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

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Optimization of the structure of targeted Daunomycin conjugates against non-small cell lung cancer

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Introduction

Cancer is one of the leading causes of death in the World and in the EU too.[1,2] Lung cancer alone was responsible for 1.7 million deaths worldwide in 2016. As tumor cells are originated from normal cells they are very similar to those. This makes it highly challenging to eliminate them without the destruction of healthy tissues. In spite of the common origin, small differences can be found in their receptors or enzyme sets. These little differences may allow a valuable increase in the selectivity of therapy and may enhance the rate of success during treatment. Thus, identification of new therapeutically useful targeting units is a hot topic.

An often used promising tool to identify tumor homing peptides is the technique of phage display.[3] These are mostly short, tumor specific peptides and can specifically accumulate into tumors. One drawback of this technique is that there is no optimization of the identified peptides. However, sometimes the optimized sequences may have more therapeutic values compared to the original ones. The chemical modification may increase both the stability and the specificity of these peptides.[4] Phage display technique was carried out to identify tumor homing peptides against non-small cell lung cancer as well.[5-7]

Tumor homing peptides can be applied to deliver antitumor drugs into tumor tissues to decrease the side effect and thus increase the efficacy. The covalent linkage, the number of drugs and the structure of conjugates may affect the efficiency.

Results

Based on the literature, two tumor homing peptides were selected for further optimization. One of them was the ARRPKLD peptide that was selected based on a selective peptide motif A(S)RXPXXX.[6] We used this peptide and motif to develop tumor-targeting drug conjugates (Table 1, conjugate **1-14**). Unfortunately, the identification of a tumor homing peptide does not give any information about the mechanism and selectivity of their cellular uptake. It is unclear, what is the main target of these peptides that causes their tumor

cell specificity. If these peptides and their conjugates can be internalized into the tumor cells by endocytosis, the conjugates can be degraded in the lysosomes. In that case an enzyme labile spacer can be used to increase the effect of conjugates. In our constructs, two different spacers – GFLG and LRRY that can be cleaved by lysosomal Cathepsin B were compared. Although the authors selected the ARRPKLD sequence, we were interested in comparing it with a peptide containing serine at the N-terminus. These kinds of peptides were the second in the enrichment assays. Furthermore, as the motif allows all amino acids in position 5 (A(S)RXP**X**XX), conjugates with Gly at this position were synthesized too. The linkage between the drug and the transporter unit may have a high influence on biological activity. Thus, the effect of two different kinds of covalent bond was studied. The Dau was coupled in some of our conjugates *via* an oxime bond while in the other *via* an amide bond (Figure 1). The latter one is stabile in physiological conditions, thus the drug is not released from these constructs, while in case of oxime bond some release may happen inside the cells.

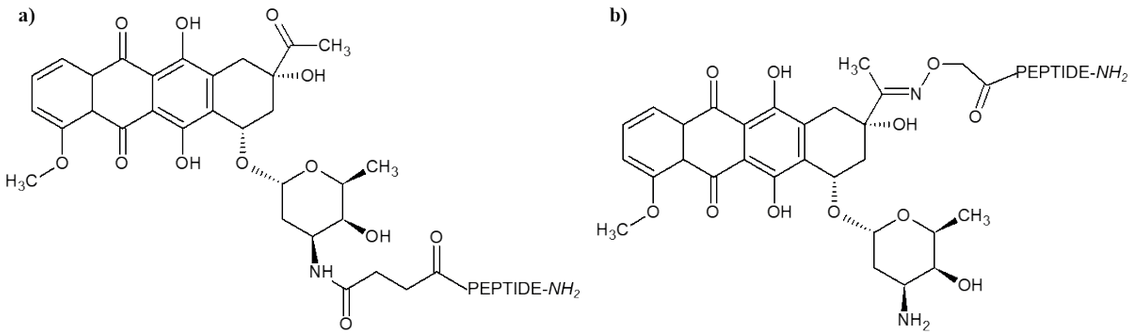

Figure 1. The structure of a) Dau-Suc conjugates and b) Dau=Aoa conjugates

The cytostatic effect of these conjugates was studied on EBC-1 cells, a human lung squamous cell carcinoma line, as a model.[8] The cells were treated with the solution of conjugates (0.83-100 μM concentration range) for 24 h. Then the cells were washed and were cultured for 48 h in serum-containing medium. The *in vitro* cytostatic effect was determined using MTT assay and IC_{50} values were determined from the viability *vs* lg(c) curve (Table 1). Among the best conjugates – their IC_{50} values are less than 10 μM – two contained Ala (conjugates **1** and **11**) while three conjugates contained Ser at the N-terminus (conjugates **2**, **4** and **6**). Three conjugates (**7**, **10** and **13**) had no or moderate cytostatic activity. All effective conjugates contained spacer, four of them (**1**, **2**, **6** and **11**) had the GFLG one. The comparison of the effect of conjugates with Lys or Gly showed that Lys is preferable at this position.

