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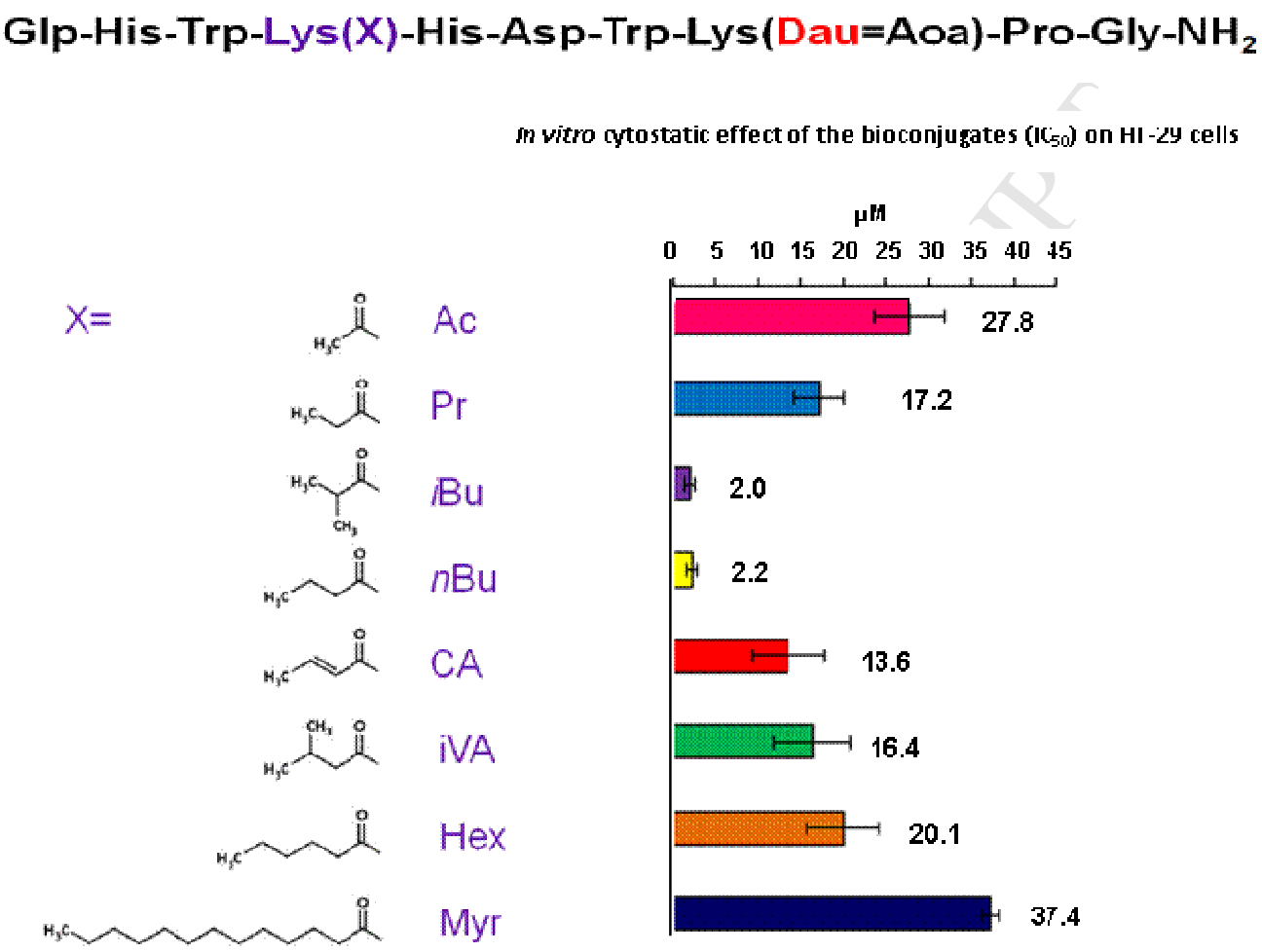
Graphical Abstract



Enhanced cellular uptake and *in vitro* antitumor activity of short-chain fatty acid acylated daunorubicin-GnRH-III bioconjugates

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ABSTRACT

Here we report on the synthesis and biochemical characterization (enzymatic stability, cellular uptake, *in vitro* antitumor activity, membrane interaction and GnRH-receptor binding affinity) of novel short-chain fatty acid (SCFA) acylated daunorubicin-GnRH-III bioconjugates, which may serve as drug delivery systems for targeted cancer chemotherapy. Ser in position 4 of GnRH-III was replaced by Lys, followed by the acylation of its ε-amino group with various fatty acids. SCFAs are potentially chemoprotective agents by suppressing the growth of cancer cells and therefore may enhance the antitumor activity of the bioconjugates. We found that all synthesized bioconjugates had high cytostatic effect *in vitro*, were stable in cell culture medium for 6 h and degraded in the presence of rat liver lysosomal homogenate leading to the formation of an oxime bond-linked daunorubicin-Lys as the smallest active metabolite. In the presence of α-chymotrypsin, all compounds were digested, the degradation rate strongly depending on the type of fatty acid. The bioconjugate containing Lys(nBu) in position 4 was taken up most efficiently by the cancer cells and exerted higher *in vitro* cytostatic effect than the previously developed GnRH-III(^{4}Lys(Ac), ^{8}Lys(Dau=Aoa)) or the parent GnRH-III(Dau=Aoa) bioconjugate. Our results could be explained by the increased binding affinity of the newly developed compound containing Lys(nBu) to the GnRH receptors.

Keywords: targeted cancer chemotherapy, daunorubicin, gonadotropin-releasing hormone, short-chain fatty acids, antitumor activity, receptor binding affinity

1. Introduction

Cancer is one of the leading causes of death worldwide. Besides lung, stomach, liver and breast cancer, colorectal cancer is still a major health problem and one of the most

common causes of cancer death in the developed countries (WHO) [1]. It has been shown that a fiber and complex carbohydrate rich diet could be preventive for colon cancer [2]. Short-chain fatty acids (SCFAs) are produced by anaerob bacterial fermentation of dietary fiber and resistant starch within the large colon. Depending on the source, the major products are acetate, propionate, butyrate, valerate, hexanoate and branched SCFAs (isobutyrate and isovalerate). These SCFAs, especially butyrate, are known for their potential to act as chemopreventive agents by slowing cell growth and activating apoptosis in colon cancer cells [3]. It is currently accepted that the effects of butyrate are mainly caused by the regulation of the expression of specific genes through its histone deacetylase inhibitor activity [4]. It regulates proliferation of mucosal epithelial cells and it can also induce apoptosis in various human cancer cell lines (HT-29, HL-60, etc) by increasing the level of pro-apoptotic protein bak and/or decreasing the level of the anti-apoptotic protein p26-bcl-2 [2]. Butyrate may contribute to the detoxification of dietary carcinogens by inducing glutathione S-transferases (GSTs) in cancer cell lines. Although GST activity was induced only by butyrate, prevention of chemoresistance was caused by SCFA mixtures containing acetate, propionate, butyrate, valerate, hexanoate and the branched SCFAs (isobutyrate and isovalerate) [5]. Therefore, SCFAs may be an important component of drug delivery systems for targeted cancer chemotherapy.

Targeted chemotherapy is a modern oncological approach designed to increase the selectivity of cytotoxic drugs and consequently to prevent their toxic side effects. Considering that gonadotropin-releasing hormone (GnRH) receptors are highly expressed on various cancer cells, GnRH analogs can be used as targeting moieties for the attachment of anticancer drugs. Apart from pituitary cells and reproductive organs, other organs express GnRH receptors only in a limited number or not at all, making them a selective target for cancer chemotherapy [6, 7].

