

# Binding and Cytotoxicity of Conjugated and Recombinant Fusion Proteins Targeted to the Gonadotropin-Releasing Hormone Receptor

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## ABSTRACT

Pokeweed antiviral protein (PAP) is a plant-derived, highly potent ribosome inactivating protein that causes inhibition of protein translation and rapid cell death. We and others have delivered this protein to various cell types, including cancer cells, using hormones to specifically target cells bearing the hormone receptor. Here, we compare binding and cytotoxicity of GnRH-PAP hormonotoxins prepared either by protein conjugation (GnRH-PAP conjugate) or through recombinant DNA technology (GnRH-PAP fusion). Although GnRH-PAP conjugate protein bound specifically to and caused cell death in cells bearing the gonadotropin-releasing hormone (GnRH) receptor, we could not detect binding or cytotoxicity using two different versions of the fusion protein in receptor-positive cells. We conclude that generation of an active GnRH-PAP fusion protein may not be feasible either because both ends of the GnRH molecule are required for receptor binding, but only the NH<sub>2</sub> terminus is free in the fusion protein and/or that more potent analogues of GnRH (inclusion of which is not feasible in the fusion protein) are needed for efficient targeting. In contrast, the GnRH-PAP conjugate shows promise as a novel anticancer agent, capable of targeting cancer cells expressing the GnRH receptor such as prostate, breast, ovarian, endometrial, and pancreatic cells. It may also be useful as a therapeutic agent to eliminate pituitary gonadotrophs, eliminating the need for chronic GnRH analogue administration to treat hormone-sensitive diseases.

## INTRODUCTION

Hormones have been used to target toxins to specific cells in the body displaying the targeted hormone receptor (1–4). The hormonotoxin binds the receptor on the cell surface, is internalized by receptor-mediated endocytosis, and upon delivery to the cytoplasm, mediates cell death. In many cases, hormonotoxins are targeted to cancer cells. Unlike chemotherapeutic agents where thousands of molecules may need to be delivered to a cancer cell for efficacy, toxin molecules are enzymes. Thus, one molecule of toxin can have a much greater intracellular influence, potentially acting irreversibly on thousands of substrate molecules within the cell.

In the past decade, administration of toxin conjugates has been carried out in dozens of human clinical studies (recently reviewed in Refs. 5–7), and perhaps 10 times this number are reported in animals. A number of significant responses have been reported approaching 40% efficacy in some cases. Among peptide hormones, cytotoxic analogues of somatostatin and bombesin and GnRH have been synthesized in a research program headed by Dr. Andrew Schally (reviewed in Ref. 8). These compounds have shown efficacy in ovarian, breast, and renal cell carcinoma cell lines and xenograft models. Food and Drug Administration approval has been achieved for at least two

fusion toxins (9, 10), Ontak (Denileukin diftitox), which targets the high-affinity interleukin 2 receptor on T cells with a fusion toxin composed of diphtheria toxin-A/modified B chain fused with interleukin 2, and Mylotarg (gemtuzumab ozogamicin), which targets calicheamicin to the CD33 receptor on acute myelogenous leukemia cells using a monoclonal antibody. These results are encouraging, given the advanced disease status of most patients who enter such trials. To date, most toxicities from this approach have been related to vascular leak syndrome; however, this occurs only in small percentage of patients at very high doses of toxin.

Bacterial toxins that have been targeted to cancer cells include *Pseudomonas* exotoxin (PE) and diphtheria toxin (11–13). However, bacterial-derived toxins are very immunogenic (14). Moreover, these toxins often display some degree of nonspecific toxicity because they are able to penetrate living cells via their cell recognition domain (3, 15). Pokeweed antiviral protein (PAP) is produced by the plant *Phytolacca americana* and belongs to the ribosome-inactivating protein family (16). This enzyme is a RNA *N*-glycosidase that specifically removes an adenine residue from a highly conserved and exposed surface region in the large rRNA of eukaryotic and prokaryotic ribosomes (17–20), inducing a conformational change in the subunit. This irreversibly inactivates the ribosomal subunit and prevents the GTP-dependent binding of elongation factor-2 to the affected ribosome (21), thus inhibiting translation and blocking protein synthesis; this, in turn, leads to cell death.

PAP is composed of 313 amino acids (22). Twenty-two amino acids at the NH<sub>2</sub> terminus correspond to the leader sequence and are posttranslationally cleaved; an additional 29 amino acids at the COOH terminus are also cleaved during posttranslational modification (23). The mature protein is 262 amino acids. PAP alone (which does not contain the cell-binding domain) is not able to penetrate living cells. Because of its specificity and extreme toxicity, PAP is an ideal candidate for targeting cell death as the toxic moiety of a hormonotoxin. PAP has been effectively targeted to human B cells to eliminate leukemic progenitor cells in patients with acute lymphoblastic leukemia (24–26).

