Luteinizing hormone-releasing hormone receptor–targeted deslorelin-docetaxel conjugate enhances efficacy of docetaxel in prostate cancer therapy

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Abstract

Docetaxel, a chemotherapeutic agent currently used for improving survival of prostate cancer patients, suffers from low therapeutic index. The objective of this study was to prepare a new docetaxel derivative conjugated to deslorelin, a luteinizing hormone-releasing hormone (LHRH) superagonist, and to determine whether it enhances docetaxel potency *in vitro* and *in vivo*. Because docetaxel is not amenable for conjugation with peptides, we introduced a -COOH group in docetaxel, forming docetaxel-hemiglutarate, and subsequently conjugated this to serine in deslorelin, forming deslorelin-docetaxel. Fourier-transform IR, [1]H-nuclear magnetic resonance, and liquid chromatography-mass spectrometry analyses confirmed deslorelin-docetaxel formation. Antiproliferative efficacy in LNCaP and PC-3 cell lines over 24, 48, and 72 hours exhibited the order deslorelin-docetaxel > docetaxel, whereas deslorelin alone had no effect, with deslorelin-docetaxel potency being 15-fold greater than docetaxel at 72 h. Further, cells pretreated with antisense oligonucleotide against LHRH receptor exhibited decreased deslorelin-docetaxel efficacy, without any change in docetaxel efficacy. Thus, deslorelin-docetaxel efficacy is likely mediated via LHRH receptor. Cell cycle analysis showed that docetaxel treatment led to arrest in G_2-M phase, whereas deslorelin-docetaxel treatment allowed greater progression to apoptosis in both cell lines, with deslorelin-docetaxel exerting 5-fold greater apoptosis compared with docetaxel in prostate cancer cell lines. Antitumor efficacy studies in PC-3 prostate xenograft-bearing mice indicated the efficacy order deslorelin-docetaxel > docetaxel ≫ deslorelin > PBS, with deslorelin-docetaxel exerting 5.5-fold greater tumor growth inhibition than docetaxel alone. Thus, deslorelin-docetaxel prepared in this study retains pharmacologic effects of both docetaxel and deslorelin while enhancing the antiproliferative, apoptotic, and antitumor efficacy of docetaxel by several folds in prostate cancer therapy. [Mol Cancer Ther 2009;8(6):1655–65]

Introduction

Prostate cancer is the most prevalent form of cancer and the second leading cause of death in Western countries (1). In 2008, an estimated 186,320 new cases and 28,660 deaths are expected to occur in the United States alone (1). First-line therapies for managing prostate cancer are local treatments such as radical prostatectomy and/or radiotherapy (2). Following first-line therapy, ~22% of the patients relapse and are treated with hormone therapies including luteinizing hormone-releasing hormone (LHRH) agonists and antiandrogens, constituting second line of therapy (3). However, acquired resistance to some of these therapies leads to enhanced metastasis (4). At this stage, median survival rate of prostate cancer patients is ~12 months and necessitates the use of chemotherapeutic agents for effective management of the disease (5–8).

Chemotherapy has been the main treatment modality in metastatic diseases, which is, however, limited by its intrinsic or acquired multidrug resistance and nonselective toxicity to normal cells (9). Several chemotherapeutic agents, including docetaxel, are currently available for treatment of prostate cancer. Docetaxel is a semisynthetic product of the European yew *Taxus baccata* (10), similar to paclitaxel. At the molecular level, docetaxel disrupts normal processes of microtubule assembly and disassembly. Docetaxel binds tubulin to promote polymerization and prevents depolymerization of microtubules in the absence of guanosine triphosphate (11). Docetaxel also acts as a mitotic spindle poison and induces a mitotic block in proliferative cells (12). Previous studies have shown that docetaxel has a broad spectrum of activity against a variety of tumors, including breast and prostate cancers (5, 13).

In 2004, the U.S. Food and Drug Administration approved docetaxel in combination with prednisone for the treatment of metastatic hormone-independent prostate cancer (5, 13). The TAX327 trial, a combination of docetaxel (75 mg/m², every 3 weeks) with prednisone (10 mg daily; survival, 18.9 months), exhibited a median survival improvement of 2.4 months over mitoxantrone (12 mg/m², every 3 weeks) and prednisone (10 mg daily; survival,

16.5 months; ref. 13). The SWOG-9916 trial, a combination of docetaxel (60 mg/m^2, day 2, every 3 weeks) with estramustine (280 mg, three times a day, days 1–5, every 3 weeks; survival, 17.5 months), exhibited a median survival improvement of 1.9 months over mitoxantrone (12 mg/m^2, day 1, every 3 weeks) combined with prednisone (5 mg, twice daily; survival, 15.6 months; ref. 5). Despite having improved survival efficacy with a docetaxel and prednisone chemotherapy, the improvement is only a progression-free survival of 3 months compared with other chemotherapy regimens available for the treatment of prostate cancer (14). Furthermore, there was an increase in gastrointestinal (nausea and vomiting) and cardiovascular (thrombosis and pulmonary embolism) toxicities in the docetaxel-treated groups (5).

It is therefore desirable to develop delivery systems that would specifically deliver docetaxel to the tumor site. The advantages of preparing such a targeting system will be (*a*) improved efficiency of drug delivery to the macrometastatic tumors and (*b*) reduction in the dosage required to achieve effective tumor growth reduction (15, 16).

Targeting can be achieved by attaching a ligand for specific receptors that are expressed preferentially on the tumor cells. The LHRH receptor (LHRH-R) is a member of the G protein–coupled receptor superfamily of receptors (17). The presence of LHRH-R on the pituitary is well characterized (17). LHRH-R is overexpressed on prostate cancer cells (18), breast cancer cells (19), ovarian cancer cells (17), and endometrial cancer cells (20, 21). There are currently no reports on targeted conjugates of docetaxel. The objective of this study was to determine whether conjugation of docetaxel to deslorelin, a LHRH superagonist, will lead to an improvement in its therapeutic efficacy *in vitro* and *in vivo* models of prostate cancer.

Deslorelin is a nonapeptide of molecular weight 1,285 Da with the amino acid sequence pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-ProNHEt (22, 23). Deslorelin contains modifications at position 6 replacing Gly with D-Trp and at position 10 replacing Gly with ethylamide (22). This makes the synthesized deslorelin 144 times more potent than the native LHRH (22), whose short half-life (24, 25) precludes its use in targeting. Deslorelin has a half-life in the order of hours in plasma and functions similar to LHRH by stimulating the release of LH and follicle-stimulating hormone following acute exposure and inhibits their release on chronic administration (26). Docetaxel, unlike some other anticancer agents, is not readily amenable to conjugation with peptides such as deslorelin. To overcome this, in this study, we synthesized a hemiglutarate derivative of docetaxel and conjugated it to deslorelin. This approach not only retained the activity of docetaxel but also enhanced it several fold. This article reports the preparation and characterization of deslorelin-docetaxel conjugate using Fourier-transform IR (FTIR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and liquid chromatography-mass spectrometry (LC-MS) analysis. Further, it reports the efficacy of the conjugate *in vitro* in LNCaP and PC-3 prostate cancer cell lines and *in vivo* in a mouse PC-3 xenograft model.

