Uptake of Branched Polypeptides with Poly[L-Lys] Backbone by Bone-Marrow Culture-Derived Murine Macrophages: The Role of the Class A Scavenger Receptor

Rita Szabó,[†,⊥] Leanne Peiser,[||,⊥] Annette Plüddemann,[‡,⊥] Szilvia Bösze,[†] Sigrid Heinsbroek,[‡] Siamon Gordon,[‡] and Ferenc Hudecz*[,†,§]

Research Group of Peptide Chemistry at Eötvös L. University, Hungarian Academy of Sciences, Budapest 112, POB 32, H-1518 Hungary, Sir William Dunn School of Pathology, Oxford University, Oxford, OX1 3RE, UK, Department of Organic Chemistry, Eötvös L. University, Budapest 112, POB 32, H-1518 Hungary, and Department of Genome Sciences, University of Washington, HSB K-116, Box 357710, 1959 NE Pacific Street, Seattle, Washington 98195. Received June 14, 2005; Revised Manuscript Received August 18, 2005

Selective delivery of antiparasitic or antibacterial drugs into infected macrophages could be a promising approach for improved therapies. Methotrexate conjugate with branched chain polypeptides exhibited pronounced anti-*Leishmania* activity in vitro and in vivo as reported here earlier. To identify structural requirements for efficient uptake of branched polypeptides, we have studied murine bone marrow culture-derived macrophages (BMMϕ) from 129/ICR mice. We report on the translocation characteristics of structurally closely related compounds labeled with 5(6)-carboxyfluorescein. We found that this process is dependent on experimental conditions (e.g. polypeptide concentration, incubation time, and temperature). Using specific inhibitors as well as macrophages from wild-type and class-A scavenger receptor knockout (SR-A −/−) mice, we demonstrated that SR-A was involved in the endocytosis of some polypeptides depending on their charge. Uptake could be blocked by unlabeled polypeptide, by SR-A inhibitors, and by specific anti-SR-A monoclonal antibody. The polyanionic polypeptide poly[Lys(Succ-Glu$_{1.0}$-DL-Ala$_{3.8}$)] (SuccEAK) with high charge density translocated more efficiently than poly[Lys(Ac-Glu$_{1.0}$-DL-Ala$_{3.8}$)] (AcEAK), which had a lower anionic charge density. On the basis of experimental data presented, SuccEAK can be considered as a potential candidate for the design of a macromolecular carrier for specific drug delivery of bioactive entities into macrophages via SR-A.

INTRODUCTION

A number of intracellular pathogens including *Mycobacterium tuberculosis* (*1*) and the unicellular parasite *Leishmania* (*2*) are capable of surviving intracellularly in macrophages within phagosomes. Macrophage-selective delivery of antibacterial or antiparasitic drugs by endocytosis via specific receptors could be an attractive strategy for more efficient therapies. This approach would require the development of therapeutic macrophage receptor-specific carriers of macromolecular nature. Scavenger receptors play a crucial role in the uptake of bacteria (*3*, *4*), apoptotic cells, and modified lipoproteins (*5*). Scavenger receptors are structurally varied transmembrane proteins binding mainly polyanions with diverse chemical composition (e.g. sulfated polysaccharides, poly(I) and poly(G)) (*6*). Reports published earlier described successful introduction of antimycobacterial *p*-aminosalicylic acid (*7*) and phthalocyanine derivatives (*8*) into macrophages or macrophage cell lines by using maleylated BSA (mal-BSA). The conjugate of *p*-aminosalicylic acid was taken up by peritoneal macrophages infected in culture by *M. tuberculosis* and was 100 times more effective in killing the intracellular organisms (*7*). J774 macrophages treated with the conjugate of phthalocyanine derivatives with mal-BSA exhibited phototoxicity higher than in nonphagocytic EMT-6 cells (*8*). Mukhopadhyay et al. found that daunomycin coupled to mal-BSA entered daunomycin resistant J774A.1 cells (*9*) in vitro. When injected into mice, the daunomycin−mal-BSA conjugate was cleared rapidly from the circulation and accumulated in the macrophage-rich tissues, liver, lung, and spleen (*10*). The same group described also that the conjugate of methotrexate and mal-BSA delivered the drug into *Leishmania donovani*-infected macrophages (*11*). Also, Prasad and colleagues coupled antisense oligonucleotide to a 10-mer poly(G) and introduced this into the J774E macrophage cell line (*12*).

Very few systematic studies have been reported on structural and functional factors required for delivery/targeting of bioactive entities by macromolecules. To this end we have initiated systematic structure−function studies for rational selection of polypeptides as synthetic macromolecular carriers. For this we prepared several groups of branched chain polypeptides with the general formula of poly[Lys(X$_i$−DL-Ala$_m$)] (XAK) (Figure 1), where $i \cong 1$, $m \cong 3-4$, and X represents an additional optically active amino acid residue (*13*, *14*). These polypeptides characterized by chemical (primary structure, conformation) and biological (in vitro cytotoxicity, biodegradation, immunoreactivity, and biodistribution) properties (*13*−

* Corresponding author. Fax: 36-1-372-2620, tel: 36-1-372 2828, e-mail: hudecz@szerves.chem.elte.hu.

† Research Group of Peptide Chemistry at Eötvös L. University.

‡ Oxford University.

§ Department of Organic Chemistry, Eötvös L. University.

|| University of Washington.

⊥ R.S., L.P., and A.P. contributed equally to this study.

10.1021/bc050168f CCC: $30.25
Published on Web 10/22/2005

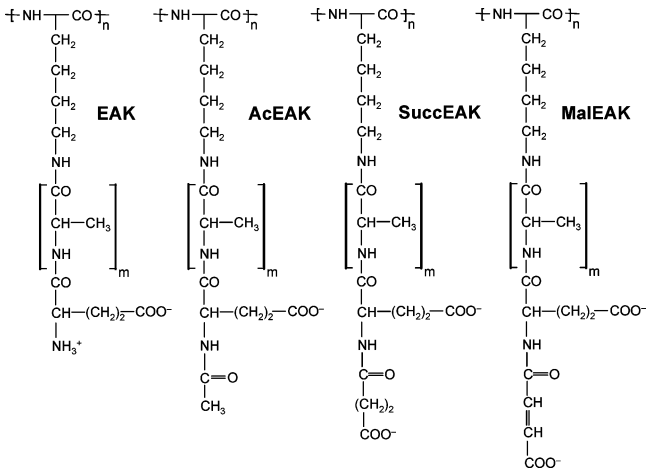

Figure 1. Chemical structure of the amphoteric EAK and its polyanionic derivatives.

15) had a marked effect on phospholipid model membranes depending on their side chain structure (*16*−*18*).

Recently we prepared several highly active bioconjugates in which an antitumor agent (*19*), radionucleide (*20*), or epitope peptide (*21*) was covalently attached to selected branched polypeptides.The coupling of an acid labile derivative of daunomycin to poly[Lys(Glu_i−DL-Ala_m)] (EAK) resulted in compensation of the immunosuppressive effect of the drug, and this conjugate in vivo was very effective in mice bearing L1210 leukemia producing 66−100% long-term (>60 days) survivors (*22*). Recently, a pronounced anti−*Leishmania donovani* effect of methotrexate (MTX) branched polypeptide conjugates was observed in vitro and in vivo (*23*). We demonstrated that the leishmanicidal activity was carrier dependent and the covalent bond between the carrier and MTX is essential. On the basis of in vitro observations, we postulated that the MTX conjugate exhibited an effect through uptake by macrophages, which was different from that of the free drug.

