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Paclitaxel conjugation with the analog of the gonadotropin-releasing hormone as a targeting moiety

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ABSTRACT

A new targeted conjugates in which paclitaxel was used as a cytostatic compound and an analog of the gonadotropin-releasing hormone (GnRH) as a targeting moiety were synthesized. The molecule of the peptide hormone GnRH was modified to allow its connection to paclitaxel *via* spacer. The conjugates were prepared as prodrugs using 2′-hydroxyl group of paclitaxel. 4-Maleimidobutyric acid and chloroacetic acid served as spacers. The structures of the prepared derivatives were analysed by NMR and HR-MS. The conjugates MP264 and MP265 were chosen and their antiproliferative effect was tested in the breast cancer cell line MCF-7 using the MTT test of cell viability and neutral red uptake test. In MCF-7 cells, conjugate MP265 showed higher antiproliferative effect than paclitaxel alone. Receptor saturation tests showed that the unconjugated peptide analog of GnRH decreased efficacy of conjugate MP265 in concentration- and time-dependent manner. In conclusion, the paclitaxel conjugate with the analog of GnRH exhibited targeted antiproliferative effect for which its further testing will be implemented.

1. Introduction

Paclitaxel (PTX) is an antineoplastic drug which is active in ovarian, breast and lung cancer (Rowinsky and Donehower, 1995). Paclitaxel is a mitotic inhibitor, which promotes the assembly of the microtubules (Schiff et al., 1979). Paclitaxel is a potent chemotherapeutic but the treatment with it has many drawbacks – the most adverse one is no cell specificity. The conjugation of paclitaxel with targeting moieties such as antibody (Safavy et al., 2003), peptide hormone (Cavallaro et al., 2007) or fatty acid (Bradley et al., 2001) were accomplished to improve the treatment. Many of these conjugates were prepared as prodrugs *via* 2′-hydroxyl group on the side chain. This group is essential for antiproliferative effect of paclitaxel therefore the prodrugs are designed to disengage this group under certain conditions (Georg et al., 1995).

GnRH (Gonadotropin-releasing hormone) is a hypothalamic decapeptide (pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly·NH_2) which controls mammalian reproduction (Baba et al., 1971; Schally et al., 1971). GnRH stimulates the biosynthesis and secretion of hormones LH (Luteinizing hormone) and FSH (Follicle stimulating hormone) from pituitary gonadotropes. GnRH receptors are present not only in the anterior pituitary but also in tumors (reviewed by Limonta et al., 2003; Harrison et al., 2004). These receptors were found in prostate cancer tissue (Limonta et al., 1999), the GnRH bindind sites were discovered in ovarian cancer tissue (Emons et al., 1990) and the GnRH receptor's mRNA in malignant breast tissue (Kottler et al., 1997). For instance, MCF-7 human breast cancer cell line possesses these GnRH binding sites (Eidne et al., 1987). Many GnRH analogs have been prepared to date and many of them have clinical applications (reviewed by Barbieri, 1992). Nowadays, it is well known which amino acids of the hormone are essential for the receptor binding and activation (reviewed by Millar, 2005; Sealfon et al., 1997). Concretely, the GnRH analog where the Gly^6 was replaced by D-Lys^6 was successfully used as a targeting moiety in conjugation with 2-pyrrolino-doxorubicin (Nagy et al., 1996).

Because GnRH receptors are expressed in ovary and breast carcinomas, where paclitaxel is an effective cytostatic compound, there is a good reason to connect these two compounds together. In this

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study, the analog of hormone GnRH was used as a targeting moiety and paclitaxel as a cytostatic agent.

2. Materials and methods

2.1. Chemicals

Paclitaxel was purchased from Cedarburg Hauser Pharmaceuticals, Inc. (Broadway, Denver, USA). N,N-Diisopropylethylamine (DIEA), N,N′-dicyclohexylcarbodiimide (DCC), N,N-dimethylformamide (DMF), 4-maleimidobutyric acid, pyridine, chloroacetic anhydride, Dulbecco's modified Eagle's medium (DMEM), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer, neutral red, dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich, s.r.o. (Prague, Czech Republic). TLC Silica gel 60 F_{254} aluminium sheets and acetonitrile (ACN) and methanol in HPLC gradient grade quality were purchased from Merck, s.r.o. (Prague, Czech Republic). The peptides were synthesized by VIDIA, s.r.o. (Jesenice u Prahy, Czech Republic). Fetal bovine serum (FBS) and gentamycin sulphate were purchased from Invitrogen (Carlsbad, CA). All reagents and solvents were used without further purification.

