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

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New GnRH-III derivative as a homing peptide for potential drug targeting in cancer therapy

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Introduction

Targeted cancer therapy is a promising tool to overcome the drawbacks of classical chemotherapy like the lack of selectivity, toxicity to healthy tissue and the development of multidrug resistance forced by high dose treatments.[1] In general, ligands with high binding affinities to tumor-specific receptors or receptors which are overexpressed on the surface of cancer cells can be used as carriers for anticancer drugs enabling the selective delivery of an effective cytotoxic agent or radionuclides to tumor cells. Many regulatory peptides (*e.g.* gonadotropin-releasing hormone (GnRH), somatostatin, bombesin, neurotensin) have membrane-bound receptors on different types of tumor.[2] These receptors are usually overexpressed on tumor cells in comparison with normal tissues. Therefore, they might be good targets in targeted tumor therapy. Based on these findings, efficient cytotoxic GnRH-I derivatives were developed in Schally's laboratory. The most prominent conjugate AEZS-108 (ZoptrexTM, previously AN-152) consists of a GnRH-I-[^{6}D-Lys] targeting moiety and the antitumor agent doxorubicin (Dox), which was conjugated to the side chain of the ^{6}D-Lys through an ester bond by insertion of a glutaryl spacer.[3] It has been demonstrated that AEZS-108 internalizes selectively in GnRH-R expressing cells followed by an intracellular release of the drug by tumor-specific carboxylesterases. Thus, the antitumor effect of AEZS-108 was intensively studied *in vitro* and *in vivo* revealing a significant tumor growth inhibition and regression of several tumor types *in vivo*.[4] Due to the positive results, preclinical studies and clinical trials were performed up to phase III. Unfortunately, AEZS-108 could not achieve its primary endpoint in clinical phase III studies on endometrial cancer, which was caused by the lack of a significant difference in the median period of overall survival of patients treated with Zoptrex™ as compared to patients treated with doxorubicin.[5] The main reason for this might be the poor enzymatic stability (against carboxylesterases) of the conjugate in circulation. Therefore, more stable oxime-linked daunomycin (Dau) – GnRH conjugates were

investigated in our laboratories.[6] Instead of GnRH-I (<EHWSYGWLPG-NH_2, where <E is pyroglutamic acid) GnRH-III (<EHWSHDWKPG-NH_2) was applied in these conjugates, because this natural GnRH isoform (isolated from sea lamprey) has good affinity to GnRH receptors on tumor cells, but its endocrine effect is significantly lower in mammals which results in lower hormonal side effects during the treatment of hormone-independent tumors (*e.g.* colon cancers).[7,8] Numerous GnRH-III – Dau conjugates had been developed up to the beginning of this project. Our lead compound was <EHWSK(Bu)DWK(Dau=Aoa)PG-NH_2 in which Ser in position 4 was replaced by side-chain butirylated Lys and the Dau was attached to the side chain of Lys in position 8 *via* an oxime linkage.[9] From this conjugate the released smallest metabolite was H-Lys(Dau=Aoa)-OH that also bound to the DNA efficiently resulting in significant tumor growth inhibition.[10] In this project our plan was to make further modifications in the sequence of the homing peptide for increasing the antitumor activity of peptide-drug conjugates.