Materials and Methods

Chemicals

Deslorelin was a gift from Balance Pharmaceuticals, Inc. Docetaxel was obtained from Chempacific. LNCaP and PC-3 cells were kindly provided by Dr. Ming-Fong Lin (University of Nebraska Medical Center). Cell culture materials and reagents and Lipofectin were obtained from Invitrogen Corp. The chemicals for the conjugation were obtained from Sigma-Aldrich.

Synthesis of Deslorelin-Docetaxel Conjugate

The synthesis of deslorelin-docetaxel conjugate and its proposed mechanism of action are shown in Fig. 1A. Initially, docetaxel-hemiglutarate was formed and then conjugated to deslorelin. Briefly, docetaxel (121.19 mg; 0.15 mmol/L), an equimolar quantity of glutaric anhydride (17.115 mg; 0.15 mmol/L), and pyridine (10 μL) were dissolved in 4 mL DMSO. The above reaction mixture was stirred at room temperature under a nitrogen stream for 72 h to form docetaxel-hemiglutarate. The resulting mixture was dialyzed against 1 L of deionized water (membrane molecular weight cutoff, 1,000) for 12 h with intermittent water exchange at 1, 2, 4, and 8 h. The final lyophilized product was characterized by ^{1}H-NMR and FTIR.

Following filtration and lyophilization, docetaxel-hemiglutarate (90 mg; 0.976 mmol/L) was dissolved in 3 mL DMSO and the carboxyl group was activated by stirring in the presence of N,N′-dicyclohexylcarbodiimide (20.14 mg), N-hydroxysuccinimide (11.23 mg), and 4-(dimethylamino)-pyridine (4.92 mg) for 2 h at room temperature. To the above reaction mixture, an equimolar quantity of deslorelin (125.43 mg; 0.976 mmol/L) was added and the reaction was left to proceed for 72 h. The resulting precipitate of dicyclohexyl urea was removed by filtration and the filtrate was dialyzed (membrane molecular weight cutoff, 2,000) against deionized water with intermittent water exchange at 1, 2, 4, 8, and 12 h. The dialysate was collected and analyzed by high-performance liquid chromatography for deslorelin as described previously (27). Dialysis was carried out until deslorelin was not detected in the dialysate (16 h). The final dialyzed mixture free from deslorelin was lyophilized to obtain as a free powder and further characterized by FTIR, ^{1}H-NMR, and LC-MS/MS.
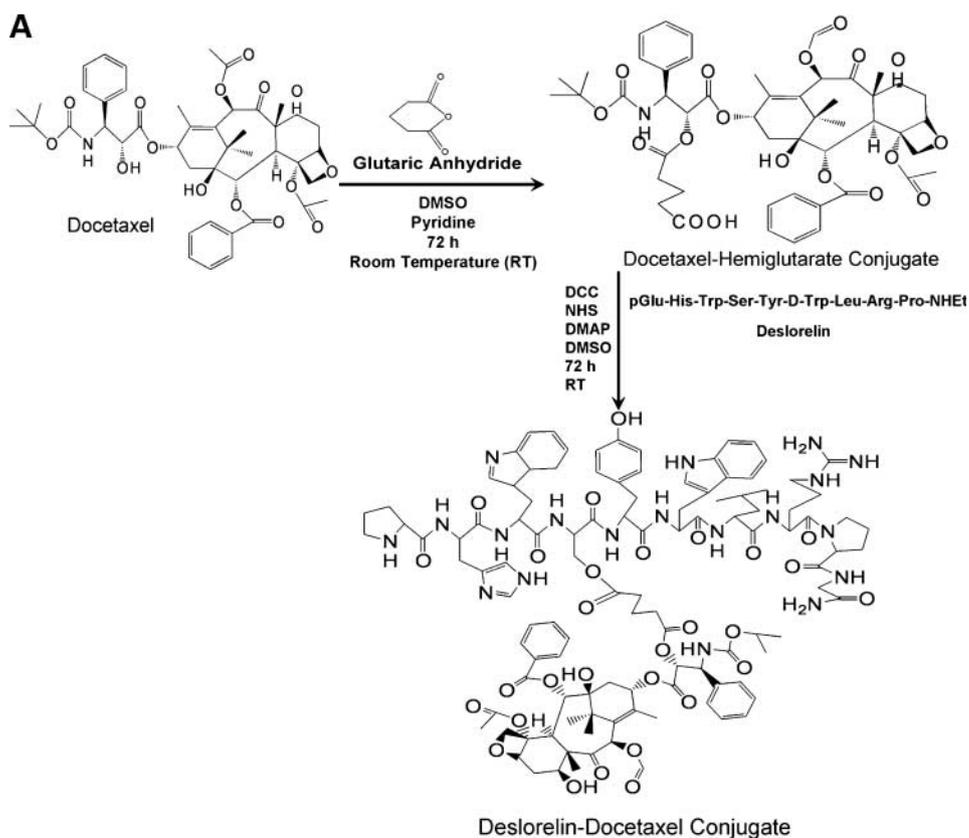
^{1}H-NMR Characterization of Deslorelin-Docetaxel Conjugate

NMR characterization of the synthesized conjugate was recorded using the Varian INOVA 500 MHz instrument (Eppley Institute, University of Nebraska Medical Center). NMR spectra of docetaxel and docetaxel-hemiglutarate were recorded in CDCl$_3$ solvent. The spectra of deslorelin and deslorelin-docetaxel conjugate were obtained in DMSO-d_6 solvent.

FTIR and LC-MS Characterization of Deslorelin-Docetaxel Conjugate

FTIR characterization was recorded using a Nicolet Avatar 360 FTIR (University of Nebraska, Lincoln, NE). FTIR

Figure 1. **A,** scheme for deslorelin conjugation with docetaxel. Docetaxel was reacted with glutaric anhydride in the presence of DMSO and pyridine to form docetaxel-hemiglutarate with a free -COOH group. This -COOH group was conjugated with the -OH group in serine of deslorelin using a carbodiimide reaction in the presence of *N,N'*-dicyclohexylcarbodiimide (*DCC*), *N*-hydroxysuccinimide (*NHS*), and 4-(dimethylamino)-pyridine (*DMAP*), forming an ester bond between deslorelin and docetaxel. All reactions were carried at room temperature. **B,** scheme representing mechanisms of action of deslorelin-docetaxel conjugates in LNCaP and PC-3 cells expressing LHRH-Rs. Following endocytosis via the clathrin-coated pits, the conjugate is expected to break down to its parent components in the cells. In cells undergoing mitosis, spindle fiber formation requires tubulin involvement. Docetaxel stabilizes the tubulin in spindle fibers, leading to a G_2-M phase arrest, which in turn causes cell death via apoptosis.

spectra were collected in the range of 4,000 to 400 cm^{-1} at a resolution of 4 cm^{-1}. A total of 32 scans were recorded for each spectrum.

