Synthesis and functional characterization of new peptide/protein bioconjugates
2013-01-01 - 2017-06-30
Final report

1. Introduction

The aim of this project was to design and prepare novel two- or three-component bioconjugates, to characterize their structural and functional properties, to establish structure-function correlation and to study the mechanism of action. For this, novel compounds including bioconjugates and also the partner molecules, their appropriate derivatives, synthetic as well as analytical procedures were developed.
Three groups of bioconjugates were prepared and characterized. In Group 1.: Conjugates with realized or new, potential chemotherapeutic properties against intracellular parasite *Leishmania donovani* detected also in Europe or against cancer cells; Group 2.: Conjugates with tumour-related or autoimmune protein derived epitope peptide (e.g. mucin [MUC] glycoproteins, desmoglein [Dsg]) with optimized structure; Group 3.: Conjugates with novel "reporter" function including intracellular (e.g. calpain) enzyme inhibitors or established/newly designed fluorophore mainly with novel ring-substituted naphthyl-oxazolone derivatives.
In the above mentioned conjugates as peptide-partner component we have incorporated mainly oligopeptides with well-established or newly discovered specific recognition unit (e.g. antigenic site, receptor binding site) or novel branched polypeptides with different side chain structure and solution conformation. In addition have identified of peptide sequences of HSV viral protein and chemotaxtic peptides as potential new recognition sites to be tested for specific targeting.

2. Synthesis of bioconjugates and component derivatives

2.1. Conjugates with realized or new, potential chemotherapeutic properties against intracellular parasite Leishmania donovani detected also in Europe or against cancer cells

Synthesis of a new set of vindoline derivatives and also their amino acid conjugates were performed using amide bond linkage[1]. Selected derivatives, Br-vindoline-(L/D)-Trp, were coupled with octaarginine cell penetrating peptide (CPP) at the *N*-terminal with amide bond[2]

Novel ferrocene-cinchona alkaloid hybrids incorporating chalcone- and 1,2,3- triazole moieties[3] as well as new *N*-ferrocenylpyridazinones were synthesized[4].

A folate antagonist (methotrexate, MTX) or its pentaglutamylated derivatives were conjugated with oligopeptides (penetratin and octaarginine as cell-penetrating peptides [CPP]) with or without spacer unit (Gly_3) and prepared by solid phase peptide synthesis[5].

MTX was also covalently attached to branched chain polypeptides XAK type (X=Ser, SAK or X= Glu, EAK) by thioether linkage using GFLGC oligopeptide spacer and thioether linkage[6].

A new group of XAK type branched polypeptides with side chain terminal (X) of Ile, Nle, Leu, Val, or Arg amino acid[7] and their methotrexate (MTX) conjugates with amide linkage were synthesized[8,9] using optimized procedures.
During the preparation of oligopeptides new synthesis strategy was developed for the deprotection of N^{ω}-nitro-Arg, by $NaBH_4$ assisted catalytic hydrogenation (solvent, metal ion catalyst) appropriate scale and was successfully utilized for protected Arg in oligopeptides[10].

2.2. Conjugates with tumour-related or autoimmune protein derived epitope peptide (e.g. mucin glycoproteins, desmoglein) with optimized structure

A set of new glycosylated peptide (^{16}PTPTGTQ^{22}) corresponding to the T_n antigen epitope of mucin-2 (MUC2) glycoprotein with *N*-acetylgalactoseamine in position Thr^{17}, Thr^{19}, Thr^{21} or on all three Thr residues were prepared[11].

A new group of peptide antigen conjugate containing multiple copies of the uniformly oriented epitope oligopeptide covering the APDTRPAP sequence of mucin-1 (MUC1) elongated with an *N*-terminal Cys and XAK (X = Leu, LAK or X=Glu, EAK) or XK (X = Glu, EK) branched polypeptide[12].

Based on prediction analysis, oligopeptides corresponding to B- or T-cell epitope sequences derived from desmoglein 1 and/or 3 proteins involved in *Pemphigus vulgaris* autoimmune diseases were prepared on pins or resin using SPPS approach[13,14,15].