Results

Twenty new GnRH-III – Dau conjugates were developed. First, Asp in position 6 was replaced by D-Asp, D-Glu and D-Trp in both the native GnRH-III (Ser in position) and in our lead compound **K2** ([^{4}Lys(Bu)]-GnRH-III(^{8}Lys(Dau=Aoa)).[11] These conjugates showed lower cytostatic effect than the basic prime conjugates which was related to the lower cellular uptake of the new conjugates both on MCF-7 human breast (hormone-dependent) and HT-29 human colon (hormone-independent) adenocarcinoma. Considering the fact that the incorporation of ^{6}D-Aaa did not lead to an improved antitumor activity of the GnRH-III-Dau conjugates, further amino acid substitutions, and their effect on cancer cell proliferation have been investigated. The applied sequence modifications have been selected based on the findings of previously reported structure-related activity studies of unconjugated GnRH-III derivatives.[12] According to this, ^{7}Trp was changed to D-Trp and/or ^{3}Trp was replaced by D-Trp or D-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid), an unnatural amino acid. D-Tic is a secondary amine (enclosed in a cycle) similarly to Pro, therefore the attachment of the following amino acid (His) was not easy, and a significant amount of His deleted peptide could be detected in the crude mixture. Therefore, we prepared conjugates from [^{2}ΔHis, ^{3}D-Tic]-GnRH-III and [^{2}ΔHis, ^{3}D-Tic, ^{4}Lys(Bu)]-GnRH-III derivatives as well. Interestingly, [^{2}ΔHis, ^{3}D-Tic, ^{4}Lys(Bu)]-GnRH-III(^{8}Lys(Dau=Aoa) conjugate showed significantly (2-5 times) higher cytostatic effect on many different tumor cell lines than our previous lead

compound (Table 1).[13] The elevated activity of the new lead compound could be explained by its higher cellular uptake by tumor cells (Figure 1).

Table 1. Anti-proliferative effect of free drug Dau and GnRH-III-Dau conjugates **K2** and **16** on various cell lines.

		IC_{50} [1] 24h + 48h			Relative efficiency[2]	
Tumor type	**Cell line**	**Dau (nM)**	**K2 (μM)**	**16 (μM)**	**K2/Dau**	**16/Dau**
Breast	MDA-MB-231	54.6 ± 7.4	5.8 ± 0.8	1.9 ± 0.2	106.2	34.8
Breast	MCF-7	63.9 ± 21.0	16.5 ± 1.2	4.0 ± 0.8	258.2	62.6
Mice breast	4T1	56.0 ± 14.7	6.3 ± 0.9	1.8 ± 0.1	112.5	32.1
Colon	HT-29	202.9 ±1.0	15.5 ± 1.7	7.3 ± 0.3	76.4	36.0
Mice colon	C26	117.5 ± 8.6	10.6 ± 0.2	2.6 ± 0.7	90.2	22.1
Prostate	DU145	16.3 ± 4.6	5.3 ± 0.4	2.1 ± 0.2	325.2	128.8
Prostate	PC-3	32.7 ± 4.7	6.3 ± 0.3	2.4 ± 0.6	192.7	73.4
Glioblastoma	U87MG	126.4 ± 53.7	9.0 ± 0.8	2.3 ± 0.1	71.2	18.2
Ovarian	A2780	10.4 ± 1.6	1.4 ± 1.1	2.1 ± 0.5	134.6	201.9
Ovarian	OVCAR-3	404.0 ± 9.4	46.0 ± 1.3	8.2 ± 0.5	113.9	20.3
Ovarian	OVCAR-8	185.6 ± 99.8	5.7 ± 0.8	9.5 ± 0.8	30.7	51.2
Liver	HepG2	22.9 ± 1.4	6.8 ± 0.3	2.2 ± 0.7	296.9	96.1
Melanoma	A2058	35.1 ± 14.9	8.4 ± 0.3	2.6 ± 0.5	239.3	74.1
Melanoma	WM983b	49.8 ± 22.9	12.7 ± 1.5	2.6 ± 0.6	255.0	52.2
Melanoma	HT168-M1/9	27.5 ± 9.1	13.5 ± 1.1	2.9 ± 0.6	490.9	105.5
Melanoma	M24	118.8 ± 25.0	16.2 ± 0.2	3.5 ± 0.6	136.4	29.5
Mice melanoma	B16	26.0 ± 8.0	3.2 ± 0.8	1.1 ± 0.2	123.1	42.3
Head and neck	PE/CA-PJ41	45.6 ± 33.5	4.7 ± 0.8	1.7 ± 0.5	103.1	37.3
Head and neck	PE/CA-PJ15	50.5 ± 38.7	7.4 ± 0.8	2.9 ± 0.6	146.5	57.4
Lung	H1975	20.9 ± 2.7	4.1 ± 0.1	2.3 ± 0.7	196.2	110.0
Lung	H1650	50.3 ± 13.4	10.5 ± 1.1	4.0 ± 0.8	208.7	79.5
Lung	A549	69.3 ± 23.5	9.7 ± 0.6	4.3 ± 0.4	140.0	62.0
Pancreas	PANC-1	525.9 ± 24.7	>100	56.4 ± 4.5	>190.2	107.2
Normal fibroblast	MRC-5	287.6 ± 35.1	41.9 ± 3.8	19.7 ± 1.2	145.7	68.5