Stock solutions were prepared in double-distilled deionized water or dimethyl sulfoxide and stored at 4 °C in the dark.

2.2. The synthesis of 2′-maleimidobutyryl-paclitaxel

4-Maleimidobutyric acid (20 mg, 109 μmol) was dissolved in 0.5 mL of dichloromethane. Triethylamine (2 μL, 14 μmol) and then DCC (5 mg, 24 μmol) were added and the mixture was stirred at room temperature for 2 h. Afterwards, the solution was evaporated and the crude mixture was dissolved in 1 mL of dichloromethane. To this mixture, paclitaxel (16 mg, 19 μmol) and pyridine (5 μL, 62 μmol) were added, and the mixture was stirred overnight at room temperature. The reaction was monitored by TLC. The product 2′-maleimidobutyryl-paclitaxel was purified by semipreparative HPLC to yield the product in 86% as a white solid.

HR ESI-MS *m/z* 1041.36321 $[M+Na]^+$ (calcd for $C_{55}H_{58}N_2O_{17}Na$, 1041.36277).

^{1}H NMR (600 MHz): δ (ppm): 1.13 (s, 3H, H-17); 1.23 (s, 3H, H-16); 1.69 (s, 3H, H-19); 1.89 (ddd, J_1 = 2.5 Hz, J_2 = 11.0 Hz, J_3 = 14.7 Hz, 1H, H-6a); 1.93 (m, 2H, H-6′); 1.95 (d, J_1 = 1.6 Hz, 3H, H-18); 2.13 (ddd, J_1 = 0.5 Hz, J_2 = 9.2 Hz, J_3 = 15.6 Hz, 1H, H-14a); 2.23 (s, 3H, OAc (C-10)); 2.33 (t, J_1 = 7.3 Hz, 2H, H-5′); 2.37 (dd, J_1 = 9.2 Hz, J_2 = 15.6 Hz, 1H, H-14b); 2.49 (s, 3H, OAc (C-4)); 2.57 (ddd, J_1 = 6.6 Hz, J_2 = 9.7 Hz, J_3 = 14.7 Hz, 1H, H-6b); 3.59 (t, J_1 = 6.9 Hz, 2H, H-7′); 3.82 (dd, J_1 = 1.0 Hz, J_2 = 7.1 Hz, 1H, H-3); 4.21 (dd, J_1 = 1.1 Hz, J_2 = 8.5 Hz, 1H, H-20a); 4.32 (dd, J_1 = 1.1 Hz, J_2 = 8.5 Hz, 1H, H-20b); 4.46 (dd, J_1 = 6.6 Hz, J_2 = 11.0 Hz, 1H, H-7); 4.99 (bdd, J_1 = 2.5 Hz, J_2 = 9.7 Hz, 1H, H-5); 5.51 (d, J_1 = 3.5 Hz, 1H, H-2′); 5.69 (d, J_1 = 7.1 Hz, 1H, H-2); 6.03 (dd, J_1 = 3.5 Hz, J_2 = 9.2 Hz, 1H, H-3′); 6.25 (tq, J_1 = 1.6 Hz, J_2 = 9.2 Hz, 1H, H-13); 6.29 (s, 1H, H-10); 6.70 (s, 2H, H-9′); 7.31–7.63 (m, 11H, arom.); 7.43 (bd, J_1 = 9.2 Hz, 1H, NH-3′); 7.77–7.79 (m, 2H, arom.); 8.14–8.17 (m, 2H, arom.).