Considering primary structure prediction analyses, 3D modelling of interaction between Herpes simplex virus type 1 (HSV-1) glycoprotein D (gD) with HVeA/nectin-1 host cell protein receptors, novel oligopeptides as well as substituted analogues from the *C*-terminal of gD glycoprotein were prepared by using Fmoc/tBu protection and SPPS strategy[16].

2.3. Conjugates with "reporter" established (e.g.5(6)-carboxyfluorescein) or newly designed fluorophore (e.g. novel ring-substituted naphthyl-oxazolone derivatives)

A new and efficient method to synthesize the precursors for the ethoxymethylene-oxazolone was developed, which works with compounds unstable in basic environment, like ring-substituted naphthyl oxazolone[17].

We have prepared 5(6)-carboxyfluorescein (Cf) labeled [Cf-K(AaAa)] (a: *D*-Ala, A: *L*-Ala) and the related MTX conjugate [Cf-K(MTX-AaAa)] as model compounds corresponding to the monomeric unit of AK and its Cf-labeled MTX conjugates for structure–activity experiments. During the synthesis of Cf-labelled branched oligopeptide conjugates on MBHA resin with Boc strategy, using Fmoc-Lys(Boc)-OH unexpected cyclopeptide-formation with the lactone-carboxylic group of the Cf was detected, when Cf was attached to the α-amino group of the Lys residue on solid phase.This was avoided by changing the order of Cf incorporation with using Fmoc/Dde strategy. Alternatively, we have built the peptide with Fmoc strategy on solid phase first and performed the labeling with Cf-OSu subsequently in solution[9,18]

For internalization studies of CPP conjugates of MTX with/without Glu_5 or Glu_5-Gly_3 oligopeptide unit 5(6)-carboxyfluorescein (Cf) labeled derivatives were prepared[5].

Fluorescent (Cf) labelled 20-mer – oligopeptide conjugates covering the 21-280 region as well as novel oligopeptides as well as substituted analogues from the *C*-terminal of D glycoprotein of Herpes simplex virus (HSV) involved in intracellular uptake of the virus were synthesized[16].

A new class of calpain inhibitors for monitoring the intracellular level of calpain enzymes were designed and prepared by incorporation of L- or D- epoxysuccinyl (Eps) group on solid phase[19]. Conjugates of two oligopeptides containing Eps as effective inhibitors of m-calpain as well as their fluorescent derivatives were produced with cell-penetrating octaarginine at their *C*-terminal[20,21].

New cell-penetrating calpain substrate with oligoarginine (Dabcyl-TPLKSPPPSPRK(Cf)R_7) was designed and prepared for detection of calpain activity in the cytosol (Bánóczi et al. manuscript in preparation).

A new group of XAK (X = Ser, Thr or Arg) and XK (X = Pro, His or Leu) cationic branched polypeptide conjugates were prepared by labelling the polypeptides with 5(6)-carboxyfluorescein (Cf) [22].

A novel group of MTX-XAK branched polypeptide conjugates with side chain terminal of Ile, Nle, Leu, Val, or Arg amino acid were coupled with 5(6)-carboxyfluorescein (Cf)[8,9].

For the synthesis of photoluminescent, red emitting gold quantum clusters oligopeptides were synthesized[23].

3. Chemical analysis and stability studies of bioconjugates

Amino acid/oligopeptide conjugates as well as their components (e.g. vindoline derivatives, calpain inhibitory oligopeptides with L- or D-Eps, oligopeptides from HSV gD, desmoglein peptides and their *N*-terminally truncated derivatives, glycopeptides corresponding to MUC2 protein epitope, ferrocene-derivatives and other intermediates etc.) were purified by chromatography (TLC, HPLC) or dialysis (branched polypeptides and conjugates).

Purity, stability as well as the primary structure, fragmentation were verified by HPLC, ESI and/or MALDI mass spectroscopy, NMR spectroscopy, amino acid- and occasionally by elemental analysis. Novel, branched polymeric polypeptides (XK, XAK) as well as the conjugates were characterized by amino acid analysis, molecular mass distribution.

The solution conformation of epitope oligopeptides (e.g. Dsg-3, HSV gD-1), MUC2 glycopeptides, branched polypeptides and MTX-polypeptide conjugates was analysed by ECD spectroscopy in water and in the ordered structure promoting TFE.

