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

Excerption from the results obtained in frame of the grant

NVKP_16-1-2016-0036

supported by the National Research, Development and Innovation Office

ISBN 978-963-489-286-1

Budapest, 2020

Optimation of homing peptide sequence selected by phage display for HT-29 colon cancer cells to improve the antitumor activity

Krisztina Kiss[1,2], Beáta Biri-Kovács[1,2], Rita Szabó[1], Kata Nóra Enyedi[1,2], Murányi József[3], Gitta Schlosser[1,2], Ivan Ranđelović[4], József Tóvári[4], Gábor Mező[1,2]

[1]MTA-ELTE Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University, Budapest, Hungary

[2]Institute of Chemistry, Eötvös L. University, Budapest, Hungary

[3]MTA-SE Pathobiochemistry Research Group, Semmelweis University, Budapest, Hungary

[4]Department of Experimental Pharmacology, National Institute of Oncology, Budapest, Hungary

Introduction

Colon cancer has become the third most commonly diagnosed cancer and the fourth leading cause of death related to cancer in the world.[1,2] Besides the conventional treatments of colon cancer (surgery, radiation therapy and chemotherapy) targeted therapy is one of the main therapeutic approaches that might have a significant role in the future.[3] The most remarkable advantages of targeted cancer therapy over the conventional chemotherapy are the specificity towards cancer cells while sparing toxicity to off-target cells and the avoidance of multi-drug resistance (MDR), which are the major obstacles in cancer chemotherapy.[4] The concept of selective drug targeting is based on the high expression of certain cell surface components on tumors or the tumor neovasculature.[5] Therefore, the search of new tumor homing peptides that recognize them is a hot topic in targeted cancer therapy.[6] One of the approaches often used to explore new peptides is a technique belonging to *in vitro* evolution methods: phage display is a useful tool to identify tumor-specific peptides that can be used efficiently for anticancer drug targeting.[7]

Zhang and his co-workers selected HT-29 human colon cancer-specific heptapeptides by phage display technology.[1] In the *in vitro* panning experiment a 7-mer phage-display peptide library containing 10^{11} pfu was used (*ca.* 100 clones belong to one peptide sequence). After 3 rounds of panning using colon cancer cell lines and 2 rounds of subtractive screening, the peptide sequences of 50 randomly picked phage clones were analyzed by cell – enzyme-linked immunosorbent assay. The heptapeptide VHLGYAT was found as the most selective peptide to HT-29 colon cancer cell line. It has to be noted that the receptor recognized by this peptide was not identified. We believed that during the random selection of 50 clones from the panned and screened phages after three rounds of panning, some compounds with higher

affinity and/or selectivity were lost. Thus, Ala scan and positional scan procedures were used to find more active conjugates in this study.

Results

Daunomycin (Dau) conjugate of VHLGYAT peptide amide (Dau=Aoa-LRRYVHLGYAT-NH_2) was prepared. Dau as an anticancer agent was attached to the homing peptide *via* oxime linkage through an aminooxyacetic moiety (Aoa) which was connected to a Cathepsin B cleavable spacer (LRRY). This spacer was investigated to avoid the release of different metabolites from the conjugates, because the oxime bond is stable, and the smallest metabolite that can be released is Dau=Aoa-Xxx-OH (where Xxx is the amino acid whose amino group is acylated with aminooxyacetic acid).[8] The peptide chain was built up by SPPS, and Dau was conjugated to the aminooxy acetylated peptide in solution (Figure 1).