The aim of this work was to analyze the uptake of structurally related branched polypeptides by murine bone marrow culture-derived macrophages (BMMϕ) from 129/ICR mice. We found that polypeptides labeled with 5(6)-carboxyfluorescein are taken up and that the process was dependent on experimental conditions (e.g. polypeptide concentration, incubation time, and temperature). Furthermore, comparing polypeptide uptake by wild-type and class A macrophage scavenger receptor (SR-A)-deficient macrophages, we demonstrated that SR-A played a crucial role in the uptake of some of the polypeptides tested. SR-A-dependent recognition and endocytosis of the polypeptides was dependent on their charge properties. Taken together, our data indicate that the polypeptide poly[Lys(Succ-$Glu_{1.0}$-DL-$Ala_{3.8}$)] (SuccEAK) could be a potential candidate for the design of a macromolecular carrier for specific drug delivery of bioactive entities into macrophages via SR-A.

EXPERIMENTAL PROCEDURES

Abbreviations. Abbreviations of 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 chain polypeptides was used in accordance with the recommended nomenclature of graft polymers (*24*). For the sake of brevity, codes of branched chain polypeptides were constructed by us using the one-letter codes of amino acids. All amino acids are L configuration unless otherwise stated. The other abbreviations in this paper are the following: Z: benzyloxycarbonyl, Boc: *tert*-butyloxycarbonyl, Pcp: pentachlorophenyl, NCA: *N*-carboxy anhydride, HOBt: 1-hydroxybenzotriazole, DMF: *N,N*-dimethylformamide, DMSO: dimethyl sulfoxide, TEA: triethylamine, DCM: dichloromethane, NMM: 4-methylmorpholine, TEA: triethylamine, TFA: trifluoracetic acid PBS: phosphate-buffered saline, Ac: acetyl, Succ: succinyl, Mal: maleyl, CF: 5(6)-carboxyfluorescein, DiI: 1,1′-dioctadecyl-1−3,3,3′,3′-tetramethylindocarbocyanine perchlorate, LDL: low-density lipoprotein.

Branched Chain Polypeptides. *Reagents and Solvents.* Amino acids used for this study, HCl, trifluoracetic acid, acetic acid anhydride, and succinic anhydride, were purchased from REANAL (Budapest, Hungary). Benzyloxycarbonyl chloride, pentachlorophenol, *N,N′*-dicyclohexylcarbodiimide, HOBt, TEA, DMSO, and NMM were from Fluka (Buchs, Switzerland). Acetonitrile was from Merck (Darmstadt, Germany). 5(6)-Carboxyfluorescein succinimide ester was obtained from Sigma-Aldrich (Budapest, Hungary).

Synthesis of Poly[Lys]·HBr. The synthesis was carried out from N^{α}-carboxy-N^{ϵ}-benzyloxycarbonyl-lysine anhydride under conditions to obtain an average degree of polymerization of approximately 100 (monomer: diethylamine initiator molar ratio = 50:1), as reported previously (*25*). Protecting groups were cleaved by HBr in glacial acetic acid (35%, m/V) and poly[Lys]·HBr was precipitated using diethyl ether. The polypeptide was purified by dialysis against distilled water using visking casing and was isolated by freeze-drying. The average relative molar masses (M_w, M_z) were determined by sedimentation equilibrium using a short column technique at 25 °C (MOM 3180 type analytical ultracentrifuge, Hungary). The number average of the relative molar mass (M_n), the polydispersity factor (M_z/M_w), and the average degree of polymerization (DP_n) values were calculated from data described earlier (*26*).

Synthesis of Poly[Lys(X_i-DL-Ala_m)] (XAK) Type Polypeptides and the Polyanionic Derivatives of Poly[Lys(Glu_i-DL-Ala_m)], (EAK). Poly[Lys(DL-Ala_m)] (AK) (*27*) and poly[Lys(X_i-DL-Ala_m)] (XAK) polypeptides, where X = Glu or Ser, were prepared as described earlier (*28*). Briefly, AK polypeptide was synthesized by grafting short oligomeric DL-Ala chains to the $^{\epsilon}NH_2$ group of lysine residues using *N*-carboxy-DL-Ala-anhydride. The terminal amino acids (X) were coupled to the $^{\alpha}NH_2$-groups of the side chains as Z−X-OPcp, where X = Glu or Ser. The reaction was performed by an in situ active ester method using an equivalent amount of HOBt dissolved in DMF as activating reagent. The pH of the solution was adjusted to 8.0 by adding NMM. Stirring was continued at room-temperature overnight. Z-protecting groups were removed with HBr using acetic acid containing 35% HBr. The polypeptides were purified by dialysis against distilled water for 2 days using a Visking tube (cutoff = 12000−14000 Da) and isolated by freeze-drying.

Poly[Lys(AcGlu$_i$-DL-Ala_m)] (AcEAK) was prepared by acetylation of poly[Lys(Glu_i-DL-Ala_m)] (EAK) as follows. 85 mg (159 μmol) of EAK was dissolved in 2 mL of distilled water and diluted with 10 mL of DMF under cooling at 0 °C. 27 μL (198 μmol) of TEA was added to this solution to neutralize the polymer. Five equivalents of freshly prepared imidazolyl-acetate was used for the acetylation (*29*), and then the product was dialyzed against distilled water followed by freeze-drying.

Poly[Lys(Succ-Glu_i-DL-Ala_m)] (SuccEAK) was prepared by succinylation and poly[Lys(Mal-Glu_i-DL-Ala_m)] (MalEAK) by maleylation of poly[Lys(Glu_i-DL-Ala_m)],(EAK) as follows. 10 mg (18.5 μmol) EAK) was dissolved in 2 mL of 0.1 M carbonate buffer (pH = 9.2). 220 μL (220 mmol) of succinic anhydride or 219 μL (220 mmol) maleic anhydride, respectively, was dissolved in DMSO (c = 100 mg/mL) and was added to this solution with continuous stirring for 30 min at RT (*30*). The pH was maintained between 9.0 and 9.2 with 0.1 M NaOH. The reaction was continued for 4 h followed by dialysis against distilled water for 2 days and then freeze-drying.

Amino Acid Analysis. The amino acid composition of the polymeric polypeptides was determined by amino acid analysis using a Beckman 6300 analyzer (Fullerton, CA). Prior to analysis, the samples were hydrolyzed in 6 M HCl in sealed and evacuated tubes at 105 °C for 24 h.

Labeling of Branched Polypeptides with 5(6)-Carboxyfluorescein. 10 mg (6×10^{-7} to 1.9×10^{-7} mol) amounts of polypeptides were dissolved in 2 mL of Na_2CO_3 buffer (0.1 M; pH 9.4). The amino-reactive dye 5(6)-carboxyfluorescein succinimide ester (5(6)-CF-SE) was dissolved in DMF (c = 10 mg/mL, 0.21 mM) and was added to the polypeptide solution in 20 μL aliquots. The reaction was continued for 1 h at room temperature with continuous stirring. The product was purified on a PD10 Sephadex G25 column using distilled water as eluent. The samples were then freeze-dried and they were resolved in PBS (0.1 M, pH 7.4) before use.