Our studies in recent years have focused on targeting toxicity via the gonadotropin-releasing hormone receptor (GnRHR). To achieve specificity, the GnRHR levels on cell types other than the targeted tumor must be considerably lower than on the tumor cells. Although normal (nontransformed) pituitary cells express GnRHR at high levels, most other normal cells express low to undetectable levels. Malignant cells that express GnRHR include breast (27), prostate (28, 29), endometrial (9–31), and ovarian (32) cancers.

In animal studies, passive immunization was achieved with anti-GnRH antibodies in nude mice with human breast cancer cell xenografts (33), and rabbits immunized with a multimer of GnRH conjugated to the receptor binding domain of PE-A exhibited ovarian degeneration (34). GnRH-PE conjugates were also shown to reduce adenocarcinoma tumor size when injected in a nude mouse xenograft model (35). In a human clinical study (36), GnRH decapeptide conjugated to diphtheria toxoid was injected into patients with locally advanced prostate cancer. In all patients, antibodies to GnRH and castrate levels of testosterone (which appeared to be reversible) were

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produced. We have injected biologically active GnRH conjugates into animals (rats, ewes) and have observed no toxicity except to targeted reproductive tissues (unpublished data). In these animal and human studies, there was no evidence for nonspecific toxicity, supporting the idea that GnRH receptor can be safely used as the target for hormonotoxins. Given that both ends of GnRH are necessary for interaction with receptor, we hypothesized that conjugating a GnRH analogue via one of its internal amino acids to a ribosome-inhibiting protein would lead to a more potent cytotoxin than a GnRH-fusion protein.

More recently, fusion proteins consisting of GnRH and PAP or PE have been produced and shown to inhibit growth of cultured tumor cells expressing GnRH receptors (37–39); however, the activity of the fusion proteins has not been directly compared with the activities of hormonotoxins produced by conjugation of a GnRH analog to the toxic protein moiety.

In the current study, we compare binding and cytotoxic activity of a GnRH-PAP conjugated protein to two different GnRH-PAP recombinant fusion proteins, corresponding to either the mature (mPAP) or the full-length (fPAP) PAP sequence. We show here that two different versions of GnRH-PAP fusion protein lacked binding and toxicity in cell types where the GnRH-PAP conjugate bound and caused specific toxicity.

## MATERIALS AND METHODS

**GnRH-PAP Conjugate.** PAP was purified from pokeweed (*Phytolacca americana*) leaves, and the conjugate of D-Lys<sup>6</sup>-GnRH-Pro<sup>9</sup>-ethyl amide (a GnRH analogue with enhanced receptor binding activity) with PAP (GnRH-PAP) prepared as described previously (40). The GnRH agonist possesses ~30 times the biological activity of native GnRH. SDS-PAGE (12% reducing gel) analysis and mass spectrometry showed that the final product (as expected) was heterogeneous and contained three major fractions: PAP with one D-Lys<sup>6</sup>-GnRH molecule attached; PAP with two D-Lys<sup>6</sup>-GnRH molecules attached; and unconjugated PAP. Unconjugated PAP in the final product was estimated to be in the range of 25–35%.

**Construction, Expression, and Purification of GnRH-PAP Fusion Protein.** cDNA encoding mature PAP (22) was amplified using reverse transcriptase-PCR from total RNA of freshly picked *P. americana*. RNA was isolated using RNeasy Plant Mini kit (Qiagen, Valencia, CA), and cDNA was prepared using SuperScript (Life Technologies, Inc./Invitrogen, Carlsbad, CA). Primers for the PAP gene were used to amplify the PAP cDNA and designed to incorporate a convenient restriction site (*Bam*HI) and fragment B of the staphylococcal protein A, FB fragment sequences as well as the full-length sequence for GnRH for cloning into the pGEX-KG expression vector (41). The sense primer sequence was as follows: (5'-gcaGGATCCAGCACTGGTCTCTGGAATGCGCCCTG-GAGCCAAGAACTGAACGACTCAGGCGCGAAGAGTGATATGGT-GAATACAATCATCATCTAC-3') (underline, *Bam*HI; bold, GnRH; italics, FB fragment). The introduction of FB fragment provides a linker between the GnRH and PAP to improve the solubility and efficiency of the fusion (42). The antisense primer (CGCAAGCTTTCAGATGCTGCTGACAGCTCCC) was designed to introduce a *Hind*III restriction site (underline) at the 3'-end of the mature PAP (mPAP) gene. We also designed another antisense primer (5'-CGCAAGCTTTCAGAAATCCTCAAATAGAT-3') to acquire the full-length cDNA of PAP (fPAP) following procedure described by Schlick *et al.* (39).

The amplified cDNA was extracted from agarose gel using the Gel Extraction kit (Qiagen), digested with *Bam*HI and *Hind*III, and then subcloned into pGEX-KG. The resultant plasmid, pGEX/GnRH-PAP, was verified by restriction endonuclease digestion and DNA sequence analysis.

