SUMMARY

Development of anticancer drug-peptide bioconjugates for combination targeted cancer chemotherapy

(OTKA K104045)

1. Cyclic-NGR and -RGD peptide derivative drug conjugates

In drug targeting NGR-peptides recognized by CD13 receptors on tumor neovasculature have got improved interest. CD13, a membrane-bound metallopeptidase, is not (or barely) expressed on endothelium of normal blood vessels but it is up-regulated in angiogenic blood vessels and has multiple functions (e.g. protein degradation, cell proliferation, cell migration, angiogenesis). This observation makes CD13 a suitable target molecule for specific targeted delivery of drugs and nanoparticles to tumor neovasculature, using NGR-peptides as a homing motif.

It is well known, that Asn deamidation through succinimide ring formation can easily occur, especially if Asn is followed by Gly on its *C*-terminus. This non-enzymatic intra-molecular reaction finally leads to the formation of isoaspartyl (*iso*Asp) and aspartyl (Asp) containing peptides of about 3:1 ratio, and depends on pH, temperature, solvent dielectric constant, primary sequence and secondary structural motives. This modification causes difficulties on *in vitro* and *in vivo* biological data interpretation as well as on NGR-peptide formulation. Furthermore, this non-enzymatic post-translational modification could be responsible for the integrin binding properties of NGR sequence containing peptides and proteins. It was shown that cyclic and linear peptides with *iso*Asp-Gly-Arg sequence can bind to $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$ and $\alpha_5\beta_1$ integrins while peptides with Asp-Gly-Arg sequence not. Therefore, stability studies of linear and cyclic NGR-peptides used as tumor-homing motives are crucial before developing such drug delivery systems.

In our experiments seven cyclic NGR-peptides of different ring sizes ($15 < n < 18$ atoms) with amide-, disulfide- and thioether bond were synthesized (c[KNGRE]-NH_2 (**1**); Ac-c[CNGRC]-NH_2 (**2**); c[CH_2CO-XNGRC]-NH_2: X = Ø (**3**), Lys (**4**) or Pro (**5**); c[CH_2CH_2CO-NGRC]-NH_2 (**6**); and c[CH_2CO-NGR*h*C]-NH_2 (**7**)). Significant differences were found in succinimide ring formation rate for the present compounds, namely **1** >> **6** ~ **2** > **5** > **7** > **4** > **3** stability order was established. The rearrangement of Asn to *iso*Asp and Asp through succinimide ring formation depended on the pH, salt concentration and temperature of the solution, but the structure of the cyclic peptides has the main influence on speed and rate of deamidation. From

the NMR data analysis, we conclude that *d*(N-C), the distance of N^{Gly} and $C\gamma^{Asn\text{-}sc}$ atoms is a relevant measures of reactivity rate, significant correlation was established. However, if Asn side chain is pointing away from N^{Gly}, and the molecular fold is locked by H-bonds in the latter conformation, chemo-stability will be high, NGR will hardly decompose as seen for **1** c[KNGRE]-NH_2. (Enyedi *et al*. J Med Chem 58(4):1806-1817 (2015)).

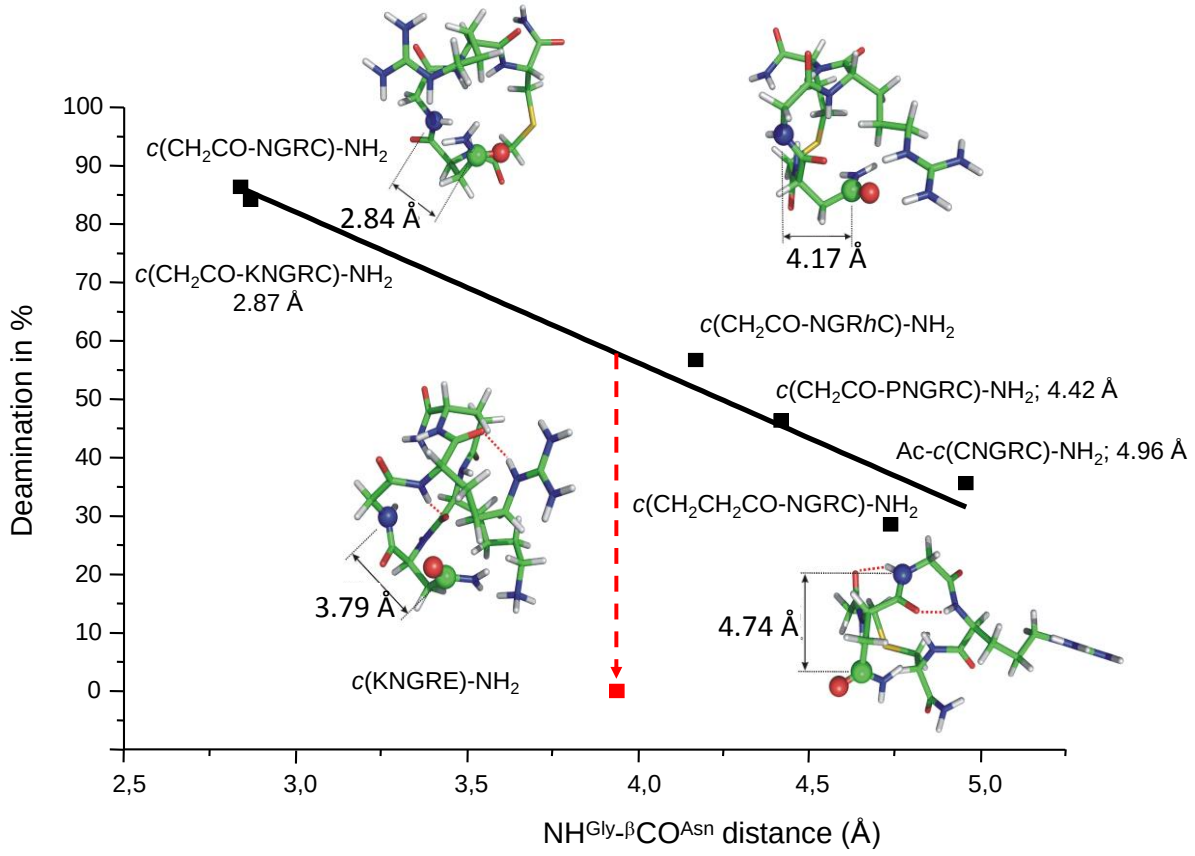


Figure 1: Correlation of NH^{Gly}-$^{\beta}CO^{Asn}$ distance and stability of cyclic NGR peptides

