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Gonadotropin-releasing hormone receptor-targeted paclitaxel–degarelix conjugate: synthesis and *in vitro* evaluation‡

Chenhong Wang,§ Yongtao Ma,§ Siliang Feng, Keliang Liu and Ning Zhou*

To increase the selectivity of chemotherapeutic agents, receptor-mediated tumor-targeting approaches have been developed. Here, degarelix [Ac-*D*-Nal-*D*-Cpa-*D*-Pal-Ser-Aph(*L*-Hor)-*D*-Aph(Cbm)-Leu-ILys-Pro-*D*-Ala-NH_2], a gonadotropin-releasing hormone antagonist, was employed as a targeting moiety for paclitaxel (PTX). Five PTX–degarelix conjugates were synthesized, in which PTX was attached via disulfide bond to the different position in the degarelix sequence. All of the PTX–degarelix conjugates exhibited a half-life greater than 10 h determined in human serum. A fluorometric imaging plate reader assay showed that the conjugates LK-MY-9 and LK-MY-10 had an antagonism efficacy similar to that of degarelix. The *in vitro* cytostatic effects of the conjugates were determined by a (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay, and the 50% inhibitory concentration value of the conjugates on 3T3 mouse embryonic fibroblast cells were one order of magnitude higher than the 50% inhibitory concentration values of the conjugates on MCF-7 human breast cancer cells and HT-29 human colon cancer cells. Receptor saturation tests further demonstrated that pre-incubation of the cells with degarelix reduced the efficacy of LK-MY-10 in a concentration-dependent manner. In conclusion, degarelix is a valid and stable moiety that has great potential for targeting chemotherapy drugs. Copyright © 2015 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: targeted drug delivery; gonadotropin-releasing hormone antagonist; paclitaxel–degarelix conjugates; *in vitro* stability

Introduction

While chemotherapy has been the main treatment modality for metastatic diseases, its efficacy has been greatly limited by intrinsic or acquired multidrug resistance and nonspecific toxicity to normal cells [1–3]. Through the use of specific cell-surface markers, targeted therapy can improve the therapeutic index of antitumor drugs [4–7]. For instance, the gonadotropin-releasing hormone receptor (GnRH-R) is a member of the G protein-coupled receptor superfamily [8] and is overexpressed in the anterior pituitary and also on the surface of tumor cells from the ovary [8], prostate [9], breast [10], and endometrium [11,12]. In contrast, expression of the GnRH-R is not detected in normal organs [10,13–15]. Thus, gonadotropin-releasing hormone (GnRH) analogs have the potential to target agents that will enhance the uptake of antitumor drugs by GnRH-R-positive cancerous cells, while sparing normal cells.

The concept of developing targeted chemotherapeutics based on human GnRH derivatives was first introduced by A.V. Schally in the late 1980s. Since then, several conjugates employing GnRH peptide analogs as targeting moieties have been synthesized and characterized [16–18]. To date, the reported GnRH-targeted antitumor conjugates generally contain GnRH agonists conjugated to antitumor drug molecules. For instance, the conjugate Dox-[Lys_6]-GnRH (AN-152), which contains an ester bond coupling, is currently in phase III clinical trials and has been shown to induce apoptosis independent of multiple-drug resistance gene-1 (MDR-1) in GnRH-R-positive human ovarian and endometrial cancer cell lines [19]. However, the stability of AN-152 still needs to be improved, and the half-life of ester bonds in the presence of carboxylesterases is 2 h in human serum and 20 min in mouse serum [20]. This rapid degradation can lead to the premature release of a cytotoxic drug, thereby resulting in reduced uptake and efficacy in cancer cells and widespread toxicity for rapidly proliferating cells [21,22]. Therefore, many anticancer drug–GnRH agonists' conjugates with higher stability in human serum were developed, and the anticancer drug was attached to the GnRH peptide through various spacers/linkers, such as oxime bond and hydrazone bond [23,24]. It is well known that GnRH antagonists exhibit a similar affinity for the GnRH-R as GnRH agonists yet they have enhanced enzymatic stability. Therefore, it is hypothesized that if GnRH antagonists are replaced with GnRH agonists, the resulting conjugates would not only retain their affinity for the GnRH-R but would also be more stable. Of the GnRH antagonists

* *Correspondence to: Ning Zhou, State Key Laboratory of Toxicology and Medical Countermeasures, Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China. E-mail: zhouning6818@163.com*

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§ *Both authors contributed equally to this work.*

State Key Laboratory of Toxicology and Medical Countermeasures, Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China

Abbreviations: *PTX, paclitaxel; GSH, glutathione; DIC, N,N′-diisopropylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DMAP, 4-dimethylaminopyridine; DIEA, N,N′-diisopropylethylamine; TFA, trifluoroacetic acid; 4Aph, 4-aminophenylalanine; Cbm, carbamyl; 4Cpa, 4-chlorophenylalanine; Hor, L-hydroorotyl; Ilys, N^{ε}-isopropyl lysine; DCM, dichloromethane; Pal, 3-pyridylalanine; MBHA, p-methylbenzhydrylamine; HBSS, Hank's balanced salt solution; OD, optical density; FLIPR, fluorometric imaging plate reader.*

developed to date, degarelix is the most recently approved by Food and Drug Administration (FDA) [25]. Degarelix has a high affinity for the GnRH-R, is enzymatically stable, and has been used for the treatment of advanced hormone-dependent prostate cancer in the USA and the European Union [26]. Therefore, the aim of the present study was to develop and define the antiproliferative effects of various conjugates that contain the chemotherapeutic agent, PTX, and the targeting moiety, degarelix.

