Effect of the Polylysine Based Polymeric Polypeptides on the Growth and Chemotaxis of *Tetrahymena pyriformis*

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ABSTRACT: Polylysine based branched polypeptides represents a group of biocompatible polymers that could be utilized as macromolecular carriers for drugs, epitopes or reporter molecules. Ten polymers with different character (amino acid composition and charge properties) were prepared: polypeptides with single amino acid in the branches (poly[Lys(X_i)]), X = His, Pro or Glu; and polymers possessing oligo[DL-alanine] side chains only (poly[Lys(DL-Ala$_m$) (AK) or with an additional amino acid residue poly[Lys(X_i-DL-Ala$_m$)] (XAK), where X = Ser (SAK), Thr (TAK), Glu (EAK), acetyl-Glu (Ac-EAK) or succinyl-Glu (Succ-EAK). were investigated. The concentration of these compounds influence the chemotaxis and survival of eukaryotic unicellular model organism, *Tetrahymena pyriformis* GL. Two types of experiments were performed. First the polymer induced chemoattractant/chemorepellent response of *Tetrahymena* cells were tested, then chemotactic selection experiments were performed. The chemotactic responses elicited by the polymers were dependent not only on chemical properties (composition, charge and the length of the side chain) of the compounds, but also on their concentration. Based on these results, the

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polymers were grouped as full-chemoattractant expressing this behavior in the full concentration range investigated (H_iK), full-chemorepellent (E_iK and Ac-EAK) and partial chemoattractant/chemorepellent with concentration dependent activity (P_iK, EAK and Succ-EAK).

KEY WORDS: polylysine based polymeric polypeptides, *tetrahymena*, chemotaxis, chemotactic selection, cytotoxicity.

INTRODUCTION

Several attempts have been made to apply synthetic polymeric polypeptides as macromolecular carriers for specific targeting of bioactive molecules to relevant cells. Promising results imply that polylysine based polymeric polypeptides can be utilized as a drug carrier for agents such as the antiparasitic, methotrexate, or the antitumor, daunomycin [1]. On the other hand, it has been reported that polycationic polymers like polylysine [2] or histidylated polylysine [3] form complexes with DNA, which are taken up by human hepatoma cells utilized for oligonucleotide delivery. In our laboratory, several branched chain polymers with polylysine backbone were developed. One type of the polymeric polypeptides provide a single amino acid as side chain attached to the ε-amino-groups of the lysine residues (poly[Lys(X_i)]), X_iK. Another group of polypeptides contain a relatively short oligo[DL-alanine] side chain and a single amino acid coupled to the α-amino group of the terminal alanine with the general formula poly[Lys(X_i-DL-Ala_m)], XAK, where $i \cong 1$ and $m \cong 3$.

The biological properties of these polymers were investigated such as the *in vitro* and *in vivo* cytotoxicity [4,5], immunogenicity [4], biodistribution [4,8], blood survival [5], immonomodulatory effect [6,7] as well as the *in vitro* stability against exo- and endopeptidases [1]. It was ascertained that the composition of the side chain, particularly, the character of the terminal amino acid significantly influenced the biological properties of the polymers and the corresponding drug-conjugates [1,4–8]. To establish a correlation between the biological effect and the chemical structure of polypeptide carriers is an important step in the process of developing effective therapeutic bioconjugates.

In this study, our aim was to examine the effect of polylysine based polymers on the chemotactic activity and proliferation of an unicellular model organism, *Tetrahymena pyriformis* GL. This eukaryotic ciliated protozoan is a frequently used subject for studying chemotaxis and also signaling and hormone–receptor interactions. These cells carry

receptors and secrete hormones (e.g. insulin) homologous to the organisms at higher rank of the evolution [9,10], and possess second messenger systems like cAMP, cGMP and calcium-calmodulin [10]. Numerous molecules induce a chemotactic response in *Tetrahymena* including amino acids, peptides, cytokines, hormones [19–23].

For the present investigation, ten polymers with different (amino acid composition and charge properties) on the side chain were prepared: three polypeptides with a single amino acid on the side-chain (poly [Lys(X_i)]), where X = His, Pro or Glu; and seven polymers possessing oligo[DL-alanine] side chains only (poly[Lys(DL-Ala_m) (AK) or with an additional amino acid residue poly[Lys(X_i-DL-Ala_m)] (XAK), where X = Ser (SAK), Thr (TAK), Glu (EAK), acetyl-Glu (Ac-EAK) or succinyl-Glu (Succ-EAK). We report the concentration dependent influence of these compounds on the survival and proliferation of *Tetrahymena* cells as well as their effect on the chemotaxis. Two types of experiments were performed. First the dose dependent chemotactic response of *Tetrahymena* cells was tested. This was followed by chemotactic selection experiments. Chemotactic selection is a technique, to determine the chemotactic capacity of different signal molecules to form subpopulations from mixed cultures of cells [23,24] by the analysis of biological response after an approximately 70th generation of *Tetrahymena* cells.

EXPERIMENTAL

Abbreviations

Abbreviations of amino acids and their derivatives follow the 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 is used in accordance with the recommended nomenclature of graft polymers [10]. For the sake of brevity codes of branched chain polypeptides were constructed using the one-letter symbols of amino acids. All amino acids were L-configuration unless otherwise stated. The other abbreviation in this paper are the following – Z: benzyloxycarbonyl, Boc: tert-butyloxycarbonyl, Pcp: pentachlorophenyl, NCA: *N*-carboxy anhydride, HOBt: 1-hydroxy-benzotriazole, DMF: *N*,*N*-dimethyl formamide, DMSO: dimethyl sulfoxide, DCM: dichloromethane, NMM: *N*-methylmorpholine, TEA: triethylamine, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PBS: phosphate buffer saline, Ac: acetyl, Succ: succinyl.