Because of the unfavorable endocrine effect of the hormone molecules in targeted cancer therapy, in particular for the treatment of hormone independent tumors, research is focused on GnRH derivatives with decreased hormonal activity. The third isoform of GnRH (GnRH-III: Glp-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2), isolated from the sea lamprey (*Petromyzon marinus*), exerts negligible endocrine activity in mammals [8], inhibits the proliferation of various cancer cells [9, 10] and binds to both type I and type II GnRH receptors [11]. To decrease the endocrine activity and increase the antitumor effect of GnRH-III, various analogs have been developed [9, 12]. It was found that not many changes in the sequence were allowed. In positions 5-7, single amino acid replacement was well tolerated for the endocrine, but not for the anticancer activity. Furthermore, the replacement of Ser by Lys or Lys(Ac) in position 4 did not have a major effect either on the endocrine activity or on the anticancer property of the peptides. In position 8, the elimination of the basic side chain function of lysine did not influence the anticancer activity, but it led to a decreased hormonal potency [9]. Therefore, in our previous studies, the side chain of ^{8}Lys was used to form symmetrical GnRH-III dimers and peptide-drug conjugates [10, 13]. Besides its anticancer activity and low hormonal effect, an ideal peptide based drug candidate should be stable in human serum and in the presence of digestive enzymes, especially if it is intended to be orally administered. Oral administration of drugs is considered to be the most convenient and economical route of treatment. It offers the advantage of self-administration with a high degree of patient acceptability and compliance [14].

We have recently reported on the synthesis and biochemical characterization of a daunorubicin-GnRH-III bioconjugate in which ^{4}Ser was replaced by Lys(Ac). This compound, GnRH-III(^{4}Lys(Ac), ^{8}Lys(Dau=Aoa)), was more stable than the parent bioconjugate GnRH-III(^{8}Lys(Dau=Aoa)). It was stable in the presence of pepsin and trypsin and its degradation by α-chymotrypsin was slower. Its *in vivo* tumor growth inhibitory effect

was evaluated on orthotopically developed C26 murine colon carcinoma bearing mice. The results showed that the bioconjugate had significantly higher antitumor activity than the parent bioconjugate [15]. Furthermore, the concentration dependent cellular uptake of GnRH-III(^{4}Lys(Ac), ^{8}Lys(Dau=Aoa)) on MCF-7 cells was significantly higher compared to the non-acetylated version [15]. Taken together these previous findings and also considering the effect of SCFAs on tumor growth inhibition, it is assumed that SCFAs on the side chain of ^{4}Lys in GnRH-III(^{4}Lys(X), ^{8}Lys(Dau=Aoa)) may increase not only the cellular uptake and enzymatic stability, but also the antitumor activity of the bioconjugates. Thus, in the present study we aimed at developing new derivatives containing SCFAs for targeted drug delivery.

Here we report on the synthesis and biochemical characterization (enzymatic stability, cellular uptake, *in vitro* antitumor activity, membrane interaction and GnRH-receptor binding affinity) of novel daunorubicin-GnRH-III bioconjugates in which Ser in position 4 was replaced by Lys, followed by the acylation of its ε-amino group with different saturated and unsaturated short-chain fatty acid chains of different length. The stability of the bioconjugates in cell culture medium was determined by high performance liquid chromatography. The degradation of the bioconjugates in the presence of rat liver lysosomal homogenate and their stability in the presence of the digestive enzyme α-chymotrypsin were investigated by liquid chromatography-mass spectrometry. Their *in vitro* cytostatic effect and the cellular uptake were evaluated on HT-29 human colon and MCF-7 human breast cancer cell lines. Radioligand binding studies were performed on normal human pituitary sample (anterior lobe) and prostate cancer specimen.

2. Results and Discussion

2.1. Short-chain fatty acid acylated daunorubicin-GnRH-III derivative bioconjugates

One of the most promising approaches in modern oncology is the targeted cancer chemotherapy, which is based on the features of cancer cells that differ from the normal cells. In this way, only the cancer cells are affected, resulting in fewer side effects. The essential advantage of this procedure is the improved selectivity and not necessarily the enhanced efficiency. It was found that receptors for various peptide hormones such as GnRH, somatostatin and bombesin are highly expressed on cancer cells; therefore, selectivity could be achieved by applying these peptide hormones as targeting moieties. One of the advantages of the GnRH analogs is that they exert an antitumor effect themselves. Therefore, GnRH derivatives as components of drug delivery systems may serve not only as targeting moieties, but also as antitumor agents. A promising GnRH containing bioconjugate, developed in A. V. Schally's laboratory (AN-152) [16], in which doxorubicin is attached to [D-^{6}Lys]-GnRH-I, is currently in Phase-II clinical trial on ovarian and endometrial cancer [17, 18].

We have previously reported on a daunorubicin-GnRH-III bioconjugate in which ^{4}Ser was replaced by Lys(Ac) and daunorubicin was attached to ^{8}Lys through an oxime bond [15]. This compound, GnRH-III(^{4}Lys(Ac), ^{8}Lys(Dau=Aoa)), had increased enzymatic stability, cellular uptake, *in vitro* cytostatic effect and *in vivo* tumor growth inhibitory activity, compared to the parent bioconjugate GnRH-III(^{8}Lys(Dau=Aoa)).

The positive effect of the incorporation of ^{4}Lys(Ac) instead of ^{4}Ser as well as the apoptosis inducing activity of SCFAs [19] prompted us to develop new GnRH-III(^{4}Lys(X), ^{8}Lys(Dau=Aoa)) bioconjugates containing SCFAs on the side chain of ^{4}Lys. This modification may also enhance their ability to be taken up by the cells by changing their lipophilicity or structure. An unsaturated SCFA, crotonic acid (crotonyl-coenzyme A is an intermediate in the fermentation of butyric acid) and a long chain fatty acid, myristic acid, were also used as acylating agents. Myristic acid can bind to human serum albumin resulting in significant accumulation of the bioconjugate in the surrounding of the tumor [20].

All peptides were synthesized by solid phase methodology using Fmoc-chemistry. Lys(ivDde) was incorporated in position 4 and Lys(Mtt) in position 8 as an orthogonal protecting scheme for lysine residues. The cleavage of the ivDde protecting group and the acylation of ^{4}Lys with different short-chain fatty acids were carried out prior to the removal of Mtt under mild acidic conditions, followed by the attachment of Boc-Aoa-OH. All compounds were cleaved from the resin with TFA in the presence of appropriate scavengers. Daunorubicin was conjugated to the aminooxyacetylated GnRH-III(^{4}Lys(X)) derivatives by oxime ligation, which was carried out under slightly acidic conditions (pH 5.0). The structures of the synthesized compounds are schematically represented in Fig. 1.

All bioconjugates were purified by semipreparative RP-HPLC and the purified compounds were characterized by analytical RP-HPLC and mass spectrometry (Table 1 and Supplementary material Fig.s S1 – S7). As previously reported [21, 22], the fragmentation of the glycosidic bond during the ESI-mass spectrometric analysis could be observed, resulting in the loss of daunosamine (-129, -147). These fragments were assigned in all mass spectra by an asterisk.

2.2. Enzymatic stability/degradation of bioconjugates

Determination of the stability or the metabolic properties of a new drug candidate is one of the most important steps during the drug discovery and development process. *In vitro* approaches, such as stability/degradation studies in the presence of gastrointestinal or lysosomal enzymes, can be used for the early estimation and prediction of the *in vivo* stability and metabolism [23]. The aim of the targeted cancer chemotherapy is to specifically deliver the chemotherapeutic agents to the cancer cells, in order to prevent the side effects. Therefore, the linkage between the anticancer drug and the targeting moiety in the bioconjugates must have high stability in circulation or gastrointestinal tract, to avoid the toxic side effects caused

by an early drug release before reaching the site of action. Nevertheless, the intracellular drug release from the bioconjugates is required for their antitumor activity [24]. In the case of the reported bioconjugates, an oxime bond was formed between the C13 keto group of daunorubicin and the aminooxyacetyl group of the GnRH-III derivatives. The oxime bond is chemically stable between pH 3 and 8 [25], as well as under *in vitro* and *in vivo* biological experimental conditions [26]. Moreover, the daunorubicin-GnRH-III bioconjugates must possess increased stability in the presence of digestive enzymes present in stomach and intestine in order to be suitable for oral administration. In our previous work, we reported that the bioconjugate GnRH-III(^{4}Lys(Ac),^{8}Lys(Dau=Aoa)) was stable in human serum and in the presence of pepsin and trypsin even after 24 h of incubation [15, 22]. In the presence of α-chymotrypsin, the -^{3}Trp-^{4}Lys(Ac)- peptide bond was cleaved in the bioconjugate; however, the degradation rate was slower than in the case of -^{3}Trp-^{4}Ser- in the parent bioconjugate. In the present study, the stability/degradation of the bioconjugates was evaluated in the presence of rat liver lysosomal homogenate, in cell culture medium and in the presence of α-chymotrypsin.