PTX is a widely used antitumor drug that interferes with the dissociation of microtubules during cell division [27]. It has been used to treat patients with lung, ovarian, breast, head, or neck cancers, as well as for patients with advanced forms of Kaposi's sarcoma [28,29]. Here, PTX was conjugated to a degarelix analog via disulfide bonds, which are stable under normal physiological conditions yet are reduced to free thiols in the presence of reducing agents, such as GSH. GSH is an ideal reducer because it is abundant in the cell cytoplasm (~10 mM) yet is present at very low levels in blood plasma (~0.002 mM) [30]. It is hypothesized that when the conjugates are exposed to the reductive environment of tumor cells, their disulfide bonds will be cleaved, thereby resulting in the release of conjugated drug. Five PTX–degarelix conjugates were synthesized and characterized. *In vitro* stability of the conjugates were assessed in human serum by liquid chromatography, and their *in vitro* cytostatic effects were evaluated using (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assays with human breast cancer, human colon cancer, and mouse embryonic fibroblast cell lines.

Materials and Methods

Materials

PTX was purchased from Beijing Zhongshuo Pharmaceutical Technology Development Co., Ltd (Beijing, China). DIC, HOBt, and all of the Boc-protected amino acids were obtained from GL Biochem Shanghai Ltd (Shanghai, China). The reagents, DMAP, DIEA, and TFA were purchased from J&K Chemical (Beijing, China). As previously described [31], 2-pyridyl disulfide *p*-nitrophenyl carbonate was prepared. Most amino acid derivatives were obtained from Chengnuo Ltd (Chengdu, China), including Boc-*D*-Ala-OH, Boc-*D*-2-naphthylalanine-OH (Boc-*D*-Nal-OH), Boc-*D*-4Cpa-OH (Boc-*D*-Cpa-OH), Boc-N^{ε}-isopropyl lysine(Z)-OH [Boc-ILys(Z)-OH], Boc-Leu-OH, Boc-Pal-OH (Boc-*D*-Pal-OH), Boc-Pro-OH, and Boc-Ser(Bzl)-OH. Boc-*L*-4Aph(Fmoc)-OH [Boc-*L*-Aph(Fmoc)-OH] and Boc-*D*-Aph(Fmoc)-OH were synthesized from Boc-*L*-4-nitro-Phe-OH and Boc-*D*-4-nitro-Phe-OH (Innochem, Beijing, China), respectively, as previously published [32]. Boc-*D*-4Aph (Cbm)-OH [Boc-*D*-Aph(Cbm)-OH] was synthesized from Boc-*D*-Aph-OH as previously published [33]. The MBHA resin was obtained from NanKai HeCheng (Tianjin, China). Human serum was obtained from the 307 Hospital (Beijing, China). CellTiter 96® AQueous One Solution Reagent was purchased from Promega Corporation (Madison, WI, USA). All reagents and solvents were of analytical grade and used without further purification.

Preparation of PTX Derivatives

PTX (0.8671 g, 1.02 mmol), 2-pyridyl disulfide *p*-nitrophenyl carbonate (0.3878 g, 1.1 mmol), and DMAP (0.1406 g, 1.15 mmol) were mixed with 15 ml of DCM. After the solution was stirred in the dark for 24 h at room temperature (RT), it was diluted with 50 ml of DCM, was washed once with a saturated solution of $NaHCO_3$ (50 ml) and brine (50 ml), and then was dried over Na_2SO_4. The solvent was evaporated under vacuum, and the residual product was purified on a silica gel column and eluted with DCM/CH_3OH (100 : 1). A white powder of 2-pyridyl disulfide PTX carbonate was obtained (0.971 g, 89% yield), with an ESI-MS mass-to-charge ratio (m/z) of 1068. 9 $[M+H]^{+1}$ (calculated for $C_{55}H_{58}N_2O_{16}S_2$, 1067.3), ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.32 (d, 1H, $J=8$ Hz, –N**H**), 8.45–7.19 (m, 19H, **H**-Ph, **H**-Py), 6.29 (s, 2H, **H**-10), 5.83 (m, 1H, **H**-13), 5.55 (t, 1H, $J=8$ Hz, **H**-3′), 5.41 (d, 1H, $J=8$ Hz, **H**-2), 5.37 (d, 1H, $J=8$ Hz, **H**-2′), 4.93 (m, 1H, $\mathbf{H}_5$), 4.65 (s, 1H, **OH**-1), 4.39 (m, 2H, –CO–CH_2–$\mathbf{CH}_2$–S–S–), 4.11–4.01 (m, 3H, **H**-7, **H**-20α, **H**-20β), 3.59 (d, 1H, $J=7.2$ Hz, **H**-3), 3.14 (t, 2H, $J=8$ Hz, –CO–$\mathbf{CH}_2$–CH_2–S–S–), 2.33 (m, 1H, **H**-6α), 2.26 (s, 3H, $\mathbf{CH}_3$CO-4), 2.12 (s, 3H, $\mathbf{CH}_3$CO-10), 1.78 (m, 1H, **H**-14α), 1.75 (s, 3H, **H**-18), 1.64 (m, 1H, **H**-6β), 1.54 (m, 1H, **H**-14β), 1.50 (s, 3H, **H**-19), 1.03 (s, 3H, **H**-16), and 1.00 (s, 3H, **H**-17).