Synthesis of Polymeric Polypeptides

Materials

Amino acids, DCM, DMF were from Reanal (Budapest, Hungary), while benzyloxycarbonyl chloride, *N*-methylmorpholine, 1-hydroxybenzotriazole and triethylamine were obtained from Fluka (Buchs, Switzerland). Pentachlorophenol was purchased from Merck (Darmstadt, Germany), while MTT from Sigma (Budapest, Hungary).

Synthesis of Poly[Lys]·HBr

The synthetic procedure for the preparation of polylysine has been described [6]. Briefly, N^{α}-carboxy-N^{ε}-benzyloxycarbonyl-lysine anhydride was polymerized under conditions to obtain a degree of polymerization of approximately 100 (monomer: diethylamine initiator molar ratio = 50 : 1) or approximately 300 (monomer: diethylamine initiator molar ratio = 150 : 1). Protecting groups were cleaved by HBr in acetic acid (35%, m/V) and poly[Lys]·HBr was precipitated with diethylether. The polymer samples were dissolved in water, purified by dialysis against distilled water and recovered by freeze-drying. Removal of protecting groups from the polymer was checked by UV spectroscopy at $\lambda = 254$ nm. The polymer samples were analyzed by sedimentation equilibrium and the average molecular weight and the relative molar masses ($\bar{M}_w$ and $\bar{M}_z$) were determined; in addition, the polydispersity factor ($\bar{M}_z/\bar{M}_w$) and the average degree of polymerization ($\bar{DP}_n$) were calculated.

Synthesis of Poly[Lys(X_i)],(X_iK) Polypeptides

The side chain amino acids were coupled to the ε-NH_2 group of lysine residue as protected pentachlorophenyl ester derivatives (Z-X-OPcp, where X = Glu or Pro; Boc-X-OPcp, where X = His) [14]. Briefly, 4 mmol of the protected and activated amino-acid derivative and equivalent amount (4 mmol, 0.54 g) of 1-hydroxy-bezotriazole dissolved in 10 mL DMF were added to the polymer dissolved in 10 mL of water: DMF (2 : 8, v/v) solvent mixture. A 50% molar excess of both Z- or Boc-X-OPcp and HOBt, calculated for the side-chains of the polymer, was used. The pH 8 of the solution was maintained by adding *N*-methylmorpholine. Stirring was continued at room temperature overnight. The solvent was removed in vacuo and the residue was triturated several times with diethylether containing 10% dichloromethane. Z or Boc protecting groups were cleaved with HBr or acetic acid containing 35% HBr (m/V). The product was dissolved in water, purified by dialysis against distilled water using appropriate tubing and isolated by freeze-drying. Removal

of protecting groups from the polymer was checked by UV spectroscopy at $\lambda = 254$ nm.

Synthesis of Poly[Lys(X_i-DL-Ala_m)],(XAK) Polypeptides

XAK type polypeptides and AK were synthesized by grafting short oligomeric DL-Ala chains to the ε-NH_2 group of lysine residues using *N*-carboxy-DL-Ala-anhydride. Poly[Lys]·HBr (0.13–0.15 g, 1.0–1.15 mmol) was dissolved in 5 mL of distilled water and neutralized by equimolar amount of triethylamine ($\sim 7\,\mu$L). To this solution 0.5 g (4 mmol) of *N*-carboxy-DL-alanine anhydride dissolved in 3 mL dioxane was added. The polymerization was continued at room temperature for 2 days. Afterwards the reaction mixture was dialyzed against distilled water for two days using Visking tube (cutoff = 12,000–14,000 Da). The filtered solution was freeze-dried. The terminal amino acids were coupled to the α-NH_2-groups of the oligo(DL-Ala) side chains as Z-X-OPcp, where X = Glu, Ser, Thr. AK (1.0 g, 2.4 mmol) ($\bar{M}_w = 24200$ and 32900; $\overline{DP}_n = 60$ and 80, respectively) was dissolved in 5 mL of distilled water and it was diluted with 25 mL of DMF. Z-X-OPcp (2.6 mmol) and equivalent amount of HOBt (0.35 g, 2.6 mmol) in 25 mL of DMF were added to the solution of AK. The pH was adjusted to 7.5 with NMM. The reaction was continued overnight at room temperature, then the solvent was removed by evaporation. The remaining oil was solidified by diethylether, and washed with 10% DCM/ether mixture. The dried material was suspended in 10 mL of acetic acid. The cleavage of Z protecting groups was performed with 35% HBr/acetic acid for 4 h at room temperature. The unprotected polymer was precipitated by diethylether, and washed with ether several times after filtration. The product was dried over P_2O_5 and KOH and dissolved in water and dialyzed in Visking tube (cutoff = 12,000–14,000 Da) for 2 days against distilled water followed by freeze-drying [6,13].

Synthesis of Polyanionic Derivatives of Poly[Lys(Glu_i-DL-Ala_m)],(EAK)

Acetylation of EAK: Poly[Lys(Glu_i-DL-Ala_m)],(EAK) (85 mg, 159 μmol) was dissolved in 2 mL of distilled water and diluted with 10 mL of DMF under cooling; then TEA (27 μL, 198 μmol) were added to this solution to neutralize the polymer. For the acetylation, 5 Eqv. freshly prepared imidazolyl-acetate was used [15]. The product was dialyzed in Visking tubes (cutoff = 12,000–14,000 Da) against distilled water for 2 days followed by lyophilization freeze-drying.