^{13}C NMR (150.9 MHz): δ (ppm): 9.59 (C-19); 14.86 (C-18); 20.83 (CH_3(OAc-C10)); 22.15 (C-17); 22.79 (CH_3(OAc-C4)); 23.77 (C-6′); 26.77 (C-16); 31.14 (C-5′); 35.47 (C-14); 35.51 (C-6); 37.03 (C-7′); 43.13 (C-15); 45.52 (C-3); 52.70 (C-3′); 58.48 (C-8); 71.76 (C-13); 72.14 (C-7); 74.47 (C-2′); 75.07 (C-2); 75.60 (C-10); 76.42 (C-20); 79.14 (C-1); 81.00 (C-4); 84.44 (C-5); 126.56 (Ar); 127.37 (Ar); 128.35 (Ar); 128.48 (Ar); 128.73 (Ar); 128.99 (Ar); 129.16 (Ar); 130.24 (Ar); 131.85 (Ar); 132.66 (C-11); 133.67 (Ar); 133.72 (Ar); 134.10 (C-9′); 136.95 (Ar); 142.90 (C-12); 167.41 (CO, C-10′); 168.05 (CO, C-1′); 169.86 (CO, OAc-C4); 170.77 (CO, C-8′); 171.30 (CO, OAc-C10); 173.00 (CO, C-4′); 203.86 (CO, C-9).

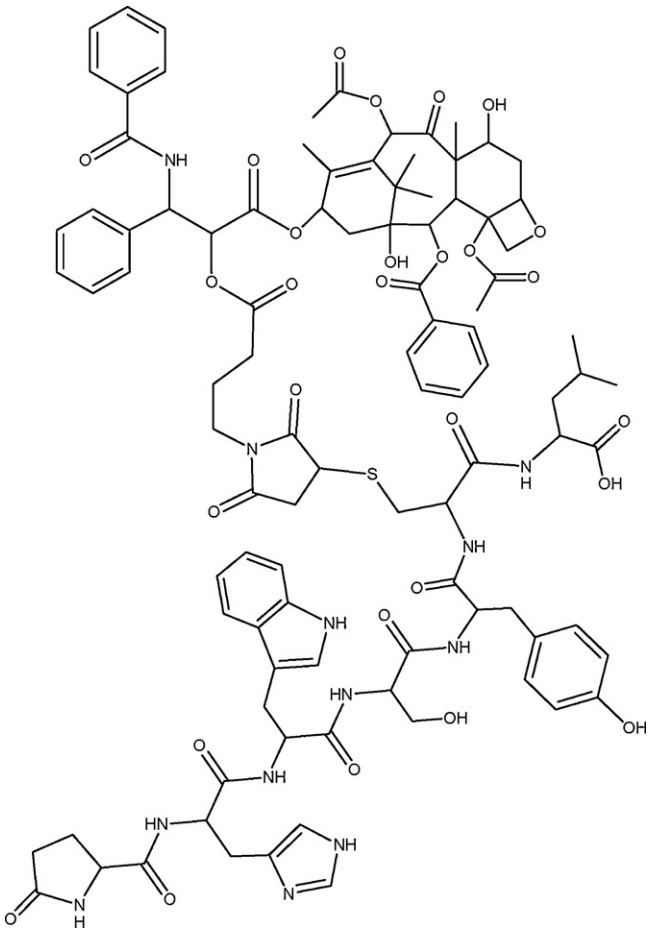

Fig. 1. The structure of the targeted conjugate MP264.

2.3. The synthesis of conjugate MP264 (Fig. 1)

2′-Maleimidobutyryl-paclitaxel (3 mg, 3 μmol) was dissolved in 1 mL of anhydrous DMF and the analog of GnRH (pGlu–His–Trp–Ser–Tyr-D-Cys–Leu–OH, 8 mg, 9 μmol) was added. The reaction was stirred overnight at room temperature and monitored by analytical HPLC. The solvent was freeze–dried and the crude product was purified by semipreparative HPLC in 85% yield as a white solid.

HR ESI-MS *m/z* 1937.7499 $[M+H]^+$ (calcd for $C_{98}H_{113}N_{12}O_{28}S$, 1937.7502).

2.4. The synthesis of 2′-chloroacetyl-paclitaxel

Chloroacetic anhydride (20 mg, 117 μmol) was dissolved in 1 mL of dichloromethane. Paclitaxel (20 mg, 23 μmol) and pyridine (5 μL, 62 μmol) were then added. DCC (5 mg, 24 μmol) was added in portions over 1 h, until the paclitaxel was reacted completely. The reaction was stirred at room temperature and monitored by TLC. When finished, the reaction was evaporated to dryness. The product was purified by semipreparative HPLC in 75% yield as "white powder".

