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Abstract

In order to establish structure–function relationship for the design of a new group of oligopeptide antigen–macromolecule conjugate, multiple copies of mucin-1 B-cell epitope peptide, APDTRPAPG were conjugated with branched chain polymeric polypeptides possessing poly[L-Lys] backbone. By the synthesis, radiolabelling (125I) and in vivo treatment of BALB/c mice with epitope conjugates containing XiK/XAK type carrier, where X = Glu (EiK or EAK) or Leu (LAK), the influence of the polypeptide structure on the blood clearance profile and on tissue distribution profile concerning the epitope delivery to relevant organs (e.g. immunocompetent or involved in excretion) were investigated. We observed significant differences in the blood clearance profiles for the conjugates, the respective polypeptide carriers and free epitope peptide. All conjugates, regardless of their charge properties exhibited longer presence in the circulation than the free oligopeptide. Tissue distribution data also showed that the structural properties (e.g. amino acid composition, charge) of the carrier polypeptide have marked influence on the tissue accumulation of the epitope peptide conjugates. In contrast to conjugates with linear (K) or branched chain (LAK) polycationic polymers exhibiting rapid blood clearance and high spleen/liver uptake, amphoteric epitope peptide conjugates with different branches, but similar charge properties (EiK or EAK) had extended blood survival and generally lower tissue accumulation. The results on this systematic investigation suggest that further studies on the immune response induced by these epitope conjugates would be needed to provide correlation between biodistribution properties (presence in the blood, level of tissue accumulation) and the capacity of these conjugates to elicit antibody production.

Keywords	MUC-1 mucin peptide antigen; polymeric polypeptide carrier; oligopeptide epitope conjugates; carrier effect; biodistribution; blood clearance – conjugate structure relationship
Taxonomy	Conjugated Systems, Peptides, Peptide Vaccine for Cancer, Biomarker Research, Structure-Function Relationship

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The effect of the branched chain polypeptide carrier on biodistribution of covalently attached B-cell epitope peptide (APDTRPAPG) derived from mucin 1 glycoprotein

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Abstract

In order to establish structure–function relationship for the design of a new group of oligopeptide antigen–macromolecule conjugate, multiple copies of mucin-1 B-cell epitope peptide, APDTRPAPG were conjugated with branched chain polymeric polypeptides possessing poly[L-Lys] backbone. By the synthesis, radiolabelling (^{125}I) and *in vivo* treatment of BALB/c mice with epitope conjugates containing X_iK/XAK type carrier, where X = Glu (E_iK or EAK) or Leu (LAK), the influence of the polypeptide structure on the blood clearance profile and on tissue distribution profile concerning the epitope delivery to relevant organs (e.g. immunocompetent or involved in excretion) were investigated. We observed significant differences in the blood clearance profiles for the conjugates, the respective polypeptide carriers and free epitope peptide. All conjugates, regardless of their charge properties exhibited longer presence in the circulation than the free oligopeptide. Tissue distribution data also showed that the structural properties (e.g. amino acid composition, charge) of the carrier polypeptide have marked influence on the tissue accumulation of the epitope peptide conjugates. In contrast to conjugates with linear (K) or branched chain (LAK) polycationic polymers exhibiting rapid blood clearance and high spleen/liver uptake, amphoteric epitope peptide conjugates with different branches, but similar charge properties (E_iK or EAK) had extended blood survival and generally lower tissue accumulation. The results on this systematic investigation suggest that further studies on the immune response induced by these epitope conjugates would be needed to provide correlation between biodistribution properties

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Keywords

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Introduction

Mucin 1 (MUC1) is a cell surface glycoprotein expressed by epithelial cells of glands, body channels, organs of the genitourinary tract (ovary, uterus, urinary tract) and mammary glands. Its function is to protect the underlying epithelium by forming the mucus. Carcinoma cells often express mucins with different structures such as defective/modified glycosylation, resulting in MUC1 glycoprotein immunologically distinguishable from that of healthy cells, as both peptide and carbohydrate neoepitopes may appear. MUC1 mucin contains a polypeptide core composed of a variable number of repeats (usually 40-80) of a 20-amino acid sequence, APDTRPAPGSTAPPAHGVTS [1], with unique sequences on the termini, including the transmembrane and intracellular regions on the C-terminus. Recent reviews on antigen presentation and molecular recognition of tumour-associated MUC1 derivatives in free or bound form emphasized the need of better understanding structure – activity phenomena [2] to develop new immunogens for the design of cancer vaccines. As an example of promising attempts, fully synthetic MUC1-derivatives with appropriate conjugates should also be mentioned [3].

The majority of MUC1 protein core specific monoclonal antibodies react with peptide epitopes of 3-5 amino acids within the hydrophilic region APDTRPAP [4-8] of the repeat unit. It has also been demonstrated that peptides of this region (SAPDTRPA [9], APDTRPAP [10]) are capable of MHC binding.

Small epitope peptides alone, although recognized by the immune system, are not suitable for inducing efficient antibody responses – these compounds are usually not immunogenic. Suitable adjuvants (added or built-in), or increasing their size by multiplication or by conjugation to macromolecules could increase the antigenic potency [11]. Multiplying the tandem repeat unit [12], coupling of mucin peptide antigens to carrier proteins such as keyhole limpet haemocyanin [13], cholera toxin [14] or immunostimulants like Cd3 complement protein [15] have been used for eliciting specific immune responses. Conjugation/ligation of mucin peptide to T-cell epitope peptides, derived from e.g. influenza

haemagglutinin [16], tetanus toxoid [17-18**],** polio virus [19], and / or to Toll-like receptor agonist Pam3Cys [2, 20-22] also resulted in higher immunogenicity. B- and T-cell epitope peptides may also be conjugated with various polymeric structures. (e.g. linear poly(N-(2-hydroxypropyl)methacrylamide) [23]. Although larger Lys-based dendrimers [24] often present solubility problems, a di-Lys-based dendrimer [25] and other dendrimer-like constructs like hyperbranched polyglycerol [17] can be used instead with success. Calixarene scaffold [26] or gold nanoparticles [15] have also been studied. Interestingly no data has been reported on the pharmacokinetic/pharmacodynamics properties (e. g. biodistribution) of these constructs developed as antitumor vaccine candidates [27].

Recently the achievements in polymeric polypeptide-based conjugate research as potential advanced drug delivery constructs were reviewed and some basic correlations between structure, properties, and the biological behaviour of these conjugates for the successful design were delineated. [28] Our research group has been working for a long time with branched chain poly-α-amino acids possessing poly[L-Lys] backbone, as synthetic macromolecules with general formulae of poly[Lys(X_i)], X_iK or of poly[Lys-(X_i-DL-Ala$_m$], XAK) [29-32]. One of the aims of designing/synthesizing these polymeric polypeptides was to perform structural and functional studies and to establish a rational approach for selection of synthetic branched polypeptides as carriers for the construction of bioconjugates with chemotherapeutic agents [e.g. daunomycin, methotrexate], "reporter" entities (e.g. radiolabels) or with haptens/oligopeptide epitopes. These studies have provided wide structural versatility derived from the amino acid composition and structure of the branches for rational carrier design, e.g. with polar *vs* apolar amino acid X resulting in cationic, acidic residues in amphoteric, or even acetylated/succinylated acidic residues in polyanionic carriers [33].