The stable compound **1** and the labile compound **4** with Lys in the cycles were applied for development of conjugates for PET (positron emission tomography). For this purpose a chelator was attached to the side chain of Lys and ^{68}Ga were chelated. The results indicated that compound **1** is suitable for efficient tumor localization and tumor visualization. This compound showed higher tumor localization that the RGD dimer derivative that is very popular in PET experiments recently. Compound 4 was less effective and when Gly was replaced by sarcosine (N-Me-Gly) the activity was lost completely. (Máté G, et al. Eur. J. Pharm. Sci. 69: 61-71, 2015; a second manuscript is in progress)

A series of cyclic and linear with Asn-Gly and Asp-Gly sequential elements were prepared for kinetic studies of rearrangement to *iso*Asp derivatives. In this NMR based experiment the importance of the structural and sequential environment of Asn/Asp-Gly were indicated. These results might be important also for understanding several illnesses related to Asn/*iso*Asp rearrangement in proteins. The manuscript (Láng A., Enyedi KN. *et al*.) is under preparation and will be sent to Nature Chemistry soon.

According to the results of stability studies of cyclic NGR peptides 4 peptides (compounds **1**, **2**, **3** and **4**) with different stability were selected for drug targeting. Daunomycin (Dau) as anti-tumor agent was attached *via* oxime linkage through an enzyme cleavable spacer (GFLG) to the cyclic peptides. Cyclic peptides were elongated on their C-terminus for the conjugation. In case of Lys containing peptide variants (**1** and **4**), the Dau=Aoa-GFLG- (where Aoa is aminooxyacetyl moiety) was attached to the side chain of Lys as well. The cytostatic/cytotoxic effects of the conjugates were studied on HT-1080 CD13+ (human fibrosarcoma) and HT-29 CD13- but integrin receptor positive (human colon carcinoma) cells. Both cell lines express RGD integrin receptors. Results are summarized in Table 1.

The results indicated that the conjugates are more selective to CD13 receptors when the drug – spacer part is attached to the side chain of Lys in the cycle (compounds **4** and **6**). However, the most effective compound was compound **5**, but it was not selective to CD13. Compound **1**, that deamidated quite fast was not active after 6 h treatment but showed some effect after 72 h treatment. Furthermore, in this case a bit higher cytostatic effect was detected on CD13 negative HT-29 cells suggesting that rather the formed *iso*Asp derivative has bioactivity on RGD integrin receptors. The results have been sent for publication to Bioconjugate Chemistry (Enyedi KN. *et al.*) (Enyedi KN., *et al.* J. Pept. Sci. 22:(52S) p. 164. (2016))

The structure – stability studies indicated that the side chain of Lys in c[KNGRE]-NH_2 do not play any role in the stabilization of the conformation. Furthermore, the synthesis of most effective conjugate (compound **5)** has difficulties because selectively removable orthogonal protecting group scheme should be applied. To overcome this 5 further conjugates were developed to identify whether the Lys in the sequence can be replaced. Ala, Ser, Leu, Pro and Nle were incorporated into the sequence instead of Lys. The Pro, Leu and Ala was accepted in this position (Pro was the best), but none of them reached the activity of Lys containing derivative. (Tripodi A., Enyedi KN., *et al.* J. Pept. Sci. 22:(52S) p. 197. (2016))

The best compounds will be tested alone and in combination with other peptide – drug conjugates on tumor bearing mice soon.

Table 1. Cytostatic and cytotoxic effects of cyclic NGR peptide – drug conjugates

Code	Compounds	IC_{50} (μM), 6 h		IC_{50} (μM), 72 h	
		HT1080	HT-29	HT1080	HT-29
	Daunorubicin	0.7±0.3	0.7±0.3	0.1±0.1	0.2±0.1
1	Dau =Aoa-GFLGK-NH_2 CH_2CO-NGRC-GG	>>50	>>50	23.8 ±10.7	20.6±5.6
2	Dau =Aoa-GFLGK-NH_2 Ac-CNGRC-GG	32.3 ±11.2	46.2±3.5	12.4 ±5.2	27.4±1.1
3	Dau =Aoa-GFLGK-NH_2 CH_2CO-KNGRC-GG	8.0 ±1.9	10.3±3.4	3.3±0.6	5.7±1.5
4	CH_2CO-KNGRC-NH_2 Dau =Aoa-GFLG	45.5±13.2	>>50	14.5±2.0	>>50
5	Dau =Aoa-GFLGK-NH_2 KNGRE-GG	5.4±0.1	8.7±1.2	1.2±0.7	3.0±0.6
6	KNGRE-NH_2 Dau =Aoa-GFLG	43.7±4.1	>>50	15.8±0.1	>>50

Two RGD based drug conjugates were developed. “Head-to-tail” cyclic [RGDfC] (where f is a one letter symbol of D-Phe) were prepared and attached to two different, chloroacetylated enzyme labile the spacer (>=Aoa-GFLGK(ClAc)-NH_2 and (>=Aoa-LRRYK(ClAc)-NH_2, where >= is isopropylidene protecting group) *via* thioether linkage. After the chemoselective ligation and removal of isopropylidene protecting group, Dau was conjugated to the homing peptide via oxime linkage formation. The in vitro cytotoxic effect of Dau-RGD cyclic peptide conjugates was studied by MTT assay on MCF7 human breast cancer and B16-F10 murine melanoma cells, while HT29 human colon cancer cells were used as negative control considering the low expression of $\alpha_v\beta_3$ receptors. Compounds dissolved in serum containing (FBS+) or serum free (FBS-) RPMI 1640 medium were added to the cells and the treatment was carried out for 72 h. The conjugates were not active on HT-29 cells, but showed some effect on MCF-7 cells and a bit higher on B16-F10 cells (IC_{50} = ~10 μM). The conjugate with GFLG spacer seems to be more potent than the other one. (Tripodi A., Enyedi KN., *et al.* J.

