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ORIGINAL ARTICLE

In vitro degradation and antitumor activity of oxime bond-linked daunorubicin–GnRH-III bioconjugates and DNA-binding properties of daunorubicin–amino acid metabolites

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Abstract Bioconjugates with receptor-mediated tumor-targeting functions and carrying cytotoxic agents should enable the specific delivery of chemotherapeutics to malignant tissues, thus increasing their local efficacy while limiting the peripheral toxicity. In the present study, gonadotropin-releasing hormone III (GnRH-III; Glp-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2) was employed as a targeting moiety to which daunorubicin was attached via oxime bond, either directly or by insertion of a GFLG or YRRL tetrapeptide spacer. The in vitro antitumor activity of the bioconjugates was determined on MCF-7 human breast and HT-29 human colon cancer cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Their degradation/stability (1) in human serum, (2) in the presence of cathepsin B and (3) in rat liver lysosomal homogenate was analyzed by liquid chromatography in combination with mass spectrometry. The results show that (1) all synthesized bioconjugates have in vitro antitumor effect, (2) they are stable in human serum at least for 24 h, except for the compound containing an YRRL spacer and (3) they are hydrolyzed by cathepsin B and in the lysosomal homogenate. To investigate the relationship between the in vitro antitumor activity and the structure of the bioconjugates, the smallest metabolites produced in the lysosomal homogenate were synthesized and their binding to DNA was assessed by fluorescence spectroscopy. Our data indicate that the incorporation of a peptide spacer in the structure of oxime bond-linked daunorubicin–GnRH-III bioconjugates is not required for their antitumor activity. Moreover, the antitumor activity is influenced by the structure of the metabolites (daunorubicin–amino acid derivatives) and their DNA-binding properties.

Keywords Gonadotropin-releasing hormone-III · Oxime bond · Daunorubicin–peptide bioconjugates · Antitumor activity · In vitro degradation/stability · DNA binding

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Introduction

A major disadvantage of anticancer drugs is their lack of selectivity for tumor tissues, which causes severe side

effects and results in low cure rates; therefore, targeted delivery of anticancer drugs is one of the most actively pursued approaches in cancer chemotherapy (Singh et al. 2008). The combination of anticancer drugs with a targeting moiety which recognizes tumor specific or overexpressed receptors on cancer cells might provide efficient chemotherapeutic agents with minimal systemic toxicity (Dubowchik and Walker 1999). Drug delivery systems are generally designed so that the bond between the drug and the targeting moiety is resistant during transport (e.g., in the blood stream, digestive tract), but susceptible to acidic pH or enzymatic hydrolysis upon internalization into the cancer cells leading to the regeneration of the active cytotoxic agent or to the formation of an active metabolite (Jaracz et al. 2005).

Targeting moieties, such as antibodies (Trail et al. 2003), lectins (Yamazaki et al. 2000), sugars (David et al. 2004), hyaluronic acid (Luo et al. 2002), folic acid (Yadav et al. 2008), peptides (Schally and Nagy 2003) have been employed for the preparation of drug delivery systems for cancer chemotherapy. Tumor targeting with peptides is an intensively investigated approach for the specific delivery of anticancer drugs. It was found that receptors for peptide hormones such as gonadotropin-releasing hormone (GnRH) and somatostatin are expressed in a higher amount on cancer cells as compared to normal cells and serve as targets for peptide ligands to which cytotoxic drugs can be linked (Schally and Nagy 2004; Mező and Manea 2010). Consequently, these peptide hormones and their analogs could be used as targeting moieties to deliver cytotoxic agents directly to tumor cells, thereby increasing the concentration of the drugs in the tumor tissue and sparing normal cells from unnecessary exposure. So far, efficient anticancer drug—hormone peptide bioconjugates have been developed in A. V. Schally's laboratory. In these compounds, doxorubicin (Dox) or 2-pyrrolino-doxorubicin were attached through an ester bond to appropriate hormone peptide derivatives that were used as targeting moieties (Schally and Nagy 2003, 2004). The bioconjugates showed high selectivity and significant tumor growth inhibition on many tumor types. However, the fast degradation of the ester bond by carboxylesterases (half life of 2 h in human serum and 20 min in mouse serum) could lead to an early release of the cytotoxic drug resulting in non-receptor-specific toxicity on rapidly proliferating cells. The main toxic side effect was the myelotoxicity that was more pronounced in case of 2-pyrrolino-Dox (Kovács et al. 2009). Therefore, anticancer drug–peptide bioconjugates having higher stability in human serum are currently being developed. Moreover, increased doses of such compounds could be used to achieve a certain therapeutic effect without toxic side effects.

On the other hand, it has been reported that the intracellular drug release from the bioconjugates is required for their antitumor activity (Malugin et al. 2007). Lysosomes, intracellular organelles that have an internal acidic pH (pH 4.8) and contain a wide variety of hydrolytic enzymes, play a crucial role in the intracellular drug release. Various spacers/linkers have been developed and employed in the preparation of drug delivery systems for targeted cancer chemotherapy. The most frequently used linkers are (1) hydrazone linker which can be cleaved under acidic conditions in lysosomes, (2) disulfide linkers cleavable inside the tumor cells through disulfide exchange with an intracellular thiol, such as glutathione, (3) peptide linkers designed for high stability in serum and enzymatic hydrolysis in lysosomes (Jaracz et al. 2005).

Examples of peptide spacers cleavable primarily by cathepsin B, a lysosomal enzyme overexpressed in cancer cells, are as follows: -Phe-Lys-, -Val-Cit-, -Gly-Gly-Gly-, -Gly-Leu-Gly-, -Gly-Phe-Leu-Gly-, -Gly-Leu-Phe-Gly- and -Ala-Leu-Ala-Leu- (Dubowchik and Firestone 1998). One of the mostly used spacers is the -Gly-Phe-Leu-Gly- (GFLG) tetrapeptide, which was introduced by Omelyanenko et al. (1998) to connect doxorubicin to HMPA (*N*-(2-hydroxypropyl)methacrylamide) copolymer. The cleavage of the spacer by cathepsin B allowed the efficient drug release from the biocompatible, but not biodegradable HMPA copolymer (Etrych et al. 2001). In one of our previous studies, a bioconjugate for chemotactic drug targeting was developed, in which the γ-carboxyl group of methotrexate (Mtx) was attached to an oligotuftsin derivative carrier through the GFLGC pentapeptide spacer (Bai et al. 2008). In the presence of cathepsin B, this spacer was completely hydrolyzed resulting mainly in the Mtx(Gly) metabolite. However, no degradation of the oligotuftsin derivative carrier was observed. When daunorubicin (Dau) was attached via an oxime bond to the aminooxyacetylated GnRH-III derivative [Glp-His-Trp-Ser-His-Asp-Trp-Lys (Aoa-Gly-Phe-Leu-Gly)-Pro-Gly-NH_2, where Aoa is aminooxyacetic acid], besides the hydrolysis of the tetrapeptide spacer, the -^{7}Trp-^{8}Lys-peptide bond within the carrier sequence was also cleaved by cathepsin B (Szabó et al. 2009).

The presence of the hydrophobic GFLG spacer in the structure of the above-mentioned Dau–GnRH-III bioconjugate led to decreased solubility in aqueous buffers. Therefore, development of peptide spacers that provide increased solubility to the drug delivery system and are cleavable by lysosomal enzymes is of high importance. It has been reported that cathepsin B has both endopeptidase and peptidyldipeptidase activity; it cleaves C-terminal dipeptides sequentially with a broad specificity. Cathepsin B also cleaves -Arg-Arg-Xxx-peptide bonds and displays a preference for large hydrophobic residues in the P1′ position (Trp, Tyr, Phe, Leu) and Tyr in the P3 position (Ménard et al. 1993; Taralp et al. 1995). Based on these findings, we designed the -Tyr-Arg-Arg-Leu- tetrapeptide

spacer and employed it in the preparation of a bioconjugate in which the anticancer drug daunorubicin was attached via oxime bond to the GnRH-III as a targeting moiety.

GnRH-III is a weak GnRH agonist peptide which was first isolated from sea lamprey (*Petromyzon marinus*) (Sower et al. 1993). It has been shown that it has lower endocrine effect in mammals than the human GnRH (GnRH-I, also called LH–RH), it binds to the GnRH receptors on cancer cells and exhibits antiproliferative effect on many types of GnRH receptor-positive tumors (Kovács et al. 2007). Chemical modifications of the ε-amino group of Lys in position 8 did not result in a significant change in the antitumor activity of the parent hormone peptide (Mező et al. 1997; Pályi et al. 1999; Herédi-Szabó et al. 2005; Mező et al. 2007). Taking into consideration, these features of the GnRH-III, we prepared drug delivery systems for targeted cancer chemotherapy in which daunorubicin was attached to the side chain of ^{8}Lys of various GnRH-III derivatives. The lack of the hydroxyl group at C-14, which differentiates daunorubicin from doxorubicin, prevents the ester bond formation between the GnRH peptides and the anthracycline derivative. Therefore, in our previous work, daunorubicin was coupled to GnRH-III through an oxime or hydrazone bond (at the C-13 oxo group) resulting in bioconjugates with antitumor activity (Mező et al. 2008; Szabó et al. 2009).

