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GnRH-III based multifunctional drug delivery systems containing daunorubicin and methotrexate

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ABSTRACT

Here we report on the design, synthesis and biochemical characterization of multifunctional bioconjugates containing two chemotherapeutic agents, daunorubicin and methotrexate, coupled to the GnRH-III decapeptide, which served as a targeting moiety. This represents a possible approach to increase the receptor mediated tumor targeting and consequently the cytostatic effect of anticancer drug-peptide bioconjugates. The multifunctional bioconjugates were prepared according to two drug design approaches recently developed by our group. Both bifunctional GnRH-III derivatives, [^{4}Lys]-GnRH-III (Glp-His-Trp-Lys-His-Asp-Trp-Lys-Pro-Gly-NH_2) and [^{8}Lys(Lys)]-GnRH-III (Glp-His-Trp-Ser-His-Asp-Trp-Lys(Lys)-Pro-Gly-NH_2), contain two free amino groups suitable for the attachment of two anticancer drugs, such as methotrexate and daunorubicin. The drugs were chosen with respect to their different mechanisms of action, with the goal of increasing the antitumor effect of the bioconjugates. The *in vitro* cytostatic effect of the bioconjugates was determined on MCF-7 human breast, HT-29 human colon and LNCaP human prostate cancer cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Their *in vitro* stability/degradation in human serum and in the presence of rat liver lysosomal homogenate was investigated by liquid chromatography in combination with mass spectrometry. The influence of the multifunctional bioconjugates on the cell adhesion and cell proliferation was studied on Mono Mac 6 human leukemic monocytes. It was found that (1) all synthesized bioconjugates had *in vitro* cytostatic effect; (2) they were stable in human serum for at least 24 h; (3) they were hydrolyzed in the presence of lysosomal homogenate and (4) they exerted a moderate cell–cell adhesion inducing effect. These results demonstrate that multifunctional bioconjugates containing two different anticancer drugs attached to the same GnRH-III targeting moiety could be successfully prepared and resulted in higher *in vitro* cytostatic effect than the monofunctional bioconjugates containing either methotrexate or daunorubicin, in particular on HT-29 human colon cancer cells.

Abbreviations: Ac, acetyl; Aoa, aminooxyacetyl; Dau, daunorubicin; DHFR, dihydrofolate reductase; Dox, doxorubicin; ESI, electrospray ionization; FH_2, dihydrofolate; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LC-MS, liquid chromatography-mass spectrometry; LH, luteinizing hormone; MM6, Mono Mac 6; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTX, methotrexate; OD, optical density; THF, tetrahydrofolate.

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1. Introduction

Chemotherapy is still one of the most important therapeutic approaches for cancer, although its ubiquitary toxicity can result in various complications and a narrow therapeutic window. However, the administration of high doses of chemotherapeutics is thought to be advantageous in order to prevent the development of drug resistance [1]. A more selective delivery of chemotherapeutic agents solely to cancer cells, e.g. through receptor-mediated endocytosis, could decrease their peripheral toxicity and circumvent the development of drug resistance.

Considering that gonadotropin-releasing hormone receptors (GnRH-Rs) were found to be highly expressed on various tumor types, including breast, ovarian, endometrial, prostate, colon, oral and laryngeal cancers, as well as melanomas and non-Hodgkin's lymphoma [2,3], GnRH derivatives can be used directly for the treatment of hormone dependent tumors (e.g., estrogen- and testosterone-dependent tumors of the reproductive tract) and also serve as targeting moieties for the specific delivery of chemotherapeutic agents. The first anticancer drug-GnRH derivative bioconjugates were developed in A.V. Schally's group, among them AN-152 consisting of the GnRH-I derivative [D-^{6}Lys]-GnRH-I as a targeting moiety and doxorubicin as an anticancer drug [4]. This bioconjugate was shown to deliver the chemotherapeutic agent doxorubicin selectively to cancer cells, thereby reducing its peripheral toxicity and overcoming the drug resistance [5–7]. However, the administration of GnRH-I based bioconjugates is limited due to their endocrine side effects which might cause an initial aggravation of the disease.

In contrast to GnRH-I, the natural isoform GnRH-III (Glp-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2), initially isolated from the brain of the sea lamprey (*Petromyzon marinus*) [8], was shown to have an insignificant effect on the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) both *in vitro* and *in vivo*, as well as a direct antiproliferative effect on cancer cells [9,10]. These features reveal the advantages of GnRH-III to be used as a targeting moiety for drug delivery. We have recently demonstrated that the type of targeting moiety, either [D-^{6}Lys]-GnRH-I or GnRH-III, had no significant influence on the *in vitro* cellular uptake and cytostatic effect of bioconjugates containing ester bond-linked doxorubicin [11]. Furthermore, drug delivery systems for targeted cancer chemotherapy containing the GnRH-III peptide as a targeting moiety and daunorubicin (Dau, also called daunomycin) as a chemotherapeutic agent were prepared in our laboratories. These Dau-GnRH-III derivative bioconjugates had both *in vitro* and *in vivo* antitumor effect without showing significant toxic side effects [12,13]. The *in vivo* tumor growth inhibitory effect of two monofunctional compounds (GnRH-III[^{8}Lys(Dau = Aoa)] and GnRH-III [^{4}Lys(Ac),^{8}Lys(Dau = Aoa)], where Aoa is aminooxyacetyl and Ac is acetyl) was recently investigated on orthotopically developed C26 murine colon carcinoma bearing Balb/c mice. GnRH-III [^{4}Lys(Ac),^{8}Lys(Dau = Aoa)] could inhibit the tumor growth by 49.3%, in comparison to only 24.4% inhibition exerted by the free drug which was used at a well-tolerated dose [13].

Despite the high antitumor activity of this monofunctional GnRH-III bioconjugate, its efficacy might be restricted due to the limited number of GnRH receptors on cancer cells [14]. Furthermore, the receptors can desensitize under continuous exposure to GnRH analogs [15], possibly resulting in a resistance of the cancer cells towards the GnRH-III targeted anticancer drug bioconjugates. A possible approach to enhance the treatment potency would be the application of multifunctional drug delivery systems in which more than one anticancer drug (identical or different) is attached to one GnRH-III molecule. A bioconjugate carrying more than one chemotherapeutic agent might be more potent due to its ability to exert an elevated cytotoxicity compared to a monofunctional compound. Ideally, the anticancer drugs attached to one GnRH-III peptide would act synergistically or additively.

We have recently reported on two drug design approaches that allowed the attachment of two anticancer drugs to one GnRH-III molecule. It was found that those multifunctional Dau-GnRH-III derivative bioconjugates were internalized into GnRH-R positive cells and exerted a high cytostatic effect on various human cancer cell lines [16]. The first drug design was based on the bifunctional [^{4}Lys]-GnRH-III (Glp-His-Trp-Lys-His-Asp-Trp-Lys-Pro-Gly-NH_2); the ε-amino groups of the two lysine residues in positions 4 and 8 were used for the coupling of daunorubicin. In the second drug design, the native GnRH-III peptide (Glp-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2) was employed as a scaffold for the anticancer drug attachment. In order to obtain two free amino groups available for conjugation, an additional lysine was coupled to the ε-amino group of ^{8}Lys. The aim of pursuing these two different synthetic strategies was to ascertain which drug design led to multifunctional bioconjugates with improved biological activity.

Because of their different mechanisms of action, two chemotherapeutic agents – daunorubicin and methotrexate (MTX) – were chosen for the preparation of multifunctional GnRH-III bioconjugates in the present study. MTX acts by reversible and competitive inhibition of dihydrofolate reductase (DHFR), an enzyme critical for the intracellular housekeeping folate metabolism. Daunorubicin, on the other hand, inhibits DNA replication and transcription by intercalation; furthermore, Dau participates in the formation of cytotoxic superoxide- and hydroxyl-radicals [17]. Taking into account the different mechanisms of action of MTX and Dau, their combination in one GnRH-III containing bioconjugate is assumed to lead to an increased cytotoxicity and possibly to enhanced therapeutic efficacy of the bioconjugates. According to the drug design approaches described above, two bioconjugates containing both methotrexate and daunorubicin were prepared. Daunorubicin was coupled *via* an oxime bond to an aminooxyacetylated GnRH-III derivative as previously described [11,12]. MTX was attached by amide bond formation between one of its two carboxyl groups (α- and γ-COOH) and an amino group of the GnRH-III peptide.