Pept. Sci. 22:(52S) p. 197. (2016)) Further studies on other type of cancer cells as well as in combination with conjugates having selectivity to other receptors are in progress.

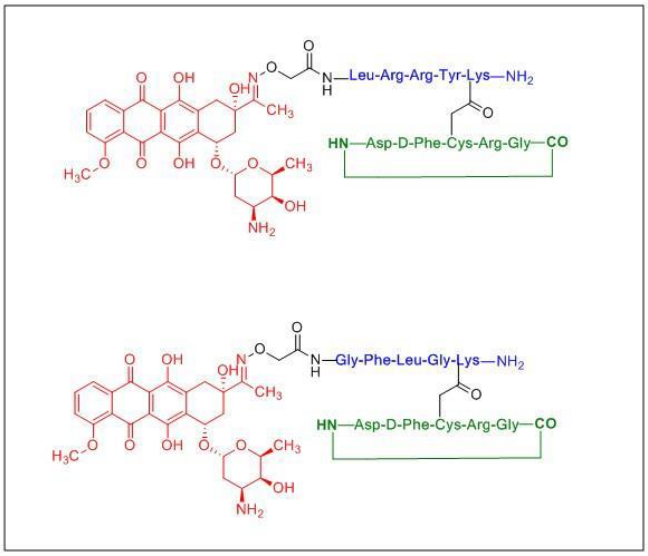


Figure 2: Structure of cyclic RGD – Dau conjugates

2. GnRH derivative drug conjugates

GnRH (gonadotropin-releasing hormone) receptors are overexpressed on many types of tumor. Therefore, it is a good target for targeted tumor therapy. In the last decade ca. 25 GnRH – drug conjugates were developed for selective drug delivery into tumor cells.

In our previous studies we selected the most efficient GnRH-III – daunomycin conjugate (<EHWK(Bu)HDWK(Dau=Aoa)PG-NH_2, where <E is pyroglutamic acid, Bu is butyric acid, and Aoa is aminooxyacetyl moiety for oxime ligation between the drug and peptide molecules) that was further studied. Recently we confirmed its receptor mediated cellular uptake by confocal microscopy and by receptor blockade with the GnRH super agonist triptorelin ([D-Trp^6]-GnRH-I) (Schuster S. et al. J. Pept. Sci. submitted 2016).

It was also indicated that this conjugate had significant tumor growth inhibitory effect not only on sub cutan transplanted tumor xenograft but also on orthotopically developed colon cancer in immunodefficient mice (40-50%). The antitumor effect of the conjugate was higher than the efficiency of the free drug in maximal tolerated dose. Furthermore, the metastases on several organs were lower in case of the application of the conjugate instead of free drug. (Kapuvári B., et al. Magyar Onkológia 59: 310-318. (2015), and Investigational New Drugs 34: 416-423. (2016))

Fourteen new GnRH-III – Dau conjugates were developed in which amino acids in position 3, 6, and 7 were replaced by D-amino acids (D-Asp, D-Glu and D-Trp in position 6 and D-Trp and D-Tic (tetrahydro-isoquinoline-3-carboxylic acid) in positions 3, 7 or in both. We studied their cytostatic effect, cellular uptake, stability and degradation. The smallest metabolites were identified in all cases. We could conclude that the antitumor effect depends not only on the cellular uptake but also on the metabolism of the conjugates. (Schuster S. et al. J. Pept. Sci. submitted 2016, J Pept. Sci 22:(52S) 195., 2016).

Shortend versions of these conjugates were prepared, too. One of the new GnRH-III – Dau conjugates showed one order of magnitude higher cytostatic effect than the previously mentioned lead compound. Therefore, the former lead compound and the best new conjugate were further developed. We attached other type of drugs and we used different linkages between the peptide and the drug instead of oxime linkage. Some biological studies are still in progress and fortunately these new conjugates can be patented in the near future. Therefore, the structures of these compounds are not presented here and they will be published after patenting them.

Conjugates with two drug molecules were also prepared. For this purpose GnRH-III dimer derivatives were synthesized (dimerization was made by disulfide bond formation) and conjugated to two Dau molecules *via* oxime linkage. Slightly improved antitumor effect was detected using the dimer conjugates compared to the monomer ones. (Schreier VN, et al., Bioorg. Med. Chem. Lett. 23: 2145-2150 (2013)) In another experiment two Dau were attached to one [Lys^4]-GnRH-III molecule in position 4 and 8 through GFLG enzyme labile spacer. Unfortunately the solubility of the conjugate was very low. Thus, oligoethylene glycol linker was incorporated between the side chain of lysines (in position 4 and 8) and the GFLG spacers. This modification resulted in significant improvement both in solubility and cytostasis that was comparable with the free drug molecules. This modification seems to be a good choice to enhance the bioavailability of our conjugates. (Hegedüs R., et al. Biopolymers Pept. Sci. 104: 167-177. (2015)) Further GnRH-III conjugates with two different (methotrexate – Dau and paclitaxel – Dau) were also prepared. Especially the later one showed high antitumor activity that was much higher than the conjugates containing only one drug molecules on the carrier peptide. (Two publications are under preparation in this topic)

