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Review paper

Design of synthetic branched-chain polypeptides as carriers for bioactive molecules

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New groups of synthetic biodegradable branched chain polypeptides have been prepared with the general formula poly[Lys–(X_i–DL-Ala$_m$)] [XAK] or poly[Lys–(DL-Ala$_m$–X_i)] [AXK], where $m \sim 3$ and $i < 1$, and used to elucidate structural and functional properties required for the selection of macromolecular carriers for (i) targeting/delivery of antitumor agents (e.g. daunomycin, methotrexate, boron derivatives), peptide hormones (e.g. GnRH antagonist) or radionuclides for imaging (e.g. ^{123}I, ^{111}In, ^{51}Cr) or therapy (e.g. ^{153}Sm, ^{131}I) or (ii) the construction of synthetic antigens with peptide epitopes of mucin or Herpes Simplex virus glycoprotein D. Principles applicable for a rational carrier design are outlined based on chemical (size, charge, solution conformation) and biological (cytotoxicity, pirogenicity, biodegradation, immunogenicity, immunomodulatory potential, biodistribution) characterization of these biopolymers and their conjugates.

Key words: Antitumor agent, branched-chain polypeptide, carrier, conjugate.

Introduction

The use of macromolecular carriers for small molecules such as drugs and hormones has a wide range of application in the field of drug targeting/delivery. Promising results have been reported concerning the alteration of pharmacological or immunological properties of biologically active compounds by their conjugation to macromolecules.[1–3] In immunology, carriers are frequently used to induce immune responses against covalently attached small, non-immunogenic epitopes for the production of epitope-specific monoclonal antibodies or for the construction of synthetic vaccines.[4–6] Conjugates of reporter molecules like radionuclides or fluorophores with macromolecules could be useful for the development of diagnostics or for antitumor therapy.[7,8]

In order to understand correlations between the chemical structure and various biological properties required for optimal carrier function of macromolecules, two new groups of biodegradable branched polypeptides were introduced by our laboratory.[9–14] These biodegradable polypeptides with the general formula poly[Lys–(X_i–DL-Ala$_m$)] (XAK) and poly[Lys–(DL-Ala$_m$–X_i)] (AXK), where $i < 1$, $m \sim 3$ and X = Glu, D-Glu, Ile, Leu, D-Leu, Phe, D-Phe, Pro, His, Lys, D-Lys or Tyr, are based on a poly[L-Lys] backbone and contain short side chains composed of about three DL-Ala residues and one other amino acid residue (X) either at the end of the branches (XAK) or at the position next to the polylysine backbone (AXK) (Figure 1). These constructs represent a significantly modified version of multichain polypeptides used for immunological studies by Sela *et al.*[15] In order to provide a simple, but versatile model system suitable for (i) primary structure–solution conformation and (ii) structure–function analysis, the length of the poly[DL-Ala] side chains has been shortened (from 22–30 to 3–4) and instead of copolymers, only single optically active amino acids (X) were introduced into the branches. Due to the limited solubility of branched polypeptides containing only short side chains of L-Ala or D-Ala, racemic oligo[DL-Ala] grafts were applied.

In order to outline principles applicable for a rational design of macromolecular carriers, much emphasis was placed on chemical characterization, especially on the analysis of conformational features[16] and on complex biological examination[17] of these structurally related synthetic polypeptides. These studies include the evaluation of cytotoxicity, pirogenicity, biodegradation, immunogenicity, immunomodulatory potential and biodistribution (Figure 2).

Selected biopolymers have been introduced by our group as carriers for targeting/delivering of anti-tumor agents (daunomycin, methotrexate, boron derivatives, GnRH antagonist),[18–21] radionuclides,[22,23] tuftsin,[24] and for the construction of

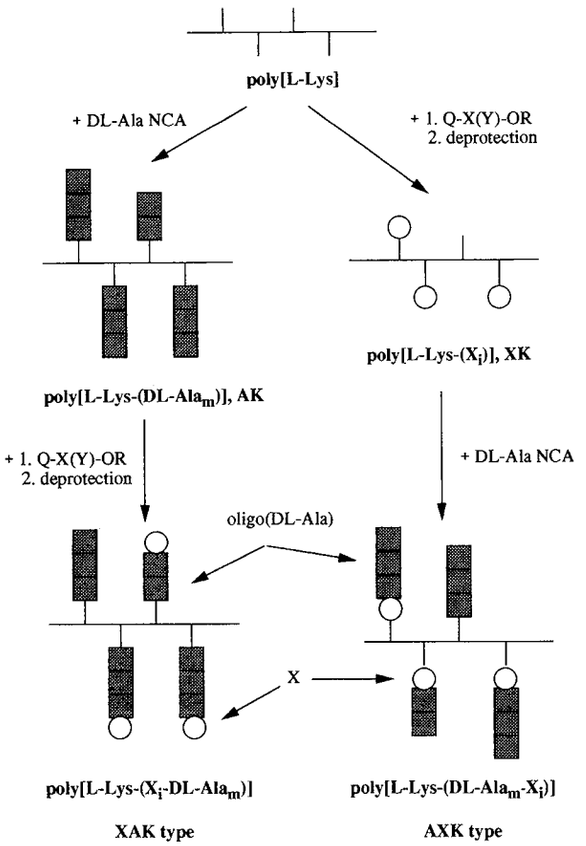

Figure 1. Synthesis and schematic presentation of XAK- and AXK-type branched polypeptides, where $m \sim 3$, $i < 1$. X = Glu, D-Glu, Ile, Leu, D-Leu, Phe, D-Phe, Pro, His, D-His, Lys, D-Lys or Tyr.

synthetic antigens with oxazolone derivative,[25–27] peptide epitopes of mucin[28–30] or Herpes Simplex virus (HSV) glycoprotein D (gD).[31–36]

In this contribution a summary will be provided to outline our results obtained during the last 15 years in the field of synthesis, and chemical and biological characterization of branched polypeptides and their antitumor agents or radionuclide conjugates. No attempt will be made to review epitope–carrier conjugates.

Synthesis of branched polypeptides

The schematic representation of the synthesis strategy of two groups of branched polypeptides is depicted in Figure 1. Two types of polypeptides (XAK and AXK) were synthesized by the substitution of the ε-amino groups of poly[L-Lys]. First poly[L-Lys] was prepared by the polymerization of N^{α}-carboxy-N^{ε}-(benzyloxycarbonyl)-lysine anhydride under conditions that allowed an average degree of polymerization of either 80–120 or 400–500. The protecting groups were cleaved by HBr/acetic acid solution.[9–11]

To produce XAK-type polypeptides, first poly[Lys–(DL-Ala$_m$)] (AK) was prepared by grafting of short oligomeric DL-Ala side chains onto the ε-amino groups of the poly[L-Lys] backbone by the aid of *N*-carboxy-DL-Ala anhydride. Then the α-amino groups of AK were reacted with suitably protected amino acid active esters [Q–X(Y)–OR]. The removal of the blocking groups was followed by UV spectroscopy.[9–11]

For the synthesis of AXK-type polypeptides, a subset of branched polypeptides with the general formula poly[Lys–(X$_i$)], where X = Phe, D-Phe, Leu, D-Leu, Ala, D-Ala, Ile, Pro, Glu, D-Glu or His, were synthesized by Mezö *et al.*[12–14] In these compounds the amino acid X is attached to the ε-amino groups of poly[Lys]. Similar polypeptides with X = Gly[37,38], Phe[38] or Leu[38] have been described before, but no preparation of derivatives comprising hydrophilic amino acids (His, Glu) or Ala, D-Ala, Ile, D-Leu, Pro or D-Phe has been published. The use of pentafluorophenyl or pentachlorophenyl ester in the presence or absence of an equimolar amount of 1-hydroxy-benzotriazole (by which these new polymeric polypeptides were produced) also provided a new approach to optimize reaction conditions.[14] After cleavage of protecting groups of amino acid X, the α-amino groups of the respective poly[Lys–(X$_i$)] polymers were reacted with *N*-carboxy-DL-Ala anhydride to produce AXK polypeptides (Figure 1).

Abbreviations for amino acids and their derivatives follow the revised recommendation of the IUPAC–IUB Committee on Biochemical Nomenclature, entitled *Nomenclature and Symbolism for Amino Acids and Peptides* (recommendations of 1983). Nomenclature of branched polypeptides is used in accordance with the recommended nomenclature of graft polymers.[39] For the sake of brevity codes of branched polypeptides were constructed by us using the one-letter symbols for amino acids (Figure 1). The abbreviations used in this paper for branched polypeptides and their conjugates are the following. XAK, poly[Lys–(X$_i$–DL-Ala$_m$)]; AXK, poly[Lys–(DL-Ala$_m$–X$_i$)]; XK, poly[Lys–(X$_i$)] cAD-XAK, poly[Lys–(cAD$_f$–X$_i$–DL-Ala$_m$)]; cAD-AXK, poly[Lys–(cAD$_f$–DL-Ala$_m$–X$_i$)]; MTX-XAK, poly[Lys–(MTX$_f$–X$_i$–DL-Ala$_m$)]; MTX-AXK, poly[Lys–(MTX$_f$–DL-Ala$_m$–X$_i$)]. Capital and small letters denote the size of polypeptides used in biodistribution studies. All amino acids are of L-configuration unless otherwise stated.

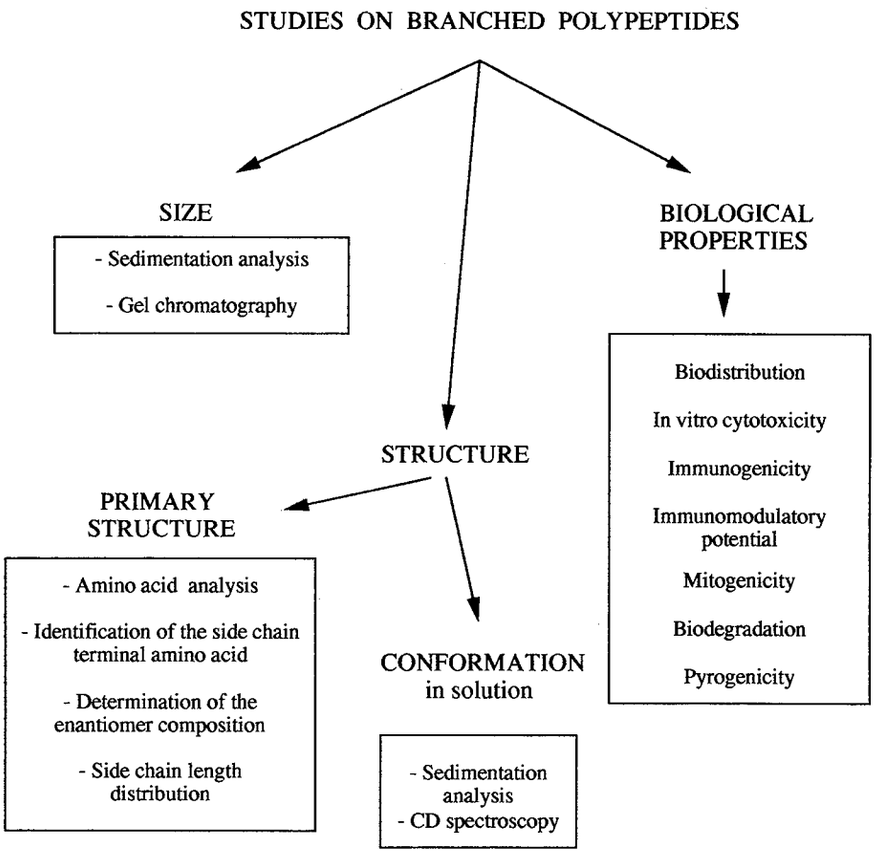

Figure 2. Structural and functional characterization of branched polypeptides.