Preparation of Degarelix Derivatives

The five degarelix derivatives were prepared manually by solid-phase peptide synthesis according to Boc/Fmoc strategy on MBHA resin (1.08 mmol/g coupling capacity). The following Boc-protected amino acid derivatives were used: Boc-*D*-Ala-OH, Boc-Pro-OH, Boc-ILys(Z)-OH, Boc-Leu-OH, Boc-*D*-Nal-OH, Boc-*D*-Cpa-OH, Boc-Leu-OH, Boc-*D*-Pal-OH, Boc-Ser(Bzl)-OH, Boc-Aph(Fmoc)-OH, and Boc-*D*-Aph(Cbm)-OH.

The derivatives were synthesized as follows: (**1**) 10% DIEA/DCM (two times, 10 min each), (**2**) *N*,*N*,-dimethylformamide (DMF) washing (three times, 2 min each), CH_3OH washing (three times, 2 min each), DCM washing (three times, 2 min each), (**3**) coupling of two equivalent Boc-protected amino acid derivatives : DIC : HOBt (1 : 2 : 1) in DMF (4 h), (**4**) DMF washing (three times, 2 min each), CH_3OH washing (three times, 2 min each), DCM washing (three times, 2 min each), (**5**) Boc deprotection with 50% TFA/DCM (25 min), and (**6**) DMF washing (three times, 2 min each), CH_3OH washing (three times, 2 min each), DCM washing (three times, 2 min each). Upon completion of the coupling of ^{5}Aph, the Fmoc-protecting group was removed from the ω-NH_2 groups of ^{5}*D*-Aph with 50% pyridine in DMF (25 min). The *L*-hydroorotic acid (*L*-Hor) residue was then coupled to the ω-amino group of ^{5}Aph according to the previously mentioned protocol.

For the synthesis of Mer-*D*-Nal-*D*-Cpa-*D*-Pal-Ser-Aph(*L*-Hor)-*D*-Aph(Cbm)-Leu-ILys-Pro-*D*-Ala-NH_2 and Mer-Gln-Arg-*D*-Nal-*D*-Cpa-*D*-Pal-Ser-Aph(*L*-Hor)-*D*-Aph(Cbm)-Leu-ILys-Pro-*D*-Ala-NH_2, Trt-protecting mercaptopropionic acid (Trt-Mpa-OH) was coupled to the *N*-terminus of the peptide according to the previously mentioned protocol.

To synthesize Ac-*D*-Nal-*D*-Cpa-*D*-Pal-Ser-Aph(*L*-Hor)-*D*-Aph(Mer)-Leu-ILys-Pro-*D*-Ala-NH_2, Ac-*D*-Nal-*D*-Cpa-*D*-Pal-Ser-Aph(*L*-Hor)-*D*-Aph(-Arg-Gln-Mer)-Leu-ILys-Pro-*D*-Ala-NH_2, and Mer-Gln-Arg-*D*-Nal-*D*-Cpa-*D*-Pal-Ser-Aph(*L*-Hor)-*D*-Aph(-Arg-Gln-Mer)-Leu-ILys-Pro-*D*-Ala-NH_2, the Fmoc-protecting group was removed from the ω-NH_2 groups of ^{6}D-Aph with 50% pyridine in DMF (25 min). Then, Trt-Mpa-OH was attached to the free ω-NH_2 groups of ^{6}*D*-Aph according to the previously mentioned protocol. For Ac-*D*-Nal-*D*-Cpa-*D*-Pal-Ser-Aph(*L*-Hor)-*D*-Aph(-Arg-Gln-Mer)-Leu-ILys-Pro-*D*-Ala-NH_2 and Mer-Gln-Arg-*D*-Nal-*D*-Cpa-*D*-Pal-Ser-Aph(*L*-Hor)-*D*-Aph(-Arg-Gln-Mer)-Leu-ILys-Pro-*D*-Ala-NH_2, prior to the coupling of Trt-Mpa-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Gln-OH were serially coupled to the free ω-NH_2 groups of ^{6}*D*-Aph. The peptide

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sequences were then prolonged using the previously mentioned protocol. An acetyl group was introduced by capping the peptide with acetic anhydride and DIEA after deprotection of the Boc group of Boc-*D*-Nal-OH in the peptide terminus.

The degarelix derivatives were cleaved from the resin using anhydrous hydrogen fluoride (HF) in the presence of a scavenger (2% anisole) in an ice bath. After 1 h, the derivatives were precipitated with ice-cold diethyl ether, were washed three times with diethyl ether, and were solubilized in 20% acetic acid prior to freeze drying. The crude peptides were purified by preparative RP-HPLC and were analyzed by MALDI-TOF MS.

Synthesis of PTX–Degarelix Conjugates

Degarelix derivatives and 2-pyridyl disulfide PTX carbonate were dissolved in DMSO; the latter was added in a 20% excess compared with the degarelix derivative. The reaction mixture was stirred for 4 h at RT, and the reaction was monitored by RP-HPLC. The resulting conjugates were separated by preparative RP-HPLC and were analyzed by MALDI-TOF MS.

