Tumor-associated receptor-binding peptides for selective tumor targeting
Final report

Introduction

ErbB2 receptor as a potential target

Members of the Epidermal Growth Factor Receptor (EGFR) tyrosine kinase family are central regulators of normal and tumor cell functions.[1] The family has four members: epidermal growth factor receptor (EGFR), ErbB2, ErbB3 and ErbB4. They have an extracellular ligand-binding region, a single membrane spanning region, and a cytoplasmic tyrosine kinase containing domain. The binding of the ligand initiates signaling by causing specific homodimeric or heterodimeric receptor formation and activation of the cytoplasmic kinase domain. A soluble ligand for ErbB2 has not been identified, but it is a heterodimerization partner of the EGFR.

ErbB2 and EGFR have been implicated in the development of many types of human cancer.[2] ErbB2 is a tumor-associated receptor responsible for tumor cell survival, proliferation and metastases in many human cancers.[3, 4] ErbB2 receptor is overexpressed in 30-50% of primary breast cancers.[5] Patients with ErbB2-overexpressing tumors have a significantly poor prognosis compared to patients whose tumors did not overexpress ErbB2.[5, 6] Breast tumors overexpressing the ErbB2 gene are less responsive to cyclophosphamide, methotrexate, 5-Fluorouracil (CMF) or epirubicin treatments.[7] Furthermore overexpression of ErbB2 receptor is associated with resistance to apoptosis.[8] All the above mentioned data suggest that ErbB2 receptor might be a promising target in cancer chemotherapy.

In order to selectively target tumor cells, several strategies can be applied. One of the possibilities is the targeting of tumor-associated or tumor-specific cell surface molecules. As the ErbB2-binding peptides are ErbB2 antagonists, they can block the interaction of ErbB2 with its native ligand(s) or with other EGFR family members (e.g., EGFR, ErbB3 or ErbB4). With the inhibition of ErbB2-EGFR/ErbB3/ErbB4 receptor complex, initiation of metastasis might be inhibited.[9]

Peptides for ErbB2 receptor targeting

As a first step of this project, ErbB2 receptor binding peptides were selected, which could be used for selective targeting. Several peptides bind to ErbB2 receptor and some of them, such as peptides LTVSPWY, KCCYSL and WTGWCLNPEESTWGFCTGSF, were chosen from phage library.[3,10,11] Another peptide (called AHNP) is derived from ErbB2 receptor binding monoclonal antibody (Trastuzumab).[12]

Using random phage peptide libraries, Shadidi and co-workers aimed to identify peptides that specifically or preferentially bind to breast cancer cells. The selected peptides did not bind to normal mammary epithelial cells, but showed preferential binding and internalization into breast cancer cell lines.[13] One of the cancer-specific oligopeptides, peptide LTVSPWY was identified as an ErbB2-binding peptide. It was also shown that its GFP-fusion derivative was specifically internalized by SK-BR-3 human breast cancer cells, which overexpress ErbB2 receptor.[8, 14]

One of my aims was the application of KCCYSL motif containing peptides as carrier molecules, because the ErbB2 receptor binding ability of this peptide was already published and it was successfully applied for imaging ErbB2-expressing ovarian carcinomas by SPECT/CT.[15]

Another group of the ErbB2-targeting peptides applied in this study is derived from EC-1 peptide, which binds to the extracellular domain of the ErbB2 and inhibits the further phosphorylation steps.[11]
Anti-HER2/neu peptide (AHNP) is derived from anti-ErbB2 therapeutic antibody, Trastuzumab.[12]

A chemotherapeutical agent: daunomycin

Daunomycin (Dau) is one of the anthracycline-type antitumor agents able to inhibit the division of the cells by intercalating to DNA and/or by inhibiting the topoisomerase-II.[16] Clinical application of Dau is limited by side effects like nausea, vomiting, lack of appetite, but the most severe side effect is cardiotoxicity.[17] The intrinsic or acquired resistance of tumor cells to daunomycin also reduces the response to the treatment.[18]
Conjugation of anthracycline drug to different type of carrier (e.g. oligo- and polypeptides[19], proteins[20], polysaccharides[21], polymers[22] and dextran[23]) could improve the selectivity and decrease the side effects by utilizing different cellular uptake mechanism(s). For this reason daunomycin-oligopeptide conjugates were synthesized at the Research Group of Peptide Chemistry Hungarian Academy of Sciences. One of these conjugates was Dau=Aoa-LTVSPWY-NH_2 in which oxime bond was formed between daunomycin and the oligopeptide. Peptide LTVSPWY can bind to ErbB2 receptor according to the literature.[8] Synthesis, chemical characterization, *in vitro* stability, cytotoxicity, cytostatic effect and cellular uptake profile of this conjugate was described. Dau=Aoa-LTSVPWY-NH_2 conjugate was found to be chemically stable and effective on different type of tumor cells with different expression levels of ErbB2.[24]