Determination of 5(6)-Carboxyfluorescein Content of Conjugates. Labeled polypeptide samples and 5(6)-carboxyfluorescein succinimide ester were hydrolyzed in 6 M HCl in sealed and evacuated tubes at 110 °C for 24 h. HCl was removed, and the hydrolysate was dissolved in DMSO. The hydrolyzed samples (20 μL) were analyzed by RP-HPLC using reversed phase Eurospher C_{18} column (3.9 × 250 mm; packed with spherical 5 μm silica with 100 Å pore size [Knauer, Bad Homburg, Germany] and gradient elution consisted of 0.1% TFA in water [eluent A] and 0.1% TFA in acetonitrile/water = 80/20 v/v [eluent B]). During chromatography the eluent B content was between 25 and 45%, in 20 min, 45−99%, in 10 min. The flow rate was 1 mL/min at room temperature. The average sample concentration applied was 0.65 μg/mL. Peaks were detected at λ_{ex} = 419 nm and λ_{em} = 519 nm using a fluorescence detector (Shimadzu, Japan). A calibration curve was constructed using 5(6)-carcoxyfluorescein in the 4.8×10^{-5} M to 1.2×10^{-5} M concentration range.

Functional Studies. *Reagents.* 1,1′-Dioctadecyl-1−3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled acetylated LDL (DiI-AcLDL) was obtained from Autogen Bioclear, and poly(I) from Signa (Reading, UK). OptiMEM medium and all other culture media were from Invitrogen (Paisley, United Kingdom). Rat anti-mouse SR-A antibody 2F8 was prepared at the Sir William Dunn School of Pathology, Oxford, as described previously (*33*). CAMPATH rat anti-mouse IgG, an isotype-control for 2F8, was a kind gift from H. Waldmann, Sir William Dunn School of Pathology, Oxford. Cy3-labeled donkey anti-rat IgG was from Jackson ImmunoResearch (Soham, UK), and rabbit and goat sera were from Sigma. Fluorescent Mounting Medium was from DakoCytomation (Glostrup, Denmark). Plastic products were purchased from Becton Dickinson Labware (Oxford, UK).

Animals. Mice deficient in SR-AI and II (SR-A −/−) were produced as previously described (*31*). SR-A −/− and SR-A +/+ control 129/ICR mice of the same sex were used at 8−12 weeks of age. All animals were handled in accordance with guidelines issued by the Home Office, United Kingdom.

Cell Isolation and Culture. BMMϕ were obtained and cultured by standard procedures described previously (4). The cells were maintained in 15 cm bacteriologic plastic (BP) Petri dishes containing RPMI 1640 with 50 IU/mL penicillin G, 50 μg/mL streptomycin, 2 mM glutamine (PSG), 10% fetal calf serum (FCS), and 15% (vol/vol) L-cell conditioned medium (LCM) as a source of macrophage colony-stimulating factor (*32*). The macrophages were harvested with PBS (0.1 M, pH 7.4) containing 10 mM EDTA and 4 mg/mL lidocaine·HCl and plated onto six-well BP plates (Greiner, Gloucester, United Kingdom). The cells were maintained in the six-well plate for further 24 h at 37 °C. The bone marrow from the femurs and tibias of one mouse gave $(1-5) \times 10^7$ Mϕ after 7 days in culture.

Cytotoxicity Assay. Prior to the experiment, cells were washed three times with PBS. Each polypeptide was added in OptiMEM at a concentration of 1 μg/mL or 20 μg/mL. BMMϕ were incubated with the polypeptide solution for 1 h and then harvested with lidocaine/EDTA. The number of living cells was determined by counting the cells in a Neubauer haemocytometer after staining with 0.4% trypan blue dissolved in PBS. The statistical significance of data was determined by Student's t test at the 95% confidence level.

Flow Cytometry Analysis of Cellular Uptake. Polypeptides labeled with 5(6)-CF dissolved in OptiMEM were used. Before adding CF-labeled polypeptides, the cells were washed three times with PBS. The CF-labeled polypeptides were incubated with the cells for 1 h at 37 °C or 4 °C. For the inhibition experiments poly(I) at 50 μg/mL (4) or 2F8 at 15 μg/mL was used (*33*). Prior to the addition of polypeptides, cells were preincubated for 30 min with inhibitor which was also maintained throughout the experiment. In some experiments unlabeled polypeptides were added at 10 times excess of labeled molecule concentration. The increase in macrophage fluorescence following incubation with labeled molecules was analyzed by flow cytometry. Data were analyzed with CellQuest 3.0 software. The mean fluorescence of untreated control cells was subtracted from the mean fluorescence obtained with treated samples under all different assay conditions applied. Statistical analysis of data was performed using Student's t test at the 95% confidence level.

Analysis of the Cellular Uptake by Confocal Microscopy. Wild-type 129/ICR BMMϕ were plated on sterile glass coverslips in 24-well tissue-culture dishes in RPMI 1640 medium supplemented PSG; 10% FCS; and 15% (vol/vol) LCM at 5×10^4 macrophages per well 24 h prior to the experiment. The cells were washed three times with PBS. CF-labeled polypeptides were incubated with the cells for 5, 15, 30, and 60 min at 37 °C. Cells were fixed with 4% formaldehyde/PBS for 15 min at room temperature then permeabilized using a blocking buffer containing 1% (m/V) BSA, 0.25% (m/V) saponin, and 1% goat and 1% rabbit serum. The cells were incubated with 2F8 (c = 10 μg/mL) or with an isotype-matched control antibody (CAMPATH 1G) in parallel for 1 h at room temperature followed by washing three times with blocking buffer. After incubation with Cy3 labeled anti-rat IgG antibody for 1 h in the dark, the coverslips were mounted on glass microscopy slides using DakoCytomation Fluorescent Mounting Medium. Cells were analyzed in a BIO RAD Radiance 2000 confocal microscope (λ_{ex} = 488 nm).

Table 1. Characteristics of Polylysine Based Branched Chain Polymeric Polypeptides with Poly[Lys(X_i-DL-Ala_m)] Formula

polypeptide	code[a]	amino acid composition[b] Lys	Ala (m)	X (i)	$\overline{DP_n}$[c]	$\overline{M_w}$[d] ± 5%	CF [%][e]
poly[Lys]	-	1.00	-	-	130	16700	-
poly[Lys(Glu_i-DL-Ala_m)]	EAK	1.00	3.80	1.00	60	31600	2.91
poly[Lys(Ac-Glu_i-DL-Ala_m)]	AcEAK	1.00	3.80	1.00	60	34500	0.46
poly[Lys(Succ-Glu_i-DL-Ala_m)]	SuccEAK	1.00	3.80	1.00	60	37900	0.53
poly[Lys(Succ-Glu_i-DL-Ala_m)]	MalEAK	1.00	3.80	1.00	60	37800	0.72

[a] Code of branched chain polymeric polypeptides, based on one-letter symbol of amino acids. [b] Amino acid composition was determined by amino acid analysis as after hydrolysis in 6 M HCl at 105 °C for 24 h. [c] Number of average degree of polymerization determined by sedimentation equilibrium measurements. [d] Average molecular mass of polymers; calculated from the average degree of polymerization (DP_n) of poly[Lys] and of the side chain composition. [e] Average degree of substitution for 5(6)-carboxyfluorescein (CF) was determined by reverse phase HPLC after hydrolysis of the samples in 6 M HCl at 105 °C for 24 h.