HR ESI-MS *m/z* 952.29350 $[M+Na]^+$ (calcd for $C_{49}H_{52}NO_{15}ClNa$, 952.29177).

^{1}H NMR (600 MHz): δ (ppm): 1.14 (s, 3H, H-17); 1.24 (s, 3H, H-16); 1.69 (s, 3H, H-19); 1.89 (ddd, J_1 = 2.3 Hz, J_2 = 11.0 Hz,

J_3 = 14.7 Hz, 1H, H-6a); 1.93 (d, J_1 = 1.5 Hz, 3H, H-18); 2.20 (dd, J_1 = 9.8 Hz, J_2 = 15.4 Hz, 1H, H-14a); 2.23 (s, 3H, OAc (C-10)); 2.38 (dd, J_1 = 9.4 Hz, J_2 = 15.4 Hz, 1H, H-14b); 2.46 (s, 3H, OAc (C-4)); 2.50 (bd, J_1 = 3.5 Hz, OH (C-7)); 2.57 (ddd, J_1 = 6.5 Hz, J_2 = 9.7 Hz, J_3 = 14.7 Hz, 1H, H-6b); 3.82 (dd, J_1 = 0.6 Hz, J_2 = 7.2 Hz, 1H, H-3); 4.15 (d, J_1 = 15.2 Hz, H-5a′); 4.19 (d, J_1 = 15.2 Hz, H-5b′); 4.21 (dd, J_1 = 1.0 Hz, J_2 = 8.3 Hz, 1H, H-20a); 4.32 (bd, J_1 = 1.0 Hz, J_2 = 8.3 Hz, 1H, H-20b); 4.45 (ddd, J_1 = 3.5 Hz, J_2 = 6.5 Hz, J_3 = 11.0 Hz, 1H, H-7); 4.98 (dd, J_1 = 2.3 Hz, J_2 = 9.7 Hz, 1H, H-5); 5.56 (bd, J_1 = 3.1 Hz, 1H, H-2′); 5.69 (d, J_1 = 7.2 Hz, 1H, H-2); 6.01 (bdd, J_1 = 3.1 Hz, J_2 = 9.2 Hz, 1H, H-3′); 6.27 (dt, J_1 = 1.5 Hz, J_2 = 9.7 Hz, 1H, H-13); 6.29 (s, 1H, H-10); 6.88 (bd, J_1 = 9.2 Hz, 1H, NH-3′); 7.34–7.46 (m, 7H, arom.); 7.49–7.54 (m, 3H, arom.); 7.59–7.64 (m, 1H, arom.); 7.72–7.75 (m, 2H, arom); 8.13–8.16 (m, 2H, arom.).

^{13}C NMR (150.9 MHz): δ (ppm): 9.58 (C-19); 14.81 (C-18); 20.81 (CH_3(OAc-C10)); 22.11 (C-17); 22.70 (CH_3(OAc-C4)); 26.82 (C-16); 35.49 (C-6); 35.49 (C-14); 40.22 (C-5′); 43.17 (C-15); 45.53 (C-3); 52.71 (C-3′); 58.52 (C-8); 72.13 (C-7); 72.22 (C-13); 75.06 (C-2); 75.33 (C-2′); 75.54 (C-10); 76.44 (C-20); 79.18 (C-1); 81.08 (C-4); 84.42 (C-5); 126.55 (Ar); 127.07 (Ar); 128.71 (Ar); 128.74 (Ar); 128.76 (Ar); 129.13 (Ar); 129.19 (Ar); 130.22 (Ar); 132.11 (Ar); 132.90 (C-11); 133.46 (Ar); 133.70 (Ar); 136.49 (Ar); 142.54 (C-12); 167.06 (CO, C-1′); 167.32 (CO, C-4′); 169.76 (CO, OAc-C10); 171.26 (CO, OAc-C4); 203.76 (CO, C-9).

2.5. The synthesis of conjugate MP265 (Fig. 2)

2′-Chloroacetyl-paclitaxel (3 mg, 3 μmol) was dissolved in 1 mL of anhydrous DMF and the analog of GnRH (pGlu–His–Trp–Ser–Tyr-D-Cys–Leu–OH, 8 mg, 9 μmol) was added together with 2 μL of DIEA (11 μmol). The reaction was stirred overnight and monitored by analytical HPLC. The solvent was freeze–dried and the crude product was purified by semipreparative HPLC in 80% yield as a white solid.

