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Cellular Uptake Mechanism of Cationic Branched Polypeptides with Poly[L-Lys] Backbone

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Abstract

Cationic macromolecular carriers can be effective carriers for small molecular compounds, drugs, epitopes or nucleic acids. Polylysine based polymeric branched polypeptides have been systematically studied on the level of cells and organisms as well. In the present study we report on our findings on the cellular uptake characteristics of nine structurally related polylysine based polypeptides with cationic side chain composed of i) single amino acid (poly[Lys(X_i)], X_iK) or ii) oligo[DL-alanine] (poly[Lys(DL-Ala_m)], AK) or iii) oligo[DL-alanine] with an additional amino acid (X) at terminal position (poly[Lys(X_i-DL-Ala_m)] (XAK)) or iv) at the position next to the polylysine backbone ((poly[Lys(DL-Ala_m-X_i)] (AXK)). *In vitro* cytotoxicity and cellular uptake were characterized on HT-29 human colon carcinoma and HepG2 human hepatocarcinoma cell lines. Data indicate that the polycationic polypeptides studied are essentially non-toxic in the concentration range studied and their uptake is very much dependent on the side chain structure (length, identity of amino acid X and on the distance between the terminal positive charge) and also on the cell lines. Our findings in uptake inhibition studies suggest that predominantly macropinocytosis and caveole/lipid raft mediated endocytosis are involved. The efficacy of their internalization is markedly influenced by the hydrophobicity and charge properties of the amino acid X. Interestingly, the uptake properties of the these polypeptides show certain similarities to the entry pathways of several cell penetrating peptides.

Introduction

Polymers including peptide based carriers are widely used for the delivery of bioactive molecules like antitumor agents[1-3], B- and T-cell epitope peptides[4, 5] or therapeutic macromolecules such as siRNA[6]. Coupling small molecular cytotoxic drugs e.g. daunomycin to a macromolecule can improve solubility, decrease side effects and can give a chance for cell or tissue specific targeting *in vivo*[7]. Considering that the tumor vasculature is more permeable and the lymphatic system is not as well developed as the lymphatic system of the healthy tissues, macromolecules are able to accumulate selectively in the tumor[8, 9] but not in the healthy tissues. The cationic macromolecule polylysine and its derivatives proved to be feasible for nucleic acid delivery over the years – including DNA[10-12], siRNA[13] and microRNA.[14] Polylysine based branched polypeptides could be effective carriers for drugs like daunomicin [7, 15], methotrexate [16, 17] as well as T cell or B cell epitope peptides [18, 19]. This group of polymeric polypeptides was methodically investigated on the level of the organism: toxicity [3, 20], blood survival [20], biodistribution [21, 22]; immunogenicity [23], immunomodulatory effect [24] as well as at cellular level: *in vitro* cytotoxicity [3] and chemotaxis [25] were studied. Interaction of the polypeptides with phospholipid bilayers was also examined. [26, 27] Results of these studies showed that the length and amino acid composition of the side chains attached to the poly[L-Lys] backbone and the charge of the terminal amino acid can determine the biological and physico-chemical properties of the polypeptides and also that of their drug/epitope conjugates. On cellular level we recently demonstrated that poly[L-Lys] based branched polypeptides with different charge properties are taken up effectively by the J774 macrophage like cell line and also by bone marrow macrophages. Polyanionic polypeptides entered the cells via class A scavenger receptor [25, 28], while the polycationic and amphoteric polypeptides most likely have no recognition structure on the cell surface. Although the cellular

uptake of daunomycin–polypeptide conjugates have already been studied and it was established that these conjugates, as well as corresponding free polypeptides, are internalized into murine macrophages and murine and human cell lines [29] and HL-60 human leukemia cells [30], we know little about the mechanism of the internalization.

In the present study we examined the cellular uptake characteristics of nine structurally related polylysine based polypeptides with cationic side chains consisting of a single amino acid (X) (poly[Lys(X_i)], X_iK) or an oligo[DL-alanine] side chain (poly[Lys(DL-Ala_m)], AK) or oligo[DL-alanine] side chains with an additional amino acid X at the *N*-terminal position (poly[Lys(X_i- DL-Ala_m)], XAK) or at position next to the polylysine backbone (poly[Lys(DL-Ala_m-X_i)], AXK) (Figure 1.). Here we report on our findings on *in vitro* cytotoxicity and cellular uptake of the polypeptides on two tumor cell lines of different origin: HT-29 human colon carcinoma and HepG2 human hepatocarcinoma cells. In addition, we also describe the results of a study concerning the cellular uptake mechanism of these polycationic compounds with different side chain architecture. By using a set of endocytic inhibitors we intended to clarify the type(s) of endocytic mechanisms potentially involved in the uptake. Data suggest that the polycationic polypeptides studied are essentially non-toxic in the concentration range studied, but their uptake is very much dependent on the side chain structure, length of the side chain, and identity of amino acid X and on the distance between the backbone and the terminal positive charge, and also on the cell lines. Our findings also suggest that macropinocytosis and caveole/lipid raft mediated endocytosis are typically involved in the uptake of the cationic polymeric polypeptides.