RESULTS

Branched Polypeptides. Branched polypeptides with the general formula poly[Lys(X_i-DL-Ala_m)], ($m \cong 4$, $i \cong 1$), which were used for these studies have short oligo-(DL-Ala) side chains and Glu at the *N*-terminal of the branches. The terminal amino acid X was coupled to the $^{\alpha}NH_2$ groups of the terminal alanine by an in situ active ester method (*28*). EAK bearing $^{\alpha}NH_2$ and also free carboxyl groups at the end of the branches possess an amphoteric character. The three additional polypeptides that were also included in our studies were the polyanionic derivatives of poly[Lys($Glu_{1.0}$-DL-$Ala_{3.8}$)] (EAK) prepared by acetylation, succinylation or maleylation of the $^{\alpha}NH_2$ groups of the terminal glutamic acid residues (Figure 1). Poly[Lys($AcGlu_{1.0}$-DL-$Ala_{3.8}$)] (AcEAK) side chains contain only one negative charge per monomer unit under conditions used for the present studies. The charge density of the succinylated (poly[Lys($SuccGlu_{1.0}$-DL-$Ala_{3.8}$)], SuccEAK) and maleylated (poly[Lys($MalGlu_{1.0}$-DL-$Ala_{3.8}$)], MalEAK) polypeptides were higher, since two negative charges were present per unit. Thus, we investigated this group of structurally related polypeptides built on a polylysine backbone with systematically altered chemical properties (charge, conformation (*13*, *30*)). The polypeptides were characterized according to their relative average molar mass, determined by sedimentation equilibrium analysis (*26*), and the composition of their side chain. The chemical characteristics of the polypeptides are shown in Table 1.

Cytotoxic Effect of Polypeptides on BMMϕ. The cytotoxicity of polypeptides labeled with CF was studied on BMMϕ cells from wild-type mice at two different concentrations (1 μg/mL and 20 μg/mL). The cytotoxicity of the polypeptides was determined by counting live cells by trypane blue exclusion. Polylysine was included as a control. Results are expressed as a percentage of untreated cells and summarized in Figure 2. We found that after 1 h incubation none of the polypeptides had a significant toxic effect on the macrophages at 1 μg/mL concentration. At 20 μg/mL poly[Lys] proved to be highly toxic, but no cytotoxic effect of the other polypeptides was observed.

Uptake of polypeptides by BMMϕ. We studied the endocytosis of polypeptides by BMMϕ from wild-type mice under different conditions. The effects of polypeptide concentration, incubation time, and temperature on uptake were analyzed. Furthermore we investigated the influence of unlabeled polypeptides on the internalization of CF-polypeptides in inhibition assays.

Influence of the Polypeptide Concentration on the Uptake. BMMϕ from wild-type mice were incubated with varying concentrations (0.1−100 μg/mL) of the CF-labeled polypeptides, and the rate of uptake was detected by flow cytometry. The amphoteric EAK and polyanionic AcEAK were internalized only at 100 μg/mL concentration. In the case of polyanionic SuccEAK, uptake was proportional with the increasing concentration in the concentration range tested, while MalEAK was taken up effectively only at 50 and 100 μg/mL (Figure 3).

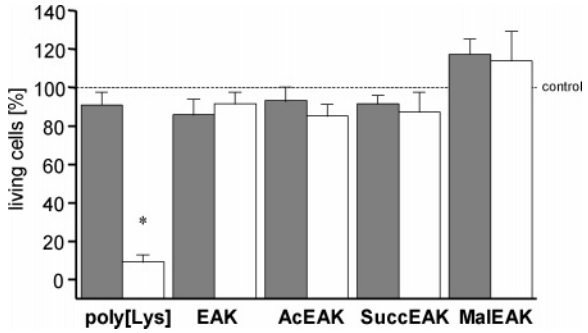

Figure 2. The effect of the branched CF-polypeptides on the survival of BMMϕ. Columns represent the number of living cells as a percentage of untreated control ± SD. Polylysine ($\overline{DP_n}$ = 130, $\overline{M_w}$ = 17000) was used as control polypeptide. ■: c = 1 μg/mL, □: c = 20 μg/mL. Statistical analysis of data was performed by Student's t test; *: $p<0.05$. Poly[L-Lys] (M_w = 17000, DP_n = 130) was used as control.

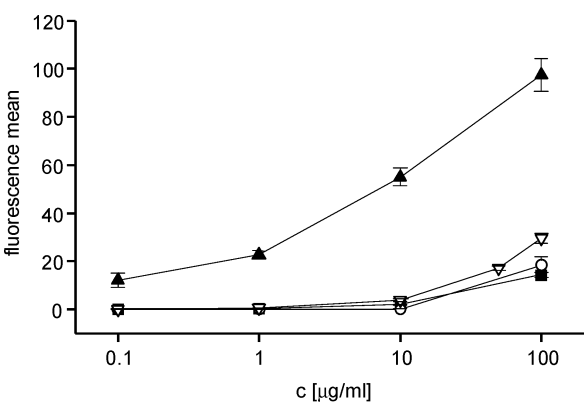

Figure 3. Concentration dependence of the uptake of the CF-polypeptides by BMMϕ after 60 min incubation with the polypeptides. Symbols represent the average of mean fluorescence values of a representative experiment of three independent assays ± SD after subtracting the control. ■: CF-EAK, ○: CF-AcEAK, ▲: CF-SuccEAK, ▽: CF-MalEAK.

Time Dependence. Time dependence of uptake was examined by flow cytometry after 1, 5, 10, 20, 30, 45, and 60 min incubation with the polypeptides (Figure 4). The internalization of the polyanionic polypeptides by the cells was in direct proportion to the duration of incubation. The uptake of neither the scavenger receptor ligand DiI-AcLDL (c = 5 μg/mL) used as positive control (6, 34) nor the CF-polypeptides was saturable during the 1 h

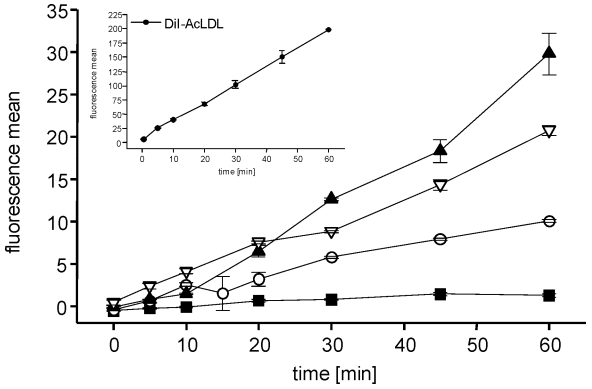

Figure 4. Time dependence of the endocytosis of polypeptides. Symbols represent the average of mean fluorescence values of a representative experiment of three independent assays ± SD after subtracting the control. ■: CF-EAK, c =100 μg/mL; ○: CF-AcEAK, c = 100 μg/mL; ▲: CF-SuccEAK, c = 50 μg/mL; ▽: CF-MalEAK, c = 100 μg/mL. Insert: time dependence of the uptake of DiI-AcLDL (c = 5 μg/mL) under the same conditions.