As one of the most important features of drug delivery systems is the stability of the chemical bond between the drug and the targeting moiety during transport (e.g., in the blood stream) and its susceptibility to acidic pH or enzymatic hydrolysis upon internalization into the cancer cells, the stability of the bioconjugates in human serum, as well as their degradation in lysosomal homogenates was investigated by liquid chromatography-mass spectrometry (LC-MS). Furthermore, the *in vitro* cytostatic effect of the bioconjugates was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on HT-29 human colon, MCF-7 human breast and LNCaP human prostate cancer cell lines. In addition to this, the influence of the bioconjugates on the adhesion of Mono Mac 6 human leukemic monocytes was assessed using the novel xCELLigence System.

2. Results and discussion

2.1. Methotrexate and daunorubicin containing GnRH-III derivative bioconjugates

In recent years, drug targeting and delivery using peptide hormones have intensively been investigated [2]. As previously reported by our groups, oxime bond-linked daunorubicin-GnRH-III derivative bioconjugates have significant *in vitro* and *in vivo* antitumor activity. To increase the cytostatic effect of these compounds, different approaches could be pursued, including

A ^{1}Glp-^{2}His-^{3}Trp-^{4}Xaa(**R$_1$**)-^{5}His-^{6}Asp-^{7}Trp-^{8}Lys(**R$_2$**)-^{9}Pro-^{10}Gly-NH$_2$

Compound	^{4}Xaa	R_1	R_2
1 GnRH-III[^{4}Lys(MTX),^{8}Lys(Ac)]	Lys	MTX	Ac
2 GnRH-III[^{4}Lys(MTX),^{8}Lys(Dau=Aoa)]	Lys	MTX	Dau=Aoa
3 GnRH-III[^{8}Lys(MTX-Lys(Dau=Aoa))]	Ser	-	MTX-Lys(Dau=Aoa)
GnRH-III[^{8}Lys(Dau=Aoa)]	Ser	-	Dau=Aoa

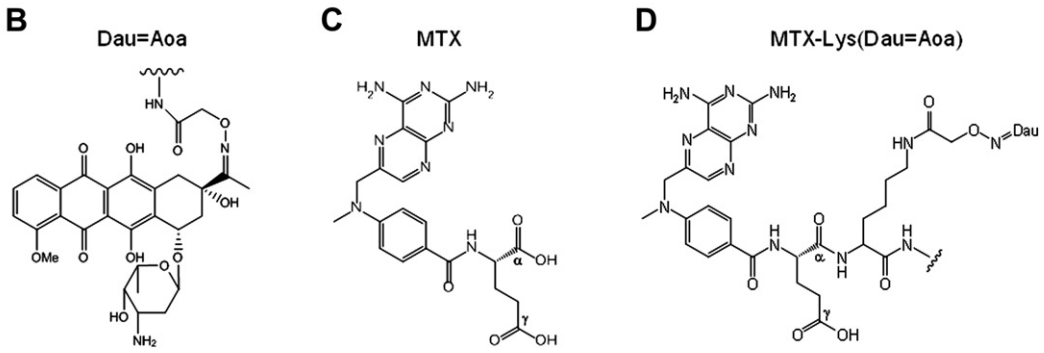

Fig. 1. Structure representation of anticancer drugs-GnRH-III derivative bioconjugates: (A) GnRH-III sequence and the corresponding modifications in positions 4 (R_1) and 8 (R_2); (B) Structure representation of Dau = Aoa; (C) Structure representation of MTX; (D) Structure representation of MTX-Lys(Dau = Aoa).

structural modifications of the targeting moiety [13] and the attachment of more than one anticancer drug to the same carrier molecule [16]. In the present study, we employed GnRH-III analogs modified in positions 4 and 8 to couple two different anticancer drugs, namely daunorubicin and methotrexate. The structures of the synthesized compounds are schematically represented in Fig. 1. The bioconjugates containing both methotrexate and daunorubicin were synthesized by a combination of solid phase synthesis and chemical ligation in solution. As already mentioned, MTX was attached by amide bond formation between one of its two carboxyl groups (α- and γ-COOH) and an amino group of the GnRH-III peptide, whereas Dau was conjugated *via* an oxime bond. The purified compounds **1**–**3** (<EHWK(MTX)DWK(Ac)PG-NH$_2$, <EHWK(MTX)DWK(Dau = Aoa)PG-NH$_2$ and <EHWSDWK(MTX-K(Dau = Aoa))PG-NH$_2$; where <E is pyroglutamic acid) were characterized by analytical RP-HPLC and mass spectrometry (Table 1 and supplementary material, Figs. S1–S3). As previously reported, the glycosidic bonds in daunorubicin are very labile under ESI-mass spectrometric conditions, resulting in the loss of daunosamine (−129 Da, −147 Da) [18]; these fragments were marked in all mass spectra by an asterisk.

In 1985, Rosowsky and colleagues showed that the free α-carboxyl group of MTX seemed to be crucial for the inhibition of the target enzyme DHFR [19]; accordingly, it would be advantageous to conjugate MTX *via* its γ-COOH group. However, site specific protection of the α-COOH group appeared to be difficult and resulted in low yields [20]. In one of our previous studies, MTX was attached to an enzymatic cleavable spacer (GFLGC-NH$_2$), leading to the formation of three different isomers ($^{\gamma}$MTX-GFLGC-NH$_2$, $^{\alpha}$MTX-GFLGC-NH$_2$ and D-$^{\alpha}$MTX-GFLGC-NH$_2$) that could be separated by RP-HPLC [21]. $^{\gamma}$MTX-GFLGC-NH$_2$ and $^{\alpha}$MTX-GFLGC-NH$_2$ were further conjugated to an oligotuftsin derivative as a targeting moiety, resulting in bioconjugates that exerted lower but significant cytotoxic effect compared to the free MTX [22]. Interestingly, the bioconjugate containing MTX attached through the α-carboxylic group had similar or even higher cytotoxic effect than the γ-conjugated compound [21,22], a result which might be explained by the higher enzymatic susceptibility of the peptide bond compared to the isopeptide bond. Therefore, in the present work, coupling of MTX to the GnRH-III *via* amide bond formation has been accomplished without protecting the α-COOH group. An off-line LC-MS analysis was performed with compound **1** (<EHWK(MTX)DWK(Ac)PG-NH$_2$) in order to determine the identity of the obtained products (Fig. 2). It is evident that a mixture of isomers (α- and γ-bioconjugates) was obtained, as the mass spectrometric analysis showed the same *m*/*z* values for all three HPLC fractions. The third peak is thought to correspond to a racemate of the α-conjugated compound, as shown by Mező et al. in case of MTX-GFLGC-NH$_2$ [21]. In order to evaluate if this mixture of α- and γ-conjugated methotrexate-GnRH-III bioconjugates is able to inhibit DHFR, a microplate DHFR enzyme inhibition assay was performed according to a protocol published by Widemann et al. [23]. This sensitive and specific assay is based on the conversion of dihydrofolate to tetrahydrofolate and the resulting oxidation of NADPH

Table 1
Chemical characteristics of anticancer drugs-GnRH-III derivative bioconjugates.

Compound	RP-HPLC[a] R_t [min]	ESI-MS[b] MW_{calc}/MW_{exp}
1, GnRH-III[^{4}Lys(MTX),^{8}Lys(Ac)]	25.74	1778.81/1778.75
2, GnRH-III[^{4}Lys(MTX),^{8}Lys(Dau = Aoa)]	28.16	2318.98/2319.10
3, GnRH-III[^{8}Lys(MTX-Lys(Dau = Aoa))]	28.21	2406.52/2406.10

[a] Column: Vydac C_{18} (250 mm × 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 at λ = 280 nm.
[b] Bruker Daltonics Esquire 3000+ ion trap mass spectrometer.