Based on the above mentioned structure–activity relationship we can conclude that Ser at the N-terminus and GFLG spacer are better choices in case of this set of conjugates.

Table 1. The *in vitro* cytostatic activity of conjugates

Code	Peptide	IC_{50} (μM)		
		EBC-1	**A2058**	**A431**
1	Dau=Aoa-GFLG-ARRPKLD-NH_2	3.1±0.4	8.1±0.5	12.2±1.0
2	Dau=Aoa-GFLG-SRRPKLD-NH_2	6.7±1.1	8.9±0.4	14.4±2.6
3	Dau=Aoa-LRRY-ARRPKLD-NH_2	50.8±36.2	n.d.	n.d.
4	Dau=Aoa-LRRY-SRRPKLD-NH_2	6,4±3.9	53.2±11.7	89.7±14.6
5	Dau=Aoa-GFLG-ARRPGLD-NH_2	40.8±27,2	n.d.	n.d.
6	Dau=Aoa-GFLG-SRRPGLD-NH_2	4.5±1.8	2.0±0.5	3.9±1.8
7	Dau=Aoa-LRRY-ARRPGLD-NH_2	100	n.d.	n.d.
8	Dau=Aoa-LRRY-SRRPGLD-NH_2	13.3±4.5	n.d.	n.d.
9	Dau-Suc-ARRPGLD-NH_2	53.8±8.8	n.d.	n.d.
10	Dau-Suc-SRRPGLD-NH_2	>100	n.d.	n.d.
11	Dau-Suc-GFLG-ARRPGLD-NH_2	4.4±2.6	>100	>100
12	Dau-Suc-GFLG-SRRPGLD-NH_2	10.2±9.8	n.d.	n.d.
13	Dau-Suc-GFLG-ARRPKLD-NH_2	>100	n.d.	n.d.
14	Dau-Suc-GFLG-SRRPKLD-NH_2	57.4±5.8	n.d.	n.d.
15	Dau=Aoa-LRRY-DWTY-NH_2	20.8±15.1	n.d.	n.d.
16	Dau=Aoa-LRRY-SYDWTY-NH_2	7.4±5.4	n.d.	n.d.
17	Dau=Aoa-LRRY-LRSYDWTY-NH_2	10.3±5.7	n.d.	n.d.
18	Dau=Aoa-LRRY-TDSILRSYDWTY-NH_2	20.7±1.2	n.d.	n.d.
Dau	Daunomycin	0.05±0.03	n.d.	n.d.

n.d no data

For studying the tumor selectivity of the best five conjugates, A2058 malignant melanoma[9] and A431 human squamous carcinoma[10] cells were treated with these conjugates as well. The cytostatic activity of conjugate **1**, **2** and **6** was very similar on these cells compared to their activity on EBC-1. On the other hand, conjugate **4** and **11** showed lower or no cytostatic activity, respectively. They differ from each other only in the presence (**4**) or absence (conjugate **1**, **2** and **6**) of LRRY spacer. Based on these results, it seems that this spacer is not appropriate in case of these two cell lines.

The three best (**1**, **2** and **6**) and one ineffective (**7**) conjugates were selected to study their cellular uptake on EBC-1 cells. The cells were treated for 3 h then were washed and their fluorescence intensity was measured by flow cytometry (Figure 2). Although conjugates **1**, **2** and **6** have very similar *in vitro* cytostatic activity, they showed different internalization. Conjugates **2** and **6** have lower cellular uptake compared to conjugate **1** (Figure 2). On the other hand, all have more pronounced internalization than that of the ineffective conjugate **6** (Figure 2), which is in accordance with the IC_{50} values. Although in case of the cytostatic effect the Ser is better, it is not preferable in the sequence if cellular uptakes are compared (at least in case of the studied conjugates). These cellular uptake results gave back clearly the

noticed success of sequences in accumulation in tumors.[6] The identified best sequence showed the highest internalization in our experiments and alteration of this "preferred" sequence by double substitution ($Lys^5 \rightarrow Gly^5$ and $Ala^1 \rightarrow Ser^1$) decreased dramatically the cellular uptake. Although only one conjugate with LRRY spacer was studied, the results may indicate that this tetrapeptide reduced the internalization to a great extent.