Methods applied

1. Peptides used for conjugation were synthesized with solid phase peptide synthesis. Several ErbB2-binding peptides were selected for synthesis and conjugation based on the literature and our previous results (e.g.: WTGWCLNPEESTWGFCTGSF, AHNP etc.).
2. Conjugation of the peptides to daunomycin was carried out with the formation of oxime-bond between the peptide and daunomycin.
3. The synthesized compounds were chemically characterized by analytical Reverse Phase High Performance Liquid Chromatography (RP-HPLC) and Electrospray-Ionization Mass Spectrometry (ESI-MS).
5. *In vitro* cytostatic effect of the compounds was characterized by MTT-assay on different tumor cells with different expression levels of ErbB2 receptor.
6. Cellular uptake of the conjugates was studied by flow cytometry.

Results

Synthesis and characterization of ErbB2-binding peptide-derivatives

All the peptides in this study were prepared manually by solid phase peptide synthesis on Rink-Amide MBHA resin (0.73 mmol/g) using Fmoc/ ^{t}Bu strategy. The protocol of the synthesis was as follows: (i) Fmoc deprotection with piperidine/DBU/DMF (2 : 2: 96 v/v) solution (4 times, 2+2+5+10 min); (ii) washing with DMF (5×1 min); (iii) coupling 3 equivalent of Fmoc-amino acid derivative-DIC-HOBt dissolved in NMP (60 min); (iv) washing with DMF (5×1 min); (v) ninhydrin or isatin test. Boc-aminooxy-acetic acid was also coupled on solid phase to the free *N*-terminal amino group of the peptides using DIC/HOBt coupling agent. The coupling time was 60 min. The peptide derivatives were cleaved from the

resin with TFA/H_2O/thioanisol/EDT/phenol (10 mL : 0.5 mL : 0.5 mL : 0.25 mL : 0.75 g) mixture (2 hrs, RT). After filtration off the resin, the peptide derivatives were precipitated with cold diethyl ether and centrifuged. Pellet was solubilized in 0.1% TFA (aqueous solution), freeze-dried and characterized by analytical RP-HPLC and ESI-MS (Bruker Esquire 3000)

In the conjugation reaction equal amounts of Dau hydrochloride and aminooxy-acetyl peptide were dissolved in a mixture of 0.2 M NaOAc/AcOH buffer (pH 5.0) and DMSO or DMF depending on the peptide sequence (85:15 v/v%). The reaction mixture was stirred at room temperature and the reaction was monitored by analytical RP-HPLC. The Dau-oxime-peptides conjugate were formed in one day.

The crude product was purified on semipreparative RP-HPLC. The purified conjugate was dissolved in 0.1% TFA (aqueous solution), freeze-dried and characterized by analytical RP-HPLC and ESI-MS.

Three peptides with KCCYSL motif were designed and synthesized with different N- and C-terminus. Two of them were subsequently conjugated to the chemotherapeutical agent, daunomycin via oxime bond. (The synthesis efficiency of the third one was lower as usual therefore the reaction has to be further optimized before conjugation.)

During the conjugation reaction, precipitation was observed in some cases probably because of the disulfide bond formation between the cysteines of the peptides. Several conditions and solvents were tested, but the problem was emerged again and again, therefore Acm-protected cysteines were built in the peptide or the cysteines were replaced by serine residues. The shortest peptide (the KCCYSL itself) was successfully conjugated to daunomycin without any additional modifications, while peptide MEGPSKCCYSLALSH was synthesized with Acm protected Cys and also Ser replacement was carried out. The conjugation of these modified peptides was more efficient, but precipitation was also observed in smaller amount.

The other group of peptides was EC-1 derivatives. This is a longer peptide with two cysteines and all together it contains 20 aminoacids (WTGWCLNPEESTWGFCTGSF), therefore some difficulties were appeared during the synthesis and conjugation. Our strategy was again the replacement of Cys residues with Ser or Acm-protected Cys, but in this case this modification decreased the efficiency of the conjugation.