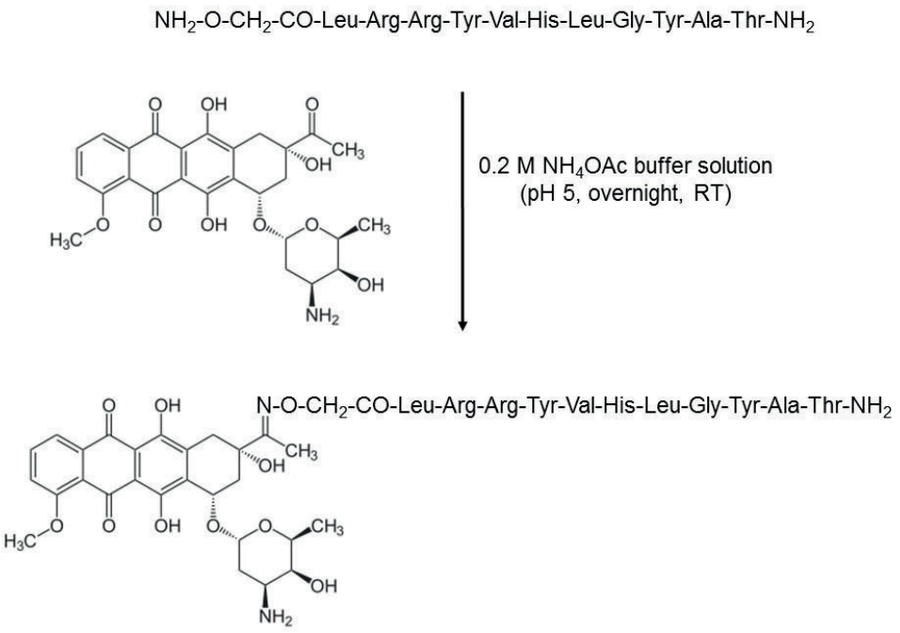

Figure 1. Conjugation of daunomycin to the aminooxyacetylated homing peptide

The conjugate showed moderate cytostatic effect (Table 1).[9] In the next step Ala scan was made and all amino acid in the basic heptapeptide sequence was replaced by Ala step by step. The cytotoxic effect of the new conjugates was measured on HT-29 colon adenocarcinoma cells. The results indicated that the replacement of Val, Leu or Tyr to Ala (V/A, L/A, Y/A) in the sequence of the homing peptide is not allowed without loss of cytostatic effect (IC_{50} > 100 μM). When Thr was changed to Ala (T/A) the anti-tumour effect

decreased a bit, while the modification of His (H/A) in the sequence resulted in a slightly more active conjugate (Table 1). The replacement of Gly to Ala (G/A) increased the cytostatic effect significantly (from IC_{50} = 46.9 ± 9.4 μM to 24.1 ± 1.6 μM) in this experiment. The differences in antitumor activity of the conjugates could be explained by their different cellular uptake propensity measured by flow cytometry. Therefore, further different types of amino acids were incorporated in this position. Lys as a basic, Glu as an acidic amino acid, Thr, Ser and Asn as polar and Phe, Leu as nonpolar (aromatic and nonaromatic) amino acids were incorporated. Furthermore, Pro that might break the conformation of the peptide was investigated.

Table 1. Characteristics of conjugates modified by Ala and positional scanning

Compounds (code)	RP-HPLC R_t (min)[a]	ESI-MS calc	ESI-MS meas[b]	IC_{50}[c] (μM)
Dau=Aoa-LRRY-**A**HLGYAT-NH_2 (**1**)	28.2	1930.1	1929.9	46.9±9.4
Dau=Aoa-LRRY-**A**HLGYAT-NH_2 (**2**)	27.7	1901.5	1901.8	>100
Dau=Aoa-LRRY-V**A**LGYAT-NH_2 (**3**)	29.0	1863.5	1864.0	36.8±0.4
Dau=Aoa-LRRY-VH**A**GYAT-NH_2 (**4**)	27.2	1887.5	1887.8	>100
Dau=Aoa-LRRY-VHL**A**YAT-NH_2 (**5**)	28.6	1943.6	1943.9	24.1±1.6
Dau=Aoa-LRRY-VHLG**A**AT-NH_2 (**6**)	27.4	1837.5	1837.8	>100
Dau=Aoa-LRRY-VHLGYA**A**-NH_2 (**7**)	28.0	1899.8	1899.9	70.9±3.8
Dau=Aoa-LRRY-VHL**K**YAT-NH_2 (**8**)	28.2	2000.7	2000.8	50.3±3.0
Dau=Aoa-LRRY-VHL**E**YAT-NH_2 (**9**)	29.3	2001.6	2001.7	29.5±6.3
Dau=Aoa-LRRY-VHL**L**YAT-NH_2 (**10**)	31.1	1985.7	1986.0	7.5±3.5
Dau=Aoa-LRRY-VHL**F**YAT-NH_2 (**11**)	31.2	2019.7	2019.8	6.6±2.9
Dau=Aoa-LRRY-VHL**S**YAT-NH_2 (**12**)	28.9	1959.6	1959.8	24.8±7.4
Dau=Aoa-LRRY-VHL**T**YAT-NH_2 (**13**)	29.0	1973.6	1974.0	21.7±6.5
Dau=Aoa-LRRY-VHL**N**YAT-NH_2 (**14**)	29.0	1986.6	1987.9	28.0±19.4
Dau=Aoa-LRRY-VHL**P**YAT-NH_2 (**15**)	31.2	1969.7	1970.1	>50
Dau=Aoa-LRRY-VHL**Y**YAT-NH_2 (**16**)	30.8	2036.2	2036.1	38.4±17.7
Dau=Aoa-LRRY-VHL**Cpa**YAT-NH_2 (**17**)	31.3	2053.6	2053.8	3.6±0.1