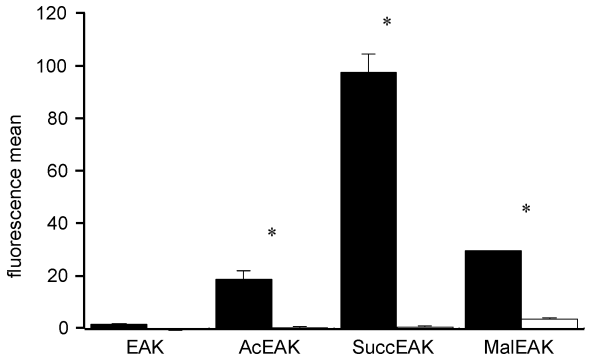

Figure 5. Uptake of polypeptides by 129/ICR BMMϕ at 4 °C and 37 °C after 1 h. Columns represent the average of mean fluorescence values of a representative experiment of three independent assays ± SD after subtracting the control. ■: 37 °C μg/mL, □: 4 °C. Statistical analysis of data was performed by Student's t test; *: $p < 0.05$.

study period, and the polyanionic polypeptides were taken up continuously by the BMMϕ during this time (Figure 4).

Temperature Dependence. The uptake of the polypeptides was also investigated at 4 °C versus 37 °C by flow cytometry. The results are summarized in Figure 5. In the case of the amphoteric EAK no significant uptake could be observed at 37 °C or 4 °C. Two polyanionic polypeptides, AcEAK and SuccEAK, were internalized only at 37 °C. In the case of MalEAK a significant difference could be observed between the uptake at 4 °C or at 37 °C.

The Effect of Pretreatment with Unlabeled Polypeptides. In this experiment the endocytosis of CF-SuccEAK was examined at the lowest concentration that could be easily detected. Macrophages were pretreated with unlabeled polypeptides at a concentration 10 times higher than the labeled polypeptide for 30 min prior to the incubation of the cells with the respective CF-polypeptides. The polyanionic polypeptides, AcEAK and SuccEAK, significantly inhibited the uptake of the labeled SuccEAK into the macrophages (Figure 6). Pretreatment of the macrophages with c = 10 μg/mL unlabeled SuccEAK inhibited the uptake of CF-SuccEAK (c = 1 μg/mL) to the control levels. After 60 min incubation with the labeled polypeptide, mean fluorescence was 1.43 ± 0.15 in the presence and 10.77 ± 0.65 in the absence of the unlabeled SuccEAK, p < 0.01. To a

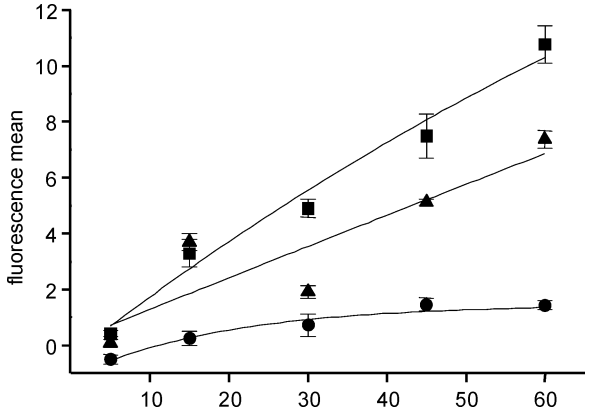

Figure 6. Effect of pretreatment with unlabeled polypeptides of similar chemical character. Symbols represent the average of mean fluorescence values of a representative experiment of three independent assays ± SD after subtracting the control. ■: uptake of CF-SuccEAK, c = 1 μg/mL; ●: uptake of CF-SuccEAK pretreated with unlabeled SuccEAK, c = 10 μg/mL, ▲: uptake of CF-SuccEAK pretreated with unlabeled AcEAK, c = 10 μg/mL.

lesser extent, unlabeled AcEAK also elicited a statistically significant inhibition of the internalization of CF-SuccEAK (mean fluorescence [%] = 7.36 ± 0.31 in the presence of AcEAK, $p < 0.01$).

The Role of the Scavenger Receptor in the Uptake of Polyanionic Polypeptides. To clarify the potential involvement of the SR-A, we examined the uptake of the polypeptides by BMMϕ from SRA −/− mice. In these studies in order to block the receptor we utilized a known general SR-A ligand, poly(I), and a specific anti-SR-A monoclonal antibody, 2F8.

Uptake of Polypeptides by Wild-Type and SR-A −/− Macrophages. The uptake of polyanionic polypeptides CF-AcEAK, CF-SuccEAK, and CF-MalEAK was tested. The scavenger receptor ligand DiI-AcLDL was used as a positive control (*34*), and the amphoteric CF-EAK polypeptide as negative control. The three polyanionic polypeptides were internalized by wild-type macrophages, but to a lower extent compared with DiI-AcLDL (Figures7 and 8). The uptake of CF-SuccEAK, with higher negative charge density, by wild-type BMMϕ was greater than CF-AcEAK having less negative charges and CF-MalEAK containing a double bond in its anionic end-group. In all cases we observed that the internalization of these polypeptides was significantly diminished in SR-A −/− cells (Figure 7). In the case of CF-SuccEAK (c = 50 μg/mL) we detected a thirteen-fold difference between the fluorescence intensities of the internalized polypeptide by the wild-type and SRA −/− BMMϕ (mean fluorescence = 52.49 ± 1.34 and 3.77 ± 0.01 respectively, $p < 0.001$). With CF-AcEAK (c = 100 μg/mL) a statistically significant difference could be demonstrated between the uptake of the wild-type and of the SR-A −/− macrophages, a 8-fold decrease in the uptake could be observed (mean fluorescence = 10.195 ± 1.43 for the wild-type and 1.235 ± 0.26 for the SRA −/− cells, $p < 0.001$) (Figure 8).

Blocking of Scavenger Receptor-A with Poly(I) and MAb 2F8. Next, wild-type and SR-A −/− BMMϕ were pretreated with the scavenger receptor ligand poly(I) (c = 50 μg/mL) (*4*, *6*) or the SR-A specific monoclonal antibody 2F8 (at c = 15 μg/mL) for 30 min before the addition of the polypeptides. Data are presented in Figure 8. We observed that the presence of these SR-A scavenger receptor ligands significantly reduced the uptake of the

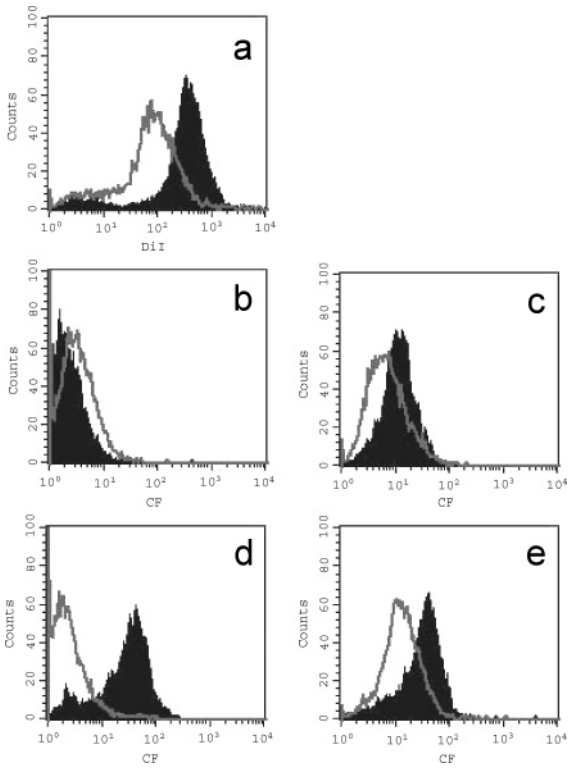

Figure 7. Uptake of polyanionic polypeptides by wild-type and SR-A −/− BMMϕ. Cells were fixed with 2% formaldehyde. Diagrams were recorded by measurig 10000 cells. Data were analyzed by CellQuest 3.0. a. Filled diagrams represent wild-type macrophages, while open lines represent *SR-A* −/− cells. (a) DiI-AcLDL, $c = 5$ μg/mL, (b) CF-EAK, $c = 100$ μg/mL, (c) CF-AcEAK: $c = 100$ μg/mL, (d) CF-SuccEAK, $c = 50$ μg/mL, (e) CF-MalEAK, $c = 50$ μg/mL.

polyanionic polypeptides and of DiI-AcLDL used as positive control.