Earlier studies with hapten/epitope–branched polypeptide conjugates have demonstrated the importance of the carrier moiety on the *in vitro* and *in vivo* immunorecognition of the covalently attached entities [11, 34-36]. For example, branched polypeptides coupled with the synthetic monovalent hapten, 4-(ethoxymethylene)-2-phenyl-5(4H)-oxazolone (Ox) induced oxazolone-specific antibody responses *in vivo* when repeatedly administered with or without Freund's adjuvant in inbred mice. Quantitative and qualitative features of the hapten- and carrier-specific T and B cell-mediated immune response were dependent on the composition of the XAK type carrier involved [36-37]. The influence of the carrier moiety on specific immune responses induced by peptide epitope derived from gD of Herpes simplex virus *in vivo* [11, 33, 37-40] was also well-documented. Furthermore, it was observed that the

branched polypeptide component of HSV type 1 gD [41] or mucin/1 [34] derived epitope peptide conjugate markedly influenced the *in vitro* antibody binding of HSV or mucin specific monoclonal antibodies, respectively. Similarly, *in vitro* T-cell immunogenicity was highly dependent on the structure of the polypeptide part of bioconjugates comprising multiple copies of T-cell epitope peptide of 16 or 38kD proteins from *M. tuberculosis* [35, 42]. It has also been demonstrated that the composition and conformational properties of branched polypeptides influence the interaction between epitope conjugate and phospholipid bilayer membrane [43].

Biodistribution studies of several of these branched polypeptides have been performed [44-47]. Conjugation of small molecules to macromolecular carriers can alter their biodistribution profile including blood-survival and tissue biodistribution. We have showed earlier that antitumour drugs (e.g. daunomycin [48], methotrexate) [49]), a gonadotropin releasing hormone antagonist [50] attached covalently to branched chain polypeptide exhibited carrier-dependent and markedly different biodistribution characteristics as compared to the free small molecular entity [33], but of hapten or epitope peptide conjugates the biodistribution properties have not been established.

In the present study a peptide corresponding to the APDTRPAPG (elongated with an N-terminal Cys for conjugation) antigenic sequence of MUC1 glycoprotein repeat unit has been selected as a linear B-cell epitope for conjugation with linear (poly[L-Lys]) and branched chain poly-α-amino acid polypeptides X_iK or of XAK, where X = Glu (E_iK or EAK) or Leu (LAK) as carriers. Here we report our findings on the relationship between the structure of this new group of bioconjugates containing multiple copies of uniformly oriented oligopeptide epitope covering the above APDTRPAPG sequence of MUC1 glycoprotein and their biodistribution profile (blood clearance, tissue distribution) after *iv* injection in mice. Comparative analysis indicated that predominantly the charge properties of the carrier polypeptide influenced the blood survival as well as the delivery of the epitope-conjugates into (immunocompetent) organs and organs of excretion.

Methods

Preparation of CAPDTRPAPG (CG) conjugates

APDTRPAPG sequence was elongated with an N-terminal Cys to result in CAPDTRPAPG (CG) peptide containing a thiol group suitable for oligopeptide conjugation to carrier polypeptide. The oligopeptide was prepared by solid phase synthesis using *p*-hydroxymethylphenoxymethyl resin with Fmoc/^{t}Bu chemistry on an ABI Automatic Peptide

Synthesiser (Model 431A), purified by RP-HPLC and characterised by ESI-MS and amino acid analysis as described [34].

CG peptide was conjugated to polycationic or amphoteric branched polypeptides as well as with poly[L-Lys], *via* the N-terminal Cys using the heterobifunctional reagent N-succinimidyl 3-(2-pyridylthio)propionate) (SPDP) (Sigma Chemical Co., Poole, UK) coupling reagent [34]. Briefly, for preparation of epitope peptide conjugates amphoteric (poly[Lys(Glu_i)], E_iK, poly[Lys-(Glu_i-DL-Ala_m], EAK) and polycationic ((poly[L-Lys], K, poly[Lys-(Leu_i-DL-Ala_m)], LAK) polymeric polypeptides were applied. In order to have conjugates with uniformly oriented peptide epitopes, a disulphide bridge was introduced between the ε-NH_2 group of poly[L-Lys] or the α-NH_2 group of the N-terminal Ala, Leu or Glu residues of LAK, E_iK or EAK carriers, respectively, and the SH group of peptide CG. In the first step, the amino-group in the side chain of the carrier was modified with SPDP to introduce protected SH groups into the polymer structure [(SSP)XK, where X = LA_n, EA_n or E_i] [51]. The extent of 2-pyridyl-disulphide group incorporation was determined spectrophotometrically from the amount of pyridine-2-thione released by reduction with DTT [36, 52].

10 mg of SSP-polypeptides containing 5–7 μmol of 2-pyridyl-disulphide group were dissolved in distilled water and mixed with CG peptide (7.5–10 μmol) in PBS, pH = 8.0 (10 mg/ml) [34]. After 30 min of stirring the reaction mixture was dialyzed against distilled water for 48 h then freeze-dried. The absence of pyridyl-disulphide groups and pyridine-2-thione in CG-polypeptide conjugates was verified by UV spectroscopy. The average degree of substitution was estimated from the amino acid analysis.

Amino acid analysis of CG, free branched polypeptides and of conjugates was performed using a Beckman 6300 automatic amino acid analyser after hydrolysis of the samples in 6M HCl in sealed and evacuated tubes at 110ºC for 24 h.

Radiolabelling

For biodistribution studies of branched chain polymers, CG peptide and the conjugates, they were labelled with ^{125}I using pre-iodinated Bolton and Hunter reagent (N-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate [53]) by a method described before [45]. Briefly, ~ 5 MBq of Bolton and Hunter reagent (Amersham International plc, Amersham, UK) in 2 μl of benzene was added to a plastic microfuge tube and evaporated to dryness under a stream of nitrogen. Peptide, polypeptide carriers or conjugates (100 μg) in 100 μl of phosphate buffered saline (PBS) at pH 8.0 were added, and the solutions were agitated periodically over a 10 min

incubation at room temperature. Subsequently the ^{125}I labelled CG peptide, free polypeptides and conjugates were purified by passage through Sephadex G-25, with PBS eluent at pH 7.2, using prepacked PD-10 columns (Pharmacia, Milton Keynes, UK) to separate unreacted Bolton and Hunter reagent.

The labelling efficacy was 35-40 %. Specific activities of the final products were ~2 MBq/mg.

Blood clearance and biodistribution

All *in vivo* studies were carried out in adult (~20 g) female BALB/c mice (Biomedical Services Unit, University of Nottingham) with appropriate licenses from the UK Home Office, and with due consideration for animal welfare. 5-10 μg of labelled compound in 0.2 ml PBS (250-500 μg/kg) were injected intravenously *via* a tail vein into the mice (groups of n=4 were used). Drinking water contained 0.1% w/v sodium iodide to block thyroid uptake of free radioiodine. Serial blood samples (10 μl) were taken from the tail tip at 1, 10 and 30 min and at 1, 2, 3 and 4 h after injection directly into microcapillary pipettes (Drummond Microcaps, Drummond Scientific Co., Broomhall, PA, USA). Blood clearance curves were constructed of the percent of the total initially injected count rates in the total blood volume against time. The total intravascular blood volumes were calculated assuming the blood volume of the mice (in ml) to be 11.2 % of the body weight (in g) [40]. Areas under curves (AUC), as percent dose x time (hours), were calculated using the trapezoidal rule [54]. 4 h after the injection the mice were killed and weighed samples of blood, visceral organs and residual carcass were assayed for radioactivity. We assumed that the ^{125}I labelled marker was associated with the polypeptides during the study period. Results of the tissue distribution analysis were expressed as a percentage of the total initially injected count rate per g of tissue.