In vitro cytostatic effect and cellular uptake of the new Dau-oxime-peptide conjugates

After chemical characterization, *in vitro* cytostatic effect of the compounds was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-assay (MTT-assay) as it is described in our previous paper.[24] Cellular uptake properties were determined by flow cytometry using the own fluorescence of Dau. For these biological assays different tumor cell lines which express ErbB2 receptor at different levels (e.g.: MDA-MB-453, HL-60) were applied. HL-60 human leukemia cell line was selected for this study, because one of the ErbB2-binding peptides studied before, showed high *in vitro* cytostatic effect and cellular uptake with this cell line.[24] SK-BR-3 cells were also planned to be used for the determination of biological activity, but because of technical difficulties, another ErbB2 receptor overexpressing breast cancer cell line, MDA-MB-453 was applied in this study.

IC_{50} value of the compounds is proportional to their effectivity: the lower IC_{50} value the higher *in vitro* cytostatic effect. IC_{50} values of all the studied conjugates were around or above 50 uM on MDA-MD-453 cells, while on HL-60 cells much higher effectivity was observed, as a representative example Dau-oxime-KCCYSL-NH_2 showed 2.06±1.65 μM IC_{50} value. Not only the *in vitro* cytostatic effect, but also the cellular uptake was lower on MDA-MB-453 cell line: the best cellular uptake was observed on this cell line if cells were treated with Dau-oxime-FSDGFYASYKDV-NH_2 (3.30% of the cells were daunomycin-positive at 4 μM concentration).

The new conjugates show relatively high *in vitro* cytostatic effect on HL-60 human leukemia cell line, but one of our previous ErbB2 targeting conjugate (Dau=Aoa-LTVSPWY-NH_2) had slightly lower IC_{50}. The *in vitro* cytostatic effect of the best conjugates is summarized in Table1.

	IC_{50} (μM)
Dau	0.02±0.01
Dau=Aoa-LTVSPWY-NH_2	0.53±0.12
Dau=Aoa-KCCYSL-NH_2	2.06±1.65
Dau=Aoa-WTGWSLNPEESTWGFSTGSF-NH_2	4.19±0.37

Table1: In vitro cytostatic effect of the best conjugates on HL-60 cells

Cellular uptake properties of the conjugates were also studied on HL-60 cells, but they could take up the new peptide conjugates less efficiently than the former studied Dau=Aoa-LTVSPWY-NH_2 conjugate. In this case only 4.6% of the cells were Dau-positive if they were treated with 4 μM conjugate, in contrast 99.2% could take up Dau=Aoa-LTVSPWY-NH_2 conjugate (Figure 1).