The stability of GnRH-III(^{4}Lys(X),^{8}Lys(Dau=Aoa)) bioconjugates in cell culture medium was monitored by analytical RP-HPLC. All bioconjugates were stable at 37°C for 6 h (under the same conditions and time period used for the treatment of cells *in vitro*), since only the intact bioconjugates could be detected in the HPLC chromatograms (supplementary material, Fig. S8).

In the presence of α-chymotrypsin, similarly to our previous results, the -^{3}Trp-^{4}Lys(X)-peptide bond was cleaved in the bioconjugates **1**-**6**. However, in case of GnRH-III(^{4}Lys(Myr),^{8}Lys(Dau=Aoa)) (**7**), because of its low solubility, only peaks of very low intensity could be observed in the LC profiles and only the intact bioconjugate could be identified (supplementary material, Fig.s S9G and S10M). The rate of chymotryptic

degradation was dependent on the nature of the fatty acid chain in the compounds, as shown in the UV chromatograms depicted in Fig. S9.

We observed a correlation between the length of the fatty acid chain and the rate of degradation, except for the GnRH-III(^{4}Lys(iBu), ^{8}Lys(Dau=Aoa)), which in comparison to the other bioconjugates was much faster degraded by chymotrypsin. After 4 h, approximately 60% of the GnRH-III(^{4}Lys(iBu), ^{8}Lys(Dau=Aoa)) was degraded and the degradation was almost complete after 6 h (90% of the compound was cleaved). After 24 h, no intact compound could be detected. Mass spectrometric analysis of the reaction mixtures led to the identification of -^{3}Trp-^{4}Lys(iBu)- cleavage site (the fragment H-K(iBu)HDWK(Dau=Aoa)PG-NH_2 was detected at *m/z* 506.8 (3+) and 759.7 (2+) - supplementary material, Fig.s S9B, S10C and S10D).

The highest degradation rate among the other bioconjugates was observed in case of the GnRH-III(^{4}Lys(Pr), ^{8}Lys(Dau=Aoa)). After 6 h, 45% of the compound was degraded and after 24 h no intact bioconjugate could be detected. The -^{3}Trp-^{4}Lys(Pr)- cleavage site was identified by mass spectrometry (H-K(Pr)HDWK(Dau=Aoa)PG-NH_2 fragment detected at *m/z* 752.9 (2+) and 502.3 (3+) – supplementary material, Fig.s S10A and S10B). By increasing the chain length of the fatty acid, the stability of the bioconjugates against chymotryptic digestion increased. Only 36% of the GnRH-III(^{4}Lys(nBu), ^{8}Lys(Dau=Aoa)) bioconjugate was cleaved after 6 h; however, after 24 h the compound was completely degraded. The -^{3}Trp-^{4}Lys(nBu)- cleavage site was identified by mass spectrometry (H-K(nBu)HDWK(Dau=Aoa)PG-NH_2 fragment detected at *m/z* 759.9 (2+) and 506.9 (3+) – supplementary material, Fig.s S9C, S10E and S10F). The bioconjugate **4** containing an unsaturated crotonic acid on the ^{4}Lys proved to be less stable than the compound **3** modified by the linear butyric acid (having also four carbon atoms in the chain). After 6 h, 50% of the compound GnRH-III(^{4}Lys(CA), ^{8}Lys(Dau=Aoa)) was degraded and after 24 h the

degradation was complete, as only the H-K(CA)HDWK(Dau=Aoa)PG-NH_2 fragment was detected at *m/z* 758.8 (2+) and 506.2 (3+) (supplementary material, Fig.s S10G and S10H). The bioconjugate **5**, acylated with an isovaleric acid (5-carbon atoms) on ^{4}Lys was more stable than the bioconjugates **1-4**; only 34% degradation was achieved after 6 h and 50% after 8 h incubation time. After 24 h, no intact bioconjugate could be detected; only H-K(iVA)HDWK(Dau=Aoa)PG-NH_2 fragment was identified by mass spectrometry at *m/z* 767.0 (2+) and 511.6 (3+) (supplementary material, Fig.s S9, S10I and S10J). The most stable bioconjugate was compound **6**. After 6 h, only 30% of the GnRH-III(^{4}Lys(Hex), ^{8}Lys(Dau=Aoa)) bioconjugate was degraded and even after 24 h, 10% of the intact compound was detected. H-K(Hex)HDWK(Dau=Aoa)PG-NH_2 fragment was identified by mass spectrometry at *m/z* 774.1 (2+) and 516.4 (3+) (supplementary material, Table S1, Fig.s S9, S10K and S10L).

Comparing the results obtained in the present study (e.g., GnRH-III(^{4}Lys(nBu), ^{8}Lys(Dau=Aoa)) - 64% intact bioconjugate after 6 h) with the previous ones (e.g., GnRH-III(^{4}Lys(Ac), ^{8}Lys(Dau=Aoa)) - 33% intact bioconjugate after 6 hours) [22], one can conclude that the acylation of ^{4}Lys with short-chain fatty acids led to increased stability of the bioconjugates in the presence of the digestive enzyme chymotrypsin; therefore, GnRH-III(^{4}Lys(X), ^{8}Lys(Dau=Aoa) might be good candidates for oral administration. In contrast, the iBu containing bioconjugate proved to be the most susceptible one (only 10% of intact compound was detected in the reaction mixture after 6 h).

The degradation of the bioconjugates in the presence of rat liver lysosomal homogenate was also investigated by LC-MS. As shown in Fig. 1 and Table S2, all seven bioconjugates were digested in the presence of lysosomal enzymes resulting in various peptide fragments; however, in case of the bioconjugate containing myristic acid, a very low amount of detectable metabolites was generated due to its low solubility. In agreement with

our previous results, no free daunorubicin was detected by LC-MS. In all cases, the smallest drug containing fragment was H-Lys(Dau=Aoa)-OH (detected in the mass spectra at *m/z* 729.5 (1+)). We have previously shown that this fragment was able to bind to DNA [22]. The mass spectra recorded after 2 and 24 h of incubation of the bioconjugates **1** – **7** with the lysosomal homogenate are shown in the Supplementary material, Table S1 and Fig.s S11-S17. Interestingly, a fragment was detected by mass spectrometry at *m/z* 581.2 (1+), which might be assigned to <EHWK-OH peptide, indicating the cleavage of the amide bond between the side chain of ^{4}Lys and the fatty acids [10, 27]. However, the unambiguous identification of this fragment and of the free SCFA release requires further investigations.

2.3. Secondary structure determination by circular dichroism spectroscopy

To investigate the effects of the ^{4}Lys-acylation with different short-chain fatty acids on the secondary structure of the bioconjugates, circular dichroism (CD) spectra were recorded in water and in trifluoroethanol. All CD spectra of aqueous solutions of bioconjugates were characterized by a strong negative band around 200 nm and a positive band at 230 nm, with a shoulder at 250 nm, indicating a predominantly unordered conformation. A negative band around 290 nm, most probably caused by interactions between aromatic residues (Trp and daunorubicin), was also present in all spectra [28]. The CD spectra recorded in trifluoroethanol were characterized by an intensive negative band around 200 nm with a shoulder at around 210 nm, a positive band at 230 nm and another one at 250 nm, also indicating a predominantly unordered structure. A negative band around 290 nm, most probably caused by interactions between aromatic residues (Trp and daunorubicin), was also present in all spectra. The spectra of bioconjugates **1-7** were compared to the spectra of the unmodified GnRH-III, GnRH-III(^{8}Lys(Dau=Aoa)) and GnRH-III(^{4}Lys(Ac),^{8}Lys(Dau=Aoa)).

The data indicated that the secondary structure of the bioconjugates was not influenced by the type of the fatty acids (supplementary material, Fig.s S18 and S19).

