Mol. Cells, Vol. 9, No. 1, pp. 31–36

Molecules and Cells

Luteinizing Hormone Releasing Hormone-RNase A Conjugates Specifically Inhibit the Proliferation of LHRH-Receptor-Positive Human Prostate and Breast Tumor Cells

Yong Song Gho† and Chi-Bom Chae*

Department of Life Science, Pohang University of Science and Technology, Pohang 790-784, Korea.

(Received on July 11, 1998)

Human prostate and breast tumor cells produce luteinizing hormone-releasing hormone (LHRH) receptors on their cell surface even when they have lost dependency on sex steroid hormones for growth. To investigate whether LHRH can be used as a cell-binding moiety to deliver toxin molecules into prostate and breast tumor cells, LHRH-bovine RNase A conjugates were constructed using the chemical cross-linking method. The treatment of the LHRH receptor-positive cells such as prostate LNCapFGC and breast MCF7 tumor cells with LHRH-RNase A conjugates resulted in a dose-dependent inhibition of growth. The cytotoxic activities of these conjugates were effectively reduced by the presence of exogenous LHRH. Either free RNase A or LHRH alone did not affect the proliferation of these cells. The LHRH-RNase A conjugates did not show cytotoxicity against FRTL5 and TM4 cells which do not express the LHRH receptors. These results suggest that LHRH can be used as a cell-binding molecule for the specific delivery of toxin molecules into the cells which express LHRH receptors on their surface. Thus, a new class of biomedicines that act as fusion proteins between LHRH and toxins will give us a new avenue for the treatment of human prostate and breast cancers, regardless of their steroid hormone dependency.

Keywords: Breast; LHRH; Prostate; RNase A; Tumor.

* To whom correspondence should be addressed.
Tel: 82-562-279-2125; Fax: 82-562-279-2199
E-mail: cbchae@postech.ac.kr

Introduction

An immunotoxin (IT) is a cytotoxic agent that consists of a cell-binding moiety and a toxin moiety. The cell-binding moiety can be an antibody, a growth factor, or a hormone that binds selectively to the cell surface molecules of certain cell types. The toxin moiety can be a naturally occurring *Pseudomonas* exotoxin, *diphtheria* toxin, or plant toxin. These immunotoxins can kill cells with selectivity in tissue culture and in animal models (Aaron and Phillip, 1981; Ahmann *et al.*, 1987). However, the toxins derived from bacteria are very immunogenic molecules and some immunotoxins are toxic to the liver because of the nonspecific uptake by the liver (Bajusz *et al.*, 1989; Beintema *et al.*, 1984). The use of a member of the human ribonuclease A superfamily such as serum protein angiogenin may alleviate the problems caused by the immunogenicity and liver toxicity (Bond, 1988; Brinkmann *et al.*, 1993; Denis and Mahler, 1990; Fekete *et al.*, 1989). When angiogenin was fused to the transferrin or anti-transferrin receptor monoclonal antibody by chemical cross-linking or recombinant methods, these immunotoxins exhibit cell type-specific cytotoxic activity (Denis and Mahler, 1990; Fekete *et al.*, 1989). In contrast, angiogenin alone does not have any cytotoxicity at appropriate concentrations.

LHRH is a decapeptide hormone released by the hypothalamus and acts on gonadotropes at the anterior pituitary to stimulate the release of the luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Potent agonists or antagonists of LHRH have been used for the