Succinylation of EAK Poly[Lys(Glu_i-DL-Ala_m)](EAK) (10 mg, 18.5 μmol) was dissolved in 2 mL of 0.1 M carbonate buffer (pH = 9.2). To this solution 220 μL (220 mmol) of succinic anhydride dissolved in DMSO ($c = 100$ mg/mL) was added in aliquots with continuous stirring

in 30 min at RT. The pH was maintained between 9.0 and 9.2 with 0.1 M NaOH. After 4 h the solution was dialyzed against distilled water for 2 days and then lyophilized.

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

Functional Studies

Cells and Culturing

Tetrahymena pyriformis GL cells were maintained in culture medium containing 1% tryptone and 0.1% yeast extract (Difco, Michigan, USA) in distilled water (pH = 7.2). Cells of logarithmic growth phase (48 h) were assayed, cell density was 10^4 cells/mL.

Proliferation Assay

Tetrahymena cells were divided into 96 well tissue-culture plates in 100 μL culture medium (1% tryptone and 0.1% yeast extract dissolved in distilled water) with initial cell number 10^3 cells/well. The polymers were dissolved in fresh culture medium and then added to the cells at final concentrations of 0.2, 2.0, 20 and 200 μg/mL. After 6 and 24 h incubation at 28°C μg the amount of the living cells was determined by MTT-assay using 10^{-3} mg/mL final concentration of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in each well. After a 3 h incubation, the absorbance was measured with ELISA-reader (Labsystems MS Reader) at λ=540 nm and 620 nm as reference wavelength [17]. Statistical analysis of data was performed by ANOVA of Origin4.

Chemotaxis Assay

The chemotactic activity of *Tetrahymena* cells was examined with a modified two-chamber capillary chemotaxis method [18,19]. Briefly, the tip of a micropipette served as the inner chamber filled with the test solution. The outer chamber was a microtitration plate well filled with the *Tetrahymena* cells (3×10^4 cells/well). After 20 min incubation, the cells that responded positively and moved to the inner chamber were fixed in 4% formaldehyde dissolved in PBS (pH = 7.4). Approximately 10–20% of untreated control cells move into the inner chamber. The number of the cells was determined by Neubauer hemocytometry. Statistical analysis of the data was performed by ANOVA of Origin4. In the present studies the dependence of the chemotactic activity

on the polymer concentration was investigated using 0.02, 0.2, 2.0 and 20.0 μg/mL concentrations, while fresh culture medium without polymer served as the control.

Chemotactic Selection

Using the chemotaxis assay described above, the positively responding cells were transferred to fresh culture medium for cultivation. Cultures were selected with polymer solution (P) as well as fresh culture medium as control (C), and selected populations obtained were transferred consecutively every 48 h for one week. Chemotactic responsiveness of the selected cultures was determined in the following combinations: (1) cells selected with control medium were coursed towards control medium (C/C); (2) cells selected with control medium towards the identical polymer (C/P); (3) cells selected with polymer solution towards control medium (P/C); (4) cells selected with polymer solution towards the identical polymer (P/P). The responder cells were counted by hemocytometry. Statistical analysis of data was performed by Student's *t*-test. From the number of responding cells chemotactic selection coefficient (Ch_{sel}) was defined [22,23] and calculated by the formula:

$$Ch_{sel} = (P/P \times C/C)/(P/C \times C/P) \quad (1)$$

Statistical analysis of data was performed by ANOVA of Origin4.

RESULTS AND DISCUSSION

Synthesis

In this study, the effect of polylysine based branched chain polymeric polypeptides on the proliferation and chemotaxis of the unicellular *Tetrahymena pyriformis* GL was investigated. Three types of branched polypeptides were synthesized. The first, poly[Lys(X_i)], (X_iK) single amino acid (Glu, His or Pro), was coupled to the ε-NH_2 groups of the lysine residue by *in situ* active ester method. The second type of polymer, poly[Lys-(DL-Ala_m)], (AK) polymer, was produced by the polymerization of DL-Ala-*N*-carboxy anhydride using the ε-NH_2 groups of polylysine as multifunctional initiator. The side chains of the third type of polymeric polypeptides, poly[Lys(X_i-DL-Ala_m)], (XAK), contained a single amino acid residue (Glu, Ser or Thr), coupled to the terminal α-NH_2 group of the oligo[DL-Ala] branches grafted to the polylysine backbone. The characteristics of the polypeptides are shown in Table 1.

Table 1. Characteristics of polylysine based branched chain polymer polypeptides with poly[Lys(X_i)] or poly[Lys(X_i-DL-Ala$_m$)] formula.

Polymer	Abbreviation[a]	Amino acid composition[b] Lys	Ala (*m*)	*X* (*i*)	DP_n[c]	M_w[d] ± 5%
Poly[Lys(Pro$_i$)]	P$_i$K	1.00	–	0.95	84	24800
Poly[Lys(His$_i$)]	H$_i$K	1.00	–	0.56	93	15400
Poly[Lys(Glu$_i$)]	E$_i$K	1.00	–	1.00	94	25700
Poly[Lys(DL-Ala$_m$)]	AK	1.00	4.50	–	80	32900
Poly[Lys(Ser$_i$- DL-Ala$_m$)]	SAK	1.00	3.80	1.00	60	29400
Poly[Lys(Thr$_i$- DL-Ala$_m$)]	TAK	1.00	3.80	0.90	60	29300
Poly[Lys(Glu$_i$- DL-Ala$_m$)]	EAK	1.00	4.30	1.00	80	44500
Poly[Lys(Ac-Glu$_i$- DL-Ala$_m$)]	Ac-EAK	1.00	3.00	0.96	61	36500
Poly[Lys(Succ-Glu$_i$- DL-Ala$_m$)]	Succ-EAK	1.00	4.00	1.00	80	51400

[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 6M 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 ($\overline{DP}_n$) of poly[Lys] and of the side chain composition.