Qualitative characterization of the deslorelin-docetaxel conjugate to confirm conjugation was done by a LC-MS analysis. A manual infusion of conjugate (10 μg/mL) as well as deslorelin and docetaxel alone (parent compounds; 5 μg/mL) in methanol was carried out. The analysis was carried out using an inbuilt syringe pump of a Q-trap 2000 LC-MS/MS system at a flow rate of 10 μL/min. Ionization mode was positive and spectrum was acquired in the range of 200 to 1,700 *m/z* (a.m.u.). The acquisition speed was 0.61 s/cycle. The acquired spectrum was then reprocessed using Bayesian peptide reconstruct software to identify the *m/z* ratio.

Cell Culture

LNCaP and PC-3 cells were grown in RPMI 1640 with 10% FCS and supplemented with L-glutamine and penicillin-streptomycin. The medium was changed every 3 d. The cells with passage numbers 30 to 36 were used for all the experiments.

Antiproliferative Effects of Deslorelin-Docetaxel Conjugate in LNCaP and PC-3 Prostate Cancer Cells

In LNCaP and PC-3 cells, antiproliferative effects of the conjugate were tested as per the methods described previously (28) with some modifications. Both LNCaP and PC-3 cells were plated onto 96-well tissue culture plates at 10,000 per well densities. Following 24-h incubation, the medium was removed and the cells were incubated with treatments in serum-free medium over 24, 48, or 72 h. Designated

columns were treated with 1 to 250 μg/mL of docetaxel (1.25–300 nmol/L), 1 to 250 μg/mL of deslorelin (0.7–200 nmol/L), and 1 to 250 μg/mL of deslorelin-docetaxel conjugate (0.19–46 nmol/L docetaxel equivalent of the conjugate). Medium alone, cells without drug as a blank control, and approximate amounts of 50% ethanol contained in the 250 μg/mL group were added to designated columns as controls. Following incubations, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL) was added and incubated over 3 h. At the end of 3 h, the medium was removed, and the formazan crystals were dissolved in DMSO. Absorbance was measured at 540 nm.

Influence of Decreased LHRH-R Expression on the Efficacy of Deslorelin-Docetaxel Conjugate

A previously described protocol was followed to determine the effect of decreased expression of LHRH-R on efficacy of deslorelin-docetaxel conjugate *in vitro* (23). Phosphorothioated antisense oligonucleotide (AON) and sense oligonucleotide (SON) directed against the LHRH-R, previously validated by Koushik et al. (23) for reducing LHRH-R expression, were synthesized for this purpose (29). Briefly, both LNCaP and PC-3 cells plated in six-well plates were treated with 10 μmol/L AON or SON and Lipofectin (10 μg/mL) in serum-free medium for 24 h, after which the cells were placed in 2% serum-containing medium for 12 h. Cells that were not pretreated were used as controls. These cells were then harvested and plated in 96-well plates and antiproliferative effects of the conjugate were tested in these cells as described above.

Cell Cycle Analysis

Cell cycle analysis following treatment with conjugate was done as previously described (30). Briefly, LNCaP and PC-3 cells at an approximate density of 50,000 per well were plated in six-well plates. The cells were allowed to attach and grow for 24 h in medium containing serum. The medium was aspirated and the cells were treated with the unconjugated drugs or deslorelin-docetaxel conjugate for 24 h at the IC_{50} concentrations from the antiproliferation studies. The medium was then removed, and the cells were trypsinized and pelleted with centrifugation at 3,000 rpm for 10 min at 4°C. The pellet was resuspended in 1 mL PBS, and 3 mL of ice-cold absolute ethanol were added drop wise with simultaneous vortexing at 10,000 rpm. The cells were incubated on ice for 1 h, harvested by centrifugation at 3,000 rpm, washed with 1 mL PBS, and resuspended in Telford's reagent overnight. Cell cycle phases were analyzed by FACSCalibur (BD Biosciences).

Animals, Tumor Cell Inoculation, and Tumor Volume Measurements

These studies were done with approval and in accordance with guidelines of the Institutional Animal Care and Use Committee at the University of Colorado Denver. Male athymic nude BALB/c mice were obtained from National Cancer Institute, acclimatized for 1 wk, and caged in groups of five. Prostate cancer PC-3 cells (10^8/100 μL) were injected s.c. in the upper-right flank of mice using a 30-gauge needle and a 1-mL syringe. The tumor length (long diameter) and width (short diameter) were measured twice a week using a Vernier caliper and volumes were calculated using the following equation: length × width^2 × 0.52. When tumor volumes measured 60 mm^3, mice were randomized into the following groups: control (vehicle treated), deslorelin (2.5 mg/kg), docetaxel (2.5 mg/kg), and deslorelin-docetaxel conjugate (2.5 mg/kg of docetaxel equivalent) for repeated weekly i.v. doses (100 μL; three doses). Tumor growth inhibition was monitored by measuring tumor volumes twice weekly until day 28.

Statistical Analysis

Experiment was carried out with an $n = 8$ wells for the MTT assays, $n = 3$ for the apoptosis studies using flow cytometry analysis, and $n = 6$ mice in PBS and docetaxel and $n = 5$ in deslorelin and deslorelin-docetaxel groups for *in vivo* studies. Data in all cases are expressed as mean ± SD. Comparison of mean values between the different treatments was carried out using two-way ANOVA followed by Tukey's post hoc analysis with the Statistical Package for the Social Sciences (version 8) software. The level of significance was set at $P < 0.05$.

Results

^{1}H-NMR Characterization of Deslorelin-Docetaxel Conjugate

The ^{1}H-NMR spectra of deslorelin, docetaxel, docetaxel-hemiglutarate, and deslorelin-docetaxel conjugate were obtained (Fig. 2). In the deslorelin spectra (Fig. 2A), characteristic -CH_3 peak of leucine at 0.95 ppm, the amide R-CONH peaks between 5.5 and 8.0 ppm, and NMR shifts of the phenolic hydroxyl group of tyrosine at 10.8 ppm were observed (31). In the docetaxel spectrum (Fig. 2B), the signal corresponding to C-2′ OH group at 3.56 ppm was observed (32). In the docetaxel-hemiglutarate spectrum (Fig. 2C), this characteristic docetaxel peak was absent and a -CH_2-C = O peak at 2.62 ppm appeared, confirming the conjugation of glutaric anhydride to docetaxel. In the deslorelin-docetaxel conjugate spectrum (Fig. 2D), characteristic peaks of both deslorelin and docetaxel were observed in addition to an ester -H-C-COOR peak at 2.2 ppm, indicating the formation of a linkage between deslorelin and docetaxel-hemiglutarate. Furthermore, the presence of the glutarate linker was observed as an overlapping peak at 2.55 ppm along with the DMSO-d_6 peak. In the deslorelin-docetaxel NMR spectra, the phenolic -OH peak shift was present at 10.8 ppm, indicating that this group was not modified in the conjugation reaction. This further supports the conjugation of docetaxel-hemiglutarate at the -CH_2-OH position of serine, the fourth amino acid in deslorelin.