When considering the advantages of the oxime chemical ligation such as (1) easy synthesis due to chemoselectivity and (2) high chemical stability of oxime bond containing bioconjugates, in the present study only oxime bond-linked daunorubicin–GnRH-III bioconjugates were synthesized, structurally and biologically characterized. In addition to the spacer-containing bioconjugates, a compound in which Dau was directly attached to the aminooxyacetylated GnRH-III was prepared, with the aim of investigating whether the incorporation of a spacer is required for the antitumor activity of the bioconjugates. Because the efficacy of these bioconjugates is related to their stability in the blood circulation and to their degradation in the target cells, we investigated the stability/degradation of the bioconjugates (1) in human serum, (2) in the presence of cathepsin B and (3) in rat liver lysosomal homogenate by liquid chromatography in combination with mass spectrometry. The in vitro antitumor activity of the bioconjugates was determined on MCF-7 human breast and HT-29 human colon cancer cells by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay (MTT assay). To evaluate the relationship between the in vitro antitumor activity and the structure of the bioconjugates, the smallest metabolites produced in the lysosomal homogenate (H–Lys(Dau=Aoa)–OH, Dau=Aoa–Gly–OH and Dau=Aoa–Tyr–OH) were synthesized and their binding to DNA was investigated by fluorescence spectroscopy. The results show that the incorporation of a peptide spacer in the structure of oxime bond-linked daunorubicin–GnRH-III bioconjugates is not required for their in vitro antitumor activity. Moreover, the in vitro antitumor activity of the bioconjugates is influenced by the DNA-binding properties of the smallest metabolites produced in the lysosomal homogenate. Furthermore, in the present study, the *E*-oxime isomer of the bioconjugate without spacer was revealed by NMR spectroscopy.

Materials and methods

Materials

All amino acid derivatives for peptide synthesis, benzotriazole-1-yloxytrispyrrolidinophosphonium hexafluorophosphate (PyBOP) and Rink-Amide MBHA resin were purchased from NovaBiochem (Läufelfingen, Switzerland) and GL Biochem Shanghai Ltd (Shanghai, China). Scavengers, coupling agents, and cleavage reagents [triisopropylsilane, 4-methylmorpholine (NMM), piperidine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), trifluoroacetic acid (TFA)] and Boc-aminooxyacetic acid (Boc–Aoa–OH) were obtained from Fluka (Buchs, Switzerland). Daunorubicin hydrochloride was a gift from IVAX (Budapest, Hungary). *N,N*-dimethylformamide (DMF), ethyl acetate (EtOAc) and diethyl ether were from Molar Chemicals Kft (Budapest, Hungary). Hydroxylamine hydrochloride (NH_2–OH·HCl), pentachlorophenol (PcpOH) and solvents for HPLC [methanol (MeOH) and acetonitrile (CH_3CN)] were purchased from Sigma-Aldrich Kft. (Budapest Hungary). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was a Sigma-Aldrich Ltd (St. Louis, MO, USA) product. All reagents and solvents were of analytical grade or highest available purity.

Methods

Synthesis of oxime bond-linked daunorubicin–GnRH-III bioconjugates

Aminooxyacetic acid (Aoa) derivatives of GnRH-III (<EHWSHDWK(Aoa)PG–NH_2, <EHWSHDWK(Aoa–GFLG)PG–NH_2, <EHWSHDWK(Aoa–YRRL)PG–NH_2, where <*E* is pyroglutamic acid) were prepared manually by solid-phase peptide synthesis according to Fmoc/*t*Bu chemistry on a Rink-Amide MBHA resin (0.38 mmol/g coupling capacity). The following Fmoc-protected amino acid derivatives were used: Fmoc–Gly–OH, Fmoc–Pro–OH, Fmoc–Lys(Mtt)–OH, Fmoc–Trp–OH, Fmoc–Asp(O*t*Bu)–OH, Fmoc–His(Trt)–OH, Fmoc–Ser(*t*Bu)–OH, Fmoc–Leu–OH, Fmoc–Phe–OH, Fmoc–Tyr(*t*Bu)–OH and

Fmoc–Arg(Pbf)–OH. The protocol of the synthesis was as follows: (1) DMF washing (4 × 1 min), (2) Fmoc deprotection with 2% DBU, 2% piperidine in DMF (15 min), (3) DMF washing (10 × 1 min), (4) coupling of 5 equiv of Fmoc-amino acid : PyBOP : NMM in DMF (60 min), (5) DMF washing (4 × 1 min). Following completion of the synthesis of GnRH-III protected peptide chain, the Mtt protecting group of the $^{\varepsilon}NH_2$ function of ^{8}Lys was removed by 2% TFA in DCM and then the peptide–resin was divided into three portions. On one part of the resin, Boc-aminooxyacetic acid was attached to the $^{\varepsilon}NH_2$ group of ^{8}Lys after pre-activation with PyBOP in the presence of NMM (3 equivalent each to the resin capacity; coupling time: 60 min). On the second part of the resin, the GFLG tetrapeptide branch was prepared by Fmoc strategy and then Boc–Aoa–OH was attached to the *N*-terminus of the branch as above mentioned. The third portion of the resin was employed for the synthesis of the compound containing the YRRL spacer.

The peptides were cleaved from the resin using a mixture of 95% TFA, 2.5% triisopropylsilane and 2.5% water (v/v/v) for 2.5 h at room temperature and then precipitated with cold diethyl ether, washed three times with diethyl ether and solubilized in 100% acetic acid prior to freeze drying. The crude products were purified by semi-preparative RP-HPLC and analyzed by mass spectrometry.

The conjugations via oxime bond were carried out in 0.2 M sodium acetate buffer, pH 5, at a peptide concentration of 10 mg/mL. Daunorubicin was used in 20% excess as compared to the aminooxy derivatives of GnRH-III. The reaction mixtures were stirred for 16 h at RT and then the bioconjugates were separated by semipreparative RP-HPLC. The purified GnRH-III(Dau=Aoa), **1**, GnRH-III(Dau=Aoa–GFLG), **2** and GnRH-III(Dau=Aoa–YRRL), **3** were characterized by analytical RP-HPLC and mass spectrometry (Table 1 and Electronic Supplementary Material S3–S4).

Synthesis of H-Lys(Dau=Aoa)–OH, Dau=Aoa–Gly–OH and Dau=Aoa–Tyr–OH metabolites

Boc–Lys–OH, H–Tyr(*t*Bu)–OH or unprotected glycine (1 mmol each) were dissolved in 10 mL of 2 M Na_2CO_3. Prior to the addition of an equivalent amount of Boc–Aoa–OPcp in 10 mL DMF, the solutions were diluted with 10 mL DMF. The coupling reactions were carried out at RT for 24 h. The solvents were removed in all cases and the remaining materials were washed with ether to remove the pentachlorophenol. The solid materials were dissolved in water and the pH was adjusted to 2 with 1 M HCl under cooling. The protected compounds were extracted with EtOAc, afterwards. The organic solutions were dried over $MgSO_4$ for 1 h, followed by filtration and evaporation of the solvent. The oily compounds were dried in a desiccator for 1 day and then the *tert*-butyl-type protecting groups were cleaved at RT for 1 h with TFA containing 5% d.i. water as a scavenger. The mixtures were concentrated in weighted round bottom flasks using a water aspirator. After that the dried compounds were dissolved in 0.2 M NH_4OAc (pH 5). Daunorubicin hydrochloride (1.5 equiv) was added to the solution of each Aoa-modified amino acid. The oxime ligation was carried out overnight at RT. The reaction mixtures were injected directly to a semi-preparative RP-HPLC column to separate the prepared metabolites and the excess of daunorubicin. Except Dau=Aoa–Tyr–OH, the conjugates Dau=Aoa–Gly–OH and H-Lys(Dau=Aoa)–OH could be well separated (10 mg crude product was purified in one run). In case of Dau=Aoa–Tyr–OH, the compound was dissolved again in 0.2 M NH_4OAc (pH 5) and an excess of hydroxylamine hydrochloride was added to this solution. Hydroxylamine reacted with the excess of daunorubicin (3 h reaction time) and then the Dau=Aoa–Tyr–OH and Dau=N–OH could efficiently be separated by RP-HPLC. The synthesized metabolites were characterized by analytical HPLC and mass spectrometry (Table 2 and Electronic Supplementary Material S13).