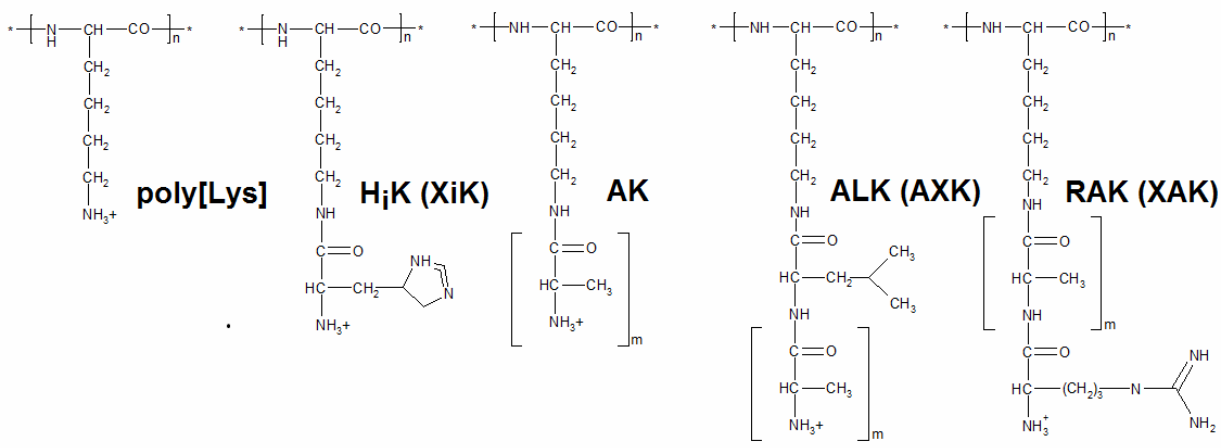

Figure 1. Schematic chemical structure of polylysine and polylysine based branched polypeptides with different side chain composition (m=1-4). The figure shows one representative of each type of polypeptides: X_iK (poly[Lys(X_i)] type H_iK, where X=His; AK (poly[Lys(DL-Ala_m)], AXK (poly[Lys(DL-Ala_m-X_i] type ALK, where X=Leu and XAK (poly[Lys(X_i-DL-Ala_m]) type RAK, where X=Arg.

Results and Discussion

Branched polypeptides with the general formula poly[Lys(X_i)], poly[Lys(DL-Ala_m)], poly[Lys(X_i-DL-Ala_m)] or poly[Lys(-DL-Ala_m-X_i)] ($m \cong 3$, $i \cong 1$) were investigated. The common feature of the group studied is their cationic charge properties. However, the polypeptides varied in the length of their side chain, the identity of the terminal amino acid and/or in the position of amino acid X in relation to the poly[Lys] backbone. First we studied the *in vitro* cytotoxicity of the compounds. This was followed by the analysis of the level of the cellular uptake and the intracellular localization of carboxyfluorescein labeled polypeptides in two different cell lines. The uptake mechanism of selected compounds with elevated ingestion was investigated by applying four different inhibitors related to various forms of endocytosis.

***In vitro* cytotoxicity**

Cytotoxicity of the unlabeled cationic polypeptides was determined at the concentration range of 6.25 μg/mL-100 μg/mL by MTT assay. Results are expressed as a percentage of untreated cells and after that, LC_{50} values were determined as shown in Table 1. Results indicate that only poly[Lys] was toxic both on HT-29 and HepG2 cells (LC_{50}=44.1±4.6 μg/mL and 32.3±10.8 μg/mL, respectively).

Polypeptide[a]	**Code**[b]	**DP_n**[c]	**M_w**[d]	**Cf [%]**[e]	**LC_{50} [μg/mL] (SD)**[f]	
					HT-29	**HepG2**
poly[Lys]	-	130	17000	15.5	44.1(4.6)	32.3(10.8)
poly[Lys($Pro_{0.95}$)]	P_iK	84	18400	18.6	>100	>100
poly[Lys($His_{0.56}$)]	H_iK	93	18900	8.7	>100	>100
poly[Lys($Leu_{0.92}$)]	L_iK	80	18500	10.5	>100	>100
poly[Lys(DL-$Ala_{3.9}$)]	AK	60	24200	10.5	>100	>100
poly[Lys($Ser_{1.0}$- DL-$Ala_{3.8}$)]	SAK	60	29400	2.4	>100	>100
poly[Lys($Thr_{0.9}$- DL-$Ala_{3.8}$)]	TAK	60	29300	14.0	>100	>100
poly[Lys(DL-$Ala_{3.0}$-$Leu_{0.97}$)]	ALK	80	34800	21.7	>100	>100
poly[Lys($Arg_{0.57}$-DL-$Ala_{3.9}$)]	RAK	60	29800	1.3	>100	>100
Arg_8	R_8	-	-	23.2		

Table 1 Chemical characteristics and cytotoxicity of polylysine based branched chain polymeric polypeptides with poly[Lys(X_i)], poly[Lys(X_i-DL-Ala_m)] or poly[Lys(DL-Ala_m-X_i)] formula.

