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Influence of the Daunomycin Position on Bioactivity in Angiopep-2 - Drug Conjugates

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Introduction

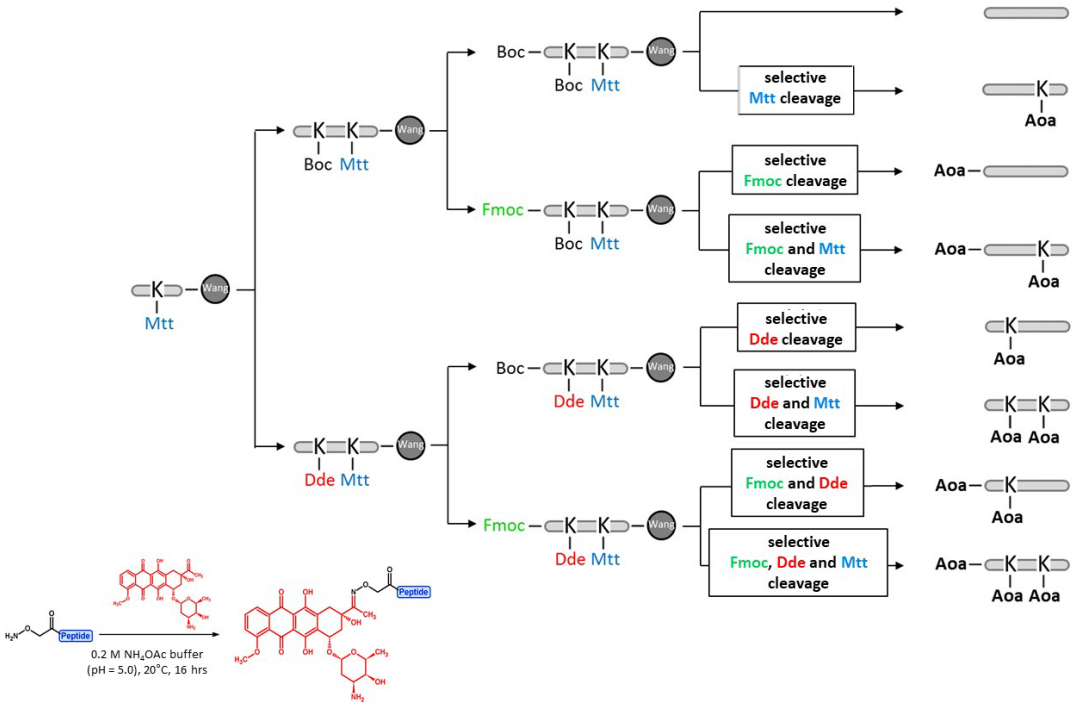
Transporting therapeutics through the blood-brain barrier (BBB) is a major challenge in the treatment of brain tumors. The BBB is a semipermeable system and therefore the majority of the active substances are poorly transported through this barrier resulting in decreased therapeutic effects [1]. Angiopep-2 (TFFYGGSRGKRNNFKTEEY) is a peptide containing 19 amino acids that was shown to be a ligand of the low-density lipoprotein receptor-related protein-1 (LRP1). This peptide can cross the blood-brain barrier *via* receptor-mediated transcytosis and simultaneously target glioblastoma, thereby dual targeting may be achieved [2]. Angiopep-2 contains three amino groups (two lysine side chains in positions 10 and 15 and the *N*-terminal), and all these groups were functionalized in previous studies to produce drug-peptide conjugates [3,4]. Using all possible conjugation sites is convenient from the synthetic point of view but is not always necessary for achieving the best effect. In this case the role and importance of each position have not yet been investigated. Hence, in this work, the number and position of the drug molecules in Angiopep-2 based conjugates were in the focus.