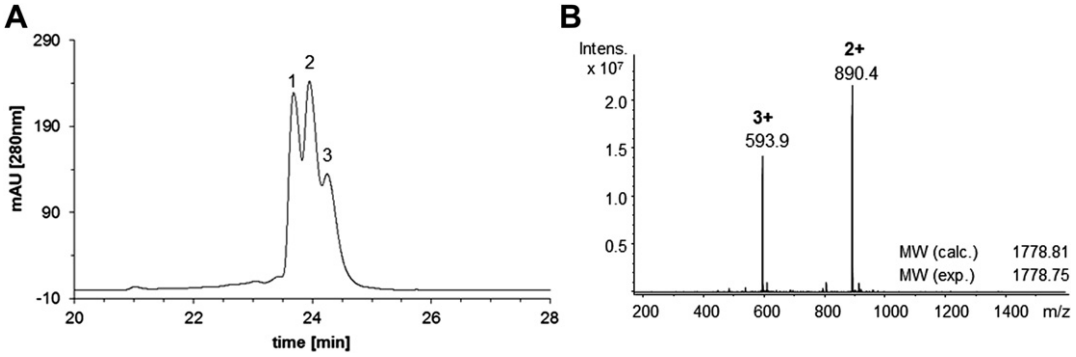

Fig. 2. Analytical RP-HPLC profile of compound **1** and ESI-ion trap mass spectrum of fraction 1. Similar mass spectra were obtained for fractions 2 and 3.

to $NADP^+$ by DHFR, which can be monitored by kinetic absorbance measurements at 355 nm. In addition to compounds **1**–**3** and free MTX, GnRH-III[^{8}Lys(Dau = Aoa)] was tested in order to determine the effect of the GnRH-III peptide on DHFR inhibition in the absence of MTX. As shown in Fig. 3, compound **1** exerted the highest DHFR inhibition in comparison to the other bioconjugates containing MTX; still, its activity was lower than that of free MTX. Compound **2** seemed to be a superior inhibitor of DHFR over compound **3**, a result that may be explained by the close proximity of Dau and MTX in compound **3**, which could lead to its diminished inhibitory activity. GnRH-III[^{8}Lys(Dau = Aoa)] did not exert any effect on DHFR.

2.2. *In vitro stability/degradation of the bioconjugates*

One important aspect in the development of targeted chemotherapeutics is their stability in human serum, since the premature release of the anticancer drug from its targeting moiety would lead to undesired toxic side effects. However, the drug should be readily released once taken up by the cancer cell. Therefore, the chemical bond between the GnRH-III and the anticancer drug should be stable in human serum, but prone to lysosomal degradation, preferably resulting in the formation of a small, active drug metabolite or in the release of the free drug.

2.2.1. *Stability of the bioconjugates in human serum*

The anticancer drugs-GnRH-III derivative bioconjugates were incubated for up to 24 h in 90% human serum at 37 °C to identify possible degradation products. LC-MS analyses of compounds **2** and **3** incubated in human serum indicated that the intact bioconjugates were detectable in the reaction mixtures even after 24 h (Fig. 4A and B show the ESI-ion trap mass spectra averaged over the chromatographic window where the compounds eluted; the *m*/*z* values corresponding to the intact bioconjugates are marked in bold; the other ions correspond to human serum components). According to the mass spectrometric analyses, no proteolytic fragments could be detected.

In order to identify ions originating from human serum, components lower than 10 kDa in molecular weight from human serum were analyzed by LC-MS (Fig. 4E). In another control experiment, aqueous solutions of the bioconjugates (c = 10 μM) were incubated at 37 °C for 24 h and then subjected to LC-MS analysis. Mass spectrometric data indicated that the bioconjugates were chemically stable under these conditions (Fig. 4C and D). Compound **1**, which only contains MTX and no Dau, also showed high stability in human serum, the intact bioconjugate being identified in the reaction mixture after 24 h of incubation with human serum at 37 °C (Fig. S4, supplementary material).

2.2.2. *Degradation of the bioconjugates in the presence of rat liver lysosomal homogenate*

The degradation of the bioconjugates in the presence of rat liver lysosomal homogenate was investigated by LC-MS, as well. The incubation of the compounds at 37 °C in the presence of lysosomal enzymes resulted in various peptide fragments presented in Table 2. Compound **1**, which only contains MTX at position 4, showed a moderate degradation in rat liver lysosomal homogenate. Apart from the large peptide fragment <EHWK(MTX)HDWK(Ac)-OH, no smaller drug-containing metabolites could be identified. In case of compound **2**, the smallest drug containing metabolite detected in the mass spectrum at *m*/*z* 729.5 (1+) was H-Lys(Dau = Aoa)-OH, which

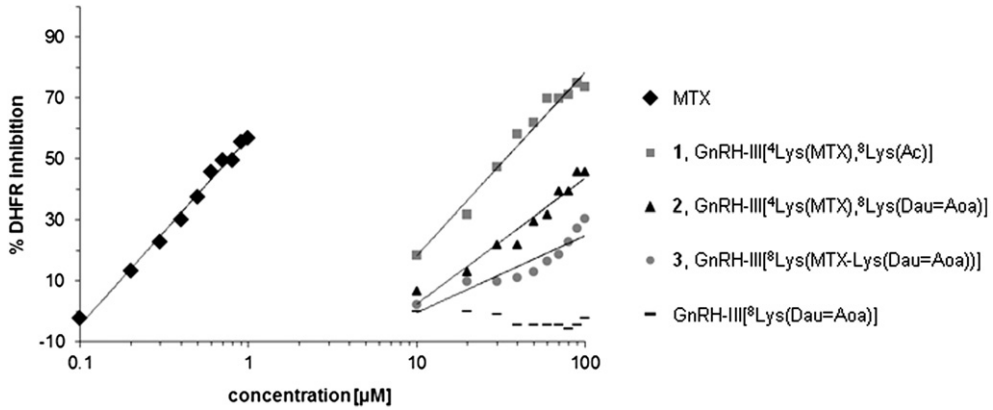

Fig. 3. Inhibition of the MTX-target enzyme dihydrofolate reductase by free methotrexate, compounds **1**–**3** and GnRH-III[^{8}Lys(Dau = Aoa)].