[a]Amino acid composition was determined by amino acid analysis following hydrolysis in 6M HCl at 105°C for 24 hours;

[b]Code of branched chain polymeric polypeptides, based on one-letter symbol of amino acids;

[c]Average degree of polymerization determined by sedimentation equilibrium measurements;

[d]Average molecular mass of each polypeptide was calculated from the average degree of polymerization of poly[Lys] and of the side chain composition

[e]Average degree of substitution (m/m%) for 5(6)-carboxyfluorescein (Cf) was determined by UV spectrophotometry at λ = 492 nm.

[f]LC_{50} values (SD) were determined by fitting a sigmoid (logistic) curve on the cytotoxicity data with Microcal Origin 9.2

Cellular uptake

Concentration dependence

Concentration dependence of the uptake of fluorescently labeled polypeptides was examined by flow cytometry. Cells were treated with the polypeptides at 0.1, 1, 10, 50 and 100 μg/mL concentrations. Earlier we investigated the kinetics of the internalization of Cf-polypeptides on J774 monocyte cell line. [25] Based on that results, we chose 60 minutes as the duration of the treatment. We observed that the uptake of the compounds increased by the elevation of the concentration in both cell lines. However, the dynamics and the level of internalization at a given concentration varied markedly. In case of HT-29 cells, Cf-RAK and Cf-P$_i$K polypeptides were internalized at 50 and 100 μg/mL concentrations, respectively. Whereas Cf-H$_i$K, Cf-L$_i$K, Cf-AK, Cf-SAK Cf-ALK and Cf-poly[Lys], polypeptides were ingested already at 10 μg/mL. Uptake of CPP Cf-Arg_8 was more effective (it was significant from 0.1 μg/mL). (Table 2). In order to

compare the efficacy of the uptake we calculated fluorescence mean data normalized to carboxyfluorescein content of each polypeptide (Figure 2A). According to these values, the uptake of Cf-Arg_8 was comparable to the polypeptides; at 50 μg/mL the most effective uptake was observed as follows: Cf-H_iK>Cf-L_iK>Cf-RAK~Cf-SAK>Cf-AK>Cf-poly[Lys]>Cf-ALK**.** The internalization profile of HepG2 cells was slightly different. Cf-RAK polypeptide entered the cells at 50 μg/mL, while Cf-H_iK, Cf-P_iK, Cf-SAK, Cf-ALK and poly[Lys] polypeptides were taken up from 10 μg/mL concentration, whereas the most effective polypeptides, Cf-L_iK and Cf-AK elicited an uptake already at 1 μg/mL concentration as well as Cf-Arg_8 peptide (Table 3). According to data normalized to Cf content of each polypeptide, the following order of the uptake could be observed at 50 μg/mL concentration: Cf-L_iK>Cf-RAK>Cf-H_iK~Cf-AK>Cf-poly[Lys]>Cf-ALK (Figure 2B). Essentially no (Cf-TAK) or minimal (Cf-P_iK) uptake was observed on either of the cell cultures. The comparison of the uptake by the two cell lines indicates some similarities. Namely, the most engulfed three polypeptides (Cf-L_iK, Cf-H_iK, and Cf-RAK) and the least ingested polypeptides (Cf-P_iK, Cf-TAK) were identical.

Cf-labeled	c [µg/mL]				
(Poly)peptide	**0.1**	**1**	**10**	**50**	**100**
poly[Lys]	1 (0)	32 (4)	1662 (79)*	4487 (238)*	7470(105)*
H_iK	113 (129)	49 (8)	4741 (376)*	11946 (65)*	14095 (1082)*
P_iK	2 (0)	78 (9)	-1 (1)	36 (14)	2139 (97)*
L_iK	9 (4)	39 (17)	566 (88)*	8991 (467)*	23332 (467)*
AK	2.5 (1.4)	14.5 (1.4)	703.5 (29.7)*	4066.5 (99)*	7349 (124)*
SAK	0 (1)	4 (1)	461 (30)*	1406 (6)*	n.d.
TAK	-1 (1)	0 (1)	4 (0)	10 (1)	36 (18)
ALK	9 (5)	30 (1)	575 (54)*	2757 (34)*	5379 (214)*
RAK	1 (1)	2 (4)	63 (12)	845 (91)*	3050 (303)*
Arg_8	9(0)*	214 (0)*	5551 (373)*	48259 (991)*	68895(2971)*

Table 2 Uptake of the fluorophore (Cf)-labeled polycationic polypeptides and cell penetrating peptide Arg_8 by HT-29 cells after 60 minutes. Data represent the average of mean fluorescence values (SD) of a representative experiment of two independent assays after subtracting the control. Statistical analysis of data was performed by Student's t-test of Origin 9.2 (* $p<0.05$).