The effect of the peptide component on the fluorescent properties of conjugates with 5(6)-carboxyfluorescein (Cf) was characterized. Uptake of labelled compounds was studied by FACS analysis as a function of concentration, incubation time. Intracellular localization of the oligo/polypeptide conjugates was visualized by fluorescent microscopy.

Furthermore, a detailed proteomic analysis of the exosomal protein profile of the HL-60 cells was performed using nano-HPLC-ESI-MS/MS analysis on a Q-TOF mass spectrometer. Quantitative analysis of differentially expressed proteins in HL-60 cells treated with drug at various concentrations was investigated by using shotgun proteomics in combination with iTRAQ labeling.

The main results related to chemical characterisation, briefly:

a) Novel RP-HPLC based method was developed for the separation of all three (α L/D and γ L) MTX oligo peptide conjugate isomers[18].
b) For the identification of citrullination (deimination) sites in epitopes derived e.g. from vimentin, fibrin, filaggrin, MS/MS fragmentation method was developed with collision-induced dissociation (CID) technique using synthetic epitope oligopeptides with various length and composition[24].
c) The secondary structure of a) glycopeptides derived from Tn antigen peptide epitope of MUC2 is dependent on the number and position of the glucose unit[11], b) branched chain polymeric polypeptides, MTX conjugates is influenced by the identity of the side chain terminal amino acid (e.g. Arg vs. Glu)[7].
d) Correlation was established between the functional properties and immuno-recognition of Dsg epitope peptides[14].

4. Functional characterization of bioconjugates

4.1. Conjugates with realized or new, potential chemotherapeutic properties against intracellular parasite Leishmania donovani or against cancer cells

I*n vitro* cytotoxic/cytostatic properties of vindoline derivatives were studied on human leukemia cells (HL-60). In contrast to free vindoline different modifications of the ring system and/or the conjugation with L- or D-tryptophan resulted in active compounds (IC_{50} = 25-80 μM)[1]. The conjugation of the Br-vindoline derivatives increased significantly the *in vitro* antitumor activity on HL-60, MCF-7 and MDA-MB-231 cells. However, *in vivo* the Br-vindoline-(L)-Trp-Arg_8 conjugate could inhibit the tumour growth but none of the conjugates increased the survival of mouse leukaemia (P388) and murine colon carcinoma (C26) bearing mice *in vivo*[2].

Two groups of small non-peptide compounds with marked bioactivity were identified for conjugation with appropriate peptide/polypeptide. *In vitro* cytotoxic/cytostatic properties of **ferrocene-derived compounds** (ferrocene-cinchona hybrids and *N*-ferrocenylpyridazinones) have been evaluated *in vitro* on human colon carcinoma (HT29) and HepG2 human hepatoma cells. It was documented that the presence of the quinchona moiety is essential[3] and the activity of *N*-ferrocenylpyridazinones couild be related to the potential in generating reactive oxygen species (ROS)[4]. Under the COST program (COST Action CM1106, Chemical Approaches to Targeting Drug Resistance in Cancer Stem Cells, 2012-2016) in a search for novel compounds to target cancer stem cells (CSCs) involved in drug resistance we have studied the *in vitro* cytotoxic/cytostatic properties of the above ferrocene-cinchona hybrid epimers. It was documented by using three different human multi-drug resistant (MDR) cancer cell lines and their sensitive counterparts (non-small cell lung carcinoma NCI-H460/R

and NCI-H460, colorectal carcinoma DLD1-TxR and DLD1, glioblastoma U87-TxR and U87) that the chirality is essential in the anti-tumour activity[25]. Under the same COST project new **substituted salicylanilide compounds** which significantly decreased viability of cultured ovarian cancer stem cells *in vitro* were identified[26].

The cytostatic effect of MTX-octaarginine conjugates with or without pentaglutamyl (Glu_5) insertion was examined on human leukemia (HL-60) cells and also on two different breast cancer cells on sensitive breast carcinoma (MCF-7) as well as MDR resistant (MDA-MB-231) cells in vitro. We found that the influence of pentaglutamylation on cytostatic effect of MTX depends mainly on the cell type. The cellular uptake of fluorescent label - CPP conjugates with or without Glu_5 was also studied on HL-60 cells by flow cytometry. The results show that the presence of Glu_5 in the conjugates decreased the internalization dramatically[5].