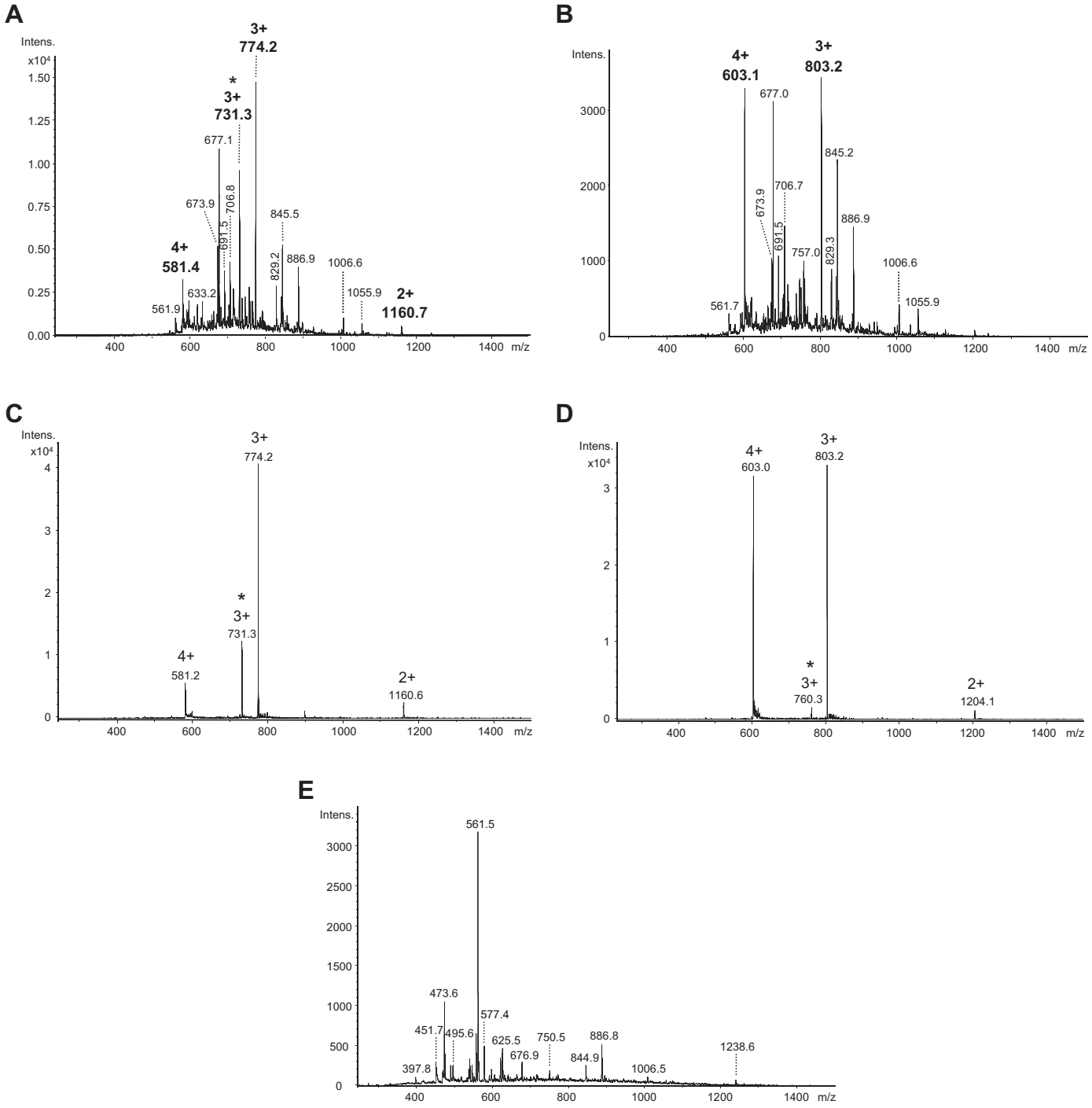

Fig. 4. Stability of the bioconjugates **2** and **3** in human serum. Shown are the ESI-ion trap mass spectra from the LC-MS analyses performed after 24 h incubation of the compounds in human serum at 37 °C. Spectra were averaged over the chromatographic window where the compounds eluted. The *m/z* values corresponding to the intact bioconjugates are marked in bold (A) GnRH-III[^{4}Lys(MTX),^{8}Lys(Dau = Aoa)] and (B) GnRH-III[^{8}Lys(MTX-Lys(Dau = Aoa))]. LC-MS analyses of aqueous solutions of the bioconjugates **2** (C) and **3** (D) incubated for 24 h at 37 °C. LC-MS analysis of the human serum prepared similarly to the bioconjugate samples was also performed (E).

has previously been shown to bind to DNA [12], an important feature for Dau to exert its cytotoxic activity. The smallest MTX-containing metabolite detectable in the reaction mixture was <EHWK(MTX)HDWK(Dau = Aoa)-OH. For compound **3**, H-Lys(MTX-Lys(Dau = Aoa))P-OH was found as the smallest metabolite at *m/z* 1391.7 (1+), a result which is in good agreement with our previously published data on a multifunctional GnRH-III derivative bioconjugate containing two daunorubicin residues at this position [16]. The detailed mass spectrometric analyses are shown in the supplementary material (Fig. S5). The LC-MS analysis of the lysosomal degradation of GnRH-III[^{8}Lys(Dau = Aoa)] has previously been published [12].

2.3. In vitro cytostatic effect

The *in vitro* cytostatic effect of anticancer drugs-GnRH-III derivative bioconjugates was determined on MCF-7 human breast, HT-29 human colon and LNCaP human prostate cancer cells.

Table 2
Fragments produced by the cleavage of anticancer drugs-GnRH-III derivative bioconjugates in the presence of rat liver lysosomal homogenate.

Compound	Fragment	MW_{calc}/MW_{exp}
1, GnRH-III[^{4}Lys(MTX),^{8}Lys(Ac)]	<EHWK(MTX)HDWK(Ac)-OH	1626.7/1626.7
2, GnRH-III[^{4}Lys(MTX),^{8}Lys(Dau = Aoa)]	<EHWK(MTX)HDWK(Dau = Aoa)-OH	2166.3/2166.5
	H-DWK(Dau = Aoa)PG-NH_2	1183.3/1184.0
	H-K(Dau = Aoa)P-OH	825.3/825.5
	H-K(Dau = Aoa)-OH	728.3/728.4
3, GnRH-III[^{8}Lys(MTX-Lys(Dau = Aoa))]	<EHWSHDWK(MTX-K(Dau = Aoa))-OH	2253.3/2253.6
	H-HDWK(MTX-K(Dau = Aoa))PG-NH_2	1883.8/1884.0
	H-HDWK(MTX-K(Dau = Aoa))-OH	1730.7/1731.0
	H-K(MTX-K(Dau = Aoa))P-OH	1390.5/1390.7

Compounds **2** and **3** showed mostly high cytostatic effects with IC_{50} values in the low micromolar range (Table 3). Interestingly, compound **1** did not exert any cytostatic effect in the studied concentration range; the determined IC_{50} values were higher than 50 μM for all three tested cancer cell lines. Most likely, this is due to the large metabolite formed after lysosomal degradation that does not allow MTX to exert its cytostatic activity.

On HT-29 cell line, the activity of the multifunctional compounds was significantly higher than that of the monofunctional compound containing only daunorubicin. The cytostatic effect of compounds **2** and **3**, which contain both daunorubicin and methotrexate, exceeded that of the GnRH-III[^{8}Lys(Dau = Aoa)] bioconjugate, confirming our assumption that the therapeutic efficacy could be increased by the attachment of a second anticancer drug to one GnRH-III molecule. Interestingly, despite of the large metabolite produced in the presence of the lysosomal homogenate, compound **3** had a comparable cytostatic effect with compound **2**. This could indicate that the large H-Lys(MTX-Lys(Dau = Aoa))Pro-OH fragment might either be further processed inside the cells or that the close proximity of methotrexate and daunorubicin does not affect their ability to interact with their intracellular targets.

Similar IC_{50} values were determined on MCF-7 human breast cancer cells, though the multifunctional bioconjugates were not significantly more effective in comparison to the daunorubicin containing monofunctional compound. On LNCaP human prostate cancer cells, compound **2** had a decreased cytostatic effect in contrast to compound **3** and the monofunctional daunorubicin-bioconjugate. For both multifunctional compounds, the IC_{50} values were lower in case of HT-29 and MCF-7 cells, while these compounds were less effective on LNCaP cells. Interestingly, the multifunctional bioconjugates showed similar cytostatic effect on two of the three tested cell lines. Only the GnRH-III [^{8}Lys(Dau = Aoa)] bioconjugate exerted a significantly different effect on the different cancer cell lines. As a further comparison, the cytostatic effects of free daunorubicin and methotrexate were also determined. The free anticancer drugs showed overall the highest activity with IC_{50} values in the range of 0.1–1.4 μM. As the free drugs are taken up by passive diffusion and do not require any intracellular processing, the higher IC_{50} values obtained for the anticancer drugs-GnRH-III derivative bioconjugates are most probably due to their uptake by receptor-mediated endocytosis and further intracellular processing.

Table 3
In vitro cytostatic effect of free daunorubicin, free methotrexate and anticancer drugs-GnRH-III derivative bioconjugates on various human cancer cell lines.

