In-vivo antitumour effect of daunorubicin–GnRH-III derivative conjugates on colon carcinoma-bearing mice

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Targeted cancer chemotherapy is a novel approach developed for the specific delivery of anticancer drugs. Tumour targeting can be achieved by combining a chemotherapeutic agent with a targeting moiety that recognizes tumour-specific or highly expressed receptors on cancer cells. We used the gonadotropin-releasing hormone-III (GnRH-III) as a targeting moiety to which the chemotherapeutic agent daunorubicin (Dau) was attached through an oxime bond either directly or by inserting a GFLG tetrapeptide spacer. The in-vivo toxicity of Dau–GnRH-III derivative conjugates was evaluated on healthy BDF-1 female mice, and their tumour growth inhibitory effect was determined on C26 murine and HT-29 human colon carcinoma-bearing mice. Both oxime bond-containing conjugates were well tolerated and exerted significant antitumour activity on C26 colon carcinoma-bearing mice at a dose of 30 mg Dau content in conjugate/kg body weight. Furthermore, the conjugates inhibited the tumour growth more than the free drug at a dose that was still not toxic. Similar tumour growth inhibitory effects were obtained on HT-29 human colon carcinoma-bearing mice using three treatments with 15 mg Dau content in conjugate/kg. The tumour growth inhibitions according to the tumour volume and the tumour weight were 44/41% and 58/50%, respectively. Considering the results, both of the investigated Dau–GnRH-III derivative conjugates were well tolerated and had significant antitumour effect on colon carcinoma-bearing mice. *Anti-Cancer Drugs* 23:90–97 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The incidence and mortality rates for most types of cancer (including lung, colorectal, prostate and female breast cancer) are decreasing in the United States and many other Western countries; however, they are increasing in several less developed and economically transitioning countries [1]. Colorectal cancer is still one of the most frequent types of cancer, with increasing incidence rates in men and women under 50 years of age (*http://www.cancer.gov/newscenter/pressreleases/ReportNation2009Release*) [2,3]. Efficient therapeutic approaches, such as surgery followed by adjuvant radiation or chemotherapy, can be implemented only in the case of an early stage diagnosis [4]; otherwise, the response to the treatment is in general temporary. When considering the incidence and mortality rates of colorectal cancer, the development of highly efficient new drugs is of utmost importance. However, the application of small organic compounds as drugs is usually limited because of their nonselectivity for cancer cells over normal cells, leading to severe side effects and low cure rates. Targeted delivery of anticancer drugs has recently become one of the most investigated approaches in cancer chemotherapy [5]. The combination of anticancer drugs with a targeting moiety that recognizes tumour-specific or highly expressed receptors on cancer cells might provide efficient chemotherapeutic agents with minimal systemic toxicity [6]. Peptide-based tumour targeting is a novel approach developed for the specific delivery of anticancer drugs [7]. It was found that receptors for peptide hormones such as gonadotropin-releasing hormone (GnRH, also called luteinizing hormone-releasing hormone) and somatostatin are highly expressed on cancer cells compared with normal cells and serve as targets for peptide ligands to which cytotoxic drugs can be linked [8,9]. On the basis of these observations, anticancer drug–hormone peptide conjugates have been developed by Bajusz *et al.* [10]. The most promising compounds consisted of doxorubicin (Dox) or 2-pyrrolino-doxorubicin attached through an ester bond to appropriate hormone peptide derivatives [e.g. (D-Lys^6)-GnRH] that were used as targeting moieties [11,12]. The antiproliferative effect of the conjugates was evaluated *in vitro* and *in vivo* on various types of tumours, including colon carcinoma (HT-29, HCT-15, HCT-116, LoVo and Colo-320DM) [12,13]. In most cases, the conjugates containing 2-pyrrolino-doxorubicin exerted the highest antitumour activity; however, their toxic side effects were higher compared with the

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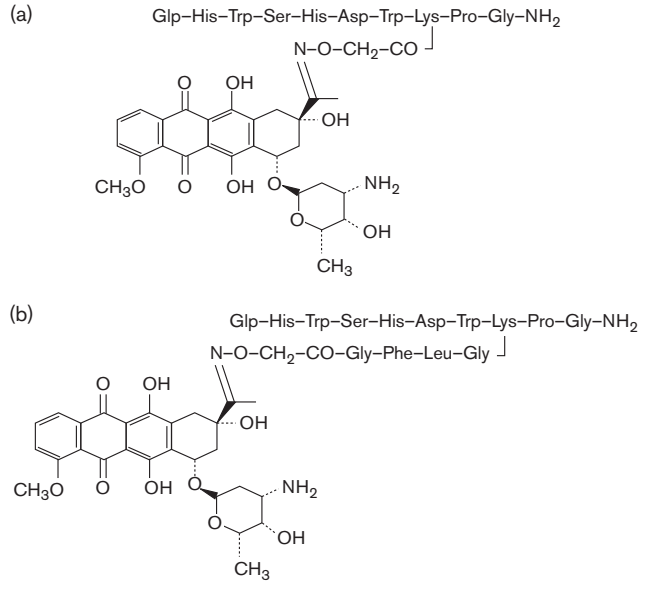
doxorubicin-containing compounds. These results might be explained by the fast drug release from the conjugates in the circulation caused by carboxylesterases [14]. It was evident that 2-pyrrolino-doxorubicin was more cytotoxic than doxorubicin. Therefore, only the ester bond-linked Dox–GnRH conjugate [AN-152, AEZS-108 (Æterna Zentaris Inc., Québec, Canada)] was further investigated in clinical trials on ovarian and endometrial cancer (*http://www.aezsinc.com*).