FTIR Characterization of Deslorelin-Docetaxel Conjugate

Characteristic peaks of deslorelin, docetaxel, docetaxel-hemiglutarate, and deslorelin-docetaxel conjugate were obtained using FTIR spectroscopy (Fig. 3A). The docetaxel-hemiglutarate spectrum exhibited a characteristic peak for C = O stretch of the carboxyl group at 1,710 cm^{-1}, which was not present in the docetaxel spectra. Further, an O-H stretch of the carboxyl group was observed at 2,750 to 3,000 cm^{-1} in the docetaxel-hemiglutarate spectrum,
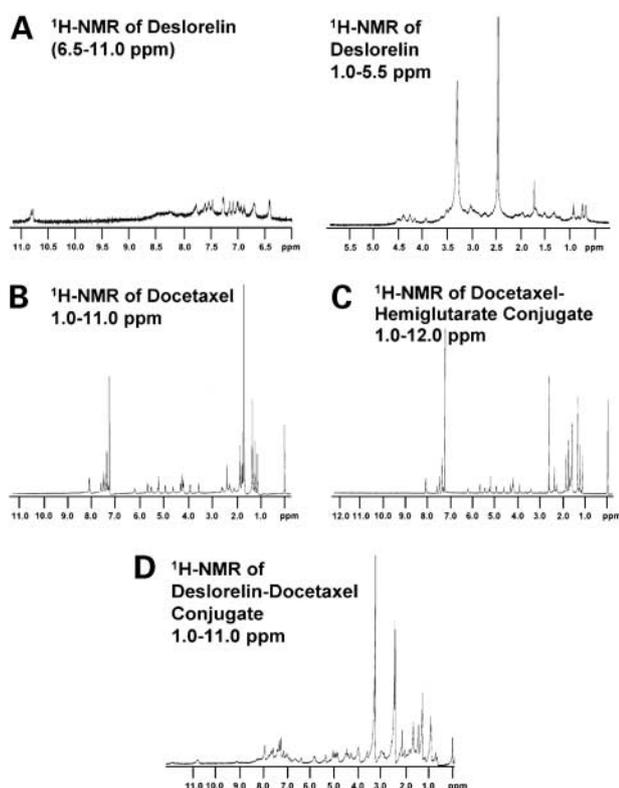
Figure 2. ^{1}H-NMR spectroscopic confirmation of the formation of deslorelin-docetaxel conjugate. **A,** deslorelin (in the 6–11 ppm and in the 0.5–6 ppm ranges). **B,** docetaxel. **C,** docetaxel-hemiglutarate. **D,** deslorelin-docetaxel conjugate spectra. Characteristic peaks for -CH$_3$ of leucine at 0.95 ppm, amide linkages of R-CONH at 5.5 to 8.0 ppm, and a shift corresponding to phenolic -OH group of tyrosine were observed in deslorelin spectra. The two panels for deslorelin were obtained by zooming into the spectrum to better visualize the peaks between 6.0 and 8.0 ppm. The absence of the C-2′ -OH peak at 3.56 ppm observed in the docetaxel spectrum and the presence of a -CH$_2$-C = O peak at 2.62 ppm in the spectrum of docetaxel-hemiglutarate confirm the conjugation of glutaric anhydride to docetaxel. The presence of a -H-C-COOR peak at 2.2 ppm in conjunction with an overlapping peak at 2.55 ppm corresponding to glutarate linker confirms the formation of the deslorelin-docetaxel conjugate. Deslorelin-docetaxel conjugates are formed via an ester bond linking the -COOH group of docetaxel-hemiglutarate with the -OH group of serine in deslorelin.

confirming the introduction of free carboxyl group in docetaxel conjugated with glutarate. The deslorelin spectrum exhibited characteristic broad amide peaks between 3,100 and 3,500 cm^{-1}. The deslorelin-docetaxel conjugate spectrum exhibited the characteristic peaks of both deslorelin (amide peaks) and docetaxel-hemiglutarate. The conjugation of peptide to docetaxel was evident from the C = O stretch of the ester linkage at 1,735 and 1,710 cm^{-1} in the conjugate spectrum. This ester linkage was not present in deslorelin, which had only amide linkages, confirming conjugation of deslorelin and docetaxel.

LC-MS Characterization of Deslorelin-Docetaxel Conjugate

Formation of the deslorelin-docetaxel conjugate was confirmed with LC-MS analysis using a manual infusion method (Fig. 3B). A peak for the conjugate was observed

at 1,078 m/z (a.m.u.). Following Bayesian reconstruct analysis, two charges were observed on the compound, which gave a peak at 1,078 m/z (a.m.u.). Hence, by the reconstruct analysis, the actual mass of the conjugate was calculated to be 2,156 a.m.u. The calculated mass of the compound is ~2,200 a.m.u. This indicates that the conjugate is indeed formed.

Deslorelin-Docetaxel Conjugate Is More Effective in LNCaP and PC-3 Cells as Compared with Docetaxel Alone

Antiproliferative effects of deslorelin-docetaxel conjugate on LNCaP and PC-3 cells are shown in Fig. 4. It is evident that docetaxel does not lose its activity by formation of the deslorelin-docetaxel conjugate. Following 24, 48, and 72 hours of exposure, it is evident that the deslorelin-docetaxel conjugate is significantly more cytotoxic compared with docetaxel alone. Further, the drug/conjugate exhibits cytotoxicities in both LNCaP (Fig. 4A) and PC-3 (Fig. 4C) cells in the order deslorelin-docetaxel conjugate > docetaxel > deslorelin at all time points tested (24, 48, and 72 hours). The IC$_{50}$ values of the drugs confirm this notion. The data were fit to a sigmoidal inhibitory E_{max} pharmacodynamic model. Deslorelin did not have any cytotoxic effects on both LNCaP and PC-3 cells even at doses as high as 100 nmol/L. IC$_{50}$ values could not be accurately determined for deslorelin.

Decreased Expression of LHRH-R Leads to a Decrease in the Effects of Deslorelin-Docetaxel Conjugate

The effects of decreased expression of LHRH-R on the cytotoxicity of the deslorelin-docetaxel conjugate in LNCaP (Fig. 4B) and PC-3 (Fig. 4D) cells are shown. It can be inferred that deslorelin does not lose its binding efficacy or LHRH-R interactive ability due to the conjugation with docetaxel. Pretreatment with AON leads to a significant decrease in the cytotoxicity of the deslorelin-docetaxel conjugate. This effect is not observed in the SON-treated groups, indicating that the LHRH-R is involved in the efficacy of the deslorelin-docetaxel conjugate. The IC$_{50}$ values were calculated using a sigmoidal inhibitory E_{max} pharmacodynamic model. Deslorelin-treated groups did not exhibit cytotoxicity in any of the groups even at doses as high as 100 nmol/L. The IC$_{50}$ values for deslorelin could not be determined.