2.4. Cellular uptake of the bioconjugates on MCF-7 human breast and HT-29 human colon cancer cells

Cellular uptake of the bioconjugates was investigated by flow cytometry on HT-29 human colon and MCF-7 human breast cancer cells. Different concentrations between 0.16 and 100 µM were applied. Samples at each concentration were measured in duplicates and each experiment was repeated twice. The cellular uptake of free daunorubicin and Dau containing bioconjugates was determined only in living cells. The bioconjugates were taken up by both cell types in a concentration dependent manner as shown in Fig. 2. In both cases, the cellular uptake could already be observed in the lower concentration range (4 µM). The bioconjugates containing isobutyrate or *n*-butyrate were taken up the most effectively. At 20 µM concentration, 96.9% (compound **2**) and 98.5% (compound **3**) of the living HT-29 cells were daunorubicin positive (Dau+ living cells); at 100 µM concentration 99.9% and 99.9%, respectively (Fig. 2A). In case of MCF-7 cells, 87.7% (compound **3**) and 93.3% (compound **2**) of the living cells were Dau+ at 20 µM concentration, while at 100 µM concentration 99.7% and 99.7% of the living cells were Dau+ (Fig. 2B). The bioconjugate containing myristic acid was taken up the most significantly by MCF-7 cells in the lower concentration range. At 4 µM, 41.3% of the living cells were Dau+. At 20 µM, it was taken up considerably (Dau+ living cells: 85.1%) compared to the other compounds, probably due to the longer fatty acid chain that might lead to altered receptor or membrane binding. These results were compared with the previously reported ones. On HT-29, GnRH-III(^{4}Lys(Ac), ^{8}Lys(Dau=Aoa)) was taken up less effectively than the bioconjugates **1**, **2**, **3**, **5**, **6**, **7**; only 5.3% of the living cells were Dau+ at 20 µM concentration and 74.2% at 100 µM

concentration. On MCF-7 cells, the cellular uptake of GnRH-III(^{4}Lys(Ac), ^{8}Lys(Dau=Aoa)) was higher than the uptake of bioconjugates **1** and **6**, but significantly lower than the other bioconjugates, both at 20 µM (Dau+ living cells: 20.1%) and at 100 µM (Dau+ living cells: 94.5%) concentration, respectively [15]. It has to be mentioned that [D-Lys^{6}(Dau=Aoa)]-GnRH-I showed similar cellular uptake as the GnRH-III bioconjugates containing the butyrate isomers (Fig.s 2C and 2D). These data suggest that not only the length of the SCFAs has an influence on the cellular uptake.

2.5. Cytostatic effect of the bioconjugates

The *in vitro* cytostatic effect 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 (MTT) assay. The IC_{50} values were determined from the dose-response curves (Table 2). The replacement of ^{4}Ser by the short-chain fatty acid acylated ^{4}Lys led to an increased cytostatic effect compared to the parent compound and the GnRH-III(^{4}Lys(Ac), ^{8}Lys(Dau=Aoa)) on both tested human cancer cell lines, whereas myristoylation of ^{4}Lys resulted in a decreased cytostatic effect. This result might be explained by its low solubility in cell culture medium. The majority of the bioconjugates had higher cytostatic effect on MCF-7 than on HT-29 cells, which is in agreement with our previously reported data [22] showing that daunorubicin containing GnRH-III bioconjugates had lower antitumor activity on HT-29 human colon cancer cell line [12]. In case of GnRH-III(^{4}Lys(iBu), ^{8}Lys(Dau=Aoa)) and GnRH-III(^{4}Lys(nBu), ^{8}Lys(Dau=Aoa)), the IC_{50} values on HT-29 and MCF-7 cells were comparable, probably due to the fact that the *n*-butyrate and isobutyrate can induce apoptosis and inhibit the proliferation of HT-29 cells [19]. On MCF-7 cells, the cytostatic effect of the bioconjugates containing *n*-butyrate or isobutyrate was slightly, but not significantly higher than of other compounds, although the cellular uptake was much higher

or comparable (bioconjugate **7**). On HT-29 cells, a significant improvement was achieved in the *in vitro* antiproliferative effect of the bioconjugates **2** and **3**; moreover, they were taken up by the cells in a significantly higher amount. This result might be explained by the antitumorigenic effects of butyrate on colon cancer cells or different intracellular routes or degradation pathways in the cells. Compared to our previous results, the modification of GnRH-III with short-chain fatty acids resulted in an enhanced cellular uptake.

Considering all these data, we can conclude that the replacement of ^{4}Ser by short-chain fatty acid acylated Lys in the GnRH-III sequence led to an increased cytostatic effect of daunorubicin-GnRH-III bioconjugates, in particular in case of butyrate and isobutyrate containing bioconjugates. The cellular uptake and the *in vitro* cytostatic effect of the newly developed bioconjugates were significantly increased compared to the compounds previously synthesized and characterized in our laboratory. We were further interested in the interaction between the bioconjugates and the cell membrane, as well as in the binding affinity of the bioconjugates to the GnRH receptors.

2.6. Characterization of the lipophilicity of the GnRH-III(^{4}Lys(X), ^{8}Lys(Dau=Aoa)) bioconjugates

To reach the targeted cancer cells, drugs have to be transported through several membranes. Therefore, the hydrophobicity of the compound, which affects its absorption, bioavailability, metabolism or its interaction with lipid membranes, is in the focus of rational drug design. In the present work, using the classical shake-flask method and HPLC detection [29], the octanol-water partition coefficient and logP of the compounds were determined.

LogP was calculated using the following equation:

Partition Coefficient, P = [octanol] / [water], where [] = area under the curve determined by RP-HPLC; $\log P = \log_{10}$ (Partition Coefficient)

During RP-HPLC analysis, a peak was detected from the organic phase only in case of the bioconjugate GnRH-III(^{4}Lys(Myr), ^{8}Lys(Dau=Aoa)). The calculated logP value was -0.596, indicating that the fatty acids did not significantly influence the lipophilicity of the bioconjugates and all of them were rather hydrophilic.

2.7. Penetration of the GnRH-III(^{4}Lys(X), ^{8}Lys(Dau=Aoa)) into a DPPC monolayer

Lipid Langmuir monolayers represent a membrane model suitable for the characterization of the molecular interactions between the bioconjugates and membrane forming lipids [30]. The membrane affinity of the bioconjugates or their compatibility with the lipid molecules is determined from the obtained quantitative information about the influence of the bioconjugates on the stability, structure and permeability of the lipid film.

In the penetration experiments, the lipid monolayer was compressed in the Langmuir balance to reach a surface pressure of 15 or 25 mN/m and the barrier was stopped. These surface pressures were chosen considering the compactness of the cell membrane close to these values [31]. A solution of the bioconjugates was injected into the subphase and the change of surface pressure was recorded for an hour. The results are shown in Table 3.

All bioconjugates penetrated either the less or the more dense monolayer, exhibiting affinity to the lipid. As expected, the degree of penetration into the denser lipid layer (25 mN/m) was smaller than the penetration into the less dense monolayer (15 mN/m).

The bioconjugates containing no fatty acid or having a chain of 1, 2, 3, 4 or 5 carbon atoms penetrated the monolayer at a quite similar rate (Table 3). Slight increase could be observed in the degree of penetration of the bioconjugate **6** containing caproic acid ($\Delta\pi$: 7.4 at 15 mN/m; $\Delta\pi$: 2.7 at 25 mN/m) and significant increase was observed in case of the bioconjugate **7** having myristic acid ($C_{14}H_{28}O_2$) on ^{4}Lys ($\Delta\pi$: 17.4 at 15 mN/m; $\Delta\pi$: 11.5 at 25 mN/m).

From the penetration experiments, we can conclude that all bioconjugates showed affinity to the lipid monolayer; increasing the chain length of the fatty acid in the bioconjugates enhanced the penetration ability significantly only in case of the bioconjugate with myristic acid. Therefore, we may conclude that the cellular uptake takes place on a receptor-mediated way and might be influenced by the receptor binding affinity.