*Escherichia coli* strain XA90 carrying recombinant pGEX/GnRH-PAP was grown in Luria-Bertani medium containing ampicillin (100 µg/ml) at 37°C until  $A_{600\text{ nm}}$  reached 0.2 and was then incubated at room temperature for another 30 min. GnRH-PAP fusion protein expression was induced by the addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for 16 h at room temperature. The induced culture was harvested by centrifugation at 4000 × *g* for 20 min at 4°C. Cell pellets were resuspended in 25 ml/liter lysis buffer [50 mM Tris-HCl (pH 8.0), 0.25 M NaCl, 1 mM DTT, and 1 mM EDTA], and then sonicated to lyse the cells. Cell lysate was centrifuged 25,000 × *g* for 60 min. Supernatant was then transferred to glutathione Sepharose 4B column (Am-

ersham Pharmacia, Piscataway, NJ), and the GnRH-PAP fusion protein was purified by affinity chromatography and treated with thrombin at 4°C to cleave the glutathione *S*-transferase-fusion protein. The affinity-purified fusion protein was loaded onto a Superdex 200 column and further purified by gel-filtration chromatography. The purified GnRH-PAP fusion protein was concentrated with a 10k centrifugal filter device (Millipore, Bedford, MA) and kept in aliquots at –80°C.

**Western Blot Analysis of GnRH Conjugate and Fusion Proteins Using Anti-PAP and Anti-GnRH.** One or 10 µg of total protein (as indicated), determined by the RC/DC Protein Assay (Bio-Rad, Hercules, CA), were separated on 12% SDS-polyacrylamide gel, then transferred to polyvinylidene difluoride membranes (Bio-Rad). Anti-GnRH antiserum was prepared in rabbits using a BSA conjugate (43); the resulting antibody against GnRH preferentially recognizes native GnRH but also recognizes the GnRH analog in the conjugate protein. Antiserum against the GnRH analogue, recognizing the D-Lys<sup>6</sup>-GnRH in the conjugate protein, was prepared in rabbits using keyhole limpet hemocyanin-conjugated leuprolide as previously described for conjugation of GnRH to keyhole limpet hemocyanin (44). Antisera were purified using protein A HiTrap affinity columns (Amersham Pharmacia) according to manufacturer's instructions. Final dilutions of purified anti-GnRH (1:100) and anti-leuprolide (1:500) were made in Tris-buffered saline supplemented with 0.1% Tween 20 and 5% nonfat dry milk. Proteins were visualized using ECL-Plus (Amersham Pharmacia).

**Preparation of Bovine Pituitary Membranes and GnRH Receptor Binding Assay.** Bovine pituitary membranes (with approximately the same GnRH receptor number and affinity as membranes from the CHO-GnRHR cells) were prepared as described previously (45). D-Ala<sup>6</sup>-desGly<sup>10</sup>-GnRH-Pro<sup>9</sup>-ethylamide was radioiodinated using a glucose-oxidase procedure, and reaction products were separated on quaternary aminoethyl Sephadex (45). Freshly prepared [<sup>125</sup>I]D-Ala<sup>6</sup>-desGly<sup>10</sup>-GnRH-Pro<sup>9</sup>-ethylamide (0.044 ng) in 50 µl of ice-cold assay buffer was added to each tube in the presence of varying concentrations of unlabeled D-Lys<sup>6</sup>-GnRH (between 0.09 and 9 nM), GnRH-PAP conjugate or fusion proteins (between 0.11 and 333.33 nM), or PAP (between 0.1 and 330 nM). Reactions were incubated on ice for 4 h and then centrifuged (16,000 rpm, 15 min, 4°C). Radioactivity in the pellet was quantified using an Apex Automatic gamma-spectrometer (Micromedex Systems, Inc., Horsham, PA).

**Inhibition of *in Vitro* Translation.** Varying concentrations of GnRH-PAP (conjugate or fusion protein, 0.2–400 pM) or PAP (0.2–400 pM; 2 µl) were added to the translation mixture. The latter contained 35 µl of rabbit reticulocyte lysate (Promega), 0.2 µl of a mixture of amino acids at a concentration of 1 mM, 1.4 µl of 2.5 M KCl, 1 µl of RNase inhibitor (40 units/µl), and 10.6 µl of H<sub>2</sub>O. The reaction was started by the addition of 1 µl of luciferase control RNA (1 mg/ml). After incubation (90 min at 30°C), protein synthesis was determined by analysis of luciferase (Promega, Madison, WI) according to the manufacturer's instructions. The experiment was performed with triplicate samples.