HPLC

The crude products were purified on a Prep LC 4000 system (Waters Corporation, Milford, MA, USA) using a preparative X-Bridge C8 column (195 mm × 250 mm) with 5 μm silica. A linear gradient elution was performed with eluent A (0.1% TFA/H_2O) and eluent B [0.1% TFA in acetonitrile–water (70 : 30, v/v)] (0 min 40% B; 5 min 55% B; 25 min 80% B; 35 min 40% B) at a flow rate of 16 ml/min. Peaks were detected at 220 nm.

Analytical RP-HPLC was performed on an LC-10AT VP Plus liquid chromatograph (Shimadzu Corporation, Kyoto, Japan) with an SPD-10A VP Plus UV–Vis detector operating at 210 nm using a Zorbax XDB-C8 column (4.6 mm × 150 mm) with silica (5 μm) as a stationary phase. Linear gradient elution (0 min 30% B; 11 min 100% B; 14 min 100% B; 17 min 30% B; 21 min 30% B) was performed at a flow rate of 1 ml/min. Peaks were detected at 210 nm.

Drug Release of PTX–Degarelix Conjugates in Dithiothreitol (DTT) Solution

The LK-MY-10 and tenfold DTT were dissolved in a mixture of phosphate buffer solution (pH = 7.4, 0.1 mM) with acetonitrile (2 : 1) and incubated at 37 °C. The reaction was monitored by RP-HPLC at different time.

Stability of PTX–Degarelix Conjugates in Human Serum

Samples were initially dissolved in DMSO, and then, human serum was added to a final peptide concentration of 100 μM and a final DMSO concentration of 2.5%. The mixtures were incubated at 37 °C, and 200 μl aliquots were removed at different time intervals. The reactions were quenched by adding 600 μl acetonitrile. Following centrifugation, 450 μl from each supernatant was analyzed by RP-HPLC (Agilent 1200, Agilent Technologies, Santa Clara, CA, USA). The residual ratio for each conjugate was examined by comparing the peak areas detected by RP-HPLC before and after treatment with human serum.

Cell Culture

MCF-7 human breast cancer cells, HT-29 human colon cancer cells, and 3T3 mouse embryonic fibroblast cells were obtained from the Cell Resource Center of Peking Union Medical College (Beijing, China) and were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin/streptomycin. CHO-K1/Gα15 cells stably expressing the GnRH-R were purchased from GenScript (Nanjing, China) and were maintained in Ham's F12 medium containing 10% fetal bovine serum, 400 μg/ml G418, and 100 μg/ml hygromycin B. Cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Antagonism Activity Assay Using FLIPR

CHO-K1/Gα15 cells stably expressing the GnRH-R were plated in 384-well tissue culture-treated, black polystyrene plates with a clear, flat bottom (BD Biosciences, San Jose, CA, USA). Briefly, 20 μl of 8×10^5 cells/ml were plated and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. After ~24 h, the medium was removed, and 20 μl of Ca3 dye (Molecular Devices Corporation, Sunnyvale, CA, USA) containing 2.5 mM freshly prepared probenecid was added according to the manufacturer's protocol. Then, 20 μl of the antagonist, AG045572 (TOCRIS), or buffer was added into each well with 20 mM HEPES buffer. Each compound was assayed in duplicate at eight concentrations that descended in half-log increments. Dye-loading solution (20 μl) was then added, and the CHO-K1 cells were placed in a 37 °C incubator. After 1 h, followed by 15 min incubation at RT, changes in intracellular Ca^{2+} were monitored using a FLIPR (Molecular Devices Corporation). The fluorescence measurements were read simultaneously from all of the wells at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. A baseline fluorescence signal was measured for the first 10 s, after which 20 μl of the eight descending concentrations of conjugates in half-log increments were added to the cell plate. Changes in fluorescence intensity were measured every second for the first minute and then were measured every 5 s for an additional minute.

Percent inhibition was calculated as follows:

$$\%\ \text{inhibition} = [1 - (\Delta RFU_{\text{Compound}} - \Delta RFU_{\text{Background}}) / (\Delta RFU_{\text{Antagonist control}} - \Delta RFU_{\text{Background}})] \times 100$$

The percent inhibition values were then plotted as a function of the log of the cumulative doses of the compounds. Relative fluorescence unit (ΔRFU) intensity values were calculated according to the maximal fluorescence units by subtracting the average baseline value. The 50% inhibitory concentration (IC_{50}) values were derived by nonlinear regression to a four-parameter logistic equation using GraphPad Prism curve fitting software (GraphPad Software, Inc., San Diego, CA, USA). The geometric mean of the IC_{50} values from at least two independent experiments is reported for each compound.

In Vitro Cytotoxicity of the PTX–Degarelix Conjugates Using an MTS Assay

MCF-7, HT29, and 3T3 cells were each seeded in 96-well cell-culture plates (Corning Life Sciences, Tewksbury, MA, USA) (5×10^3 cells/well). After 24 h, the cells were washed twice with HBSS and were incubated with 100 μl of PTX or the conjugates at the indicated concentrations at 37 °C. After 72 h, the cells were washed with HBSS again. To each well, 80 μl of HBSS and 20 μl of CellTiter 96® AQueous One Solution Reagent (Promega Corporation) were added. After 2 h, the OD for each well was measured at 490 nm using a SpectraMax® M5 microplate reader (Molecular Devices

Corporation). HBSS alone was used as a negative control. The percentage of cytotoxicity was calculated using the following equation:

$$\text{Cytotoxicity } \% = [1 - (OD_{\text{treated}} / \text{OD}_{\text{control}})] \times 100$$

where OD_{treated} and OD_{control} correspond to the OD values of the treated and control cells, respectively. The percentage of cytotoxicity was plotted as a function of concentration, and these points were fitted to a sigmoid curve. The IC_{50} values were determined from these curves. The experiment was repeated five times for each concentration tested.