Beside the comparative cytotoxic and cellular uptake studies of GnRH-I (<EHWSYk(Dau=Aoa)LRPG-NH_2), GnRH-II (<EHWSHk(Dau=Aoa)WYPG-NH_2), and GnRH-III (<EHWSHDWK(Dau=Aoa)PG-NH_2), their effect on apoptosis markers were determined. For this purpose a 23 apoptosis marker-containing kit was used. It was indicated

that the GnRH analogs as homing peptides had significant and different influence on the different apoptosis markers. (Szabó I., et al. J Pept. Sci. 21: 426-435 (2015) and Hegedüs R., Lajkó E., et al. Oncotarget (will be submitted soon))

Cellular uptake of carboxyfluorescent labelled GnRH-I (<EHWSYk(FITC)LRPG-NH_2), GnRH-II (<EHWSHk(FITC)WYPG-NH_2), and GnRH-III (<EHWSHDWK(FITC)PG-NH_2) derivatives was also compared on different cancer cells. The results indicated that the preferred GnRH conjugate depends on cell type. Our experiments presented first, that Detroit-562 pharynx cancer cells express GnRH receptors in a high amount and the cells take up the GnRH conjugates efficiently. It was also observed that GnRH-III conjugate had the highest solubility while GnRH-II conjugate had the lowest one. (Murányi J., et al. J. Pept. Sci. 22: 552-560 (2016) and Murányi J., et al. JoVE (Journal of Visualized Experiments) (submitted for publication), 2016).

Table 2. Total uptake of [D-Lys^6(FITC)]-GnRH-I (I), [D-Lys^6(FITC)]-GnRH-II (II) and [Lys^8(FITC)]-GnRH-III (III)

cell line	[D-Lys^6(FITC)]-GnRH-I*		[D-Lys^6(FITC)]-GnRH-II*		[Lys^8(FITC)]-GnRH-III*	
	1 hour	5 hours	1 hour	5 hours	1 hour	5 hours
MDCK	0.08±0.06	0.11±0.02	0.04±0.06	0.05±0.01	0.09±0.03	0.15±0.02
LNCaP	0.20±0.05	0.56±0.10	0.34±0.03	1.11±0.05	0.16±0.02	0.54±0.08
MCF-7	0.31±0.10	0.57±0.08	0.35±0.13	0.73±0.14	0.24±0.08	0.50±0.07
BxPC-3	0.14±0.09	0.09±0.05	0.05±0.05	0.07±0.01	0.03±0.04	0.01±0.02
HT-29	0.10±0.05	0.19±0.07	0.12±0.06	0.21±0.05	0.17±0.02	0.30±0.07
Detroit-562	0.32±0.02	0.61±0.03	0.27±0.04	0.67±0.03	0.33±0.05	0.82±0.08

*Applied concentration: 1 µM; incubation time: 1 hour and 5 hours; results were presented as SD, N=3

Because Detroit-562 showed high GnRH receptor expression and efficient cellular uptake of GnRH(FITC) conjugates, furthermore pharynx tumor might be treated by photodynamic idtherapy protoporphyrin IX was conjugated to the similar GnRH-I, -II, and -III derivatives and their efficiency was identified after light irradiation at 490 nm. The results confirmed the suitability of such type of conjugates in photodynamic therapy. (Nassima Kram MSc. thesis)

These studies are still in progress. Model peptide – porphyrin derivatives were also developed to study the DNA – porhyrin and protein – porphyrin interactions. (Orosz Á., et al. BiophysChem 177-178: 14-23, 2013; Orosz Á., et al. BBA General Subjects submitted)
From these experiments it can be concluded that the antitumor effect of GnRH – drug conjugates depends not only on cellular uptake and their degradation profile but also on their influence on apoptosis markers.

3. Somatostatin

Previously we developed a daunomycin (Dau) containing somatostatin analog (TT232) in which Dau was attached to the N-terminal D-Phe. To increase the free drug release or its active metabolite new conjugates were prepared. Hydrazone bond formation was used instead of oxime bond in one case resulting the Dau=N-NH-CO-$(CH_2)_2$-CO-TT232 conjugate. In case of two other derivatives, the sequence of TT232 was elongated by enzyme labile spacers, that also increased the water solubility of the conjugates and Dau was ligated *via* oxime bond to these spacers (Dau=Aoa-YRRL-TT232, Dau=Aoa-LRRY-TT232, where Aoa is aminooxyacetyl). The study of the cytostatic effect of these compounds indicated that the incorporation of LRRY spacer increased the cytostatic effect while YRRL showed similar activity as Dau=Aoa-TT232 that was prepared earlier. This is probably due to the different metabolites released from the conjugates. Recently we prepared somatostatin derivatives with oxime linkage instead of disulfide bond in the cycle. The cellular uptake of these conjugates were lower, but the absence of disulfide bridge in the molecule give opportunity for other type of drug conjugation (e.g. disulfide bridge formation or application of self imolative spacer) resulting in the release of the free drug molecule. These studies are in progress now.

4. Structure optimization of peptide selected from phage display library for efficient drug targeting

The goal of the combined targeted tumor therapy is to increase the anti-tumor activity by the application of a combination of different peptide – drug conjugates that can enter the cells using different receptors. One of the mostly studied cancer cell line in our laboratory is HT-29 human colon carcinoma cells. Therefore, we chose homing peptides from the literature that recognize selectively this type of cancer cell.
Phage display is a molecular diversity technology that allows the presentation of a large number of peptides permitting the selection of peptides with high affinity and selectivity for almost any target. A phage display-7 peptide library that contained 10^{11} pfu was applied and

phage clones that bind to colon cancers cells were isolated by 3 rounds of positive panning. Approximately 50 phage clones were randomly picked for further analysis. Peptide sequence VHLGYAT showed the highest binding activity for HT-29 colon cancer cell line. Therefore, we chose this heptapeptide as targeting moiety to develop peptide – drug conjugates for targeted tumor therapy.