[1] IC_{50} values (average ± SD). [2] Relative potency = IC_{50} conjugate / IC_{50} Dau.

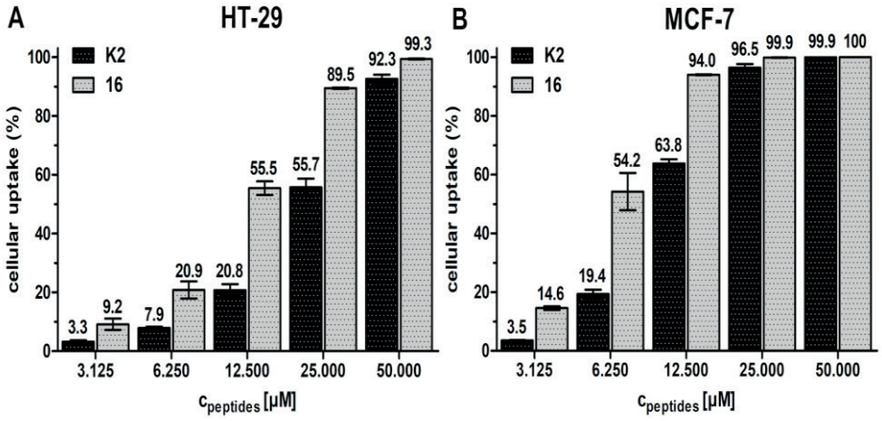

Figure 1. Cellular uptake of GnRH-III-Dau conjugates [^{4}Lys(Bu)]-GnRH-III(^{8}Lys(Dau=Aoa) **K2** and [^{2}ΔHis, ^{3}D-Tic, ^{4}Lys(Bu)]-GnRH-III(^{8}Lys(Dau=Aoa) **16** by flow cytometry. **A**: HT-29 and **B**: MCF-7 cancer cells after 6 h treatment.

In metabolism studies, it was also indicated that the modification resulted in the improvement of enzyme stability of the new conjugates, but the efficiency of the active metabolite release was not influenced by this sequence modification. These two conjugates were investigated for *in vivo* studies, too. Mice with orthotopically developed HT-29 colon carcinoma were treated with free Dau once a week (3 times, 1 mg/kg dose) and with conjugates two times/week (7 times, 10 mg Dau content/kg dose).[13] The mice in free Dau treated group exhibited a significantly decreased bodyweight, whereby the experiment was terminated on day 23 after tumor transplantation (day 17 of treatment). The bodyweight of the mice in the control group was significantly decreased on day 30 after tumor transplantation which was the reason for experiment termination (Figure 2A). Tumor weights indicated similar growth inhibition in all treated groups compared to the control (84.3% for Dau (one week earlier stage), 80.8% for **K2** and 87.1% for **16** (Figure 2B). However, the conjugates did not show liver toxicity in comparison with the free Dau treatment (Figure 2C).