Colocalization of SR-A and CF-polypeptides in the Cells. The time-dependence of the uptake of CF-SuccEAK and its colocalization with SR-A were further investigated by confocal microscopy. We detected SR-A in cells, using 2F8- and Cy3-labeled anti-rat IgG secondary antibody. We found that CF-SuccEAK was internalized by the cells. Vesicles containing the polypeptide were observed in the cytoplasm (Figure 9). We detected colocalization of CF-SuccEAK (green) and SR-A (red) within 5 min of incubation.

DISCUSSION

Class A scavenger receptors are membrane glycoproteins that are present mainly on cells of the macrophage lineage (*35*). Therefore these receptors are potential targets for macrophage-specific delivery of macromolecules and their drug or epitope conjugates. The scavenger receptor ligands described in the literature are polyanionic compounds with diverse chemical structure and composition (e.g. sulfated polysaccharides such as fucoidin, AcLDL, maleylated proteins, poly(I), and poly(G) (*6*).

The present studies are the first attempt at systematic examination of the interaction between SR-A-bearing cells and a chemically related polypeptide family with different charge and structure. Our previous results with these polypeptides imply that chemical structure, the charge and length of the side chains, have a pronounced effect on various biological and physicochemical properties (*16*, *17*). Results presented in this paper indicate that the uptake of branched polypeptides by murine BMMϕ depends on the composition of the branches, indicating the importance of both charge and the structure of the terminal amino acid derivative. As assessed by the fluorescence intensity of CF-labeled polypeptides, we found a significant difference between the rate of uptake of the polyanionic polypeptides. Although both SuccEAK and MalEAK contain two carboxyl groups at the end of the side chain, whereas AcEAK possesses only one in the terminal glutamic acid, SuccEAK was internalized by BMMϕ to a greater extent than MalEAK. This observation could be explained by the presence of a C−C double bond in MalEAK, which is in conjugation with the C−O double bond influencing the charged state of the terminal carboxyl group.

To analyze the mechanism of uptake we performed inhibition studies with the polypeptide internalized most efficiently. The translocation of CF-SuccEAK was investigated in the presence of unlabeled SuccEAK and also AcEAK. The results of this study showed that unlabeled SuccEAK markedly inhibited the internalization of the CF-labeled polypeptide compound, but AcEAK exhibited only a partial inhibitory effect. These data suggest that AcEAK and SuccEAK share the same receptor, but AcEAK binds with lower affinity or that there are multiple receptors for SuccEAK some of which are shared.

To clarify if SR-A was involved in the endocytosis, we investigated the uptake of the polyanionic polypeptides on wild-type and SR-A −/− BMMϕ. Results show that SR-A −/− macrophages internalize these compounds at a substantially lower level compared with wild-type cells. Blocking of the SR-A with receptor antagonist, poly(I), or with an anti-SR-A monoclonal antibody, resulted in a clear reduction in uptake. In addition, confocal microscopic studies provided independent evidence of colocalization of CF-SuccEAK with SR-A soon after internalization into cells. Taken together, these results suggest that CF-SuccEAK is taken up by SR-A-mediated endocytosis and indicate that the receptor recognizes polyanionic polypeptides and has an important role in their uptake.

It is known that not all polyanionic molecules are able to bind SR-A due to the presence of an undetermined structural requirement for recognition by the receptor. Using our systematic modification of polylysine, we were able to demonstrate that we could enhance binding to SR-A. Thus, the use this approach provides us a powerful new tool to study SR-A binding in further detailby structural manipulation of the polylysine to enhance or reduce binding to the receptor and by modeling how the changes alter the charge distribution and spatial arrangement.

We showed that SuccEAK had higher accumulation in macrophages compared to AcEAK. Interestingly, our previous data on the blood clearance and biodistribution of branched polypeptides showed that AcEAK had prolonged blood survival in Balb/c mice, while SuccEAK was cleared rapidly from the circulation (*36*). This difference in the blood clearance, which was not due to enzymatic degradation, could be related to the difference of macrophage uptake between AcEAK and SuccEAK. Thus, the enhanced uptake by macrophages, mainly through SR-A, could contribute to the rapid clearance of SuccEAK.

Several previous papers have reported on the uptake of modified proteins and conjugates by macrophages. Majumdar et al. demonstrated that the binding of PAS-MalBSA labeled with ^{125}I to peritoneal macrophages was saturable, indicating a limited number of binding sites. Addition of unlabeled conjugate to medium of the macrophages eliminated binding of the labeled compound.