Statistics

For tissue accumulation, standard deviation of the four samples was calculated. For tissue blood ratios standard deviation was calculated according to the rules of propagation of error. Levels of statistical difference between groups of animals were assessed by Student's t test.

Results and Discussion

A radiolabelled linear (K) and three radiolabelled branched chain polymeric polypeptides (E_iK, EAK, LAK), and their respective conjugates with oligopeptide epitope CG of MUC1

glycoprotein were prepared, also the ^{125}I-labeled CG peptide as control. The peptide CG was coupled *via* its N-terminal cysteine to the side chains of the macromolecular carrier to ensure uniform orientation. Chemical characteristics of the macromolecular carriers and peptide-conjugates based on amino acid analysis are shown in Table 1. With these characterised constructs, it was possible to evaluate the influence of structure of the polypeptide carrier upon the blood survival as well as on tissue distribution of the conjugate comprising a short, linear synthetic antigenic peptide.

Blood clearance and whole-body retention

The blood clearance profiles of CG-polypeptide conjugates and their corresponding free components (macromolecular carrier and oligopeptide) are depicted in Figure 1, the area under the curve (AUC 0-4 h) as well as the whole-body retention (WBR) values are presented in Table 2. We found significant differences in the blood clearance profiles of the free carrier polypeptides as well as the respective conjugates, and also between the free CG oligopeptide and its conjugates. All four CG-conjugates showed longer presence in the circulation than the free CG oligopeptide, detectable in < 3% even two hours after injection. Similarly, the low WBR value (9.2 %) corresponding to the oligopeptide indicates the quick elimination of CG not only from the circulation, but also from the body after 4h. The presence of amphoteric polypeptides E_iK and especially EAK possessing epitope CG resulted in significantly elongated blood survival (Figure 1.C, D) and higher WBR (9.2 % for CG *vs* ~ 33 % for the conjugates). Even at the second hour after injection ~ 20 % of E_iK-CG and > 40 % of EAK-CG conjugate was present in the circulation. The blood clearance curve of CG attached to linear or branched polycationic polypeptide (K or LAK) displayed increased blood survival compared to the free epitope peptide (Figure 1.A, B), but it was markedly shorter than conjugates of the amphoteric E_iK or EAK. By the third hour the presence of LAK-CG conjugate decreased to < 10%, and the presence of K-CG was less than 5 % even after the second hour. Interestingly, the WBR values for the conjugates were also higher as compared to the free CG, but the increase was structure dependent: conjugation with the linear polycationic carrier (K) resulted in a modest change (1.6 fold), while the LAK conjugate exhibited significantly higher WBR value (5.6 fold).

In case of the polycationic compound family the free peptide (CG) and the free carrier (K or LAK) showed similar blood clearance curves, while the CG-conjugates remained longer in the circulation. The WBR values for both free carriers were high (43.5 % and 39.2 %, respectively), while interestingly in case of their conjugates these values were 15% and 51 %,

respectively. On the other hand, when amphoteric carriers (E_iK or EAK) were used the profiles of the free carriers and the CG-conjugates were similar, though not identical (EAK and its conjugate displayed markedly higher blood retention). The difference observed might be attributed to the length of the branches (short in E_iK, longer in EAK). The WBR values at 4 h were also higher than in case of the free CG peptide (~3.5 fold for both amphotheric conjugates). These findings could indicate that probably the amphoteric nature of the conjugate with Glu at the end of the branches, together with their larger size, is responsible for the slower blood clearance kinetics, as described earlier for unconjugated EAK vs LAK carriers [46]. The hydrophilic, amphoteric characteristics of CG peptide with low molecular mass, together with the larger size of the respective conjugate compared to the free polymer and the higher degree of branching may be responsible for the delayed clearance of conjugates *vs* polymers. Similar effect of the covalently attached antitumor drug entities (daunomycin and methotrexate) was observed earlier [48-49].

The blood clearance profiles of EAK and EAK-CG were essentially identical, after 4 hours ~ 34 % of the total injected dose of both the free carrier and the conjugate are still present in the circulation (Figure 1.D). In the case of E_iK its clearance has been slightly slowed by the conjugation with the peptide (Figure 1.C).

The quick disappearance of decapeptide CG could be expected, predominantly due to the low molecular mass. The free polypeptides with higher average molecular mass (M_w = 20 – 46 kDa) were present in the circulation for a significantly longer period of time, as compared with the free oligopeptide, but their blood clearance was mainly dependent on their charge properties. The polycationic and amphoteric polypeptides exhibited different blood clearance profile: in contrast to the polycationic pair (K and LAK), the amphoteric polypeptides (E_iK and EAK) remained much longer in the circulation as demonstrated in Figure 1 (1.A and B *vs* 1.C and 1.D). These findings are in harmony with previously published data for LAK *vs* EAK carriers [46]. Conjugation of CG peptide with polycationic polypeptides resulted in different blood clearance profiles and also whole-body retention (Table 2). It should be noted that the CG peptide contains one acidic (Glu, D) and one basic (Arg, R) side chain function. Therefore, its attachment to the carrier modified the charge characteristics of the unconjugated polypeptides (Figure 1.A and 1.B). On average, 12% (in CG-K) or 30% (in CG-LAK) of the positive side chain charges were reduced by the incorporation of 12 or 30 copies of the CG oligopeptides. Therefore, the conjugates were markedly less polycationic as compared with the free carrier.

Coupling of CG with amphoteric polypeptide resulted in only minor changes in the blood survival of the carrier (Figure 1.C and D). These data could be interpreted by the substitution of the amino groups of the branches of amphoteric polypeptides. 18 % (in E_iK) or 30 % (in EAK) of the branch terminal amino groups were modified by CG peptides and caused essentially no significant changes in the charge properties. Thus, in both cases the fate of the conjugates was essentially determined by the charge properties of the carrier component.

In conclusion the hydrophilic, amphoteric characteristics of CG epitope peptide, together with the larger size of the conjugate compared to the free polymer and the degree of substitution may be responsible for the elongated clearance of conjugates *vs* free polymers. On the other hand, the covalent attachment of the decapeptide epitope (CG) with balanced charge distribution to polymeric polypeptide carrier resulted in altered charge properties and consequently its longer presence in the blood circulation. The blood clearance was markedly shorter when the partner was polycationic (K and LAK) as compared with that of amphoteric (E_iK and EAK).

Thus, the conjugation of an antigenic oligopeptide with epitope properties in multiple copies with polymeric polypeptide macromolecule could significantly influence the blood circulation profile of the covalently attached entity as it was observed earlier in case of antitumour drugs [48-49]. By appropriate selection of the structural properties (e.g. amino acid composition, sequence and length of the branch) of the carrier polypeptide there is a possibility to modulate the blood clearance profile as well as whole-body retention of the epitope containing conjugate in mice.