In our work, GnRH-III [a weak GnRH agonist peptide that was first isolated from the sea lamprey (*Petromyzon marinus*)] was used as a targeting moiety [15]. It has been shown that GnRH-III (Glp-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2) binds to GnRH receptors [16] and that it has antitumour activity on many tumour types [17] and much lower endocrine effect in mammals than the human GnRH [18]. The lysine in position 8 of GnRH-III can be modified (e.g. by cyclization, dimerization or conjugation) without a significant decrease in the biological activity [19–21]. We have previously shown that the attachment of daunorubicin (Dau) to GnRH-III via an oxime bond resulted in a conjugate having in-vitro and in-vivo antitumour activity [22]. In this compound, a GFLG tetrapeptide spacer cleavable by cathepsin B was incorporated between the drug and the GnRH-III. The in-vivo antitumour activity of GnRH-III(Dau=Aoa-GFLG) conjugate (where Aoa is aminooxyacetyl) was evaluated in C26 murine colon carcinoma-bearing mice, and it was dependent on the number and start of the treatments. The highest tumour growth inhibition (46%) was obtained when the mice were treated on days 4 and 7 after tumour transplantation with the conjugate at a dose of 62.5 mg/kg body weight (15 mg Dau content, 26.6 µmol). A significant increase in the survival of the mice was also observed. The above-mentioned amount of conjugate was not toxic on healthy mice, whereas free Dau was toxic at a lower dose (5 mg/kg body weight). We have recently demonstrated that the incorporation of the GFLG tetrapeptide spacer in the structure of the compound is not necessary for the in-vitro cytostatic effect [23]. The degradation of both conjugates (Fig. 1), GnRH-III(Dau=Aoa-GFLG) and GnRH-III(Dau=Aoa), in the presence of rat liver lysosomal homogenate did not result in the release of free Dau. Moreover, the resulting metabolites [Dau = Aoa-Gly-OH and H-Lys (Dau = Aoa)-OH] had similar binding affinities to DNA, an outcome that might explain their bioactivity.

Fig. 1



Structure representation of (a) GnRH-III(Dau=Aoa) and (b) GnRH-III(Dau=Aoa-GFLG) conjugates. Dau, daunorubicin; GnRH, gonadotropin-releasing hormone.

Here we report on the further optimization of the treatment schedule with Dau–GnRH-III derivative conjugates and their in-vivo antitumour activity on HT-29 human colon carcinoma-bearing severe combined immunodeficiency (SCID) mice. Our data indicate that both investigated compounds, GnRH-III(Dau=Aoa-GFLG) and GnRH-III(Dau=Aoa), are well tolerated and have significant antitumour activity.

Materials and methods

Synthesis of Dau–GnRH-III derivative conjugates

The synthesis of Glp-His-Trp-Ser-His-Asp-Trp-Lys(Dau = Aoa)-Pro-Gly-NH_2 (1) and Glp-His-Trp-Ser-His-Asp-Trp-Lys(Dau = Aoa-Gly-Phe-Leu-Gly)-Pro-Gly-NH_2 (2) (where Glp is pyroglutamic acid) was described previously [22,23]. In brief, the aminooxyacetylated hormone peptide derivatives were prepared by solid-phase peptide synthesis on a Rink-Amide MBHA resin according to the Fmoc/^{t}Bu strategy. The attachment of Dau to the cleaved and purified peptides was carried out in solution (0.2 mol/l NaOAc, pH 5).

Acute toxicity of Dau and Dau–GnRH-III derivative conjugates

First-generation hybrid BDF_1 (C57BL/6 female and DBA/2 male) adult female mice, weighing 22–24 g, specified pathogen free from the Department of Exprimental Pharmacology, National Institute of Oncology, Budapest, Hungary, were used (colonies were used in these experiments). The animals were kept in macrolon cages at 22–24°C (40–50% humidity), with a lighting regimen of 12/12 h light and dark. They had free access to tap water and were fed with a sterilized standard diet (Akronom Kft., Budapest, Hungary) *ad libitum*. The animals used in these studies were cared for according to the 'Guiding Principles for the Care and Use of Animals' based on the Helsinki declaration and they were approved by the local ethics committee.

Dau, GnRH-III(Dau=Aoa) and GnRH-III(Dau=Aoa-GFLG) conjugates were dissolved in distilled water at a concentration that allowed the dose (2 or 10 mg/kg for Dau and 30 mg Dau content/kg for conjugates) to be given in a volume of 0.1 ml/10 g body weight. In this experiment, groups of 7–7 mice were used. The conjugates were administered intraperitoneally on days 1, 3, 5 and 8. The toxicity was evaluated on the basis of life span and body weight. The survival time was followed over 19 days.

