# DEVELOPMENT OF BIOCONJUGATES AND THEIR MODUL CONSTRUCTS FOR TARGETED THERAPY OF CANCERS WITH HIGH MORTALITY

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# Development of novel cyclic NGR peptide–daunomycin conjugates with dual targeting property

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## Introduction

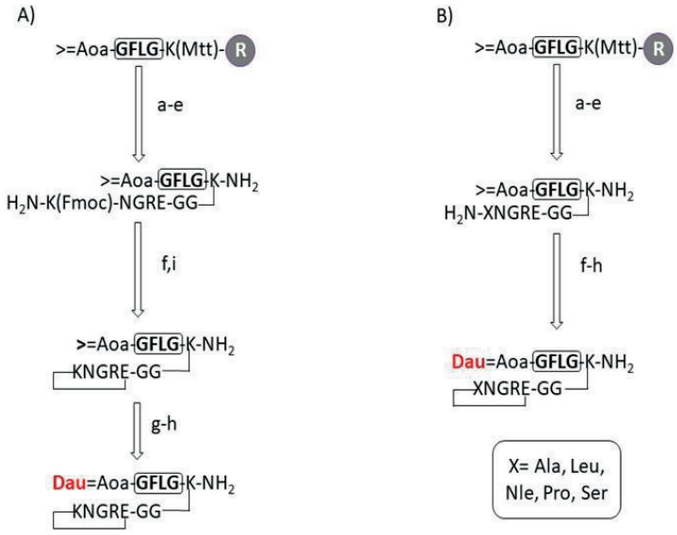
Targeted chemotherapy is one of the most promising approaches for selective cancer treatment that may decrease the toxic side effects of anticancer drugs. This therapeutic approach is based on the fact that tumor-specific receptors are highly expressed on cancer cells/tissues. NGR-motif (Asn-Gly-Arg) containing peptides identified by phage display are suitable candidates for selective drug delivery. NGR peptides bind to CD13-receptors on tumor cells and tumor-related angiogenic blood vessels.[1] However, it is known that the Asn-Gly moiety is subject to Asn deamidation through succinimide formation leading to isoaspartic acid (*iso*Asp, *iso*D) and aspartic acid derivatives usually in a ratio of 3:1 after hydrolysis.[2] *Iso*DGR peptides are bound to RGD-integrin receptors with high affinity.[3] Due to their function in tumor proliferation, metastasis and angiogenesis, integrin-receptors are also promising targets for cancer therapy. Thus, NGR-peptide homing devices may provide dual-targeted delivery of anticancer drugs.

In our previous studies, cyclic NGR derivatives with different bonds (amide, disulfide or thioether) in the cycle were applied for drug targeting. Special attention was paid to the chemostability and *in vitro* biological activity of the compounds.[4,5] Our results indicated that the conjugates had an antitumor effect on both the CD13(+) HT-1080 human fibrosarcoma cells and the CD13(−) HT-29 human colon adenocarcinoma cells. Both cell lines were integrin positive. Moreover, we showed that the toxicity and the selectivity of the conjugates highly depended on their structure, cellular uptake and propensity to deamidation. The most active conjugate was Dau=Aoa-GFLGK(c[KNGRE]-GG-)-$NH_2$. In this conjugate, the cyclic NGR peptide was attached through a Gly-Gly dipeptide spacer to the lysine side chain connected to the Cathepsin B labile GFLG spacer that allows lysosomal drug release. Dau was conjugated to the GFLG spacer *via* oxime linkage through an incorporated

aminooxyacetyl (Aoa) moiety. The preparation of the conjugate required a sophisticated synthetic route and the use of orthogonal protecting groups (Figure 1A). Previous studies indicated that the free ε-amino group of Lys does not have an impact on biological activity.[6] To prove our assumption, a set of novel cyclic NGR peptide-Dau conjugates were developed in which the Lys was replaced by different amino acids (Ala, Leu, Nle, Pro and Ser). The main goal of the present study was to investigate whether the exchange of the lysine in the cycle has any influence on the chemostability, selectivity and anti-tumor activity of the conjugates.

**Results**

Five new conjugates were developed by the replacement of Lys in the cycle in the conjugate Dau=Aoa-GFLGK(c[KNGRE]-GG-)-$NH_2$ (**1**) with different amino acids (Nle (**2**), Ala (**3**), Leu (**4**), Pro (**5**) and Ser (**6**)).[7] These new conjugates could be prepared with better yield in comparison with conjugate **1** because of the simpler synthetic route (Figure 1).




a) Mtt-cleavage: 2% TFA/DCM; b) Fmoc-Aaa(X)-OH coupling; c) Fmoc-cleavage 2% piperidine/2% DBU/DMF, 0.1 M HOBt; d) cleavage from resin 2.5% TIS/ 2.5% $H_2O$/ 95% TFA (RT, 3 h); e) salt exchange Pyr.HCl 10 eq/MeOH (1 h); f) cyclization: BOP 3eq/HOBt 3eq/DIPEA 6eq/DMF (c=0.5 mg/mL, RT, 24 h); g) deprotection of aminooxyacetic acid 0.2 M $NH_4OAc$ solution (pH 5.0)/1 M methoxylamine (RT, 1 h); h) daunorubicin conjugation (RT, 24 h) in 0.2 M $NH_4OAc$ solution (pH 5.0); i) Fmoc-cleavage 4% hydrazine/DMF (RT, 2 h).