Tissue distribution

Results of the tissue distribution analysis showed that the properties of the carrier polypeptide have a marked influence not only on the blood clearance profile, but also on the tissue accumulation of the epitope peptide conjugates (Figure 2, Table 2.). The tissue accumulation of the conjugates was also compared to their respective polypeptide carriers. Poly[L-Lys] (K), known for its toxicity [55], accumulated in most organs (spleen: 20.9 %, kidney: 11.9 %, liver: 14.6 %, lung: 13.4 %/ g of tissue). The liver accumulation of polycationic LAK was also high (17.2 %). On the other hand, E_iK and EAK amphotheric carriers caused no outstanding tissue deposition, similarly to earlier studies performed with LAK and EAK polypeptides [46].

The tissue accumulation values were significantly lower in the case of K-CG conjugate compared to the carrier, while in the case of LAK-CG *vs* LAK we observed somewhat higher conjugate accumulation. E_iK-CG also showed slightly higher (although still relatively low) accumulation in all tissues than E_iK. The EAK-CG conjugate showed similar tissue accumulation pattern to its respective carrier (Figure 2., Table 2).

Typically, low tissue accumulation could be observed with the free as well as CG-containing amphotheric E_iK and EAK carriers, while both polycationic polypeptides and their CG-conjugates were present at higher level in spleen and liver. It is interesting to note that marked accumulation of the polycationic conjugate (CG-LAK) with hydrophobic N-terminal amino acid (Leu, L) was observed in the liver.

In agreement with the above observations the analysis of tissue/blood ratios showed similar findings. The tissue to blood ratio values of unconjugated polypeptides and conjugates were far the lowest in the case of the amphoteric carriers (E_iK and EAK) and conjugates (E_iK-CG and EAK-CG), all tissue/blood values were under 2 (Figure 3).

Tissue/blood ratios of free (K and LAK) and conjugated polypeptide (K-CG and LAK-CG) were higher. In the case of K-CG tissue/blood ratios were slightly lower than those of free K, but still 10-20 values could be calculated.

LAK-CG conjugate showed tissue/blood ratios between 2 and 10, which are between those of K-CG and the amphoteric conjugates. High value (> 50) was documented for the unconjugated, Leu containing polypeptide LAK in harmony with tissue distribution (see above). Thus, conjugates with amphoteric polypeptide had generally lower tissue accumulation than those with polycationic ones.

Data outlined above clearly indicate that the structural features of the epitope conjugates affecting tissue distribution were similar to those altering blood clearance.

Conclusions

Taken together, among the CG epitope peptide–carrier 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 explained, on the one hand, by its charge properties; compared to the conjugates of polycationic polypeptide carriers this conjugate had a lower number of free amino groups, and on the other hand by having a neutral oligoalanine chain, separating the poly[L-Lys] backbone from the N-terminal Glu, compared to E_iK-CG conjugate. The effect of lower polarity, the addition of apolar residues to the carrier can also be observed in comparing K-CG and LAK-CG conjugates, the latter

showing longer blood survival and higher tissue accumulation. Further studies on the immune response induced by these epitope conjugates will be initiated to establish correlation between biodistribution properties (presence in the blood clearance, level of tissue accumulation) and the capacity of these conjugates to elicit antibody production.

In summary, to the best of our knowledge this is one of first reports on biodistribution of polymeric polypeptide conjugates which contain epitope peptide of an immunogenic protein (e.g. MUC1 protein) attached to various, but structurally related polypeptide carriers. In the light of the limited current understanding about the pharmacokinetics–pharmacodynamics in vaccine immunogenicity/vaccination related research [27-28], data presented here on biodistribution of epitope-conjugates could be important i) to identify structural elements of the carrier (e.g. amino acid composition, length and sequence of the branches, charge) for the design of appropriate synthetic immunogens with desired blood clearance, tissue distribution etc. and ii) be relevant for the design and construction of suitable synthetic immunogens, vaccines.

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References

[1] S. Gendler, J. Taylor-Papadimitriou, T. Duhig, J. Rothbard, J. Burchell, A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats, J. Biol. Chem. 263 (1988) 12820-12823.

[2] N. Martínez-Sáez, J.M. Peregrina, F. Corzana. Principles of mucin structure: implications for the rational design of cancer vaccines derived from MUC1-glycopeptides. Chem Soc Rev. 46 (2017) 7154-7175. doi: 10.1039/c6cs00858e.

[3] L. Shi, H. Cai, Z.H. Huang, Z.Y. Sun, Y.X. Chen, Y.F. Zhao, H. Kunz, Y.M. Li, Synthetic MUC1 Antitumor Vaccine Candidates with Varied Glycosylation Pattern Bearing R/S-configured Pam3 CysSerLys4, Chembiochem 17 (2016) 1412-1415. doi: 10.1002/cbic.201600206. Epub 2016 Jun 15.

[4] G. Denton, M. Sekowski, M.R. Price, Induction of antibody responses to breast carcinoma associated mucins using synthetic peptide constructs as immunogens, Cancer Lett. 70 (1993) 143-150.

[5] M.R. Price, F. Hudecz, C. O'Sullivan, R.W. Baldwin, P.M. Edwards, S.J.B Tendler, Immunological and structural features of the protein core of human polymorphic epithelial mucin, Mol. Immunology 27 (1990) 795-802.

[6] P.-X. Xing, K. Reynolds, G.A. Pietersz, I.F.C. McKenzie, Effect of variations in peptide sequence on anti-human milk fat globule membrane antibody reactions, Immunology 72 (1991) 304-311.

[7] P.-X. Xing, J. Prenzoska, I.F.C. McKenzie, Epitope mapping of anti-breast and anti-ovarian mucin monoclonal antibodies, Mol. Immunology 29 (1992) 641-650.

[8] J. Burchell, J. Taylor-Papadimitriou, M. Boshell, S. Gendler, T. Duhig, A short sequence, within the amino acid tandem repeat of a cancer-associated mucin, contains immunodominant epitopes, Int. J. Cancer 44 (1989) 691-696.

[9] E. Lazoura, J. Lodding, W. Farrugia, P.A. Ramsland, J. Stevens, I.A. Wilson, G.A. Pietersz, V. Apostolopoulos, Enhanced major histocompatibility complex class I binding and immune responses through anchor modification of the non-canonical tumour-associated mucin 1-8 peptide, Immunology 119 (2006) 306-316.

[10] B. Agrawal, B.M. Longenecker, MUC1 mucin-mediated regulation of human T cells, Int. Immunol. 17 (2005) 391-399.

[11] K. Uray, F. Hudecz, Peptide epitopes: Identification and structural modifications of synthetic antigens, in: M. Ryadnov, E. Farkas (Eds.), Amino Acids, Peptides and Proteins, The Royal Society of Chemistry, Cambridge, 2015, pp. 68-113. (Specialist Periodical Reports – Amino Acids, Peptides and Proteins, ISSN:1361-5904; 39.)

[12] Y. Kotera, J.D. Fontenot, G. Pecher, R.S. Metzgar, O.J. Finn, Humoral immunity against a tandem repeat epitope of human mucin MUC-1 in sera from breast, pancreatic, and colon cancer patients, Cancer Res. 54 (1994) 2856-2860.