2.8. Binding of daunorubicin-GnRH derivative bioconjugates to GnRH receptors

In order to determine the potency of GnRH-III(^{4}Lys(nBu), ^{8}Lys(Dau=Aoa)) to displace radiolabeled [D-Trp^{6}]GnRH-I from GnRH receptors on human pituitary and human prostate cancer specimens, heterologous displacement experiments were performed. The receptor binding affinity of GnRH-III(^{4}Lys(nBu), ^{8}Lys(Dau=Aoa)) was compared with the affinity of [D-Lys^{6}(Dau=Aoa)]-GnRH-I, GnRH-III(Dau=Aoa) and GnRH-III(^{4}Lys(Ac), ^{8}Lys(Dau=Aoa)), respectively. The [D-Lys^{6}(Dau=Aoa)]-GnRH-I bioconjugate was used as a reference compound. Similarly to the GnRH-III based bioconjugates, it contains daunorubicin attached via an oxime bond to the targeting moiety, which is the widely used strong GnRH-I agonist, namely [D-Lys^{6}]-GnRH-I. D-Lys in position 6 was employed as a conjugation site. The results summarized in Table 4 indicate that the binding affinity of all bioconjugates was higher on human prostate cancer than on human pituitary tissue. The concentrations required to inhibit 50% of the specific [^{125}I][D-Trp^{6}]-GnRH-I binding (IC_{50}) were the lowest (1.62 nM on pituitary and 0.89 nM on prostate cancer, respectively) in case of the [D-Lys^{6}(Dau=Aoa)]-GnRH-I. The GnRH-III(Dau=Aoa) showed one order of magnitude lower binding affinities (22.87 nM and 16.51 nM, respectively). The replacement of Ser in position 4 by Lys(Ac) resulted in slightly elevated binding affinities (18.86 nM and 15.73 nM). However, the incorporation of *n*-butyrate instead of acetate to the side chain of ^{4}Lys led to a significant improvement in binding affinities (9.03 nM and 7.41 nM, respectively). Although the

bioconjugate [D-Lys^{6}(Dau=Aoa)]-GnRH-I had higher binding affinity than the GnRH-III(^{4}Lys(nBu), ^{8}Lys(Dau=Aoa)), its antiproliferative effect was not better than that of GnRH-III(^{4}Lys(nBu), ^{8}Lys(Dau=Aoa)), probably due to different intracellular processing (Table 2). Taken together, these results suggest that not only the binding of the compounds to the GnRH receptors has an influence on their efficacy, but also the proper drug release (metabolite formation), which might not be very efficient in case of oxime bond-linked daunorubicin attached to D-Lys.

Comparing the investigated bioconjugates containing GnRH-III derivatives as targeting moieties, it can be concluded that the GnRH-III(^{4}Lys(nBu), ^{8}Lys(Dau=Aoa)) has the highest affinity to the GnRH receptors both on human pituitary and human prostate cancer.

3. Conclusions

In the present work, GnRH-III modified with SCFAs in position 4 was employed as a targeting moiety to which daunorubicin was attached *via* an oxime bond leading to the formation of drug delivery systems for targeted cancer chemotherapy.

All investigated bioconjugates were stable at least for 6 h in cell culture medium. The stability of the compounds was also evaluated in the presence of the digestive enzyme of the intestinal tract, α–chymotrypsin. According to the LC-MS analyses of the digestion mixtures, all bioconjugates were digested and the degradation rate strongly depended on the type of fatty acid. Moreover, the stability of all bioconjugates except the bioconjugate **2,** was enhanced compared to the most efficient compound GnRH-III(^{4}Lys(Ac,), ^{8}Lys(Dau=Aoa)) previously developed in our laboratories.

The degradation of the bioconjugates in the presence of rat liver lysosomal homogenate was also investigated. In all cases, the smallest drug containing fragment

identified by LC–MS was H-Lys(Dau=Aoa)-OH; however, no free daunorubicin release was detected.

All bioconjugates showed enhanced cytostatic effect on MCF-7 human breast and HT-29 human colon cancer cell lines *in vitro*. The highest *in vitro* cytostatic effect was observed in case of bioconjugates **2** and **3**. These two compounds were also most effectively taken up by both tested cancer cell lines. The compound GnRH-III(^{4}Lys(nBu),^{8}Lys(Dau=Aoa)) was more stable against chymotrypsin than the GnRH-III(^{4}Lys(iBu),^{8}Lys(Dau=Aoa)). In conclusion, based on the results of the cellular uptake, cytostatic effect *in vitro*, enzymatic stability and receptor binding affinity studies, GnRH-III(^{4}Lys(nBu), ^{8}Lys(Dau=Aoa)) was selected for further investigation of its tumor growth inhibitory effect *in vivo*.

4. Experimental Section

4.1. Chemicals

All amino acid derivatives and Rink-Amide MBHA resin were purchased from Iris Biotech GmBH (Marktredwitz, Germany). Boc-aminooxyacetic acid (Boc-Aoa-OH), (aminooxy)acetic acid, scavengers, coupling agents and cleavage reagents (1-hydroxybenzotriazole hydrate (HOBt), *N*,*N*′-diisopropylcarbodiimide (DIC), triisopropylsilane (TIS), piperidine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), trifluoroacetic acid (TFA)), diisopropylethylamine (DIPEA), acetic anhydride (Ac_2O), 2,2,2-trifluoroethanol (TFE), *n*-octanol, methanol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propionic anhydride, *n*-butyric anhydride, isobutyric anhydride, isovaleric anhydride, caproic anhydride and crotonic acid chloride were obtained from Sigma-Aldrich Kft (Budapest, Hungary). Daunorubicin hydrochloride was a gift from IVAX (Budapest, Hungary). Dimethylformamide (DMF), dichloromethane (DCM) and

diethyl ether were purchased from Molar Chemicals Kft (Budapest, Hungary) and solvents for HPLC methanol (MeOH) and acetonitrile (MeCN) were purchased from Sigma-Aldrich Kft. (Budapest, Hungary).

All reagents and solvents were of analytical grade or highest available purity.

4.2. Synthesis of oxime bond-linked GnRH-III([4]Lys(X), [8]Lys(Dau=Aoa)) bioconjugates

[[4]Lys]-GnRH-III acylated with SCFAs in position 4 and aminooxyacetylated (Aoa) in position 8 (<EHWK(X)HDWK(Aoa)PG-NH_2, where X = propionyl (Pr), *n*-butyryl (nBu), isobutyryl (iBu), crotonyl (CA), isovaleryl (iVA), caproyl (Hex), myristyl (Myr) and <E is pyroglutamic acid) were prepared manually by solid phase peptide synthesis (SPPS) according to Fmoc/*t*Bu chemistry on a Rink-Amide MBHA resin (0.64 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 and Fmoc-Lys(ivDde)-OH. The protocol of the synthesis was as follows: (i) DMF washing (4 × 1 min), (ii) Fmoc deprotection with 2% DBU, 2% piperidine in DMF (4 times; 2 + 2 + 5 + 10 min), (iii) DMF washing (8 x 1 min), (iv) coupling of α-Fmoc-protected amino acid derivative : DIC : HOBt (3 eq each to the resin capacity) in DMF (1 x 60 min), (v) DMF and DCM washing (2-2 x 1 min), (vi) ninhydrine test.

After completion of the synthesis of the protected decapeptides, the ivDde-protecting group of the ε-NH_2 group of [4]Lys was removed by 4% hydrazine in DMF (12 x 5 min); afterwards, the acylation of the ε-NH_2 group with short-chain fatty acids of different chain length was achieved using 3 eq fatty acid (propionic, *n*-butyric, isobutyric, isovaleric, caproic) anhydride or crotonic acid chloride: DIPEA (1:1, mol/mol) for 2 h. In case of myristic acid, the carbonic acid was coupled using DIC, HOBt coupling reagents (3eq each to the resin capacity). After removing the Mtt-protecting group from the ε-NH_2 group of [8]Lys (2% TFA,

2% TIS (triisopropylsilane) in DCM for 6 × 5 min), Boc-Aoa-OH was coupled for 2 h using DIC, HOBt coupling reagents (3 eq each to the amino group).