**Cell Culture.** Chinese hamster ovary (CHO) cells were transfected with cDNA for the murine GnRH receptor fused to green fluorescence protein and yellow fluorescence protein to create a cell line expressing high levels of GnRH receptor (CHO-GnRHR; 46). Functional characteristics of the GnRH-GFP/YFP receptors appeared to be identical to native receptors (47). These CHO-GnRHR cells were a generous gift from Dr. Colin Clay (Colorado State University, Fort Collins, CO). CHO-GnRHR cells were maintained in DMEM (Sigma, St. Louis, MO) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 10% fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, CA), and 1% nonessential amino acids (Life Technologies, Inc., Grand Island, NY). Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

**Clonogenic Assay.** Clonogenic survival was defined as the ability of the cells to maintain their clonogenic capacity and to form colonies. Briefly, 300 cells (either CHO control or CHO-GnRHR) were seeded into 6-well dishes in 2 ml of medium. Two days later, varying amounts of the test compounds (GnRH-mPAP fusion protein, GnRH-PAP conjugate, and PAP only) were each added to the dishes, which were then maintained for 6–7 days in a CO<sub>2</sub> incubator. Cells were fixed with methanol-acetic acid (3:1) solution and then stained with crystal violet. The number of colonies, containing a minimum of ~50 cells, was counted using a dissection microscope. The number and area of colonies in treated cultures were expressed as a percentage of those in control cultures. Each experiment was performed in triplicate.

**Apoptosis Assay.** The induction of programmed cell death was assayed using Vybrant Apoptosis Assay kit no. 2 (Molecular Probes, Eugene, OR).

Briefly,  $5 \times 10^4$  cells/well were seeded into 6-well plates and incubated for 24 h to permit attachment. After appropriate treatment with PAP or GnRH-PAP conjugate, cells were trypsinized, washed, and resuspended in 100  $\mu$ l of Annexin binding buffer. Annexin V (5  $\mu$ l/sample) and  $1 \times$  propidium iodide were added, and samples were mixed gently and incubated for 15 min at room temperature. Four-hundred  $\mu$ l of Annexin binding buffer were then added, and samples were processed for fluorescent activated cell sorting (BD FACS Calibur). Using flow cytometry with the 488-nm line of an argon-ion laser for excitation after staining a cell population with Alexa Fluor 488 Annexin V and propidium iodide, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence. Additional plates identically treated, except for no addition of PAP or conjugate, were analyzed for cell number with a hemocytometer to normalize the values for cell numbers, and results were expressed relative to untreated control samples. Each treatment was performed in triplicate.

**Statistical Analysis.** Values for inhibition of protein translation, cell survival by clonogenic assay, and apoptosis assays are presented as mean  $\pm$  SD. Differences, as determined by Student's *t* test, were considered significant at  $P < 0.05$ .

## RESULTS

### Purification of GnRH-PAP Conjugate and Fusion Proteins.

Conjugate and fusion proteins were prepared as described in "Materials and Methods." The two different fusion proteins correspond to the full-length (**f**, amino acids 23–313) sequence and the mature (**m**, amino acids 23–284). Both versions of the fusion protein were constructed to determine whether the 29 amino acids cleaved from the COOH terminus in the mature protein were necessary for toxin activity.

Fig. 1 shows a protein gel of PAP (Fig. 1, Lane A), GnRH-PAP conjugate (Fig. 1, Lane B), and the two versions of GnRH-PAP fusion protein, GnRH-mPAP (Fig. 1, Lane C) and GnRH-fPAP (Fig. 1, Lane D). Coomassie blue stain of the gel shows the expected products for the conjugate (estimated to be 65–75% conjugate, with the remainder unconjugated PAP; see "Materials and Methods") and the two fusion proteins. Western blot analysis of the same samples using anti-PAP, anti-GnRH, and anti-leuprolide (as indicated in the left margin) confirms that the conjugate and fusion proteins have GnRH and PAP moieties as expected. Anti-PAP antiserum cross-reacted with all four samples, and