In-vivo anti-tumour effect of Dau and Dau–GnRH-III derivative conjugates on murine C26 colon carcinoma-bearing mice

Balb/c inbred female mice (albino mouse strain was originally developed by Halsey J. Bagg of Memorial Hospital, New York, in 1920), weighing 22–24 g and kept under the same conditions as described above, were used in this experiment. Tumour tissue fragments (Colon-26 mouse carcinoma, SRI, Birmingham, Alabama, USA; 3–4 mm^3, ~25 mg weight) were transplanted subcutaneously into one side of the intrascapular region of the mice using a tweezers. The treatments started on day 4 after tumour transplantation by intraperitoneal administration of the compounds dissolved in distilled water (7–7 mice/group were used). Except for the control group, the mice were treated with either free Dau [twice 2 mg (3.55 μmol)/kg body weight on days 4 and 7 after tumour transplantation] or GnRH-III(Dau=Aoa) and GnRH-III(Dau=Aoa-GFLG) conjugates [twice 104 and 125 mg/kg, respectively (53.2 μmol; 30 mg Dau content) on the same days]. An additional group of mice (5–5 mice/group) was treated four times with the same dose of Dau or GnRH-III(Dau=Aoa-GFLG) conjugate per treatment on days 4, 7, 10 and 15 after tumour transplantation. Using a digital calliper, the tumour volume was determined seven times within 3 weeks following the tumour transplantation. The tumour volume was calculated using the following formula: $V = a^2 \times b \times \pi/6$ (where a and b represent the shortest and the longest diameter, respectively, of a given tumour) [24]. The changes of the body weight and the survival time were also determined (the survival probability was calculated according to the Kaplan–Meier curve, which was drawn using the MedCalc 10 program).

Routine histological examination was carried out in order to evaluate the toxic side effects of the conjugates and the free drug on heart, liver, kidney and spleen of the treated C26 colon carcinoma-bearing mice.

In-vivo antitumour activity of Dau and Dau–GnRH-III derivative conjugates on human HT-29 colon carcinoma-bearing SCID mice

The immunodeficient SCID mice on a C.B.-17 background were bred in our Specific Opportunistic and Pathogen Free isolator breeding rooms. The breeding pairs originated from Jackson Laboratories. The breeding isolator was supplied with 25 kGry γ-irradiated corn-cob bedding and standard VRF1 rodent chow, and with acidified (pH 3) sterilized distilled water. The mice from the breeding rooms were used for the xenograft transplantation. They were held in filter-top boxes in the experimental barrier rooms, and every box-opening was performed under a Class 100 laminar-flow hood by an operator dressed in sterilized surgical attire. The gloves were disinfected before opening the boxes. The animal housing density was in accordance with the international recommendations.

The cage components, corn-cob bedding and food (VRF1 from Special Diet Services) were steam-sterilized in an autoclave (121°C, 20 min). The distilled water was acidified to pH 3 with hydrochloric acid to control the bacterial count, mainly *Pseudomonas* species. Representative mice from the breeding colony were submitted to a health-state check twice a year according to the annual FELASA recommendation. Additional cultures were performed to check for opportunistic microorganisms.

The xenografts were established by injecting subcutaneously into one side of the intrascapular region, *c.* 10^6 HT-29 colon carcinoma cells. The cells were from ATCC and cultured according to their standards. Female SCID mice weighing 25–28 g were used in this study. The mice with palpable tumours were killed by cervical dislocation and disinfected with iodine, and the subcutaneous tumour was dissected out aseptically. Tumour pieces of 2–3 mm^3 were transplanted subcutaneously, into one side of the intrascapular region, under aseptic conditions into narcotized SCID mice (65 mg/kg pentobarbital sodium intraperitoneally). In all groups, 7–7 mice were used. The treatments started when the tumours were well established at a size of around 200 mm^3. The first treatment was carried out on day 13 after tumour transplantation using conjugates at a dose of 15 mg/kg body weight Dau content [GnRH-III(Dau=Aoa): 52 mg/kg and GnRH-III(Dau=Aoa-GFLG): 62.5 mg/kg; 26.6 μmol in both cases]. The treatment with the same doses was repeated on days 23 and 30. The tumour size was measured as described above. The experiment was terminated on day 35 after tumour transplantation because of the bad condition of some mice from the control group. The mice from all groups were killed by cervical dislocation; their tumours were then removed and weighed in all cases.

In the case of the treatment with free Dau, doses of 2.5 mg/kg (4.43 μmol) or 5 mg/kg (8.87 μmol) were administered. After the first treatment, mice died within 10 or 7 days, respectively. Therefore, no more treatments were performed in these groups.

Routine histological examination

The removed and fixed tissue samples were dehydrated in a graded series of ethanol, infiltrated with xylene and embedded into paraffin. Histological sections were

stained with Harris haematoxylin and eosin (10:1, v/v, 1% solutions) in acidified 70% ethanol and mounted. The histological samples were examined under a light microscope (Olympus CH30; Olympus Optical, Tokyo, Japan).

Determination of the proliferation index

Before ending the experiment, the tumour-bearing animals were injected intraperitoneally with 5-bromo-2′-deoxyuridine (BrdU, 200 mg/kg; Sigma-Aldrich Kft, Budapest, Hungary). After 6 h, 5–7-μm-thick frozen sections of fresh tumours were used to detect BrdU-positive cells using an anti-BrdU monoclonal antibody (Becton Dickinson Hungary Kft., Budapest, Hungary) according to the manufacturer's protocol. Positive cells were visualized with TRITC-conjugated anti-mouse IgG (1 : 100, Sigma). To discriminate tumour cells from endothelial cells, the vessels were stained with a rat anti-mouse CD31 antibody followed by biotinylated anti-rat IgG and streptavidin–FITC (Vector Laboratories, Burlingame, California, USA), and the nuclei were stained with the Hoechst 33342 dye (Molecular Probes, Eugene, Oregon, USA). The labeling index of HT-29 tumour cells and the number of CD31-positive vessels inside the living tumour mass were determined in 10 independent areas of five different tumours.

Statistical analysis

The statistical analyses were performed by Statistica 7.0 (StatSoft, Tulsa, Oklahoma, USA) using the nonparametric Kruskal–Wallis test with post-hoc analysis. P values under 0.05 were considered statistically significant.

