SYNTHESIS AND IN VITRO APPLICATION OF BIOCONJUGATES FOR DRUG DELIVERY

PhD thesis

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INTRODUCTION

In the developed countries, cancer is overtaking cardiovascular disease as the leading cause of death. The disease can be treated by surgery, chemotherapy, radiation therapy, immunotherapy, or other alternative methods. The choice of therapy depends on the location and grade of the tumor as well as the general state of the patient. The effectiveness of chemotherapy is limited by toxicity to other tissues in the body.

In the field of targeted drug delivery, numerous bioconjugates have been developed to enhance the efficiency and specificity of novel antitumor therapeutics. These kinds of drug delivery systems (DDSs) usually consist of a carrier, a drug and targeting moieties. A major disadvantage of anticancer drugs is their lack of selectivity for tumor tissue, which causes severe side effects and results in low cure rates. To avoid these side effects and increase the effectiveness of the cancer chemotherapy, during the past decade, several carrier systems (e.g. liposomes, polymers etc.) have been involved depending on the target organ. Forasmuch the receptor mediated endocytosis may provide the appropriate pathway for cellular uptake, targeting moieties have amended the structure of DDSs.

AIMS

My aim was to develop targetable oligopeptide-based chemotactic drug delivery systems (CDDS) for the treatment of cancer. In these conjugates, tetratuftsin derivatives (OT20 or Tp20) were used as carriers and formyl-tripeptides or tuftsin derivatives (For-MLF, For-NleLF, TKPR, For-TKPR, TKPKG, Ac-TKPKG) were investigated as chemoattractant targeting sequences. Methotrexate (Mtx) or Daunomycin (Dau) were applied as anticancer agents and were attached to the carrier *via* an enzyme (lysosomal Cathepsin B) labile pentapeptide (GFLGC) spacer. The drug-spacer conjugate was synthesized by solid phase peptide synthesis followed by conjugation with the carrier through thioether bond in liquid phase (*Figure 1.*).

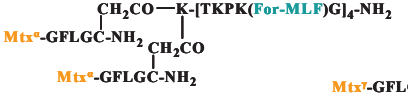
The following topics were planned to be examined:

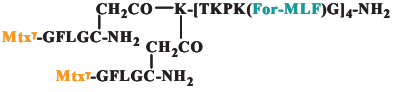
- Effect of the topology of Mtx coupling (γ vs. α-amide bond) for the biological activity

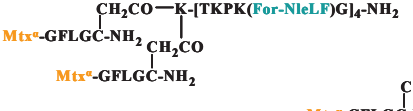
- The effect of the various targeting sequences for chemotaxis and internalization of the cells.
- Influence of the number and position of the targeting moieties for the biological activities
- The effect of the type of the carriers (OT20 or Tp20) on the biological activities
- The effect of the coupling of the drug-containing spacer to the chemotactic branched peptides
- The differences in the effectiveness between the two drugs (Mtx and Dau) in the conjugates
- Release of the drugs from the drug-conjugates

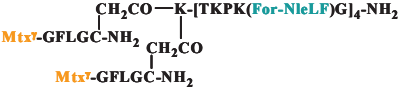
For the cellular uptake studies, in case of the Mtx-containing DDS, the synthesis of CF-labeled conjugates was planned.

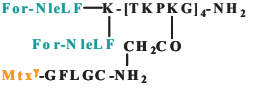
Synthesis, purification and *in vitro* application (chemotaxis, cellular uptake, apoptosis and MTT-assay) of 17 drug-containing conjugates and 7 types of CF-labeled conjugates was planned. The planned drug-containing conjugates:











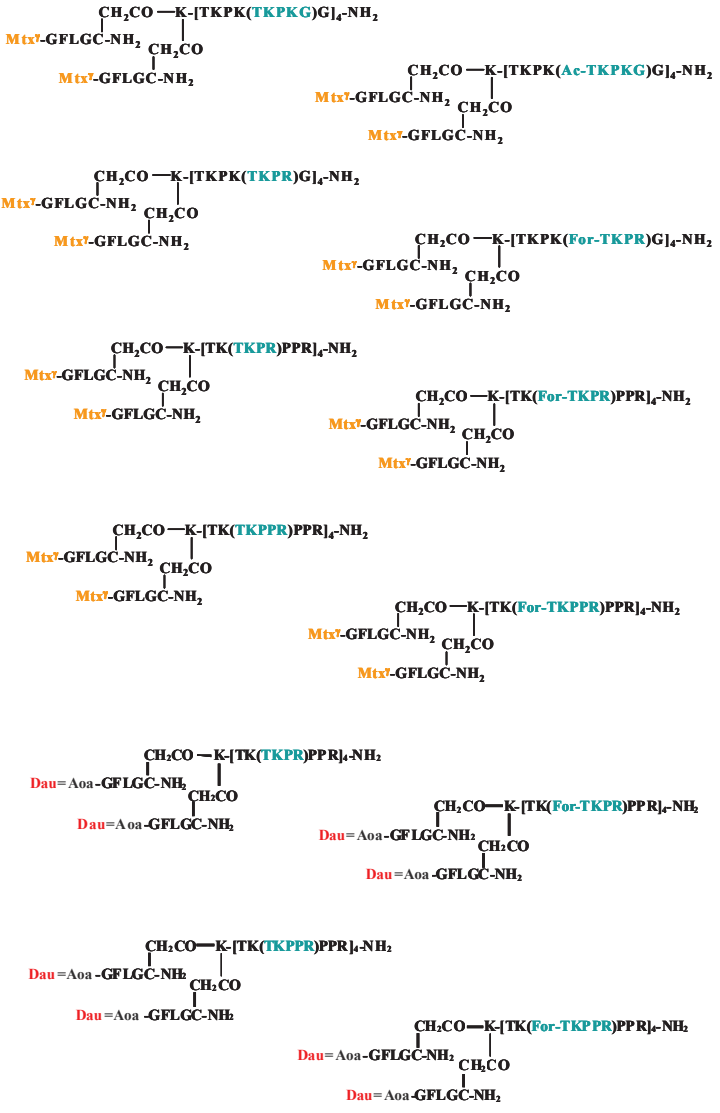


Figure 1.: Schematic structure of the conjugates

APPLIED METHODS

Carriers with tuftsin-like targeting moieties in branches and the drug-containing GFLGC spacer peptide were synthesized by SPPS using mixed Boc and Fmoc strategies. The crude products were purified by RP-HPLC and the pure compounds were characterized by analytical HPLC and ESI-MS.

In vitro biological assays (chemotaxis, cellular uptake studies, apoptosis and MTT-assay) were performed on the prepared conjugates and their components on *Tetrahymena pyriformis* cells, on MonoMac6 and THP-1 human tumor cell lines.

The chemotactic ability of *Tetrahymena* cells was evaluated using a two-chamber multichannel capillary chemotaxis assay; the chemotactic ability of MonoMac6 and THP-1 cells was evaluated using a 96-well modified Boyden chamber (NeuroProbe chamber).

Cellular uptake of the CF-labeled analogues and of the Dau-containing conjugates was studied on MonoMac6 and THP-1 human tumor cell lines by flow cytometry.

The cytotoxicity of the drugs (Mtx and Dau) and drug-conjugates was investigated via apoptosis or MTT assay on the tumor cell lines.

To determine the intracellular drug release, enzymatic digestions with Cathepsin B, a lysosomal enzyme, were carried out with Mtx-spacer and an arbitrarily chosen conjugate.

THESIS

Carriers with targeting moieties in branches and the drug- or CF-containing spacer peptides were synthesized by SPPS using mixed Boc and Fmoc strategies. The Mtx and CF were coupled through amide bond and the Dau was coupled *via* oxime bond to the spacer peptide (GFLGC). The branched peptides were conjugated to the drug- or CF-containing spacer peptide in liquid phase forming thioether bond.

The crude products were purified by RP-HPLC and the pure compounds were characterized by analytical HPLC and ESI-MS.

Altogether 17 drug-conjugates and 7 CF-labeled conjugates were prepared.

In vitro biological assays were performed on the prepared conjugates and their components on *Tetrahymena pyriformis* model cells, on MonoMac6 and THP-1 human leukemia tumor cell lines.

