists dictates the susceptibility of cells to an apoptotic stimulus (12). Caspase 3 activity can be induced by proapoptotic Bax and inhibited by the antiapoptotic Bcl-2. Activation of caspase family enzymes results in cleavage of cytoskeletal and nuclear proteins, and nucleosomal fragmentation of DNA (14). Convergent events in apoptosis center on mitochondria. Release of caspase activators, such as cytochrome *c*, changes in electron transport, loss of mitochondrial transmembrane potential, altered cellular oxidation-reduction, and participation of Bcl-2 family proteins lead to apoptosis (11). However, other studies have shown that inhibiting caspases does not always reduce cell death induced by Bcl-2 proapoptotic markers (18, 19).

Thus, these data are consistent with increased apoptotic induction by AN-207 in cells that synthesize recombinant LH-RH-Rc. This model system may be useful in future studies examining the pharmacology and efficacy of novel LH-RH conjugated analogues, as well as the ability of LH-RH antagonists such as Cetrorelix to block the apoptotic effects of such conjugates. In addition, because the LH-RH-Rc is expressed by using an inducible vector system, cell lines can be manipulated to test the apoptotic effect of conjugates as a function of receptor density, which may be considered when designing targeted cytotoxic analogs.

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