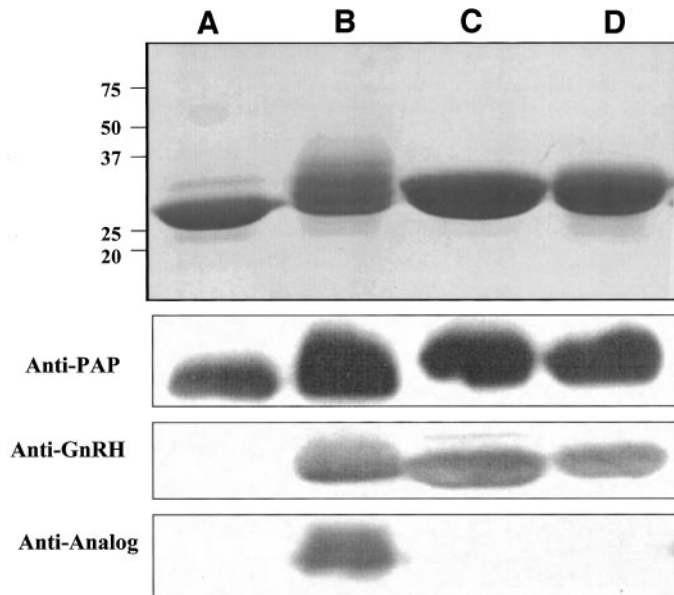


Fig. 1. Protein gel analysis of pokeweed antiviral protein (PAP; Lane A), gonadotropin-releasing hormone (GnRH)-PAP conjugate (Lane B), GnRH-mature PAP fusion protein (Lane C), and GnRH-full-length PAP fusion protein (Lane D). As indicated in the left margin, bands are either from proteins stained with Coomassie or Western blots using sera made against PAP or native or analogue GnRH. For Westerns, either 10  $\mu$ g (for anti-PAP and anti-GnRH) or 1  $\mu$ g (for anti-analogue) total protein was loaded on the gel.

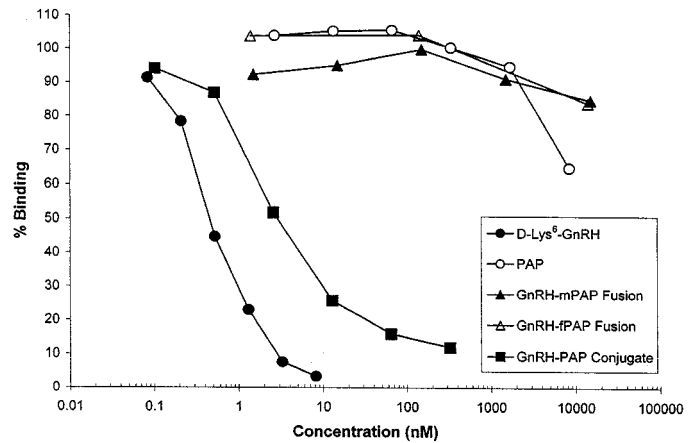


Fig. 2. Binding assays for conjugate and fusion proteins to gonadotropin-releasing hormone (GnRH) receptor. Binding of GnRH-pokeweed antiviral protein (PAP) conjugate, GnRH-mature PAP (mPAP), and GnRH-full-length PAP (fPAP) fusion proteins, and control D-Lys<sup>6</sup>-GnRH control was evaluated using bovine pituitary membranes.

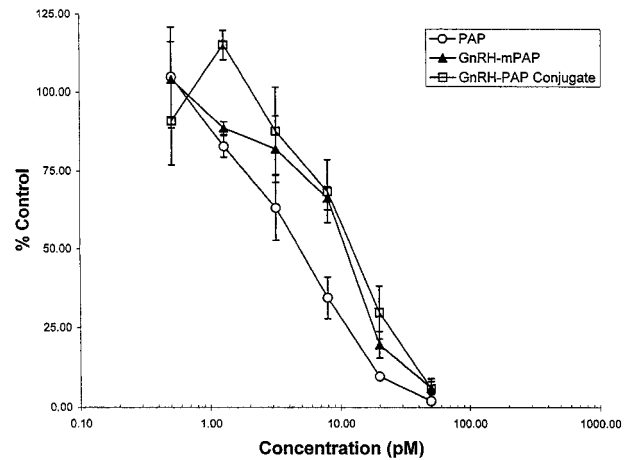


Fig. 3. Inhibition of protein translation in a cell-free system by pokeweed antiviral protein (PAP), gonadotropin-releasing hormone (GnRH)-PAP conjugate, and GnRH-mature PAP (mPAP) fusion protein. All three proteins inhibited protein synthesis, demonstrating that toxicity from the PAP moiety in the fusion protein was still intact. Values are averaged from triplicate samples with SD indicated.

antiserum against native GnRH cross-reacted with conjugate and both fusion proteins but not with PAP. However, antiserum made against the GnRH analogue, leuprolide, cross-reacted only with the conjugate protein and not with either of the fusion proteins. This is because the antiserum specifically recognizes the molecular conformation of the analogue, which differs from that of the native GnRH molecule.

### Binding of GnRH, PAP, and GnRH-PAP Conjugate and Fusion Proteins to GnRH Receptor in Bovine Pituitary Membranes.

Binding studies were done to determine whether the ability of the conjugate or fusion proteins to bind to GnRHR was impaired. Fig. 2 shows that although the GnRH-PAP conjugate bound to GnRHR, albeit at somewhat higher concentrations (3 *versus* 0.6 nM was needed to reduce binding of [<sup>125</sup>I]D-Ala<sup>6</sup>-desGly<sup>10</sup>-GnRH-Pro<sup>9</sup>-ethylamide by 50%) compared with control GnRH alone (D-Lys<sup>6</sup>-GnRH), neither of the fusion proteins (GnRH-fPAP or GnRH-mPAP) were able to inhibit binding of the radioligand even at 10,000 nM. Because neither fusion protein showed any binding in this assay, we chose to continue with only the mature GnRH-mPAP protein for subsequent assays.