HR ESI-MS *m/z* 1834.6849 $[M+Na]^+$ (calcd for $C_{92}H_{105}N_{11}O_{26}SNa$, 1834.6845).

2.6. Analysis by analytical HPLC

The progress of the reactions and the purity of products were monitored by analytical HPLC which was carried out on analytical HPLC system, using Phenomenex Luna 5 μm Phenyl-Hexyl column (250 mm × 4.60 mm; 5 μm particle size) and a gradient program with the eluent system of 40% B to 100% B (A: 20% methanol/13% acetonitrile/67% water; B: 20% methanol/53% acetonitrile/27% water) with a linear gradient for 60 min; flow rate 1 mL/min; temperature 20 °C.

2.7. Purification by semipreparative HPLC

The final purification of all the crude products was carried out on semipreparative HPLC system, using Phenomenex Luna 5 μm Phenyl-Hexyl column (250 mm × 10 mm; 5 μm particle size) and a gradient program was the same as for analytical method; flow rate 2.5 ml/min; temperature 20 °C.

2.8. TLC analysis

TLC plates coated with silica gel 60 F_{254} were used as stationary phase and a mixture of chloroform:ethanol at a ratio 9:1 (v/v) was used as a mobile phase. A solution of vanillin (15 g) in ethanol (250 mL) and conc. sulfuric acid (2.5 mL) was used as a visualizing agent.

2.9. NMR measurements

^{1}H and ^{13}C NMR spectra were measured on a FT NMR spectrometer Bruker AVANCE-600 (600.1 MHz for ^{1}H and 150.9 MHz for

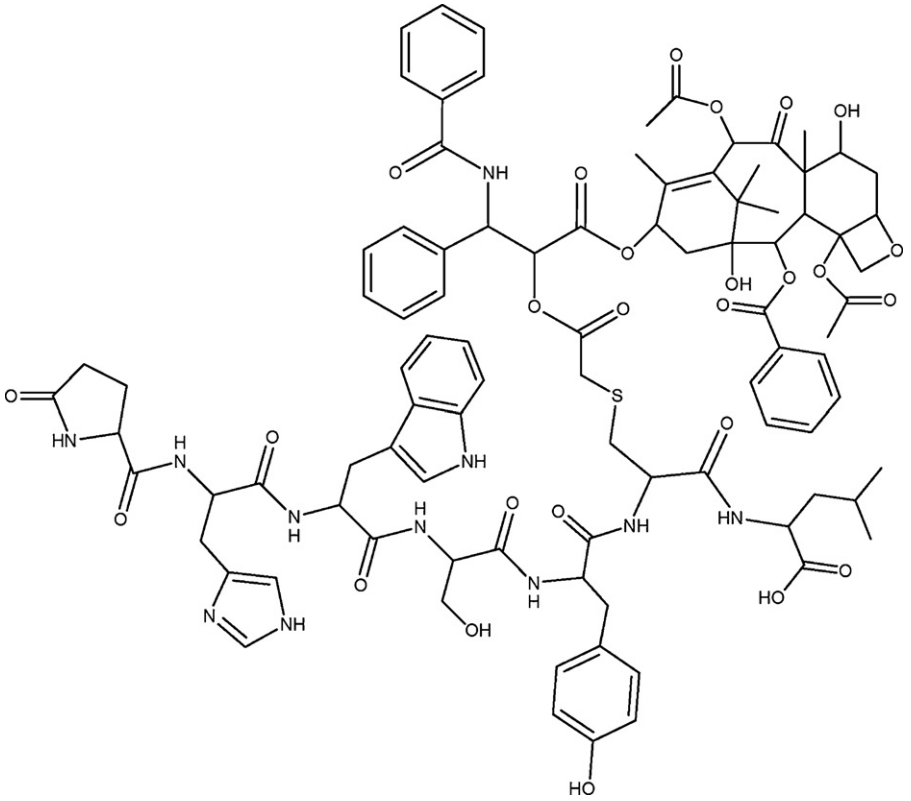

Fig. 2. The structure of the targeted conjugate MP265.