The cytotoxicity/cytostatic effect, anti *Leishmania* parasite effect as well as chemotactic properties of the MTX-branched chain polymeric polypeptide conjugates (MTX-EAK and MTX-LAK) were comparatively studied under *in vitro* conditions. Data suggest that chemotaxis produced by the MTX-XAK polypeptide conjugates depends on specific chemical characteristics. For example, the identity of the *N*-terminal amino acid (Ser [SAK], or Glu [EAK]) at the branch significantly influences the elicited chemotaxis and also the attachment site of MTX in the conjugates (alpha- vs. gamma-carboxylic groups) affect their chemotactic activity[6].

All XAK polypeptides with hydrophobic amino acid (Ile, Nle, Leu or Val) at the terminal position were essentially non-toxic on murine bone marrow-derived macrophages from Balb/c mice, whereas the polypeptide with terminal Arg (RAK) proved to be markedly cytotoxic[7].

Among the MTX-XAK conjugates, also the Arg containing elicited pronounced cytotoxicity to the macrophages after 24 hours[8,9]. Anti-parasitic effect of the polypeptides and MTX-conjugates was studied on *L. donovani* promastigotes and *L. pifanoi* amastigotes *in vitro*. Mechanism of the leishmanicidal effect in case of selected compounds was also examined. Results indicate that coupling of MTX to the polypeptide carrier could enhance the anti-parasitic effect in case of *L. donovani* promastigotes. Methotrexate conjugate with Nle containing carrier elicited an effective uptake by macrophages and simultaneously proved to be effective anti-parasitic agents, showing selectivity to the parasites. The position of Leu in the side chain influences also the effect of the polypeptides (LAK vs ALK) as well as the MTX-conjugates (MTX-LAK vs MTX-ALK)[9,27].

Chemotaxis, adhesion and proliferation of J774 murine macrophage cells induced by tetra (SXWS) and pentapeptide libraries (WSXWS) were investigated. Results indicated that amino acid X had a marked influence on chemotaxis, adhesion as well as on proliferation induced by (W)SXWS peptides. Elongation of SXWS sequence with a tryptophan at the *N*-terminus altered pronouncedly all the physiological responses of the cells. Peptides with chemoattractant properties are considered as potential targeting unit of bioconjugates[28].

4.2. *Conjugates with tumour-related or autoimmune protein derived epitope peptide (e.g. mucin glycoproteins, desmoglein) with optimized structure*

We have described that glycosylation in position 19 (^{16}PTPT(GalNAcα)GTQ^{22}) in T_n antigen epitope of MUC2 glycoprotein resulted in enhanced antibody recognition and significantly altered secondary structure, while glycosylation in position 21 completely demolished the binding[12].

Binding properties of the monoclonal antibody (MAb 996) recognizing peptide ^{16}PTPTGTQ^{22} with GalNAcα on the first (Thr^{17}) or second (Thr^{19}), but not on the third (Thr^{21}) Thr residue are different and could be utilized to identify human colon carcinoma tissues vs normal epithelia and may prove to be a valuable tool in the diagnosis and monitoring of colon carcinoma[29].

By ^{125}I-radiolabelled MUC1 oligopeptide peptide (CAPDTRPAPG [CG]) antigen conjugate with XAK (X = Leu, LAK or X=Glu, EAK) or XK (X = Glu, EK) branched polypeptide, the biodistribution was comparatively studied. Among the conjugates EAK-CG promised to be the most effective, both in remaining in the circulation for the longest time after injection and having generally the lowest tissue accumulation. This may be due to its amphoteric charge properties; compared to the conjugates of polycationic character, and also due to possess a neutral oligoalanine chain compared to EK-CG conjugate. The amphoteric characteristics of the conjugated CG peptide seem to have lowered the blood clearance and tissue accumulation of the cationic polypeptides in K-CG and LAK-CG. Therefore among the conjugates of the CG MUC1 mucin epitope peptide, EAK-CG conjugate may be the most suitable candidate for vaccination studies[12].