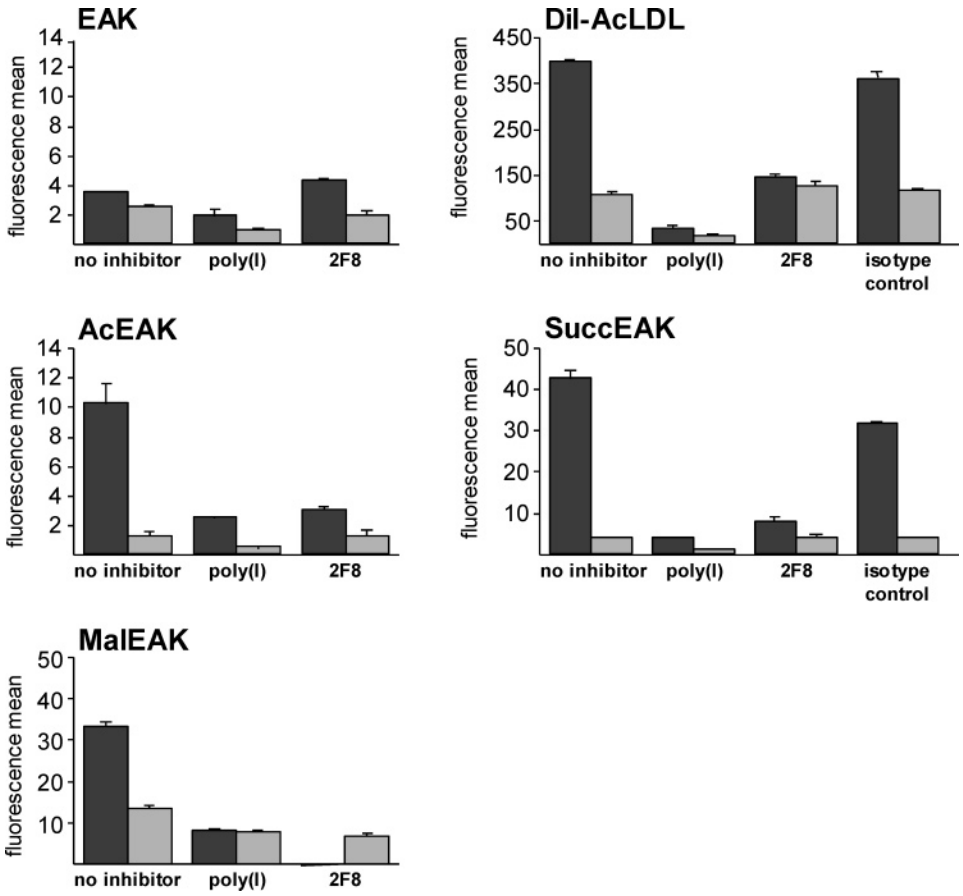

Figure 8. Uptake of polyanionic polypeptides by wild-type and SR-A −/− BMMϕ, CF-AcEAK: $c = 100$ μg/mL. CF-SuccEAK, $c = 50$ μg/mL,MalEAK, $c = 50$ μg/mL DiI-AcLDL, $c = 5$ μg/mL. Columns represent the average of mean fluorescence values of a representative experiment of three independent assays ± SD after subtracting the control. ■: 129/ICR, gray: SR-A −/−.

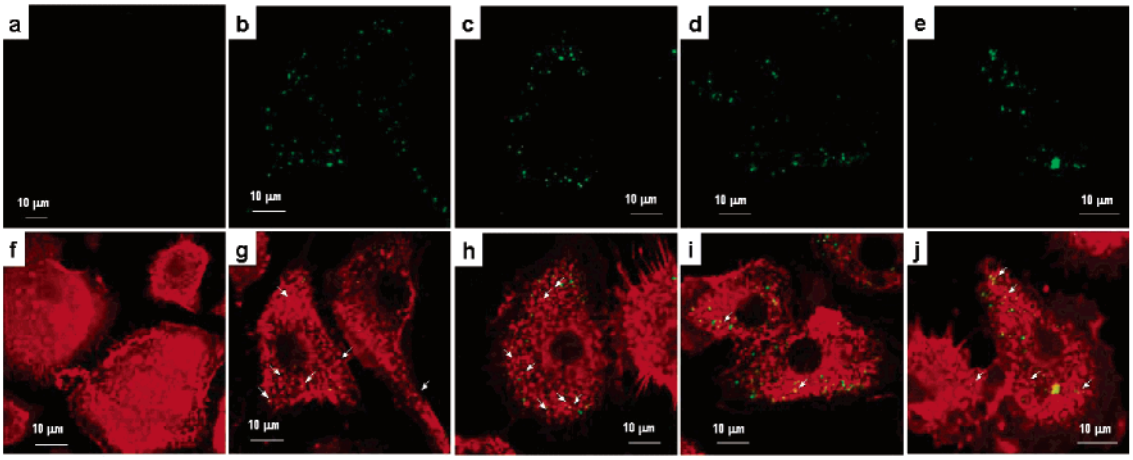

Figure 9. Colocalization of SR-A (red) and CF-SuccEAK (green). SR-A was detected by the SR-A specific monoclonal antibody 2F8 and a Cy3-labeled secondary antibody. The cells were analyzed by confocal microscopy ($\lambda_{ex} = 488$ nm). (a) isotype control of 2F8, (b) CF-SuccEAK 5 min, (c) CF-SuccEAK 15 min, (d) CF-SuccEAK 30 min, (e) CF-SuccEAK 60 min, (f) control cells not reated with polypeptide, SR-A detected by 2F8 and a Cy3 labeled secondary antibody, (g) CF-SuccEAK 5 min and SR-A: merge, (h) CF-SuccEAK 15 min and SR-A: merge, (i) CF-SuccEAK 30 min and SR-A: merge, (j) CF-SuccEAK 60 min and SR-A: merge. Arrows show examples of colocalization.

The authors also demonstrated the uptake of the conjugate at 37 °C, which was followed by its lysosomal degradation (*7*). Brasseur et al. described the specific uptake of phthalocyanine-malBSA conjugates followed by photoinactivation of J774 macrophages. The receptor binding was demonstrated by competition assay between labeled and nonlabeled conjugates (*8*). These studies showed no direct evidence of the involvement of scavenger receptors in the processes described. Similarly, the articles of Mukhopadhyay et al. contained no experimental data on the involvement of scavenger receptors in the uptake and cytotoxicity of daunomycin or methotrexate conjugates with mal-BSA (*9*−*12*). Recognition of a 10-mer poly(G)-tethered antisense oligonucleotide by scavenger receptors was assessed by a competition assay. In this the degradation of ^{125}I-labeled mal-BSA by J774E

cells in the presence or absence of different polyanionic molecules as competitors exhibited the characteristic scavenger receptor profile (*12*).

To the best of our knowledge this is the first report to show that systematic chemical modification of polylysine-based polypeptides greatly influences the rate and mechanism of uptake by BMMϕ. We also demonstrated for the first time that the internalization of polyanionic synthetic polypeptides, especially SuccEAK, could be considered a SR-A mediated process. Thus, this polypeptide seems to be appropriate as a macromolecular carrier for specific drug delivery into macrophages and they can become the basis for development of further carrier molecules and bioconjugates.

ACKNOWLEDGMENT

Experimental work summarized in this paper was supported by grants from the British-Hungarian Intergovernmental Program (2/2003), from the Hungarian Research Fund (OTKA No. T-045679), and from the Hungarian Ministry of Education (Medichem 2, NKFP). Work in the laboratory of S.G. was supported by a grant from the Medical Research Council, UK. The authors thank Dr. Hedvig Medzihradszky-Schweiger for the amino acid analyses.

LITERATURE CITED

(1) Hostetter, J., Steadham, E., Haynes, J., Bailey, T., and Cheville, N. (2003) Phagosomal maturation and intracellular survival of *Mycobacterium avium* subspecies paratuberculosis in J774 cells. *Comput. Immun. Microbiol. Infect. Dis. 26*, 269−283.

(2) Bogdan, C., and Röllinghoff, M. (1998) The immune response to *Leishmania*: mechanisms of parasite control and evasion. *Int. J. Parasitol. 28*, 121−134.

(3) Peiser, L., M. de Winther, P. J., Makepeace, K., Hollinshead, M., Coull, P., Plested, J., Kodama, T., Moxon, E. R., and Gordon, S. (2002) The class A macrophage scavenger receptor is a major pattern recognition receptor for *Neisseria meningitidis* which is independent of lipopolysaccharide and not required for secretory responses. *Infect. Immun. 70*, 5346−5354.

(4) Peiser, L., Gough, P. J., Kodama, T., and Gordon, S. (2000) Macrophage class A scavenger receptor-mediated phagocytosis of *Escherichia coli*: Role of cell heterogeneity, microbial strain, and culture conditions *in vitro*. *Infect. Immun. 68*, 1953−1963.

(5) Sambrano, G. R., Parthasarathy, S., and Steinberg, D. (1994) Recognition of oxidatively damaged erythrocytes by a macrophage receptor with specificity for oxidized low-density lipoprotein. *Proc. Natl. Acad. Sci. U.S.A. 91*, 3265−3269.

(6) Krieger, M., and Herz, J. (1994) Structures and function of multiligand lipoprotein receptors − macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu. Rev. Biochem. 63*, 601−637.