Compound	MCF-7 IC_{50} [μM]	LNCaP IC_{50} [μM]	HT-29 IC_{50} [μM]
Daunorubicin	0.4 ± 0.1	0.1 ± 0.1	0.2 ± 0.2
Methotrexate	n.d.	0.3 ± 0.2	1.4 ± 0.6
1, GnRH-III[^{4}Lys(MTX),^{8}Lys(Ac)]	>50	>50	>50
2, GnRH-III[^{4}Lys(MTX),^{8}Lys(Dau = Aoa)]	5.8 ± 1.1	28.8 ± 4.7	3.6 ± 1.5
3, GnRH-III[^{8}Lys(MTX-Lys(Dau = Aoa)]	5.4 ± 0.7	11.3 ± 0.5	5.6 ± 3.0
GnRH-III[^{8}Lys(Dau = Aoa)]	6.7 ± 1.9	15.2 ± 2.4	29.9 ± 0.6

2.4. Antiproliferative/cytotoxic effect

The antiproliferative/cytotoxic effects of free daunorubicin and methotrexate as well as of their GnRH-III containing bioconjugates were determined on Mono Mac 6 human leukemic monocytes for up to 72 h in the 10^{-9}–10^{-6} M concentration range. The decrease in the number of viable cells was normalized to the control (cells treated with culture medium) and this value was given as "inhibition index" in percent (%inh). The viability parameter was expressed as a ratio of the control.

After 24 h of treatment, the bioconjugates exerted a significant antiproliferative effect on the monocytes. The inhibition indices (% inh) at a concentration of 10^{-6} M for compounds **2** (%inh: 88%, viability: 1.06) and **3** (%inh: 82%, viability: 1) were similar to that of methotrexate (inh%: 80%, viability: 0.95) (Table 4). At a lower concentration of 10^{-7} M, only daunorubicin exerted an antiproliferative effect (%inh: 59%, viability 0.85).

A longer incubation time of 48 h led to a higher antiproliferative effect in case of GnRH-III[^{8}Lys(MTX-Lys(Dau = Aoa))] (%inh: 63%, viability: 0.9 at 10^{-6} M), while the inhibitory effect of GnRH-III [^{4}Lys(MTX),^{8}Lys(Dau = Aoa)] did not increase at the same concentration (%inh: 86%, viability: 0.98) (Table 4). The treatment with the free anticancer drugs resulted in a decreased number of cells in a wider concentration range. The free daunorubicin exerted a high cytotoxic effect (%inh: 36–26%, viability: 0.79–0.45) in the concentration range of 10^{-9}–10^{-6} M, whereas methotrexate seemed to be slightly less effective on the tested cell line (10^{-7}–10^{-6} M; %inh: 52%, viability: 0.98).

After 72 h of treatment, the determined inhibition index of compound **3** (%inh: 38%, viability: 0.77) was about two times lower compared to that of compound **2** (%inh: 76%, viability: 0.95) at a concentration of 10^{-6} M (Table 4). Of all tested compounds, daunorubicin had the highest cytotoxic effect in the 10^{-9}–10^{-6} M concentration range (%inh: 21–16%, viability: 0.64–0.49). Methotrexate also exerted a cytotoxic effect on monocytes, with a maximum at 10^{-7}–10^{-6} M (inh%: 27–29%, viability: 0.76–0.77).

Taken together, these data indicate that the antiproliferative/cytotoxic effect of the free drugs, especially of daunorubicin, is higher than that of the bioconjugates. However, the inhibitory activity of the bioconjugates increased over time and after 72 h compound **3** (GnRH-III[^{8}Lys(MTX-Lys(Dau = Aoa))]) exerted a significant cytotoxic effect (%inh: 38%, viability: 0.77) at 10^{-6} M concentration, comparable to the effect of free methotrexate (%inh: 29%, viability: 0.77). It is worth mentioning that the monofunctional bioconjugate GnRH-III[^{8}Lys(Dau = Aoa)] could elicit only a moderate antiproliferative effect on Mono Mac 6 cells (%inh: 84%) at 10^{-6} M after 72 h [24].

Comparing the results obtained from the tested cell lines, it was observed that both multifunctional bioconjugates **2** and **3** had similar cytostatic effects on MCF-7 and HT-29 cell lines, while the cytotoxic effect of compound **3** on monocytes was twice higher than that of compound **2**. Several factors might be responsible for the observed differences in the growth inhibitory effects of the

Table 4
Antiproliferative/cytotoxic effect of free anticancer drugs and their GnRH-III containing bioconjugates on Mono Mac 6 human monocytic cell line (%inh: inhibition index as a percentage of the control; significance levels correspond to *x*: $p < 0.05$, *y*: $p < 0.01$, *z*: $p < 0.001$).

Compound	%inh (10^{-9} M)			%inh (10^{-8} M)			%inh (10^{-7} M)			%inh (10^{-6} M)		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Daunorubicin	97	36^{z}	21^{z}	95	35^{z}	17^{z}	59^{z}	26^{z}	14^{z}	46^{z}	27^{z}	16^{z}
Methotrexate	101	97	96	102	94	60^{y}	89	52^{y}	27^{z}	80^{x}	52^{y}	29^{z}
2, GnRH-III[^{4}Lys(MTX),^{8}Lys(Dau = Aoa)]	108	89	81	103	92	103	98	94	104	88^{x}	86	76^{x}
3, GnRH-III[^{8}Lys(MTX-Lys(Dau = Aoa))]	116	93	99	128^{y}	91	97	105	97	94	82^{x}	63^{y}	38^{z}

bioconjugates; e.g., the experimental conditions, the cellular milieu (ligand-selectivity of the cells) and conjugation site, which can markedly influence the release of the active drug and/or metabolite.

2.5. Effect of the bioconjugates on the adhesion of Mono Mac 6 human leukemic monocytes

It is widely accepted that cell adhesion and migration might play an important role in the development of cancer metastasis. Therefore, it is important to investigate not only the anti-proliferative/cytotoxic effect of the bioconjugates, but also their influence on the adhesion of cancer cells. In our work, Mono Mac 6 human leukemic monocytes were used as a model system for the cell adhesion studies.

Significant differences were determined between the cell adhesion modulator effects of the bioconjugates and those of the free anticancer drugs. In the studied concentration range between 10^{-9} and 10^{-6} M, compound **2** developed rapidly (in less than 2 h) a moderate, but long lasting adhesion inducing effect (120–135%), in a concentration dependent manner with a maximum effect at 10^{-9} M (135%) (Fig. 5A). Compound **3** showed no effect on cell adhesion in the concentration range of 10^{-7}–10^{-6} M. At a lower concentration of 10^{-9} M, the adhesion of the Mono Mac 6 cells even decreased to 77.6%, with long-term characteristic (Fig. 5B).

Compared to the non-treated control cells, MTX induced a significant increase of cell adhesion (~140–160%) over the concentration range of 10^{-9}–10^{-6} M (Fig. 6A). This adhesion enhancement character of methotrexate was also significantly expressed out of the presented concentration range, namely 10^{-11}–10^{-12} M (data not shown). An adhesion enhancing effect could also be detected for daunorubicin in the concentration range between 10^{-8} and 10^{-6} M (121–131%); nevertheless, the positive effect (142%) of 10^{-7} M daunorubicin was detectable only in the first 1.5 h incubation time (Fig. 6B).