Abbreviations: EDC, 1-ethyl-3-(3-dimethylamino propyl) carbodiimide·HCl; FSH, follicle-stimulating hormone; IT,

treatment of sex steroid hormone-dependent tumors such as prostate and breast cancers (Isaacs and Coffey, 1981). The suppression of gonadal steroid secretion by hormonal therapy with these agonists and antagonists retards the growth of these hormone-dependent tumors. However, these hormone analogs may also act directly on the tumors. Human prostate and breast cancer cells produce LHRH receptors on their cell surface even when they have lost their dependency on sex steroid hormones for growth, and LHRH agonists or antagonists can inhibit the growth of some breast cancer cell lines (Jennes *et al.*, 1988; Miller *et al.*, 1985; Newton *et al.*, 1994). Highly potent metallopeptide analogs of LHRH show selective cytotoxicity against human prostate and breast cancer cell lines *in vitro*, and LHRH analogs containing cytotoxic radicals inhibit the growth of estrogen-independent MXT mouse mammary carcinoma cells *in vivo* (Newton *et al.*, 1996; Pai *et al.*, 1992). The LHRH analogs containing cytotoxic agents may be useful for the treatment of human prostate and breast cancer, especially for the hormone-independent tumors because hormonal therapy does not prevent the growth of hormone-independent cells (Pai *et al.*, 1990). These results implicate that LHRH can be used as a cell-binding moiety to deliver toxin molecules such as ribonuclease into prostate and breast tumor cells regardless of their steroid hormone dependency for growth.

To investigate this possibility, LHRH-bovine RNase A conjugates were constructed using the chemical cross-linking method. According to our preliminary experiments, these conjugates show cell type-specific cytotoxicity, and the cytotoxicity of these conjugates is abolished by the presence of excess LHRH. The results suggest that decapeptide LHRH can be successfully used as a cell-binding molecule for the specific delivery of toxin molecules into the cells which express LHRH receptors on their surface. Thus, construction of the fusion protein between LHRH and a member of the human ribonuclease superfamily such as pancreatic RNase A, angiogenin and eosinophil-derived neurotoxin will give us a new avenue to treat the human prostate and breast cancers regardless of their steroid hormone dependency.

Materials and Methods

Chemical conjugation of LHRH and RNase A Bovine pancreatic RNase A (2 mg, Sigma) and LHRH free acid (5 mg, Bachem California) were dissolved together in 0.8 ml of water, and the pH of the mixture was adjusted to 7–8. Next, 1-ethyl-3-(3-dimethylamino propyl) carbodiimide·HCl (100 mg, EDC, Pierce) freshly dissolved in 0.2 ml of water, was added. The reaction was permitted to proceed with gentle agitation at room temperature for 2 h and terminated by gel filtration on a PD10 column (Pharmacia) to remove the unreacted EDC and LHRH. aliquoted and stored at −70°C until use. The amount of conjugated protein was quantified by Bio-Rad protein assay reagents (Bio-Rad Laboratories) using bovine serum albumin as a standard.

Assay of ribonucleolytic activity The ribonucleolytic activity was determined in a reaction mixture (0.1 ml) containing 4 mg/ml of yeast tRNA (type X, Sigma), 10 µM of PMSF, 0.1 mg/ml of human serum albumin (Calbiochem), 33 mM HEPES/NaOH pH 7.5, 33 mM NaCl, and appropriate concentrations of RNase A or LHRH-RNase A conjugates. After 15 min incubation at 37°C, the reaction was terminated by the addition of 0.24 ml of 3.4% ice-cold perchloric acid. The samples were kept on ice for 10 min and then centrifuged for 10 min at 4°C. Absorbance of the supernatant was determined at 260 nm (Ramakrishnan *et al.*, 1992). The activity of LHRH-RNase A conjugates was compared with that of non-conjugated RNase A. All experiments were carried out in duplicates.

Cell culture LNCaP·FGC human prostate adenocarcinoma cells (ATCC CRL 1740) were grown in RPMI 1640 with 10% fetal bovine serum. MCF7 human breast adenocarcinoma cells (ATCC HTB 22) were cultured in Eagle's MEM with 10% fetal bovine serum. TM3 mouse Leydig cells (ATCC CRL 1714) and TM4 mouse Sertoli cells (ATCC CRL 1715) were grown in a 1:1 mixture of Ham's F12 medium with Dulbecco's modified Eagle's medium containing 5% horse serum and 2.5% fetal bovine serum. FRTL-5 rat thyroid cells (ATCC CRL 8305) were cultured in Coon's modified Ham's F12 medium with 5% bovine calf serum. Cells were subcultured after trypsinization, and the medium was changed every 2–3 d.