^{13}C) equipped with TCI cryoprobe 5 mm in deuterochloroform (99.8 atom %D, Aldrich). For standardization internal signal of tetramethylsilane was used (δ 0.0 for ^{1}H). ^{13}C NMR spectra were taken using the broadband decoupling technique, the central line of solvent signal as internal standard was used (δ 77.00 for ^{13}C). For unambiguous assignment of both ^{1}H and ^{13}C signals 2D NMR ^{1}H, ^{13}C gHSQC and gHMBC spectra (Hurd and John, 1991) were measured using standard parameters set and pulse program delivered by producer of the spectrometer.

2.10. Mass spectrometry analysis

High resolution mass spectra (HR-MS) were measured on LTQ ORBITRAP XL from Thermo Fisher Scientific (MA, USA). Ions were generated by API (ESI). 90% methanol was used as a mobile phase.

2.11. Cell culture

The MCF-7 human breast cancer cell line was used (purchased in 2004 from the European Collection of Cell Cultures (ECACC) Catalogue No. 86012803). Cells were multiplied in three passages, frozen in aliquots and stored in liquid nitrogen. The absence of mycoplasma in all cell lines used in laboratory is periodically checked by Generi Biotech s. r. o. For each set of experiments (lasted 3–9 weeks), new storage cells were resuscitated. The cells were maintained in Dulbecco's Modified Eagle's Medium with phenol red and $NaHCO_3$. The culture medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) and gentamicin sulphate (10 mg/mL). Cells were grown in T-75 cm^2 culture flasks in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.12. Antiproliferative effect of conjugates MP264 and MP265 in MCF-7 cells

The MCF-7 cells were exposed to various concentrations of paclitaxel, MP264 and MP265 in culture medium. The cytostatics were pre-dissolved in DMSO. The cells cultured in medium without cytostatics were used as control samples. After exposition, the viability of cells was assayed using the following tests. The measurements were carried out in three independent experiments. Four to eight parallel samples were used in each experiment.

2.12.1. The MTT assay

The mitochondrias of living cells are able to reduce yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan. Tested compounds were pre-dissolved in DMSO. The concentration of DMSO in culture medium was 0.1%, the tested concentrations were 1, 10, 25 and 50 nM. MCF-7 cells in 96-well-plates were exposed for 48-h to culture medium with tested compounds. The control cells were exposed to culture medium with 0.1% DMSO. After exposure, 25 μL of MTT solution (2.5 mg of MTT in 1 mL of 0.1 M phosphate buffer, pH 7.4) was added to each well. Plates were incubated at 37 °C for additional 2 h, then the medium was removed and formed formazan was dissolved in 50 μl of 0.08 M HCl in isopropanol by 30-min shaking. The absorbance in each well was quantified by measuring at 570 and 690 nm using spectrophotometer Tecan.

2.12.2. The neutral red uptake test

The cells were cultured in 96-well plates. After 48-h exposure, the medium was removed and the cells were washed two-times with 50 μL phosphate buffer. Then 200 μL of neutral red-containing medium was added into each well and plates were incubated at 37 °C for additional 3 h. The cells were fixed in a solution of 0.5% formaldehyde/1% calcium chloride for 15 min. The neutral red dye was extracted from the viable cells with a solvent (50% ethanol/1% acetic acid) at room temperature on a microplate shaker for 30 min. The absorbance of solubilized dye was measured with a spectrophotometric microplate reader at wavelength 540 nm. Each sample was assayed in 6 parallels and three independent experiments were performed. The viabilities of treated cells were expressed as percentage of untreated controls (100%).

2.13. Receptor saturation tests

The MCF-7 cells were seeded in 96-well plates. After 24-h, the culture medium was removed and the fresh one without or with unconjugated analog of GnRH (pGlu–His–Trp–Ser–Tyr-D-Cys–Leu–OH) in various concentration (10, 100, 250 or 500 nM) was added. This pre-exposition lasted 30 min. In the first experiment, various concentrations of MP265 or PTX were then added and cells were exposed for 48 h. In the second experiment, the pre-exposition medium with the analog of GnRH in various concentrations (10, 100 or 500 nM) was removed from some wells, medium with 10 nM MP265 was added and the cells were exposed for 48 h. In third experiments, the cells were exposed to unconjugated analog of GnRH in various concentrations for 48 h. After exposition, the viabilities of cells were assayed using MTT test. The measurements were carried out in three independent experiments. Six parallel samples were used in each experiment. The cells cultured in medium without cytostatics and without unconjugated analog of GnRH were used as control samples.