The daunorubicin containing reference compound (GnRH-III [^{8}Lys(Dau = Aoa)]) elicited a strong negative effect on the cell adhesion at all tested concentrations [24]. Compared to that, the MTX-containing bioconjugates showed an overall positive effect on cell adhesion, possibly due to the presence of the additional methotrexate residue in the multifunctional compounds. These

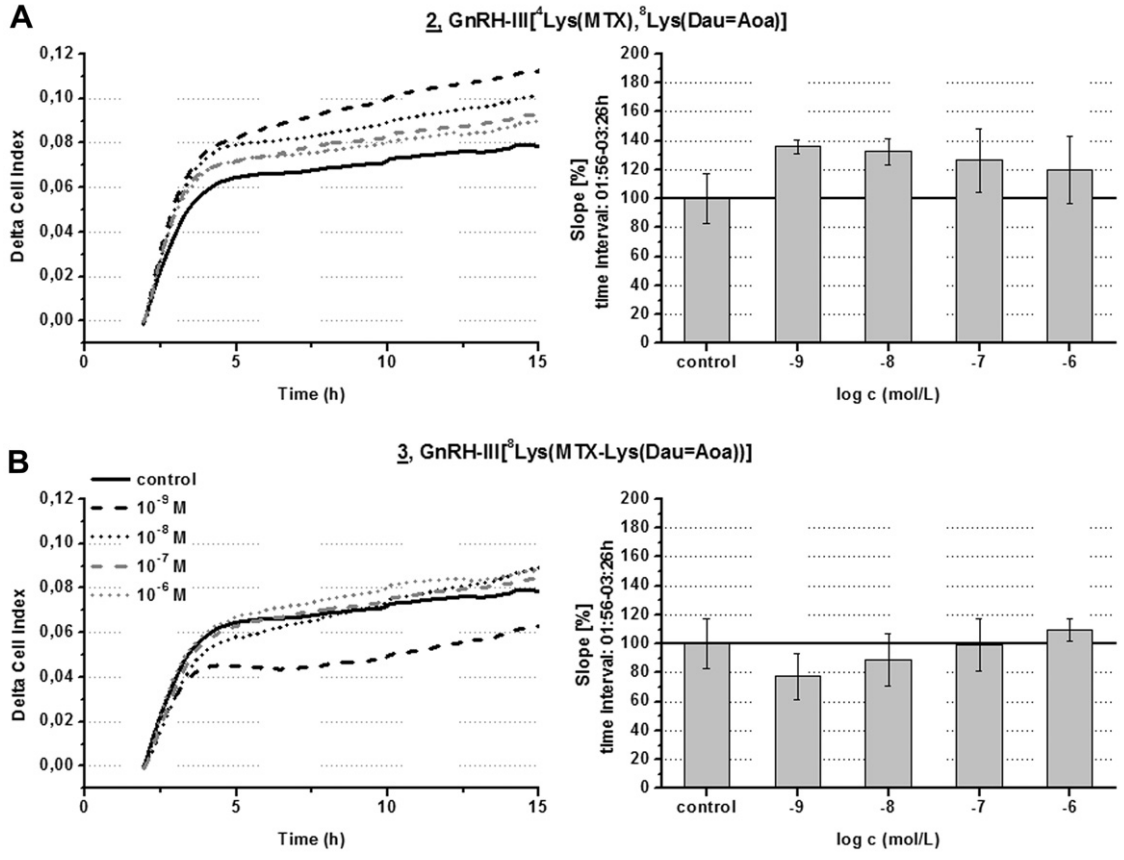

Fig. 5. Adhesion induced by anticancer drugs-GnRH-III derivative bioconjugates on Mono Mac 6 human monocytic cells: (A) GnRH-III[^{4}Lys(MTX), ^{8}Lys(Dau = Aoa)] and (B) GnRH-III [^{8}Lys(MTX-Lys(Dau = Aoa))].

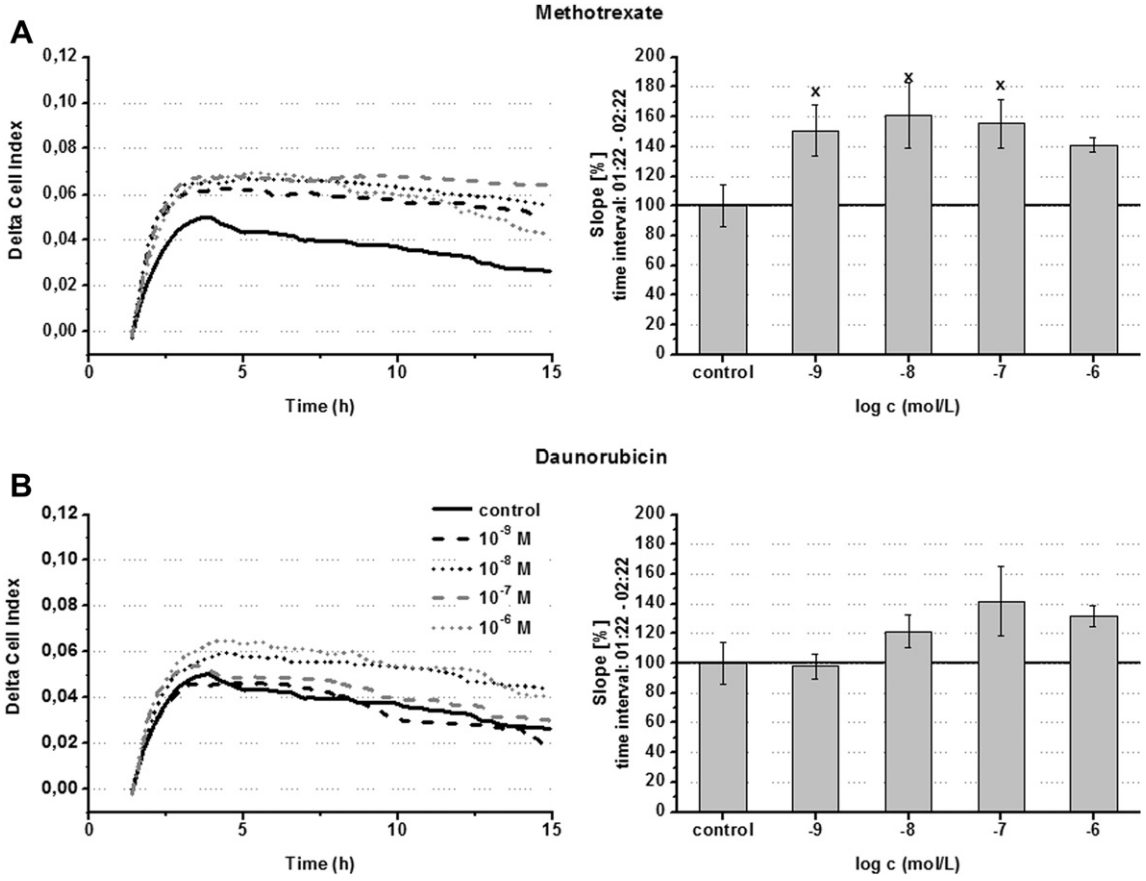

Fig. 6. Adhesion inducing effect of (A) free methotrexate and (B) free daunorubicin on Mono Mac 6 human monocytic cell line. Significance levels correspond to *x*: $p < 0.05$.

positive effects on cell adhesion of the multifunctional compounds, along with their long-term antiproliferative/cytotoxic effect, could be promising features to be further explored in targeted cancer chemotherapy, in particular to inhibit metastasis formation of a primary tumor.

3. Conclusions

To our knowledge, this is the first study reporting the design, synthesis and biochemical characterization of multifunctional bioconjugates containing two different anticancer drugs attached to a peptide as a targeting moiety. The combination of two different anticancer drugs, methotrexate and daunorubicin, attached to the same GnRH-III targeting moiety resulted in bioconjugates with enhanced cytostatic effect, in particular on HT-29 human colon cancer cells, confirming the assumption that the effect of two drugs incorporated in one bioconjugate might be synergistic. On MCF-7 human breast and LNCaP human prostate cancer cells, they were as effective as a monofunctional GnRH-III bioconjugate containing oxime bond-linked daunorubicin. Possibly, the combination of two anticancer drugs in one bioconjugate could be of special advantage in the treatment of hormone independent tumors such as human colon carcinoma. On all three tested cancer cell lines, all compounds had higher cytostatic effect than the monofunctional methotrexate-GnRH-III bioconjugate **1**. Besides their notable antiproliferative/cytotoxic activity, the adhesion inducing effect of the multifunctional GnRH-III bioconjugates (in particular that of GnRH-III[^{4}Lys(MTX),^{8}Lys(Dau = Aoa)]), suggests their possible inhibitory action on metastasis formation of a primary tumor and possible future use in targeted cancer chemotherapy as drug delivery systems with both antimetastatic and anticancer effects.