Cytotoxicity assay Each well of a tissue culture plate (96 well, Nunc) was plated with 10^4 cells. After incubation at 37°C for 2 d, 100 µl of varying amounts of LHRH-RNase A conjugates, LHRH or RNase A samples in culture medium was added to each well. After incubation for two additional days, the number of viable cells was determined using Cell Titer 96 Non-Radioactive Proliferation assay kit (Promega) according to the manufacturer's instructions. Each experiment was carried out in triplicates, and an average value was used to determine percent viability.

A time course of the action of LHRH-RNase A conjugates against LNCapFGC and MCF7 cells was investigated as follows. Cells were placed in a 96-well tissue culture plate at 10^3 cells per well and incubated for 2 d. The cells were grown for 2, 5, and 9 additional days in the presence of 1 µM of LHRH-RNase A conjugates.

Results

Preparation of LHRH-RNase A conjugates Natural LHRH does not have any free carboxyl or amino groups (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2). Therefore, bovine pancreatic ribonuclease A (RNase A) was coupled to LHRH free acid which contains a Gly-free carboxyl group at the C-terminus instead of Gly-amide

conjugates from RNase. RNase A was reacted with a 35-fold molar excess of LHRH free acid. The unreacted LHRH and EDC were separated from the conjugates by gel filtration on a PD 10 desalting column. Successful conjugation between the reactants was confirmed by SDS-PAGE and Western blotting using monoclonal antiLHRH antibody (data not shown). The ribonucleolytic activity of LHRH-RNase A was 10-fold lower than that of RNase A. This reduction of enzymatic activity of the conjugates may be due to the modification of some lysine at the active center of RNase A (Rodriguez *et al.*, 1993).

The cell type-specific cytotoxic effect of LHRH-RNase A conjugates To investigate whether LHRH-RNase A conjugates can inhibit proliferation of the cells which retain LHRH receptors on their surface, human prostate adenocarcinoma LNCapFGC cells and human breast adenocarcinoma MCF7 cells were treated with varying amounts of conjugates for 2 d. As shown in Figs. 1 and 2, the conjugates inhibit the proliferation of these cells in a concentration-dependent manner, and the concentrations of conjugates giving a 50% inhibition of cell proliferation were found to be ~0.5 μM and ~0.7 μM for LNCapFGC and MCF7 cells, respectively. However, RNase A and LHRH alone do not affect the growth of both cells at up to 10 μM. Therefore, the chemical attachment of LHRH to RNase A is needed for the cytotoxicity of these conjugates.

To demonstrate that the cytotoxicity of LHRH-RNase A conjugates was due to the binding to the LHRH receptor, two types of experiments were carried out. First, the cytotoxic activity of varying concentrations of conjugates against LNCapFGC and MCF7 cells was investigated in the presence of 10 nM of LHRH. Exogenous LHRH effectively reduces the cytotoxicity of LHRH-RNase A conjugates against these cells (Figs. 1 and 2). Secondly, we investigated whether LHRH-RNase A conjugates could be delivered into specific cells which express LHRH receptors on their surface. The conjugates had no cytotoxic activities against LHRH-receptor-negative Sertoli TM4 cells and thyroid FRTL5 cells (Fig. 3). However, the proliferation of Leydig TM3 cells which express LHRH receptors on their cell surface was also inhibited by the presence of conjugates.