[13] L.G. Durrant, E. Jacobs, M.R. Price, Production of monoclonal antibodies recognising the peptide core of MUC2 intestinal mucin, Eur. J. Cancer 30A (1994) 355-363.

[14] J. Pinkhasov, M.L. Alvarez, L.B. Pathangey, T.L. Tinder, H.S. Mason, A.M. Walmsley, S.J. Gendler, P. Mukherjee, Analysis of a cholera toxin B subunit (CTB) and

human mucin 1 (MUC1) conjugate protein in a MUC1-tolerant mouse model, Cancer Immunol. Immunother. 59 (2010) 1801-1811.

[15] R.P. Briňas, A. Sundgren, P. Sahoo, S. Morey, K. Rittenhouse-Olson, G.E. Wilding, W. Deng, J. Barchi Jr, Design and synthesis of multifunctional gold nanoparticles bearing tumor-associated glycopeptide antigens as potential cancer vaccines, J. Bioconjugate Chem. 23 (2012) 1513-1524.

[16] G. Denton, F. Hudecz, J. Kajtár, A. Murray, S.J. Tendler, M.R. Price, Sequential order of T and B cell epitopes affects immunogenicity but not antibody recognition of the B cell epitope, Pept. Res. 7 (1994) 258-264.

[17] M. Glaffig, B. Palitzsch, S. Hartmann, C. Schüll, L. Nuhn, B. Gerlitzki, E. Schmitt, H. Frey, H. Kunz, A fully synthetic glycopeptide antitumor vaccine based on multiple antigen presentation on a hyperbranched polymer, Chemistry 20 (2014) 4232-4236.

[18] D. Straßburger, M. Glaffig, N. Stergiou, S. Bialas, P. Besenius, E. Schmitt, H. Kunz: Synthetic MUC1 Antitumor Vaccine with Incorporated 2,3-Sialyl-T Carbohydrate Antigen Inducing Strong Immune Responses with Isotype Specificity, Chembiochem 19(11) (2018) 1142-1146.

[19] W.G. Bessler, L. Heinewetter, K.H. Wiesmueller, G. Jung, W. Baier, M. Huber, A.R. Lorenz, U.V.D. Esche, K. Mittenbuehler, P. Hoffman, Bacterial cell wall components as immunomodulators – I. Lipopeptides as adjuvants for parenteral and oral immunization, Int. J. Immunopharmacol. 19 (1997) 547-550.

[20] A. Kaiser, N. Gaidzik, T. Becker, C. Menge, K. Groh, H. Cai, Y.-M. Li, B. Gerlitzki, E. Schmitt, H. Kunz, Fully synthetic vaccines consisting of tumor-associated MUC1 glycopeptides and a lipopeptide ligand of the Toll-like receptor 2, Angew. Chem. Int. Ed. 49 (2010) 3688-3692.

[21] V. Lakshminarayanan, P. Thompson, M.A. Wolfert, T. Buskas, J.M. Bradley, L.B. Pathangey, C.S. Madsen, P.A. Cohen, S.J. Gendler, G.J. Boons: Immune recognition of tumor-associated mucin MUC1 is achieved by a fully synthetic aberrantly glycosylated MUC1 tripartite vaccine, Proc Natl Acad Sci U S A. 109(1) (2012) 261-266.

[22] A-B. M. Abdel-Aal, V. Lakshminarayanan, P. Thompson, N. Supekar, J. M. Bradley, M. A. Wolfert, PA Cohen, SJ Gendler, GJ Boons: Immune and anticancer esponses elicited

by fully synthetic aberrantly glycosylated MUC1 tripartite vaccine modified by a TLR2 or TLR9 agonist, Chembiochem 15 (2014) 1508–1513.

[23] L. Nuhn, S. Hartmann, B. Palitzsch, B. Gerlitzki, E. Schmitt, R. Zentel, H. Kunz, Water-soluble polymers coupled with glycopeptide antigens and T-cell epitopes as potential antitumor vaccines, Angew. Chem. Int. Ed. 52 (2013) 10652.

[24] J.P. Tam, Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system, Proc. Natl. Acad. Sci. USA 85 (1988) 5409-5413.

[25] T. Becker, A. Kaiser, H. Kunz, Synthesis of Dendrimeric Tumor-Associated Mucin-Type Glycopeptide Antigens, Synthesis 7 (2009) 1113-1122.

[26] C. Geraci, G.M. Consoli, G. Granata, E. Galante, A. Palmigiano, M. Pappalardo, S.D. Di Puma, A. Spadaro, First self-adjuvant multicomponent potential vaccine candidates by tethering of four or eight MUC1 antigenic immunodominant PDTRP units on a calixarene platform: synthesis and biological evaluation, Bioconjug. Chem. 24 (2013) 1710-1720.

[27] J.-P. Amorij, G.F.A. Kersten, V. Saluja, W.F. Tonnis, W.L.J. Hinrichs, B. Slütter, S.M. Bal, J.A. Bouwstra, A. Huckriede, W. Jiskoot, Towards tailored vaccine delivery: Needs, challenges and perspectives, J. Controlled Release 161 (2012) 363–376.

[28] O. Zagorodko, J.J. Arroyo-Crespo, V.J. Nebot, M.J. Vicent, Polypeptide-Based Conjugates as Therapeutics: Opportunities and Challenges, Macromol Biosci. 17(1) (2017) 1600316**.**

[29] F. Hudecz, H. Votavova, D. Gaál, J. Sponar, J. Kajtár, K. Blaha, M. Szekerke, Branched polypeptides with a poly(L-lysine) backbone: synthesis, conformation and immunomodulation, in: Ch.G. Gebelein, Ch.E. Carraher (Eds.), Polymeric Materials in Medication, Plenum Press, New York, 1985, pp. 265-289.

[30] G. Mező, F. Hudecz, J. Kajtár, G. Szókán, M. Szekerke, The influence of the side chain sequence on the structure-activity correlations of immunomodulatory branched polypeptides. Synthesis and conformational analysis of new model polypeptides, Biopolymers 28 (1989) 1801-1826.

[31] F. Hudecz, S. Kutassi-Kovács, G. Mező, M. Szekerke, Biodegradability of synthetic branched polypeptide with poly(L-lysine) backbone, Biol. Chem. Hoppe-Seyler 370 (1989) 1019-1026.

[32] F. Hudecz: Synthesis of peptide bioconjugates. In: J. Howl (Ed) Methods in Molecular Biology, vol.298: Peptide Synthesis and Applications Humana Press, Totowa, NJ, USA, 2005, pp. 209-224.

[33] F. Hudecz, Branched polymeric polypeptides with poly[Lys], in: M. Ryadnov, F. Hudecz (Eds.), Amino Acids, Peptides and Proteins, The Royal Society of Chemistry, Cambridge, 2019, (Specialist Periodical Reports – Amino Acids, Peptides And Proteins), in press.

[34] F. Hudecz, M.R. Price, Monoclonal antibody binding to peptide epitopes conjugated to synthetic branched chain polypeptide carriers. Influence of the carrier upon antibody recognition, J. Immunol. Methods 147 (1992) 201-210.