The Effect of the Polypeptides on the Proliferation of *Tetrahymena pyriformis*

Tetrahymena pyriformis cells were incubated in the presence of branched chain polypeptides with different charges. Cells were treated with polymers at various concentrations (0.2–200 μg/mL) for 6 or 24 h. Control cells were incubated with solvent without polypeptide for the same period of time. Proliferation of cells was calculated from the absorbance measured by the MTT assay by the formula: -[1-(A_{540} treated cells/A_{540} untreated control)]×100 and expressed as percentage of control. The data obtained are summarized in Figures 1 and 2. Among the polylysine derivatives with a single amino acid in the side chain, no polymers induced cytotoxicity in the concentration range studied (Figure 1a). Poly[Lys(His$_i$)], (H$_i$K), poly[Lys(Pro$_i$)], (P$_i$K) and amphoteric poly[Lys(Glu$_i$)], (E$_i$K) exhibited weak (up to 20 %, H$_i$K and P$_i$K) or no (E$_i$K) proliferative effect of the cells after 6 h. A small influence on proliferation was observed after incubation for 24 h with H$_i$K and P$_i$K (Figure 2a). No cytotoxic effect of poly[Lys(DL-Ala$_m$)], AK and poly[Lys(Ser$_i$-DL-Ala$_m$)], SAK was registered after 6 and 24 h (Figures 1b,2b), while the treatment with poly[Lys(Thr$_i$-DL-Ala$_m$)], TAK showed a slight cytotoxicity after 6 h (up to 15 %) and a small but statistically significant effect at 2.0 μg/mL after 24 h incubation (Figures 1b,2b). After a 24 h exposure besides TAK at 200 μg/mL, the three polymers containing short oligomeric side-chains had no marked effect on

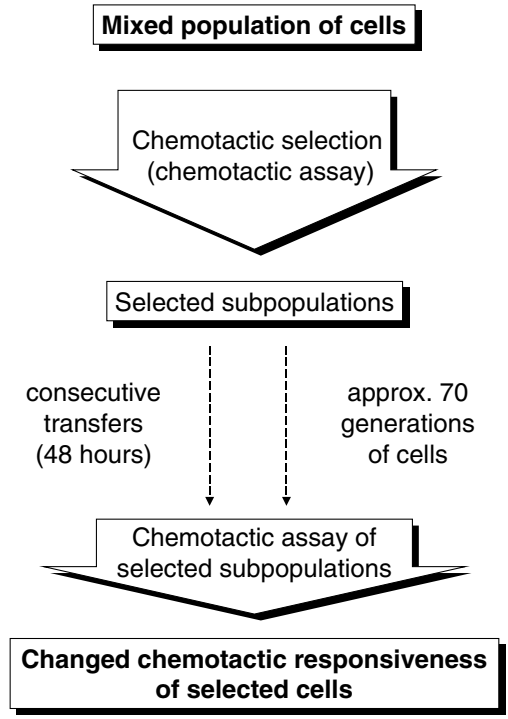

Figure 1. The schematic outline of the chemotactic selection.

proliferation. Amphoteric poly[Lys(Glu$_i$-DL-Ala$_m$)], EAK, and polyanionic poly[Lys(Ac-Glu$_i$-DL-Ala$_m$)], Ac-EAK and poly[Lys(Succ-Glu$_i$-DL-Ala$_m$)], Succ-EAK were not toxic to the *Tetrahymena pyriformis* cells after 6 or 24 h and expressed limited increase (up to 20%) or no influence on cell proliferation (Figures 2c, 3c). Taken together, neither cytotoxic nor proliferative effects by the polymers on the *Tetrahymena pyriformis* cells had surpassed the 20% value for the wide range of polymer concentration applied. Therefore, based on these observations, it seems that the branched polypeptides essentially have no marked effect on the viability and proliferation of *Tetrahymena pyriformis* cells.

The Effect of the Polypeptides on the Chemotaxis of *Tetrahymena pyriformis*

The chemotactic responses, of *Tetrahymena pyriformis* to branched polypeptides are shown in Figures 4–7. Among the polymers containing single amino acids in the side chain, polycationic H$_i$K, induced significant