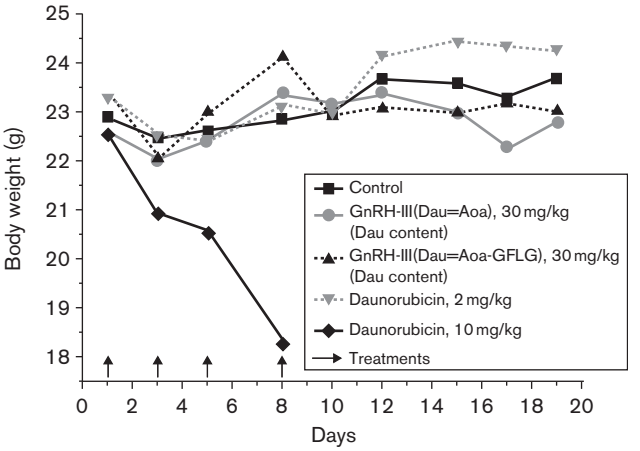
Results and discussion

Dau–GnRH-III derivative conjugates

The concept of targeted cancer chemotherapy based on human GnRH derivatives was introduced by A.V. Schally *et al.* in the late 1980s. The most promising compound developed by Schally's group, in which doxorubicin-14-*O*-hemiglutarate was conjugated to [D-Lys^6]-GnRH-I [AN-152, AEZS-108 (Æterna Zentaris Inc.,)], is currently in a phaseII clinical trial on ovarian and endometrial cancer (*http://www.aezsinc.com*). This compound was also tested *in vivo* on colon cancer-bearing mice [13]. In one experiment, 2 mm^3 pieces of HT-29 tumour tissue were transplanted subcutaneously into male athymic nude mice. The treatment of HT-29 tumour-bearing mice started on day 14 after tumour transplantation and continued for 55 days. The compound, at a dose of 20.7 μmol/kg, was administered intraperitoneally on days 1 and 28. The final tumour volume and weight in the case of the treated animals were 735 ± 158 mm^3 and 944 ± 218 mg, respectively, compared with those of the control group (1219 ± 372 mm^3 and 1236 ± 374 mg, respectively), indicating 25–40% tumour growth inhibition.

We have recently developed Dau–GnRH-III derivative conjugates containing an oxime bond between the drug and the targeting moiety [22,23,25]. GnRH-III was selected and used as a targeting moiety because of its much lower endocrine effect in mammals compared with GnRH-I analogues. Dau was conjugated to the aminooxyacetylated GnRH-III derivatives by oxime ligation, resulting in a chemical bond that was stable in human serum, thus preventing early drug release. According to our present knowledge, no free Dau could be released from these conjugates in the presence of lysosomal homogenates [23].

Fig. 2



Effect of free Dau and Dau–GnRH-III derivative conjugates on changes in the body weight and survival of healthy BDF-1 female mice (seven animals per group, values report the average). Dau, daunorubicin; GnRH, gonadotropin-releasing hormone.

In this work, the in-vivo toxicity and antitumour activity of two oxime bond-linked Dau–GnRH-III derivative conjugates are reported. The chemotherapeutic agent was attached to the targeting moiety either directly or by insertion of a GFLG tetrapeptide spacer (Fig. 1).

In-vivo toxicity of Dau–GnRH-III derivative conjugates and their antitumour activity on murine C26 colon carcinoma-bearing mice

We have previously reported that GnRH-III(Dau = Aoa-GFLG) conjugate was well tolerated by healthy BDF-1 female mice at a dose of 62.5 mg/kg body weight (15 mg Dau content in conjugate) administered once. A similar amount of Dau was highly toxic, according to the rapid loss of the body weight and early death of the mice [22]. In this study, we investigated whether the maximum tolerated dose of the GnRH-III(Dau=Aoa) and GnFH-III(Dau=Aoa-GFLG) conjugates could be elevated. Therefore, the mice were treated four times on days 1, 3, 5 and 8 with 104 mg GnRH-III(Dau=Aoa)/kg and 125 mg GnRH-III(Dau=Aoa-GFLG)/kg, respectively (53.2 μmol; 30 mg Dau content). Both conjugates were not toxic at the tested dose (the body weight of the treated mice did not differ significantly from that of the

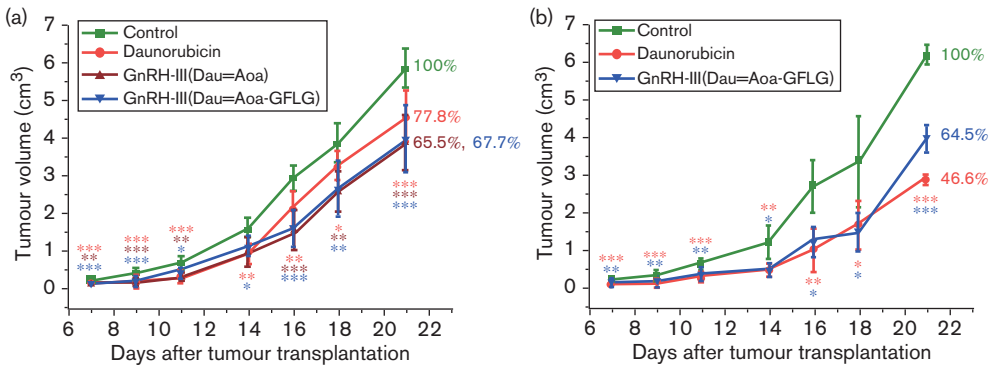
control animals) (Fig. 2). However, the mice treated four times with 10 mg Dau/kg died within 10 days, whereas four times administration of 2 mg Dau/kg was well tolerated.