High-performance liquid chromatography

Analytical RP-HPLC was performed on a Knauer (H. Knauer, Bad Homburg, Germany) or on a Dionex system (Dionex, Idstein, Germany) using either a Vydac C_{18} or a Phenomenex Jupiter C_{18} column (250 × 4.6 mm) with

Table 1 Characteristics of oxime bond-linked daunorubicin–GnRH-III bioconjugates

Compound number	Code	RP-HPLC R_t (min)[a]	ESI-MS[b] MW_{calc}/MW_{exp}
1	GnRH-III(Dau=Aoa)	26.5	1,840.75/1,841.05
2	GnRH-III(Dau=Aoa–GFLG)	29.6	2,215.25/2,215.45
3	GnRH-III(Dau=Aoa–YRRL)	27.5	2,429.11/2,429.06

RP-HPLC profiles and mass spectra are presented in the Electronic Supplementary Material, S3–S4

[a] Column: Vydac C_{18} (250 × 4.6 mm) with 5 μm silica (300 Å pore size); gradient: 0 min 0% B; 5 min 0% B; 50 min 90% B; eluents 0.1% TFA in water (A) and 0.1% TFA in acetonitrile–water (80:20, v/v) (B); flow rate: 1 mL/min; detection: $\lambda = 220$ nm

[b] Bruker Daltonics Esquire 3000 + ion trap mass spectrometer

Table 2 Chemical characteristics of daunorubicin and its derivatives

Compound	RP-HPLC R_t (min)[a]	ESI–MS M_{calc}/M^b_{exp}
Daunorubicin (Dau)	29.7	527.2/527.4
Dau=N–OH	28.3	542.2/542.3
Dau=Aoa–OH[c]	31.6	601.2/601.2
Dau=Aoa–Gly–OH	27.1	657.2/657.5
Dau=Aoa–Tyr–OH	28.7	763.3/763.5
H-Lys(Dau=Aoa)–OH	25.1	728.3/728.4

[a] RP-HPLC: column: Phenomenex Jupiter C_{18} (250 × 4.6 mm, 5 μm, 300Å); eluents: 0.1% TFA in water (A), 0.1% TFA in acetonitrile–water (80:20, v/v) (B); flow rate: 1 mL/min; gradient: 0% B (0 min), 0% B (5 min), 90% B (50 min); detection at 220 nm

[b] Bruker Daltonics Esquire 3000 + ion trap mass spectrometer

[c] Dau=Aoa–OH was prepared as previously described (Szabó et al. 2009)

5 μm silica (300 Å pore size) as a stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 50 min 90% B) with eluent A (0.1% TFA in water) and eluent B [0.1% TFA in acetonitrile–water (80:20, v/v)] was used at a flow rate of 1 mL/min. Peaks were detected at 220 nm.

The crude Dau–GnRH-III bioconjugates were purified on a Dionex HPLC system (Dionex, Idstein, Germany) using a semipreparative Vydac C_{18} column (250 × 10 mm) with 10 μm silica (300 Å pore size). Linear gradient elution (0 min 10% B; 5 min 10% B; 55 min 60% B) with eluent A (0.1% TFA in water) and eluent B [(0.1% TFA in acetonitrile–water (80:20, v/v)] was used at a flow rate of 4 mL/min. Peaks were detected at 220 and 280 nm. The crude metabolites were purified on a semipreparative Phenomenex Jupiter C_{18} column (250 × 10 mm) with 10 μm silica (300 Å pore size) using a methanol–water (90:10, v/v) solvent mixture.

Mass spectrometry (MS)

Electrospray (ESI)-mass spectrometric analyses were carried out on an Esquire 3000 + ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Spectra were acquired in the 100–2,500 *m/z* range. Samples were dissolved in a mixture of 50% methanol, 48% water and 2% acetic acid.

Liquid chromatography–mass spectrometry (LC–MS) was carried out on an Esquire 3000 + ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany). Peptides were separated on a Vydac MS C_{18} column (150 × 1 mm; 300 Å, 3 μm) using a linear gradient from 90% solvent A [0.1% formic acid in water (v/v)] and 10% solvent B [0.1% formic acid in acetonitrile (v/v)] to 70% solvent B over 60 min and a flow rate of 50 μL/min. Spectra were recorded in positive ion mode in the 100–2,500 *m/z* range.

Cathepsin B catalyzed hydrolysis of GnRH-III(Dau=Aoa) and GnRH-III(Dau=Aoa–YRRL) bioconjugates

Digestion of GnRH-III(Dau=Aoa) and GnRH-III(Dau=Aoa–YRRL) bioconjugates by cathepsin B was performed similarly to the digestion of GnRH-III(Dau=Aoa–GFLG) as previously described (Szabó et al. 2009). In brief, the compounds were dissolved at a concentration of 0.1 μg/μL in 0.1 M NaOAc buffer containing 0.01 M DTT (pH 5), and then human liver cathepsin B (Calbiochem, Germany; $c = 0.4$ μg/μL in 20 mM NaOAc buffer, pH 5, containing 1 mM EDTA) was added at an enzyme to substrate ratio of 1:50, w/w. The reaction mixtures were incubated at 37°C, and aliquots of 20 μL were taken after 5 min, 2, 4, 8 and 24 h (the reaction was quenched by adding 2 μL of acetic acid and followed by LC–MS analysis). A control experiment in which bioconjugate solutions ($c = 0.1$ μg/μL in 0.1 M NaOAc buffer, pH 5 containing 0.01 M DTT) were incubated at 37°C for 24 h was also performed.

Degradation of daunorubicin–GnRH-III bioconjugates in rat liver lysosomal homogenate

The rat liver lysosomal homogenate was prepared according to the following procedure: livers from two male rats were collected and homogenized in two volumes of ice cold 0.3 M sucrose with 10 strokes at 15 g. The homogenate was diluted with three volumes of 0.3 M sucrose. The nuclei and cell debris were centrifuged at 700*g* for 10 min. The supernatant was washed with 0.3 M sucrose solution and centrifuged again at 700*g* for 10 min. After that the supernatant was centrifuged at 10,000*g* for 10 min to sediment the crude lysosomal–mitochondrial fraction. The sediment was re-homogenized in 20 mL of 0.3 M sucrose containing $CaCl_2$ (final $CaCl_2$ concentration 1 mM). The homogenate was incubated at 37°C for 5 min for the mitochondria swallowing and then 20 mL of 50% Percoll were added to the solution, followed by the centrifugation of the homogenate at 10,000*g* for 10 min. The supernatant was removed, while the pellet was resuspended in 0.3 M sucrose and centrifuged again at 10,000*g* for 10 min. The hard brown pellet was the lysosomal fraction, which was diluted 1:2 with 0.3 M sucrose for the best pipetting.

The protein concentration was determined by Pierce BCA protein assay (bicinchoninic acid) according to the manufacturer's protocol (Thermo Fisher Scientific, Rockford, IL, USA) and it was 16.6 μg/μL.

The degradation of the daunorubicin–GnRH-III bioconjugates **1**, **2** and **3** in the rat liver lysosomal homogenate was determined as follows: 100 μg of lysosomal homogenate

was added to 100 μg of bioconjugates dissolved in 1 mL 0.2 M sodium acetate buffer, pH 5.0 (bioconjugates: lysosomal homogenate ratio = 1:1, w/w). The reaction mixtures were incubated at 37°C and aliquots of 50 μL were taken at 5 min, 1, 2, 3, 4, 6, 8, 24, 48 and 72 h. The reactions were quenched by adding 5 μL of acetic acid and followed by LC–MS analysis. Control experiments were performed with solutions of bioconjugates in 0.2 M sodium acetate buffer, pH 5, which were incubated at 37°C for 24, 48 and 72 h and then analyzed by LC–MS.

Stability of daunorubicin–GnRH-III bioconjugates in 90% human serum

GnRH-III(Dau=Aoa), **1**, GnRH-III(Dau=Aoa–GFLG), **2** and GnRH-III(Dau=Aoa–YRRL), **3** were first dissolved in water and then the human serum was added (final peptide concentration 10 μM). The mixtures were incubated at 37°C. Aliquots of 100 μL were taken after 5 min, 2, 4, 8 and 24 h in the case of bioconjugates **1** and **2** and after 5, 10, 15, 30 min, 1, 3 and 6 h in the case of bioconjugate **3**. The reactions were quenched by adding 10 μL of acetic acid. Before mass spectrometric analysis, the larger human serum proteins were removed using Microcon centrifugal devices, cut off 3 kDa (Millipore Corporation, Bedford, MA, USA) and the lower molecular weight fraction was analyzed by LC–MS.