Characterization of branched polypeptides

Size

The size of poly[L-Lys] and of branched polypeptide AK has been estimated in two ways: by sedimentation analysis,[40] and by gel permeation layer[9] and column[40,41] chromatography. The average relative molar masses ($\bar{M}_w$, $\bar{M}_z$) were determined by sedimentation equilibrium using a short column technique, and calculated by the van Holde and Baldwin method. The number average of the relative molar mass ($\bar{M}_n$), the polydispersity factor ($\bar{M}_z/\bar{M}_w$) and the average degree of polymerization ($\overline{DP}_n$) values were computed from data described above.[40]

In the case of XAK- and AXK-type polymers, the average relative molar masses were estimated from the average degree of polymerization ($\overline{DP}_n$) of the polylysine backbone and the equivalent relative molar mass of the side chains per one lysine residue obtained by quantitative amino acid analysis of the polypeptide.[9–14]

The second approach was based on gel permeation chromatography (GPC) using Sephadex G-150 superfine gel in thin layer experiments (TLG),[9] or Sephadex G-100, G-150, G-150 superfine[40] or Sephacryl S-300[41] gels in column chromatography. Comparing the average relative molar mass values obtained by TLG and those derived from sedimentation measurements, a good correlation was observed only when the value of the sedimentation constant was high, indicating that only more compact structures are suitable for reliable size estimation by TLG.[9]

In Sephadex GPC experiments, poly[L-Lys] and AK samples ($\bar{M}_w$ = 36–94 000) were completely excluded from the gel matrix under nearly physiological conditions.[40] Similarly ^{111}In-labeled poly[Lys–(Glu$_t$–DL-Ala$_m$)] [EAK] ($\bar{M}_w$ = 46 000) eluted from the Sephacryl column before labeled transferrin ($\bar{M}_w$ = 88 000).[41] This anomaly could be due to the conformation of these molecules, which is known from circular dichroism (CD) studies to be relatively unordered.[42] It has been found that the elution profile from GPC columns can be effectively used for a quick characterization of the relative molar mass distribution of branched polypeptides, but it is not suitable for the determination of average relative

molar mass with conventional protein standards.[40,41] Our data obtained through sedimentation analysis and GPC experiments indicate that both polylysine and the branched polypeptides produced in our laboratory possess a fairly narrow distribution of relative molar mass.

Primary structure

The amino acid composition and sequence of the side chains proved to be important structural features, which can influence *in vitro* cytotoxicity,[43] blood clearance[44] or immunoreactivity.[45,46] Consequently much emphasis was put on the characterization of the primary structure of branched polypeptides. The amino acid composition was determined by amino acid analysis and the presence of N-terminal amino acid X in the side chains (in XAK-type compounds) was verified by its identification using 1-dimethylamino-5-naphtalenesulfonyl chloride (Dns-Cl). Isocratic HPLC studies of the hydrolyzates of dansylated polypeptides indicated that (i) amino acid X is present and (ii) the Lys:Ala:X molar ratio is in good agreement with the respective values derived from amino acid analysis.[47] These studies also showed that the number of unsubstituted lysines in AK polypeptides was under 5%.

The enantiomer composition of the branches might be an additional option to manipulate various biochemical, immunological and pharmacological properties (e.g. tissue distribution or proteolytic processing). In order to elucidate the enantiomer composition of branched polypeptides, a new method was developed by the aid of a chiral derivatizing agent, *N*-(5-fluoro-2,4-dinitrophenyl)-L-alanine amide (Marfey's reagent).[48,49] After the reaction with this reagent the quantities of D- or/and L-amino acid residues, present in the hydrolyzates of branched polypeptides, were determined as diastereomers by reversed phase HPLC. So far no studies have been performed to provide data concerning the stereoisomer composition of branched-chain polypeptides. The results from this study show that (i) the polymerization initiated by the polylysine backbone is not stereo-specific or stereo-selective and (ii) no detectable racemization occurs during the coupling of amino acid X active ester to the poly[L-Lys][13,49] or the poly[Lys–(DL-Ala_m)] (AK).[50,51]

Due to the nature of the synthetic procedure used for the preparation of AK, the length of the oligo [Ala] side chains is not identical. In order to examine the potential influence of the random side chain length distribution on conformational or biological features, AK was sequenced by automated Edman degradation. Data were used to calculate the length and the frequency of each given length of oligo[DL-Ala] side chains grafted to the polylysine backbone. Similar studies have been carried out on immunogenic branched polypeptides with side chains of random amino acid sequence [(Phe, G)–A—L, (T, G)–A—L] and of defined amino acid sequence [GGT–A—L and TG–A—L].[52] Data concerning side chain length distribution and primary structure of the branches enabled the interpretation of results from mice immunization experiments.[53]

In our study we found that AK with an Ala:Lys = 3.5:1 mol/mol ratio ($\bar{M}_w$ = 75 000) has a defined range of side chain length, which predominates the distribution pattern (Figure 3). Of the branches, 65% consist of two to four Ala residues, a significant portion of the side chains is longer than four amino acid residues (~ 25%) and approximately 10% of the ε-amino groups of the Lys residues are substituted by only one Ala residue.[51] Based on these investigations, it has been concluded that the polylysine backbone of poly[Lys–(DL-$Ala_{3.5}$)] is almost completely covered by the oligo-[DL-Ala] side chains introduced by the polymerization of *N*-carboxy-DL-Ala anhydride.

These studies proved that the synthetic methods applied for the preparation of XAK- and AXK-type branched polypeptides produce compounds whose side chain length distribution is characteristic and the enantiomer compositions are in good agreement with calculated expectations. These findings could be useful for the reliable interpretation of various biological phenomena observed in connection with the presence of L- or D-amino acid residues in the side chains.

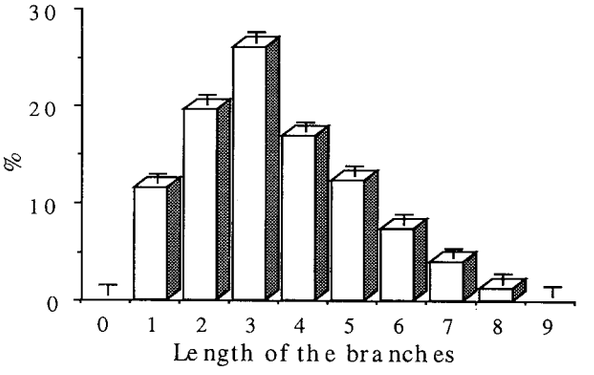

Figure 3. Length distribution of oligo[DL-Ala] chains in poly[Lys–(DL-$Ala_{3.5}$)] (AK) polypeptide.[51]

Conformation in solution

Due to the simplicity of the side chain structure of the XAK- and AXK-type branched polypeptides developed in our laboratories, these groups of synthetic polymers represents a versatile model system for the assessment of correlation between primary structure and solution conformation. Conformation of polypeptides and an octameric unit of AK was studied by CD spectroscopy in water solutions of various pH values (2–12) and ionic strength (0–2.0 M NaCl) and in water–alcohol or SDS mixtures.[11–14,40,42,54–56] CD spectra used for the detection of elements of secondary structure indicated marked dependence of the polypeptide conformation on the identity, hydrophobic/hydrophilic nature, charge configuration and sequential position of amino acid X in the side chain, and on the number of these residues present. (It should be noted that no ordered conformation of the short branches can be expected, therefore we mean mainly the conformation of the backbone by the term 'ordered conformation'.)

In the group of XAK-type polypeptides it has been found that substitution at the end of the DL-Ala$_m$ side chain of AK with hydrophobic amino acids (Leu, Phe, Ile, Nle) resulted in CD spectra demonstrating the presence of an α-helix under nearly physiological conditions (Figure 4A). In contrast hydrophilic (His, Glu, Lys) side chain termini did not affect the unordered conformation of AK[11,12,42,54,55] (Figure 4B).

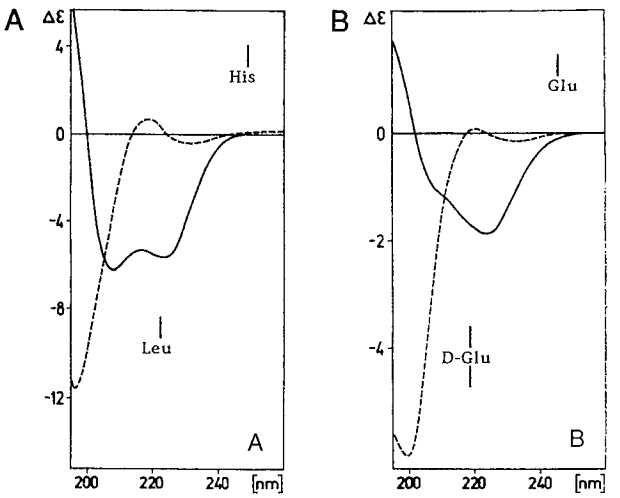

Figure 4. Influence of the side chain structure on the solution conformation of branched polypeptides. (A) CD spectra of polycationic poly[Lys–(His$_i$–DL-Ala$_m$)] (HAK) (- - - -) and poly[Lys–(Leu$_f$–DL-Ala$_m$)] (LAK) (———) at pH 7.3 in 0.2 M NaCl. (B) CD spectra of amphoteric poly[Lys–(Glu$_f$–DL-Ala$_m$)] (EAK) (- - - -) and poly[Lys–(DL-Ala$_m$–D-Glu$_i$)] (A-D-EK) (———) at pH 7.3 in 0.2 M NaCl.