Daunomycin was attached directly or through enzyme (Cathepsin B overproduced in cancer cells) labile spacers (GFLG, LRRY) and the cytostatic effect of the conjugates was measured on HT-29 cells. The results indicated that the conjugate with LRRY spacer (Dau=Aoa-LRRY-VHLGYAT-NH_2) the highest anti-tumor effect. Thus, this conjugate was used for further structure optimization.

Because the homing peptide was randomly selected from the phage display we believed, that there is a possibility to find better construct for drug targeting. First Ala-scan was made on the sequence. The *in vitro* cytostatic effect and cellular uptake studies indicated that the replacement of Gly by Ala is allowed and the new conjugate showed higher cytostatic effect which is correlated with the increased cellular uptake. In the second experiment further eight amino acids with different character was incorporated into this position. Replacement of Gly by Pro completely destroyed the effect probably due to the conformational changes (to confirm this CD measurements are in progress). Also the incorporation of positively charged Lys decreased the cytostatic effect. Ser was as good as Ala derivatives but showed much better solubility. The best conjugates were the Phe and Leu substituted conjugated suggesting that a large apolaric side chain in this position increase the activity of the conjugate. Dau=Aoa-LRRY-VHL**F**YAT-NH_2 showed one order of magnitude lower IC_{50} value, than the starting compound while the selectivity did not change significantly. The identification of the receptor recognized by these conjugates is in progress. (Two thesis for Student's Scientific Competition were prepared by Krisztina Kiss and the first one received 3rd place on the national competition (OTDK) in 2015; Kiss K., et al. J. Pept. Sci. 22:(52S) 183. (2016); preparation of a full paper is in progress).

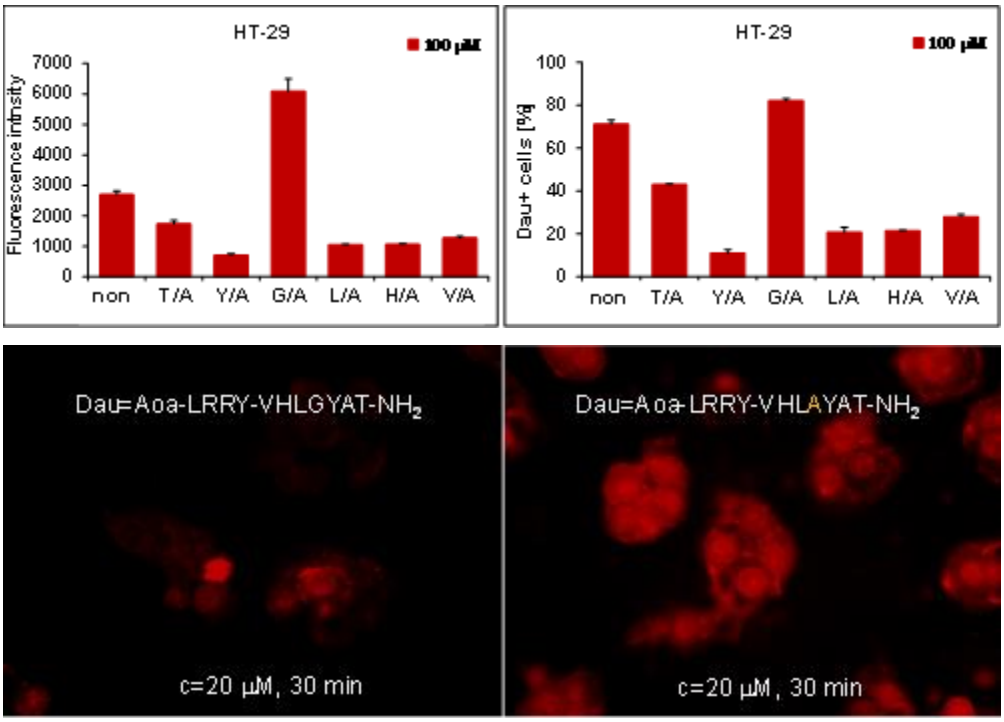


Figure 3: Cellular uptake studies by flow cytometry and fluorescent microscopy

Altogether, it is worth to optimize the peptide sequences selected by phage display technique to get very efficient targeting moiety suitable for targeted tumor therapy. This will be applied in a new NKFIH grant (K_16 119552) obtained our research group recently to follow our research on the development of bioconjugates for targeted tumor therapy of cancer types leading to high mortality.

Table 2: Cytostatic effect of conjugates with the positional scanning

Conjugates	**Cytostatic effect 24 h (IC_{50} ± SD; µM)**
Dau=Aoa-LRRY-VHLGYAT-NH_2	39.4 ± 5.9
Dau=Aoa-LRRY-VHLAYAT-NH_2	27.9 ± 6.4
Dau=Aoa-LRRY-VHLKYAT-NH_2	50.3 ± 3.0
Dau=Aoa-LRRY-VHLEYAT-NH_2	29.5 ± 6.2
Dau=Aoa-LRRY-VHLLYAT-NH_2	7.5 ± 3.5
Dau=Aoa-LRRY-VHLFYAT-NH_2	6,6 ± 2,9
Dau=Aoa-LRRY-VHLSYAT-NH_2	24.8 ± 7.4
Dau=Aoa-LRRY-VHLTYAT-NH_2	21,7 ± 6.5
Dau=Aoa-LRRY-VHLNYAT-NH_2	28.0 ± 19.4
Dau=Aoa-LRRY-VHLPYAT-NH_2	>50