Results and Discussion

Conjugates containing one, two, and three daunomycin molecules *via* oxime linkage were prepared. The peptides for conjugation were prepared by SPPS using Fmoc/*t*Bu strategy starting from one batch of Rink-Amide MBHA resin. Before the coupling of the second lysine derivative, the resin was split, and orthogonal protecting schemes were further applied to create the proper conjugation sites (Scheme 1). Before the cleavage of the peptides from the resin, one or more protecting groups were removed selectively, and the resulting free amino groups were modified by Boc-protected aminooxyacetyl (Aoa) moiety. After the peptide cleavage the derivatives containing different number of Aoa were purified by RP-HPLC and used for conjugation with daunomycin (Dau) under slightly acidic condition (0.2 M NH_4OAc at pH 5 for overnight). In this way three conjugates with one Dau in different positions, three conjugates with 2 Dau and one with 3 Dau in all possible conjugation sites were produced.

The cytostatic effect of the conjugates was investigated on U87GM human glioblastoma cell line. The cells were incubated with the conjugates in serum free medium for 24 h and after washing the cells were further incubated in fresh serum containing medium for additional 48 h The results indicated significant differences in the antitumor activity of the conjugates, which was not related to the number but rather the position of the drug(s) on the homing peptide (Figure 1). The free Angiopep-2 did not show any effect on tumor cells up to 100 μM concentration. Among the conjugates with only one Dau compound **3** in which Dau was attached to the *N*-terminus showed the highest antitumor effect on glioblastoma cells (IC_{50}: 10.9 ± 2.8 μM). When the Dau was conjugated to the Lys side chain in position 15 (compound **2**) the effect decreased significantly (IC_{50}: 30.2 ± 6.4 μM) and conjugate **4** also was not significantly better (IC_{50}: 24.0 ± 5.9 μM). The conjugates with two drug molecules similar tendency was observed. Compound **7** with Dau at the *N*-terminus and on the Lys side chain in position 10 showed the highest activity (IC_{50}: 7.8 ± 6.3 μM). When the Lys side chains were used as conjugation sites, the formed compound (**6**) had a moderate activity on U87 cells (IC_{50}: 21.6 ± 5.4 μM). Surprisingly, conjugate **5** with Dau at the *N*-terminus and Lys side chain in position 15 showed the lowest potency on cells (IC_{50}: 32.3 ± 8.1 μM). Compound **8** with three Dau had moderate activity as well (IC_{50}: 23.6 ± 6.3 μM). The data indicated that the side chain of Lys in position 15 is an inadequate

conjugation site for drug delivery. In contrast, the substitution of the *N*-terminal amino group with a drug molecule might be the most efficient choice for drug targeting with Angiopep-2.



Scheme 1. Synthetic protocol of different Angiopep-2 – daunomycin conjugates using orthogonal side chain protecting groups.

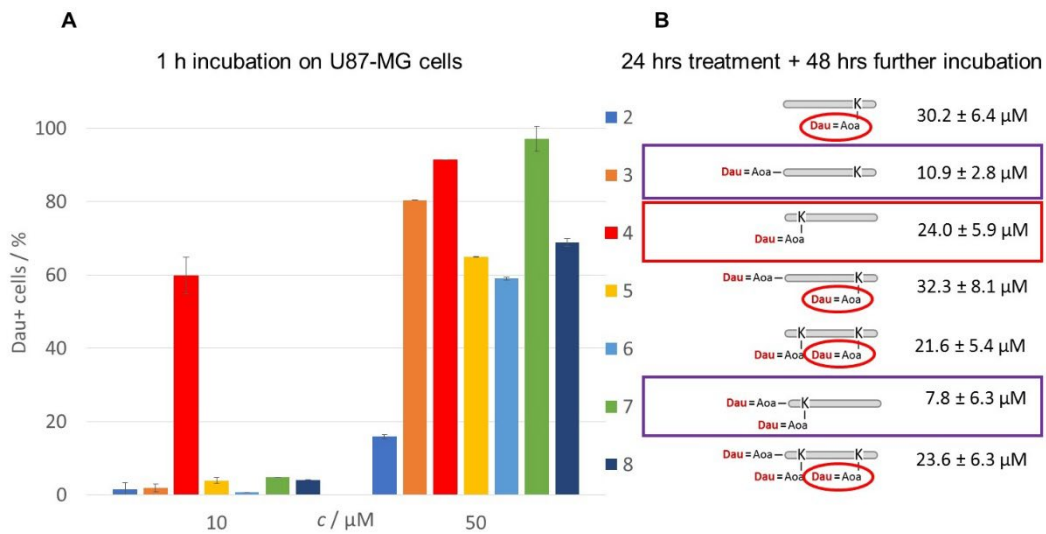


Fig. 1. Cellular uptake (A) and cytostatic effect (B) of Angiopep-2 − daunomycin conjugates.