**Inhibition of Translation in a Cell-Free System by PAP and GnRH-PAP Conjugate and Fusion Proteins.** Fig. 3 shows that the GnRH-PAP conjugate and GnRH-mPAP fusion proteins both inhib-

ited translation to a similar extent as PAP alone in a cell-free rabbit reticulocyte translation system. For all proteins tested, 50% inhibition was observed in the range of 5–10 pM. This demonstrates that the PAP molecule in the conjugate and fusion proteins have retained toxicity and are nearly as toxic as the control PAP. Thus, any difference in their cytotoxicity when tested in cell survival/clonogenic assays cannot be attributed to disruption of PAP function.

**Cytotoxic Activity of GnRH-PAP Conjugate and Fusion Proteins on CHO and CHO-GnRHR Cells.** To evaluate the ability of GnRH-PAP to inhibit growth of cells expressing GnRHR on their surface, cells were treated with increasing amounts of PAP or GnRH-PAP conjugate or fusion protein. Results from a clonogenic assay are shown in Fig. 4 in CHO (Fig. 4A) and CHO-GnRHR (Fig. 4B) cells. Six-well dishes showing stained colonies from a representative clonogenic experiment with PAP and GnRH-PAP fusion and conjugate proteins are shown in Fig. 4C. As seen in the figure, PAP and GnRH-mPAP fusion showed only nonspecific toxicity, with similar

results in both CHO and CHO-GnRHR cells at all concentrations ( $P > 0.1$ , comparing GnRH-mPAP to PAP alone at  $1 \times 10^{-8}$  M). Fifty percent inhibition in both cell types with these proteins was observed only at concentrations  $> 100$  nM. In contrast, GnRH-PAP conjugate protein showed specific toxicity in CHO-GnRHR cells ( $P < 0.002$  at  $1 \times 10^{-8}$  M compared with PAP alone), with 50% inhibition seen at  $\sim 2$  nM; in CHO control cells, toxicity from the conjugate protein was within the same concentration range as the fusion protein and PAP ( $P > 0.09$  at  $1 \times 10^{-8}$  M). These results show that the conjugate protein was  $\sim 50$ -fold more cytotoxic to CHO-GnRHR cells compared with CHO control cells. The fusion protein, however, did not show any specific toxicity to CHO-GnRHR cells. This finding is consistent with the lack of fusion protein binding to GnRHR (Fig. 2).

**GnRH-PAP Conjugate Protein Induces Apoptosis in CHO-GnRHR Cells.** CHO control and CHO-GnRHR cells were assayed for apoptosis using the Annexin V assay following exposure for varying times to  $3 \times 10^{-8}$  M PAP or GnRH-PAP conjugate. As seen