Cf-labeled	**c [μg/mL]**				
(Poly)peptide	**0.1**	**1**	**10**	**50**	**100**
poly[Lys]	-3 (1)	44 (18)	1489 (1)*	6584 (1048)*	9346 (308)*
H_iK	8 (8)	185 (194)	1717 (28)*	4945 (1488)*	13596(740)*
P_iK	-22 (11)	17 (4)	337 (40)*	745 (102)*	1242 (70)*
L_iK	47 (7)	140 (18)*	1501 (305)*	8570 (94)*	13146(290)*
AK	2 (2)	76 (1)*	1897 (36)*	6582 (59)*	9354 (235)*
SAK	-4 (1)	12 (1)	106 (11)*	100 (11)*	554 (58)*
TAK	-3 (1)	-3 (4)	3 (4)	24 (1)	45 (1)*
ALK	1 (6)	2 (1)	418 (39)*	7043 (2553)*	16786(1963)*
RAK	1 (0)	12 (2)	274 (49)	1006 (222)*	2639 (780)*
Arg_8	4,5 (2)	390 (17)*	4577 (405)*	10663 (774)*	15976 (214)*

Table 3 Uptake of the fluorophore (Cf)-labeled polycationic polypeptides and cell penetrating peptide Arg_8 by HepG2 cells after 60 minutes. Data represent the average of mean fluorescence values (SD) of a representative experiment of two independent assays after subtracting the control. Statistical analysis of data was performed by Student's t-test of Origin 9.2 (* $p<0.05$).