Prolonged inhibition of cell proliferation by LHRH-RNase A conjugates We investigated a time course of the cytotoxic activity of LHRH-RNase A conjugates against LNCapFGC and MCF7 cells by growing the cells for 2, 5, and 9 d in the presence of conjugates. The inhibition of the growth of LNCapFGC and MCF7 cells continued for 9 d. This effect is not due to the possible deterioration of culture medium since the control cells

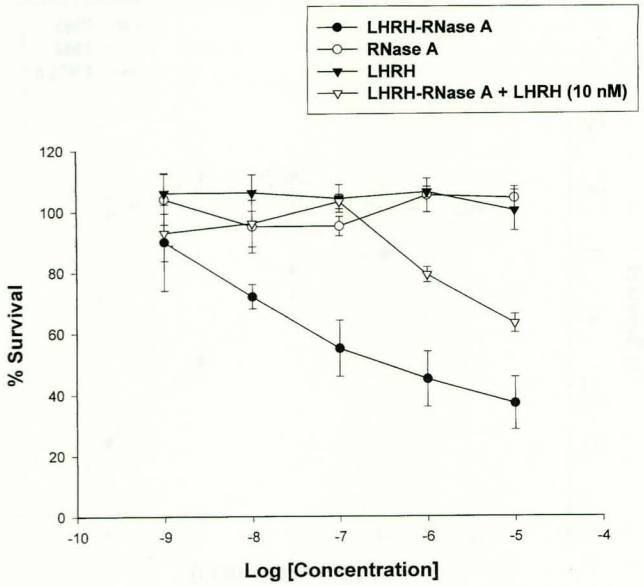

Fig. 1. Cytotoxicity of LHRH-RNase A conjugates against LNCapFGC prostate cancer cells. LNCapFGC cells were treated with varying concentrations of LHRH-RNase A, RNase A and LHRH for 2 d and percentage viability was determined as described in Materials and Methods. The cytotoxic activity of varying concentrations of LHRH-RNase A conjugates against LNCapFGC cells was also investigated in the presence of 10 nM of LHRH.

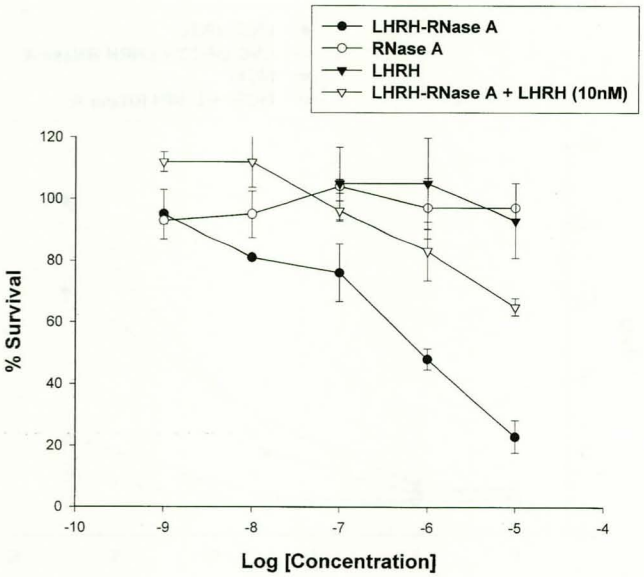

Fig. 2. Cytotoxicity of LHRH-RNase A conjugates against MCF7 breast cancer cells. MCF7 cells grown in culture were treated with varying concentrations of LHRH-RNase A, RNase A and LHRH for 2 d and percentage viability was determined as described in Materials and Methods. The cytotoxic activity of varying concentrations of LHRH-RNase A conjugates against