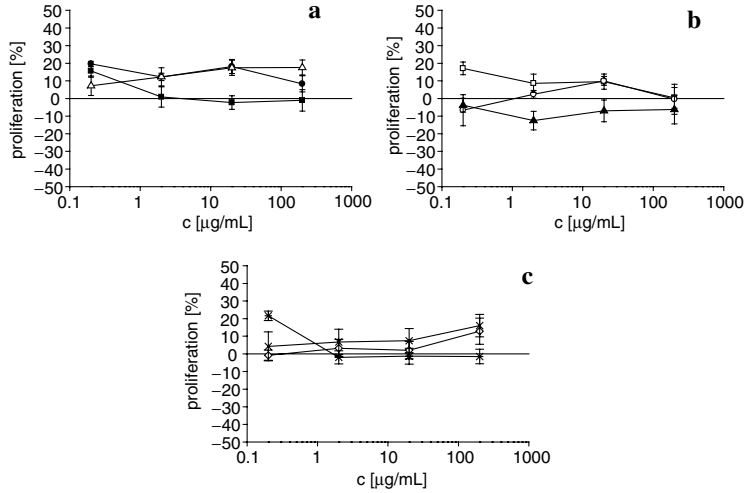

Figure 2. Proliferation of *Tetrahymena pyriformis* treated with polylysine based polymers after 6 h: H_iK –●–, P_iK –△–, E_iK —■—(a); AK–□–, SAK –○–, TAK –▲– (b); EAK –◇–, Ac-EAK –×–(c) and Succ-EAK –⋆–(d). Relative amount of cells was determined by MTT assay. The proliferation index was calculated by the formula-[1-(A_{540} treated cells/A_{540} untreated control)]×100 and expressed as percentage of untreated control. Statistical analysis of data was performed by Student's *t*-test.

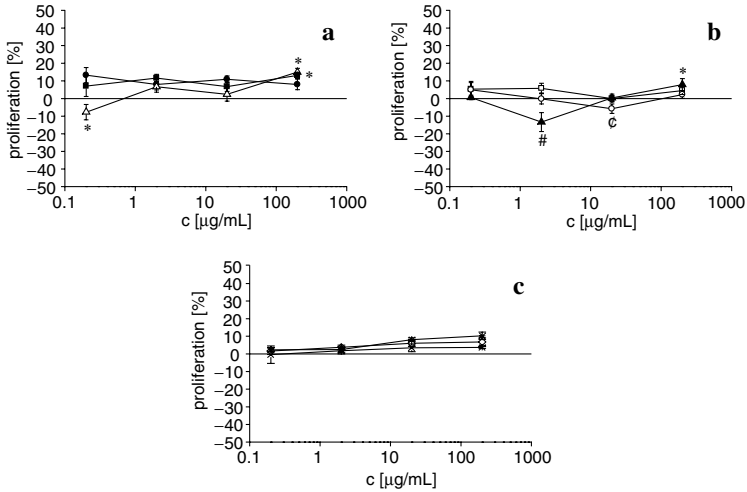

Figure 3. Proliferation of *Tetrahymena pyriformis* treated with polylysine based polymers after 24 h: H_iK –●–, P_iK –△–, E_iK —, (a); AK —□–; SAK –○–, TAK –▲–, (b); EAK –◇–, Ac-EAK –×– and Succ-EAK –⋆–, (c). Relative amount of cells was determined by MTT assay. The proliferation index was calculated by the formula -[1-(A_{540} treated cells/A_{540} untreated control)]×100 and expressed as percentage of untreated control. Statistical analysis of data was performed by Student's *t*-test (*, $p<0.01$, #, $p<0.001$).

positive chemotaxis in a wide concentration range (0.02–2 μg/mL) (Figure 4a). The polycationic P_iK was a weak attractant at 0.02–2 μg/mL, but proved to be repellent with a chemotaxis index of 25% at $c = 20$ μg/mL (Figure 4b), while the amphoteric E_iK elicited a chemorepellent response (30–75%) by the cells; this was more pronounced at higher concentrations (Figure 4c). In the group of polycationic polypeptides containing oligo[DL-Ala] in their side chain, the chemotactic response was different. Polymer AK induced essentially no chemotactic response (Figure 5a) SAK exhibited marked chemorepellent effect (50%) on cells at low concentration (0.02 μg/mL) (Figure 5b). while the hydroxy amino acid (Thr) was chemoattractant at the highest concentration studied (20 μg/mL)(Figure 5c). Summarized in Figure 6 are results obtained by amphoteric and polyanionic polymers. The amphoteric EAK polypeptide (Figure 6a) exhibited statistically significant chemoattractant properties at low and high concentrations (0.02 and 20 μg/mL, respectively). A curve with two peaks surprising since the leukocyte-attractant peptide formyl-Nle-Leu-Phe [21] and bradykinin

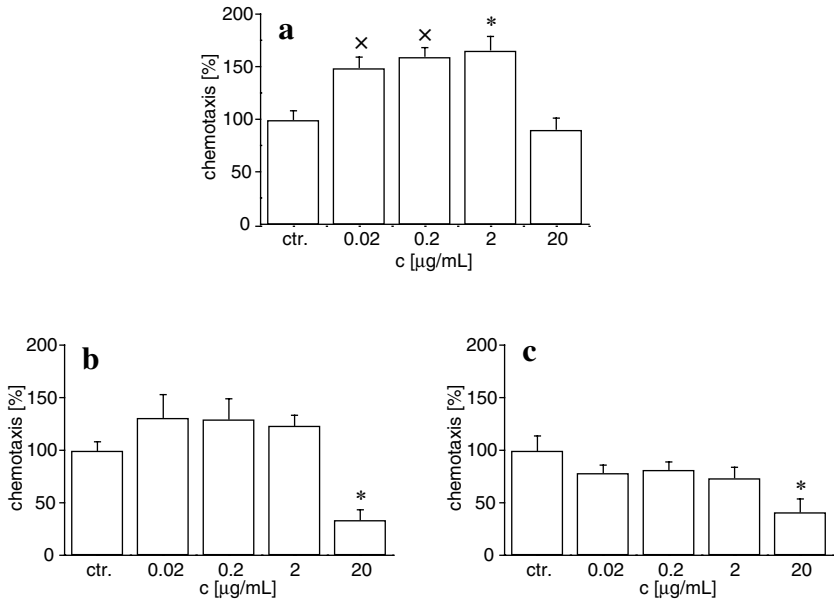

Figure 4. The effect of polylysine based branched chain polymers on the chemotaxis of *Tetrahymena pyriformis* GL: H_iK (a); P_iK (b); E_iK (c). The number of migrated cells was counted in Neubauer hemocytometer. The chemotactic index was expressed as percentage of control. Statistical analysis of data were performed by Student's *t*-test (×, $p<0.05$, *, $p<0.01$).