**Figure 2:** Schematic representation of the synthesis of cyclic KNGRE (A) and XNGRE (B) drug-conjugates.

**Table 1.** Stability of cyclic NGR peptide-daunorubicin conjugates

| Ratio of Asn-/Asp-/isoAsp-derivatives (DMEM CM, 37 °C) | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Code** | **Aaa in position X of the conjugates** | **6 h** | | | **72 h** | | |
| | | **NGR** | **DGR** | ***iso*DGR** | **NGR** | **DGR** | ***iso*DGR** |
| 1 | Lys | 100 | 0 | 0 | 100 | 0 | 0 |
| 2 | Nle | 93 | 1 | 6 | 58 | 9 | 33 |
| 3 | Ala | 96 | 0 | 4 | 58 | 11 | 31 |
| 4 | Leu | 93 | 0 | 7 | 54 | 11 | 35 |
| 5 | Pro | 73 | 14 | 13 | 19 | 46 | 35 |
| 6 | Ser | 93 | 0 | 7 | 56 | 12 | 31 |

The best yield could be observed in the case of conjugate **2** that was five times higher (overall yield was 10.6%) than in the case of the original compound.

Chemical stability of cyclic NGR bioconjugates was investigated under the treatment conditions used for the *in vitro* cytotoxicity experiments. Deamidation rate was evaluated by HPLC-MS at 0 min, 6 h and 72 h. In contrast to the control conjugate (**1**) that showed high stability in our previous study, the novel conjugates suffered rearrangement in time. The results presented similar *iso*Asp/Asp (~3:1) rates after deamidation of conjugates **2**, **3**, **4** and **6** calculated from the area under the curve. After 6 h, the reasonable rearrangement was observed, which increased over time. Nevertheless, 54-58% of the parent cyclic NGR conjugates was still intact after 72 h (Table 1). Decreased stability was detected in the case of the Pro-containing conjugate (**5**) with faster deamidation and a higher ratio of DGR. Except for deamidation, no other decomposition could be observed during the entire execution of the study.

The antitumor effects of bioconjugates were examined *in vitro* on CD13(+) HT-1080 human fibrosarcoma and on CD13(−) HT-29 human colon adenocarcinoma cells. Both cell types are integrin receptor positive. It seems that the replacement of Lys by the hydrophilic amino acid Ser (**6**) is not favored (Table 2). However, the incorporation of hydrophobic amino acids was well tolerated. The conjugate with a bulky side chain in this position (Leu, **4**) had higher $IC_{50}$ values that might be explained by steric hindrance. The bioconjugates with Nle (**2**) or Ala (**3**) showed the best antitumor activity on both cell lines. Conjugate **2** showed similar activity on HT-1080 and higher activity on HT-29 cells compared to the control conjugate **1**. To further characterize the biological activity of the conjugates, their lysosomal degradation

and cellular uptake were also investigated. The degradation of the conjugates resulted in Dau=Aoa-Gly-OH in all cases in rat liver lysosomal homogenate and no significant difference in the speed of the metabolite release could be detected. Therefore, this cannot be the reason for the different antitumor activity.