Deslorelin-Docetaxel Conjugate Leads to Higher Rates of Apoptosis as Compared with the Parent Compounds Alone

Cell cycle analysis of LNCaP and PC-3 cells exposed to docetaxel and deslorelin-docetaxel conjugate for 24 hours is shown in Fig. 5. It is evident that deslorelin-docetaxel conjugate induces significantly greater apoptosis in both LNCaP and PC-3 cells compared with docetaxel alone. Deslorelin alone had no effect on cell cycle in both LNCaP and PC-3 cells. In LNCaP cells, docetaxel exhibited significant arrest in G$_2$-M phase, whereas deslorelin-docetaxel conjugate exhibited a significant decrease in G$_2$-M phase arrest with a corresponding increase in apoptosis. In PC-3 cells, docetaxel exhibited a significantly higher arrest in G$_2$-M phase, whereas deslorelin-docetaxel conjugate exhibited similar arrest in
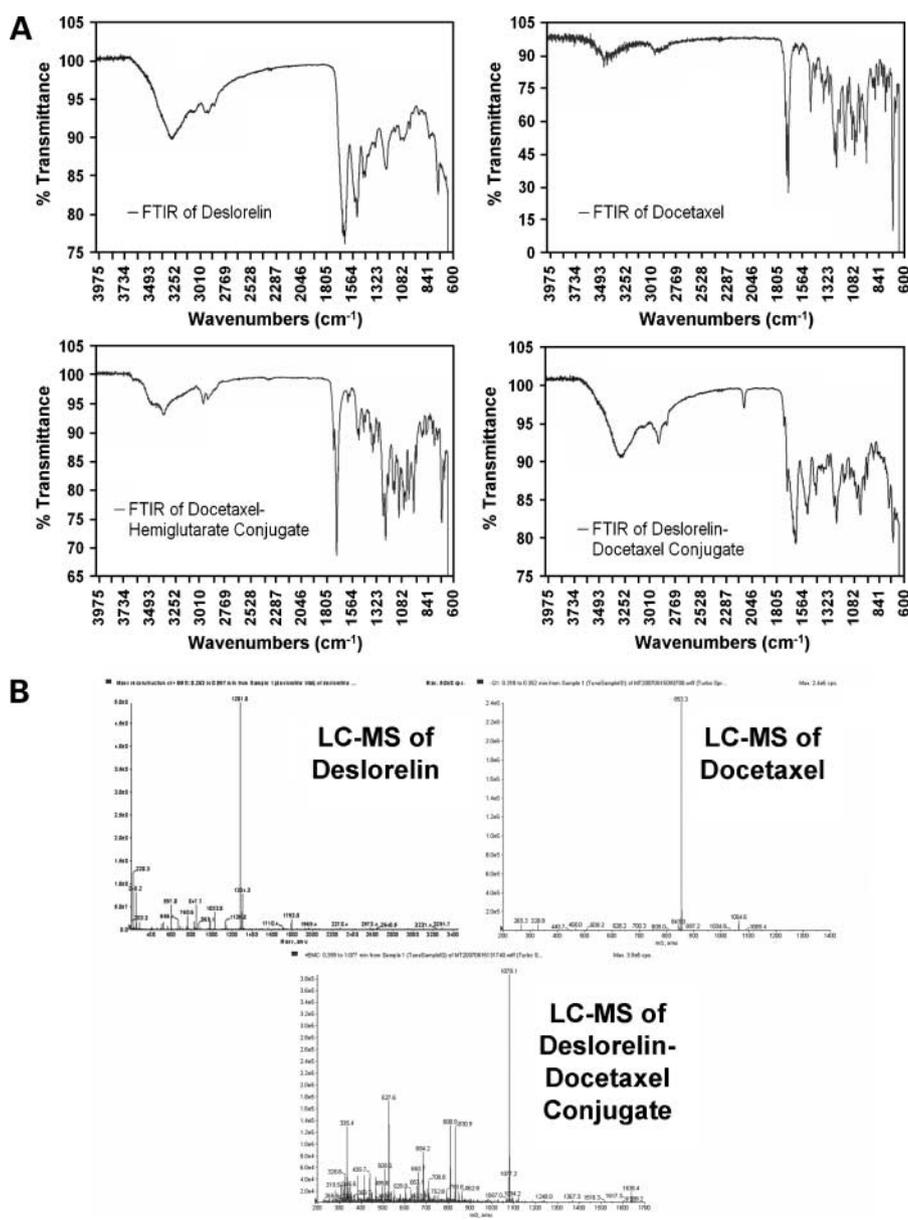
Figure 3. Spectroscopic confirmation of the formation of deslorelin-docetaxel conjugate. **A,** FTIR spectra. **B,** LC-MS spectra. **A,** includes FTIR spectra of deslorelin, docetaxel, docetaxel-hemiglutarate, and deslorelin-docetaxel conjugate. Deslorelin exhibited characteristic broad amide peaks at 3,100 to 3,500 cm^{-1}. Formation of docetaxel-hemiglutarate is characterized by the presence of a peak for the C = O stretch of the carboxyl group at 1,710 cm^{-1} not present in the docetaxel spectra. It is further confirmed by the peak observed at 2,750 to 3,000 cm^{-1} characterizing an O-H stretch of the carboxyl group. Conjugation of docetaxel-hemiglutarate and deslorelin is confirmed by the presence of a characteristic peak at 1,735 and 1,710 cm^{-1} corresponding to the C = O stretch of the ester linkage, which is absent in deslorelin as well as the docetaxel-hemiglutarate spectra. This confirms the formation of the deslorelin-docetaxel conjugate. **B,** includes the LC-MS spectra of deslorelin, docetaxel, and deslorelin-docetaxel conjugate. The peak at 1,078 a.m.u. represents deslorelin-docetaxel conjugate. Deslorelin peak was observed at 1,281.2 a.m.u., whereas docetaxel peak was observed at 853.3 a.m.u. Based on the mass of deslorelin and docetaxel as well as the mass of the glutarate linker, the calculated mass of the deslorelin-docetaxel conjugate is 2,200 a.m.u. Based on a Bayesian reconstruct analysis on the peak observed at 1,078 *m/z* (a.m.u.) for deslorelin-docetaxel conjugate, the actual mass of the conjugate was calculated to be 2,156. This confirms the formation of deslorelin-docetaxel conjugate.

G$_2$-M phase but higher apoptosis induction. The percentage of cells reaching apoptosis is significantly greater in the deslorelin-docetaxel group compared with the docetaxel group. Based on cell cycle analysis, both LNCaP and PC-3 cells following treatment for 24 hours exhibit percent apoptosis in the following order: deslorelin-docetaxel conjugate > docetaxel > deslorelin ≥ untreated controls.

