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

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Synthesis and *in vitro* evaluation of drug-containing melanoma-specific peptide conjugates

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Introduction

Increasing mortality rate of tumorous diseases is reported by the World Health Organization. Skin cancer is the most increasing incidence of cancer diseases especially in the case of young adults. Although melanoma is only about 1% of skin cancers, it causes a large majority of skin cancer derived mortality. However, in the case of melanoma, using chemotherapy, radiotherapy or immunotherapy (the main procedures for the treatment of advanced or metastatic cancers) is recommended due to its localization and rapid progression. Thus, the high and continuously rising rate of death among melanoma patients has encouraged new research focusing on therapies and drugs with increasing efficiency, especially against metastatic melanoma. Selective and targeted approaches might improve the impact of anticancer therapies with fewer side effects.

Melanoma is originated from the malignant transformation of melanocytes which are the melanin-producing cells of the skin, hair and eyes. It is formed either by dysfunction of dysplastic nevi or a single melanocyte.[1] Melanocytes are located with keratinocytes in the basal layer of the epidermis and they form a very stable population, as they proliferate extremely rarely under normal circumstances. Not only the outer layer (epidermis), but also the inner layer (dermis) of the skin, which involves hair roots, blood and lymph-vessels and nerves, includes melanocytes, though they are biologically different population than epidermis located ones. Based on this evidence, cutaneous melanoma is a heterogeneous tumour, which involves the wide population of melanocytes with different origin and differentiation stages (from undifferentiated, cancer stem-like cells with self-renewal capacity and high proliferation and differentiation ability to functional melanocytes). Among all type of skin cancers, melanoma has the highest rate of the metastatic effect. Based on its location, it has a high potential to spread rapidly through other body sites by entering the lymphatic system and bloodstream.

Cell surface receptors with altered expression levels are not the only specific and selective targets for tumor cells. Markedly, modified proteoglycan expression and structure

can also be observed during tumor development and growth.[2] The melanoma-associated chondroitin sulfate proteoglycan (MCSP, also called NG2) is a type I single-pass transmembrane proteoglycan,[3] which is widely expressed in several different tumors, including glioblastomas, chondrosarcomas, melanomas and some leukemias.[4-7] Functionally, it promotes tumor vascularization[8] due to binding to collagen VI and it promotes cell survival and adhesion.[9] Antibodies against NG2 inhibit melanoma cell growth both *in vitro* and *in vivo*.[10-12] Moreover, targeting NG2 *in vivo* in a highly malignant tumor model reduced tumor growth and angiogenesis was determined.[13] Burg *et al*. identified two novel decapeptide ligands for the NG2 proteoglycan using phage display. Based on the structure, the two sequences are clearly different (Peptide1: TAASGVRSMH; Peptide2: LTLRWVGLMS), but they may act as mimotopes of each other on the basis of small areas of similarity (ASG *vs* LTL; VR *vs* VG). Both peptides have high affinity and specificity to NG2 proteoglycan, and they can bind to similar sites on NG2.[14] Guan *et al*. developed Peptide1 conjugated biodegradable nanoparticles loaded with docetaxel, as a new nanomedicine. The construct achieved controlled drug release, and it had significant *in vitro* and *in vivo* antitumor effect which was mediated *via* NG2 receptor.[15]

Based on the above-mentioned facts, drug-containing melanoma-targeting peptide conjugates were designed, synthesized and their *in vitro* evaluation was performed in order to establish structure-activity relationships and to find the most promising construct for melanoma targeting.

Results

Melanoma targeting phage display peptides (Peptide1 and Peptide2) were synthesized by solid-phase peptide synthesis using Fmoc/^{t}Bu strategy. For both peptides, the native sequence and their modified versions were also synthesized. The following modifications were performed; i) methionine was replaced by norleucine to avoid the unwanted oxidation; ii) enzyme labile GFLG spacer was used to elongate the peptides at the N-terminus to enhance the release of the drug from the construct; and iii) scrambled and truncated peptide sequences were synthesized to establish the optimal effective sequence. The peptides were built up on Rink Amide MBHA resin using Fmoc protected amino acid derivatives. At the end of the synthesis, Boc protected aminooxyacetic acid was coupled to the N-terminus in order to functionalize the peptides for drug conjugation. Unfortunately, synthesis of GFLG spacer containing Peptide1 was unsuccessful, due to its hydrophobic feature; it forms aggregates immediately after the cleavage. Therefore, its daunomycin conjugate cannot be prepared. But

in the case of truncated Peptide1 analogs, this aggregation was not observed. Before the conjugation, peptides were cleaved from the resin and purified by preparative RP-HPLC and characterized by analytical RP-HPLC and ESI-MS (data not shown). Daunomycin as a drug was coupled to the aminooxy acetylated peptides *via* oxime bond. Conjugation was carried out in slightly acidic condition (250 mM NaOAc buffer, pH 5.2). The conjugates were purified by preparative RP-HPLC and characterized by analytical RP-HPLC and ESI-MS (Table 1). The yield of conjugation was strongly dependent on the peptide sequence. In the case of Peptide1 based conjugates due to their poor solubility and high aggregation ability, the yield was only 10-15%. In case of Peptide2, conjugates were formed almost quantitatively.