Two control experiments were performed: (1) compounds with molecular weight lower than 3,000 from human serum were separated and analyzed by LC–MS and (2) aqueous solutions of bioconjugates **1**, **2** and **3** ($c = 10$ μM) were incubated at 37°C for 24 h and then analyzed by LC–MS.

Cells

MCF-7 human breast adenocarcinoma cells were maintained in DMEM (Sigma Ltd., St. Louis, MO) medium containing 10% FCS (fetal calf serum, Sigma Ltd.), L-glutamine (2 mM), gentamicin (160 μg/mL), 1 mM pyruvate and non-essential amino acids (Sigma Ltd.). HT-29 human colon carcinoma cells were maintained in RPMI-1640 medium containing 10% FCS, L-glutamine (2 mM) and gentamicin (160 μg/mL). Both cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

In vitro cytostatic effect of bioconjugates determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay)

In vitro cytostatic effect of the bioconjugates was determined by MTT assay (Collins et al. 1977). 5×10^3 cells per well were plated on 96-well plates. After 24 h of incubation at 37°C, cells were treated for 6 h with the compounds dissolved in serum-free RPMI-1640 medium (2.6×10^{-4}–10^2 μM concentration range). Cells treated for 6 h with serum-free medium were used as a control. After treatment and incubation, cells were washed twice with serum-free medium and cultured in serum containing medium for 72 h. On the fourth day, the MTT assay was carried out. 45 μL MTT solution (2 mg/mL) were added to each well (final concentration 367 μg/mL) and during 3.5 h incubation purple crystals were formed by mitochondrial dehydrogenase enzyme present in the living cells. Cells were centrifuged for 5 min at 863*g* and the supernatant was removed. Crystals were dissolved in DMSO and the optical density (OD) of the samples was determined at $\lambda = 540$ and 620 nm using an ELISA Reader (Labsystems MS reader, Helsinki, Finland). OD_{620} was subtracted from OD_{540}. The percent of cytostasis was calculated using the following equation:

$$\text{Cytostasis}\,\% = [1 - (OD_{\text{treated}}/OD_{\text{control}})]100$$

where OD_{treated} and OD_{control} correspond to the optical densities of treated and control cells, respectively. Cytostasis% was plotted as a function of concentration fitted to a sigmoidal curve and the 50% inhibitory concentration (IC_{50}) value was determined from these curves (Table 3).

Fluorescent properties of daunorubicin, daunorubicin–amino acid derivatives and GnRH-III(Dau=Aoa) bioconjugate

Ground-state absorption spectra were recorded with 1 nm steps and 2 nm bandwidth using a Cary 4E spectrophotometer (Varian, Mulgrave, Australia). Spectra of Dau and its derivatives were recorded at 10 μM concentration (solutions prepared in 20 mM Tris–HCl, 50 mM NaCl buffer, pH = 7.4). The fluorescence intensity exhibited by the compounds in the absence and in the presence of chicken erythrocyte DNA (Reanal, Hungary) was determined. Fluorescence titration experiments were performed on a FluoroLog®-3 spectrofluorometer (Jobin–Yvon, Longjumeau, France) at ambient temperature. Samples were excited at $\lambda = 488$ nm and emission spectra were

Table 3 In vitro cytostatic effect of oxime bond-linked daunorubicin–GnRH-III bioconjugates on human MCF-7 and HT-29 cancer cell lines

Compounds	Cytostasis (MCF-7) IC_{50} (μM)	Cytostasis (HT-29) IC_{50} (μM)
GnRH-III(Dau=Aoa)	2.2 ± 1.2	14.2 ± 3.2
GnRH-III(Dau=Aoa–GFLG)	3.9 ± 1.2	19.4 ± 3.1
GnRH-III(Dau=Aoa–YRRL)	1.8 ± 0.5	28.6 ± 5.5

recorded between 495 and 800 nm. The ratio of the integrated fluorescence intensity of daunorubicin in the absence of DNA (I_0) and in the presence of DNA (I) was used to calculate the amount of bound compound according to the following equation:

$$c_{\text{free}} = c_t(I/I_0 - P)/(1 - P) \quad (1)$$

where c_t is the known added amount of compound and P is the ratio of the observed quantum yield of fluorescence of the totally bound compound to that of the free one; c_{bound} was then obtained by difference (Chaires et al. 1982). The ratio $P = I_\infty/I_0$ was obtained from the initial value of I and the plateau value of I at the highest DNA concentration.

Determination of apparent binding constant

Apparent binding constants were determined by the neighbor exclusion model (McGhee and Von Hippel 1974) using the equation

$$\frac{r}{c_{\text{free}}} = K(1 - nr)\left[\frac{1 - nr}{1 - r(n-1)}\right]^{n-1}, \quad (2)$$

where r is the number of moles of bound compound per mole of DNA base pair, K is the intrinsic binding constant and n is the exclusion parameter in base pairs.

NMR structure analysis

NMR spectra were acquired at room temperature on a Bruker Avance III 600 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a TCI-H/C/N triple resonance cryoprobe. 10 mg of GnRH-III(Dau=Aoa) were dissolved in H_2O and supplemented with 5% D_2O (v/v). Trimethylsilylpropionic acid-d4 was used as an internal standard. The pH of the 500 µL solution was adjusted to 4. If applicable, solvent suppression was achieved by excitation sculpting (Hwang and Shaka 1995). 2D NOESY and 2D TOCSY mixing time was 300 and 80 ms, respectively, the latter using the MLEV17 (Bax and Davis 1985) sequence at 8.9 kHz spinlock field strength. Coherence transfer pathways were selected using pulsed field gradients in all experiments. Spectra were processed and analyzed by Bruker Topspin 2.1 and CARA (Keller 2004).

Results and discussion

Oxime bond-linked daunorubicin–GnRH-III bioconjugates

The concept of targeted cancer chemotherapy based on human GnRH derivatives was introduced by A.V. Schally et al. in the late 1980s (Bajusz et al. 1989). The most promising compound developed by Schally's group, in which doxorubicin-14-*O*-hemiglutarate was conjugated to the [D-Lys^6]-GnRH-I (AN-152, AEZS-108 (Æterna Zentaris Inc)] (Nagy et al. 1996) is currently in phase II clinical trial on ovarian and endometrial cancer (http://www.aezsinc.com).

However, it has been reported that the ester bond connecting the doxorubicin to the GnRH-I derivative used as a targeting moiety was easily cleaved by carboxylesterases in vitro, in mouse and human sera, resulting in the release of the free drug. The half life time of the bioconjugate was shorter in mouse serum (19.49 ± 0.74 min) than in human serum (126.06 ± 3.03 min) due to the higher content of carboxylesterases in mouse serum (Nagy et al. 2000). Moreover, toxic side effects were caused by the release of free doxorubicin from the bioconjugate in mice. To elevate the maximum tolerated dose of the ester bond-linked Dox-[D-Lys^6]-GnRH-I bioconjugate, diisopropyl fluorophosphate (DFP) was used in that study to inhibit the activity of the carboxylesterases (CE). In this way, the half life time of the bioconjugate in mouse serum increased to 69.63 ± 4.44 min.

The major goal of our work was to synthesize, structurally and biologically characterize anthracycline–GnRH-III derivative bioconjugates that have antitumor activity and increased stability in human serum. Taking into consideration that daunorubicin has lower cardiotoxicity than doxorubicin (Gilladoga et al. 1976; Minotti et al. 1995), in the present study, we employed daunorubicin for the preparation of drug delivery systems for targeted cancer chemotherapy. GnRH-III, a weak agonistic hormone peptide with low endocrine effect and own antiproliferative activity was used as a targeting moiety. As already mentioned, because of the lack of OH group at the C-14 of the aglycon part, daunorubicin was conjugated via oxime bond to the GnRH-III. It has been shown that the acylation of the amino group of daunosamine by GnRH-III peptide resulted in the loss of the antitumor activity (Mező et al. 2008), therefore, the oxo group at the C-13 position of daunorubicin was used as a conjugation site. An oxime bond was formed between the keto group of daunorubicin and the aminooxyacetyl group of the GnRH-III derivatives. Because the oxime bond is chemically stable between pH 3 and 8 (Shao and Tam 1995), as well as under in vitro and in vivo biological experimental conditions (pH 7.4) (Braslawsky et al. 1991), the incorporation of an enzymatic cleavable spacer between the anticancer drug and the targeting moiety might be necessary for the efficient drug release and antitumor activity. In one of our previous studies, the GFLG tetrapeptide spacer cleavable by cathepsin B was incorporated between Dau and GnRH-III leading to the formation of a bioconjugate which had in vitro and in vivo antitumor effect (Szabó et al. 2009).