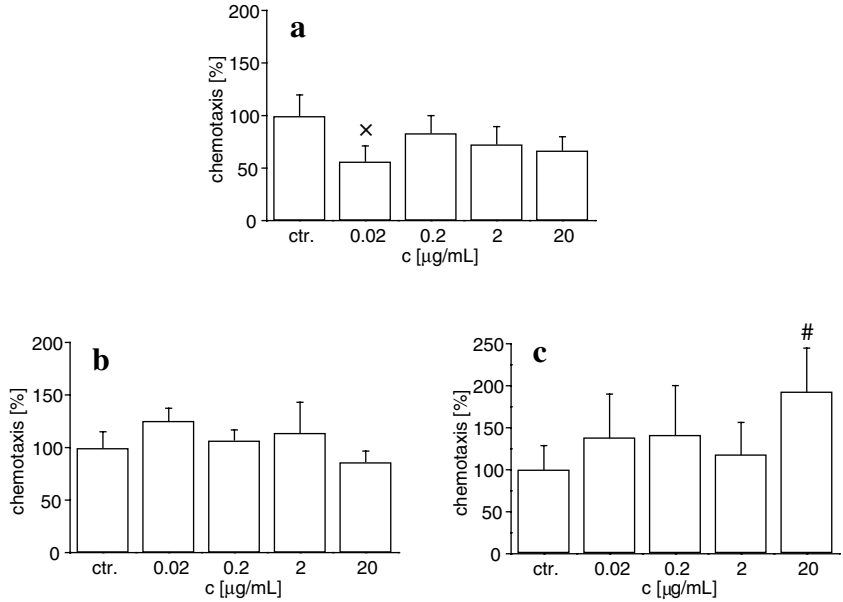

Figure 5. The effect of polylysine based branched chain polymers on the chemotaxis of *Tetrahymena pyriformis* GL: AK (a); SAK (b); TAK (c). The number of migrated cells was counted in Neubauer hemocytometer. The chemotactic index was expressed as percentage of control. Statistical analysis of data were performed by Student's *t*-test (×, $p<0.05$, #, $p<0.001$).

[22] also have this bi-phase profile in chemotaxis. This phenomenon could be explained by the assumption that there are two types of binding sites for EAK with different affinities. However, it is more likely, that the response observed at high EAK concentration is nonspecific. The polyanionic Ac-EAK, with a single negative charge per unit, was repellent with a chemotaxis index of 70% in the whole concentration range (Figure 6b). In contrast, the dose–response curve for Succ-EAK, with two negatively charged groups per side chain, shows a bell-shape correlation with pronounced chemoattractant activity peaking at $c = 2\,\mu g/mL$ and with a chemotaxis index of $176 \pm 25\%$ (Figure 6c).

Chemotaxis of the Selected *Tetrahymena* Populations

A mixed *Tetrahymena* population were selected with the chemoattractant polymers (polycationic H_iK, P_iK, and TAK, amphoteric EAK and polyanionic Succ-EAK) used at the lowest effective concentration; the results are shown in Figure 7. The cells selected with control medium displayed a significant positive chemotaxis towards H_iK

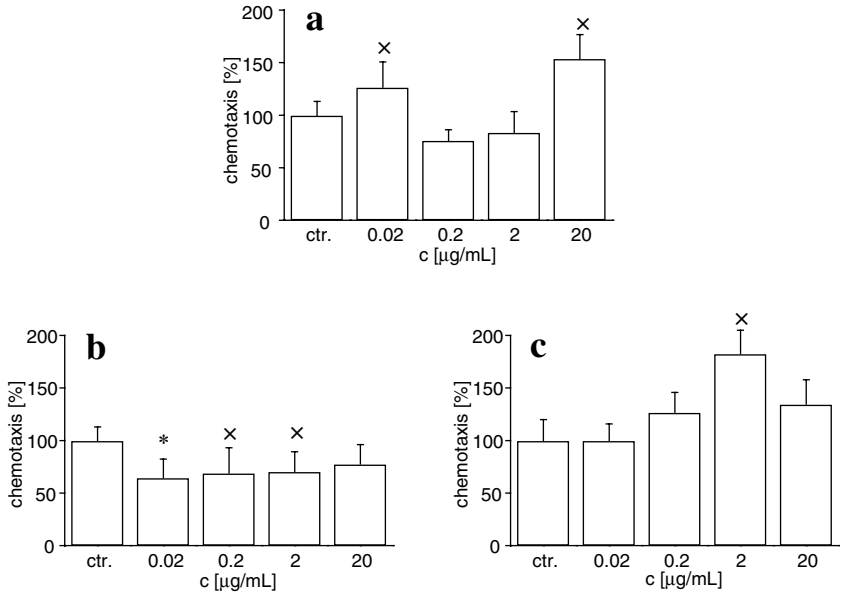

Figure 6. The effect of polylysine based branched chain polymers on the chemotaxis of *Tetrahymena pyriformis* GL: EAK (a), Ac-EAK (b), Succ-EAK (c). The number of migrated cells was counted by Neubauer hemocytometry. The chemotactic index was expressed as percentage of control. Statistical analysis of data were performed by Student's *t*-test (×, $p<0.05$, *, $p<0.01$).