Receptor Saturation Tests

MCF-7 cells were seeded in 96-well cell-culture plates (5×10^3 cells/well). After 24 h, the cells were treated with or without 10 μM degarelix at 37 °C. After 12 h, the MCF-7 cells were washed twice with HBSS and were incubated with PTX or LK-MY-10 (100 μl) at the indicated concentrations. After 48 h, cell viability was then measured by MTS assay, as described earlier.

Statistical Analysis

Data were analyzed using Student's *t*-test for comparisons. A value of $p < 0.05$ was considered statistically significant in all cases.

Results and Discussion

Synthesis of PTX–Degarelix Derivatives

The GnRH-R, also known as a luteinizing hormone-releasing hormone receptor, is a member of the seven-transmembrane G protein-coupled receptor family. Both GnRH and its receptor have been detected in extrapituitary tissues and have also been shown to have a role in the progression of some cancers, including breast, prostate, endometrial, ovarian, and pancreatic and hepatocellular carcinomas [34]. Therefore, drug molecules with a high affinity for the GnRH-R may exhibit excellent tumor-targeting ability. In this study, PTX was selected as the chemotherapy agent to be tested based on its widespread use and established efficacy against a variety of solid tumors. Targeting moieties were attached to PTX via the 2-hydroxyl group on one of its side chains. In order to release the chemotherapeutic agents within cancer cells, a disulfide bond was selected for the linker between the chemotherapeutic agents and targeting moieties to undergo reduction by GSH and release the conjugated agent. To conjugate PTX to the peptide, 2-pyridyl disulfide *p*-nitrophenyl carbonate was appended to PTX with thiopyridine termination to give 2-pyridyl disulfide PTX carbonate (Figure 1).

Here, degarelix, a GnRH-R antagonist with high GnRH-R affinity and enzymatic stability, was investigated as a targeting moiety conjugate for PTX. Previous studies of the structure–activity relationship of GnRH analogs [35–37] demonstrated that the *N*-terminus and position 6 of degarelix are not essential for receptor binding and, thus, are good candidates for the attachment of PTX. To enhance the solubility of these peptides, in the peptide sequences, MY-3, MY-4, and MY-5, the Arg-Gln chain was introduced. In total, five degarelix derivatives were designed and synthesized using solid-phase peptide synthesis on MBHA resin with the Boc strategy as previously described [38]. Therefore, five PTX–degarelix derivatives were generated after coupling the PTX derivates (Figure 2). All compounds were purified by preparative HPLC and were analyzed by analytical RP-HPLC and MS (Table 1).

To assess the release of PTX from the conjugates, LK-MY-10 was incubated in the presence of a tenfold molar excess of the disulfide-reducing agent, DTT, in the mixture of 0.1 mM phosphate buffer solution (pH = 7.4) and acetonitrile. The thiol resulting from DTT reduction was expected to cyclize into the proximate carbonyl group of the linker, leading subsequently to the release of free PTX (Figure 3). The HPLC profile showed (Figure 4) that the PTX was quickly released when LK-MY-10 encountered the DTT ($T = 0$ min) and the disulfide bond was completely cleaved within 1 h with concomitant release of PTX from the conjugates ($T = 1$ h).

Stability of the PTX–Degarelix Conjugates in Human Serum

The proteolytic stability of each of the conjugates in human serum for different time was evaluated using RP-HPLC, and the half-life of the conjugates were calculated and listed in Table 2. As expected, all

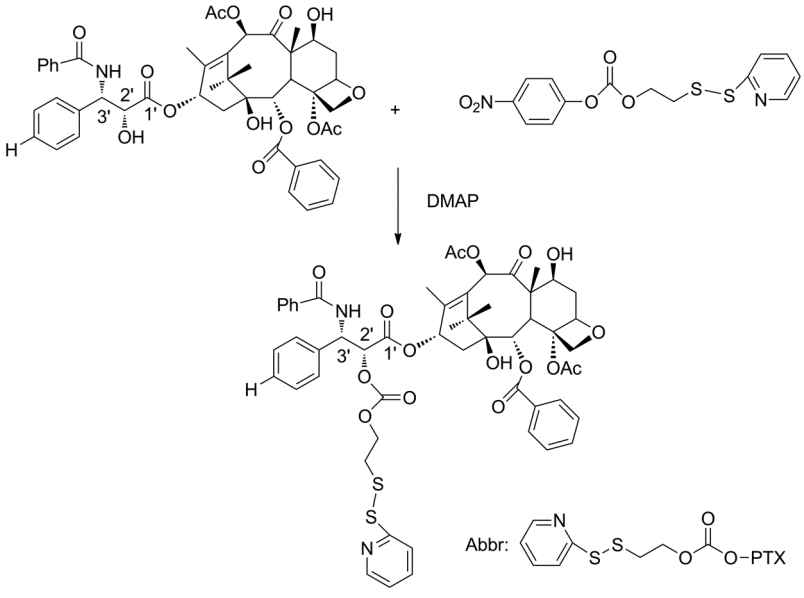

Figure 1. Synthesis of the PTX derivatives.