Chemotaxis assays were carried out with the compounds and the branched peptides, as well as with the drug-conjugates. Each of the branched peptides and the drug conjugates could trigger a significant chemotactic effect on the tumor cell lines.
Cellular uptake of the conjugates was studied. The mean fluorescence intensity of cells was determined by flow cytometry. We observed concentration-dependent accumulation of fluorescence signal within the cells indicating rapid internalization of each conjugate.
MTT and apoptosis assay was also carried out -using the conjugates and the free drugs (Mtx or Daunomycin) to determine the cytostatic or apoptotic effect of the conjugates.

The following topics were examined:

- Effect of the topology of Mtx coupling (γ vs. α-amide bond) for the biological activity

Methotrexate was coupled to the chemotactic branched peptides *via* spacer peptide (GFLGC) through α- and γ-carboxylic group of its glutamic acid residue. Compounds with Mtx attached to the carrier through the γ-amide bond were more effective in the chemotaxis assays. As the bioconjugates were developed for chemotactic drug targeting (CDT), for the further studies the γ variants were synthesized and examined in the biological assays.

- The effect of the various targeting sequences as branches in the conjugates for chemotaxis and internalization of the cells.

Results of the chemotaxis and the internalization assays suggest that the conjugates with tuftsin analogues in the branches were more effective. In case of chemotaxis the conjugates with H-TKPR or H-TKPPR branches could trigger strong chemoattractant responses. Although the formylation of the branches (resulted in the For-TKPR and For-TKPPR branched conjugates) decreased this attractant effect, but increased the cellular uptake of these components.

- Influence of the number and position of the targeting moieties on the biological activities

Although the modification of the position and number (from 4 to 2) of the targeting sequences decreased the attractant effect of the conjugates, they remained still effective. As the conjugates with 4 targeting sequences in the branches had more advantageous biological properties than the conjugates containing 2 targeting sequences, the first construction with 4 targeting sequences was synthesized for the further experiments.

- The effect of the type of the carriers (OT20 or Tp20) for the biological activities

The results of the biological assays confirmed the feasibility of both carriers (OT20 and Tp20) to deliver the drug molecules

- The effect of the coupling of the drug-containing spacer to the chemotactic branched peptides

The coupling of the Mtx- or Dau-containing spacer peptides (which were chemorepellent) to the chemoattractant branched peptides resulted in much more effective drug-containing conjugates in the most cases. Moreover these conjugates could be taken up by the cells and could kill the cells as effectively as the free drugs.

- The differences in the effectiveness between the two kind of drugs (Mtx and Dau) in the conjugates

The Daunomycin-containing conjugates were more effective than the Mtx-containing conjugates in case of chemotaxis, but as the conjugates were taken up by the cells, both drugs could kill the cells.

- Release of the drugs from the drug-conjugates

To determine the intracellular drug release enzymatic digestions with Cathepsin B, a lysosomal enzyme, were carried out with the Mtx-spacer (Mtx^{γ}-GFLGC-NH_2) and with one of the Mtx-containing conjugates. Our results suggested that the main cleavage site is between the Gly and Phe amino acid residues in the spacer sequence. The Mtx could be released in a Gly-coupled form (Mtx-Gly); thus, the drug can be released from the conjugates by Cathepsin B in the lysosomes and can kill the cells.

First and last, if we draw a parallel between the free drugs and the conjugates, we can say that the conjugates have many advantages in contrast to Mtx or Dau. The conjugates can attract the leukemia cells in certain concentrations. Beside the chemotaxis, the conjugates can be internalized rapidly as much as the free drugs, but the mechanism of internalization is different. Mtx and Dau can simply diffuse through the membrane without any specificity in contrast to the conjugates, which can be taken up by the cells through receptor-mediated endocytosis. Furthermore, the conjugates are at least as cytotoxic as the free drugs.

CONCLUSION

Most of the conjugates had advantageous chemotactic properties; they can be internalized rapidly and could trigger toxic effect on the cells, therefore our results suggest that these novel oligopeptide-based chemotactic drug delivery systems might be potential candidates for targeted cancer chemotherapy.

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