The comparative conformational analysis of XAK- and AXK-type polypeptides suggests that the tendency to form an ordered structure is determined by the identity and the position of the chiral amino acid X in the sequence of the branches. Generally, the presence of a hydrophilic (Glu, His) at the N-terminal of the branches (in XAK polypeptides) or next to the poly[L-Lys] backbone (in AXK polypeptides) results in an unordered structure under nearly physiological conditions. Due to the presence of charged α-amino groups at the end of the side chains in polycationic, hydrophobic XAK or AXK polypeptides (X = Leu, Phe) no ordered structure formation could be expected. In contrast, it has been found that XAK polypeptides adopt a partially ordered conformation and the reversed sequence in AXK polypeptides, when a hydrophobic amino acid residue (Leu, Phe) is directly joined to the poly[L-Lys] backbone, significantly enables the formation of ordered conformation.[12–14]

It has been also demonstrated that XAK or AXK branched polypeptides containing a mixture of randomly distributed DL-Ala$_m$ and DL-Ala$_m$–X$_i$ or X$_f$–DL-Ala$_m$ ($m \sim 3$, $i < 1$, X = Leu, Phe) side chains at various pH and ionic strength adopt an almost identical conformation to those polypeptides that comprise the respective tetrapeptide branches (DL-Ala$_3$–X or X–DL-Ala$_3$) coupled to the ε-amino groups of polylysine.[14]

In a separate study of poly[Lys–(X$_i$)]-type polypeptides, the influence of the identity of amino acid X on the conformation was investigated. It has been demonstrated that the introduction of an amino acid residue with either an (ar)alkyl side chain (Ala, Leu, Phe) or a negatively charged side chain (Glu) markedly promotes the formation of ordered structure of the poly[Lys–(X$_i$)]-type polypeptides. No such effect could be observed when X = His.[13]

Taken together it can be concluded that the ordered structure of a branched polypeptide might be stabilized by hydrophobic interaction between apolar side chains (in the case of XAK or AXK polypeptides when X = Leu, Phe) or by ionic attraction (like between the positively-charged N-terminal amino groups and the negatively-charged carboxyl groups of the glutamic acid residues in poly[Lys–(X$_i$)]) between the side chains of amino acid X. However, the unordered conformation of XAK (X = Glu), in which these two groups are well-separated by space, suggests that groups involved in ionic interaction must be situated within

a defined distance. These results give rather conclusive evidence that the interaction between the side chains of amino acid X can dominate over the repulsive forces of the charges of the N-terminal amino groups.[11–14]

Based on the correlation between the primary structure (side chain composition, sequence, length) and solution conformation outlined here it is feasible to design branched polypeptides with a predicted secondary structure (Figure 4). By proper selection of the identity (hydrophobic versus hydrophilic), configuration (L or D) and sequential position of amino acid X in the branches one can synthesize polycationic or amphoteric polypeptides with ordered (α-helical) or unordered (random) solution conformation.

Labeling of branched polypeptides

In order to design for drug/enzyme/epitope targeting or delivery, it is important to determine the biodistribution of the unsubstituted carrier. If these carriers can be labeled with an appropriate radionuclide (i) their structural heterogeneity (i.e. size or charge) could be detected with high sensitivity and (ii) their biodistribution could be followed by scintigraphy. In this case imaging might give important information on the pharmacokinetics (blood clearance, tissue distribution) and eventually have a role in the delivery of radionuclide for clinical applications such as diagnosis and/or therapy.

For these studies methods were developed for labeling branched polypeptides with different radionuclides. Since these polypeptides lack tyrosine, tryptophan, phenylalanine or histidine into which iodine is incorporated by conventional oxidative labeling, an alternative method was developed. Considering the high number of branches with free amino groups at the side chain, the terminal positions these polypeptides were reacted with *N*-succinimidyl 3-(4-hydroxyphenyl)propionate (Bolton and Hunter reagent) prelabeled with ^{125}I.[57] The labeled polypeptides were purified by gel filtration chromatography (G-25 Sephadex column) and electrophoresis on native polyacryamide gel with a continuous 8–25% gradient was applied to assess the low molecular weight labeled product content of the preparation. The labeling efficacy was 35–40%.[44]

An indirect labeling procedure was introduced in order to incorporate radiometals such as ^{111}In[22,41,58] or ^{51}Cr[23] into branched polypeptides. The amino groups on the terminal amino acid residues of branched polypeptides were reacted first with a chelating agent, diethylenetriamine pentaacetic acid anhydride (DTPAA), under lightly alkaline conditions.[59] The conjugate was purified by gel filtration and the ratio of modified side chains was determined by DTNB or fluorecamine assay. The derivatized branched polypeptides were then labeled by chelation with ^{111}In[22] or ^{51}Cr[23] using the strong interaction between the negatively-charged carboxyl groups of DTPA and the positively-charged metal ions. Free radiometal ions were removed by desalting and the radiochemical purity was assessed by silica gel thin-layer chromatography. Labeling efficacy was 70–80%.

Using the same procedures we have also shown that it is possible to label branched polypeptides conjugated to anti-cancer drugs (such as methotrexate and daunomycin) with similar efficacy.[18,19,60,61]

Our results suggest that gamma-emitters can be incorporated into polymeric branched polypeptides as well as their antitumor drug conjugates with high specific activity, offering potential for the use of scintigraphy in chemical and/or clinical pharmacokinetic studies.

Biological properties of branched polypeptides

Biodistribution

In respect of the possibility of *in vivo* application of synthetic branched polypeptides (and their conjugates), studies were designed to elucidate correlations between the structural features of these compounds and their biodistribution profile in normal or tumor bearing mice. We examined the blood clearance, whole body survival (WBS) and tissue distribution of branched polypeptides. In view of the importance of the side chain structure in α-helix formation[11,13,42] or in cytotoxicity[43] and in immunological properties,[45,46] we measured the biodistribution of polypeptides containing (i) amino acid residues of different identity (e.g. X = Leu, Pro or Glu), (ii) amino acid residues of different absolute configuration (X = L- or D-Glu, L- or D-Leu), or (iii) amino acid residue X at the side chain end (XAK polypeptides) or at the position next to the polylysine backbone (AXK polypeptides). These comparative studies were used to identify factors which could influence the biodistribution of the branched polypeptides.

Biodistribution in normal size. In order to dissect the effect of size from that of other molecular characteristics, two groups of such polypeptides with basically identical side-chain composition but with different size were studied.[44]

Blood clearance. The blood clearance profiles from both the large (157–213 kDA) and small (34–46 kDa) relative molecular mass series, up to 6 h after i.v. injection with ^{125}I-labeled polypeptides, are shown in Figure 5(A). There was no significant difference between the blood survival of the large and small relative molecular mass versions of

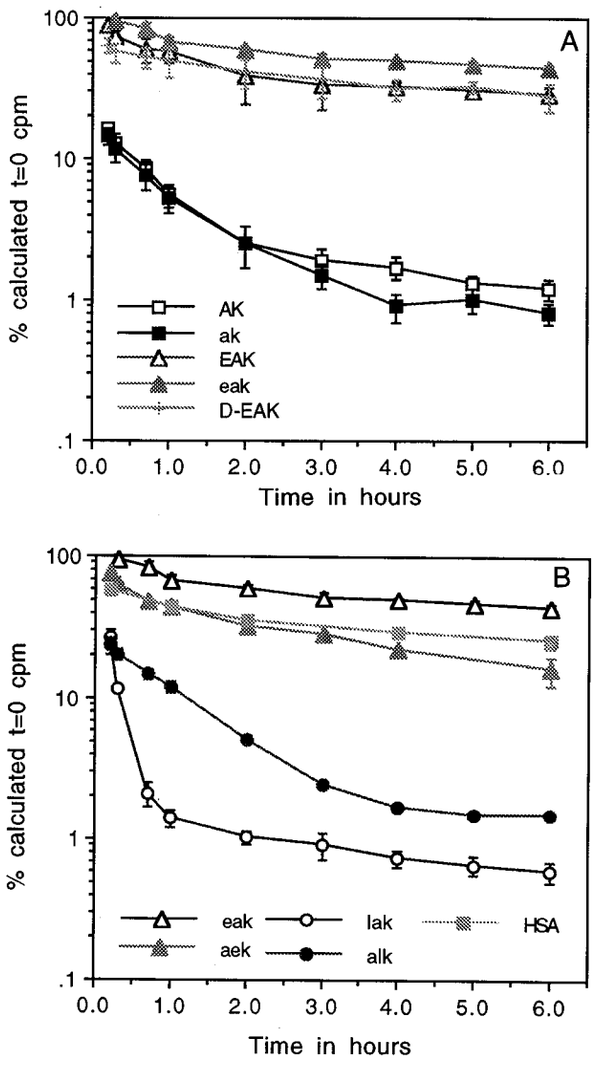

Figure 5. Blood clearance profile of ^{125}I-labeled branched polypeptides and HSA following i.v. administration to BALB/c mice.[44] (A) Influence of the polypeptide size and of the identity (Ala versus Glu) and configuration (L versus D) of the side chain terminal. (B) Influence of amino acid sequence of the side chain and comparison with HSA.

polylysine with only DL-Ala side chains (AK/ak) or their leucine substituted derivatives (LAK/lak). However, for the amphoteric glutamic acid substituted polypeptide pair (EAK/eak) the blood survival of eak was longer than that for EAK. This was reflected in a significantly higher ($p < 0.01$) AUC_{0-6h} for eak compared with that for EAK.

The incorporation of the D-amino acid into the polypeptide (EAK versus D-EAK) had no significant effect on blood survival (Figure 5A). Similar results were obtained with the polycationic LAK/D-LAK pair. In contrast, changes in the side chain terminal amino acid in the branched polypeptides, resulting in a change in the overall charge of the compounds, had a dramatic effect on blood survival (Figure 5A and B). The AUC_{0-6h} for the relatively hydrophobic polycation lak was significantly ($p < 0.05$) lower than for ak. Moreover, a 10-fold increase in AUC_{0-6h} was observed when glutamic acid was substituted in the terminal position in the Ala side chain (eak). This conferred an almost neutral charge to the polypeptide. At 24 h after i.v. injection there was over 70 times more eak remaining in the circulation compared with ak.

The effect of altering the position of amino acid X on the blood survival of the polypeptides was also examined. For the Glu containing amphoteric pair of polypeptides eak/aek there was a 2-fold reduction in AUC_{0-6h} when Glu is linked directly onto the polylysine backbone compared with substitution in the terminal side chain position (eak) (Figure 5B). In contrast, for the polycationic polypeptide pair lak/alk a 2-fold increase in AUC_{0-6h} was observed (alk versus lak). Twenty-four hours following injection there was a 4-fold difference in the amount of polypeptide surviving in the circulation.

These data show that the blood survival for branched polypeptides was primarily dependent on charge and side chain amino acid sequence. The glutamic acid containing amphoteric polypeptides showed the longest blood survival.[44]

Similar blood kinetic studies were performed with ^{111}In-labeled EAK,[22] which showed suitable biodistribution properties not only unconjugated,[41,44] but also conjugated to anti-cancer drugs such as methotrexate[19] and daunomycin.[18,61] The clearance of ^{125}I- or ^{111}In-labeled EAK in mice was essentially biphasic. There was an initial, rapid loss of some material from the blood, followed by a slower clearance of the majority of the polypeptide preparation. In order to determine the precise short-term blood pharmacokinetics without the sacrifice and dissection of groups of animals at numerous time points and to define the site of clearance from the blood,

imaging studies were performed.[41] Scintigraphic evaluation of the gamma emitter (^{111}In)-labeled EAK indicated that the initial decline in blood levels was due to rapid renal clearance, and subsequent urinary excretion, of about 30% of the injected polymers. The elution profile of gel filtration of the [^{111}In]polymer on Sephacryl S-300 clearly indicated that this preparation has a relatively wide relative molar mass distribution. Images of mice acquired 20 min after injection of the S-300 fractions showed that the early eluting material with high molar mass polypeptide was retained in the blood pool, the intermediate resulted in some urinary clearance and the late containing lower molar mass polymer was rapidly excreted. These findings emphasize the value of gamma-scintigraphy for biodistribution studies of polymeric branched polypeptides by showing the rapid excretion of subcomponents of the preparation which would not be readily detectable by simple blood pharmacokinetic analysis or even by dissection analysis of experimental animals.