**Table 2:** *In vitro* cytostatic/cytotoxic effects of compounds on HT-29 and HT-1080 cells

| Compounds | HT-1080 (6 h) $IC_{50}$ (μM) | HT-29 (6 h) $IC_{50}$ (μM) | HT-1080 (72 h) $IC_{50}$ (μM) | HT-29 (72 h) $IC_{50}$ (μM) |
|---|---|---|---|---|
| **Daunorubicin** | **1.4 ± 0.6** | **0.3 ± 0.2** | **0.5 ± 0.2** | **0.1 ± 0.1** |
| **Dau=Aoa-GFLGK(c[KNGRE]-GG-)-$NH_2$ (1)** | **5.7 ± 0.5** | **8.7 ± 1.2** | **1.4 ± 0.7** | **3.0 ± 0.6** |
| **Dau=Aoa-GFLGK(c[NleNGRE]-GG-)-$NH_2$ (2)** | **5.5 ± 0.3** | **2.2 ± 0.2** | **2.3 ± 0.6** | **1.3 ± 0.2** |
| **Dau=Aoa-GFLGK(c[ANGRE]-GG-)-$NH_2$ (3)** | **8.9 ± 0.8** | **4.3 ± 0.5** | **3.6 ± 0.7** | **3.2 ± 0.8** |
| **Dau=Aoa-GFLGK(c[LNGRE]-GG-)-$NH_2$ (4)** | **57.5 ± 6.3** | **47.0 ± 5.4** | **20.6 ± 0.4** | **14.1 ± 0.7** |
| **Dau=Aoa-GFLGK(c[PNGRE]-GG-)-$NH_2$ (5)** | **9.4 ± 4.0** | **14.6 ± 4.7** | **3.5 ±1.0** | **3.7 ± 0.8** |
| **Dau=Aoa-GFLGK(c[SNGRE]-GG-)-$NH_2$ (6)** | **>100** | **64.7 ± 4.9** | **63.7 ± 9.5** | **39.4 ±2.9** |

The cellular uptake of Dau containing conjugates can be followed by flow cytometry (Figure 2). The accumulation of conjugates **2**, **4** and **5** increased significantly in HT-29 cells than HT-1080 in comparison with the other conjugates. The low cytostatic/cytotoxic effects of conjugate **6** can be deducted by the results of cellular uptake study. The Ser-containing conjugate did not enter HT-1080 cells, while a slightly higher cellular uptake was detected in HT-29 cells, although the uptake for this conjugate was still much lower than in the case of the other tested bioconjugates.

From these findings, we could conclude that replacement of Lys in the Dau=Aoa-GFLGK(c[KNGRE]-GG-)-$NH_2$ (**1**) conjugate by different amino acids provides a more convenient and cost-effective synthetic route. Among the new cyclic NGR peptide – daunorubicin conjugates the most effective compound was Dau=Aoa-GFLGK(c[NleNGRE]-GG-)-$NH_2$ (**2**), which showed similar activity on HT-1080 CD13(+) cells to Dau=Aoa-GFLGK(c[KNGRE]-GG-)-$NH_2$, and a significantly higher antitumor effect on HT-29 CD13(−) cells. Therefore, these two conjugates were further studied *in vivo*.

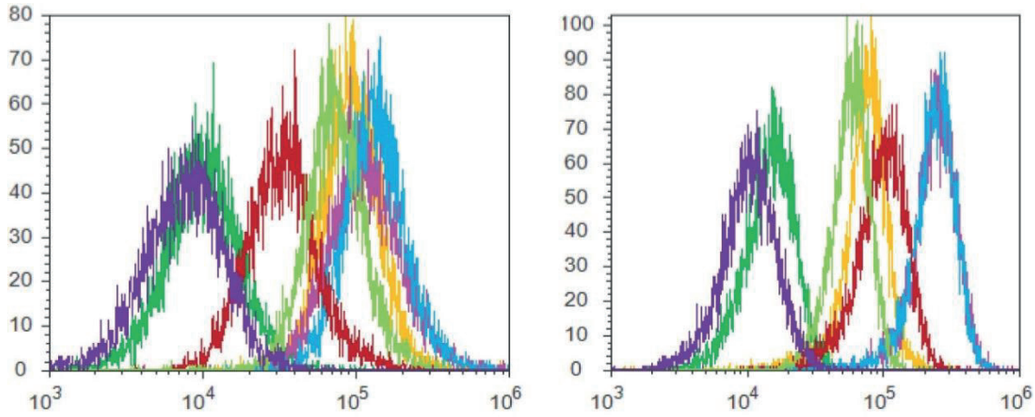



**Figure 3**: Direct uptake of Dau of conjugate **1** (yellow); **2** (light blue) **3** (light green); **4** (red); **5** (pink); **6** (green) by (left) HT1080 and (right) HT-29 cells, using 10 μM conjugate for each sample. Untreated control is marked with purple detecting the autofluorescence of the cells.

Kaposi sarcoma (KS) is one of the most deadly and aggressive types of tumors in HIV-1-infected people.[8] KS tumor cells express a high level of CD13 and this is a key factor for the contribution of increased vascularization of the tumor. Thus, the conjugates were studied on mice with subcutan developed KS tumors. The tumor growth inhibition of conjugate **1** was higher (37.7%) than the observed inhibition in the case of the free drug and conjugate **2**.[9] In contrast conjugate **2** was more potent on orthotopically developed HT-29 colon carcinoma (low CD13 but high integrin expression) bearing mice (45.7% *vs.* 16.9%).[9] The results suggested that conjugate **1** has a significantly higher affinity to CD13 receptors than to integrins because of its stability (no significant rearrangement to *iso*Asp derivative), while conjugate **2** was rather potent on HT-29 tumor that can be explained by the relatively fast Asn/*iso*Asp switch in the sequence.

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