3. Results

3.1. Synthesis and characterization of targeted conjugates

The conjugates MP264 (Fig. 1) and MP265 (Fig. 2) were synthesized and purified as described in Section 2. The structure of these compounds was identified by HR ESI-MS and the intermediates also by NMR.

3.2. The results of MTT and NR tests

The antiproliferative effect of paclitaxel and targeted conjugates MP264 and MP265 was determined in MCF-7 cells using the MTT assay and the neutral red uptake test. The results are summarized in Fig. 3. The results denoted that the conjugate MP264 had similar antiproliferative effect as paclitaxel in the neutral red uptake test and lower antiproliferative effect than paclitaxel in MTT test, on the contrary to MP265 which exhibited in concentrations 10, 25 and 50 nM higher antiproliferative effect than paclitaxel in both tests.

3.3. Receptor saturation test

The results of receptor saturation tests are demonstrated in Figs. 4 and 5. In MCF-7 cells, the combinations of MP265 and the unconjugated analog of GnRH had lower antiproliferative efficacy than MP265 alone. On the other hand, combinations of 1 nM PTX and 10 nM GnRH analog had opposite effect. When higher concentrations of PTX were used, the unconjugated GnRH analog did not affect the antiproliferative efficacy of PTX (Fig. 4). The results in Fig. 5 show that higher concentrations of the GnRH analog more protect MCF-7 cells against MP265 and that this protective effect depends on duration of cells exposition to this peptide. The GnRH analog alone had no effect on viability of MCF-7 cells (data not shown).

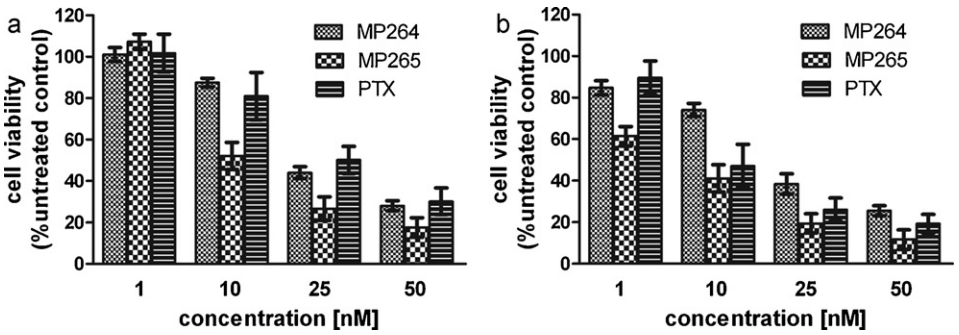

Fig. 3. The effect of MP264, MP265 and paclitaxel (PTX) on the viability of MCF-7 cells after a 48-h exposition. Evaluations were performed using the NRU test (a) or using MTT assay (b). The viabilities were expressed as a percentage of untreated controls (100%).

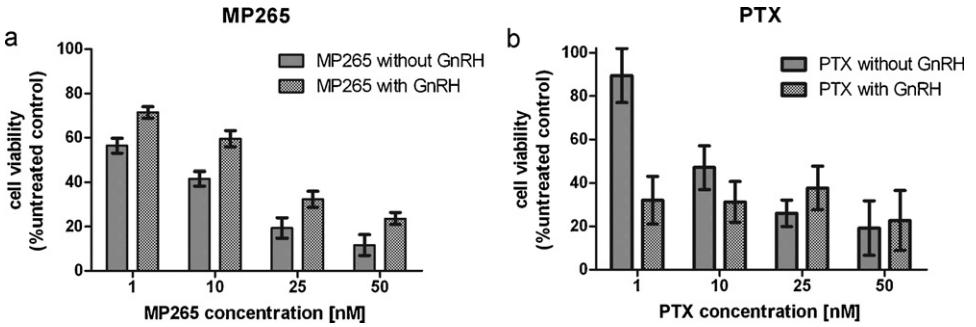

Fig. 4. The effect of unconjugated GnRH analog on the anti-proliferative efficacy of MP265 (a) and PTX (b) in MCF-7 cells. The cells were exposed to MP265 or PTX alone or to combinations of MP265 or PTX and GnRH analog for 48 h. The concentrations of GnRH analog were 10-times higher than concentration of MP265 or PTX. The viabilities were expressed as a percentage of untreated controls (100%).