Tissue distribution. The structural features of the branched polypeptides affecting tissue distribution were found to be similar to those altering blood clearance.[44] The data expressed as a tissue to blood ratio show no significant difference between the large and small relative molecular mass compounds of each individual polypeptide, but changes in the amino acid X had a dramatic effect on tissue distribution. The tissue distribution of eak, 24 h after i.v. administration, was very different from that of the other branched polypeptides studied. Polycationic polypeptides (Ak/ak, LAK/lak and PAK/pak) were taken out of circulation, primarily into spleen, kidney and liver. Much higher tissue to blood ratios were observed for LAK/lak and PAK/pak than for Ak/ak. However, the glutamic acid substituted polypeptides (EAK/eak) were not preferentially taken up by any of the visceral organs.

In contrast to observations in blood clearance studies, incorporation of the D-amino acid into the terminal side chain position resulted in an increase in the amount of polypeptide retained in the liver and spleen. This was seen for both amphoteric (EAK/D-EAK) and polycationic (LAK/D-LAK) peptides. This probably reflects the inability of the body to catabolize D-amino acids.

The effect of altering the position of amino acid X in the branches on the tissue distribution of the branched polypeptides was in keeping with blood survival data. For the amphoteric glutamic acid containing pair eak/aek the tissue distribution profile was very similar although the whole body survival for eak was significantly higher than for aek (Figure 6B). For the polycationic leucine containing polypeptides the percentage injected dose/g of alk in liver and spleen was much higher than for lak. It should be noted that although the blood clearance profiles of lak and alk were very similar (Figures 5), these two polypeptides showed dramatically different tissue distribution profiles at 24 h. The amount of alk in spleen and liver was between 4- and 6-fold higher than for lak.

Whole body survival. The WBS of the polypeptides 24 h after i.v. injection is summarize in Figure 6. There was no significant difference in the WBS of the large and small relative molecular mass versions of polycations (AK/ak and LAK/lak). However, a significant ($p < 0.01$) 2-fold increase in WBS was

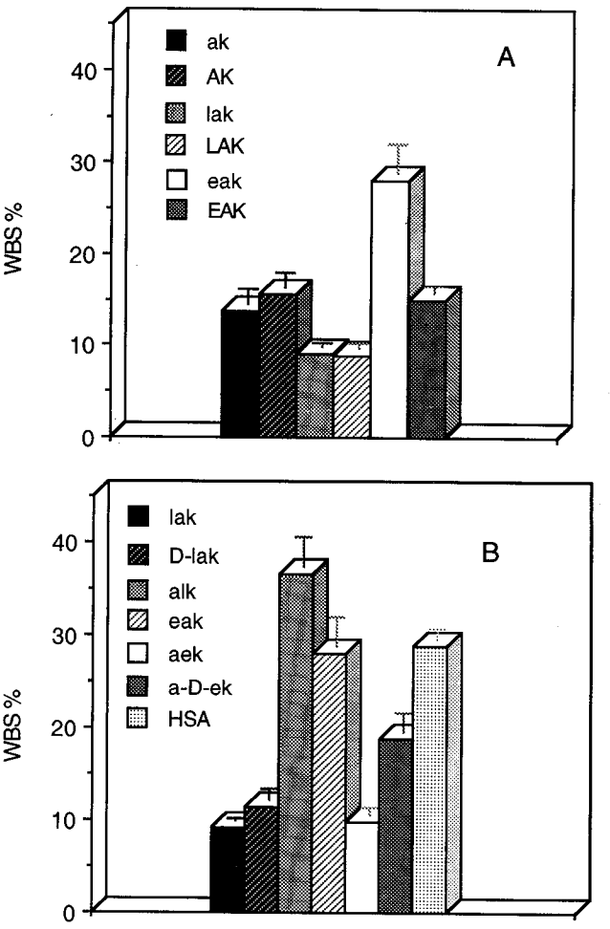

Figure 6. Whole body survival of branched polypeptides 24 h after injection of BALB/c mice. (A) Influence of the polypeptide size and of the identity (Ala, Leu versus Glu) of the side chain terminal. (B) Influence of sequence and configuration (L versus D) of amino acid in the side chain and comparison with HSA.

observed with the small relative molecular mass polypeptides containing glutamic acid (eak/EAK) (Figure 6A).

The effect of alterations in charge, configuration of terminal amino acid and side chain sequence on the tissue distribution of the branched polypeptides were reflected in the WBS observed for these compounds. Indeed, as would be expected from the tissue distribution data the amphoteric polypeptides (EAK/eak) exhibited the highest WBS. Incorporation of the D-amino acid at the side chain terminal position resulted in a 30–50% increase in WBS for both polycationic and amphoteric polymers. The dramatic alteration in the blood clearance and tissue distribution profile observed with lak/alk was also reflected in a much higher WBS for alk compared with lak (Figure 6B).

In summary, these studies have indicated that it is primarily changes in the charge of the branched polypeptides, resulting from alterations in the identity of amino acid X in the branches, which contributed to the blood clearance and tissue distribution profiles *in vivo*. The amino acid sequence of the side chains of the branched polypeptides was also an important factor for both the polycationic and the amphoteric peptides studied. There was no correlation between conformation and biodistribution of the polypeptides, ordered (LAK/lak) and unordered polycations (PAK/pak) both being cleared rapidly.

Biodistribution in tumor bearing mice. The pharmacokinetic parameters of branched polypeptides with different charge properties were studied in tumor bearing mice. Besides AK, LAK and EAK, their succinylated or acetylated derivatives (Suc-AK, Ac-AK, Ac-EAK, Suc-EAK and Suc-LAK) were synthesized and included in this study.[23] Ddy mice were injected subcutaneously with 10^6 Sarcoma 180 cells maintained in ascites form in mice. Groups of animals with palpable tumors received a single injection of [^{51}Cr]DTPA-labeled polypeptide. Serial blood samples were taken between 10 min and 48 h after injection, and their radioactivity was determined. Mice were killed and dissected at set times, and the tissues were assayed for radioactivity.

Blood clearance. The results showed that the polycationic branched polypeptides (AK or LAK) exhibit rapid blood clearance not only in normal,[44] but also in tumor bearing mice. Similarly the blood half-life of amphoteric polypeptides (EAK) was significantly longer in both groups than that for polycationic compounds. Acetylation of AK had no pronounced effect, but the succinylation of AK or LAK resulted in a markedly prolonged blood half-life in the circulation. Blocking the amino group of EAK by the introduction of an acetyl group, producing polyanionic Ac-EAK, had no significant effect on the blood clearance profile. The succinylated polypeptide (Suc-EAK) with high negative charge density was cleared from the circulation rapidly.

Accumulation in tumor tissue. The tissue distribution 24 h after i.v. administration was also studied. No significant difference was observed between the two polycations (AK and LAK), which were taken up predominantly by the liver and spleen, and only 1% of the injected dose/g was detected in the tumor. However, EAK was not preferentially taken up by any of the visceral organs. Interestingly enough the presence of 3.1% of the injected dose/g could be demonstrated in the tumor tissue. It was found that acetylation of EAK could elevate the amount of labeled polypeptide in the tumor (3.4%/g). In sharp contrast, succinylation resulted in a dramatic decrease of tumor uptake (1.2%/g).

These preliminary results indicate that branched polypeptides with a high density of positive or negative charges were cleared rapidly from the circulation and their accumulation in tumor tissue was not favored.

Conclusion. In conclusion, branched polypeptides with a poly(L-Lys) backbone provide a relatively simple system with which to identify factors (molecular size, ionic charge, primary structure) influencing the biodistribution of carrier macromolecules and would allow suitable carriers to be selected according to their intended use. The amphoteric Glu containing polypeptides are good candidates for conjunction to cytotoxic drugs with potential use for site-specific drug delivery, either simply as drug–polypeptide conjugates or linked to monoclonal antibodies. This application is currently under investigation. The polycationic peptides, exhibiting rapid blood clearance and high spleen uptake, have potential uses as carriers for haptens in antibody production and in the construction of synthetic vaccines.

Cytotoxicity

In vitro *cytotoxicity.* Cytotoxicity of branched polymers and polylysines (poly-α-lysine {poly[Lys]} and poly-ε-lysine {poly[Lys(Lys$_n$)]} was analyzed in three *in vitro* systems using rat liver, mouse spleen and HeLa cells. Viability of isolated cells as well as the growth of HeLa cells in the presence

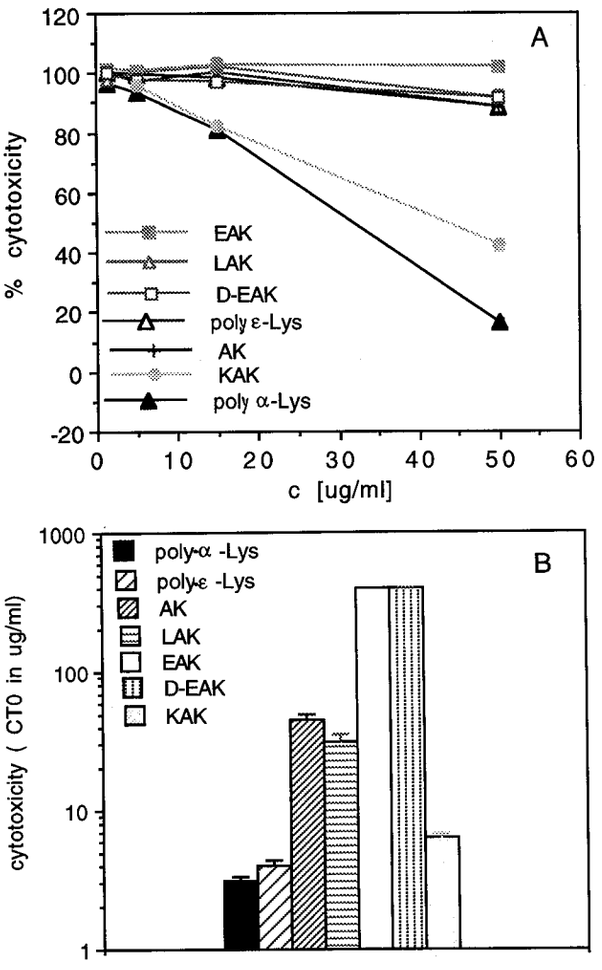

Figure 7. Effect of amphoteric and polycationic branched polypeptides on the viability of isolated rat liver cells (A) and on HeLa cell growth (B), and comparison with poly-α-Lys and poly-ε-Lys.[43]

of the polypeptides at various concentration was determined.

Cytotoxicity against isolated rat liver and mouse spleen cells in vitro. Polypeptides at various concentrations (1.5–50.00 μg/ml) were incubated with rat liver cells or with mouse spleen cells, and the percentage of cell viability as compared with controls (taken as 100%) was determined. Results of these experiments are demonstrated in Figure 7(A). As described previously, poly[Lys] was shown to retard the growth of tumor cells[25,62] and to be rather toxic to human lymphocytes,[63] to rat liver cells[64] and even more toxic to mouse spleen cells.[64] Poly[D-Lys] seemed to be even more toxic.[64] In contrast, poly[-Lys(Lys$_n$)], containing α-amide bonds, decreased only slightly the viability of liver cells (Figure 7A) and spleen cells (15%).