5. **EGFR binding peptide – drug conjugates**

Epidermal growth factor receptor (EGFR) overexpression was found by more than 60% of the human tumor cells, therefore it is a promising target for drug delivery systems. In frame of this project EGFR targeting conjugates were prepared for targeted tumor therapy. GE11 (YHWYGYTPQNVI) and D4 (LARLLT) as homing peptides were conjugated to Dau directly or through enzyme labile spacers (GFLG and YRRL). However, some of the conjugates, especially the GFLG spacer containing ones showed very bad solubility that prevent their efficient application in *in vitro* biological studies. To overcome the solubility problems hiperbranched polyglycerol (HbPG) a polyether-polyol was attached to the conjugates. Its water solubility is extraordinary, it does not induce the immune system and its biocompatibility was approved by FDA. Pentaglycine linker containing analogs (Gly_5 located between the homing peptide and HbPG) were synthesized as well to study the significance of the peptide-polymer distance. Conjugates containing PEG were also used as controls. Solubility problems could be solved by the conjugation of polymers to the peptide-drug conjugates. HbPG could increase the solubility more than PEG. Due to the branched structure the hydrodynamic volume (V_h) of HbPG is lower than the V_h of PEG with similar molecular weight that can maybe cause lower receptor binding hindrance. This can be the explanation that the HbPG containing conjugates have higher cytostatic effect on HT-29 cells in most cases. (Pethő L., et al. J. Pept. Sci. 22:(52S) 183. (2016))

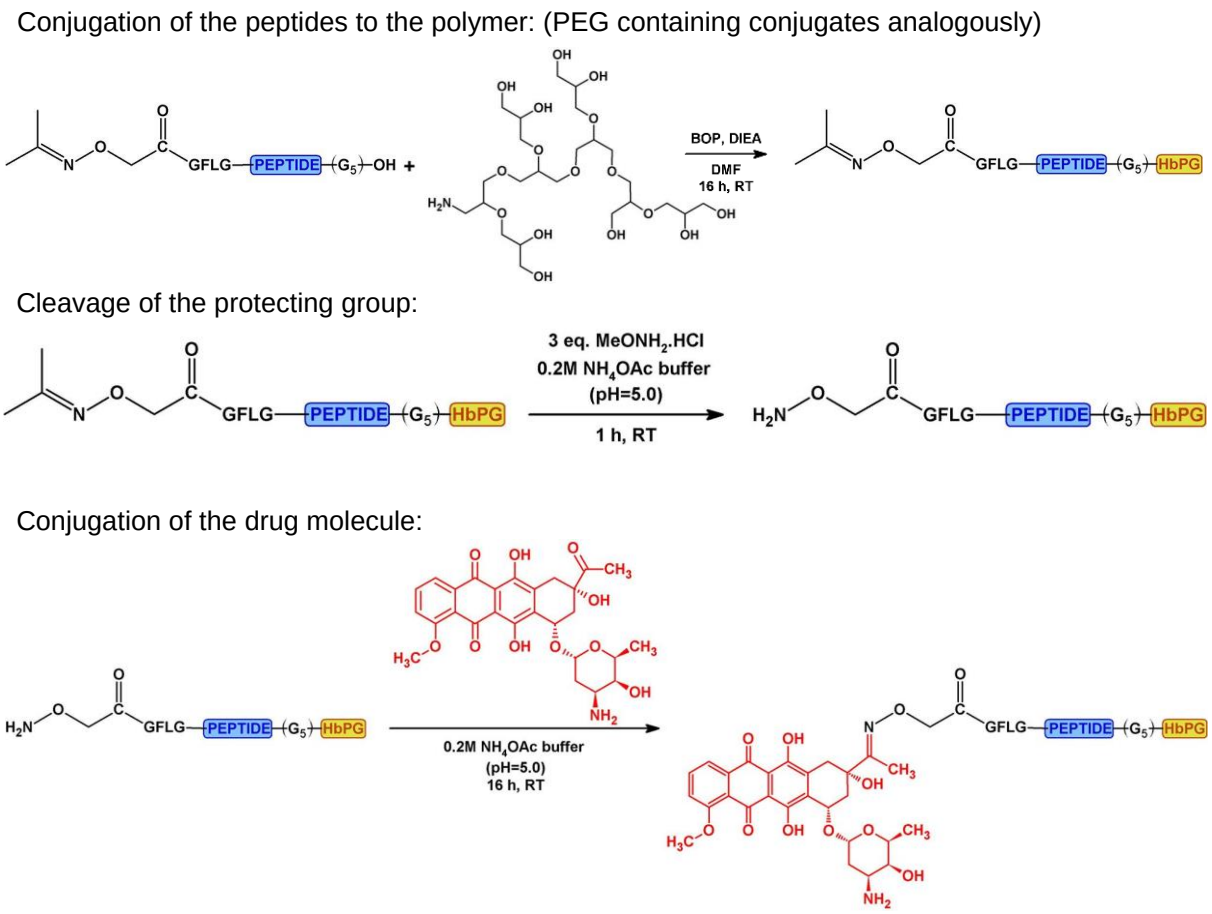
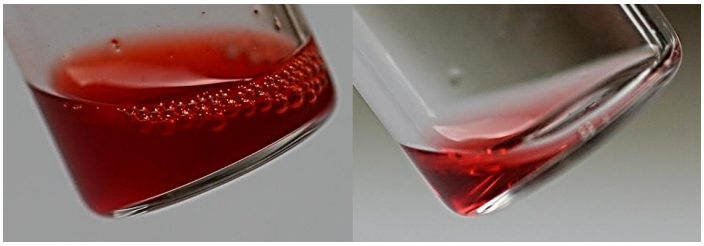