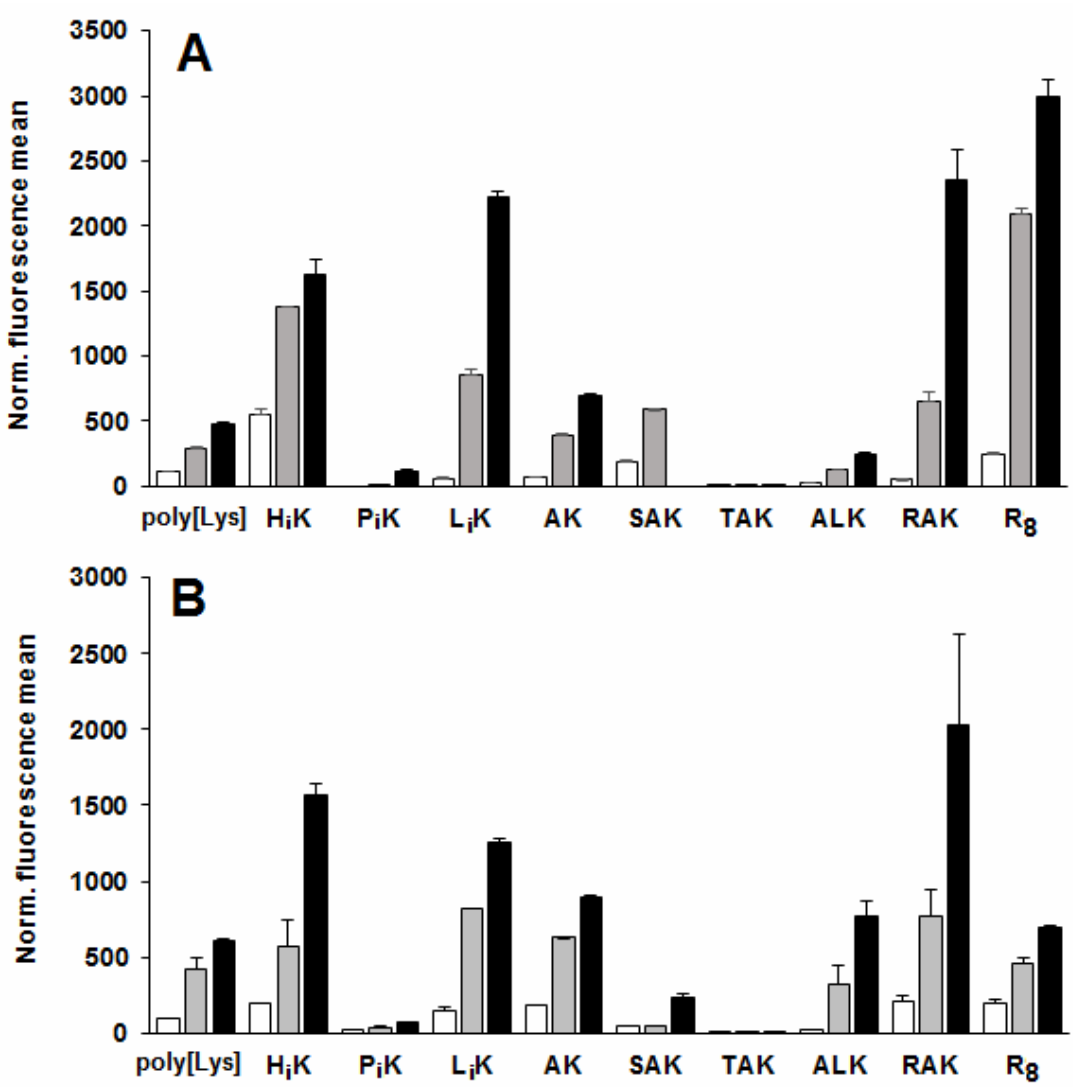

Figure 2. Concentration dependence of the uptake of the Cf-labeled polypeptides by HT-29 (A) and HepG2 (B) cells after 60 minutes. Symbols represent the average of normalized mean fluorescence values ± SD of two independent assays. Data were normalized to carboxyfluorescein content of each Cf-(poly)peptide. ■: 100 μg/mL, ■: 50 μg/mL, □: 10 μg/mL

Intracellular localization

Intracellular localization of the labeled polypeptides was investigated by confocal laser scanning microscopy. Cells were treated with the Cf-polypeptides at 100 μg/ml and with Cf-Arg_8 at 50 μg/ml concentration for 60 minutes. Results indicated that Cf-polypeptides could be detected within the cells; most of the compounds were localized in vesicles in the cytoplasm of the living cells. Cf-poly[Lys] could be observed also in the nucleus in case of both cell lines (Figure 3 and 4), partly in dead cells in the samples due to the toxicity of the compound. Cf-H_iK also was localized in the nuclei of HepG2 cells (Figure 4). Different type of vesicles could be observed depending on the side chain composition of the polypeptides and also on the cell type. In HT-29 cells, cationic and hydrophobic Cf-H_iK and Cf-L_iK polypeptides were localized in small vesicles in the cells (Figure 3). Vesicles of Cf-ALK could be observed mostly at the plasma membrane, which suggests slower uptake of this polypeptide. Larger vesicles like in case of L_iK, AK and ALK may appear due to the aggregation of the polypeptides following of prior to the uptake.

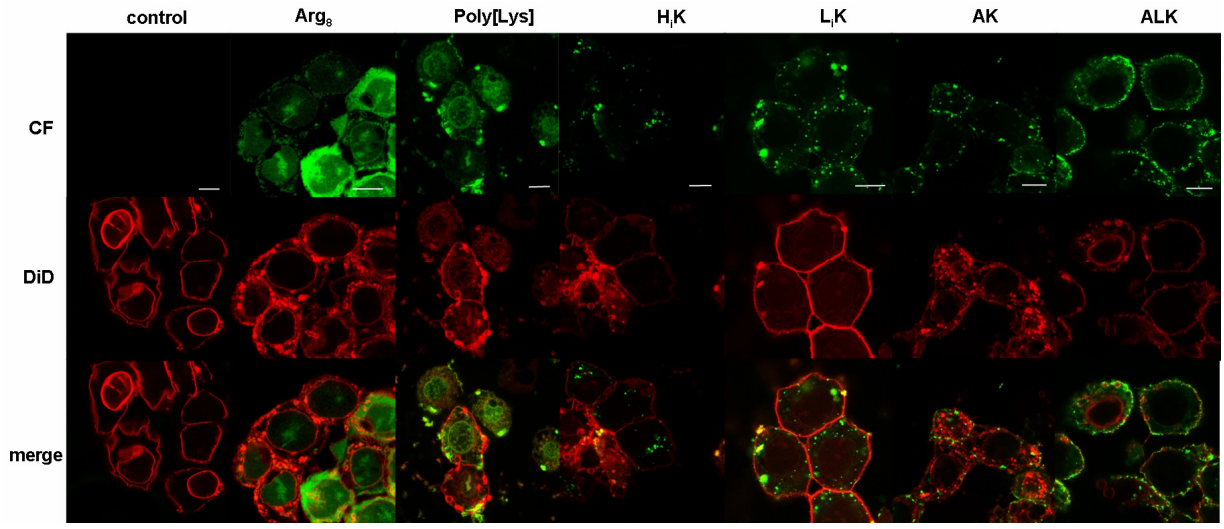

Figure 3 *Intracellular localization of Cf-polypeptides (green) in HT-29 human colon carcinoma cells (c=100 μg/mL) after 60 minutes. Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4). Membrane structures were labeled with DiD perchlorate (red). Each bar represents 10 μm.*

In case of HepG2 cells, Cf-ALK polypeptides showed a more diffuse pattern in the cytoplasm compared to HT-29 cells, whereas vesicles containing Cf-AK were localized at the plasma membrane.