[35] S. Bősze, F. Hudecz, Proteins and peptides for the immunodiagnosis and therapy of Mycobacterium tuberculosis infections, in: M. Ryadnov, E. Farkas (Eds.), Amino Acids, Peptides and Proteins, The Royal Society of Chemistry, Cambridge, 2016, pp. 146-198. (Specialist Periodical Reports – Amino Acids, Peptides And Proteins, ISSN:1361-5904; 40.)

[36] F. Hudecz, Alteration of immunogenicity and antibody recognition of B-cell epitopes by synthetic branched chain polypeptide carriers with poly[L-lysine] backbone, Biomed. Pept. Prot. Nucleic Acids 1 (1995) 213-220.

[37] É. Rajnavölgyi, A. Lányi, F. Hudecz, I. Kurucz, K. Kiss, G. László, M. Szekerke, J. Gergely, Structural characteristics influencing the carrier function of synthetic branched polypeptides based on poly/Lys-(DL-Ala)m/ backbone, Molec. Immunol. 26 (1989) 949-958.

[38] F. Hudecz, Á. Hilbert, G. Mező, I. Mucsi, J. Kajtár, Sz. Bősze, I. Kurucz, É. Rajnavölgyi, Epitope mapping of the 273-284 region of HSV glycoprotein D by synthetic branched polypeptide-carrier conjugates, Peptide Res. 6 (1993) 263-271.

[39] Hilbert, Á., Hudecz, F., Mező, G., Mucsi, I., Kajtár, J. Kurucz, I., Rajnavölgyi, É.: The influence of the branched polypeptide carrier on the immunogenicity of predicted epitopes of HSV-1 glycoprotein D. Scan. J. Immunol. 40 (1994) 609-617.

[40] G. Mező, B. Dalmadi, I. Mucsi, Sz. Bősze, É. Rajnavölgyi, F. Hudecz, Peptide based vaccine design: Synthesis and immunological characterisation of branched polypeptide conjugates comprising the 276-284 immunodominant epitope of HSV-1 glycoprotein D, J. Peptide Science 8 (2002) 107-117.

[41] G. Mező, E. de Oliveira, D. Krikorian, M. Feijlbrief, A. Jakab, V. Tsikaris, C. Sakarellos, S. Welling-Wester, D. Andreu, F. Hudecz, Synthesis and Comparison of Antibody Recognition of Conjugates Containing Herpes Simplex Virus type 1 Glycoprotein D Epitope VII, Bioconjugate Chemistry 14 (2003) 1260-1269.

[42] K.A. Wilkinson, F. Hudecz, H.M. Vordermeier, J. Ivanyi, R.J. Wilkinson, Enhancement of the T cell response to a mycobacterial peptide by conjugation to synthetic branched polypeptide, Eur. J. Immunol. 29 (1999) 2788-2796.

[43] I.B. Nagy, M.A. Alsina, I. Haro, F. Reig, F. Hudecz, Phospholipid-model membrane interactions with branched polypeptide conjugates of a Hepatitis a virus peptide epitope, Bioconjugate Chem. 11 (2000) 30-38.

[44] M.V. Pimm, A.C. Perkins, S.J. Gribben, G. Mező, D. Gaál, F. Hudecz, Gamma scintigraphy of 111In-labelled branched chain polypeptides (BCP) with a poly(L-lysine) backbone in mice with mammary carcinoma: effect of charge on biodistribution and tumour imaging potential, Ann. Nucl. Med. 94 (1995) 247-251.

[45] M.V. Pimm, S.J. Gribben, G. Mező, F. Hudecz, Strategies for labelling branched polypeptides with a poly(L-lysine) backbone with radioiodines (123I, 125I, 131I) and radiometals (111In, 51Cr) for biodistribution studies and radiopharmaceutical development, J. Labelled Comp. Radiopharm. 36 (1995) 157-172.

[46] J.A. Clegg, F. Hudecz, G. Mező, M.V. Pimm, M. Szekerke, R.W. Baldwin, Carrier design: Biodistribution of branched polypeptides with a poly(L-lysine) backbone, Bioconjugate Chem. 2 (1990) 425-430.

[47] M.V. Pimm, S.J. Gribben, K. Bogdan, F. Hudecz, The effect of charge on the biodistribution in mice of branched polypeptides with a poly(L-lysine) backbone labelled with 125I, 111In or 51Cr, J. Controlled Release 37 (1995) 161-172.

[48] F. Hudecz, J. A. Clegg, J. Kajtar, M. J. Embleton, M. Szekerke, R. W. Baldwin, Synthesis, conformation, biodistribution and in vitro cytotoxicity of daunomycin-branched polypeptide conjugates, Bioconjugate Chem. 3(1) (1992) 49–57.

[49] F. Hudecz, J.A. Clegg, J. Kajtár, M.J. Embleton, M.V. Pimm, M. Szekerke, R.W. Baldwin, Influence of carrier on biodistribution and in vitro cytotoxicity of methotrexate-branched polypeptide conjugates, Bioconjugate Chem. 4 (1993) 25-33.

[50] G. Mező, I. Mező, M.V. Pimm, J. Kajtár, J. Seprődi, I. Teplán, M. Kovács, B. Vincze, I. Pályi, M. Idei, M. Szekerke, F. Hudecz, Synthesis, conformation, biodistribution, and hormone-related in vitro antitumor activity of a gonadotropin-releasing hormone antagonist-branched polypeptide conjugate, Bioconjugate Chem. 7 (1996) 642-650.

[51] J. Carlsson, H. Drevin, R. Axen, Protein thiolation and reversible protein-protein conjugation, Biochem. J. 173 (1978) 723-737.

[52] I.B. Nagy, A. Dancs, G. Kóczán, G. Mező, F. Hudecz, Conjugation of HS-oligopeptides with polymeric branched chain polypeptides containing multiple amino groups, J. Bioactive Compatible Polymers 15 (2000) 139-154.

[53] A.E. Bolton, W.M. Hunter, The labelling of proteins to high specific radioactivities by conjugation to a 125I-containing acylating agent, Biochem. J. 133 (1973) 529-539.

[54] T. N. Tozer, M. Rowland, Assessment of area, in Introduction to pharmacokinetics and pharmacodynamics: the quantitative basis of drug therapy. Lippincott Williams & Wilkins, Baltimore (2006) pp.275-277.