When considering that the presence of the hydrophobic GFLG spacer in the structure of the above-mentioned Dau-GnRH-III bioconjugate led to decreased solubility in aqueous buffers, in the current study, we designed and employed a new YRRL tetrapeptide spacer, which provided increased solubility to the drug delivery system and it was cleaved by cathepsin B. Moreover, we were interested in answering the question whether the presence of an enzymatic cleavable spacer between daunorubicin and GnRH-III is required for the antitumor activity of the bioconjugates. For these purposes, three bioconjugates were synthesized in which daunorubicin was attached via oxime bond to the aminooxyacetylated GnRH-III derivative, either directly or by insertion of a GFLG or YRRL tetrapeptide spacer (Fig. 1). All bioconjugates were purified by HPLC and the purified compounds were analyzed by analytical HPLC and mass spectrometry (Table 1 and Electronic Supplementary Material S3–S4).

Cathepsin B catalyzed hydrolysis of GnRH-III(Dau=Aoa) and GnRH-III(Dau=Aoa–YRRL) bioconjugates

Cathepsin B, a lysosomal enzyme overexpressed in cancer cells (Sibrian-Vazquez et al. 2008), plays an important role in the intracellular digestion of proteins taken up by endocytosis. Its cleavage specificity can be used as a basis for the development of drug delivery systems for targeted cancer chemotherapy that provide controlled intracellular drug release (Dubowchik et al. 2002). We have previously shown that the GnRH-III(Dau=Aoa–GFLG) bioconjugate was cleaved by cathepsin B preferentially within the spacer sequence, namely, the peptide bonds -Gly-Phe-, -Phe-Leu- and -Leu-Gly-. Furthermore, cleavages of the isopeptide bond -Lys(Gly)- and of the peptide bond-^{7}Trp-^{8}Lys- in the GnRH-III sequence were determined by LC–MS (Szabó et al. 2009).

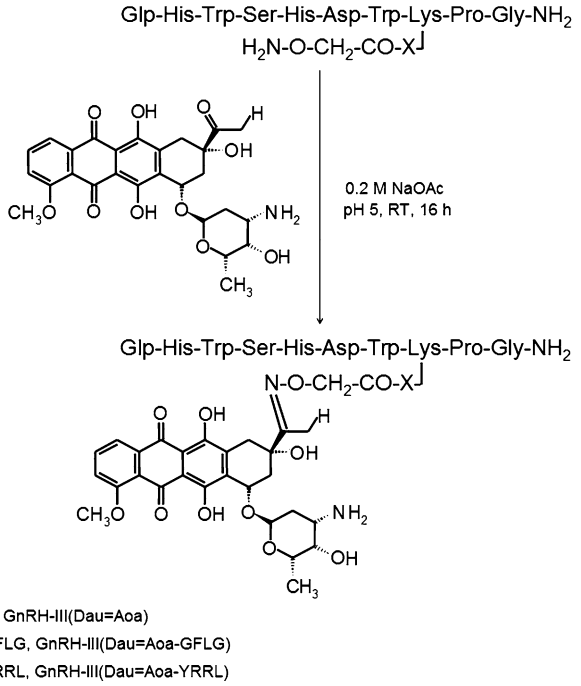

Fig. 1 Conjugation of daunorubicin to the aminooxyacetylated GnRH-III derivatives

Incubation of GnRH-III(Dau=Aoa) with cathepsin B, followed by LC–MS analysis of the reaction mixture at different time points, led to the identification of the following cleavage sites: -^{3}Trp-^{4}Ser-, -^{4}Ser-^{5}His- and -^{7}Trp-^{8}Lys- (Fig. 2; Electronic Supplementary Material S5—Fig. 4a). In case of GnRH-III(Dau=Aoa–YRRL) bioconjugate, cathepsin B catalyzed the hydrolysis of -Tyr-Arg-, -Arg-Arg- and -Arg-Leu-peptide bonds within the spacer sequence and of -^{3}Trp-^{4}Ser- and -^{4}Ser-^{5}His-peptide bonds in the GnRH-III sequence (Fig. 3; Electronic Supplementary Material S5—Fig. 5b). The fragments resulted after the cleavage of -^{3}Trp-^{4}Ser- and -^{4}Ser-^{5}His-peptide bonds, namely <EHW–OH and <EHWS–OH, formed noncovalent dimers $[<\text{EHW–OH}]_2$ and $[<\text{EHWS–OH}]_2$ that were detected by mass spectrometry at *m/z* 905.40 (1+) and 1,079.40 (1+), respectively. No cleavage of the oxime bond was determined after 24 h incubation at 37°C in the presence or absence of the enzyme. Furthermore, in the absence of cathepsin B, no cleavage of the peptide bonds was detected (Electronic Supplementary Material S6). It is important to note the fragmentation of the glycosidic bond during the mass spectrometric analysis resulting in the loss of daunosamine (−129, −147), which could not be completely prevented by lowering the capillary exit voltage. Daunosamine and the fragments resulted from the loss of daunosamine were marked in all mass spectra by an asterisk (Electronic Supplementary Material S3–S12).

Degradation of oxime bond-linked daunorubicin–GnRH-III bioconjugates in rat liver lysosomal homogenate

The degradation of GnRH-III(Dau=Aoa), GnRH-III(-Dau=Aoa–GFLG) and GnRH-III(Dau=Aoa–YRRL) bioconjugates was also investigated in rat liver lysosomal homogenate. Analyses of the reaction mixtures after 8 h are presented in the Electronic Supplementary Material S7–S8, in which the LC–ESI–mass spectra summed over the chromatographic window where the compounds eluted are shown. Six cleavage sites were identified in case of GnRH-III(Dau=Aoa) bioconjugate: -^{2}His-^{3}Trp-, -^{4}Ser-^{5}His-, -^{6}Asp-^{7}Trp-, -^{7}Trp-^{8}Lys-, -^{8}Lys-^{9}Pro- and -^{9}Pro-^{10}Gly-. All these cleavage sites were located in the GnRH-III sequence and no cleavage of the oxime bond was

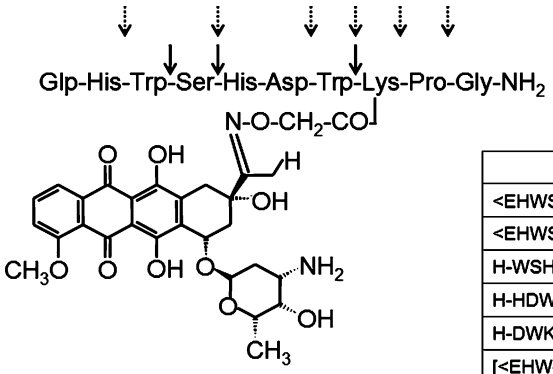

Fig. 2 Cleavage sites and the corresponding fragments produced by the proteolysis of GnRH-III(Dau=Aoa) bioconjugate in the presence of cathepsin B (*full-line arrows*) and rat liver lysosomal homogenate (*dotted-line arrows*)

Fragment	MW_{calc} / MW_{exp}
<EHWSHDWK(Dau=Aoa)PG-NH_2	1841.92/1842.20
<EHWSHDWK(Dau=Aoa)-OH	1687.63/1687.80
H-WSHDWK(Dau=Aoa)-OH	1439.54/1439.20
H-HDWK(Dau=Aoa)-OH	1166.43/1166.10
H-DWK(Dau=Aoa)-OH	1029.37/1029.10
[<EHW-OH]$_2$	904.36/904.30
H-K(Dau=Aoa)PG-NH_2	882.68/882.40
H-K(Dau=Aoa)P-OH	825.31/825.10
H-K(Dau=Aoa)-OH	728.26/728.10
<EHWS-OH	539.21/539.20
<EHW-OH	452.18/452.10

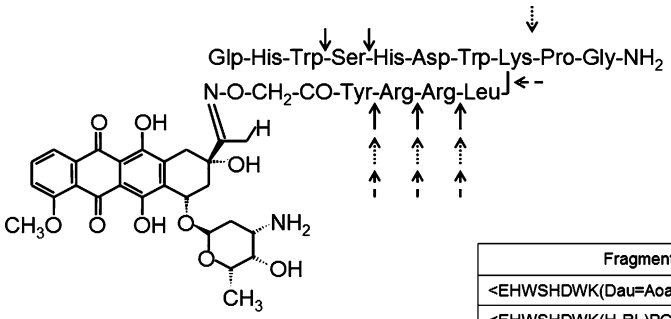

Fig. 3 Cleavage sites and the corresponding fragments produced by the proteolysis of GnRH-III(Dau=Aoa–YRRL) bioconjugate in the presence of cathepsin B (*full-line arrows*), rat liver lysosomal homogenate (*dotted-line arrows*) and human serum (*dashed-line arrows*)