4. Discussion

We have prepared and described new targeted conjugates of paclitaxel with the analog of the hormone GnRH. This conjugates might improve the treatment with paclitaxel by delivery of this anticancer drug to cancer cells which express the GnRH receptors. The peptide GnRH was chosen as a targeting moiety, because of the presence of its receptors in tumors such as prostate, ovary, breast and endometrium (Limonta et al., 2003; Harrison et al., 2004). We used shortened analog of GnRH (Hlavacek et al., 2000), in which the amino acid glycine in position 6 was replaced by D-amino acid (Sealfon et al., 1997), so the resulting analog was pGlu–His–Trp–Ser–Tyr-D-Cys–Leu–OH. D-Cysteine was chosen as a suitable D-amino acid because its free thiol group is suitable for fast and selective connection with the chloro- or maleimide-group on spacers.

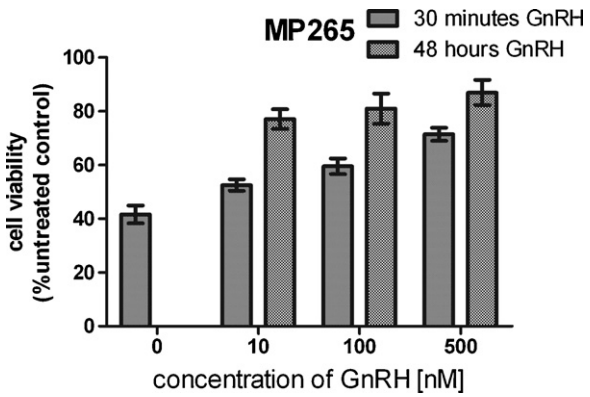

Fig. 5. The effect of the concentration of unconjugated GnRH analog (0, 10, 100 and 500 nM) and the time of GnRH analog exposition (30 min or 48 h) on the anti-proliferative efficacy of MP265 in MCF7 cells. The cells were exposed to 10 nM MP265 with or without GnRH analog for 48 h. The viabilities were expressed as a percentage of untreated controls (100%).

Two promising conjugates MP264 and MP265 were prepared. In these conjugates, paclitaxel and the GnRH analog were connected through a different spacer. As spacers, chloroacetic acid and maleimidobutyric acid were used. In both targeted conjugates the spacers were connected with paclitaxel *via* ester bond with 2′-hydroxyl group, which is suitable for preparing prodrugs. Reactive chloro- and maleimide-group, respectively, allows the connection with the GnRH analog in good yield. The structure of the conjugates was analysed by HR-MS and the structure of intermediates was also confirmed by NMR.

The MCF-7 human breast cancer line was chosen as an appropriate model cancer cells, because the GnRH binding sites are present there and the transport of conjugates might be achieved *via* them. The antiproliferative effect of paclitaxel and both targeted conjugates were tested in the MCF-7 cells using the MTT assay and the neutral red uptake test.

Results of both tests showed that the conjugate MP265 was more effective in decrease of cell proliferation than paclitaxel alone and the conjugate MP264. The higher antiproliferative effect of the conjugate MP265 compared to the conjugate MP264 might be caused by faster release of paclitaxel in the cells, due to different structure of the spacer employed.

The results of receptor saturation test demonstrated that the GnRH receptors play role in the antiproliferative effect of the conjugate MP265.

According to very promising results with MP265 conjugate, the synthesis of new paclitaxel conjugates bearing other spacers is under investigation. *In vivo* tests with MP265 conjugate will also be carried out in the near future.

5. Conclusion

In this study, the synthesis of two conjugates in which paclitaxel was linked to the analog of GnRH was described. This conjugates were tested in MCF-7 human breast cancer cell line for their antiproliferative effect. One of these conjugates was more effective in decrease of cell proliferation than paclitaxel alone. It was also demonstrated, that the GnRH receptors play role in the antiproliferative effect of this conjugate.

Acknowledgments

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