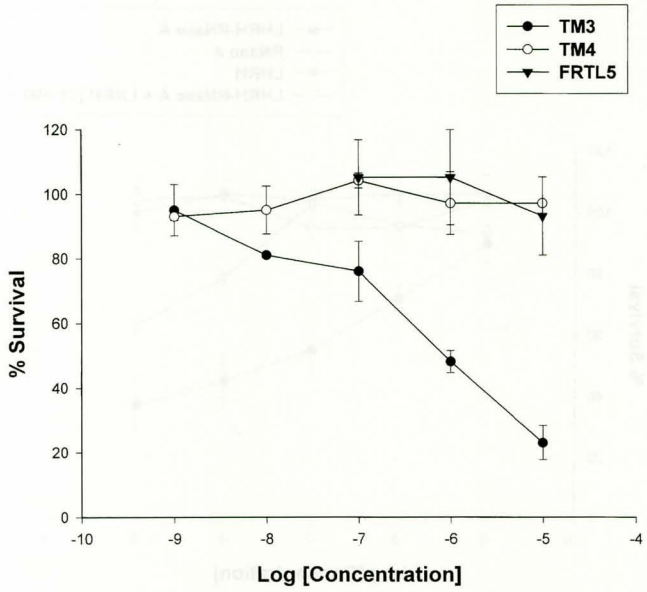

Fig. 3. Cell type-specific cytotoxic activities of LHRH-RNase A conjugates. Leydig TM3, Sertoli TM4, and thyroid FRTL5 cells grown in culture were treated with varying amounts of LHRH-RNase A conjugates for 2 d and percentage viability was determined as described in Materials and Methods.

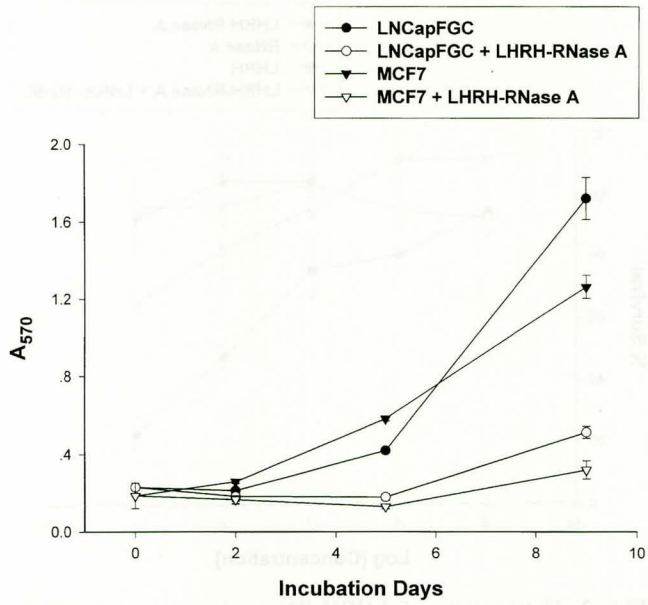

Fig. 4. Time course of the action of LHRH-RNase A conjugates. LNCapFGC and MCF7 cells were treated with 1 μM of LHRH-RNase A for 2, 5, and 9 d and viable cells were determined as described in Materials and Methods. For control experiments, LNCapFGC and MCF7 cells were grown in the absence of

Discussion

Prostate and breast cancers are the major cancers leading to death in American men and women, respectively. The functions of the prostate and breast are regulated by steroid hormones such as androgen and estrogen, respectively. Furthermore, these sex hormones are necessary for the growth of hormone-dependent human prostate and breast tumors. Available options for the treatment of prostate and breast cancers are surgical orchiectomy, radiation therapy, hormone treatment and cytotoxic chemotherapy. Administration of analogs of the natural decapeptide LHRH stimulates the secretion of LH and FSH by gonadotropes. When chronic administration is continued, LH and FSH secretion is decreased by desensitization of the LHRH receptors in the gonadotropes, leading to a decrease of circulating steroid hormones and thus inhibiting the growth of prostate and breast cancers (FitzGerald and Gordon, 1992; Isaacs *et al.*, 1981). However, surgical and hormonal combination therapy to eliminate the testicular testosterone or the ovarian estrogen leaves an opportunity for the adrenal androgen and estrogen secreted by adrenal glands to stimulate the growth of prostate and breast cancers. Furthermore, hormone-independent prostate cancer (androgen-independent) and breast cancer (estrogen-independent) can still grow after these types of combinational treatments have been applied. In the case of cytotoxic chemotherapy, the standard cytotoxic agents that are normally used against other cancers primarily act against rapidly growing tumor cells, but prostate cancer is not a rapidly growing tumor. It is not clear whether some combination of chemotherapeutic agents which is effective for the treatment of hormone-independent tumors will be found. It seems that it is time to try some new avenues. In this sense, the hormone-toxin approach is intriguing.