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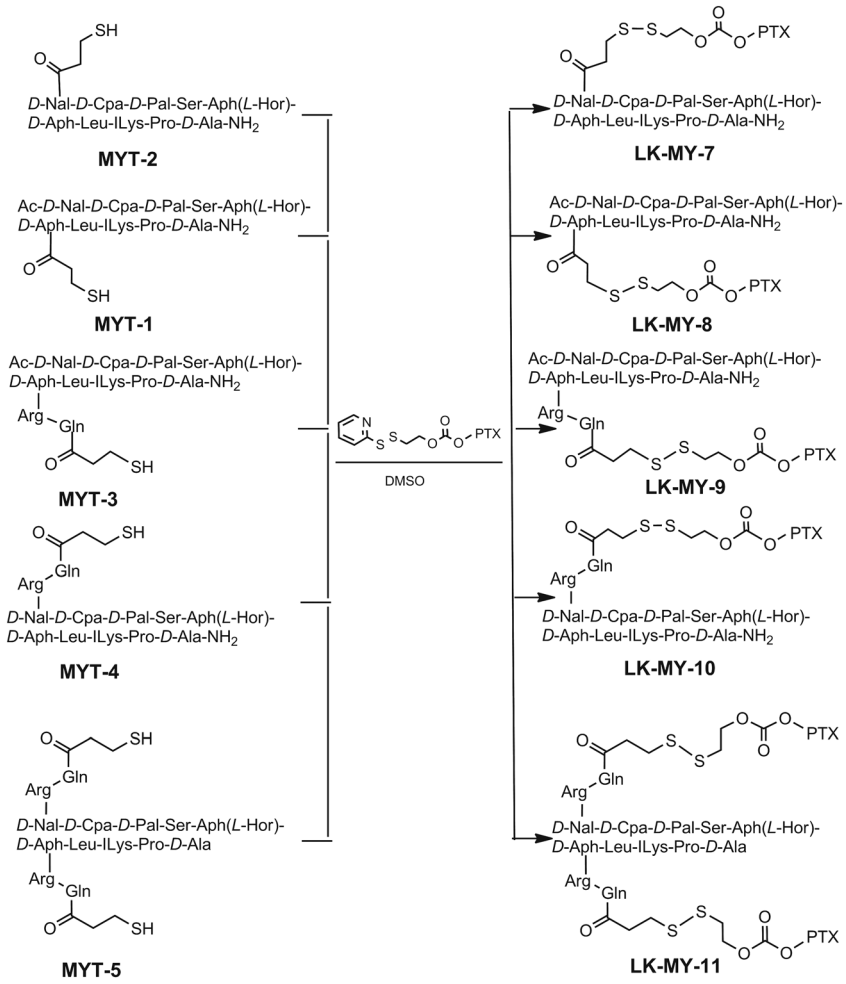

Figure 2. Synthesis of the PTX–degarelix derivatives.

Table 1. Chemical characteristics of the degarelix derivative conjugates

Compound	MALDI-TOF MS MW_{cal}/MW_{exp}	RP-HPLC R_t (min)[a]	Purity (%)[a]	Yield (%)
MYT-1	1677.36/1677.80	8.86	85.6	26.0
MYT-2	1678.35/1678.00	9.06	86.8	41.8
MYT-3	1961.68/1961.84	7.99	84.1	25.1
MYT-4	1962.67/1963.03	8.45	92.4	30.2
MYT-5	2292.08/2292.52	7.56	91.1	29.5
LK-MY-7	2634.10/2634.37	13.10	96.0	54.5
LK-MY-8	2632.79/2633.38	13.24	96.8	38.2
LK-MY-9	2917.61/2917.70	11.86	92.2	61.5
LK-MY-10	2919.10/2918.68	11.60	92.3	55.7
LK-MY-11	4202.85/4204.12	12.46	97.3	45.5

[a]RP-HPLC: Column: Zorbax XDB-C8 (4.6 mm × 150 mm) with 5 μm silica; gradient: 0 min 30% B; 11 min 100% B; 14 min 100% B; 17 min 30% B; 21 min 30% B; eluents 0.1% TFA in water (A) and 0.1% TFA in acetonitrile–water (70 : 30, v/v) (B); flow rate: 1 ml/min; detection: $\lambda = 210$ nm.

of the conjugates were relatively stable, and all had a $t_{1/2} > 10$ h, and the $t_{1/2}$ of AN-152 was 2 h in human serum [20]. Thus, PTX–degarelix conjugates have at least six times longer $t_{1/2}$ compared with the AN-152. Among the peptide sequences of the five conjugates, the introduction of Arg-Gln has decreased the stability of the peptides in human serum from the half-lives data. It may be that the Arg-Gln enhances the polarity of the peptides and decreases the binding ability with human serum protein, reducing the stability of the peptides. Furthermore, these data demonstrate that these degarelix conjugates are stable enough to allow sufficient time for cancer cell targeting.