[a]RP-HPLC: column: Phenomenex Aeris Peptide XB-C18 column (250 mm x 4.6 mm) with 3.6 μm; eluents: 0.1% TFA in water (A) and 0.1% TFA in MeCN-water (80:20, v/v) (B); gradient: 0 min 0% B, 5 min 0% B, 50 min 90% B; flow rate: 1 mL/min; detection: λ= 220 nm.
[b]ESI-MS: Esquire 3000+ ion trap mass spectrometer
[c]MTT assay on HT-29 colon adenocarcinoma cells; 24 h treatments + 48 h further incubation

All conjugates except the Pro containing one (**15**) showed cytostatic effect in the measured range. The incorporation of Lys (**8**) in this position decreased the cytostatic effect (IC_{50} = 50.3 ± 3.0 μM) compared to both G/A, and the native conjugates. No significant difference in cytostatic effect was observed when the Ala was replaced Glu, Ser, Thr, Asn (**9**,

12, **13**, **14**). However, it is worth mentioning that the change of Ala to Ser (**12**) increased the water solubility of the conjugate that might be useful in drug development. The results indicated that the incorporation of Leu (**10**) or Phe (**11**) gives the best conjugates having 4-5 times higher cytostatic effect than the G/A (IC_{50} = 6.6 ± 2.9 μM and 7.5 ± 3.5 μM, respectively). When the Phe was substituted with OH group (Tyr, **16**) the antitumor effect was decreased, while Cl substitution (Cpa: *p*-chloro-phenylalanine, **17**) enhanced the activity to IC_{50} = 3.6 ± 0.1 μM (but decreased the solubility of the conjugate). Thus Phe was applied in this position in the further studies. It was also indicated that the efficiency of cellular uptake is the main factor for the antitumor activity of the conjugates.

The parent conjugate (Dau=Aoa-LRRYVHLGYAT-NH_2) and the Phe substituted version (Dau=Aoa-LRRYVHLFYAT-NH_2) were investigated for *in vivo* studies on orthotopically developed HT-29 colon tumor bearing mice. It was indicated that the conjugate with modified sequence have higher antitumor effect also *in vivo* (89% tumor growth inhibition) compared to the conjugate with unmodified sequence (65% inhibition), and its inhibition effect was similar to the activity of the free Dau (84%) (Figure 2A). However, Dau decreased the liver weight by 28% while the conjugates did not show significant toxic side effect suggesting the suitability of conjugates over the free drug in tumor therapy (Figure 3B). The difference in antitumor effect of the conjugates can be explained by the tumor proliferation index calculated in tumor tissues treated with the different conjugates (58% *vs.* 86%). Dau did not influence significantly the proliferation of tumor tissues (94%).