The purpose for the construction of LHRH-RNase A conjugates by chemical conjugation was to evaluate the potential of these conjugates to kill human prostate and breast cancer cells which express LHRH-receptors. We found that the LHRH-RNase A conjugates can selectively kill the LHRH receptor-positive cells such as Leydig TM3, prostate LNCapFGC and breast MCF7 tumor cells but not FRTL5 and TM4 cells which do not express the LHRH receptors. Extracellular RNase A or LHRH alone is not cytotoxic toward LNCapFGC and MCF7 cells. Also, the cytotoxic activities of these conjugates can be effectively reduced by the presence of exogenous LHRH. These results clearly indicate that LHRH-RNase A conjugates can kill the specific cell types which express the LHRH-receptors and that the cell type-specific cytotoxic activities are due to the delivery of RNase A to the cytosol via LHRH receptor-mediated endocytosis.

The relatively high IC_{50} value of these conjugates might

A conjugates are heterogeneous and that the ribonuclease activity of the conjugate was 10-fold lower than that of natural RNase A. According to previous reports, the recombinant fusion protein between ribonuclease and the monoclonal antibody to the transferrin receptor was a thousand times as potent as the chemically linked hybrid (Bond, 1988; Denis and Mahler, 1990). Thus, construction of the fusion protein between LHRH and a member of the human ribonuclease superfamily such as pancreatic RNase A, angiogenin and eosinophil-derived neurotoxin eliminates the heterogeneity of chemically cross-linked LHRH-RNase A conjugates and may increase the cell type-specific cytotoxicity. Although several kinds of immunotoxins which show the cytotoxic activity against human prostate and breast cancer *in vitro* and *in vivo* have been developed as a fusion protein between the specific antibody for target cells and bacterial or plant toxin as a toxin moiety (Rybak *et al.*, 1991; 1992; Sharoni *et al.*, 1989), recombinant LHRH-RNase toxins may have several potential therapeutic advantages to immunotoxins for the following reasons. In contrast to previously developed immunotoxins, these new biomedicines may not cause immunogenesis and liver toxicity. These LHRH-RNase toxins may penetrate easily into the solid tumor due to their small size. Both Leydig cells in the testis and Granulosa cells in the ovary also express the LHRH-receptor on their surfaces (Isaacs *et al.*, 1981). These cells are major sources of androgen and estrogen which are needed for the growth of prostate and breast cancer cells, respectively. Thus, LHRH-RNase toxins may not only selectively kill the prostate and breast cancer cells, but also effectively eliminate the circulating androgen and ovarian estrogen by eliminating either the Leydig cells in men, or the Granulosa cells in women. LHRH receptors are also present in the central nervous system (Szepeshazi *et al.*, 1992). However, LHRH agonist has been used to treat human prostate and breast cancers and LHRH-cytotoxic radicals produce significant inhibition of tumor growth without visible effect on the brain function (Pai *et al.*, 1992; Vitetta *et al.*, 1983). This means that the circulating LHRH agonist and LHRH-toxin may not penetrate the brain-blood barrier. Similarly, LHRH-RNase toxins may not reach the brain nor cause any side effect on the brain function.

In conclusion, LHRH-RNase A conjugates which may not have immunogenic problems or liver toxicity will give us a new avenue for the treatment of human prostate and breast cancers regardless of their steroid hormone-dependency. In addition, its combination with surgical and hormonal therapy which have been used for the treatment of these cancers may increase their therapeutic potency.

Acknowledgments This work was supported in part by Pohang University of Science and Technology and the Korea

References

Aaron, J. W. and Phillip, B. C. (1981) Extrapituitary actions of gonadotropin-releasing hormone. *Endocrinology* **2**, 437–461.