In the first step, the *in vitro* cytostatic effect of the conjugates was determined on A2058 (ATCC® CRL-11147) human melanoma cell culture. The most effective conjugates were also tested on A431 (ATCC® CRL-1555) human epithelial (but not melanoma originated) cell culture in order to investigate the selectivity of these conjugates. In case of Peptide1 conjugates, replacement of Met to Nle (**Conj3**) has no significant effect on the activity; both conjugates have moderate *in vitro* cytostatic effect (62.8±22.1 μM and 22.1 ±12.1 μM, respectively) on A2058 cells. Incorporation of GFLG enzyme labile spacer in both cases – native (**Conj2**) and Nle substituted (**Conj4**) ones – significantly increases the *in vitro* efficacy (5.2±2.4 μM and 2.3±1.3 μM, respectively). Truncation of Peptide1 sequence is allowed; not only the first two (**Conj5**), but the first four (**Conj6**) N-terminal amino acids can also be eliminated without reduction of activity (1.3±0.6 μM and 3.1±1.1 μM, respectively) on A2058 cells. Interestingly, the scrambling of the targeting Peptide1 sequence (**Conj5**) has not influenced the *in vitro* activity compared to Nle-substituted one (**Conj4**) on A2058 cells (2.3±1.3 μM and 1.3±0.6 μM, respectively).

In case of Peptide2, not only the replacement of Met by Nle (**Conj9**), but also the incorporation of GFLG spacer (**Conj10**) decreased the *in vitro* cytostatic effect (17.5±3.3 μM and 26.5±17.6 μM, respectively) on A2058 cells. Scrambling of the targeting Peptide2 sequence (**Conj11**) does not influence the *in vitro* activity compared to the native sequence (**Conj8**) on A2058 cells (4.3±1.9 μM and 2.5±0.6 μM, respectively). Truncation of Peptide2 sequence is moderately allowed; the first two N-terminal amino acids (LT, **Conj12**) can be eliminated without reduction of cytostatic effect. However, further elimination of additional two amino acids, LR (**Conj13**) is not allowed, it can significantly decrease the *in vitro* activity. These modifications do not only influence the *in vitro* cytostatic activity, but they have also effect on the selectivity. While the native sequence (**Conj8**) proves to be melanoma

specific (4.3±1.9 μM on A2058 and 14.0±0.09 μM on A431), this selectivity is absent in case of the scrambled one (**Conj11**; 2.5±0.6 μM on A2058 and 2.6±0.9 μM on A431).

Compared to Peptide2 conjugates, unfortunately, none of the Peptide1 conjugates showed melanoma specificity, all of the Peptide1 conjugates have similar IC_{50} values on A2058 and A431 cells.

Table 1. Chemical characterization of melanoma-specific conjugates and their IC_{50} values on A2058 and A431 cells

Code	Conjugates	t_R (min)[a]	M_{av} (Da)[b]		IC_{50} (μM)[c]	
			calc	meas	A2058	A431
Conj1	Dau=Aoa-TAASGVRSMH-NH_2	10.6	1596.6	1596.5	62.8±22.1	n.t*
Conj2	Dau=Aoa-GFLG-TAASGVRSMH-NH_2	13.5	1970.5	1970.9	5.2±2.4	n.t*
Conj3	Dau=Aoa-TAASGVRSNleH-NH_2	13.9	1578.1	1578.8	22.1 ±12.1	n.t*
Conj4	Dau=Aoa-GFLG-TAASGVRSNleH-NH_2	13.7	1952.5	1953.1	2.3±1.3	1.2±0.5
Conj5	Dau=Aoa-GFLG-ARASNleHSTGV-NH_2	13.7	1952.5	1952.2	1.3±0.6	2.2±2.1
Conj6	Dau=Aoa-GFLG-ASGVRSNleH-NH_2	13.9	1780.4	1780.5	3.1±1.1	1.9±1.4
Conj7	Dau=Aoa-GFLG-GVRSNleH-NH_2	14.1	1622.2	1622.3	2.5±1.1	1.2±0.5
Conj8	Dau=Aoa-LTLRWVGLMS-NH_2	14.4	1756.1	1756.2	4.3±1.9	14.0±0.0
Conj9	Dau=Aoa-LTLRWVGLNleS-NH_2	15.1	1737.5	1737.2	17.5±3.3	n.t*
Conj10	Dau=Aoa-GFLG-LTLRWVGLNleS-NH_2	18.1	2112.3	2112.4	26.5±17.6	n.t*
Conj11	Dau=Aoa-VGLMWSLTRL-NH_2	17.1	1756.3	1756.5	2.5±0.6	2.6±0.9
Conj12	Dau=Aoa-GFLG-LRWVGLMS-NH_2	16.1	1933.6	1933.4	4.6±2.5	4.3±0.7
Conj13	Dau=Aoa-GFLG-WVGLMS-NH_2	17.4	1663.9	1663.8	10.3±7.1	16.7±1.3
Dau	Daunomycin·HCl				<0.16	<0.16