Binding properties of immobilized synthetic peptide conjugates derived from Dsg proteins were analyzed using serum autoantibodies from autoimmune patients/healthy subjects and modified ELISA. Based on these data we identified five possible B-cell epitope regions within the extracellular part of the Dsg 1, and four possible B-cell epitope regions within that of the Dsg 3 protein involved in *Pemphigus vulgaris* autoimmune diseases[13,15]. We have described that two peptides (Dsg3/206-222, Dsg3/342-358) of Dsg 3 protein of *Pemphigus vulgaris* with ordered solution conformation stimulated markedly T-cells *in vitro.* It was also demonstrated that these T-cell epitope peptides are recognized more efficiently by PBMC cells from diseased individuals, than from healthy donors. This observation could indicate correlation between the ability of Dsg3 peptides to adopt ordered secondary structure and the T-cell response inducing capacity[14,15]. Based on these findings, conjugates of the peptides covering the identified B- and/or T-cell epitope with appropriate synthetic carrier could be designed and utilized for the diagnosis or monitoring the progression/treatment of the disease[15].

Protein expression profile of HL-60 human leukemia cells treated with Dau or Dau-oligopeptide conjugates was found to be dependent on the conditions (e.g. concentrations corresponding to 0, IC_{25}, $IC_{37.5}$ and IC_{50} values) as well as on the peptide component (previously reported Erb2 receptor ligand vs cell penetrating oligoarginine). The expression of cytoskeletal, metabolic proteins and proteins involved in signaling processes were altered[30].

4.3. Conjugates with "reporter" established (e.g. 5(6)-carboxyfluorescein) or newly designed fluorophore (e.g. novel ring-substituted naphthyl-oxazolone derivatives)

By using 5(6)-carboxyfluorescein (Cf) labelled set peptides covering 21-280 region, *C*-terminally truncated and Trp/Phe substituted derivatives from glycoprotein D (gD) from Herpes simplex virus type 1 (HSV-1) we have observed marked differences in the uptake profile of Cf-labelled 20-mer oligopeptides by flow cytometry. There peptide conjugates within the 244-272 region were identified as efficiently engulfed compounds even at c=10 μM by SH-SY5Y neuroblastoma cells. The shortest peptide for internalization seems to have the 253-268 part of gD. The corresponding sequence of the peptide is situated in the region of the nectin-1 receptor binding region. ECD studies showed that 20-mer peptides within the 253-280 region could adopt 3D arrangements in solution similar to that of the same sequence in the gD protein determined by X-ray. Peptide 253-268 has partially α-helical solution conformatuion[16].

Cellular uptake and intracellular localization of of nine structurally related cationic fluorophore-branched chain polypeptides of XAK (X = Ser, Thr or Arg) and of XK (X = Pro, His or Leu) were comparatively studied under *in vitro* conditions. The efficacy of their internalization is markedly influenced by the hydrophobicity and charge properties of the amino acid X. Interestingly, the uptake properties of the these polypeptides show certain similarities to the entry pathways of several cell penetrating peptides. Our findings in uptake inhibition studies suggest that predominantly macropinocytosis and caveole/lipid raft mediated endocytosis are involved[22] .

The cellular uptake by murine bone marrow-derived macrophages (from Balb/c mice) of the Cf-labelled XAK polypeptides with hydrophobic amino acid (Ile, Nle, Leu or Val) at the terminal position as well as the Cf-MTX conjugates proved to be dependent on the concentration, although a decreased internalization could be observed after coupling MTX to the polypeptide carriers. The polypeptides and conjugates were localized in the cytoplasm of the macrophages following to the uptake. Cellular uptake was influenced by the side chain composition of the polypeptide carriers and on the distance of amino acid X (and MTX) from the polylysine backbone[8].

Data suggest that the inhibitory activity after conjugation of calpain inhibitors (see above) with cell-penetrating octaarginine was preserved on relevant isolated enzymes. The internalization ability of the fluorophore labeled conjugates was evaluated by cellular uptake experiments using FACS analysis. We have also identified five calpain specific inhibitory oligopeptide containing Eps group with different extent of enzyme selectivity on isolated enzyme[20].

Data suggest that in the new cell-penetrating calpain substrate (Dabcyl-TPLKSPPPSPRK(Cf)R_7) conjugate (see above) the substrate property as well as the cell penetrating ability were preserved after conjugation with cell-penetrating heptaarginine. Thus by this conjugate it was possible to monitor the intracellular calpain activity in HL-60 cells.

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