In addition, *in vivo* experiments in orthotopic 4T1 mice breast carcinoma and in MDA-MB-231 human breast carcinoma bearing mice indicated significant inhibition (40-50%) of macro- and micrometastasis formation compared to the control that was higher than in the case of free Dau treatment. Conjugate **16** showed slightly better antimetastatic effect than conjugate **K2** suggesting that the new lead compound can be more effective not only *in vitro*, but also *in vivo*. In addition, we can conclude that the oxime linked Dau – GnRH-III conjugates might be good candidates for targeted tumor therapy.[13]

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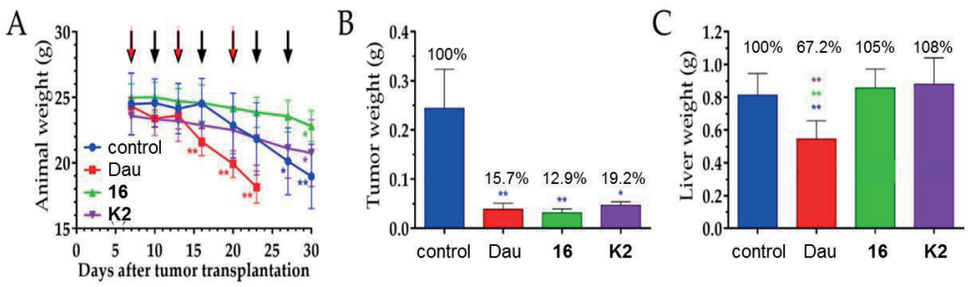

Figure 2. Effect of Dau and conjugate **16** and **K2** on animal weight (A), tumor growth (B) and their toxicity on the liver (C)

Considering the favorable results of conjugates **16** and **K2**, the corresponding peptide sequences have been selected as targeting moieties for the further conjugates, and the classical anticancer drugs Dau and PTX were used as payloads. The drugs were attached to the homing peptides through self-immolative linker (Val-Ala-PABC and Val-Cit-PABC, where Cit is citrulline and PABC is *para*-aminobenzyloxy carbonyl) that allow the release of free drugs in the presence of lysosomal enzyme Cathepsin B.[14] In addition conjugates with non-cleavable linkers were investigated for comparative studies (Figure 3).[15]

The *in vitro* cytostatic effect of the conjugates indicated that PTX-containing conjugates are more potent on both A2780 ovarium carcinoma (high GnRH-R level) and

PANC-1 pancreatic cancer (low GnRH-R level) than the Dau conjugates (Table 2.). The free PTX has also one order of magnitude higher antitumor effect on the cells than Dau. There was a significant difference between the antitumor activity of conjugates with self-immolative spacer and non-cleavable spacer in all cases. However, the type of self-immolative linker and the homing peptide had no significant influence on the effect in the case of PTX conjugates, while Val-Ala-PABC linker and the [^{2}ΔHis, ^{3}D-Tic, ^{4}Lys(Bu)]-GnRH-III homing peptide resulted in higher cytostatic effect compared with the application of Val-Cit-PABC and the [^{4}Lys(Bu)]-GnRH-III carrier. Interestingly the Dau conjugates with self-immolative linker did not show higher antitumor activity *in vitro* than the oxime linked version which does not serve the free drug release. This could be explained by the binding affinity of the conjugates to GnRH receptors. The oxime linked Dau conjugate had higher affinity to the receptor than the conjugate with a self-immolative linker. The reason might be the steric hindrance of the larger Val-Ala/Cit-PABC spacer over the aminooxyacetyl moiety.