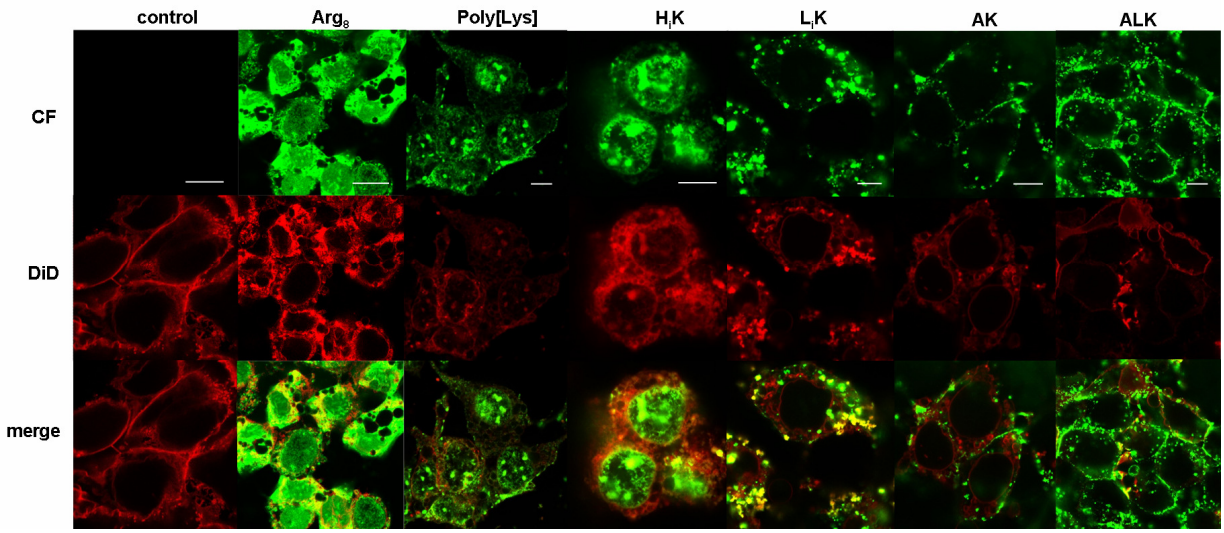

Figure 4. *Intracellular localization of Cf-polypeptides (green) in HepG2 human hepatocarcinoma cells (c=100 μg/mL) after 60 minutes. Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4). Membrane structures were labeled with DiD perchlorate (red). Each bar represents 10 μm.*

The intracellular localization of Arg_8 peptide at c=50 μg/mL concentration was different from that of the polypeptides. The CPP was observed in the nucleus as well as diffusely in the cytoplasm in both cell lines (Figure 3 and 4).

Endocytosis inhibition

In order to shed light on the mechanism of the internalization of the polypeptides, cells were pre-treated with colchicine, the inhibitor of pinocytosis [31], MβCD, inhibitor of caveole/lipid raft mediated endocytosis [32], EIPA and cytochalasin D for inhibiting macropinocytosis [33, 34]. These

experiments were performed on both cell lines, but only with polypeptides that elicited an effective internalization (Cf-poly[Lys], Cf-H$_i$K, Cf-L$_i$K, Cf-AK, Cf-ALK and Cf-RAK) and poly[Lys] a control. A different inhibition pattern could be observed in case of the two cell lines. Uptake of some Cf-polypeptides by HT-29 was significantly inhibited by cytochalasin D (Figure 5A) and by MβCD (Figure 5C), but essentially no inhibition of the ingestion of any studied polypeptide was observed after treatment of the HT-29 cells with EIPA (Figure 5B) or colchicine (Figure 5D). Inhibition of the uptake both by cytochalasin D (Figure 5A) and by MβCD (Figure 5C) was marked for Cf-H$_i$K and for Cf-AK. It is interesting to note that only cytochalasin D blocked the uptake of Cf-L$_i$K and Cf-AK (Figure 5A) and only MβCD was capable to prevent the process with Cf-poly[Lys] to some extent (Figure 5C). In case of HepG2 cells, only Cf-H$_i$K showed a decreased uptake by the cells pre-treated with more than one inhibitor (cytochalasin D) (Figure 6A), EIPA (Figure 6B) and MβCD (Figure 6C). It should be noted that cytochalasin D was also capable to influence the internalization of Cf-L$_i$K, Cf-RAK and Arg$_8$ peptide (Figure 6A). Similarly to the findings with HT-29 cells no effect on polypeptide uptake could be observed after administration of colchicine (Figure 6D). Inhibition data are summarized in Table 4. Based on the comparative analysis of the results we can presume that macropinocytosis is involved in the uptake of Cf-H$_i$K, Cf-L$_i$K, Cf-AK and Cf-ALK polypeptides by HT-29 colon carcinoma cells. Results indicate that caveole/lipid raft mediated endocytosis can be involved in the internalization of Cf-poly[Lys], Cf-H$_i$K, and Cf-ALK by HT-29 cells. Thus combined mechanism (macropinocytosis + caveole/lipid raft mediated endocytosis) could be considered in case of polypeptides H$_i$K and ALK, while the most positively charged polypeptide, RAK can be engulfed mainly by the caveole/lipid raft mediated endocytosis. In case of HepG2 cell line, macropinocytosis as well as caveole/lipid raft mediated endocytosis can both play a role in the

uptake of Cf-H$_i$K polypeptide, whereas Cf-L$_i$K and Cf-RAK polypeptides may enter HepG2 cells predominantly by macropinocytosis. In several cases pre-treatment of the cells with the endocytosis inhibitors resulted in a significant increase in the uptake (e.g. Arg$_8$ peptide and AK polypeptide in the presence of EIPA and cytochalasin D or L$_i$K polypeptide in the presence of EIPA in case of HT-29 cells showed a more pronounced uptake compared to their uptake without inhibitor. These results also suggest that not a single mechanism mediates the internalization of a compound, as one endocytic route is inhibited, other pathways can be activated.