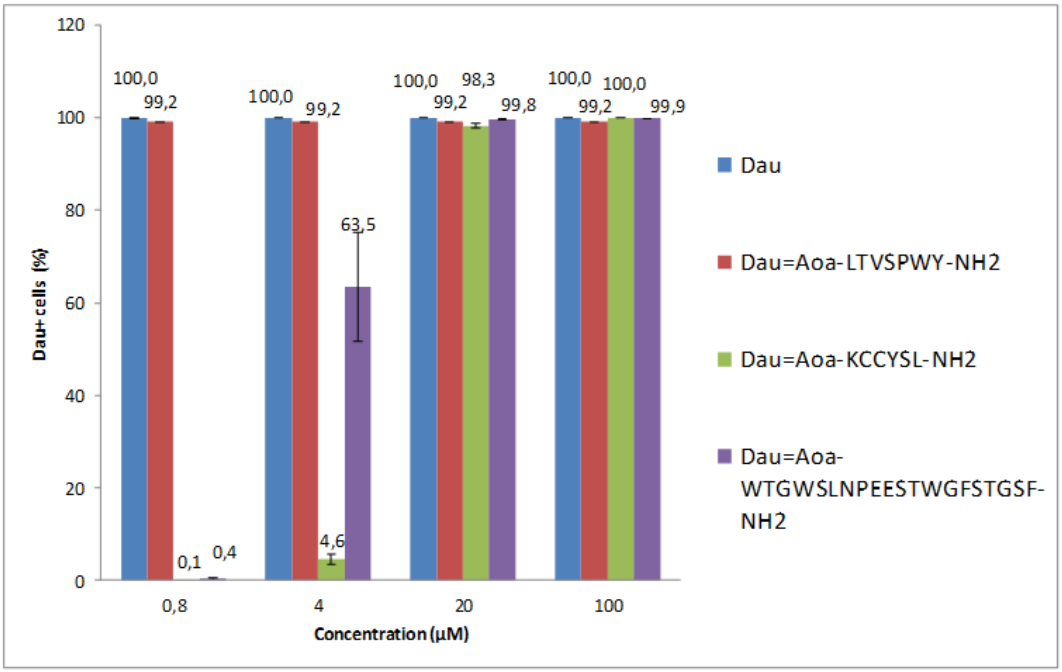

Figure 1. Cellular uptake of daunomycin-conjugates on HL-60 human leukemia cells

Summary

In this project several new daunomycin-oxime-oligopeptide conjugates were successfully synthesized containing ErbB2 receptor-binding peptides. Both drug-containing and non-containing conjugates mentioned above were chemically characterized by analytical RP-HPLC and ESI-MS. *In vitro* cytostatic effect of the synthesized conjugates was determined on different tumor cell lines with different expression levels of ErbB2 receptor and the highest effectivity was observed on HL-60 human leukemia cells. In addition cellular uptake

properties of the new daunomycin-oligopeptide conjugates were determined on different tumor cell lines. Although relatively low cellular uptake was observed, the *in vitro* cytostatic effect of the new conjugates on HL-60 cells is promising, so with further optimization these peptides could be utilized for more detailed research.

During this one year period of the project several ideas came to my mind, which could not be realized due to technical and/or time limitation. To my opinion further optimization of the peptide sequences could improve their *in vitro* cytostatic effect and cellular uptake properties. In addition the conjugation of these peptide-derivatives to peptides involved in apoptotic pathways could further improve their efficiency (as it was described in the original proposal for the second and third years).

References

1. Iivanainen, E.; Elenius, K., ErbB targeted drugs and angiogenesis. *Curr Vasc Pharmacol* **2010,** *8* (3), 421-31.
2. Hynes, N. E.; MacDonald, G., ErbB receptors and signaling pathways in cancer. *Curr Opin Cell Biol* **2009,** *21* (2), 177-84.
3. Shadidi, M.; Sioud, M., Selective targeting of cancer cells using synthetic peptides. *Drug Resist Updat* **2003,** *6* (6), 363-71.
4. Hung, M. C.; Hortobagyi, G. N.; Ueno, N. T., Development of clinical trial of E1A gene therapy targeting HER-2/neu-overexpressing breast and ovarian cancer. *Adv Exp Med Biol* **2000,** *465*, 171-80; Alaoui-Jamali, M. A.; Qiang, H., The interface between ErbB and non-ErbB receptors in tumor invasion: clinical implications and opportunities for target discovery. *Drug Resist Updat* **2003,** *6* (2), 95-107.
5. Klapper, L. N.; Kirschbaum, M. H.; Sela, M.; Yarden, Y., Biochemical and clinical implications of the ErbB/HER signaling network of growth factor receptors. *Adv Cancer Res* **2000,** *77*, 25-79.
6. Bourguignon, L. Y.; Gilad, E.; Peyrollier, K., Heregulin-mediated ErbB2-ERK signaling activates hyaluronan synthases leading to CD44-dependent ovarian tumor cell growth and migration. *J Biol Chem* **2007,** *282* (27), 19426-41.
7. Yu, D.; Hung, M. C., Role of erbB2 in breast cancer chemosensitivity. *Bioessays* **2000,** *22* (7), 673-80.
8. Wang, X. F.; Birringer, M.; Dong, L. F.; Veprek, P.; Low, P.; Swettenham, E.; Stantic, M.; Yuan, L. H.; Zobalova, R.; Wu, K.; Ledvina, M.; Ralph, S. J.; Neuzil, J., A peptide conjugate of vitamin E succinate targets breast cancer cells with high ErbB2 expression. *Cancer Res* **2007,** *67* (7), 3337-44.
9. Britten, C. D., Targeting ErbB receptor signaling: a pan-ErbB approach to cancer. *Mol Cancer Ther* **2004,** *3* (10), 1335-42.
10. Karasseva, N. G.; Glinsky, V. V.; Chen, N. X.; Komatireddy, R.; Quinn, T. P., Identification and characterization of peptides that bind human ErbB-2 selected from a bacteriophage display library. *J Protein Chem* **2002,** *21* (4), 287-96.
11. Pero, S. C.; Shukla, G. S.; Armstrong, A. L.; Peterson, D.; Fuller, S. P.; Godin, K.; Kingsley-Richards, S. L.; Weaver, D. L.; Bond, J.; Krag, D. N., Identification of a small peptide that inhibits the phosphorylation of ErbB2 and proliferation of ErbB2 overexpressing breast cancer cells. *Int J Cancer* **2004,** *111* (6), 951-60.
12. Park, B. W.; Zhang, H. T.; Wu, C.; Berezov, A.; Zhang, X.; Dua, R.; Wang, Q.; Kao, G.; O'Rourke, D. M.; Greene, M. I.; Murali, R., Rationally designed anti-HER2/neu peptide