Antagonism Activity

The antagonism activity of the conjugates toward the GnRH-R were determined *in vitro* using the FLIPR assay with CHO-K1 cells expressing the human GnRH-R and Gα15 protein [39]. Agonist or antagonism-evoked changes in the levels of intracellular calcium were detected by calcium-sensitive dyes. Table 3 shows the IC_{50} values for the antagonism activity of the conjugates that were tested, and these values ranged from 1.4 to 131.0 nM. In comparison, the biological effects of a native GnRH peptide and degarelix were evaluated on CHO-K1/GnRH-R/Gα15 cells, and the EC_{50} agonist activity and IC_{50} antagonism activity values were 41.4 and 0.2 nM, respectively. These data demonstrate that all of the conjugates exhibited high levels of antagonism and some of the conjugates had IC_{50} values close to those of degarelix (Table 3). Taken together, these results indicate that these conjugates would likely gain entry into tumor cells via receptor-mediated endocytosis.

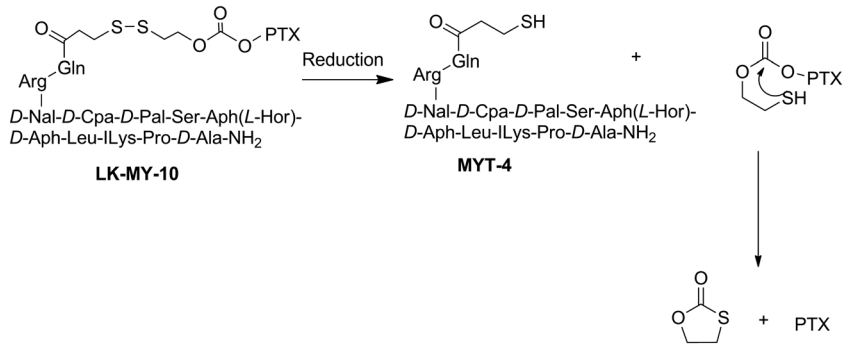

Figure 3. Disulfide-mediated release of PTX from the PTX–degarelix analog carbonate linker.

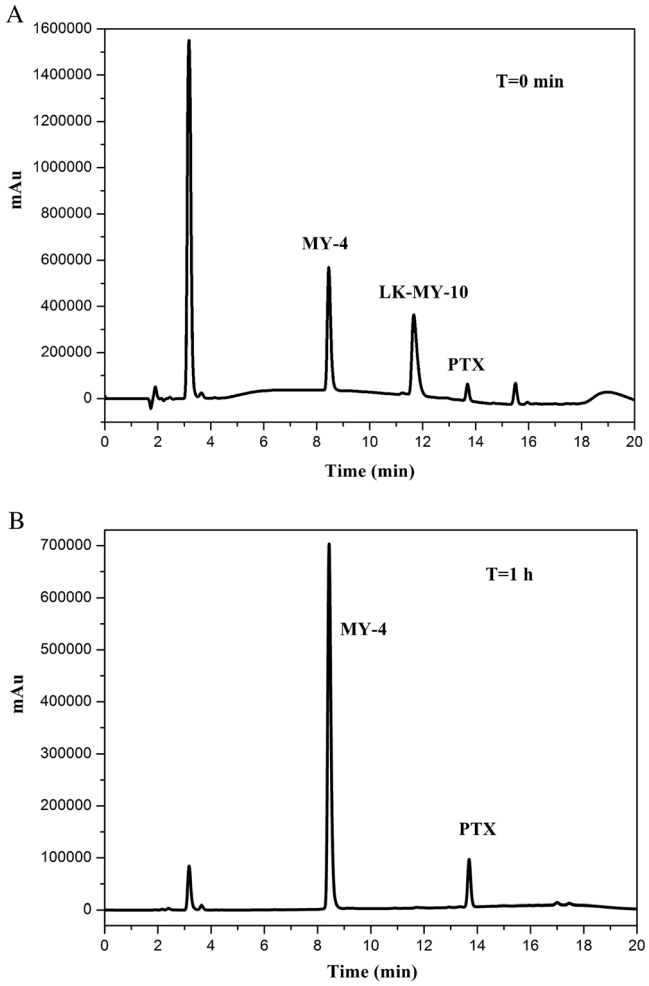

Figure 4. Triggered release of PTX from LK-MY-10 by disulfide reduction. LK-MY-10 and a tenfold excess of DTT were analyzed using RP-HPLC (*Abs* = 210 nm) in different time.

Table 2. Stability of the PTX–degarelix conjugates in human serum

Compound	Half-life in human serum($t_{1/2}$, h)
AN-152	2.10[a]
LK-MY-7	52.03
LK-MY-8	64.86
LK-MY-9	13.91
LK-MY-10	16.23
LK-MY-11	13.02

[a]The data were from Reference [20].

Table 3. GnRH-R antagonism activity of the conjugates tested

Compound	Antagonism activity(IC_{50}, nM)
Degarelix	0.2
LK-MY-7	89.2
LK-MY-8	102.0
LK-MY-9	1.4
LK-MY-10	6.4
LK-MY-11	131.0

Table 4. The *in vitro* cytostatic effects of the conjugates on MCF-7, HT-29, and 3T3 cells

Compound	MCF-7(IC_{50}, nM)	HT-29(IC_{50}, nM)	3T3(IC_{50}, nM)
PTX	7.1 ± 2.0	15.4 ± 1.3	15.7 ± 6.8
Degarelix	>1 000 000	>1 000 000	>1 000 000
LK-MY-7	17.3 ± 3.7	44.2 ± 7.0	960.8 ± 112.0
LK-MY-8	14.4 ± 2.3	54.9 ± 5.7	261.8 ± 28.3
LK-MY-9	49.1 ± 14.7	55.3 ± 11.2	142.5 ± 45.7
LK-MY-10	59.1 ± 14.2	69.7 ± 10.6	369.2 ± 12.8
LK-MY-11	33.7 ± 5.3	82.8 ± 13.5	257.2 ± 43.4