4. Experimental section

4.1. Chemicals

All amino acid derivatives, benzotriazol-1-yl-oxytrispyrrolidinophosphonium-hexafluoro-phosphate (PyBOP), bis-Boc-aminooxyacetic acid (bis-Boc-Aoa-OH) and Rink-Amide MBHA resin were purchased from NovaBiochem (Läufelfingen, Switzerland) and GL Biochem ShanghaiLtd (Shanghai, China). Scavengers, coupling agents and cleavage reagents (triisopropylsilane (TIS), 4-methylmorpholine (NMM), piperidine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), trifluoroacetic acid (TFA)), as well as daunorubicin (Dau), methotrexate (MTX), *N*-diisopropylethylamine (DIPEA), acetic anhydride (Ac_2O) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma–Aldrich Ltd. (St. Louis, MO, USA). *N*,*N*-Dimethylformamide (DMF) and acetonitrile were purchased from Acros Organics (Geel, Belgium), while ethanol and diethyl ether were from Riedel deHäen (Seelze, Germany). All reagents and solvents were of analytical grade or highest available purity.

4.2. Synthesis of GnRH-III(MTX, Dau) derivative bioconjugates

The linear protected GnRH-III derivatives (Glp-His(Trt)-Trp(Boc)-Ser(*t*Bu)-His(Trt)-Asp(O*t*Bu)-Trp(Boc)-Lys(Mtt)-Pro-Gly-R, Glp-His(Trt)-Trp(Boc)-Lys(Dde)-His(Trt)-Asp(O*t*Bu)-Trp(Boc)-Lys(Mtt)-Pro-Gly-R, Glp-His(Trt)-Trp(Boc)-Lys(Dde)-His(Trt)-Asp(O*t*Bu)-Trp(Boc)-Lys(Boc)-Pro-Gly-R; where R = resin) 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(Boc)-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Lys(Dde)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-His(Trt)-OH and Fmoc-Ser(*t*Bu)-OH. Pyroglutamic acid (Glp or <E) was attached to the peptide chain without any protection.

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 (10 × 1 min), (iv) coupling of 5 equiv α-Fmoc-protected amino acid derivative:PyBOP:NMM (1:1:2) in DMF (1 × 60 min), (v) DMF washing (4 × 1 min).

In order to synthesize < EHWK(MTX)DWK(Ac)PG-NH_2 (compound **1**) and <EHWK(MTX)DWK(Dau = Aoa)PG-NH_2 (compound **2**), the Dde-protecting group of the ε-NH_2 function of ^{4}Lys was selectively removed using 2% hydrazine in DMF (2 × 15 min). MTX (2.5 equiv) was coupled to the ε-amino group of the lysine residue in the presence of PyBOP:HOBt:NMM (1:1:2); coupling time added up to 60 min.

For the preparation of <EHWSDWK(MTX-K(Dau = Aoa))PG-NH_2 (compound **3**), an additional lysine residue was coupled to the ε-amino group of ^{8}Lys. Therefore, the Mtt-protecting group of ^{8}Lys was selectively removed from the protected linear peptide on the resin with 2% TFA in DCM (6 × 5 min), followed by the coupling of Fmoc-Lys(Mtt)-OH according to the above mentioned protocol. After removal of the Fmoc-protecting group of this additional lysine residue, MTX was coupled to the α-amino group of the branched lysine residue as described above.

After coupling of MTX, the ε-amino groups of ^{8}Lys (compound **2**) and of the branched Lys (compound **3**) were aminooxyacetylated. Therefore, the Mtt-protecting group was removed from the ε-NH_2 function and then bis-Boc-Aoa-OH (2.5 equiv) was attached to the free ε-NH_2 group after preactivation with 2.5 equiv PyBOP in the presence of 5 equiv NMM (1 × 45 min).

The ε-amino group of ^{8}Lys of compound **1** was acetylated after removal of the Mtt-protecting group using a mixture of Ac_2O, DIEA in DMF (1:1:1 v/v/v) for 1 h.

The side chain modified peptides were cleaved from the resin using a mixture of 95% TFA, 2.5% TIS and 2.5% water (v/v/v) for 2.5 h at room temperature and then precipitated with ice-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 semipreparative RP-HPLC and analyzed by mass spectrometry.

The conjugation of daunorubicin to the aminooxyacetylated GnRH-III(MTX, Aoa) derivatives was carried out in 0.2 M sodium acetate buffer (pH 5.0), at a peptide concentration of 10 mg/mL. Daunorubicin was used in 30% excess compared to the aminooxyacetylated GnRH-III(MTX) derivatives. The reaction mixtures were stirred at room temperature for 24 h and then subjected to RP-HPLC purification. The purified bioconjugates, GnRH-III [^{4}Lys(MTX),^{8}Lys(Ac)] (**1**), GnRH-III[^{4}Lys(MTX),^{8}Lys(Dau = Aoa)] (**2**) and GnRH-III[^{8}Lys((MTX)Lys(Dau = Aoa))] (**3**) were characterized by analytical RP-HPLC and mass spectrometry.

4.3. RP-HPLC

The crude products were purified on an UltiMate 3000 HPLC system (Dionex, Idstein, Germany) using a semipreparative Vydac C_{18} column (250 mm × 10 mm) with 10 μm silica (300 Å pore size). Linear gradient elution (0 min 20% B; 5 min 20% B; 55 min 70% 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 nm and 280 nm. Analytical RP-HPLC was performed on an UltiMate 3000 system (Dionex, Idstein, Germany) using a Vydac C_{18} column (250 mm × 4.6 mm) with 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 280 nm.

4.4. 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 (Agilent, Waldbronn, Germany) and a diode array detector. Peptides were separated on a Vydac MS C_{18} column (150 mm × 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 50–2500 *m*/*z* range.

4.5. Stability of bioconjugates in human serum

After dissolving the bioconjugates in water at a concentration of 100 μM, human serum was added to a final peptide concentration of 10 μM. The mixtures were incubated at 37 °C and aliquots of 100 μL were taken after 5 min, 8 h and 24 h (the reactions were quenched by adding 10 μL of acetic acid). Prior to mass spectrometric analysis, the larger human serum proteins were removed using Microcon centrifugal devices, cut-off 10 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 10 kDa from human serum were separated and analyzed by LC-MS and (2) aqueous solutions of bioconjugates (c = 10 μM) were incubated at 37 °C for 24 h and then analyzed by LC-MS in order to investigate their stability in the absence of human serum enzymes.

4.6. Degradation of bioconjugates in the presence of rat liver lysosomal homogenate

The rat liver lysosomal homogenate was prepared as previously described [12] and the protein concentration was determined by Pierce BCA (bicinchoninic acid) protein assay according to the manufacturer's protocol (ThermoFisher Scientific, Rockford, IL, USA). The degradation of the bioconjugates in the presence of rat liver lysosomal homogenate was determined as follows: bioconjugates were dissolved in 0.2 M sodium acetate buffer (pH 5.0) at a concentration of 0.1 μg/μL and then the rat liver lysosomal homogenate was added at a 1:1 (w/w) ratio. The reaction mixtures were incubated at 37 °C and aliquots of 50 μL were taken after 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. Control experiments were performed with 0.1 μg/μL solutions of bioconjugates in 0.2 M sodium acetate buffer (pH 5.0), which were incubated at 37 °C for 24 h and then analyzed by LC-MS in order to assess their chemical stability under these experimental conditions.