mimetic disables P185HER2/neu tyrosine kinases in vitro and in vivo. *Nat Biotechnol* **2000,** *18* (2), 194-8.
13. Shadidi, M.; Sioud, M., Identification of novel carrier peptides for the specific delivery of therapeutics into cancer cells. *FASEB J* **2003,** *17* (2), 256-8.
14. Seoane, S.; Montero, J. C.; Ocaña, A.; Pandiella, A., Effect of multikinase inhibitors on caspase-independent cell death and DNA damage in HER2-overexpressing breast cancer cells. *J Natl Cancer Inst* **2010,** *102* (18), 1432-46.
15. Deutscher, S. L.; Figueroa, S. D.; Kumar, S. R., In-labeled KCCYSL peptide as an imaging probe for ErbB-2-expressing ovarian carcinomas. *J Labelled Comp Radiopharm* **2009,** *52* (14), 583-590.
16. Takimoto, C. H.; Calvo, E., Principles of Oncologic Pharmacotherapy. In *Cancer Management: A Multidisciplinary Approach*, 11 ed.; 2008.
17. Von Hoff, D. D.; Rozencweig, M.; Layard, M.; Slavik, M.; Muggia, F. M., Daunomycin-induced cardiotoxicity in children and adults. A review of 110 cases. *Am J Med* **1977,** *62* (2), 200-8.
18. Riehm, H.; Biedler, J. L., Cellular resistance to daunomycin in Chinese hamster cells in vitro. *Cancer Res* **1971,** *31* (4), 409-12.
19. Gaal, D.; Hudecz, F., Low toxicity and high antitumour activity of daunomycin by conjugation to an immunopotential amphoteric branched polypeptide. *European Journal of Cancer* **1998,** *34* (1), 155-161.
20. Baurain, R.; Masquelier, M.; Deprezde, C. D.; Trouet, A., TARGETING OF DAUNORUBICIN BY COVALENT AND REVERSIBLE LINKAGE TO CARRIER PROTEINS - LYSOSOMAL HYDROLYSIS AND ANTI-TUMORAL ACTIVITY OF CONJUGATES PREPARED WITH PEPTIDIC SPACER ARMS. *Drugs under Experimental and Clinical Research* **1983,** *9* (4), 303-311.
21. Nogusa, H.; Yano, T.; Kashima, N.; Yamamoto, K.; Okuno, S.; Hamana, H., Structure-activity relationships of carboxymethylpullulan-peptide-doxorubicin conjugates--systematic modification of peptide spacers. *Bioorg Med Chem Lett* **2000,** *10* (3), 227-30.
22. Shiah, J. G.; Sun, Y. G.; Peterson, C. M.; Straight, R. C.; Kopecek, J., Antitumor activity of N-(2-hydroxypropyl)methacrylamide copolymer-mesochlorin e(6) and adriamycin conjugates in combination treatments. *Clinical Cancer Research* **2000,** *6* (3), 1008-1015; Kamada, H.; Tsutsumi, Y.; Yoshioka, Y.; Yamamoto, Y.; Kodaira, H.; Tsunoda, S.; Okamoto, T.; Mukai, Y.; Shibata, H.; Nakagawa, S.; Mayumi, T., Design of a pH-sensitive polymeric carrier for drug release and its application in cancer therapy. *Clinical Cancer Research* **2004,** *10* (7), 2545-2550.
23. Hurwitz, E., SPECIFIC AND NONSPECIFIC MACROMOLECULE DRUG CONJUGATES FOR THE IMPROVEMENT OF CANCER-CHEMOTHERAPY. *Biopolymers* **1983,** *22* (1), 557-567.
24. Orbán, E.; Manea, M.; Marquadt, A.; Bánóczi, Z.; Csík, G.; Fellinger, E.; Bősze, S.; Hudecz, F., A New Daunomycin-Peptide Conjugate: Synthesis, Characterization and the Effect on the Protein Expression Profile of HL-60 Cells in Vitro. *Bioconjug Chem* **2011,** *22* (10), 2154-2165.