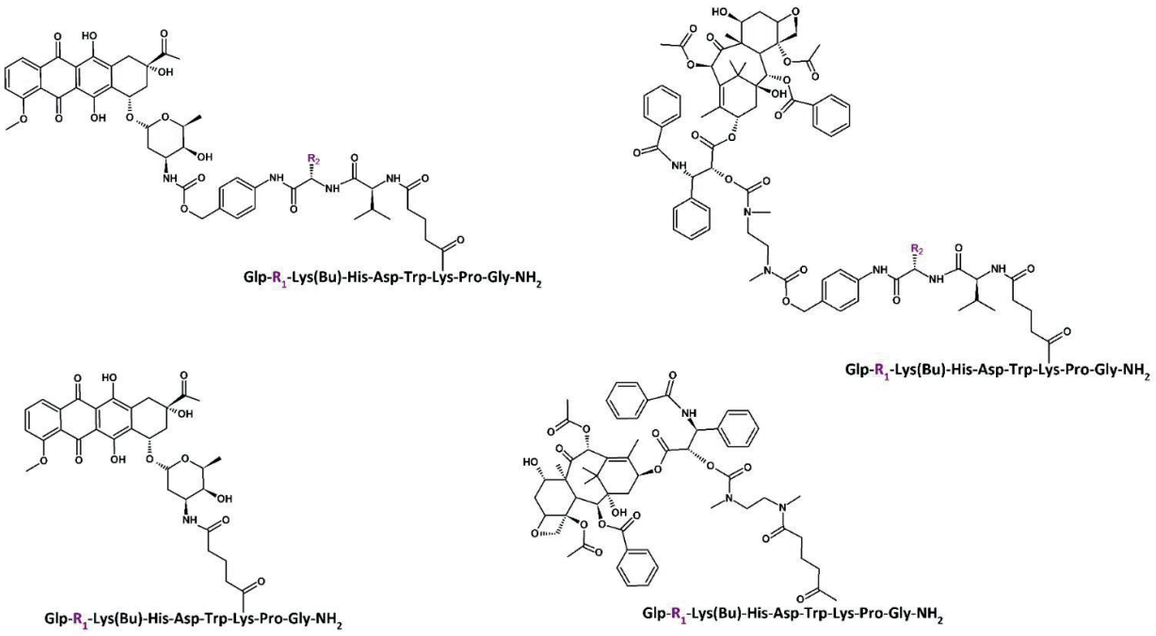

Figure 3. Chemical structure of Dau- and PTX-GnRH-III derivative conjugates with self-immolative spacers and non-cleavable spacers (R_1 is D-Tic or His-Trp and R_2 is Val-Ala or Val-Cit)

In conclusion, these experiments indicated that there are many factors that can influence the antitumor effect of the conjugates developed for targeted tumor therapy. These can be the efficacy of the drugs, the structure of the homing peptide, the binding affinity and cellular uptake of the peptide – drug conjugate, the release of the free drug or active metabolite from the conjugates in lysosomes (linker strategy). Therefore, during the development of drug

delivery systems, we have to study all of these factors to get appropriate drug candidates for targeted tumor therapy.

Table 2. Cytostatic effect of GnRH-III derivative Dau (left) and PTX (right) conjugates with the self-immolative linker and the non-cleavable linker

Code	R_1	Cleavage site	A2780 (+) IC_{50} [μM] (ovarian)	Panc-1 (-) IC_{50} [μM] (pancreatic)
Dau			0.21±0.01	2.43±0.58
Sch79	His-Trp	-Val-Cit-	11.18±0.38	85.57±24.33
Sch90	His-Trp	-Val-Ala-	7.48±0.66	56.19±17.28
Sch107	His-Trp	none	67.88±25.36	>100
Sch89	D-Tic	-Val-Cit-	4.24±1.09	>100
Sch91	D-Tic	-Val-Ala-	2.85±0.90	>100
Sch108	D-Tic	none	48.14±0.47	>100

Code	R_1	Cleavage site	A2780 (+) IC_{50} [μM] (ovarian)	Panc-1 (-) IC_{50} [μM] (pancreatic)
PTX			0.02±0.001	0.17±0.01
Sch93	His-Trp	-Val-Cit-	0.67±0.07	5.03±1.91
Sch100	His-Trp	-Val-Ala-	0.66±0.18	4.89±1.08
Sch112	His-Trp	none	41.52±9.83	> 100
Sch99	D-Tic	-Val-Ala-	0.77±0.08	8.15±3.22
Sch101	D-Tic	-Val-Cit-	0.51±0.11	6.44±1.22
Sch113	D-Tic	none	> 100	> 100

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