Fragment	MW_{calc} / MW_{exp}
<EHWSHDWK(Dau=Aoa-YRRL)PG-NH_2	2430.64/2430.60
<EHWSHDWK(H-RL)PG-NH_2	1527.76/1527.60
<EHWSHDWK(H-RL)-OH	1374.67/1374.60
<EHWSHDWK(H-L)PG-NH_2	1372.67/1372.60
<EHWSHDWK(H-L)PG-NH_2	1371.65/1371.20
<EHWSHDWKPG-NH_2	1258.54/1258.50
<EHWSHDWK(H-L)-OH	1218.56/1218.40
[<EHWS-OH]$_2$	1078.42/1078.40
Dau=Aoa-YRR-OH	1075.43/1075.00
Dau=Aoa-YR-OH	919.36/919.40
[<EHW-OH]$_2$	904.36/904.40
Dau=Aoa-Y-OH	763.26/763.40
<EHWS-OH	539.21/539.20
<EHW-OH	452.18/452.10

detected (Fig. 2, Electronic Supplementary Material S7—Fig. 6a). The smallest fragment containing daunorubicin was identified at *m/z* 729.1 (1+) and it was H–Lys (Dau=Aoa)–OH.

In contrast to GnRH-III(Dau=Aoa), the degradation pattern of the bioconjugates containing a tetrapeptide spacer between Dau and GnRH-III was different. LC–MS analysis of GnRH-III(Dau=Aoa–YRRL) incubated for 8 h with the rat liver lysosomal homogenate led to the identification of (1) cleavage sites within the spacer sequence (-Tyr-Arg-, -Arg-Arg- and -Arg-Leu-) and (2) cleavage of -^{8}Lys-^{9}Pro-peptide bond in the GnRH-III sequence (Fig. 3; Electronic Supplementary Material S7 Fig. 6b). Several metabolites containing daunorubicin were produced, the smallest one being Dau=Aoa–Tyr–OH fragment [*m/z* 764.2 (1+) and 382.6 (2+)].

A similar degradation pattern, shown in Fig. 4 and Electronic Supplementary Material S8—Fig. 6c, was obtained in case of GnRH(Dau=Aoa–GFLG). The spacer sequence was primarily hydrolyzed in the presence of lysosomal enzymes (-Gly-Phe-, -Phe-Leu- and -Leu-Gly-); however, the cleavage of the -^{7}Trp-^{8}Lys- and -^{8}Lys-^{9}Pro-peptide bonds within the GnRH-III sequence was also identified. In this case, Dau=Aoa–Gly–OH was the smallest metabolite containing daunorubicin [*m/z* 658.1 (1+)] which was produced.

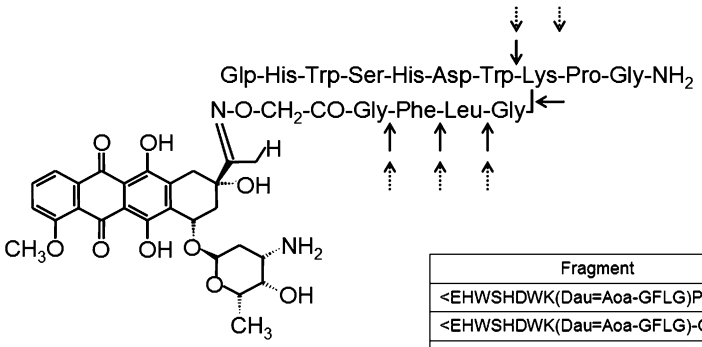

Fig. 4 Cleavage sites and the corresponding fragments produced by the proteolysis of GnRH-III(Dau=Aoa–GFLG) bioconjugate in the presence of cathepsin B (*full-line arrows*) and rat liver lysosomal homogenate (*dotted-line arrows*)

Fragment	MW_{calc} / MW_{exp}
<EHWSHDWK(Dau=Aoa-GFLG)PG-NH_2	2215.25/2215.50
<EHWSHDWK(Dau=Aoa-GFLG)-OH	2061.83/2062.20
<EHWSHDWK(H-FLG)PG-NH_2	1576.75/1577.35
<EHWSHDWK(H-G)PG-NH_2	1316.43/1316.65
<EHWSHDWK(H-LG)-OH	1275.58/1275.30
<EHWSHDWKPG-NH_2	1259.35/1259.65
H-K(Dau=Aoa-GFLG)PG-OH	1256.31/1256.60
<EHWSHDWK(H-G)-OH	1162.50/1162.20
H-K(Dau=Aoa-GFLG)-OH	1102.46/1102.35
Dau=Aoa-GFL-OH	917.34/917.30
Dau=Aoa-GF-OH	804.26/804.30
Dau=Aoa-G-OH	657.19/657.10
H-FL-OH	278.16/278.00

For all three bioconjugates, no additional fragments were identified after longer incubation times, 24–72 h (Electronic Supplementary Material S9, S10).

Stability/degradation of daunorubicin–GnRH-III bioconjugates in human serum

The stability of daunorubicin–GnRH-III bioconjugates in 90% human serum was determined by LC–MS. GnRH-III(Dau=Aoa) and GnRH-III(Dau=Aoa–GFLG) were stable in human serum at least for 1 day. LC–MS analysis of the bioconjugates incubated for 24 h at 37°C with human serum revealed the presence of intact compounds (*m/z* 921.8 (2+), 614.9 (3+) in case of GnRH-III(Dau=Aoa) and *m/z* 1,108.9 (2+), 739.7 (3+) in case of GnRH-III(-Dau=Aoa–GFLG); see Electronic Supplementary Material S11) and did not result in the identification of degradation products.

In contrast to GnRH-III(Dau=Aoa) and GnRH-III(Dau=Aoa–GFLG), GnRH-III(Dau=Aoa–YRRL) compound was very fast degraded in human serum. After 5 min of incubation with human serum at 37°C, the cleavage of the -Arg-Arg- peptide bond within the spacer sequence was identified by mass spectrometry [resulted peptide fragments: <EHWSHDWK(H–RL)PG–NH_2 at *m/z* 510.30 (3+), 383.10 (4+) and Dau=Aoa–YR–OH at *m/z* 919.30 (1+)]. The intact bioconjugate could be detected in the reaction mixture after 1 h, but not after 3 h. The LC–MS analysis of the aliquot taken out from the reaction mixture after 3 h led to the identification of the following cleavage sites: -Arg-Arg-, -Tyr-Arg-, -Arg-Leu- peptide bonds and -Lys(Leu)- isopeptide bond (Fig. 3; Electronic Supplementary Material S12).

In vitro cytostatic effect of daunorubicin–GnRH-III bioconjugates

In vitro cytostatic effect of GnRH-III(Dau=Aoa), GnRH-III(Dau=Aoa–GFLG) and GnRH-III(Dau=Aoa–YRRL) bioconjugates was determined on human MCF-7 and HT-29 cancer cell lines by MTT assay and the calculated IC_{50} values were presented in Table 3. All investigated compounds had antitumor activity, which was higher on MCF-7 cells compared with HT-29. However, no significant difference was observed between the cytostatic effect of the compounds with or without spacer. On MCF-7 cells, IC_{50} values of all compounds were between 1 and 5 μM. On HT-29 cells, GnRH-III(Dau=Aoa) was the most effective bioconjugate ($IC_{50} = 14.2 \pm 3.2$ μM) and GnRH-III(Dau=Aoa–GFLG) had higher cytostatic effect ($IC_{50} = 19.4 \pm 3.1$ μM) compared with GnRH-III(Dau–YRRL) ($IC_{50} = 28.6 \pm 5.5$ μM). Possible explanations of these results might be the higher number of GnRH receptors on MCF-7 cells (Mező et al. 2008) and/or the different intracellular route of the compounds in HT-29 cells compared to MCF-7 cells (Herédi-Szabó et al. 2006).

The IC_{50} values of the bioconjugates are one order of magnitude higher on both cell lines than those determined for the free daunorubicin. We have previously shown that daunorubicin was taken up in a much higher amount by cells than the bioconjugates (Szabó et al. 2009). The difference between the IC_{50} values of free Dau and

Dau–GnRH-III bioconjugates could be explained by their differential cellular uptake. Another explanation could be provided by the binding of various metabolites to DNA.