The in-vivo antitumour activity of the compounds was determined on C26 colon carcinoma-bearing Balb/c female mice (seven mice per group). C26 murine colon carcinoma represents a good model for the rapid evaluation of the antitumour activity of peptide–drug conjugates, as it is an aggressive, rapidly proliferating tumour (tumour volume doubling time is approximately 3 days) and it contains GnRH receptors [22]. Furthermore, the in-vitro cytostatic effect of Dau and Dau–GnRH conjugates on C26 cells was similar to that determined on HT-29 human colon carcinoma cell lines [26]. According to our previous data [22] and the results of recent toxicological studies, the treatments of the tumour-bearing mice were carried out on days 4 and 7 after tumour transplantation using 104 mg GnRH-III(Dau=Aoa)/kg and 125 mg GnRH-III(Dau=Aoa-GFLG)/kg, respectively (53.2 μmol; 30 mg Dau content), and 2 mg Dau/kg. Furthermore, two groups of animals (five mice per group) were treated with the GnRH-III(Dau = Aoa-GFLG) and Dau two more times, on days 10 and 15. These treatment schedules have been designed on the basis of our previous study [22], in which C26 murine colon carcinoma-bearing Balb/c mice were treated either with free Dau (5×2 mg/kg body weight or 1×5 mg/kg) or with GnRH-III(Dau=Aoa-GFLG) conjugate (5×5 mg Dau content in conjugate/kg). The conjugate had no significant tumour growth inhibitory effect (15% inhibition), whereas the administration of free Dau at the dose of 5×2 mg/kg led to 23% tumour growth inhibition. At a higher dose, the free drug exerted very high toxicity. Thus, in this work, we decided not to decrease the dose of the conjugate and to increase the dose of the free drug in order to evaluate their tumour growth inhibitory effect at the same dose. The tumour volume as an indicator of the inhibitory effect of the drug and conjugates was measured every second or third day until day 21, when the first animal from the control group died. In the case of all treated groups (either with the free drug or the conjugate), significant tumour growth inhibition was observed, especially until the third day after the last injection (Fig. 3). However, after finishing the treatment, the tumour started growing rapidly. At the end of the experiments, 30–40% tumour growth inhibition was determined in mice treated with the conjugates. There was no significant difference between the groups treated with the non-spacer-containing or spacer-containing conjugates (Fig. 3a).

The tumour growth inhibitory effect persisted in the group treated four times with the GnRH-III(Dau=Aoa-GFLG) (50–60% inhibition on day 18), and it was significantly higher than the inhibition observed in the case of mice treated only twice. However, on day 21, the tumour volumes were not significantly different (35% inhibition) (Fig. 3b). All the groups treated with the conjugates showed 15–20% longer survival (the median survival was calculated according to the Kaplan–Meier curve) than the controls (Fig. 4). The tumour growth inhibition was lower (22%) in the group treated two times with the free drug (Fig. 3a). The survival time was also not significantly higher in this group compared with the controls (Fig. 4a). The elevation of the number of treatments with Dau (four treatments) led to increased tumour growth inhibition (53%) (Fig. 3b). However, three of five mice from this group died on day 21 because of the high toxicity of Dau at the applied dose (Fig. 4b).

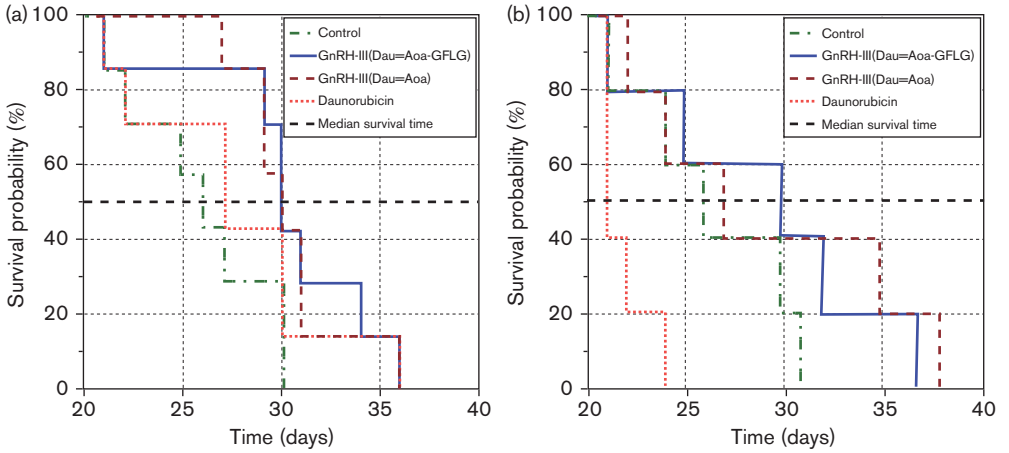
Routine histological examination of heart, liver, kidney and spleen tissues was carried out in order to determine the toxic side effects of the conjugates (two times treatment with 30 mg Dau content in conjugates/kg) or the free drug (two times treatment with a dose of 2 mg/kg).