Figure 4. Synthesis of EGFR binding peptide – drug – polymer conjugates



Dau=Aoa-GFLG-D4-PEG Dau=Aoa-GFLG-D4-HbPG

Dau=Aoa-GFLG-GE11-PEG Dau=Aoa-GFLG-GE11-HbPG

Figure 5. Comparison of the solubility of PEG and HbPG conjugates

Table 3. IC_{50} values of the synthesized drug-peptide-polymer conjugates

Conjugate	IC_{50} / μM	Conjugate	IC_{50} / μM
Dau=Aoa-GFLG-GE11-HbPG	0.26	Dau=Aoa-GFLG-D4-HbPG	6.47
Dau=Aoa-GFLG-GE11-G_5-HbPG	0.96	Dau=Aoa-GFLG-D4-G_5-HbPG	1.17
Dau=Aoa-GFLG-GE11-PEG	1.08	Dau=Aoa-GFLG-D4-PEG	6.32
Dau=Aoa-GFLG-GE11-G_5-PEG	0.12	Dau=Aoa-GFLG-D4-G_5-PEG	26.00

These conjugates will be used in combined targeted tumor therapy with GnRH peptide based conjugates. However, it is mentioned in the literature that the treatment of cancer cells with GnRH-I analogues causes dramatic decrease of EGFR on them. Therefore, we studied the probable changes of receptor content on cancer cells after the treatment with peptides. Neither the GnRH-III treatment nor the GE11 treatment decreases each other's receptors. According to these results we could conclude that these conjugates can be used in combination. The optimization of the experiments is in progress. The first results will be ready soon.

6. Oligotuftsin derivative drug conjugates

Two different tuftsin receptors have been identified yet. One of them is the well-known tuftsin receptor on immune cells (monocytes, macrophages), which can be a target molecule for targeted tumor therapy in case of leukemia. Furthermore, it has recently been shown that tuftsin is the ligand of the neuropilin-1 receptor (NRP-1), which is the co-receptor of VEGF receptors and play a crucial role in vascularization. Blocking NRP-1 might be very important to inhibit the tumor growth. Tuftsin (TKPR) and its agonist (e.g. TKPKG) as well as antagonist (*e.g.* TKPPR) derivatives are good candidates for this purpose. It is worth mentioning that TKPPR tuftsin antagonist binds to NRP-1 with 4-times affinity than tuftsin itself.

Tuftsin based peptide – drug conjugates were prepared for in vitro biological investigations. All conjugates were prepared without or with an enzyme-labile spacer located between the drug and the targeting moiety. GFLG is cleavable by cathepsin B, a lysosomal enzyme overexpressed in cancer cells.

In vitro cytostatic effect of the prepared tuftsin analog conjugates was determined on human leukemia (HL-60) cells by MTT assay. TKPR and TKPPR based conjugates had better cytostatic effect, than the TKPKG based conjugates. The free carboxyl group at the C-terminal seems to be crucial in case of short peptides. The results also indicate that the presence of the GFLG spacer is necessary for the effectiveness. Dimer and tetramer forms,

furthermore conjugates with a branched form did not significantly influence the efficacy, however two drug molecules can increase the *in vitro* effectiveness.

In vitro cellular uptake of five selected conjugates was also examined by flow cytometry on HL-60 cells. The results show, that the conjugates based on TKPR and TKPPR had higher cellular uptake rate, then the conjugates based on TKPKG. The length of the peptide chain influences the uptake rate (tuftsin oligomers showed significantly higher cellular uptake), while the presence of the GFLG spacer did not have any effect on it.

The effect on cell vitality of five selected conjugates having the best cytostatic effect was assessed using the XCELLigence System (Roche Applied Science). MDA-MB-231 human breast cancer cells and HT-29 human colorectal adenocarcinoma cells were treated with the conjugates, but cytostatic/cytotoxic effect was shown only by the conjugate containing two daunomycin residues.

The results of the biological studies indicated that conjugates based on TKPR or TKPPR sequences have better *in vitro* efficacy, than the conjugates based on TKPKG. We also observed that the enzyme-labile GFLG spacer is necessary for the effective drug release, and two drug molecules increase the in vitro antitumor effect. (Pethő L., et al. J. Pept. Sci. 20: S284-S285. (2014), a full paper from the results will be published soon)

Table 4: Cytostatic effect of tuftsin – daunomycin conjugates

Conjugate	*IC_{50} (μM)*	*Conjugate*	*IC_{50} (μM)*
Dau=Aoa-TKPR-OH	20.9±0.8	Dau=Aoa-GFLG-TKPR-OH	1.1±0.6
Dau=Aoa-TKPPR-OH	37.4±11.3	Dau=Aoa-GFLG-TKPPR-OH	0.5±0.2
Dau=Aoa-TKPKG-NH_2	>50	Dau=Aoa-GFLG-TKPKG-NH_2	11.4±3.2
H-TK(Dau=Aoa)PR-OH	15.65±0.5	H-TK(Dau=Aoa-GFLG)PR-OH	1.65±0.2
Dau=Aoa-K(Dau=Aoa)-[TKPKG]$_4$-NH_2	21.95±0.4	Dau=Aoa-GFLG-K (Dau=Aoa-GFLG)-[TKPKG]$_4$-NH_2	0.75±0.1

The identification of the conjugates by mass spectrometry was difficult because of the loss of sugar moiety from daunomycin. This MS fragmentation is extremely high when peptides contain many positively charged amino acids in the sequence like in case of tuftsin derivatives. To confirm the MS fragmentation in contrast to chemical decomposition, a new condition was developed for MS measurement that allow very low MS fragmentation also in

this cases. (Pethő L., et al. manuscript will be sent to Rapid Communication in Mass Spectrometry in this year)

7. Nanoparticle - bioconjugate compositions

Development of biocompatible and biodegradable nanofibrous drug delivery system for ensuring sustained drug release:

The aimed nanofibrous drug delivery system was planned to be produced by means of electrospinning method. During this research period 4 types of biodegradable and biocompatible polymer were tested, namely polylactic acid (PLA), polylactic-co-glycolic acid (PLGA), polycaprolactone (PCL) and poly-hydroxy-butyrate-co-hydroxy-valerate (PHBHV). These polymers are good candidates to achieve sustained drug release owing to their insolubility in water-based media. (The solvents used were chloroform, methanol, dimethylformamide and their mixtures)

At first the electrospinnability of the chosen polymers was tested and optimized. Optimal mixtures of organic solvents were used to dissolve and electrospin the polymers without active ingredients. In the next step, model drugs were incorporated in the polymer fibres for optimization of the dissolution characteristics. In the cases when promising compositions were found direct experiments with anticancer drug were performed as well. Finally it was only the PLA matrix that provided slow enough dissolution of both the model and anticancer drugs that can meet the medical aims of the project. The dissolution form any other polymer seems to be too fast (max 1-2 day) under in vitro circumstances. (Sóti P.L., et al. Eur. Polymer J. 68: 671-679 (2015))

Decision was made to perform the subsequent dissolution studies from PLA matrices. Therefore the physical chemical structure of its mixture with drugs and drug conjugates were investigated. The drug release was studied from the texture. The results showed high drug release at the beginning then a slow continuous release in time. This can mimic the initial and maintenance treatment in chemotherapy. Therefore, this texture might be especially applicable for covering skin cancer.

Long chain fatty acids were attached to GnRH and tuftsin derivatives and this conjugates were incorporated in liposomes. Liposomes were loaded with drug molecules and the effect of these targeted nanoparticles was investigated. Up to now liposomes modified with tuftsin homing peptide showed high efficiency on *mycobacterium tuberculosis* infected macrophages. This is not a topic of this project, but the results showed that this structure might be efficient in drug targeting. Experiments on cancer cells are in progress.

8. **Optimization of the synthesis of 2-pyrrolino-daunorubicin and its effect on cancer cells**

A new synthetic route was developed for the synthesis of 2-pyrrolino-daunorubicin. In this case 3-bromobutyraldehyde was prepared from THF instead of the synthesis of 4-iodobutyraldehyde from 2-(3-chloropropyl)-1,3-dioxalane. The application of 3-bromobutyraldehyde resulted in 2-pyrrolino-daunorubicin with better yield and also its dimerization could be avoided. Furthermore, this synthetic route needs much cheaper chemicals. This procedure allows to prepare high amount of PyDau that is necessary for the synthesis of peptide – drug conjugates for *in vivo* studies.

The cytotoxic effect of 2-pyrrolino-daunorubicin (PyDau) and daunorubicin (Dau) was compared on Mes (Dau sensitive)/Dx5 (Dau resistant) uterine based cancer cells and on KB3 (Dau sensitive)/KBV (Dau resistant) HeLa based cancer cells. According to the results PyDau has two order of magnitude higher toxicity than Dau and it was not a substrate of efflux pumps (PyDau - IC_{50} Mes: 1.0 nM; Dx5: 2.1 nM; Dau – IC_{50} Mes: 0.3 μM; Dx5: 1.4 μM and PyDau – IC_{50} KB3: 3.1 nM; KBV: 2.95 nM; Dau – IC_{50} KB3: 0.65 μM; KBV: 8.99 μM).

PyDau was loaded into liposomes and the *in vivo* toxicity and antitumor activity were investigated. The results will be concluded soon.

PyDau was conjugated to different homing peptides mentioned above *via* oxime linkage and the cytostatic effect of the conjugates were measured on different cancer cells. In conclusion, the conjugates with PyDau one order of magnitude higher antitumor effect than the Dau conjugates with the same targeting peptides and comparable with the free Dau. The *in vivo* toxicity studies and the measurements their tumor growth inhibitory effect are in progress.

Publications (2 or 3) from these experiments will be done later.

9. **Publications**

15 manuscripts and one book chapter have been published (3 in Hungarian) and further 4 have been submitted for publication so far. However, our results and some ongoing experiments predestinate at least 10 further publications from the summarized topics in the near future. These results were presented in different conferences during the last years. One PhD thesis were done and two more will be finished in 2017 that present the results summarized above. Several BSc. and MSc. thesis and thesis for Student's Scientific Competition were also made.

10. Future and perspectives

According to the results presented here and previous publications on the field of targeted tumor therapy Prof. Gábor Mező (Research Group of Peptide Chemistry, Eötvös Loránd University) and Dr. József Tóvári (National Institute of Oncolgy) were invited to a Horizon 2020 project (MSCA-ITN-2014-ETN: Marie Skłodowska-Curie Innovative Training Networks (ITN-ETN) Grant Agreement number: 642004) in 2015. The name of the project is MAGICBULLET symbolize the topic of targeted tumor therapy. Six universities (two German, two Italian, a Finish and ELTE (Hungary), pharmaceutical companies like Bayer, Heidelberg Pharma, PharmItalia and Health institutes are involved in this project. The fact that we can have a very close contact with pharmaceutical companies give a chance that our results and compounds will be further developed for cancer medicines. Two new PhD students at ELTE (a German and an Italian) and a Serbian PhD student at NIO (all together 15 in different universities and institutes) are employed on this project.

Furthermore, the PI won a new NKFIH project (K_16, 119552) "Development of bioconjugates for targeted tumor therapy of cancer types leading to high mortality". In frame of this project we would like to develop selective drug – peptide conjugates to lung, pancreas, and brain cancer as well as for melanoma and its metastases. One PostDoc will be employed on this project.

In addition drug, linker and homing peptide libraries will be developed in frame of a NVKP_16 project. The goal of this project is to prepare functionalized drugs and homing peptides that can be attach to each other in a very high combination by the aid of different orthogonal bifunctional linkers. In this way hundreds of different conjugates can be prepared easily by LEGO-technic that can be applied in personal targeted tumor therapy. Roughly 10 new jobs will be offered in this project.