In the present work, we showed that the degradation of bioconjugates in the presence of cathepsin B or in lysosomal homogenate resulted in different metabolites (H–Lys(Dau=Aoa)–OH in case of **1**, Dau=Aoa–Gly–OH from **2**, and Dau=Aoa–Tyr–OH from **3**) that could influence the biological activity, such as the binding to DNA.

Synthesis of daunorubicin–amino acid derivatives

To investigate the binding to DNA of the smallest metabolites produced in lysosomal homogenate, H-Lys(Dau=Aoa)–OH, Dau=Aoa–Gly–OH and Dau=Aoa–Tyr–OH were synthesized (Fig. 5). Boc–Aoa–OPcp prepared in our laboratory (Mező et al. 2010) was used to functionalize the amino acids for further oxime ligation. During the coupling reactions, the carboxyl groups of the amino acids were protected as sodium salts. The coupling occured with good yield in case of glycine; however, the application of tyrosine without protecting the phenolic hydroxyl group resulted in side products. Therefore, the hydroxyl group was protected with *tert–butyl* group that could be removed simultaneously with the Boc protecting group of Aoa. For the synthesis of H–Lys(Aoa)–OH, Boc–Lys–OH was used. Considering that the Aoa-containing derivatives are highly sensitive to aldehydes and ketones (Buré et al. 2000), the compounds were used in the reaction with daunorubicin immediately after removing the *tert*-butyl-type protecting groups. Oxime bond-linked Dau-amino acid derivatives could be well separated from the excess of free Dau, except for Dau=Aoa–Tyr–OH. However, when free Dau was modified by hydroxylamine, the resulted Dau=N–OH could be removed from Dau=Aoa–Tyr–OH by RP-HPLC using methanol–water solvent mixture. It has to be mentioned that Dau=N–OH was not stable in the eluent system used for purification (0.1% TFA/methanol–water) and that the free Dau was released in time under acidic conditions and it could be recovered.

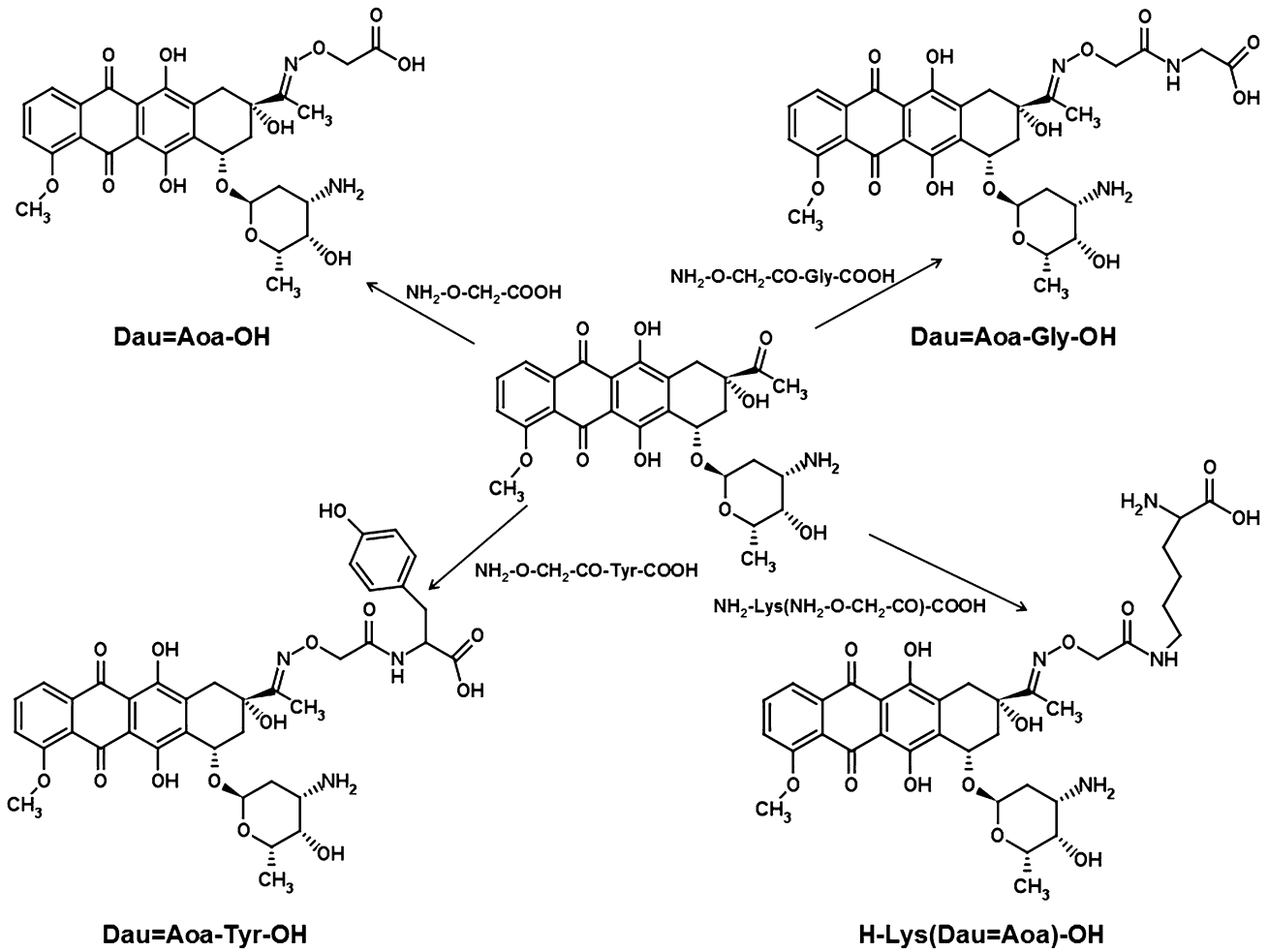

Fig. 5 Outline of the synthesis of the smallest daunorubicin containing metabolites

Determination of apparent binding constants of daunorubicin and its derivatives

The interaction of daunorubicin with DNA is thought to be a crucial step in the molecular mechanism by which it inhibits the DNA replication (Barcelo et al. 1988). The binding of daunorubicin to DNA is most appropriately described by the neighbor exclusion model of McGhee and Von Hippel (1974), taking into consideration the apparent non-linearity in binding isotherms. This model provides an analytical expression which may conveniently be used to extract the binding constant and exclusion parameter from the experimental data. We determined the apparent binding constants (K) of Dau and its derivatives and the data were presented in Table 4. It is known that the binding constant of daunorubicin is influenced by the ionic strength of the environment and the base composition of the nucleic acid (Chaires 1990).

It is important to note that in some cases, at low binding ratios, our experimental points deviated from the fit obtained by the non-cooperative model. A similar phenomenon was observed by Graves and Krugh (1983) using a phase partition method, when reported positive cooperativity at low binding ratios for the interaction of daunorubicin with calf thymus DNA. In the present work, we cannot answer this question; however, we are aiming the detailed investigation later.

A direct relationship between the affinity toward nucleic acids and biological activity of Dau was established (Valentini et al. 1985). In other instances, there were many deviations and a direct simple correlation between binding parameters and biological activity could not be obtained (Krohn 2008).

The K of free Dau determined here ($K = 11.7 \times 10^5\ M^{-1}$) is in good agreement with the K values previously obtained (Barcelo et al. 1988) under similar experimental conditions ($K = 9.0 \times 10^5\ M^{-1}$ and $K = 25.0 \times 10^5\ M^{-1}$ in 100 and 50 mM NaCl solutions, respectively). Free Dau showed the highest binding to DNA. GnRH-III (Dau=Aoa) could not bind very well to DNA ($K = 0.83 \times 10^5\ M^{-1}$), result which could be explained by the size and conformation of the bioconjugate. According to the mass spectrometric analysis, various metabolites are formed in the lysosomal homogenate and these metabolites should bind to the DNA and not necessarily the whole bioconjugate. Our data show that all metabolites could bind to DNA better than the bioconjugate and there was no significant difference between the DNA-binding properties of H-Lys(Dau=Aoa)–OH ($K = 6.80 \times 10^5\ M^{-1}$) and Dau=Aoa–Gly–OH ($K = 6.74 \times 10^5\ M^{-1}$). The DNA-binding properties of the two metabolites might explain the similar in vitro cytostatic effect of the parent bioconjugates. Interestingly, the Dau=Aoa–OH which was not found as a metabolite in our experiment showed much lower binding to DNA ($K = 2.21 \times 10^5\ M^{-1}$). These results suggest that the distance between Dau and a free carboxyl group might be important for an effective binding to DNA.