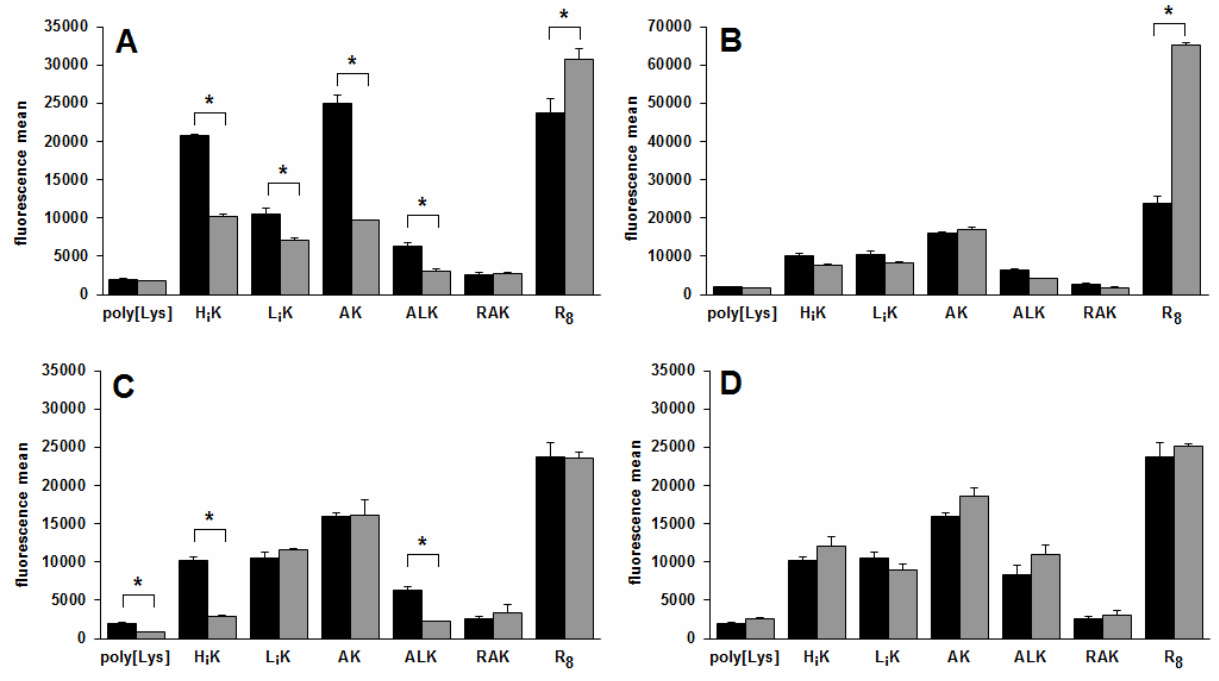

Figure 5 *Uptake of the Cf labeled polypeptides and Arg$_8$ by HT-29 cells in the presence of endocytosis inhibitors. Columns represent the average of mean fluorescence values of a representative experiment of two independent assays ± SD after subtracting the control. Black columns: internalization of the Cf-polypeptides ((c=100 µg/mL, Cf-poly[Lys]: c= 50 µg/mL)) and Cf-Arg$_8$ (R$_8$, c=50 µg/ml) without inhibitor; grey columns: internalization in the presence of*

*A: cytochalasin D (5 μg/ml), B: EIPA (75 μM), C: MβCD (2.5 mM), D: colchicine (10 μM). Statistical analysis of data was performed by Student's t test; *: $p<0.05$.*