The antitumor activity of any peptide-drug conjugate is influenced by many factors. One of the main effects is the cellular uptake of the conjugates. Therefore, we compared the cellular uptake of the different conjugates on U87GM glioblastoma cells, which were incubated with the conjugates for 1 h, by flow cytometry. The cellular uptake of the conjugates was concentration dependent. The correlation between the uptake and the cytostatic effect could be established in most cases. Conjugates **3** and **7** entered the cells very efficiently at 50 μM concentration (80% and 97% of cells were Dau+, respectively) while conjugate **2** showed very low uptake (15%) (Figure 1). Interestingly, conjugate **4** that showed moderate cytostatic effect was taken up the most effectively. 90% of the cells were Dau positive at 50 μM concentration, while this value was 60% at a concentration of 10 μM. In contrast, all other compounds showed lower cellular uptake (< 10%) at this concentration. In case of the conjugate with three Dau (**8**) only 70% of the cells were Dau+ at 50 μM concentration.

Since we discovered contradictions between the cellular uptake and cytostatic effect of some conjugates, we studied the degradation of the conjugates in lysosomal homogenates (isolated from rat liver). It is worth mentioning that oxime linkage is quite stable at neutral pH, therefore, there is no early release of drug from the conjugate in the blood circulation in contrast with conjugates containing an ester linkage. The disadvantage of the oxime linkage is that it does not decompose in cells either to release the free drug. However, the smallest metabolites that contain daunomycin linked to one amino acid through Aoa established linker can bind to DNA resulting in antitumor activity [5]. This is the reason that this type of conjugates can be used in higher amount *in vivo* providing higher antitumor effect in comparison with free Dau at the maximum tolerated dose.

The lysosomal degradation was followed by HPLC-MS. The results indicated that in case of compound **3**, the Dau=Aoa-Thr-OH fragment appeared within 1 h, at 6 h it was the main drug containing metabolite and at 72 h, this Dau containing fragment could only be detected (Figure 2). This time dependent pattern was also observed in case of conjugate **2**, where Dau was attached to the side chain of Lys in position 15, and the formed smallest metabolite was H-Lys(Dau=Aoa)-OH. Since this metabolite binds very efficiently to DNA [5], a strong cytostatic effect could be expected in case of compound **2**. As we observed the opposite, we can conclude that the low activity of this compound is related to the low cellular uptake. In contrast, this metabolite was not efficiently released from compound **4**. It was not detectable at 1 h and only a low amount could be observed after 6 h. Even after 72 h the main metabolites were H-Gly-Lys(Dau=Aoa)-Arg-OH and H-Gly-Lys(Dau=Aoa)-OH. Though the efficient cellular uptake, this observation might explain the lower anticancer activity of the conjugate.