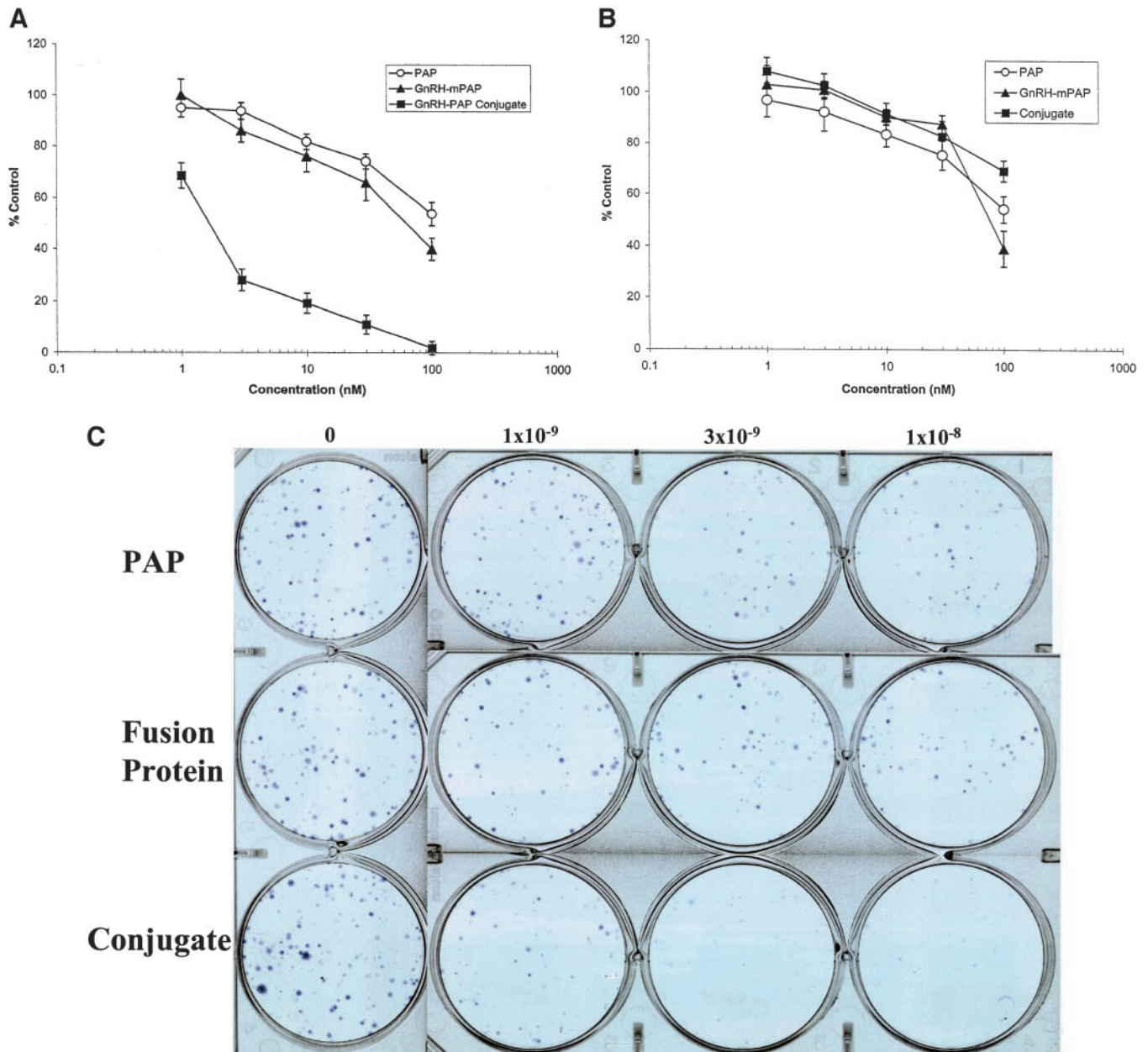


Fig. 4. Clonogenic assay in CHO-GnRHR (A) or CHO control (B) cells exposed to varying concentration of PAP, GnRH-mature PAP (mPAP) fusion protein, or GnRH-PAP conjugate. C shows crystal violet staining of plated GnRHR cells exposed to the varying levels of proteins. SD is shown from triplicate samples within each assay.

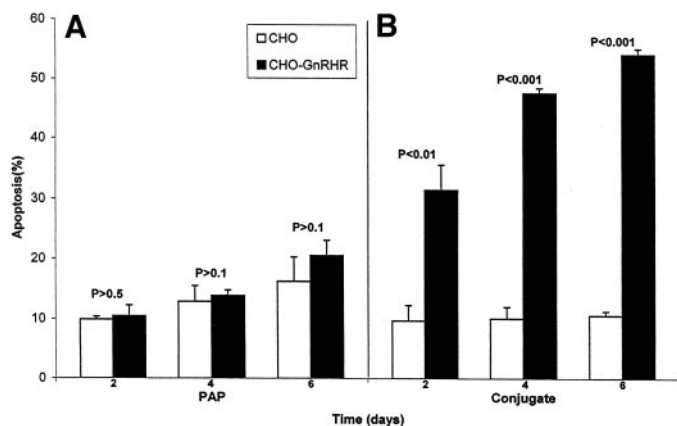


Fig. 5. Annexin V (apoptosis) assay in CHO and CHO-GnRHR cells exposed to  $3 \times 10^{-8}$  M pokeweed antiviral protein (PAP; A) or gonadotropin-releasing hormone (GnRH)-PAP conjugate (B). Assays were done 2, 4, and 6 days after exposure to protein. SD is shown from triplicate samples within each assay. The *P*s in the diagram, determined by Student's *t* test, correspond to the difference between CHO and CHO-GnRHR cells at each day assayed.

in Fig. 5A, PAP alone induced only low levels of nonspecific apoptosis in treated cells, equivalent in CHO control and CHO-GnRHR cells, and consistent with that observed in clonogenic assays (Fig. 4). In contrast, GnRH-PAP conjugate (Fig. 5B) specifically induced apoptosis in CHO-GnRHR cells but not in CHO control cells. Percentage of apoptotic cells ranged from  $\sim 30\%$  (at day 2 of treatment) to  $50\%$  (at day 6 of treatment). The difference between apoptosis in CHO control *versus* CHO-GnRHR cells was highly significant, as indicated in *P*s in the figure, on all 3 days. These data show that GnRH-PAP conjugate induces apoptosis selectively and to a high degree in cells bearing the GnRH receptor.

## DISCUSSION

We compared GnRH-PAP conjugate to fusion protein for binding and cytotoxic activity in GnRH receptor-positive cells. The GnRH conjugate contained an average of 0.7 GnRH molecules/PAP molecule (data not shown), and due to the chemical procedures used for linkage, the GnRH molecule could be located at several different sites along the PAP molecule. Because recombinant fusion proteins are not chemically synthesized, they should always have one and only one GnRH associated with each PAP molecule, and it is always at the same site. For this reason, the uniformity of GnRH-PAP fusion preparations should be much better than for conjugates, making the generation of large amounts of GnRH-PAP for clinical studies more feasible.