(519 ± 64%) (Figure 7a) and P_iK (142 ± 10%) (Figure 7b). The cells selected with H_iK showed an increased chemotactic activity towards the control medium (264 ± 47%) as well as towards the polymer itself (338 ± 47%) (Figure 7a) In the case of cells selected with TAK (Figure 7c), amphoteric EAK (Figure 7d), and anionic Succ-EAK (Figure 7e), a decreased chemotaxis was observed towards the control medium (C/polymer) and the identical polymer (polymer/polymer) combinations. In order to compare the chemotactic properties of these polypeptides on selected cell populations, the selection quotients (Ch_{sel}) were calculated and presented in Figure 7 [22,23]. Ch_{sel} can be considered as a measure of chemoattractant ($Ch_{sel} > 1.0$) or chemorepellent ($Ch_{sel} < 1.0$) activity, providing a sensitive criterion for distinguishing long and short term selection. Among polymers studied, EAK had a high selection quotient ($Ch_{sel} = 1.12$). The polypeptides with polycationic (H_iK, P_iK and TAK) or anionic (Succ-EAK) charge properties were not able to select positive responder subpopulations from the mixed culture of *Tetrahymena*. This lack of responsiveness was determined by the

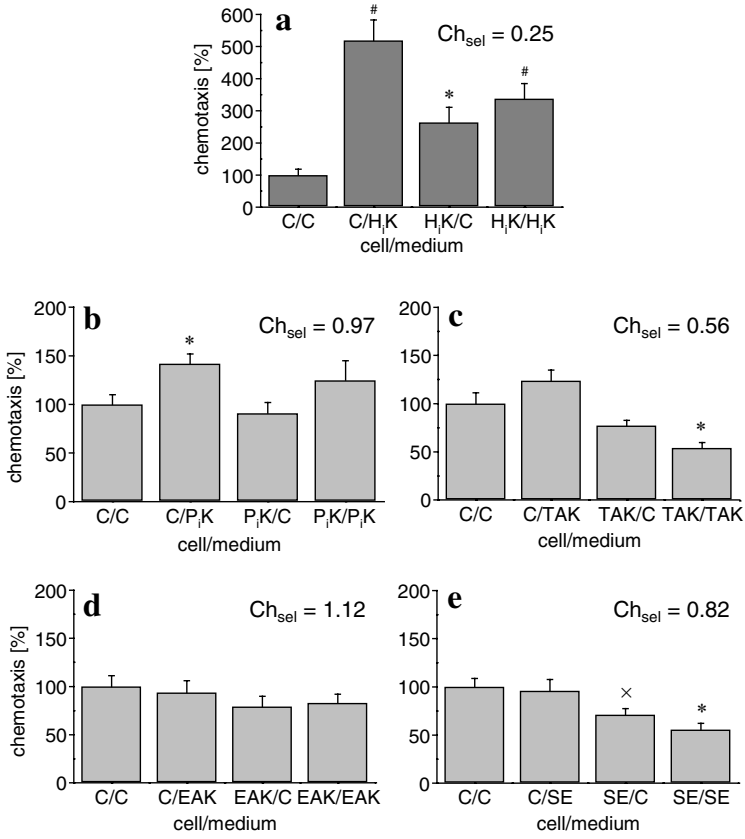

Figure 7. Chemotactic selection of mixed *Tetrahymena pyriformis* population by polylysine based polymeric polypeptides: (SE: Succ-EAK). Cells selected with control medium were coursed towards control medium (C/C), cells selected with control medium towards the polymer (C/P), cells selected with polymer solution towards control medium (P/C), cells selected with polymer solution towards polymer (P/P). The number of migrated cells was counted by Neubauer hemocytometry. The chemotactic index was expressed as percentage of C/C. Statistical analysis of data were performed by Student's *t*-test (×, $p<0.05$, *, $p<0.01$, #, $p<0.001$).

reduced values of Ch_{sel}, which were 0.97 for P_iK, 0.82 for Succ-EAK, and 0.56 for TAK, respectively. This data indicates that a short-term effect is characteristic of the interaction between these polymers and *Tetrahymena pyriformis* cells. In case of H_iK, the selection coefficient ($Ch_{sel} = 0.25$) was low; however, a pronounced positive selection was observed. This could indicate the involvement of a ligand-specific, but *in situ* assembled receptor on *Tetrahymena* cells.

CONCLUSION

This study showed that the composition and the length of the side chain on the polylysine based branched chain polymers, can effect a marked influence, on chemoattractant/chemorepellent effects on *Tetrahymena pyriformis* cells. The chemotactic responsiveness elicited by these polymers was dependent not only on the chemical properties (e.g. charge) of the compounds studied, but also on their concentration. Based on these results, these polymers can be grouped as: (a) full-chemoattractant, expressing this behavior in the full concentration range investigated (e.g. H_iK), (b) full-chemorepellent (e.g. E_iK, Ac-EAK) and (c) partial chemoattractant/chemorepellent with concentration dependent activity (e.g. P_iK, and Succ-EAK). Considered homologies between chemotaxis of *Tetrahymena* and cells from organisms at higher rank of the phylogeny, these data could be helpful for the analysis of structural features required for the design of biocompatible polymers with or without chemoattractant/chemorepellent activity.