Table 4 Apparent equilibrium binding constants (K) and the exclusion parameters (n) of daunorubicin and its derivatives on chicken erythrocyte DNA

Compound	K ($\times 10^5$) (M^{-1})	n
Dau	11.7	2.5
H–Lys(Dau=Aoa)–OH	6.80	3
Dau=Aoa–Gly–OH	6.74	3.5
Dau=Aoa–OH	2.21	3.8
Dau=Aoa–Tyr–OH	4.40	3.5
GnRH-III(Dau=Aoa)	0.83	4.2

Cellular uptake and in vitro cytostatic effect of daunorubicin, daunorubicin–amino acid derivatives and GnRH-III(Dau=Aoa) bioconjugate on MCF-7 cells

We were also interested in determining the in vitro cytostatic effect of the Dau–amino acid derivatives. However, the IC_{50} values determined by MTT assay would be relevant only in case of a similar (rather identical) uptake of the compounds by the cells. Therefore, the cellular uptake of daunorubicin, daunorubicin–amino acid derivatives and GnRH-III(Dau=Aoa) bioconjugate by MCF-7 cells was investigated by flow cytometry and expressed as the percent of Dau-positive cells. In the Electronic Supplementary Material, S14–S16, the in vitro cytostatic effect and cellular uptake of the compounds are presented. When considering that the cellular uptake of Dau-amino acid derivatives by MCF-7 cells is different, the IC_{50} values determined by MTT assay cannot be compared.

NMR structure analysis

In our previous work, the E-oxime isomer in a simple model compound, Dau=Aoa–OH, was determined by NMR spectroscopy (Szabó et al. 2009). However, the influence of the peptide chain on the structure has not been investigated yet. Therefore, in the present study, the GnRH-III (Dau=Aoa) bioconjugate was subjected to NMR spectroscopic analysis.

A nearly complete assignment was achieved by combining 2D homonuclear (COSY, TOCSY and NOESY) and heteronuclear (^{13}C-^{1}H-HSQC, ^{13}C-^{1}H-HMBC and ^{15}N-^{1}H-HSQC) experiments (Fig. 6b–d; chemical shifts are shown in Table 2 in the Electronic Supplementary Material, S17). All NH, CH, CH2, and CH3 groups were assigned.

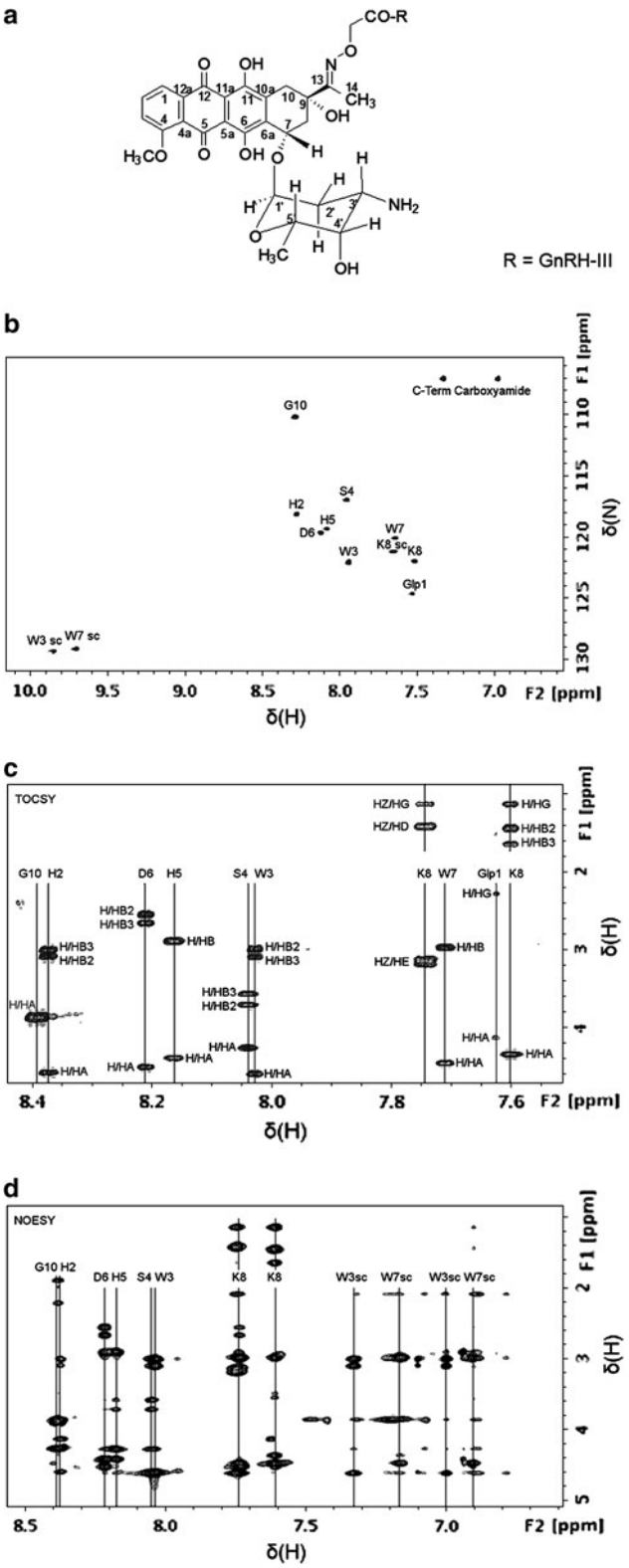

Fig. 6 NMR structure analysis of GnRH-III(Dau=Aoa) bioconjugate. ▶ **a** Numbering of atoms used for NMR assignments (arbitrary), **b** assigned ^{15}N-^{1}H-HSQC spectrum of GnRH-III(Dau=Aoa) bioconjugate. The side chain amide proton of Lys^{8} (K^{8} sc) is stabilized by the isopeptide bond with the daunorubicin–aminooxyacetyl group, **c** assigned TOCSY spectrum of GnRH-III(Dau=Aoa) bioconjugate in the region of backbone amides, **d** intense NOE signals between the amide and aromatic protons (direct dimension) and aliphatic protons (indirect dimension) imply a non-covalent oligomerization of the bioconjugate

Diastereotopic groups were not assigned stereospecifically. Some quaternary carbons of the anthracycline scaffold could not be unambiguously assigned from the HMBC spectra. Their chemical shifts were obtained by comparison with the published data of daunorubicin (Kim et al. 2000).

The *E*-oxime isomer was assigned by the upfield-shifted carbon signal of C-14 (12.86 ppm). The signal would be expected at around 16–17 ppm in a *Z*-oxime isomer (Parthiban et al. 2008). Interestingly, the bioconjugate exhibited quite intense NOEs (Fig. 6d), implying a noncovalent oligomerization under the employed experimental conditions. Thus, the identified NOEs could correspond to both intramolecular and intermolecular proximities of protons.

The vicinal H^{N}–H^{α}-couplings of the peptide backbone range between 4.3 and 9.4 Hz (Table 2 in the Electronic Supplementary Material). A number of these couplings indicate conformational averaging and suggest, therefore, that the bioconjugate does not adopt a rigid structure; however, three H^{N}–H^{α}-couplings $\geq$8 Hz indicate a preference for an extended, β-sheet-like structure of the peptide.

Conclusions

In our study, gonadotropin-releasing hormone-III was employed as a targeting moiety to which daunorubicin was attached via oxime bond, either directly or by insertion of a GFLG or YRRL tetrapeptide spacer, leading to the formation of drug delivery systems for targeted cancer chemotherapy.

Two of the investigated bioconjugates, GnRH-III (Dau=Aoa) and GnRH-III(Dau=Aoa–GFLG), were found to have increased stability in human serum (at least for 24 h), whereas the bioconjugate containing the YRRL tetrapeptide spacer was degraded within 3 h. In the presence of both cathepsin B and lysosomal homogenate, all bioconjugates were decomposed; however, no free daunorubicin was released. Using LC–MS, different metabolites were identified in which the aminooxy derivative of Dau was connected to the α-amino group of Gly or Tyr or to the ε-amino group of Lys. All three bioconjugates had in vitro antitumor effect on MCF-7 human breast and HT-29 human colon cancer cell lines. However, their antitumor activity was lower than that of free Dau, results that could be explained by the lower cellular uptake of the bioconjugates, the lack of free drug release and by different DNA-binding properties of the resulted metabolites.

When considering all these data, we conclude that (1) the C-13 keto group of daunorubicin can be used for the conjugation with various targeting moieties without leading to the loss of the antitumor effect and (2) the incorporation of a peptide spacer between Dau and GnRH-III is not required for the antitumor activity of the bioconjugates.

Based on the results obtained in this study, the in vivo antitumor activity of only GnRH-III(Dau=Aoa) and GnRH-III(Dau=Aoa–GFLG) bioconjugates is currently evaluated on colon carcinoma bearing mice.

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