Deslorelin-Docetaxel Conjugate Exhibits the Greatest Tumor Inhibition *In vivo*

Treatment efficacy following i.v. administration is shown in Fig. 6. Treatment of PC-3 prostate cancer xenograft-bearing mice with docetaxel (*P* = 0.002) and deslorelin-docetaxel (*P* = 0.006) exhibited significant tumor growth inhibition compared with vehicle-treated mice alone at the end of 28 days, with the differences between conjugate and docetaxel being significant throughout the study. Deslorelin-treated mice exhibited significant difference until day 18 (*P* = 0.002) only. On day 28, deslorelin-treated mice did not exhibit significant difference (*P* = 0.47) compared with vehicle-treated mice. Prostate cancer PC-3 xenograft-bearing mice treated i.v. with various treatment groups exhibited significant (*P* ≤ 0.001) tumor inhibition [measured as area under the tumor volume-time curve (AUC) until day 28] in the following order: deslorelin-docetaxel > docetaxel ≫ deslorelin > PBS. One animal in deslorelin group and another in deslorelin-docetaxel group died on day 11 due to excessive bleeding related to injections. These animals exhibited similar tumor reduction compared with animals in the respective groups. These animals were excluded in Fig. 6.
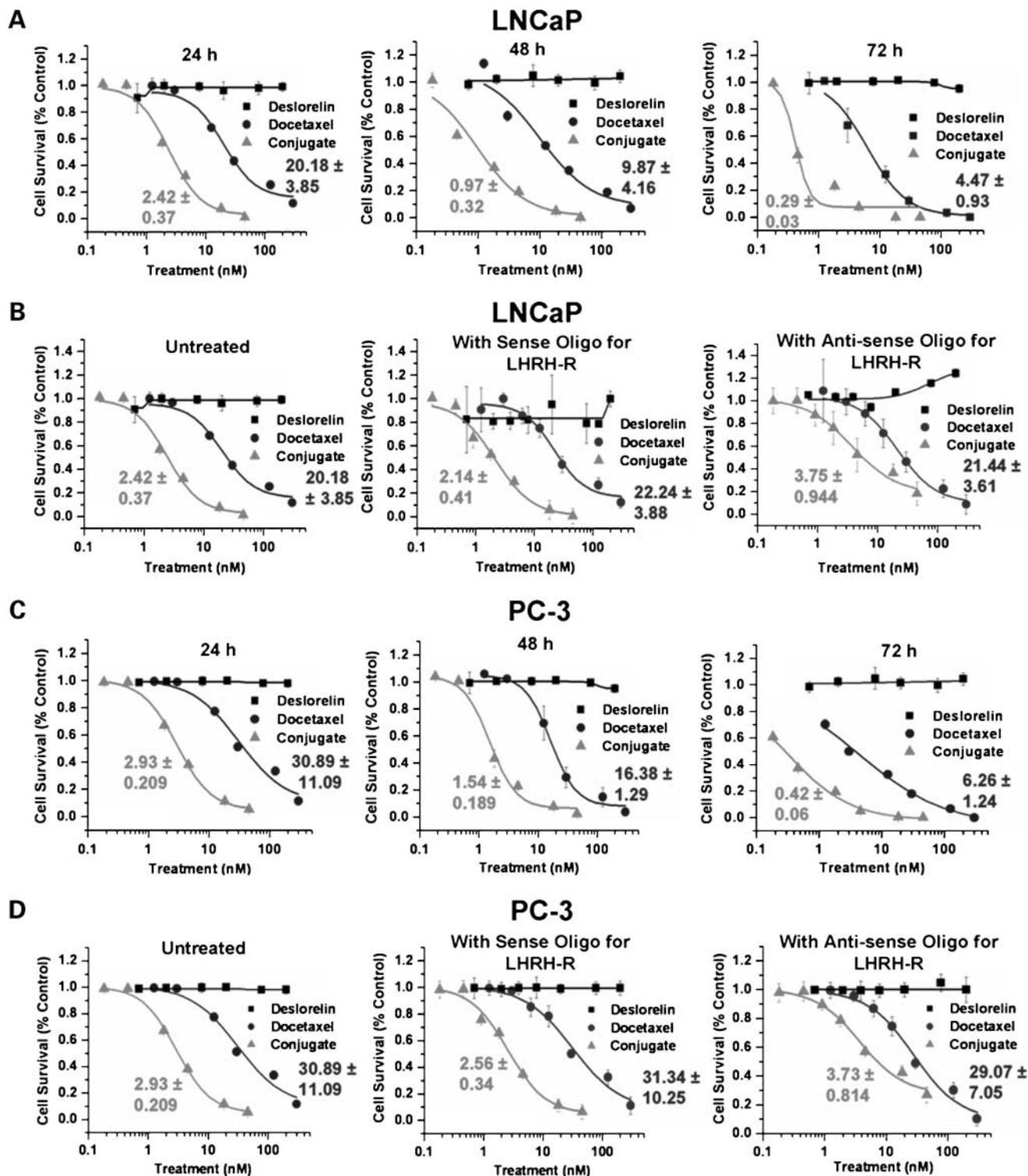
Figure 4. *In vitro* assessment of the antiproliferative effects of deslorelin-docetaxel conjugates in LNCaP and PC-3 prostate cancer cells. **A** and **B,** LNCaP. **C** and **D,** PC-3 cells. The LNCaP (**A**) and PC-3 (**C**) prostate cancer cell lines were treated with various concentrations of deslorelin, docetaxel, and deslorelin-docetaxel conjugate for 24, 48, or 72 h. Further, LNCaP (**B**) and PC-3 (**D**) cells were pretreated with SON and AON followed by treatment with various concentrations of deslorelin, docetaxel, and deslorelin-docetaxel conjugate for 24 h. The antiproliferative effect of these treatments was determined using a MTT assay. *Points,* mean ($n = 8$); *bars,* SD. The IC_{50} values were calculated using the inhibitory sigmoid E_{max} model. The lines were fit using this model.
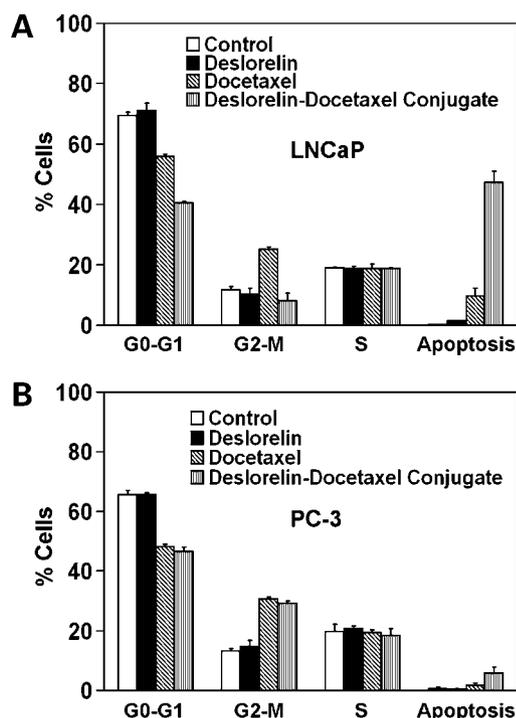
Figure 5. *In vitro* assessment of the effect of deslorelin-docetaxel conjugate on cell cycle in LNCaP and PC-3 prostate cancer cells. The cells were treated with approximate IC_{50} concentrations of docetaxel and deslorelin-docetaxel conjugate for 24 h. The cells were fixed with 70% ethanol and stained with propidium iodide. DNA content was then analyzed by flow cytometry. **A,** LNCaP cells. **B,** PC-3 cells. *Columns,* mean ($n = 3$); *bars,* SD.