Modification of poly[Lys] by substitution of short oligo[DL-Ala] side chains significantly reduced its cytotoxicity in both experimental systems. A 50% decrease was observed in the case of liver cells and 10% with spleen cells.[64] Further elongations of these branches by the same amino acid residues (up to 8.5 DL-Ala residues on average per side chains) resulted in a further increase in cell viability. This AK polypeptide was found to be marginally toxic (for liver cells) or non-toxic (for spleen cells) (Figure 7A).

Alteration of the ε-amino groups of poly[Lys] by the introduction of oligopeptidic side chains composed of DL-Ala and amino acid residue X (XAK and AXK polypeptides) could also reduce cytotoxicity. Comparison of viability values at a concentration of 50 μg/ml showed that this reduction was about 2.5- to 6.0-fold in the case of liver cells and about 19.0- to 33.0-fold with spleen cells. The extent of the reduction in both test systems differed according to the structure of the branches.

The identity of the terminal amino acid in the side chains of XAK polypeptides had a marked influence on cytotoxicity. The presence of Phe, Pro, Leu and His at this position resulted in 0–10% loss of viable liver or spleen cells, but only at the highest concentration (Figure 7A for LAK). No toxic effect of these polypeptides was found on mouse spleen cells at high polypeptide concentration, neither at 1 nor 24 h incubation periods.[64] In the case of polypeptides containing L- or D-Lys at the end of the side chains (KAK and D-KAK), a more pronounced cytotoxic effect was observed in both assays. In sharp contrast, no toxic effect could be demonstrated with polypeptides containing one or three (on average) L- or D-Glu residues attached to AK (Figure 7A).

The importance of the sequence of the amino acids in the side chain was also investigated, using Leu or Glu containing alanylated polylysines. The cytotoxicity of the polycationic ALK was not considerably different from that of LAK. For the amphoteric pair of polypeptides there was only a small decrease in viability, when glutamic acid is linked directly onto the poly[Lys] backbone (AEK) compared with substitution in the terminal side chain position (EAK).[43]

No significant changes were observed in liver cell cytotoxicity of polypeptides with preformed tetrapeptide side chains as compared with the corresponding polymers containing a mixture of oligo[DL-Ala] and DL-Ala$_m$–Leu (ALK) or Leu–DL-Ala$_m$ (LAK) branches.[43]

Effect on growth of HeLa cells. Estimation of cytotoxicity was extended to the evaluation of growth of HeLa cells in the presence of various amounts of polymers. Concentrations causing 50% (CT_{50}) and 0% (CT_0) (Figure 7B) death of cells were determined and used for classification of test compounds as follows: 'high' $c < 10$ μg/ml, 'medium' $10 < c < 100$ μg/ml and 'low' $100 < c$ μg/ml toxic activity.[43,65]

In correlation with published data, poly[Lys] was found to be highly toxic to HeLa cells.[25,62] It should be noted that poly[Lys(Lys$_n$)] displayed a similar character (Figure 7B).

Cytotoxic activity of branched polypeptides could be categorized according to side chain structure. The majority of polycationic polymers studied were toxic at an upper (PAK, D-HAK and LAK) or a lower (AK, HAK, ALK, D-LAK, FAK, D-FAK) medium level. The treatment of HeLa cells with polypeptide containing L- or D-Lys at the end of the side chains (KAK versus D-KAK) resulted in a similar increase of cell death as compared with polylysines (Figure 7B). As in the case of the above described *in vitro* systems, no cytotoxic effect of amphoteric polypeptides (EAK versus D-EAK) was observed (Figure 7B).

Conclusion. Our data clearly suggest that there is a strong correlation between charge and cell killing activity of branched polypeptides. Amphoteric polypeptides with balanced charge distribution (α-amino and γ-carboxyl groups in amphoteric molecules) have no (EAK, D-EAK) or have marginal (AEK, $E_{2.8}AK$, D-E_3AK) growth inhibitory or cytotoxicity activity. Among polycations, two groups could be identified. Macromolecules with free α-amino groups with relatively low $pK = 8.95–9.7$ at the end of the branches—almost regardless of their sequence, composition and configuration—exhibit moderate cytotoxicity. In the second subset, polycationic polymers with both α- and ε-amino groups of higher $pK = 10.53$ at the branch terminal position are more toxic to spleen or liver cells and inhibit HeLa cell growth similarly to poly-α- or poly-ε-lysines. These findings were related to a possible perturbation of cell surfaces by the charged ε-amino groups of this poly-α-amino acid, resulting in an elevated release of ions.[62,63]

It should also be noted that the HeLa cells were much more sensitive to polymers with mono- or bivalent cationic side chains than those of liver or spleen cells. These results can be interpreted in that the appropriate substitution at the side chain terminal provides a feasible tool for the reduction of the cytotoxicity of these compounds.

In vivo *toxicity.* In vivo toxicity of AK, D-LAK and HAK was evaluated by observing mice for 30 days after i.p. injections at doses of up to 25 mg/kg body weight. All mice were alive at the end of this time period, and no body weight loss or any noticeable toxic effect was detected.[64]

Immunogenicity

Several XAK-type synthetic branched polypeptides have been evaluated to study of the relationships between the chemical structure (charge, primary structure, conformation) and immunological properties. The size and inside area of branched polypeptides selected for this study were identical; however, the identity, the configuration of the chain-terminating amino acids (X) (X = Leu, D-Leu, Phe, D-Phe, Glu or D-Glu) and, in consequence, the charge and the solution conformation of the macromolecules were different.[27,43,45,46]

The immune response induced by polypeptides was studied in four inbred mouse strains of different H-2 haplotype and Igh-1 allotype. The qualitative and quantitative features of the antibody response were characterized by IgM- and IgG-type antibody levels, isotype distribution, and fine specificity of antibodies produced during the primary and memory response in BALB/c, CBA, ABY and C57Bl/6 mice. To test the specificity of T cell recognition, the intensity of the delayed-type hypersensitivity (DTH) reaction induced by these polypeptides was measured.[43,45] The suitability of the branched polypeptides for immunological carrier functions has been analyzed with the aid of polypeptide–hapten conjugates, in which an oxazolone derivative[27,66] or synthetic peptide epitopes from HSV (type I) glycoprotein D[33–36] or from mucin[29–31] were applied as antigenic determinants.

Antibody response. The antibody response to polycationic branched polypeptides (AK, LAK and D-LAK) administered in saline indicated the appearance of IgM- and IgG-type antibodies on day 6 only in the case of D-LAK.[67] The appearance of D-LAK induced IgM- and IgG-type serum antibodies showed typical time kinetics of a T cell-dependent B cell response represented by the production of all IgG subclasses in the course of the memory response. Combined use of Freund's adjuvant and the synthetic polypeptides affected isotype distribution of IgG-type antibodies but did not significantly augment production of D-LAK specific antibodies.

When synthetic polypeptides LAK, D-LAK, FAK and D-FAK emulsified in Freund's complete adjuvant were injected to four inbred mouse strains the high immunogenicity of D-LAK could be observed even following a single as well as repeated injections in all strains.[45] Comparing the relative level of serum antibodies in C57B1/6, BALB/c and CBA mice the following order of immunogenicity of branched polypeptides could be demonstrated: D-LAK $\gg$ FAK > LAK > D-FAK. In ABY mice the order was slightly different: D-LAK $\gg$ FAK = D-FAK > LAK.

In a separate experiment using an identical immunization protocol the antibody response of two amphoteric polypeptides containing L- or D-glutamic acid at the side chain terminal (i.e. EAK and D-EAK) was analyzed.[43] As a reference the most immunogenic D-LAK polypeptide was used and the magnitude of the IgG-type antibody response was measured at different points of time, i.e. 7 days after the primary and 7 days as well as 14 days after the secondary antigen challenge. As is summarized in Figure 8, D-LAK induced an intense antibody response following secondary antigen challenge in the three mouse strains studied. In contrast to the most immunogenic D-LAK, polypeptides with glutamic acid residue (EAK and D-EAK) induced IgG-type immune responses in BALB/c mice only. A relatively high level of D-EAK specific IgG-type antibodies could be detected in the sera of these mice while immunization with EAK or LAK resulted in the appearance of a low level of polypeptide specific antibodies.

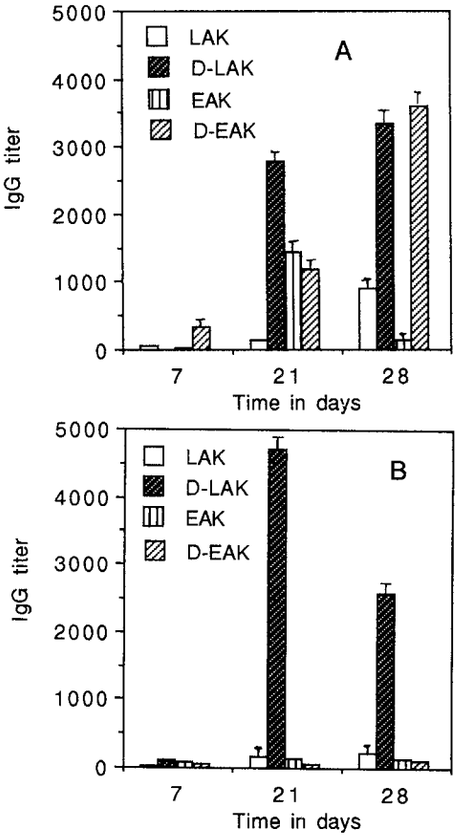

Figure 8. Immunogenicity of branched polypeptides. The time dependence of IgG-type antibody response measured in the serum of immunized BALB/c (A) and CBA (B) mice.

Analysis of the fine specificity of the polyclonal IgM and IgG response to the most immunogenic D-LAK proved that the fine specificity of D-LAK specific antibodies in BALB/c and CBA mice is different.[45] In BALB/c mice the majority of antibodies are directed against the D-LA_3 tetrapeptide while in CBA, D-LAK specific antibodies show strong cross-reactivity with LAK, FAK and D-FAK suggesting the recognition of common, AK backbone related determinants.

The cross-reactivity pattern of EAK and D-EAK or D-LAK specific antibodies from BALB/c mice show that in contrast to the monospecificity of D-LAK specific antibodies, D-EAK specific antibodies were reactive not only with D-EAK, but also with EAK and D-LAK. This considerable degree of cross-reactivity indicates the dominance of AK backbone related common epitopes and shows that Glu or D-Glu are not able to create a discrete B cell epitope as it has been proven for D-LAK.[43]

Despite the strong similarity in their chemical structure, marked differences in immunogenicity and antigenicity of the synthetic polypeptides were observed. Our results revealed that the magnitude of the IgG-type memory antibody response to different synthetic polypeptides, built up on a common AK backbone, is highly dependent on the nature, identity and absolute configuration of the side chain terminating amino acid (X). These data strongly suggest that branched polypeptides carry at least two distinct antigenic determinants. One is related to the tetrapeptide side chain and its specificity is determined by the terminal amino acid residue X. In the case of immunogenic polypeptides the expression/accessibility of this epitope is dependent on the conformation (mainly on the backbone-originated helix content) of the overall molecule.[45]

Consequently the less folded structure (e.g. D-LAK) proved to be positively affecting the antigenic potential of such an antigenic determinant. The other antigenic determinant, which is less conformation dependent, could be localized to the common inside area (AK) of the polypeptides.