Aminooxyacetylated peptides were cleaved from the resin using a mixture of 95% TFA, 2.5% TIS and 2.5% water (v/v/v) in the presence of 10 eq free aminooxyacetic acid as "carbonyl capture" reagent for 1.5 h at room temperature and then precipitated with ice-cold diethyl ether, washed three times with diethyl ether and solubilized in 10% acetic acid prior to lyophilization [32]. The crude products were purified by semipreparative RP-HPLC, analyzed by mass spectrometry and immediately used in the next synthetic step after evaporation of the solvent. The conjugation of daunorubicin to the aminooxyacetylated GnRH-III(^{4}Lys(X), ^{8}Lys(Aoa)) derivatives was carried out in 0.2 M ammonium acetate buffer (pH 5.0), at a peptide concentration of 10 mg/mL. The reaction mixtures were stirred at room temperature for 16 h and then the bioconjugates were separated by semipreparative RP-HPLC. The purified bioconjugates, GnRH-III(^{4}Lys(X),^{8}Lys(Dau=Aoa)) (where X= Pr (**1**), iBu (**2**), nBu (**3**), CA (**4**), iVA (**5**), Hex , (**6**) Myr (**7**) were characterized by analytical RP-HPLC and mass spectrometry. The content of the bioconjugates in the lyophilized product was 70-75% in all cases.

4.3. Synthesis of [D-Lys^{6}(Dau=Aoa)]GnRH-I bioconjugates

The oxime bond-linked [D-Lys^{6}(Dau=Aoa)]GnRH-I bioconjugate, as a reference compound, was also prepared. The [D-Lys^{6}(Aoa)]GnRH-I peptide was synthesized similarly to the aminooxyacetylated GnRH-III derivative peptides. The following Fmoc-protected amino acid derivatives were used: Fmoc-Gly-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-D-Lys(Mtt)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Ser(*t*Bu)-OH, and Fmoc-Trp-OH.

After completion of the synthesis of the protected peptide the Mtt-protecting group was removed from the ε-NH_2 group of ^{6}Lys and Boc-Aoa-OH was coupled.

The aminooxyacetylated peptide was cleaved from the resin as described above (see 4.2.). The crude product was purified by semipreparative RP-HPLC, analyzed by mass spectrometry and used for chemical ligation similarly to the other compounds. The bioconjugate was separated by semipreparative RP-HPLC. The pure [D-Lys^6(Aoa=Dau)]GnRH-I bioconjugate was characterized by analytical RP-HPLC and mass spectrometry.

4.4. RP-HPLC

The crude peptides and the bioconjugates were purified on a KNAUER 2501 HPLC system (H. Knauer, Bad Homburg, Germany) using a semipreparative Phenomenex Luna C18 column (250 mm x 10 mm) with 10 μm silica (100 Å pore size) (Torrance, CA, USA). Linear gradient elution (0 min 20% B; 5 min 20% B; 50 min 100% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in MeCN-H_2O (80:20, v/v)) was used at a flow rate of 4 mL/min. Peaks were detected at 280 nm.

Analytical RP-HPLC was performed on a KNAUER 2501 HPLC system using a Phenomenex Luna C18 column (250 mm x 4.6 mm) with 5 μm silica (100Å pore size) or Agilent Zorbax SB-CN column (150 mm x 4.6 mm) with 5 μm silica (300Å pore size) (Agilent Technologies, CA) (in case of the stability studies in cell culture medium) as a stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 50 min 90% B or 0 min 0% B; 2 min 0% B; 25 min 100% B) at a flow rate of 1 mL/min with eluents described above. Peaks were detected at 220 nm.

4.5. Mass spectrometry

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 50 - 2500 *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 and a diode array detector (Agilent, Waldbronn, Germany). Peptides were separated on a Vydac MS C18 column (150mm x 1 mm; 300 Å, 3 µm) (Hesperia, CA) 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 minutes at a flow rate of 50 µL/min. Spectra were recorded in positive ion mode in the 100 - 2500 *m/z* range.

4.6. Stability of GnRH-III(^{4}Lys(X),^{8}Lys(Dau=Aoa)) bioconjugates in cell culture medium

Bioconjugates **1 - 7** were dissolved in water (20% of the final volume) and then the solutions were diluted with serum free DMEM cell culture medium (final bioconjugate concentration: 0.5 mg/mL). The mixtures were incubated at 37°C for 6 h and samples of 50 µL were taken every hour. The stability of the samples was monitored using analytical RP-HPLC under the conditions described above.

4.7. Degradation of GnRH-III(^{4}Lys(X),^{8}Lys(Dau=Aoa)) bioconjugates in rat liver lysosomal homogenate

The rat liver lysosomal homogenate was prepared as previously described [22]. The protein concentration was determined by Pierce BCA (bicinchoninic acid) protein assay according to the manufacturer's protocol (ThermoFisher Scientific, Rockford, IL, USA) and it was 17.4 µg/µL.

The bioconjugates were dissolved in 0.2 M NaOAc, pH 5.0, at a concentration of 0.1 μg/μL and then the rat liver lysosomal homogenate was added (bioconjugate: 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 h, 2 h, 4 h, 6 h, 8 h and 24 h. The reactions were quenched by adding 5 μL of acetic acid and followed by LC-MS analysis.

4.8. Stability of GnRH-III(^{4}Lys(X),^{8}Lys(Dau=Aoa)) bioconjugates in the presence of digestive enzyme α-chymotrypsin

The GnRH-III(^{4}Lys(X),^{8}Lys(Dau=Aoa)) bioconjugates **1 - 7** were incubated with α-chymotrypsin (Promega Inc. Madison, WI, USA). In each case, the enzyme to substrate ratio was 1:50 (w/w). The chymotryptic digestion was carried out in 100 mM Tris-HCl / 10 mM $CaCl_2$, pH 7.8, at 25°C. Aliquots were taken after 5 min, 1 h, 2 h, 4 h, 6 h, 8 h and 24 h. Reactions were quenched by the addition of acetic acid. All samples were analyzed by LC-MS.

4.9. Cells

MCF-7 (ATCC:HTB-22) human breast adenocarcinoma cells were maintained in DMEM (Sigma Ltd., St. Louis, MO, USA) 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, USA). HT-29 (ATCC:HTB-38) human colon carcinoma cells were maintained in RPMI-1640 medium containing 10% FCS, L-glutamine (2 mM) and gentamicin (160 μg/mL). All cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

4.10. Cellular uptake of GnRH-III(^{4}Lys(X),^{8}Lys(Dau=Aoa)) bioconjugates

To examine the cellular uptake of the bioconjugates by MCF-7 human breast and HT-29 human prostate cancer cell lines, cells were plated at a number of 1 x 10^5 cells per well on 24-well plates in 1 mL of complete medium. After 24 h incubation at 37°C, the cells were centrifuged 5 min at 1000 rpm and the supernatant was removed. Thereafter, 250 µL of bioconjugate solutions (in serum-free medium) were added onto the cells, concentrations ranging from 0.16 µM to 100 µM. The control cells were treated with serum-free medium. The cells were incubated with the bioconjugate solutions at 37°C for 6 h. After that, the solutions were removed from the cells, 100 µL trypsin/EDTA were added per well and incubated for 10 min at 37°C. After adding 700 µL of 10% FCS in HPMI, the cells were transferred to FACS tubes and centrifuged for 5 min at 1000 rpm. After removing the supernatant, the cells were resuspended in 500 µL FCS-free HPMI. The fluorescence intensity of the cells was determined by flow cytometry (BD LSR II, BD Bioscience, San Jose, CA). Samples at each concentration were measured in duplicates and each experiment was repeated twice. Data were analyzed with the FACSDiVa software and the percentage of daunorubicin positive living cells was calculated.

4.11. In vitro cytostatic effect of GnRH-III(^{4}Lys(X),^{8}Lys(Dau=Aoa)) bioconjugates

In vitro cytostatic effect of the bioconjugates was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay). One day before the treatment with the bioconjugates 5×10^3 cells per well were plated on 96-well plates. After 24 h 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; samples at each concentration were applied in six parallels). 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 863g and the supernatant was removed. Crystals were dissolved in DMSO and the optical density (OD) of the samples was determined at λ = 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:

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

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. Each experiment was repeated three times and the average IC_{50} values are presented in Table 2.

4.12. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco J-715 spectropolarimeter at 25°C in quartz cells of 0.05 cm path length, under constant nitrogen flush. The instrument was calibrated with 0.06% (w/v) ammonium-d-camphor-10-sulfonate (Katayama Chemical, Japan) in water. The bioconjugates were dissolved in water or trifluorethanol at a concentration of 500 µM. The spectra were averages of six scans in a wavelength range of 180 – 400 nm. The results were expressed in terms of mean molar ellipticity (deg cm^2 $dmol^{-1}$) after subtracting the solvent baseline.