Discussion

Targeting docetaxel specifically to tumors can potentially improve its efficacy and minimize its side effects. Our studies were aimed at designing a docetaxel conjugate that can specifically target prostate cancer cells and have better efficacy than docetaxel alone. Overexpression of LHRH-R is observed in prostate tumor as well as prostate cancer cell lines. We therefore proposed that deslorelin conjugated to docetaxel could confer selectivity and enhanced efficacy to docetaxel. Based on our studies, we report the following key findings: (*a*) deslorelin conjugate of docetaxel is feasible, (*b*) the docetaxel-deslorelin conjugate formed is a viable molecule retaining the pharmacologic effects of deslorelin and cytotoxic effects of docetaxel, and (*c*) the conjugation of deslorelin and docetaxel improves *in vitro* and *in vivo* therapeutic efficacy of docetaxel. To the best of our knowledge, this is the first study to report a docetaxel-peptide conjugate and its efficacy in cancer models.

Deslorelin, a LHRH agonist, is 144 times more potent and has a longer elimination half-life of 2.5 hours compared with native LHRH. We therefore chose to conjugate deslorelin to docetaxel instead of native LHRH. Docetaxel has four hydroxyl (-OH) groups at C-1, C-7, C-10, and C-2′ that were suitable for conjugation purpose. In addition, deslorelin has hydroxyl (-OH) in serine moiety that could be used for

conjugation. However, both these molecules did not possess functional groups such as carboxyl (-COOH) suitable for direct conjugation with the other molecule. Hence, a free carboxyl group (-COOH) was introduced in docetaxel at C-2′ position by conjugating glutaric anhydride. Although four hydroxyl groups were present in docetaxel, 2′-hydroxyl and 7-hydroxyl groups are more amenable for structural modifications (33). Due to the steric hindrance at the 7-hydroxyl position, 2′-hydroxyl group is more readily available for modifications (34). Indeed, Liu et al. (35) reported polyethyleneglycol-docetaxel conjugate with conjugation occurring at the 2′-hydroxyl position.

The NMR and FTIR studies also confirmed conjugation at the 2′-hydroxyl position in this study. Furthermore, the glutarate linker is longer compared with other possible linkers
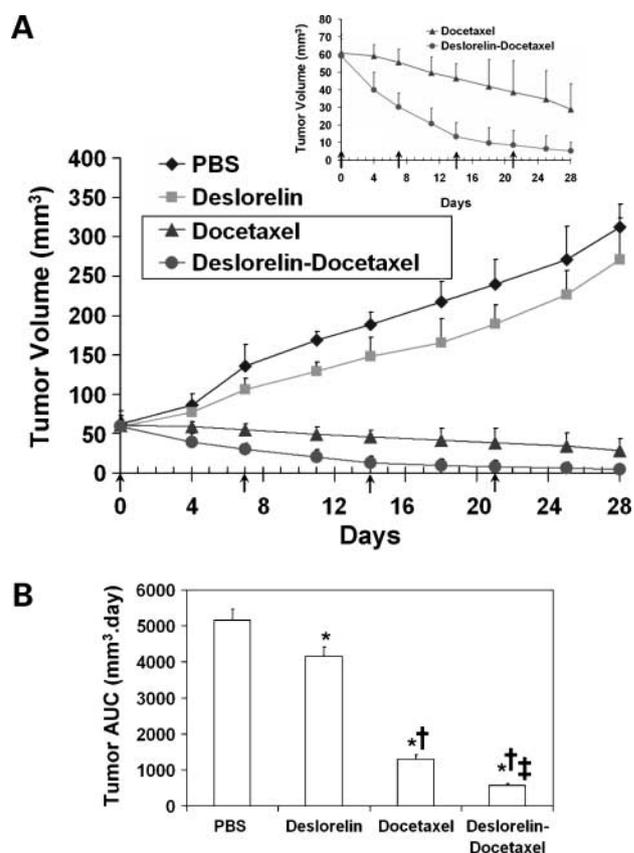

Figure 6. *In vivo* assessment of the effect of deslorelin-docetaxel conjugates on tumor growth in PC-3 prostate cancer xenografts in athymic nude mice. **A,** tumor volumes. **B,** AUC. **A,** tumor growth inhibition. The mice were treated with deslorelin and docetaxel at 2.5 mg/kg and deslorelin-docetaxel at 2.5 mg/kg equivalent of docetaxel. Controls received vehicle (PBS). All treatments were administered i.v. *Arrows,* days when treatments were administered. Tumor volumes were measured using a Vernier caliper and the following formula: length × width^2 × 0.52. The inset compares tumor volumes following treatment with docetaxel and deslorelin-docetaxel. **B,** AUC (mm^3 · day) calculated for each treatment group from the tumor volumes of mice in each treatment group. *Columns,* mean of six mice in the PBS and docetaxel groups and five mice in the deslorelin and deslorelin-docetaxel groups; *bars,* SD. *, $P < 0.05$, compared with AUC of the PBS group; †, $P < 0.05$, compared with AUC of the deslorelin group; ‡, $P < 0.05$, compared with AUC of the docetaxel group.

such as succinate and allows more room for deslorelin to bind the receptor. Studies have shown that both ends of the LHRH peptide or its analogues are a prerequisite for its receptor binding (6, 36). The reaction was therefore designed and carried out such that the deslorelin conjugation occurred at an intermediate amino acid, leaving the end terminals free for receptor interactions. ^{1}H-NMR and FTIR studies at each step of the conjugation process confirmed the formation of the expected intermediates and final products. Thus, formation of docetaxel-hemiglutarate was confirmed. Some earlier conjugates of anticancer drugs such as doxorubicin to LHRH analogues, where the end groups of peptide were free, retained LHRH analogue activity (37). The conjugation in this study was further confirmed with a LC-MS analysis. Both deslorelin (peptide) and docetaxel are ionized in positive mode of ionization of mass spectrometer because amino group in the structure of both compounds gives $[M+1]^+$ ion. The molecular weight range for mass spectrometer was 1,700 *m/z* (Q-Trap 2000 LC/MS/MS). The formation of conjugate was therefore monitored by Bayesian peptide reconstruct software to calculate the mass of multiple charged ions. The deslorelin-docetaxel conjugate peak was observed at 1,078.1 a.m.u. with two positive charges and no peak at 853 and 1,281.8 a.m.u. The *m/z* analysis of multiple charged ions using Bayesian reconstruct showed that the molecular weight of the conjugate is 2,156.2 a.m.u., thereby confirming the formation of deslorelin-docetaxel conjugate. In addition, a reverse-phase high-performance liquid chromatography analysis as per a method reported earlier (27) indicated the absence of free deslorelin in the product. The purified deslorelin-docetaxel conjugate was used in subsequent experiments.