In Vitro Cytostatic Effects of the PTX–Degarelix Conjugates

Using MCF-7 human breast cancer, HT-29 human colon cancer, and 3T3 mouse embryonic fibroblast cells, MTS assays were used to evaluate the *in vitro* cytostatic effects of the five PTX–degarelix conjugates. The calculated IC_{50} values are presented in Table 4. Free PTX exhibited a similar cytostatic effect as the conjugates in the lower nanomolar range on all tested cell lines. In contrast, degarelix alone did not have any effect on the cells, consistent with previous reports [40]. With MCF-7 and HT-29 tumor cells, both of which express the GnRH-R, the IC_{50} cytostatic values of the conjugates were several times higher than those of PTX. However, no significant differences were observed between the cytostatic effects of the conjugates with different coupling sites on degarelix in the same cell lines. On 3T3 cells, which do not express the GnRH-R, the IC_{50} values of the conjugates were one order of magnitude

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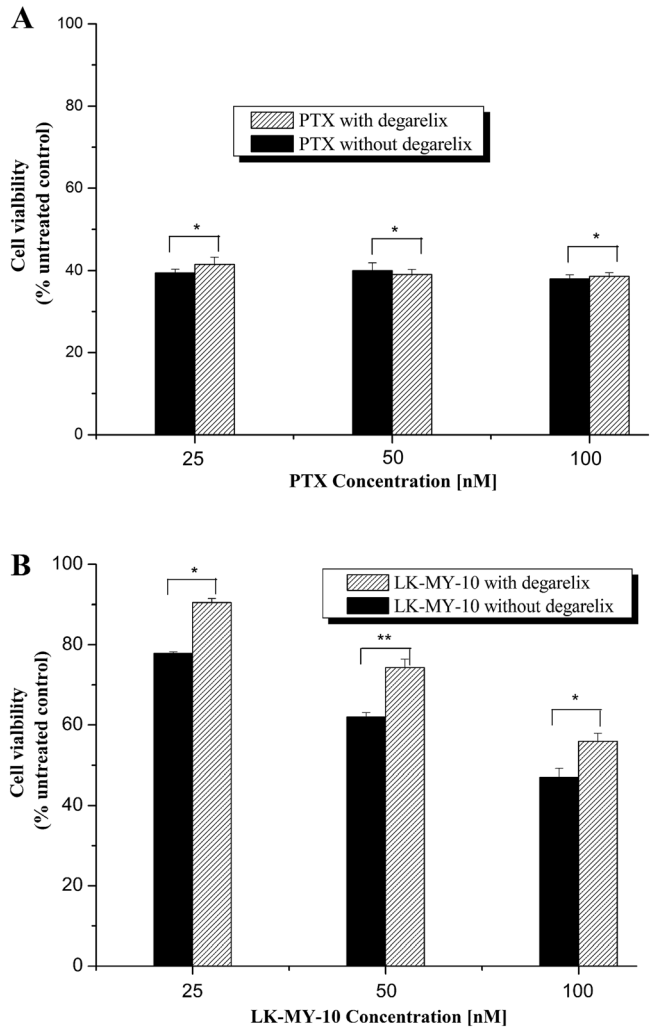

Figure 5. The effect of degarelix (10 μM) on the antiproliferative efficacy of (A) PTX or (B) LK-MY-10 in MCF-7 cells. The cells were exposed to PTX and LK-MY-10 individually or in combination with degarelix for 48 h. Cell viability was expressed as a percentage of the viability detected for untreated controls (100%). Data presented as mean ± standard deviation ($n = 5$); *$p < 0.01$, **$p < 0.05$.

higher than those determined for the MCF-7 and HT-29 cancer cell lines. Thus, the conjugates are predicted to confer selective toxicity to cancer cells *versus* normal cells.

Receptor Saturation Test

The cellular uptake of conjugates depends on their binding efficacy to the targeting ligand and the number of receptors present on the surface of a cell. To confirm that targeting of the PTX–degarelix conjugate to cancer cells is mediated by the GnRH-R at the cell surface, MCF-7 cells expressing the GnRH-R were pretreated with 10 μM degarelix for 12 h, followed by an incubation period with either LK-MY-10 or PTX. In the presence of degarelix, the antiproliferative efficacy of PTX was unaffected (Figure 5A). However, pretreatment of the cells with degarelix did reduce the antiproliferative effects induced by LK-MY-10 (Figure 5B). These results suggest that the cytotoxic effects of the conjugates tested are due to the binding and uptake of the conjugates via the GnRH-R. This evidence also demonstrates that degarelix binding is not eliminated by its conjugation to PTX, and these results are consistent with the binding affinity data described earlier.

Conclusions

In this study, five PTX–degarelix conjugates were synthesized and analyzed. All conjugates were found to be at least six times more resistant to proteolytic degradation in human serum compared with AN-152 and were also more cytostatic to cancer cells than to normal cells. These results demonstrate that degarelix conjugates are stable and efficacious targeting moieties that could be further optimized for use in targeted chemotherapy.

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