Fig. 3



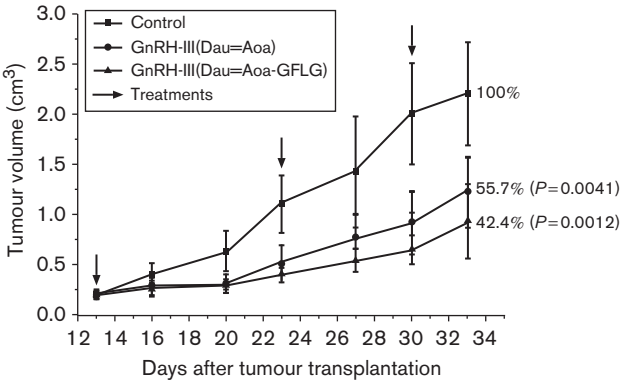
In-vivo antitumour effect of Dau and Dau–GnRH-III derivative conjugates on murine C26 colon carcinoma-bearing Balb/c mice: (a) compounds were administered twice on days 4 and 7; (b) compounds were administered four times on days 4, 7, 10 and 15. Significance: *$P<0.05$; **$P<0.01$; ***$P<0.0001$. Dau, daunorubicin; GnRH, gonadotropin-releasing hormone.

Fig. 4



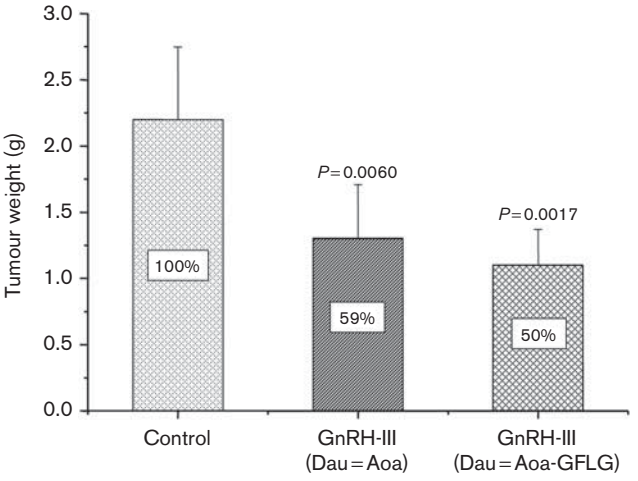
Survival probability according to the Kaplan–Meier analysis. Untreated murine C26 colon carcinoma-bearing Balb/c mice (control group) were compared with the mice treated with Dau or Dau–GnRH-III derivative conjugates (a) twice on days 4 and 7 or (b) four times on days 4, 7, 10 and 15. Dau, daunorubicin; GnRH, gonadotropin-releasing hormone.

Fig. 5



In-vivo antitumour effect of Dau–GnRH-III derivative conjugates on human HT-29 colon carcinoma-bearing SCID mice. Dau, daunorubicin; GnRH, gonadotropin-releasing hormone; SCID, severe combined immunodeficiency.

Fig. 6



In-vivo antitumour effect of Dau–GnRH-III derivative conjugates on human HT-29 colon carcinoma-bearing SCID mice on day 35 (Mann–Whitney test). Dau, daunorubicin; GnRH, gonadotropin-releasing hormone; SCID, severe combined immunodeficiency.

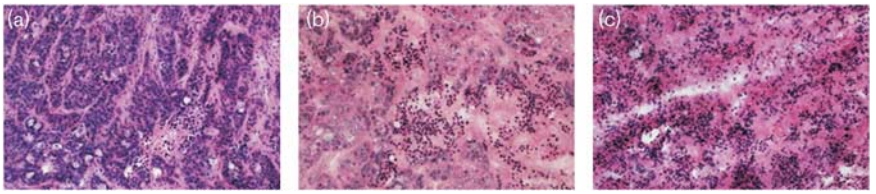
There were no significant changes on the vital organs studied that could suggest toxic side effects of the conjugates. No differences on the tissues of different organs were observed between the treated animals.

Overall, oxime bond-containing Dau–GnRH-III derivative conjugates had low toxicity and significant antitumour activity on C26 colon carcinoma-bearing mice at a dose of 30 mg Dau content/kg. Furthermore, the presence of the GFLG tetrapeptide spacer between the targeting moiety and the drug had no influence on the biological activity (the conjugate without a spacer had an antitumour activity that was similar to that of the conjugate). The average value of the tumour growth inhibition was slightly lower than that determined in our previous work when only half the amount of conjugate per treatment was used [22]. However, the significance of the inhibitory effect between the treated and nontreated animals was higher in this study. The results also revealed that the conjugates inhibited tumour growth more than the free drug at a dose that was still not toxic.

In-vivo antitumour activity of conjugates on human HT-29 colon carcinoma-bearing SCID mice

The in-vivo antitumour activity of the conjugates on human HT-29 colon carcinoma was determined on SCID mice as good hosts for the implantation of tumour xenografts. This mouse strain was functionally depleted of T and B cells and lacked precursors of lymphokine-

Fig. 7



Effect of Dau–GnRH-III derivative conjugates on subcutaneously growing HT-29 tumour xenografts (HE stain). Untreated HT-29 tumour tissue (a), HT-29 tumour treated with GnRH-III(Dau=Aoa) (b) and HT-29 tumour treated with GnRH-III(Dau=Aoa-GFLG) (c). Magnification: × 200. Dau, daunorubicin; GnRH, gonadotropin-releasing hormone.