4.13. Langmuir film experiments

The preparation of the lipid layers and the penetration experiments were carried out using a Langmuir trough [30], KSV MiniMicro, (5×20×0.6 cm), with two barriers to provide symmetric film compression. The surface pressure was recorded with an accuracy of ± 0.05 mN/m with the aid of a Wilhelmy plate made of chromatography paper (Whatman Chr1) connected to a force transducer. The trough was made of teflon, while the barrier from polyoxymethylene (POM), as suggested for lipid layers [33], and cleaned carefully with dichloromethane, methanol and water. The trough was placed on a thermo regulated plate and into a box of plexiglass in order to minimize air turbulence and possible contaminations. All measurements were performed at 23 ± 0.5^{o}C. Doubly distilled water was used as the subphase (double distilled water was checked by its conductivity (< 5 mS) and surface tension (> 72.0 mN/m at 23 ± 0.5°C) values).

For the preparation of the Langmuir layers, the lipid (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPC) solution in chloroform (Fischer Chemicals; 1.0 g/L) was spread onto the aqueous subphase. A volume of 13 μL solution was applied dropwise by a Hamilton syringe and the solvent was allowed to evaporate for 10 minutes before compression. The lipid layer was compressed and expanded at a barrier speed of 10 cm^2/min to obtain the surface pressure-area isotherms. Then the monolayer was compressed to a given surface pressure of 15 or 25 mN/m. The barrier was stopped and a fixed amount of aqueous solution of the GnRH-III(^{4}Lys(X), ^{8}Lys(Dau=Aoa)) bioconjugates was injected below the lipid layer to reach a final concentration of 0.002 mg/mL in the subphase. The change in the surface pressure as an indicator of lipid/drug interaction (adsorption/penetration) was recorded as a function of time for one hour. The result of penetration into the monolayer (the saturation value of the increase of surface pressure, $\Delta\pi$) was given as the average of two independent measurements with a standard deviation of ± 0.5 mN/m.

4. 14. Radioligand binding studies

In order to assess the potency of daunorubicin-GnRH derivative bioconjugates to displace radiolabeled [D-Trp^6]GnRH-I (Triptorelin) from the GnRH-receptors on human pituitary and human prostate cancer cells, displacement experiments were performed [34].

Normal pituitary sample (anterior lobe) was collected at autopsy and human prostate cancer specimen was obtained from a patient at the time of initial surgical treatment. The local Institutional Ethics Committee approved the collection and use of these specimens for these studies. Preparation of membranes for receptor binding studies was performed as previously described [35]. Briefly, the samples were thawed and cleaned, and then homogenized in 50 mM Tris·HCl buffer (pH 7.4), supplemented with protease inhibitors (0.25 mM phenylmethylsulfonyl fluoride, 0.4% (v/v) aprotinin and 2 µg/mL pepstatin A (Sigma-Aldrich Ltd. (St. Louis, MO)) using an Ultra-Turrax tissue homogenizer (IKA Works, Wilmington, NC) on ice. The homogenate was centrifuged at 5,00×g for 10 minutes at 4°C to remove the nuclear debris and lipid layer. The supernatant containing the crude membrane fraction was ultracentrifuged (Beckman L8-80M) twice at 70,000×g for 50 min at 4°C after resuspending in fresh buffer. The final pellet was resuspended in homogenization buffer and stored at -80°C until assayed. Protein concentration was determined by the method of Bradford using a Bio-Rad assay kit (Bio-Rad Laboratories, Hercules, CA).

Radioiodinated derivative of [D-Trp^6]GnRH-I was prepared by the chloramines-T method and purified by RP-HPLC in our laboratory [35]. GnRH-I receptor assays were carried out as reported [35] using *in vitro* ligand competition assays based on binding of [^{125}I][D-Trp^6]GnRH-I as radioligand to human pituitary and human prostate cancer membrane fractions. In brief, membrane homogenates containing 50-160 µg protein were incubated in duplicate or triplicate with 60-80,000 cpm [^{125}I][D-Trp^6]GnRH-I and increasing concentration (10^{-12}-10^{-6} M) of nonradioactive bioconjugates as competitors in a total volume of 150 µL of

binding buffer. At the end of the incubation, 125 µL aliquots of suspension were transferred onto the top of 1 mL of ice-cold binding buffer containing 1.5% bovine serum albumin in siliconized polypropylene microcentrifuge tubes (Sigma, St. Louis, MO). The tubes were centrifuged at 12,000×g for 3 minutes at 4°C (Beckman J2-21M, Beckman Coulter, Inc., Brea, CA). Supernatants were aspirated and the bottoms of the tubes containing the pellet were cut off and counted in a gamma counter (Packard Cobra II. Gamma Counter (Perkin-Elmer, Waltham, MA)).

4.15. Data Analysis

Specific ligand-binding capacities and affinities were calculated by the Ligand-PC computerized curve-fitting program of Munson and Rodbard [36].

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All authors declare no conflict of interest.

Author contributions

Designed the experiments: RH, GM, MM, ÉK, GH

Performed the experiments: RH, EO, SZI, ÉK, ÉS, GH, MM

Analyzed and interpreted the data: RH, MM, ÉK, GH, GM

Wrote the paper: RH, MM, GH, GM,

Revised critically the paper: all authors

All authors read and approved the final version of the manuscript.

Appendix A, Supplementary Data

Supplementary data related to this article can be found online.

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- Drug delivery systems.
- Structures of short-chain fatty acid acylated daunorubicin-GnRH-III bioconjugates.
- *In vitro* cytostatic effect of the bioconjugates.
- Colon and breast cancer cell lines.

Table 1. Chemical characteristics of GnRH-III(^{4}Lys(X), ^{8}Lys(Dau=Aoa)) bioconjugates

Code	Compound	[a]RP-HPLC R_t [min]	[b]ESI-MS MW_{calc} / MW_{exp}
1	GnRH-III(^{4}Lys(Pr), ^{8}Lys(Dau=Aoa))	26.4	1939.07 / 1938.70
2	GnRH-III(^{4}Lys(iBu), ^{8}Lys(Dau=Aoa))	27.8	1953.10 / 1952.70
3	GnRH-III(^{4}Lys(nBu), ^{8}Lys(Dau=Aoa))	27.8	1953.10 / 1952.70
4	GnRH-III(^{4}Lys(CA), ^{8}Lys(Dau=Aoa))	27.7	1951.08 / 1950.40
5	GnRH-III(^{4}Lys(iVA), ^{8}Lys(Dau=Aoa))	28.2	1967.13 / 1966.40
6	GnRH-III(^{4}Lys(Hex), ^{8}Lys(Dau=Aoa))	30.7	1981.15 / 1980.60
7	GnRH-III(^{4}Lys(Myr), ^{8}Lys(Dau=Aoa))	37.1	2093.37 / 2092.80
	[D-Lys^6(Dau=Aoa)]-GnRH-I	24.9	1836.20/1836.00

[a]Column: Phenomenex Luna C18 column (250 mm x 4.6 mm) with 5 μm silica (100 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 at 220 nm.