The biological activities of both carrier and cytotoxic molecule need to be maintained at the target cells. This is a very important consideration in the design and synthesis of targeted anticancer agents. To confirm the activity of the conjugate, we further tested its efficacy in LNCaP, a hormone-dependent cell line, and PC-3, a hormone-independent cell line, two cell lines overexpressing the LHRH-R (38). Our antiproliferative studies showed that the deslorelin-docetaxel conjugate is significantly more cytotoxic in both cell lines compared with docetaxel alone. These studies suggest that conjugation does not hinder docetaxel activity. Further, deslorelin by itself did not have any effect on the cells, which is consistent with the previous observations indicating lack of cytotoxicity of LHRH in various LHRH-R–expressing cell lines (39). Deslorelin likely serves as a targeting moiety for enhanced uptake in these cells overexpressing LHRH-R. Our previous studies indicated the ability of deslorelin to undergo receptor-mediated uptake in cells expressing LHRH-R (23).

The conjugate efficacy depends on binding efficacy of the targeting ligand as well as the number of receptors present on the surface of a cell. To determine whether the deslorelin-docetaxel conjugate is indeed effective via LHRH-R, LNCaP and PC-3 cells were treated with deslorelin-docetaxel conjugate following pretreatment with AON against the LHRH-R. Previous studies from our laboratory have used the AON

approach to selectively reduce LHRH-R expression (29). Using this AON, previous studies have shown a decrease in the LHRH-R mRNA expression by ~73 ± 10.2% and LHRH-R protein expression by 42 ± 4.8% in a lung cancer cell line (23). In this study, a decrease in cytotoxicity by the deslorelin-docetaxel conjugate was observed in both the LNCaP and PC-3 cells treated with AON against LHRH-R. Further, it was interesting to note that, although there is a significant decrease in cytotoxicity in the AON-treated group, the deslorelin-docetaxel conjugate is still significantly more cytotoxic compared with docetaxel alone. The decrease in efficacy of the conjugate in the AON-treated group could be the result of a decrease in the LHRH-R expression. The decrease in efficacy of the conjugate in AON-treated cells indeed suggests that the cytotoxic effects observed following treatment with the deslorelin-docetaxel conjugate are due to binding and uptake of the conjugate via the LHRH-R. This bears evidence that deslorelin binding is not eliminated by its conjugation to docetaxel.

Antiproliferative or cytotoxic effect of chemotherapeutic agents is both dose and time dependent. Higher doses and longer exposure duration lead to improved effects (6, 36). Increasing the exposure time of both docetaxel as well as deslorelin-docetaxel in our experiments led to an improvement in efficacy over a period of 72 hours, with the effects being more dramatic with the conjugate compared with docetaxel alone. The enhanced efficacy of the deslorelin-docetaxel conjugate can be attributed to overexpression of LHRH-R in the cell lines used in the study.

Docetaxel induced microtubule damage results in a marked and prolonged G_2-M arrest and subsequent apoptosis (40). Flow cytometric analysis of docetaxel-treated cells therefore exhibits an increase in the number of cells in the G_2-M phase, suggesting a G_2-M block and an increase in sub-G_0-G_1 phase, indicating the hypodiploid or apoptotic cells. Consistent with previous findings, we observed that, following treatment with docetaxel, there was an increase in the number of apoptotic cells in both PC-3 and LNCaP. Greater apoptotic cell death was observed following treatment with deslorelin-docetaxel conjugate when compared with docetaxel alone. Conjugates of LHRH were previously shown to exhibit higher apoptosis compared with their unconjugated counterparts (41). The higher apoptosis observed with the deslorelin-docetaxel conjugate is likely due to enhanced uptake of docetaxel via the LHRH-R and more effective countereffects against cellular antiapoptotic factors known to prevent the translation of cell damage to apoptosis (41, 42). Further, the higher effects observed with the conjugate might also be due to the inhibition of cdc25 phosphatase enzyme preventing cell cycle progression (43).

The IC_{50} of the conjugate in antiproliferative studies was ~1 nmol/L, which is 15 times lower than the IC_{50} of docetaxel at 72 h. Thus, with the conjugate, lower amounts of drug will be required to achieve the same effect as docetaxel alone. Such lower amounts while achieving similar efficacy are expected to lower side effects of the drug. Alternatively, the conjugate at doses comparable with clinical doses of

docetaxel would likely allow greater tumor reduction. Our *in vivo* studies confirm this notion. Following repeated i.v. injections, we observed that deslorelin-docetaxel exhibited a 59.6-fold tumor growth inhibition, whereas docetaxel alone exhibited an 11.2-fold growth inhibition compared with vehicle-treated group on day 28. Furthermore, deslorelin-docetaxel achieved a 51.7- and 5.5-fold tumor growth inhibition compared with deslorelin and docetaxel, respectively. Deslorelin alone exhibited only a 1-fold lower tumor volume compared with the vehicle-treated group. This is probably because deslorelin is not cytotoxic to the cells. However, LHRH superagonists such as deslorelin might inhibit tumor growth after prolonged treatment by suppressing production of testosterone, which is implicated in tumor growth (44). Other studies have shown that LHRH analogues act on tumor cells by interfering with the tyrosine kinase activity (45). Overall, our results confirm that deslorelin-docetaxel is indeed more effective than docetaxel or deslorelin *in vivo*. This efficacy could be attributed to its targeting features that allow for better localization of treatment doses in tumor tissue and cells compared with its nontargeted counterparts.

In summary, we have synthesized a deslorelin-docetaxel conjugate and characterized its efficacy *in vitro* in cancer cell lines (Fig. 1B) and *in vivo* in prostate cancer xenograft models. Our results show that the deslorelin-docetaxel conjugate is feasible via an ester linkage between docetaxel-hemiglutarate (-COOH) group and the fourth amino acid, serine (-OH) group. The conjugate formed is an effective molecule and retains the efficacy of both docetaxel as well as deslorelin. Deslorelin-docetaxel conjugate is significantly more effective compared with docetaxel alone with respect to its cytotoxic effects and its effects on cell cycle arrest and apoptosis induction *in vitro*. Further, it exerts several folds greater tumor inhibition compared with docetaxel or deslorelin. In conclusion, our results show that deslorelin-docetaxel conjugate is a useful alternative for enhanced efficacy of docetaxel in treating prostate and other cancers.

Disclosure of Potential Conflicts of Interest

Patent application pending.

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