Table 1 BrdU labeling index and the vessel number inside the living tumour areas

	BrdU index			CD3-positive vessel number		
	Control	Conjugate1	Conjugate2	Control	Conjugate1	Conjugate2
Mean ± SD	34.6 ± 6.7	34.1 ± 7.1	35.6 ± 8.9	26.3 ± 7.9	22.0 ± 8.9	12.3 ± 6.5
P value		0.879	0.768		0.304	0.001

Conjugate1, GnRH-III(Dau=Aoa); Conjugate2, GnRH-III(Dau=Aoa-GFLG); Dau, daunorubicin; GnRH, gonadotropin-releasing hormone.

activated killer cells. Colon carcinoma seemed to have a higher acceptance in SCID mice than in nude mice, resulting in a faster tumour growth [27]. Furthermore, the incidence of splenic tumourigenesis and liver metastases was significantly greater in SCID mice for all colon cancer xenografts in comparison with nude mice [28]. The treatment schedule of HT-29 colon carcinoma-bearing SCID mice was designed according to the results obtained in the studies on C26 tumour-bearing mice. Because no improvement in the tumour growth inhibition was observed when the conjugate was used at a dose of 30 mg Dau content/kg instead of 15 mg/kg, we decided to apply the lower dose in this experiment. Three treatments were carried out, but in less frequent injections because the HT-29 tumour grows at a much slower rate than the C26. In contrast to published data [13], the first treatment was carried out only when the tumour volume reached 200 mm^3 (13 days after the tumour transplantation). We believe that this size corresponds better to the stage of a diagnosed tumour than to a tumour size below 50 mm^3. The second and third treatments were carried out on days 23 and 30. Two different doses of Dau (2.5 and 5 mg/kg) were also applied in a comparative study. The tumour growth was inhibited in both cases. However, all mice treated with 5 mg Dau/kg died within a week after the treatment. Furthermore, mice treated with 2.5 mg Dau/kg died on day 10 after the treatment, before the second injection. The experiment was terminated on day 35 after tumour transplantation, because some mice from the control group were in a bad condition. The mice from all groups were killed. Their tumours were then removed and weighed (Fig. 5). Both the tumour volume (the last measurement was performed on day 33) and the tumour weight measurements indicated slightly, but not significantly, a higher tumour growth inhibition in the case of spacer-containing conjugate [GnRH-III(Dau=Aoa-GFLG)] in comparison with the compound without spacer [GnRH-III(Dau=Aoa)]. The tumour growth inhibition according to the tumour volume (Fig. 6a) and the tumour weight (Fig. 6b) were 58/50 and 44/41%, respectively.

Histological examination

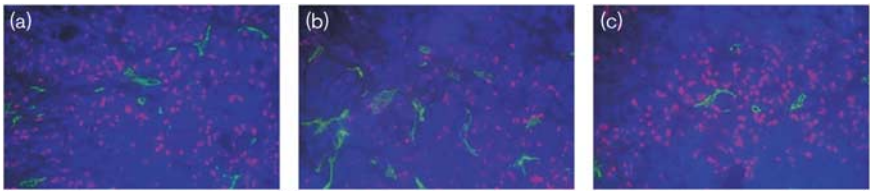
The treatment of HT-29 xenograft-bearing mice with GnRH-III(Dau=Aoa) and GnRH-III(Dau=Aoa-GFLG) conjugates induced a massive tumour regression. The living tumour mass was fragmented and massive lymphocyte infiltration was detected around the tumour cell islands of the treated animals (Fig. 7). This was not observed in the case of tumours derived from the control group (Fig. 7a). In the case of GnRH-III(Dau-Aoa-GFLG), no significant amount of living tumour cells could be detected inside the lymphocyte infiltration area (Fig. 7c).

The treatments had no effect on the BrdU labeling index (representing the proliferation capacity of the tumour cells); however, the vessel number inside the living tumour areas in the tumours treated with GnRH-III(Dau-Aoa-GFLG) (Table 1, Fig. 8c) was significantly smaller compared with the controls and the ones treated with GnRH-III(Dau=Aoa) (Table 1, Figs. 8a and b).

Conclusions

Oxime bond-linked Dau–GnRH-III derivative conjugates exerted a significant in-vivo tumour growth inhibitory effect on murine C26 and human HT-29 colon carcinoma-bearing mice. The treatment with the conjugates increased the lymphocyte infiltration inside the HT-29 tumours significantly. The slightly higher antitumour effect of the spacer-containing conjugate [GnRH-III(DAu=Aoa-GFLG)] might

Fig. 8



Effect of conjugates on BrdU labeling index and the vessel number of human HT-29 xenograft. Immunofluorescence microscopy: BrdU (red), CD31 (green) and Hoechst 33342 (nuclear staining, blue) triple labeling of control (a); GnRH-III(Dau=Aoa)-treated (b) and GnRH-III(Dau=Aoa-GFLG)-treated (c) HT-29 tumours. Dau, daunorubicin; GnRH, gonadotropin-releasing hormone.

be explained by the lower blood vessel formation in the tumours. These effects should be further investigated. The conjugates were not toxic at a dose of Dau content that was much higher than the maximum tolerated dose of the free drug.

Although the lack of a comparative study in the same in-vivo system does not allow us to draw a conclusion, it seems that the oxime bond-linked Dau–GnRH-III derivative conjugates have similar tumour growth inhibitory effect on subcutaneously transplanted HT-29 colon carcinoma as it was observed in the case of nude mice treated with AN-152.

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Conflicts of interest

There are no conflicts of interest.