(7) Majumdar, S., and Basu, S. K. (1991) Killing of *Mycobacterium tuberculosis* by receptor-mediated drug delivery. *Antimicrob. Agents. Chemother. 35*, 135−140.

(8) Brasseur, N., Langlois, R., La Madeleine, C., Ouellet, R., and Van Lier, J. E. (1999) Receptor-mediated targeting of phthalocyanines to macrophages via covalent coupling to native or maleylated bovine serum albumin. *Photochem. Photobiol. 69*, 345−352.

(9) Mukhopadhyay, A., Mukhopadhyay, B., and Basu, S. K. (1995) Circumvention of multidrug resistance in neoplastic cells through scavenger receptor mediated drug delivery. *FEBS Lett. 376*, 95−98.

(10) Mukhopadhyay, B., Mukhopadhyay, A., and Basu, S. K. (1993) Enhancement of tumouricidal activity of daunomycin by receptor-mediated delivery. In vivo studies. *Biochem. Pharmacol. 46*, 912−924.

(11) Mukhopadhyay, A., and Basu, S. K. (1990) Enhanced intracellular delivery of methotrexate by a receptor mediated process. *Biotechnol. Appl. Biochem. 12*, 529−36.

(12) Prasad, V., Hashim, S., Mukhopadhyay, A., Basu, S. K., and Roy, R. P. (1999) Oligonucleotides tethered to a short poly(G)uanylic acid stretch are targeted to macrophages: Enhanced antiviral activity of a vesicular stomatitis virus-specific antisense oligonucleotide. *Antimicrob. Agents Chemother. 43*, 2689−2696.

(13) Hudecz, F. (1995) Design of synthetic branched-chain polypeptides as carriers for bioactive molecules. *Anti-Cancer Drugs 6*, 171−193.

(14) Mezö, G., Kajtár, J., Nagy, I. B., Szekerke M., and Hudecz, F. (1997) Carrier design: synthesis and conformational studies of poly(l-lysine) based branched polypeptides with hydroxyl groups in the side chains. *Biopolymers 42*, 713−730.

(15) Hudecz, F., Pimm, M. V., Rajnavölgyi, É., Mezö, G., Fabra, A., Gaál, D., Kovács, A. L., Horváth, A., and Szekerke, M. (1999) Carrier design: new generation of polycationic branched polypeptides containing OH groups with prolonged blood survival and diminished in vitro cytotoxicity. *Bioconjugate Chem. 10*, 781−790.

(16) Nagy, I. B., Haro, I., Alsina, M. A., Reig, F. and Hudecz, F. (1998) Interaction of branched chain polymeric polypeptides with phospholipid model membranes. *Biopolymers 46*, 169−179.

(17) Nagy, I. B., Hudecz, F., Alsina, M. A., and Reig, F. (2003) Physicochemical characterisation of branched chain polymeric polypeptide carriers based on a poly-Lysine backbone. *Biopolymers 70*, 323−335.

(18) Szabó, R., Hudecz F., and Reig F. (2003) Interfacial interactions between poly[L-Lysine] based branched polypeptides and phospholipids model membranes. *J. Colloid Interface Sci. 267*, 18−24.

(19) Hudecz, F., Kóczán, Gy., and Reményi, J. (2002) Peptide or protein based delivery and targeting. In Molecular pathomechanisms and new trends in drug research (Keri, Gy., and Toth, I., Eds.) pp 553−578, Harwood Academic Publishers, Taylor and Francis Group, London.

(20) Perkins, A. C., Frier, M., Pimm, M. V., and Hudecz, F. (1998) Tc^{99m}-branched chain polypeptide (BCP): a potential synthetic radiopharmaceutical. *J. Labelled Compd. 41*, 631−638.

(21) Hudecz, F. (2001) Manipulation of epitope fuction by modification of peptide structure: A minireview. *Biologicals 29*, 197−207.

(22) Gaál, D., and Hudecz, F. (1998) Low toxicity and high antitumour activity of daunomycin by conjugation to immunpotential amphoteric branched polypeptide. *Eur. J. Cancer 34*, 155−161.

(23) Kóczán, Gy., Ghoose, A. C., Mookerjee, A., and Hudecz, F. (2002) Methotrexate conjugate with branched polypeptide influences *Leishmania donovani* infection in vitro and in experimental animals. *Bioconjugate Chem. 13*, 518−524.

(24) IUPAC−IUB Commission on Biomedical Nomenclature. (1984) *Eur. J. Biochem. 138*, 9−37.

(25) Hudecz, F., and Szekerke, M. (1980) Investigation of drug−protein interactions and the drug-carrier concept by the use of branched polypeptides as model systems. Synthesis and characterization of the model peptides. *Collect. Czech. Chem. Commun. 45*, 933−940.

(26) Hudecz, F., Kutassi-Kovács, S., and Kajtár J. (1984) GPC, CD and sedimentation analysis of polylysine and branched chain poly-Lys-poly-L-Ala polypeptides. *Colloid Polym. Sci. 262*, 208−212.

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

(28) Mezö, G., Kajtár, J., Hudecz, F., and Szekerke, M. (1993) Carrier design: Conformational studies of amino acid (X) and oligopeptide (X-DL-Ala_m) substituted poli(l-lysine). *Biopolymers 33*, 873−885.

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

(30) Hudecz, F., Kojima, Y., Miyamoto, Y., Kajtár, J., and Maeda, H. (1994) The effect of charge on the biodistribution of synthetic branched polypeptides in tumour bearing mice. *J. Controlled Release 28*, 301−302.

(31) Suzuki, H., Kurihara, Y., Takeya, M., Kamada, N., Kataoka, M., Jishage, K., Ueda, O., Sakaguchi, H., Higashi, T., Suzuki, T., Takashima, Y., Kawabe, Y., Cyn-shi, O., Wada, Y., Honda, M., Kurihara, H., Aburatani, H., Doi, T., Matsumoto, A., Azuma, S., Noda, T., Toyoda, Y., Itakura, H., Yazaki, Y., and Kodama, T. (1997) A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature 386*, 292−296.

(32) Hume, D. A., and Gordon, S. (1983) Optimal conditions for proliferation of bone marrow-derived mouse macrophages in culture: the roles of CSF-1, serum, Ca21, and adherence. *J. Cell. Physiol. 117*, 189−194.

(33) Fraser, I., Hughes, D. A., and Gordon, S. (1993) Divalent cation-independent macrophage adhesion inhibited by monoclonal antibody to murine scavenger receptor. *Nature 364*, 343−346.

(34) Voyta, J. C. Via, D. P., Butterfield, C. E., and Zetter, B. R. (1984) Identification and isolation of endothelial cells based on their increased uptake of acetylated-low-density lipoprotein. *J. Biol. Chem. 99*, 2034−2040.

(35) Naito, M., Kodama, T., Matsumoto, A., Doi, T., and Takahashi, K. (1991) Tissue distribution, intracellular localization, and in vitro expression of bovine macrophage scavenger receptors. *Am. J. Pathol. 139*, 1411−1423.

(36) Pimm, M. V., Gribben, S. J., Bogdán K., and Hudecz, F. (1995) The effect of charge on the biodistribution in mice of branched polypeptides with a poly(l-lysine) backbone labeled with ^{125}I, ^{111}In or ^{51}Cr. *J. Controlled Release 37*, 161−172.

BC050168F