The difference in the potency of EAK compared with D-EAK or of LAK to D-LAK to elicit IgG-type antibodies suggests that the introduction of chain terminating D-amino acids can significantly enhance immunogenicity of the tetrapeptide related B cell epitope.

T cell response. To test the specificity of T cell recognition the intensity of the DTH reaction induced by these polypeptides was measured. AK as well as the four polycationic analogs (LAK, D-LAK, FAK and D-FAK) selected for comparative studies elicited a strong DTH reaction in BALB/c, C57B1/6 and CBA mice. The potency of AK to induce a T cell response and its inability to exert a B cell response strongly suggest that T cell recognizable epitope(s) are related to the common inside core of these molecules.[27,66] This assumption was supported by our experiments with EAK and D-EAK which were able to provoke an intense *in vitro* T cell response (Rajnavölgyi, unpublished observations), but did not show extreme potency to induce an IgG-type antibody response. One may consider that Glu and D-Glu amino acids as chain terminating substituents may alter the distribution, possible accumulation or clearance of these amphoteric molecules in the organism,[44] and/or influence biodegradation in a way which favors antigen presentation and T cell recognition.

Conclusion. These results suggest that the chain terminating amino acid (X) influences the quantitative and qualitative features of the antibody response to polycationic or amphoteric representatives of the group of synthetic branched polypeptides. Further studies are in progress to describe the AK backbone-related T cell epitope structure. Immunological properties summarized here established the usefulness of branched polypeptides as carrier molecules and/or potential immunomodulators in cases where (i) the low level of carrier specific antibody response versus that directed against the newly introduced epitopes is required (e.g. synthetic peptide vaccines against cancer) or (ii) compensation of immunosuppression caused by anticancer drugs or irradiation is needed (e.g. drug targeting).

Results presented here proved that our polypeptide model system enables the analysis and potentially reliable interpretation of the correlation between the chemical structure and the immunogenic/antigenic properties.

Immunomodulation

The marginal cytotoxic effect of some of the synthetic polypeptides at high concentrations as well as their polycationic nature suggested the control of their immunomodulatory effect in a T cell dependent B cell response. AK, HAK, LAK and D-LAK, possessing different chemical and biological properties, were involved in these studies and their effect on the primary sheep red blood cell (SRBC) specific immune response induced in BDF_1 mice was analyzed by the direct plaque forming cell (PFC) assay. The immunomodulatory activity of these polypeptides was compared with that of a well characterized agent in clinical use, levamisol (LEV), under identical conditions. The dose-dependence of the enhancement by these polypeptides of the host immune response to SRBC was investigated in the dose range 0.02–20 mg/kg. HAK, LAK and D-LAK but not AK exerted a dose-dependent stimulatory effect at a wide dose range (0.02–10 mg/kg) administered in aqueous solution by a single or repeated i.p. injection in saline together with SRBC priming.[46] The maximum increase (3-fold) in PFC levels was observed at 0.02 mg/kg for D-LAK, at 0.2 mg/kg for HAK and at 1.0 mg/kg for LAK. The extent of PFC formation by LEV was similar, although it was effective over a narrower dose range.[46] It should be noted that EAK had no significant effect on the anti-SRBC response as assessed by the PFC assay (D. Gaál, unpublished results).

The antibody response to SRBC was also dependent on the treatment schedule. Using the optimal doses of immunostimulatory polypeptides (HAK, LAK and D-LAK), several peaks of PFC response were observed. All three compounds increased the PFC number (3.5-fold) when they were administered simultaneously with SRBC. Polypeptide treatment on day 4 or 6 prior to SRBC immunization had an enhancing effect, but no change in PFC formation was detected when polypeptides were given after immunisation. No time-dependent effect of AK was observed during the time interval tested.[46]

The stimulatory effect could also be observed when the anti-SRBC response of mice was suppressed by various antitumor drugs [vincristine (VCR), 5-fluorouracil (5-FU) or 1,2–5,6-dianhydrogalactitol (DAG)].[64,68] Drugs used at doses corresponding to 25% of LD_{50} were administered together with immunomodulators (HAK, LAK, D-LAK or LEV) three times on days 7, 4 and 1 before or on days 1, 2 or 3 after SRBC immunization. Repeated injection of free drugs resulted in significant immunosuppression (30–60% before or 80–90% after SRBC injection).

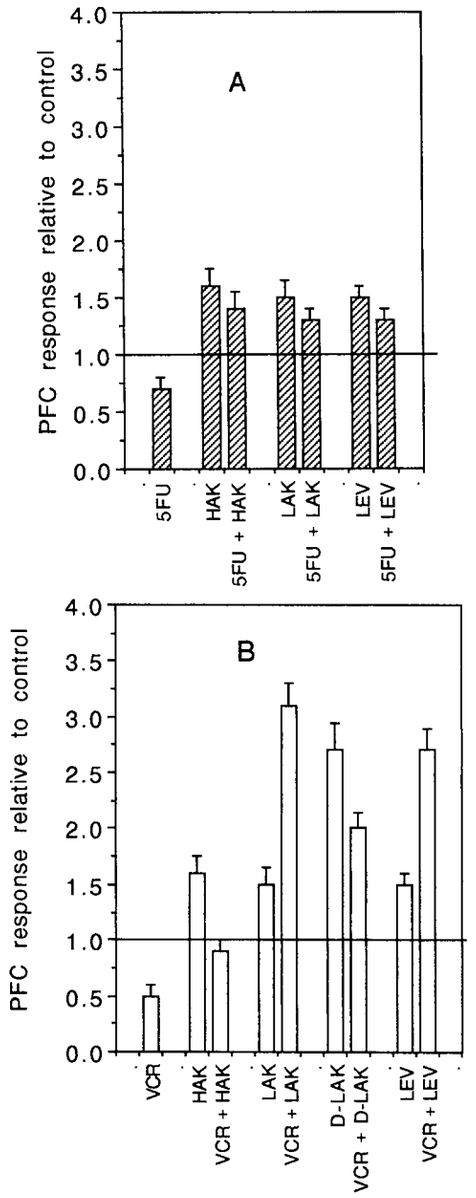

Figure 9. Compensation of the immunosuppressive effect of antitumour agents by branched polypeptides or levamisole. Immune response to SRBC was evaluated by PFC assay. BDF_1 mice were administered three times each on days 7, 4 and 1 before SRBC immunization by VCR (A) or 5-FU (B) and their combinations with branched polypeptides or levamisole.

The combined treatment of cytotoxic drug and polypeptide or LEV prior to immunization not only compensated for the immunosuppressive effect of drugs, but also resulted in a significantly increased antibody response in most cases (Figure 9). The result in Figure 9 suggest that the degree of this compensatory effect depends equally on the choice of the drug (VCR or 5-FU) and of the polypeptide. In post-immunization treatment polypeptides were able to stimulate anti-SRBC response up to a normal (control) level only when a reduced amount of drug (10% of LD_{50}) was applied.[64] Protection against the immunosuppressive effect of irradiation was also observed in BDF_1 mice that received whole body irradiation (2.5 Gy).[68] The combined application of polypeptides (AK, HAK, LAK, D-LAK and EAK) and various antitumor drugs (VCR, 5-FU, DAG, daunomycin and cisplatin) was tested in BDF_1 mice inoculated with P388 ascitic lymphoid leukemia. The increase of life span observed was found to be dependent on the combination and on its time schedule.[69] These studies provide evidence for the first time that chemically well-characterized synthetic branched polypeptides are capable of augmenting the antibody response to SRBC sufficiently to compensate for the immunosuppressive effect of various antitumor drugs and irradiation in normal and tumor bearing mice. Although no data are yet available for the mechanisms by which these polymers can influence the antibody response to SRBC antigen, the fact that they do not suppress but rather enhance a T cell mediated antibody response is promising for their use as carriers for anticancer drugs.

Biodegradation

Proteolytic processing of carrier molecules is an important event in drug delivery. Therefore detailed investigation was carried out to elucidate the biodegradation of poly[Lys–(DL-$Ala_{3.5}$)] (AK) polypeptide[70] ($\bar{M}_w$ = 40 000). The enantiomer composition of the branches indicated that the molar ratio between L-Ala and D-Ala was 1:1.[49]

Enzymatic hydrolysis was performed by an exopeptidase (aminopeptidase M, EC 3.4.11.2) with a capability of splitting both N-terminal α- and ε-peptide bonds and by an endopeptidase (trypsin, EC 3.4.21.4) which could act on endoamide bonds or by a 1:1 (w/w) mixture of these enzymes. Metabolites were separated and identified by the synthesis of oligopeptides representing the potential fragments [DL-Ala_{25}, DL-Ala_3, Lys(DL-Ala_m), m = 1–3] of AK. The kinetics and the degree of enzymatic degradation were determined during the 96 h incubation with enzymes.

The kinetics of proteolytic digestions indicate that (i) AK is degradable by these enzymes and (ii) the enzyme mixture hydrolyzes faster, resulting in approximately 30% degradation of the polymer within

72 h, whereas trypsin alone could cause only marginal (5.3%) fragmentation within 96 h. Poly[Lys] and poly[Lys(Lys$_n$)] containing α- and ε-amide bonds, respectively, were more rapidly cleaved than the branched polypeptide AK. Both polylysines were completely hydrolyzed within 24 h even when treated only with aminopeptidase M.

Degradation of breakdown products of AK [DL-Ala$_2$, DL-Ala$_3$ and Lys(DL-Ala$_m$), m = 1–3] indicated that (i) the α-peptide bonds of DL-Ala$_m$ peptides were split off with similar efficiencies by aminopeptidase M (36% degradation in 72 h) and (ii) Lys(DL-Ala$_m$) (m = 1–3) peptides containing α- and ε-peptide bonds were cleaved more rapidly than DL-Ala$_2$ or DL-Ala$_3$. The cleavage of polylysines and of the breakdown oligopeptides with aminopeptidase M indicates that this enzyme could split both α- and ε-peptide bonds. The ε-amide bond in Lys(DL-Ala) was cleaved more slowly than in poly[Lys(Lys$_n$)]. This is very likely due to the replacement of Lys by Ala and/or due to the presence of D-Ala in some dipeptides. (The introduction of D-amino acids to the peptide chain increases resistance to proteolytic degradation.[71]) These results clearly demonstrate that the hydrolysis proceeds stepwise at multiple sites on the AK polypeptide as indicated by the appearance of side chain fragments. The kinetics of the process could be controlled by the presence of ε-peptide bonds and of D-Ala in the branches. The degradation is significantly retarded compared with α- or ε-polylysines or to proteins with similar size. Information gained from these studies can be useful for more rational design of macromolecular carriers. It is feasible (i) to synthesize new AK analogs with altered L-/D-Ala ratios in order to slow down (increased D-Ala content) or to accelerate (increased L-Ala content) the rate of proteolysis and (ii) to analyze the structure–function relationship of AK degradation products in a polypeptide specific T cell response in order to clarify their role in the T cell activation process.