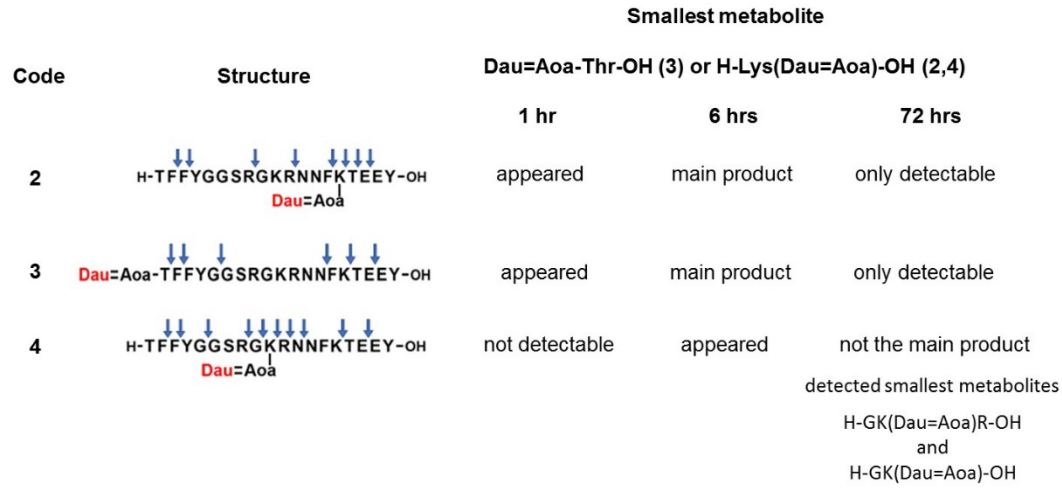


Fig. 2. Lysosomal degradation of Angiopep-2 – Dau conjugates with one drug molecule.

To increase the release of the active metabolite in this position, Cathepsin B (lysosomal enzyme) labile peptide spacers were incorporated between the side chain of Lys in position 10 and Dau. For this purpose, GFLG, VA and VAGG were selected. The application of GFLG spacer in conjugate **9** showed an efficient cellular uptake and a moderate improvement in cytostatic effect (IC_{50}: 16.9 ± 5.6 μM) compared to compound **4** (Figure 3). Unfortunately, the release of Dau=Aoa-Gly-OH was also slow and the detected main metabolite at 6 h time point was Dau=Aoa-Gly-Phe-OH. The investigation of VA spacer (conjugate **11**) showed that the antitumor activity was completely lost. This effect could be explained both by the decrease of cellular uptake and lysosomal degradation. Only negligible Dau=Aoa-Val-OH could be detected even after 72 h. The incorporation of two Gly between VA and the Lys side chain (compound **10**) resulted in higher cytostatic effect but there was no significant improvement in comparison with compound **4**. Although the Dau=Aoa-Val-Ala-OH fragment appeared after 1 h and Dau=Aoa-Val-OH was also detected in high amount after 6 h, but the cellular uptake of this conjugate was much lower than that of the conjugates **4** and **9**. According to these results we can conclude that the substitution of Lys side chain in position 10 with an appropriate enzyme labile spacer (GFLG) has a positive effect on the cellular uptake, but the drug release is still hindered. Therefore, we plan to use other properly designed enzyme labile spacers to increase the release of the active metabolite.

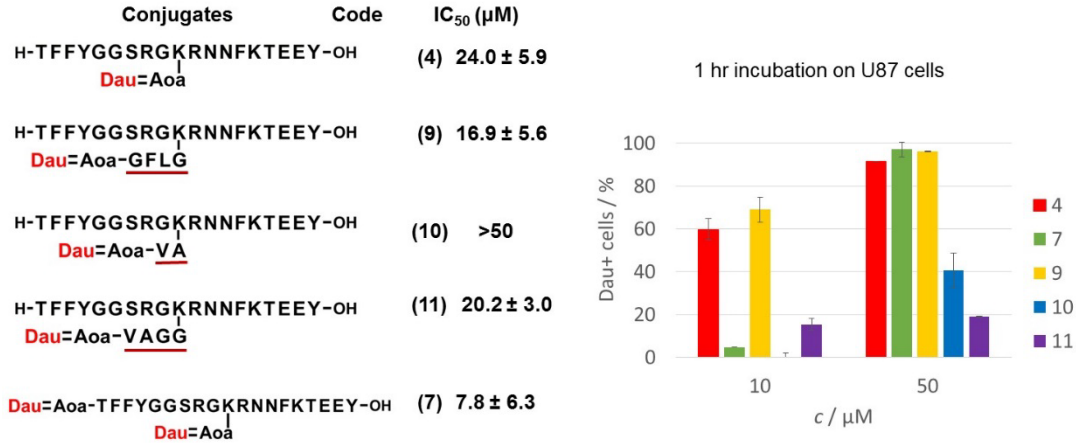


Fig. 3. Cytostatic effect and cellular uptake of Angiopep-2 – daunomycin conjugates with spacers between the homing peptide and the drug molecule.

As summary, we can draw the conclusion that the position of the drug molecule in an Angiopep-2 conjugate has higher influence on the antitumor activity than the number of the conjugated drugs.

Acknowledgments

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