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

Table 2. *In vitro* cytostatic effect of GnRH-III(^{4}Lys(X), ^{8}Lys(Dau=Aoa)) bioconjugates on various human cancer cell lines

Compound	Cytostasis (MCF-7) IC_{50} (μM)	Cytostasis (HT-29) IC_{50} (μM)
GnRH-III(^{4}Lys(Pr), ^{8}Lys(Dau=Aoa)) (**1**)	3.6 ± 0.7	17.2 ± 2.9
GnRH-III(^{4}Lys(iBu), ^{8}Lys(Dau=Aoa)) (**2**)	2.2 ± 0.0	2.0 ± 0.6
GnRH-III(^{4}Lys(nBu), ^{8}Lys(Dau=Aoa)) (**3**)	0.7 ± 0.2	2.2 ± 0.6
GnRH-III(^{4}Lys(CA), ^{8}Lys(Dau=Aoa)) (**4**)	1.6 ± 0.2	13.6 ± 4.2
GnRH-III(^{4}Lys(iVA), ^{8}Lys(Dau=Aoa)) (**5**)	2.5 ± 0.5	16.4 ± 4.5
GnRH-III(^{4}Lys(Hex), ^{8}Lys(Dau=Aoa)) (**6**)	1.5 ± 0.4	20.1 ± 4.2
GnRH-III(^{4}Lys(Myr), ^{8}Lys(Dau=Aoa)) (**7**)	6.8 ± 2.9	37.4 ± 1.0
GnRH-III(^{8}Lys(Dau=Aoa))	6.5 ± 1.8	27.8 ± 4.2
GnRH-III(^{4}Lys(Ac), ^{8}Lys(Dau=Aoa))	3.1 ± 1.7	7.4 ± 2.6
GnRH-III(^{4}Lys, ^{8}Lys(Dau=Aoa))	2.6 ± 0.8	8.1 ± 2.6
[D-Lys^{6}(Dau=Aoa)]GnRH-I	4.1 ± 0.4	20.2 ± 5.0

Table 3. Penetration values Δπ of GnRH-III(^{4}Lys(X), ^{8}Lys(Dau=Aoa)) bioconjugates into DPPC monolayers with surface pressures of 15 mN/m and 25 mN/m at a temperature of 23 ± 0.5 °C as a function of fatty acid chain length (Cn)

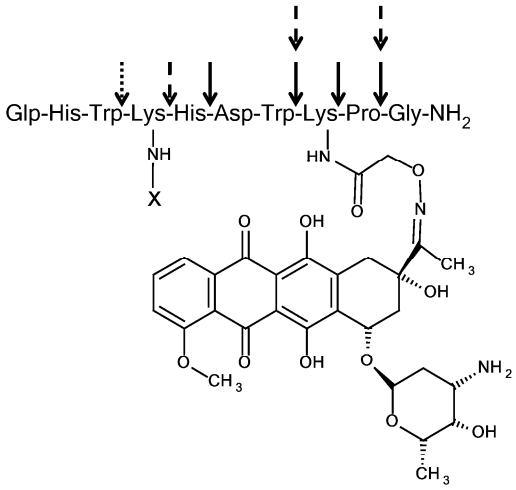
Compound	**Cn**	Surface pressure of lipid films	
		15 mN/m	25 mN/m
		Δπ at 23°C (mN/m)	
GnRH-III(^{4}Lys, ^{8}Lys(Dau=Aoa))	0	3.1	2.0
GnRH-III(^{4}Lys(Ac), ^{8}Lys(Dau=Aoa))	2	3.8	1.9
GnRH-III(^{4}Lys(Pr), ^{8}Lys(Dau=Aoa)) (**1**)	3	3.3	2.1
GnRH-III(^{4}Lys(iBu), ^{8}Lys(Dau=Aoa)) (**2**)	4	3.8	2.1
GnRH-III(^{4}Lys(nBu), ^{8}Lys(Dau=Aoa)) (**3**)	4	3.7	1.2
GnRH-III(^{4}Lys(CA), ^{8}Lys(Dau=Aoa)) (**4**)	4	3.8	1.3
GnRH-III(^{4}Lys(iVA), ^{8}Lys(Dau=Aoa)) (**5**)	5	5.4	1.7
GnRH-III(^{4}Lys(Hex), ^{8}Lys(Dau=Aoa)) (**6**)	6	7.4	2.7
GnRH-III(^{4}Lys(Myr), ^{8}Lys(Dau=Aoa)) (**7**)	14	17.4	11.5

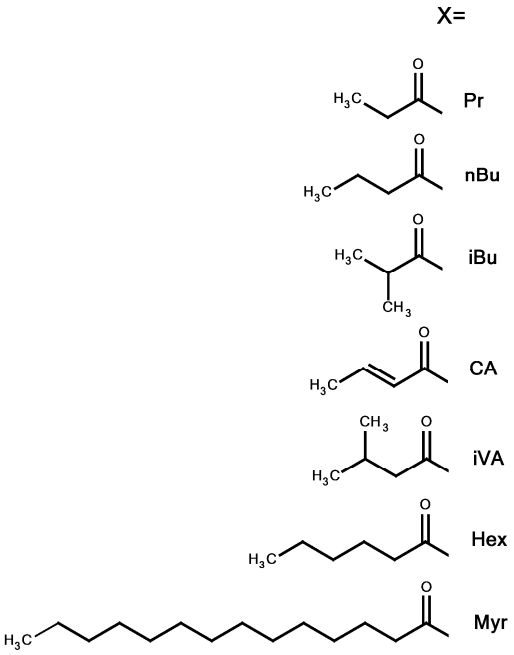
The standard deviation is ± 0.5 mN/m

Table 4. Inhibition of [^{125}I][D-Trp^{6}]GnRH-I binding to the membranes of human pituitary and human prostate cancer specimens by daunorubicin-GnRH derivatives[a]

Compound	IC_{50} (nM)	
	human pituitary	human prostate cancer
[D-Lys^{6}(Dau=Aoa)]GnRH-I	1.62 ± 0.32	0.89 ± 0.09
GnRH-III(Dau=Aoa)	22.87 ± 1.14	16.51 ± 1.08
GnRH-III(^{4}Lys(Ac), ^{8}Lys(Dau=Aoa)	18.86 ± 1.75	15.73 ± 1.24
GnRH-III(^{4}Lys(nBu), ^{8}Lys(Dau=Aoa)	9.03 ± 0.78	7.41 ± 0.55

[a]IC_{50} values were calculated by computerized curve-fitting program [37] from displacement experiments as described. IC_{50} is defined as the dose causing 50% inhibition of specific binding of [^{125}I][D-Trp^{6}]GnRH-I to the membranes. Values shown are mean ± SE of two to three independent experiments (n), each performed in duplicate or triplicate tubes.





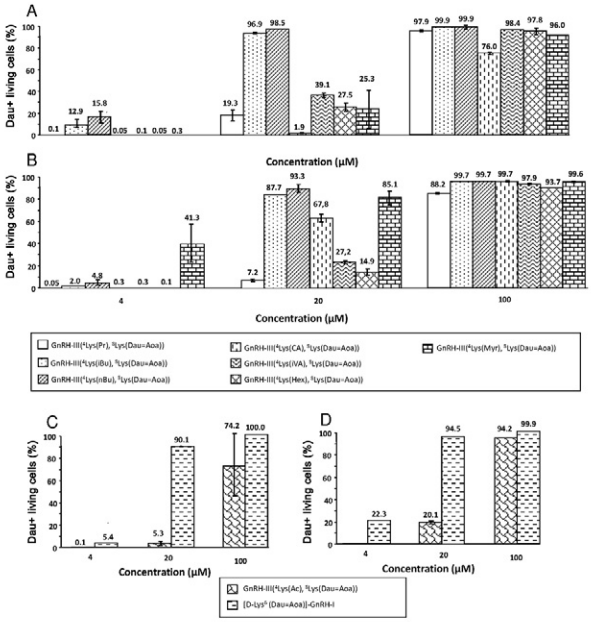


Figure 1. Cleavage sites produced by the proteolysis of bioconjugates in the presence of rat liver lysosomal homogenate (full-line arrows) chymotrypsin (dotted-line arrows) and lysosomal cleavage sites in case of GnRH-III(^{4}Lys(Myr), ^{8}Lys(Dau=Aoa)) (striped arrow)

Figure 2.

A Cellular uptake of GnRH-III(^{4}Lys(X), ^{8}Lys(Dau=Aoa)) bioconjugates by HT-29 human colon carcinoma cells

B Cellular uptake of GnRH-III(^{4}Lys(X), ^{8}Lys(Dau=Aoa)) bioconjugates by MCF-7 human breast cancer cells

C Cellular uptake of [D-Lys^{6}(Dau=Aoa)]GnRH-I and GnRH-III(^{4}Lys(Ac), ^{8}Lys(Dau=Aoa)) bioconjugates by HT-29 human colon carcinoma cells

D Cellular uptake of [D-Lys^{6}(Dau=Aoa)]GnRH-I and GnRH-III(^{4}Lys(Ac), ^{8}Lys(Dau=Aoa)) bioconjugates by MCF-7 human breast cancer cells

Values shown are mean ± SD of two independent experiments, each performed in duplicates.