Ahmann, F. R., Citrin, D. L., and DeHaan, H. A. (1987) Zoladex: a sustained release luteinizing hormone-releasing hormone analog for the treatment of advanced prostate cancer. *J. Clin. Oncol.* **5**, 912–917.

Bajusz, S., Janaky, T., Csernus, V. J., Bokser, L., Fekete, M., Srkalovic, G., Redding, T. W., and Schally, A. V. (1989) Highly potent metallopeptide analogues of luteinizing hormone-releasing hormone. *Proc. Natl. Acad. Sci. USA* **86**, 6313–6317.

Beintema, J. J., Wietzes, P., Weickman, J. L., and Glitz, D. G. (1984) The amino acid sequence of human pancreatic ribonuclease. *Anal. Biochem.* **136**, 48–64.

Bond, M. D. (1988) An *in vitro* binding assay for angiogenin using placental ribonuclease inhibitor. *Anal. Biochem.* **173**, 166–173.

Brinkmann, U., Gallo, M., Brinkmann, E., Kunwar, S., and Pastan, I. (1993) A recombinant active on prostate cancer cells and that is composed of the Fv region of monoclonal antibody PR1 and a truncated form of *Pseudomonas* exotoxin. *Proc. Natl. Acad. Sci. USA* **90**, 547–551.

Denis, L. and Mahler, C. (1990) Prostatic cancer: An overview. *Rev. Oncol.* **3**, 665–677.

Fekete, M., Redding, T. W., Comaru-Schally, A. M., Pontes, J. E., Connelly, R. W., Srkalovic, G., and Schally, A. V. (1989) Luteinizing hormone-releasing hormone, somatostatin, prolactin, and epidermal growth factor in rat and human prostate cancers and in benign prostate hyperplasia. *Prostate* **14**, 191–208.

Isaacs, J. T. and Coffey, D. S. (1981) Adaptation versus selection as the mechanism responsible for the relapse of prostatic cancer to androgen ablation therapy as studied in the dunning R-3327-H adenocarcinoma. *Cancer Res.* **41**, 5070–5075.

Jennes, L., Dalati, B., and Conn, P. M. (1988) Distribution of gonadotropin releasing hormone agonist binding sites in the rat central nervous system. *Brain Res.* **452**, 156–164.

Miller, W. R., Scott, W. N., and Morris, R. (1985) Growth of human breast cancer cells inhibited by a luteinizing hormone-releasing hormone agonist. *Nature* **313**, 231–233.

Newton, D. L., Nicholls, P. J., Rybak, S. M., and Youle, R. J. (1994) Expression and characterization of recombinant human eosinophil-derived neurotoxin and eosinophil-derived neurotoxin-anti-transferrin receptor sFv. *J. Biol. Chem.* **269**, 26739–26745.

Newton, D. L., Xue, Y., Olson, K. A., Fett, J. W., and Rybak, S. M. (1996) Angiogenin single-chain immunofusions: Influence of peptide linkers and spacers between fusion protein domains. *Biochemistry* **35**, 545–553.

Pai, L. H., Batra, J. K., Fitzgerald, D. J., Willingham, M. C., and Pastan, I. (1992) Antitumor effects of B3-PE and B3-LysPE40 in a nude mouse model of human breast cancer and the evaluation of B3-PE toxicity in monkeys. *Cancer Res.* **52**, 3189–3193.

Pai, L. H., FitzGerald, D. J., Tepper, M., Schacter, B., and Pastan, I. (1990) Inhibition of antibody response to *Pseudomonas* exotoxin and immunotoxin containing *Pseudomonas* exotoxin by 15-Deoxyspergualin in mice. *Cancer Res.* **50**, 7750–7753.

Ramakrishnan, S., Fryxell, D., Mohanraj, D., Olson, M., and Li, B.-Y. (1992) Cytotoxic conjugates containing ribosomal inhibitory proteins. *Annu. Rev. Pharmacol. Toxico.* **32**, 579–621.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.