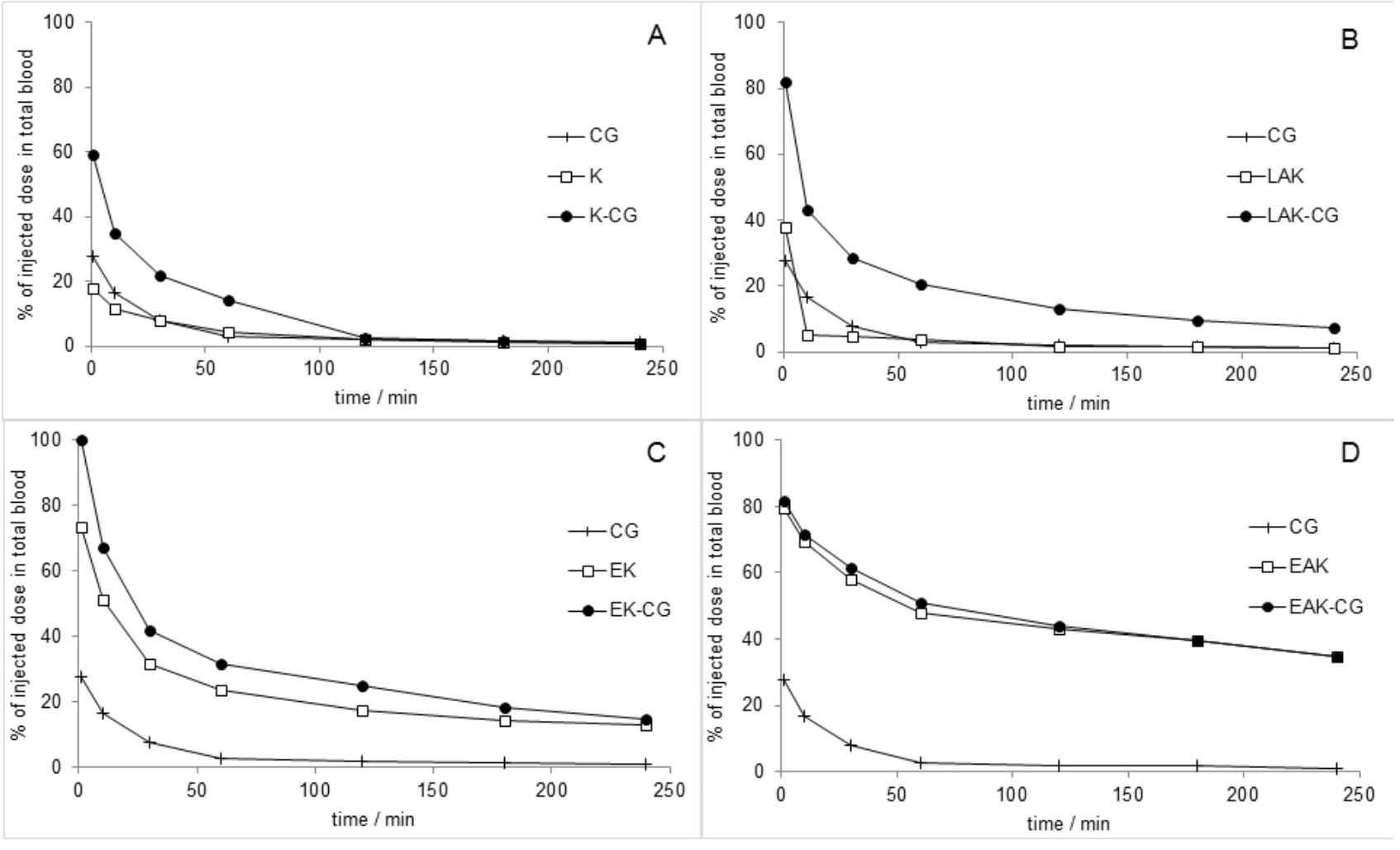
[55] F. Hudecz, D. Gaál, I. Kurucz, S. Lányi, A.L. Kovács, G. Mező, É. Rajnavölgyi, M. Szekerke, Carrier design: cytotoxicity and immunogenicity of synthetic branched polypeptides with poly(L-lysine) backbone, J. Controlled Release, 19 (1992) 231-243.

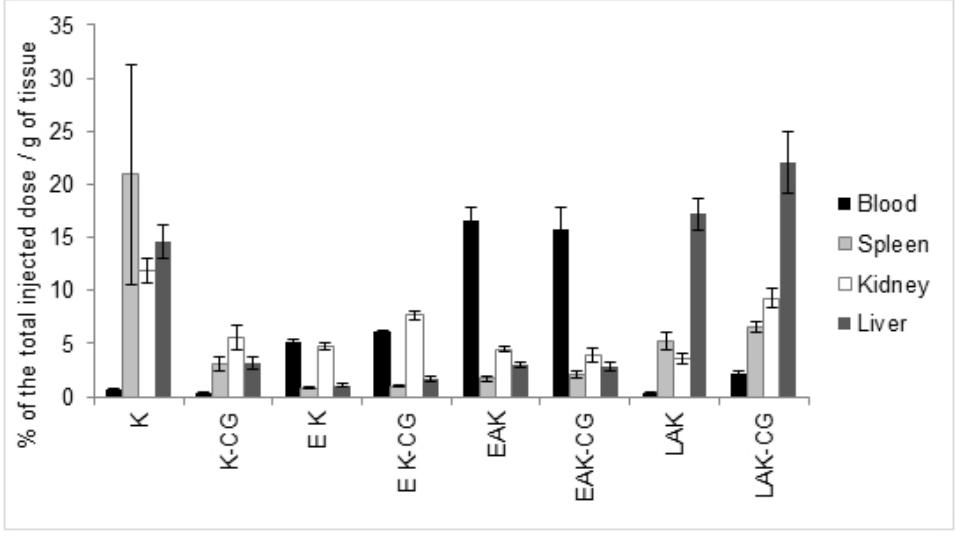
Figure Captions

Figure 1. Blood clearance profiles of ^{125}I labelled peptide CG, ^{125}I labelled polymers and their conjugates following *iv* administration to BALB/c mice. (A) K and K-CG, B) LAK and LAK-CG, C) E_iK and E_iK-CG, D) EAK and EAK-CG). AUC0-4h calculated from these data are given in Table 2. Results are expressed as mean standard deviation for groups of four animals. Standard deviation was always below 15 %.

Figure 2. Tissue distribution of ^{125}I-branched polypeptides, free polymers and of CG oligopeptide in Balb/c mice 4 h after *iv* administration. Results are expressed as % of total injected dose / g of tissue, mean for groups of four animals.

Figure 3. Tissue / blood ratio of carriers and conjugates in mice 4 h after injection. Tissue distribution of ^{125}I-branched polypeptides and free polymers in Balb/c mice 4 h after *iv* administration. Results are expressed as tissue / blood ratio as mean for groups of four animals.


A
% of injected dose in total blood
time / min
CG
K
K-CG
B
% of injected dose in total blood
time / min
CG
LAK
LAK-CG
C
% of injected dose in total blood
time / min
CG
EK
EK-CG
D
% of injected dose in total blood
time / min
CG
EAK
EAK-CG


% of the total injected dose / g of tissue
35
30
25
20
15
10
5
0
K
K-CG
E K
E K-CG
EAK
EAK-CG
LAK
LAK-CG
Blood
Spleen
Kidney
Liver