Antitumor agent–branched polypeptide conjugates

The aim of these studies was to provide a rational approach for the selection of synthetic branched polypeptide carriers for the construction of drug–macromolecule conjugates. In order to achieve this various antitumor agents [daunomycin (DAU),[18] methotrexate (MTX),[19] boron derivatives[20] or GnRH antagonist[21]] have been attached to structurally related polypeptides with different primary[9–11,13] and secondary structure,[11,14,55] and immunological[45–47] and pharmacological[43,44] properties.

Here we summarize the synthesis and conformation analysis of MTX- or DAU-branched polypeptide conjugates (Figure 10). The common feature of these conjugates was that MTX or cAD, an acid labile, *cis*-aconityl derivative of DAU, were attached to the macromolecule by an amide bond. In order to determine predominant structural features of the carrier polypeptide influencing the conjugate's biodistribution and *in vitro* cytotoxicity, we performed comparative studies on a range of MTX and cAD conjugates with different size and side chain characteristics. Conjugates have been tested and compared with the free drug and protein [human serum albumin (HSA) or monoclonal antibody]-based conjugates.

Synthesis of antitumor drug–branched polypeptide conjugates

MTX has been attached to various synthetic macromolecules such as poly[L-Lys],[72–75] poly[D-Lys],[76]

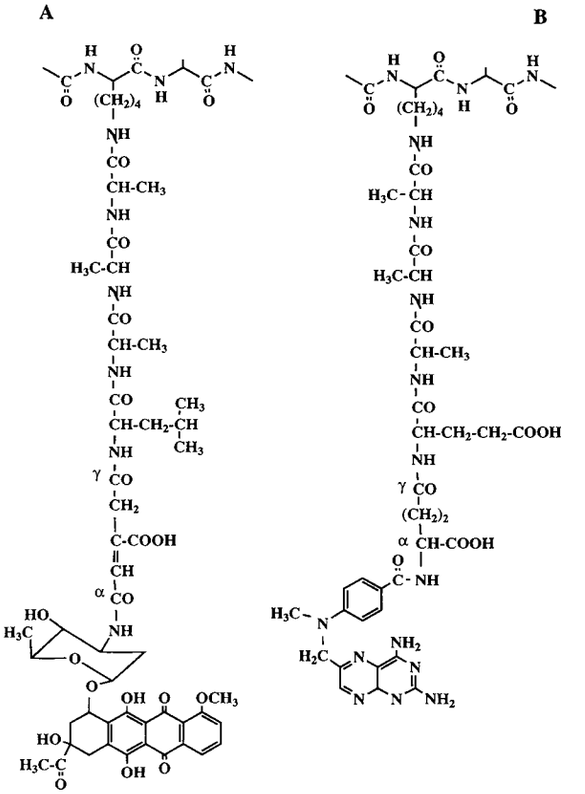

Figure 10. Simplified structure of cAD-LAK (A) and MTX-EAK (B) conjugates.

poly[Orn],[77] poly[iminoethylene],[75] poly[vinyl alcohol],[75] (carboxymethyl)-cellulose,[75] dextran,[72,78–80] aminodextran[81] or poly[divinyl ether-comaleic anhydride].[75] The methods for conjugation have involved predominantly the glutamic acid moiety. Activation of α- and γ-carboxyl groups has been achieved by (i) *N,N'*-dicyclohexyl-carbodiimides[75,79–81] or (ii) active ester formation with *N*-hydroxysuccinimide.[73,81] It should be noted that none of the above reported methods has been used for selective derivatization of α- or γ-carboxy groups of MTX. Even though the 2- and 4-amino groups of the pteridine ring of the drug have not proven to be nucleophilic, it is postulated that the aminodextran conjugate of MTX was linked through the amines.[78]

DAU has been covalently attached to synthetic carriers such as poly-α-amino acids (poly[Lys],[74,82,83] poly[Asp],[84] poly[Glu],[85] poly[Glu(N_2H_3)]),[86] dextran,[87] poly[divinyl ether-comaleic anhydride],[88] *N*-(2-hydroxypropyl)-methacrylamide (HPMA)[89] or poly[acryloy-2-amido-2-(hydroxymethyl)-1,3-propanediol.[90] These conjugates were prepared by using (i) the α-amino group of daunoseamine reacting with an anhydride[8] or active ester[89] of the polymer's carboxyl groups or applying a cross-linking reagent (e.g. carbodiimide);[85] (ii) cleavage of the bond between C-3 and C-4 of the amino sugar;[87] and (iii) the methyl ketone side chain of the aglycon by nucleophilic substitution of its 14-bromo derivative.[83,84,86] Conjugates have been produced by insertion of leucyl or aspartyl,[90] maleyl or *cis*-aconityl,[82] and succinyl or other diacidic[74] spacers.

The coupling of MTX or cAD to branched polypeptides was achieved by a carbodiimide method in which one carboxyl group of the drug molecule was linked to the α-amino group of the side chain terminal amino acid to provide covalent α-amide bonding.[18,19] First, the carboxyl groups of MTX or cAD were activated by water-soluble carbodiimide using a 1.5 times molar excess of carbodiimide at pH 5.0. In the second step, the carboxyl-activated derivative was added to the amino component (polypeptide) and the coupling reaction allowed to proceed in alkaline solution (pH 9.0). It should be emphasized that no precipitate was detected during the synthesis of conjugates under these conditions. The conjugates composed of cAD or MTX and XAK- or AXK-type polypeptides were purified by gel filtration and a new reverse phase HPLC method (with a 300 Å pore size column and isocratic elution) was developed for the detection of free drug. Characteristic values of the conjugates (cAD/MTX content, $\bar{M}_w$) show that the amount of cAD or MTX incorporated depends on the size of the polymer and on the identity of the terminal amino acid residue of the side chain.

The average molar substitution ratio was in the range of 4.0–18.3 MTX or 4.5–16.0 cAD per carrier molecule of the small relative molar mass polypeptides ($\overline{DP}_n$ = 100, $\bar{M}_w$ = 34–46 kDA) and in the range of 20.3–72.0 MTX or 20.8–70.0 cAD in the case of the large relative molar mass polymers ($\overline{DP}_n$ = 450, $\bar{M}_w$ = 157–213 kDA). Interestingly enough, no marked differences have been observed between the respective average degree of molar substitution (*DS*) values of polypeptide pairs of different size.[18,19]

We have demonstrated that the structure of the carrier polypeptide and the drug could also influence the composition of the conjugates. In case of MTX conjugates, the highest coupling efficacy was obtained with polypeptides containing only oligo(DL-Ala) side chains (AK) or Pro at the terminal positions of the side chains (PAK) ($\overline{DS}_{AK}$ = 18.3%, $\overline{DS}_{PAK}$ = 16.0%), while the highest degree of substitution in cAD conjugates was achieved with polypeptides comprising amino N-terminal Pro (PAK) or Glu (EAK) ($\overline{DS}_{PAK}$ = 16.0%, $\overline{DS}_{EAK}$ = 13.8%).[18,19]

Conformation of antitumor drug–branched polypeptide conjugates

The conformation of the conjugates in water solution was studied by CD spectroscopy in the wavelength region 190–250 nm.[18,19] The CD spectra of conjugates showed significant differences correlating with the identity of the side chain terminating amino acid, but only minor changes could be related to the replacement of L-amino acid by its D-isomer. [Considering the relatively low drug content of the conjugates (4.5–18.3%), the CD contribution of MTX or cAD proved to be negligible.]

Polycationic conjugates (LAK-MTX or -cAD, D-LAK-MTX) investigated under physiological circumstances assumed an essentially α helical secondary structure, while the CD spectrum of the amphoteric conjugates corresponded to only a partially (D-EAK-MTX) or marginally (eak-cAD) ordered conformation. It should be noted that the CD spectra of drug–polypeptide conjugates were very similar to those of unconjugated polypeptides (LAK, D-LAK, EAK and D-EAK). However, the ordered structure content of the conjugates' spectra was slightly increased as compared with free polypeptide.[55] Consequently, the incorporation of MTX or cAD molecules up to *DS* = 18.5 or 16%, respectively,

could have an affect on the solution conformation of the free carrier by promoting its transition toward an ordered secondary structure.

These conjugates as well as the free drug also have optical activity in the 250–400 nm absorption range, which is distinct from the amide chromophores and corresponds only to the structure of MTX or cAD. CD spectra in the region of 250-400 nm were found to be suitable for monitoring and verifying the presence of covalently coupled drugs.[18,19]

Biological properties of drug–branched polypeptide conjugates

Cytotoxicity against tumor cells in vitro. Branched polypeptide conjugates have been tested on osteogenic sarcoma cell line 791T,[18,19] and their *in vitro* cytotoxicity compared with that of free drug and of protein conjugates like cAD/MTX–HSA or cAD–mAB (791T/36). Conjugation of MTX or DAU in its cAD form to branched polypeptide or protein carriers significantly reduced their cytotoxicity against 791T cells compared with that of the free drug (Table 1). This observation is not unique for DAU or MTX, but has also been described for several protein or polymer conjugates of antitumor drugs.[91] It should be noted that modification of daunomycin by substitution of a *cis*-aconityl group even without further conjugation resulted in about 8-fold reduction of the 50% inhibitory concentrations (IC_{50}).

The decrease of cytotoxic activity was found to be dependent of the drug (cAD and MTX) and of the structural features (identity and position of amino acid X), but essentially independent of the relative size of the branched polypeptide carrier.[18,19] The identity of amino acid X in the side chain has a marked influence on the cytotoxic potential of both cAD and MTX. In the group of polycationic polypeptides, the position and the configuration of amino acid X in the branches have a modest influence on cytotoxic efficacy with up to 1.1- to 2.0-fold differences in IC_{50} values. In contrast, amphoteric polypeptide–drug conjugates — depending on the position and configuration of amino acid X — were more toxic [6.0- to 6.8-fold differences in IC_{50} values of cAD conjugates and 1.5- to 4.5-fold differences in MTX conjugates (Table 1)].

In the case of cAD conjugates, covalent attachment to polycationic polymers resulted in a 5- to 14-fold reduction of cAD cytotoxicity. A control conjugated of cAD prepared with HSA as carrier showed similarly decreased activity (5.6-fold reduction). In contrast, amphoteric conjugates (EAK-cAD) bearing L-Glu at the side chain terminal position produced an agent with cytotoxicity similar to that of free cAD.[18]

Conjugation of MTX to amphoteric polymers, depending on the configuration and position of amino acid X (where X = Glu or D-Glu), resulted in roughly a 200- to 1000-fold reduction of anti-791T cell activity of the free drug. However, polycationic conjugates bearing L-Leu at the side chain terminal position (LAK-MTX) produced a compound with cytotoxicity only about 60 times less effective than free MTX.[19]

The data summarized here indicate that (i) the cytotoxic activity of antitumor drugs can be sustained after synthetic branched polypeptide conjugation and it is comparable with the frequently used HSA–drug constructs and (ii) it is feasible to define structural elements of the branched polypeptide carrier molecule (e.g. side chain structure, charge) which are required for increased cytotoxicity of MTX and DAU against tumor cells *in vitro*.