4.7. Dihydrofolate reductase (DHFR) enzyme inhibition assay

The DHFR inhibition assay was performed on Costar 96-well flat-bottom microplates (BioRad Laboratories GmbH, Munich, Germany), according to a protocol published in 1999 by Widemann et al. [22]. Different stock solutions were prepared in advance:

a stock solution of 25 mg dihydrofolate (FH_2) in 1.5 mL 2-mercaptoethanol and 6.0 mL buffer A (0.5 M Tris, pH 7.5); aliquots of 125 μL stock solution were stored at −80 °C. Another stock solution was prepared of 50 mg NADPH in 10 mL buffer A; aliquots of 91 μL were stored at −80 °C. The reaction solutions were prepared immediately prior to the assay and kept on ice. The FH_2 reaction solution consisted of a 125 μL aliquot of FH_2 stock solution in 4.0 mL buffer B (0.05 M Tris, pH 7.5), yielding a final FH_2 concentration of 104 mg/L. The NADPH/DHFR reaction solution consisted of a 91 μL NADPH stock aliquot, 227.3 μL DHFR (=0.027 U) in 1.38 mL buffer B, yielding a final working concentration of 290 mg NADPH/L and 15 U DHFR/L. For a standard curve, solutions of MTX in buffer A were prepared at concentrations ranging from 0.1 to 1 μM and stored at 4 °C. The bioconjugates were first dissolved in water and then diluted with buffer A to a final concentration of 0.1 mM; serial dilutions from 100 to 10 μM were used. The general protocol for the microplate DHFR inhibition assay is described below: 130 μL of FH_2 reaction solution was added to each well of the 96-well flat-bottom plate (the outer wells of the plate were not used). MTX calibrators or bioconjugate solutions were added to duplicate wells (20 μL per well). The plate was shaken for 60 s; after that, the NADPH/DHFR reaction solution (50 μL) was added to each well. The plate was shaken again for 60 s and the absorbance was read at wavelengths of 355 nm and 490 nm as a reference on a VICTOR^2 (PerkinElmer, Rodgau, Germany), using the kinetic mode with a reading interval of 1 min for a time period of 25 min. After subtracting the absorbance at 490 nm from the absorbance at 355 nm, the mean value was calculated. The linear decrease of absorbance between 1 and 25 repeats was used for each calibrator and plotted against the concentration to obtain a calibration curve. The percentage of DHFR inhibition was calculated as

$$\%\mathrm{DHFR} = \frac{\text{blank rate} - \text{standard rate}}{\text{blank rate}} \times 100\%$$

where blank rate is the rate in the absence of MTX and standard rate is the rate in the presence of MTX or MTX-containing bioconjugates.

4.8. Cells

MCF-7 human breast cancer cell line was maintained in DMEM GlutaMAX-I (Sigma Ltd., St. Louis, MO, USA) medium containing 10% FCS (fetal calf serum, Sigma Ltd.) and gentamicine (160 μg/mL). HT-29 human colon cancer cell line was maintained in RPMI 1640 (GIBCO Invitrogen, Germany) supplemented with 10% FCS and 1% penicillin/streptomycin. LNCaP human prostate cancer cell line was maintained in RPMI 1640 supplemented with 10% FCS, 1% penicillin/streptomycin and 10 nM testosterone. Cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Mono Mac 6 (MM6) cells are human cells constitutively expressing phenotypic and functional features of mature monocytes [25]. Cultures of MM6 cells were maintained in RPMI 1640 (Sigma Ltd., USA) containing 10% FCS (Lonza Group Ltd., Switzerland), L-glutamine (2 mM) (Gibco®/Invitrogen Corporation, New York, NY, USA), 100 μg/mL penicillin/streptomycin (Gibco®/Invitrogen Corporation) at 37 °C in a humidified 5% CO_2 atmosphere.

4.9. In vitro cytostatic effect of the bioconjugates determined by MTT assay

The *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 compounds, 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 either with the bioconjugates (used in the 0.4–50 μM concentration range) or with the free drugs (used in the 0.0003–20 μM concentration range). The solutions were prepared in serum-free medium. Cells treated for 6 h with 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 performed. MTT was added to each well (final concentration: 367 μg/mL) and during 3.5 h incubation at 37 °C purple crystals were formed by mitochondrial dehydrogenase enzyme present in the living cells. After that, cells were centrifuged for 5 min at 2500 rpm and the supernatant was removed. The crystals were dissolved in 100 μL DMSO and the optical density (OD) was determined at $\lambda = 540$ and 620 nm using an ELISA Reader (SpectraFluor Plus, Tecan, Switzerland). OD_{620} was substracted from OD_{540} and the percentage of cytostasis was calculated using the following equation:

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

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 sigmoid curve and the 50% inhibitory concentration (IC_{50}) value was determined from these curves.

4.10. Cell proliferation/cytotoxicity assay

To analyze the antiproliferative/cytotoxic effects of the bioconjugates, the cells were counted by the CASY TT® professional cell analyzer and counter system (Roche Applied Science, Indianapolis, IN, USA). The effects of the compounds on the logarithmic phase of Mono Mac 6 cultures (10^5 cells/mL) were investigated at 10^{-9}–10^{-6} M concentrations prepared in normal cell culture medium of monocytes. The control group was only treated with culture medium. The cell number was determined after 24, 48 and 72 h of incubation with the compounds. For the analysis, 100 μL cell suspension from each group were diluted in 5 mL CASYton® buffer, and a 400 μL aliquot was analyzed using a 150 μm pore size capillary. Each measurement was carried out in triplicates. CASYexcell 2.3 was used for data evaluation. The decrease in the number of viable cells was normalized to the control and this value was given as "inhibition index" in percent (%inh). The viability parameter was expressed as a ratio of the control.

4.11. Cell adhesion assay on Mono Mac 6 cells

The effects of the free anticancer drugs MTX and Dau and the corresponding GnRH-III bioconjugates on the adhesion of MM6 cells were assessed using the novel xCELLigence System (Roche Applied Science, Indianapolis, USA). Alterations of cell adhesion were monitored by measuring the electrical impedance (Z) across interdigitated gold microelectrodes integrated on the bottom of a specially designed tissue culture plate (E-plate, ACEA Biosciences, Ind., USA). The xCELLigence System measures the change in impedance of gold microelectrodes to alternating current flow in real time. In the absence of cells, the impedance is constant and determined by the background. During the attachment of the cells, due to their insulating plasma membrane, an increase in the impedance could be registered. The detected impedance depends on the local ionic environment, the number and spreading of cells adhered to the surface of the electrodes. The change in impedance

is represented as Cell Index (CI), a relative and dimensionless value which is calculated as:

$$CI = \frac{(Z_i - Z_0)}{F}$$

where Z_i is the impedance at an individual time point, Z_0 is the impedance at the start of the experiment and F is a constant depending on the applied frequency.

The experimental procedure was as follows: the electrodes were first coated with human fibronectin (Chemicon International Inc., Temecula, Canada). A mixture of 1 μg/cm^2 human fibronectin in 0.1% gelatine (Sigma Ltd. St. Louis, USA) was added to the bottom of each well. After 20 min incubation at 4 °C, the protein solution was removed and the wells were desiccated for 5 min at room temperature. To register the background value, 100 μL culture medium were added to each well and the impedance was detected for 2 h. The given time interval was sufficient in each experiment to obtain constant background curves of impedance. In the next two steps, GnRH-III containing bioconjugates or free anticancer drugs (in the concentration range of 10^{-9}–10^{-6} M) were added and then the wells were loaded with MM6 monocytes (10^4 cells/100μL/well). Compound free wells were used as a control. The impedance was recorded at 10 kHz, with 20 s interval for 24 h. Each measurement was carried out in triplicates. The slope parameter was used to statistically evaluate the changes in the Cell Index after treatment with a particular compound. This parameter describes the steepness and changing rate of an adhesion curve within a given time interval and calculated by linear regression analysis of the integrated software (RTCA 1.2).

4.12. Statistical analysis

Statistical analysis was performed using the ANOVA algorithm (OriginPro 8.0). Histograms obtained from CASY were further analyzed by the Kolmogorov–Smirnov test (XLSTAT module of MS Excel). The slope analysis was performed by RTCA 1.2. Significance levels correspond to x: $p < 0.05$; y: $p < 0.01$; z: $p < 0.001$.

Conflict of interest

None.

Author contributions

Conceived and designed the experiments: MM, GM, LK.
Performed the experiments: UL, EL, EO.
Analyzed and interpreted the data: UL, MM, EL.
Contributed to the cell cultures and MTT assay: PÖ.
Contributed to the mass spectrometric analyses: AM.
Wrote the paper: UL, MM, GM, EL, LK.
Revised critically the paper: all authors.
All authors read and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2012.03.016.

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