References

1 Jemal A, Center MM, DeSantis C, Ward EM. Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiol Biomarkers Prev* 2010; **19**:1893–1907.
2 Meyer JE, Narang T, Schnoll-Sussman FH, Pochapin MB, Christos PJ, Sherr DL. Increasing incidence of rectal cancer in patients aged younger than 40 years: an analysis of the surveillance, epidemiology, and end results database. *Cancer* 2010; **116**:4354–4359.
3 Yiu HY, Whittemore AS, Shibata A. Increasing colorectal cancer incidence rates in Japan. *Int J Cancer* 2004; **109**:777–781.
4 Aranha O, Benson AB III. Adjuvant therapy for colon cancer. *Curr Gastroenterol Rep* 2007; **9**:415–421.
5 Singh Y, Palombo M, Sinko PJ. Recent trends in targeted anticancer prodrug and conjugate design. *Curr Med Chem* 2008; **15**:1802–1826.
6 Reubi JC. Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocr Rev* 2003; **24**:389–427.
7 Schally AV, Nagy A. Cancer chemotherapy based on targeting of cytotoxic peptide conjugates to their receptors on tumours. *Eur J Endocrinol* 1999; **141**:1–14.
8 Schally AV, Nagy A. Chemotherapy targeted to cancers through tumoural hormone receptors. *Trends Endocrinol Metab* 2004; **15**:300–310.
9 Mező G, Manea M. Receptor-mediated tumour targeting based on peptide hormones. *Expert Opin Drug Deliv* 2010; **7**:79–96.
10 Bajusz S, Janáky T, Csernus VJ, Bokser L, Fekete M, Srkalovic G, *et al.* Highly potent analogues of luteinizing hormone-releasing hormone containing D-phenylalanine nitrogen mustard in position 6. *Proc Natl Acad Sci USA* 1989; **86**:6318–6322.
11 Nagy A, Schally AV, Armatis P, Szepesházi K, Halmos G, Kovács M, *et al.* Cytotoxic analogs of luteinizing hormone-releasing hormone containing doxorubicin or 2-pyrrolinodoxorubicin, a derivative 500-1000 times more potent. *Proc Natl Acad Sci USA* 1996; **93**:7269–7273.
12 Nagy A, Schally AV. Targeting of cytotoxic luteinizing hormone-releasing hormone analogs to breast, ovarian, endometrial, and prostate cancers. *Biol Reprod* 2005; **73**:851–859.
13 Szepesházi K, Schally AV, Halmos G. LH-RH receptors in human colorectal cancers: unexpected molecular targets for experimental therapy. *Int J Oncol* 2007; **30**:1485–1492.
14 Kovács M, Szepesházi K, Schally AV. Endocrine and antineoplastic effects of antagonistic and cytotoxic analogs of luteinising hormone-releasing hormone. In: Kovács M, Merchenhalter I, editors. *Neuropeptides and peptide analogs*. Kerela, India: Research Signpost. 2009; pp. 33–57.
15 Sower SA, Chiang Y-C, Lovas S, Conlon JM. Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. *Endocrinology* 1993; **132**:1125–1131.
16 Millar RP. GnRHs and GnRH receptors. *Anim Reprod Sci* 2005; **88**: 5–28.
17 Lovas S, Pályi I, Vincze B, Horváth J, Kovács M, Mező I, *et al.* Direct anticancer activity of gonadotropin-releasing hormone-III. *J Pept Res* 1998; **52**:384–389.
18 Kovács M, Vincze B, Horváth JE, Seprődi J. Structure-activity study on the LH- and FSH-releasing and anticancer effects of gonadotropin-releasing hormone (GnRH)-III analogs. *Peptides* 2007; **28**:821–829.
19 Mező I, Lovas S, Pályi I, Vincze B, Kálnay A, Turi G, *et al.* Synthesis of gonadotropin-releasing hormone III analogs. Structure-anti-tumour activity relationships. *J Med Chem* 1997; **40**:3353–3358.
20 Mező G, Czajlik A, Manea M, Jakab A, Farkas V, Majer Z, *et al.* Structure, enzymatic stability and anti-tumour activity of sea lamprey GnRH-III and its dimer derivatives. *Peptides* 2007; **28**:806–820.
21 Pályi I, Vincze B, Lovas S, Mező I, Pató J, Kálnay A, *et al.* Gonadotropin-releasing hormone analogue conjugates with strong selective anti-tumour activity. *Proc Natl Acad Sci U S A* 1999; **96**:2361–2366.
22 Szabó I, Manea M, Orbán E, Csámpai A, Bősze S, Szabó R, *et al.* Development of an oxime bond containing daunorubicin-gonadotropin-releasing hormone-III conjugate as a potential anticancer drug. *Bioconjug Chem* 2009; **20**:656–665.
23 Orbán E, Mező G, Schlage P, Csik G, Kulic Z, Ansorge P, *et al.* In vitro degradation and anti-tumour activity of oxime bond-linked daunorubicin-GnRH-III bioconjugates and DNA-binding properties of daunorubicin-amino acid metabolites. *Amino Acids* 2010; **41**:469–483.
24 Tomayko MM, Reynolds CP. Determination of subcutaneous tumour size in athymic (nude) mice. *Cancer Chemother Pharmocol* 1989; **24**: 148–154.
25 Mező G, Manea M, Szabó I, Vincze B, Kovács M. New derivatives of GnRH as potential anticancer therapeutic agents. *Curr Med Chem* 2008; **15**:2366–2379.
26 Mező G, Orbán E, Szabó I, Hegedűs R, Bősze S, Tejeda M, *et al.* GnRH based drug delivery systems for targeted tumour therapy. *Collections Symp Series* 2009; **11**:72–76.
27 Kubota T, Yamaguchi H, Watanabe M, Yamamoto T, Takahara T, Takeuchi T, *et al.* Growth of human tumour xenografts in nude mice and mice with severe combined immunodeficiency (SCID). *Surg Today* 1993; **23**:375–377.
28 Furukawa T, Kubota T, Watanabe M, Nishibori H, Kuo T-H, Saikawa Y, *et al.* A suitable model for experimental liver metastasis of human colon cancer xenografts using mice with severe combined immunodeficiency. *J Surg Oncol* 1993; **52**:64–67.