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REFERENCES

1. Hudecz, F. (1995). Design of Synthetic Branched-chain Polypeptides as Carriers for Bioactive Molecules, *Anti-Cancer Drugs*, **6**: 171–193.
2. Wu, G.Y. and Wu, C.H. (1988). Evidence for Targeted Gene Delivery to HepG2 Cells in vitro. *Biochemistry*, **27**: 887–892.
3. Mahajoub, B.R. and Midoux, P. (2001). Histidylated Polylysine as DNA Vector: Elevation of the Imidazole Protonation and Reduced Cellular Uptake without Change in the Polyfection Efficiency of Serum Stabilized Negative Polyplexes, *Bioconjugate Chem.*, **12**: 92–99.
4. Hudecz, F., Gaál, D., Kurucz, I., Lányi, Á., Mezö, G., Rajnavölgyi, É. and Szekerke, M. (1992). Carrier Design: Cytotoxicity and Immunogenicity of Synthetic Branched Polypeptides with Poly(L-lysine) Backbone, *J. Controlled Release*, **19**:231–244.
5. 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.

6. 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: Gebelein, Ch.G. and Carraher, Ch.E. (eds.), *Polymeric Materials in Medication*, pp. 265–289, Plenum Press, New York.
7. Gaál, D. Hudecz, F. Kovács, A.L., and Szekerke, M. (1986). Immunomodulatory Effect of Synthetic Branched Polypeptides. II, *J. Biol. Response Modifiers*, **5**: 148–159.
8. Clegg, J.A., Hudecz, F., Mezö, G., Pimm, M.V., Szekerke, M. and Baldwin, R.W. (1990). Carrier Design: Biodistribution of Branched Chain Polypeptides with Poly(L-lysine) Backbone, *Bioconjugate Chem.*, **2**: 425–430.
9. Christopher, G.K. and Sundermann, C.A. (1995). Isolation and Partial Characterization of the Insulin Binding Sites of Tetrahymena Pyriformis, *Biochem. Biophys. Res. Commun.*, **212**: 515–523.
10. Csaba, G. (1994). Phylogeny and Ontogeny of Chemical Signaling: Origin and Development of Hormone Receptors. *Intl. Rev. Cytol.*, **155**: 1–47.
11. (1984). IUPAC-IUB Commission on Biomedical Nomenclature *Eur. J. Biochem.*, **138**: 9–37.
12. Mezö, G., Reményi, J., Kajtár, J., Barna, K., Gaál, D. and Hudecz, F. (2000). Synthesis and Conformational Studies of Poly(L-lysine) Based Branched Polypeptides with Ser and Glu/Leu in the Side Chains, *J. Controlled Release*, **63**: 81–95.
13. Mezö, G., Kajtár, J., Nagy, I., 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**: 719–730.
14. 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 Poly(L-lysine), *Biopolymers*, **33**: 873–885.
15. Mezö, G., Mezõ, I., Pimm, M.V., Kajtár, J., Seprõdi, J., Teplán, I., Kovács, M., Vincze, B., Pályi, I., Idei, M., Szekerke, M. and Hudecz, F. (1996). Synthesis, Conformation and Hormone-Related in vitro Antitumor Activity of a Gonadotropin-Releasing Hormone Antagonist-Branched Polypeptide Conjugate, *Bioconjugate Chem.*, **7**: 642–650.
16. Nagy, I.B., Dancs, A., Kóczán, Gy., Mezö, G. and Hudecz, F. (2000). Conjugation of HS-Oligopeptides with Polymeric Branched Chain Polypeptides Containing Multiple Amino Groups. *Compatible and Bioactive Polymers*, **15**: 139–154.
17. Scudiero, D.A., Shoemaker, R.H. Paull, K.D. et al. (1988). Evaluation of a Soluble Tetrazolium/Formazan Assay for Cell Growth and Drug Sensitivity

in Culture Using Human and Other Tumor Cell Lines, *Cancer Res.*, **48**: 4827–4833.

18. Leick, V. and Helle, J. (1983). A Quantitative Assay for Ciliate Chemotaxis, *Anal. Biochem.*, **135**: 466–469.
19. Köhidai, L., Kovács, P. and Csaba, G. (1996). Chemotaxis of the Unicellular Green Alga *Dunaliella salina* and the Ciliated *Tetrahymena pyriformis* – Effects of Glycine, Lysine and Alanine and their Oligopeptides, *Bioscience Receptors*, **16**(6): 467–476.
20. Köhidai, L., Soós, P. and Csaba, G. (1997). Effects of Dipeptides Containing the Amino Acid, Proline on the Chemotaxis of *Tetrahymena pyriformis*. Evolutionary Conclusions on the Formation of Hormone Receptors and Hormones, *Cell. Biol. International*, **21**(6): 341–345.
21. Köhidai, L. Kovács, P. and Csaba, G. (1994). Chemotactic Response of Unicellular *Tetrahymena* to a Leukocyte Attractant Peptide and its Repellent Derivative: Evolutionary Conclusions, *Cell. Biol. International*, **18**(2): 119–122.
22. Köhidai, L. and Csaba, G. (1998). Chemotaxis and Chemotactic Selection Induced with Cytokines (IL-8, RANTES and TNFα) in the Unicellular *Tetrahymena pyriformis*, *Cytokine*, **10**: 481–486.
23. Köhidai, L., Schiess, N. and Csaba, G. (2000). Chemotactic Selection of *Tetrahymena pyriformis* GL Induced with Histamine, di-iodotyrosine or Insulin, *Comp. Biochem. Physiol.*, **126**: 1–9.