[a]Analitical RP-HPLC, Agilent Eclipse XDB C8, 5 μm, 80Å, 4.6 x 150 mm, HPLC column, gradient: 5% B, 2 min; 5-100% B, 20 min.
[b]Bruker Daltonics Esquire 3000plus (Bremen, Germany) ion trap mass spectrometer. Spectra were acquired in the 50–2000 m/z range
[c]IC_{50} values were determined by a computerized curve-fitting program (Origin 7.5). Values shown are mean ± SE of four independent experiments, each performed in four parallels.
*n.t.: not tested

Cellular uptake profile was determined by flow cytometry using A2058 human melanoma and A431 human epithelial cells. Based on the IC_{50} values, Peptide1 originated conjugates do not seem to be melanoma-cell specific *in vitro*. Therefore, **Conj4**; **5**; **6**; **7** were selected for cellular uptake studies to confirm this hypothesis. These conjugates have the same structure; the GFLG enzyme labile linker is located between the drug and the targeting unit. In these cases, the release of the drug might occur in a similar manner (the same

metabolite can be formed), suggesting that only the targeting unit is responsible for the *in vitro* efficacy.

In all cases, the concentration-dependent cellular uptake profile was detected. Although the detected fluorescence intensity of A2058 cells was approx. twice as high as that of A431, the cellular uptake profile of the conjugates is similar. **Conj4** can be taken up the most effectively and **Conj7**, with the shortest targeting sequence, is the least internalized one. The scrambled sequence containing **Conj5** and the two amino acids truncated one (**Conj6**) have similar internalization ability on both cell cultures (Figure 1).

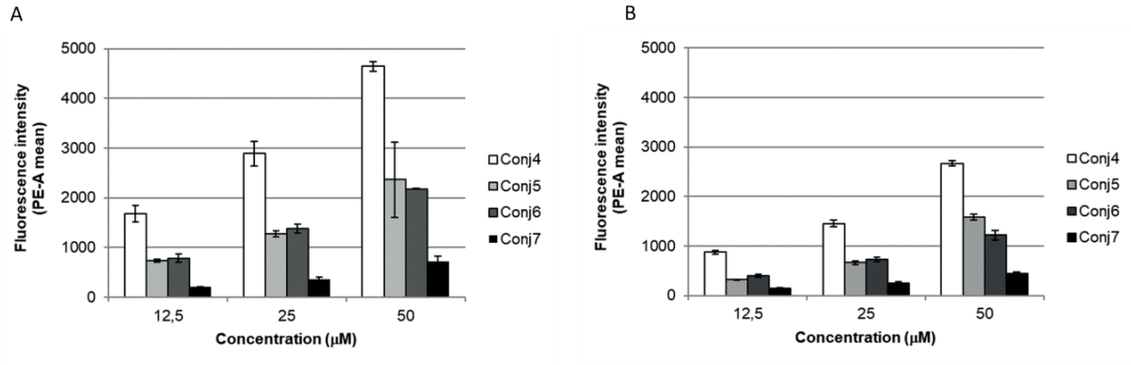

Figure 1. Cellular uptake profile of **Conj4**, **Conj5**, **Conj6** and **Conj7** on A2058 (**A**) and on A431 (**B**) cell cultures. The intracellular fluorescence intensity of A2058 or A431 cells was monitored by flow cytometer (BD LSR II) which is proportional to the cellular uptake. Data were analyzed with FACSDiVa 5.0 software.

In conclusion, drug-containing melanoma-specific peptide-based conjugates were designed, synthesized and investigated *in vitro*. Although most of the conjugates were very promising based on their *in vitro* cytostatic activity; the *in vivo* and further therapeutic application of these targeting peptides themselves might be very limited due to their poor solubility and decreased melanoma specificity. However, their application as targeting units for nanoparticles appears to be promising.

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