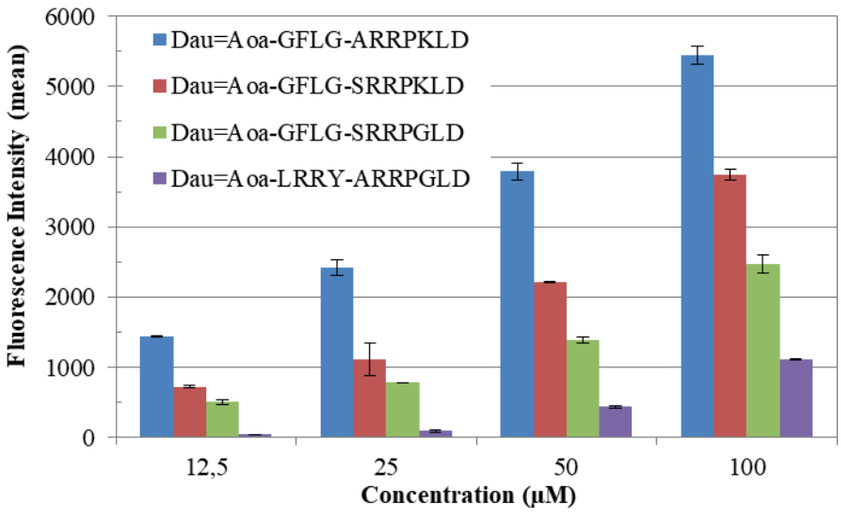

Figure 2. Cellular uptake of conjugates containing XRRPYLD peptides on EBC-1 cells (where X is Ala or Ser and Y is Gly or Lys).

The other tumor homing peptide that was used in our study was the TDSILRSYDWTY sequence. It was also determined that the WTY region is important for cell binding.[5] In our study truncated peptides were used to identify the minimal sequence which is necessary for the tumor targeting. For this purpose, four conjugates were synthesized (Table 1, conjugates **15-18**), the original peptide with LRRY as enzyme labile spacer and its shorter derivatives. In all conjugates Dau was coupled to the peptide via oxime bond. Two of them were very promising based on their *in vitro* cytostatic activity (Table 1). The longest and the shortest ones (conjugate **15** and **18**) have the same activity, suggesting that one of them is too short while the other is too long that might not have a favorable structure for receptor binding. The most effective conjugate was the Dau-Aoa-LRRY-SYDWTY, while increasing the length of the peptide (Dau-Aoa-LRRY-LRSYDWTY) resulted in a slight decrease in the cytostatic activity.

The internalization of conjugates was measured by flow cytometry on EBC-1 cells. The cells were treated for 3 h then were washed and their fluorescence intensity was measured by flow cytometry (Figure 3). Conjugate **17** showed the highest cellular-uptake ability, although it did not have the highest cytostatic activity. The internalization of the most

cytostatic conjugate (**17**) was not so outstanding. These findings suggest that not only the internalization, but other factors (*e.g.* stability, the intracellular fate) could be important in the biological activity.

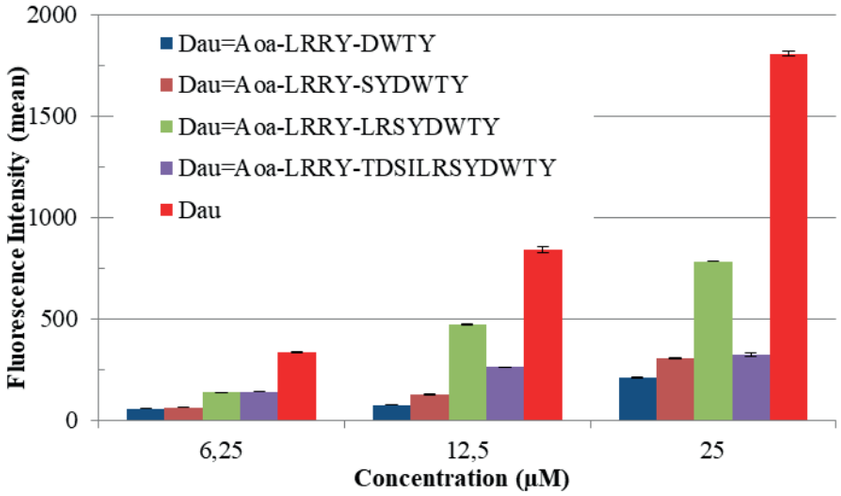

Figure 3. Cellular uptake of conjugates containing TDSILRSYDWTY sequence and its truncated derivatives on EBC-1 cells.

Our results proved that the tumor homing peptides may be suitable for the synthesis of conjugates with antitumor activity, but their structure can be further optimized to increase their effect. Our optimized conjugates showed improved antitumor activity and may be used in tumor therapy.

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