Although the fusion protein would be superior to the conjugate protein for production and reproducibility, we hypothesized that the conjugate protein would be more cytotoxic to GnRH receptor-positive cells for the following reasons: (a) there is considerable evidence that both ends of the GnRH molecule are required for receptor binding (48–51), but they are not likely to both be accessible in a fusion protein; and (b) the incorporation of a D-amino acid in position 6 of GnRH that is known to enhance receptor binding affinity  $\sim 30$ -fold is impossible in fusion proteins. Although the native GnRH sequence was used in the fusion proteins while the analogue GnRH was used in the conjugate protein, the difference in binding affinity (30-fold) by itself is not sufficient to explain the three to four log difference in binding between conjugate and fusion GnRH-PAP proteins in Fig. 2, nor the approximate two log difference in cytotoxicity in Fig. 3A.

In support of our hypothesis that the conjugate would be more cytotoxic than the fusion protein, we showed that although the con-

jugate bound and caused specific toxicity to GnRHR-positive cells, the GnRH-PAP fusion proteins were inactive in both of these regards. The two different versions of fusion protein tested corresponded to either full (f, containing posttranslationally modified sequences) or mature (m, without these sequences). GnRH-fPAP was tested because a study by Schlick *et al.* (39) demonstrated that a GnRH-PAP fusion protein containing these posttranslationally modified sequences was cytotoxic to Ishikawa cells (an endometrial cell line).

There are several possibilities that could account for the different results in our study as compared with Schlick *et al.* (39). Although our GnRH-fPAP fusion protein and the one described by Schlick *et al.* (39) appear to have the same amino acid sequence, the folding and/or three-dimensional structure may be subtly altered due to different purification procedures. Second, different cell lines were used in the two studies. We tested the fusion proteins in a cell line with high levels of high affinity GnRH receptors (40); the Schlick *et al.* study used Ishikawa cells and MCF-7 cells. In other studies from our laboratory, we have shown that Ishikawa and MCF-7 cells have 3–50-fold lower levels of GnRHR compared with CHO-GnRHR cells (40). Thus, Ishikawa and MCF-7 cells would not be expected to be as sensitive to specific cell killing using a GnRHR-targeted protein but might be more sensitive to nonspecific killing compared with the CHO control cells we used. In support of this possibility is the observation that the Schlick *et al.* study required relatively high levels of the fusion protein for cytotoxicity (the  $ID_{50}$  was 15 nM, compared with the  $ID_{50}$  of 1.8 nM for the GnRH-PAP conjugate in the current study, Fig. 4B).

As one potential mechanism by which GnRH-PAP inhibited cell growth and viability in targeted cells, the GnRH-PAP conjugate specifically and very significantly induced apoptosis in GnRHR-positive cells but not in GnRHR-negative cells. Although Annexin V is an early indicator of apoptosis, our previous data demonstrated that reduction in cell viability induced by the GnRH-PAP conjugate depended on the length of exposure and required several days for maximum effect (40). In the current study, by day 6 of treatment, apoptosis was observed in  $\sim 50\%$  of cells treated with GnRH-PAP conjugate but not in cells treated with PAP alone. Other immunotoxins, including anti-CD19-PAP and anti-CD22 linked to momordin, PAP, and saporin-S, were shown to cause apoptosis in cell lines expressing the receptor and to decrease tumor incidence in a mouse transplant model (52, 53). Additionally, an anti-CD19-PAP immunotoxin caused apoptosis in radiation-resistant human B-cell precursor leukemia cells and conferred extended survival in a mouse xenograft model (54).

Dr. A. Schally *et al.* (55, 56) have examined a series of promising targeted cytotoxic analogues using small molecules such as bombesin and doxorubicin in preclinical studies. A fusion toxin comprised of GnRH-PE, licensed from investigators at Hebrew University in Jerusalem and developed by Boston Life Sciences, shows promising preliminary results at targeting adenocarcinomas, including colon cancer (37).

Our results show that a GnRH-PAP conjugate protein is active and highly specific in killing cells bearing the GnRH receptor. We believe the receptor targeted in these studies is the GnRHR I receptor, not the GnRHR II receptor, because we have observed binding only in pituitary cells or in cells transfected with the GnRH I receptor (Refs. 40, 46 and unpublished data); we have not observed binding of GnRH-PAP in cells where the GnRHR II receptor has been shown to be expressed (57, 58). The strategy described here could provide an efficient and irreversible mechanism for targeting toxicity to cancer cells and normal pituitary cells bearing GnRHR. Prostate, breast, endometrial, ovarian, and pancreatic are among the cancer types known to express GnRHR, and this approach could extend the spec-

trum of use of GnRH-toxins to hormone-resistant cancers not thought of as hormone responsive but which nonetheless express the targeted receptor.

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