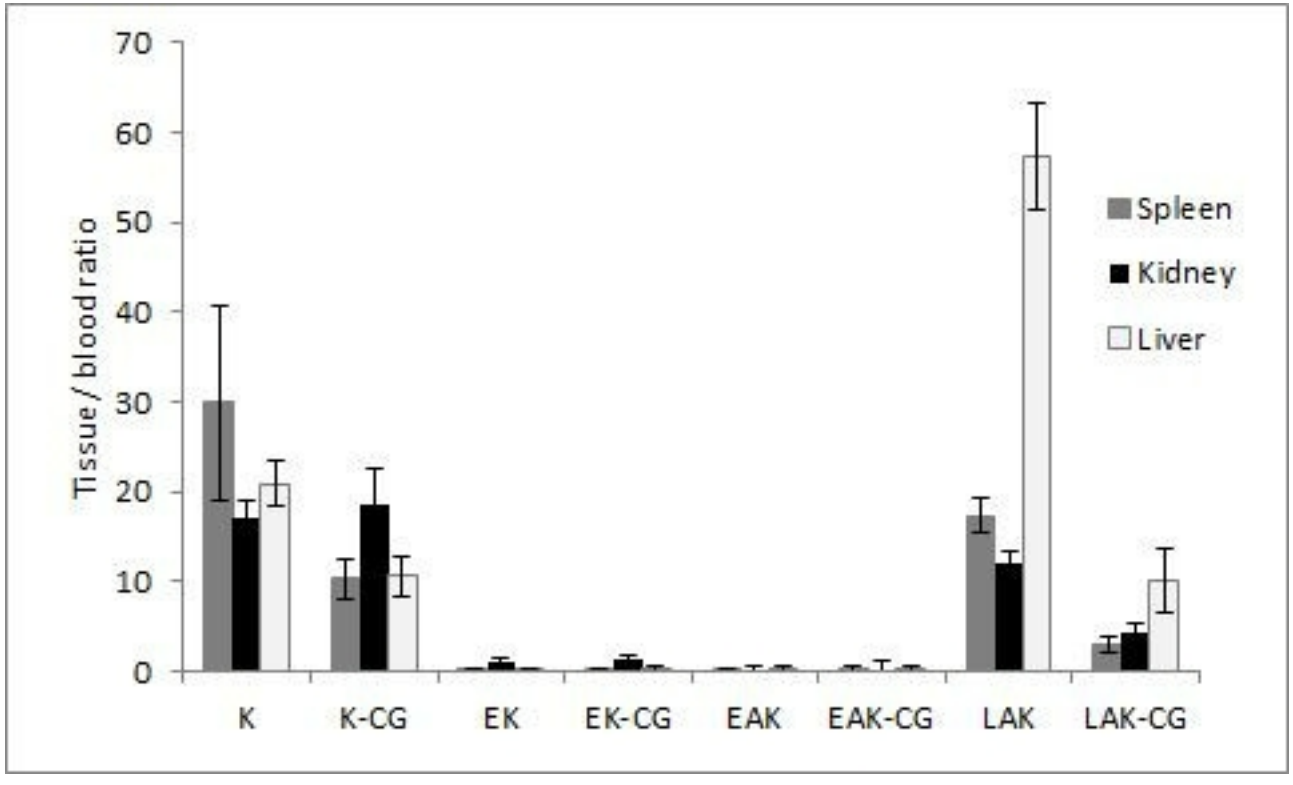

70
60
50
40
30
20
10
0
Tissue / blood ratio
Spleen
Kidney
Liver
K
K-CG
EK
EK-CG
EAK
EAK-CG
LAK
LAK-CG

Table 1. Characteristics of branched polypeptides and their CAPDTRPAPG epitope peptide conjugates

Compound	**Amino acid composition[1]** X_i : Ala_m : Lys	**Code[2]**	**Average degree of substitution[3] peptide/polymer (%)**	**Mw[4] (±5%)**
Poly[Lys]	-	K	-	20 800
Poly[Lys(CAPDTRPAPG)$_j$]	-	K-CG	12	32 600
Poly[Lys(Glu$_i$)]	0.98 : 1.0	EK	-	35 000
Poly[Lys(Glu$_i${CAPDTRPAPG}$_j$]	-	EK-CG	18	43 400
Poly[Lys(Glu$_i$-DL-Ala$_m$)]	0.93 : 2.94 : 1.0	EAK	-	45 800
Poly[Lys({CAPDTRPAPG}$_j$-Glu$_i$-DL-Ala$_m$)]	-	EAK-CG	32	77 300
Poly[Lys(Leu$_i$-DL-Ala$_m$)]	0.81 : 2.94 : 1.0	LAK	-	35 000
Poly[Lys({CAPDTRPAPG}$_j$-Leu$_i$-DL-Ala$_m$)]	-	LAK-CG	30	72 300

[1] Molar ratio calculated from the amino acid composition determined by amino acid analysis.
[2] Based on the single letter code of poly[L-Lys] and branched chain polypeptides, and that of the peptide
[3] Average degree of substitution expressed as % of modified side chains of the carrier polypeptide calculated from the average degree of polymerisation of the poly[L-Lys] (DP_n=100) and from the side chain composition of the conjugate.
[4] Average molecular weight of the macromolecule, calculated from DP_n=100 for poly[L-Lys] and from the side chain composition.

Table 2. Biodistribution of branched chain polypeptides and their CAPDTRPAPG epitope peptide conjugates in Balb/c mice 24 h after *iv* administration. Results are expressed as mean for groups of four animals.

Code	AUC 0-4 hours [% dose × hours ± SD]	Whole body retention (WBR) at 4 hr [% dose ± SD]	Percent of the total injected dose (± SD) / gram of tissue						
			Blood	Spleen	Kidney	Liver	Lung	Heart	Carcass
K	18.4 ± 1.6	43.5 ± 2.6	0.7 ± 0.1	20.9 ± 10.3	11.9 ± 1.2	14.6 ± 1.5	13.4 ± 1.2	0.2± 0.1	0.7 ± 0.0
K-CG	36.4 ± 5.0	15.1 ± 3.4	0.3 ± 0.2	3.1 ± 0.7	5.6 ± 1.1	3.2 ± 0.6	4.4 ± 1.5	3.5± 0.3	0.4 ± 0.2
E_iK	83.1 ± 7.2	22.3 ± 1.5	5.1 ± 0.3	0.8 ± 0.1	4.8 ± 0.3	1.1 ± 0.1	1.7 ± 0.1	1.3± 0.1	0.9 ± 0.1
E_iK-CG	108.1 ± 2.3	32.5 ± 3.8	6.1 ± 0.2	1.0 ± 0.1	7.7 ± 0.4	1.7 ± 0.3	1.9 ± 0.1	1.1± 0.1	1.3 ± 0.2
EAK	178.8 ± 17.5	31.3 ± 0.8	16.6 ± 1.2	1.7 ± 0.3	4.5 ± 0.2	3.0 ± 0.2	4.5 ± 0.3	2.8± 0.3	1.3 ± 0.1
EAK-CG	180.9 ± 25.7	32.2 ± 6.0	15.8 ± 2.0	2.1 ± 0.4	3.9 ± 0.7	2.8 ± 0.4	4.1 ± 0.9	3.0± 0.2	1.4 ± 0.3
LAK	9.2 ± 0.4	39.2 ± 2.3	0.3 ± 0.1	5.2 ± 0.8	3.6 ± 0.5	17.2 ± 1.5	1.1 ± 0.2	0.4± 0.1	0.2 ± 0.1
LAK-CG	67.2 ± 5.9	51.5 ± 3.5	2.2 ± 0.2	6.6 ± 0.5	9.3 ± 0.9	22.0 ± 2.9	5.3 ± 1.0	0.2± 0.1	0.4 ± 0.1
CG	15.8 ± 2.2	9.2 ± 2.8	0.6 ± 0.1	0.2 ± 0.1	0.6 ± 0.2	1.5 ± 0.2	0.5 ± 0.1	0.4 ± 0.1.	0.3 ± 0.1