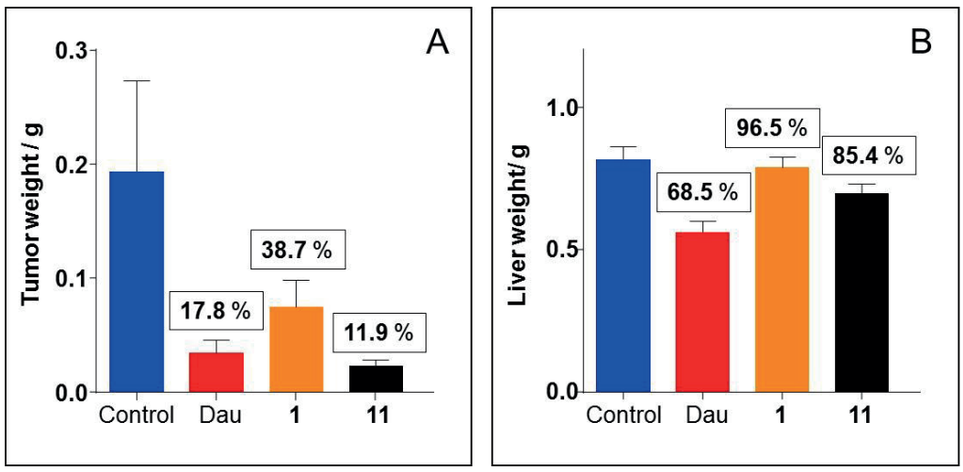

Figure 2. Antitumor effect and liver toxicity of Free Dau and conjugates **1** and **11**

The *in vitro* cytostatic effect of these two conjugates was measured on 22 different cancer cell lines and on MRC-5 normal fibroblast as a negative control. On all cancer cells the

Phe containing conjugate showed 2-5 times higher activity than the parent conjugate, and its effect was significantly higher on tumor cells than on MRC-5, indicating the tumor selectivity of the conjugates. Interestingly, the conjugates were active not only on HT-29 but also on other types of tumors, especially on lung, oral and prostate cancers and melanomas. Therefore we made effort to figure out the cell surface compartment that is recognized by this peptide sequence. By the aid of affinity chromatography and proteomic methods, Hsp70 which takes part in the immune recognition of tumors could be identified as a potential receptor for cell targeting of the conjugates. In addition, several HSP70 binding peptide sequences (A6R (AS**HLG**L**A**R) and HbS (**VHL**TPVEK)) showing sequential similarity to our lead peptide could be found in the literature.[10]

In conclusion, in this study, we demonstrated that homing peptides selected by phage display can be improved by sequence modification for more efficient targeted tumor therapy. Furthermore, it seems that the oxime linked Dau conjugates are potential drug candidates for tumor treatment. In addition, it was indicated that membrane-bound Hsp70 is a potential target in targeted tumor therapy.

References

1. Zhang Y, Chen J, Zhang Y, Hu Z, Hu D, Pan Y, Ou S, Liu G, Yin X, Zhao J, Ren L, Wang J. *J Biomol Screen* **12:**429-435 (2007)
2. Lin C, Ng HL, Pan W, Chen H, Zhang G, Bian Z, Lu A, Yang Z. *Int J Mol Sci* **16:** 26936-26952 (2015)
3. Mishra J, Dromund J, Quazi SH, Karanki SS, Shaw JJ, Chen B, Kumar N. *Crit Rev Oncol Hematol* **86:** 232-250 (2013)
4. Padma VV. *Biomedicine (Taipei)* **5:** e46 (2015)
5. Baudino TA*Curr Drug Discov Technol* **12:** 3-20 (2015)
6. Kapoor P, Singh H, Gautam A, Chaudhary K, Kumar R, Raghava GPS. TumorHoPe: *PLoS ONE* **7:** e35187 (2012)
7. Hyvönen M, Laakkonen P. *Methods Mol Biol* **1324:** 205-222 (2015)
8. Orbán E, Mező G, Schlage P, Csík G, Kulić Z, Ansorge P, Fellinger E, Möller HM, Manea M. *Amino Acids* **41:** 469-483 (2011)
9. Kiss K, Biri-Kovács B, Szabó R, Ranđelović I, Enyedi KN, Schlosser G, Orosz Á, Kapuvári B, Tóvári J, Mező G. *Eur J Med Chem* **176:** 105-116 (2019)
10. Fourie AM, Sambrook JF, Gething MJ. *J Biol Chem* **269:** 30470-30478 (1994)