Biodistribution in normal mice. The fate of these conjugates after introduction into the organism was studied in detail. The biodistribution of drug–branched polypeptide conjugates in mice has been characterized by blood clearance, WBS and tissue distribution 24 h after i.v. administration.[18,19] The blood clearance data are presented in Figure 11, while WBS results are in Table 1.

Effect of carrier size. The disappearance of conjugates from the circulation and their tissue distribution profiles revealed no significant correlation with the average molecular mass. (Table 1). However, the WBS of conjugates with low average molecular mass was usually elevated by 35–60% as compared with conjugates with high average molecular mass. It should be noted that the tissue distribution pattern of conjugates was influenced by the drug attached to branched polypeptides. cAD conjugates were generally taken up by the spleen, kidney and liver, while MTX conjugates were predominantly accumulated in the spleen and liver.

Effect of side chain structure. The elimination of drug–polypeptide conjugates from the blood could be significantly prolonged by using either amphoteric polypeptides containing a glutamic acid residue in the side chain (eak or aek) or a carefully selected polycationic one (alk). It should be noted that the retarded blood survival of amphoteric and polycationic conjugates was not accompanied by similar tissue distribution. Conjugates with a polycationic polypeptide, regardless of their drug con-

Table 1. WBS and cytotoxicity of drug–branched polypeptide conjugates[18,19]

Conjugate	WBS at 24 h (% dose ± SD)		IC_{50}[a] (pmol/ml)
MTX	NT[b]		9.0×10^0
ak-MTX	12.3	0.7	1.2×10^3
AK-MTX	7.7	0.7	3.4×10^3
lak-MTX	20.1	1.9	NT
LAK-MTX	12.8	1.4	5.4×10^2
D-LAK-MTX	30.1	0.9	1.1×10^3
apk-MTX	21.9	2.4	3.7×10^3
pak-MTX	19.6	0.5	5.8×10^3
PAK-MTX	NT		6.1×10^3
eak-MTX	22.9	1.5	6.2×10^3
aek-MTX	9.7	0.5	2.1×10^3
a-D-ek-MTX	36.1	2.1	1.0×10^4
EAK-MTX	16.9	1.7	6.2×10^3
D-EAK-MTX	13.6	0.6	9.5×10^3
HSA-MTX	5.6	2.2	1.2×10^3
DAU	NT		9.3×10^1
cAD	NT		7.1×10^2
ak-cAD	7.6	0.7	5.3×10^3
AK-cAD	7.5	1.6	9.6×10^3
lak-cAD	14.2	2.6	$> 6.0 \times 10^3$
alk-cAD	31.4	2.2	NT
LAK-cAD	NT		4.3×10^3
D-lak-cAD	11.4	0.7	$> 5.0 \times 10^3$
D-LAK-cAD	NT		3.8×10^3
pak-cAD	NT		3.3×10^3
PAK-cAD	NT		3.0×10^3
eak-cAD	22.6	1.0	1.2×10^3
aek-cAD	8.6	0.4	7.2×10^3
EAK-cAD	15.3	1.2	1.2×10^3
D-EAK-cAD	16.8	1.9	8.1×10^3
HSA-cAD	22.6	0.7	4.0×10^3
791T/36-cAD	58.0	0.8	9.6×10^3

[a] IC_{50} was determined against 791T osteogenic sarcoma cells and expressed in terms of molar MTX content of conjugates.
[b] NT, not tested.

tent and blood clearance, were directed predominantly to the spleen, liver and kidney, while conjugates with Glu at the end of the branches were taken up by the lung, kidney and liver.

The effect of the configuration of amino acid X on the fate of the conjugate was found to be dependent on its identity or position and on the nature of attached drug moiety. In the case of cAD conjugates, the presence of a D-amino acid in the terminal position of the side chain (d-lak-cAD versus lak-cAD or D-EAK-cAD versus EAK-cAD) had an effect on WBS.[18] In contrast, MTX conjugates comprising a hydrophobic D-amino acid residue at the same position (D-LAK-MTX versus LAK-MTX) or a hydrophilic amino acid residue adjacent to the polylysine backbone (a-D-ek-MTX versus aek-MTX) resulted in an elevated WBS. It is interesting to note that the introduction of D- or L-Glu at the terminal position (D-EAK-MTX versus EAK-MTX) had no marked influence on WBS.[19]

Conclusion. In conclusion, these studies have indicated that synthetic branched polypeptides can be considered as potential candidates for constructing suitable conjugates for delivery of DAU[18] and/or MTX.[19] The proper combination of elements of the primary/secondary structure of the branched polypeptide carrier molecule with the selected drug can

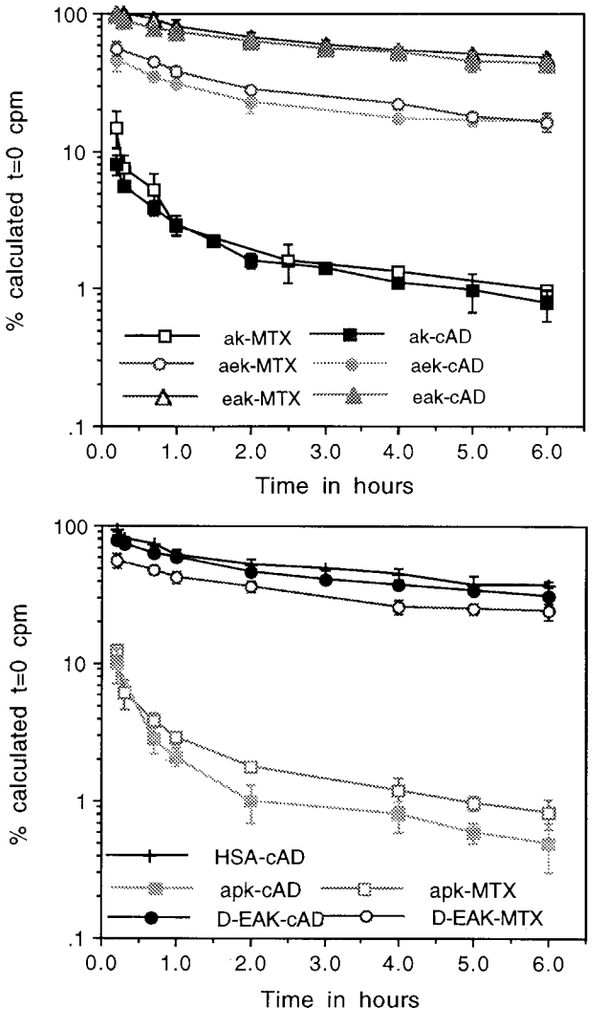

Figure 11. Blood clearance profile of ^{125}I-labeled conjugates of cAD and MTX with branched polypeptides following i.v. administration to BALB/c mice.[18,19] (A) Influence of the drug (cAD versus MTX) and of amino acid sequence of the carrier side chain. (B) Influence of the drug (cAD versus MTX) and of the charge [amphoteric (D-EAK) versus polycationic (apk)] and comparison with HSA.

result in conjugates with increased WBS and prolonged half-life in the blood. These studies showed that (i) a branched polypeptide–MTX or –cAD conjugate constructed from a polycationic or even an amphoteric carrier can sustain the cytotoxic activity of the drug comparable with the frequently used HSA–drug or mAb–cAD constructs, and (ii) branched polypeptide conjugates can be present in the circulation (amphoteric conjugates) and/or in the body (both amphoteric and polycationic conjugates) for a longer (cAD conjugates) or for much longer (MTX conjugates) periods of time than HSA–drug conjugates or free drug. In comparative experiments MTX immunoconjugates produced less impressive responses *in vitro* and *in vivo* than similar conjugates of doxorubicin, DAU or mitomycin C.[92] Taken together, our study suggests that it is feasible to beneficially alter the body distribution and the *in vitro* toxicity of MTX and DAU by logical combination of side chain sequence, configuration and identity of amino acid X in the branches of the carrier molecule. We are currently studying the therapeutic efficacy of the conjugates reported here in tumor bearing mice.

Synthesis and characterization of boron compound–branched polypeptide conjugates

Boron neutron capture therapy (BNCT) has been proposed as an alternative of conventional radiation therapy of cancer.[93] In BNCT thermal neutrons interact with boron via a ^{10}B (n,α) ^{7}Li reaction to produce short range (less than 10 mm) heavy charged particles. In order to achieve selective accumulation of ^{10}B within the tumor, boron compounds with potential localizing properties were developed[94,95] and conjugated directly to monoclonal antibodies.[96] For the construction of a ternary system containing boron derivatives, monoclonal antibodies and intermedier carrier, various groups have used dextran[97] or linear polyamino acids, such as poly[Lys], poly[Orn], poly[Arg] or poly[DL-Lys].[98] Since the number of studies with polymers is limited and polylysines are rather toxic, the design of an adequate polypeptide carrier for targeting boron compounds with or without monoclonal antibodies is an important problem to be solved.

A few representatives of branched polypeptides (AK, EAK and LAK with $\overline{DP}_n$ = 120 and $\bar{M}_w$ = 35–51 kDa) were conjugated with the polyhedral borane $Cs_2B_{12}H_{11}SH$ (BS)[94] or with *p*-dihydroxyboryl-phenylalanine (Bph).[95] Coupling of BS to AK or EAK was achieved by using heterobifunctional coupling reagents like *N*-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) or *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS).[20,98] The degree of substitution was controlled by the input BS/polypeptide ratio. In the case of AK conjugates, molar substitution above 25% resulted in insoluble derivatives, while BS–EAK conjugates prepared with SPDP afforded more soluble compounds.[20] The N-Boc derivative of Bph prepared for the first time in our laboratory was attached to AK and LAK. The highest degree of substitution could be achieved

with the *in situ* active ester procedure.[20,99] The coupling of Bph to branched polypeptides resulted in conjugates with much better solubility; however, with a relatively lower content of boron atoms as compared with polyhedral boron containing conjugates.[99]

Only Bph–AK was conjugated to an IgG2a-type monoclonal antibody raised against a human melanoma cell line, using carbodiimide as a coupling agent. It has been demonstrated that the binding activity of the monoclonal antibody was preserved in the ternary system.[99,100] Preliminary studies to evaluate the therapeutic efficacy of a mAb–AK-Bph conjugate have been performed on human melanoma xenografts. These results have shown that a 60–70% decrease in tumor growth can be achieved by this conjugate in BNCT experiments.[101]

Concluding remarks

Results with branched polypeptides and their conjugates demonstrate (i) the value of branched polypeptides in studying the correlation between structure (primary and secondary) and biological function relevant for targeting/delivery (e.g. toxicity, biodistribution or features of the immune responses) and (ii) that certain properties (e.g. biodistribution) of a conjugate are determined by the characteristics of the carrier polypeptide. These studies also suggest that the appropriate amino acid composition and sequence of the side chains provide a feasible tool for modulation of cytotoxic properties and immunoreactivity and biodistribution of carrier polypeptides and, consequently, the characteristics of their conjugates. Based on these experimental data, polypeptides can be selected for macromolecular carrier functions such as (i) delivering of antitumor agents using non-toxic, immunostimulatory polypeptides with a prolonged life time in the circulation or (ii) construction of synthetic vaccines using non-toxic branched polypeptides with low antibody response to carrier epitopes.

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