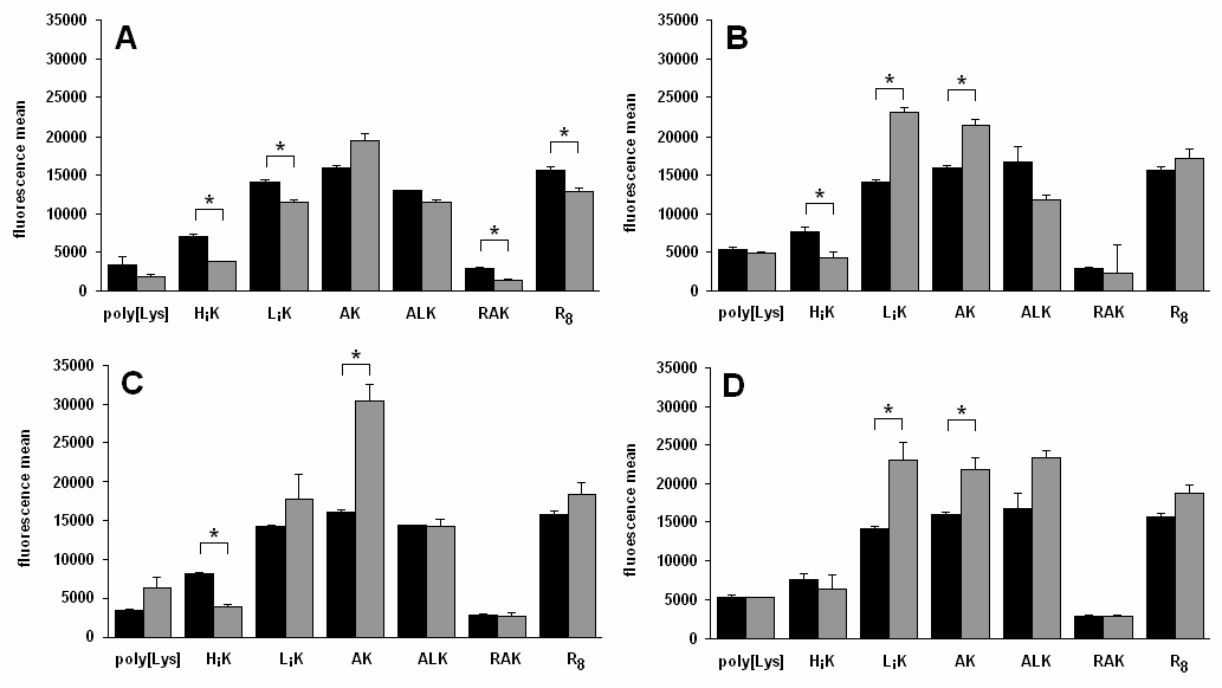

Figure 6. *Uptake of the Cf labeled polypeptides and Arg_8 by HepG2 cells in the presence of endocytosis inhibitors. Columns represent the average of mean fluorescence values of a representative experiment of two independent assays ± SD after subtracting the control. Black columns: internalization of the Cf-polypeptides (c=100 μg/mL, Cf-poly[Lys]: c= 50 μg/mL) and Cf-Arg_8 (R_8, c=50 μg/ml) without inhibitor; grey columns: internalization in the presence of A: cytochalasin D (5 μg/ml), B: EIPA (75 μM), C: MβCD (2.5 mM), D: colchicine (10 μM). Statistical analysis of data was performed by Student's t test; *: p<0.05.*

Several cationic oligopeptides like bradykinin [35] or the opioid peptide TAPA [36] are recognized by specific cell surface receptors. Internalization of other cationic peptides including cell penetrating oligopeptides was also thoroughly investigated. No single and well defined mechanism was determined so far, but in some cases multiple uptake mechanisms were reported [37-39]. Among the entry pathways several passive as well as endocytic mechanisms were described: macropinocytosis, lipid raft or clathrin mediated processes were equally proposed [40, 41]. Several endocytic mechanisms were studied by Lühmann *et al.* in case of poly[Lys] containing (PLL-g-PEG-DNA) nanoparticles: it was established that the uptake of these constructs is energy dependent and endocytic inhibitors could elicit some decrease in the uptake, but no single classical endocytosis pathways could be defined [42]. In the present study we analyzed the uptake of a structurally related group of polymeric polylysine based polypeptides. We investigated if polypeptides enter two human tumor cell lines, HT-29 colon carcinoma and HepG2 hepatocarcinoma, *via* endocytosis and we also tried by using appropriate inhibitors to find similarities in the mechanism of internalization with cell penetrating peptides already studied. Our data indicate that the efficacy of the internalization can be influenced by the composition of the side chain, and hydrophobicity and charge properties of the amino acid X and perhaps by the distance of the positively charged groups from the backbone. The polypeptides most effectively internalized contained a positively charged $^{\alpha}NH_2$-group and another cationic group (imidazole group in H_iK and guanidino group in RAK polypeptide) in their side chains or a positively charged $^{\alpha}NH_2$-group and hydrophobic amino acid (Leu in L_iK) next to the polylysine backbone. In case of P_iK polypeptide, which contains an imino group (instead of $^{\alpha}H_2N$-group) in its side chain; polypeptides TAK and SAK carrying a hydrophilic terminal amino acid and polypeptide

ALK with an $^{\alpha}NH_2$-group at the side chain terminal position, at a longer distance from the backbone, the uptake was much less pronounced. Taken together, those polypeptides elicited the most effective uptake, that carry more than one positively charged group in their side chains close to the polylysine backbone. The uptake of the polypeptides proved to be the same order of magnitude as CPP Arg_8 applied as a positive control.

The mechanism of the uptake proved to be dependent also on the cell type. Most of the cationic polypeptides entered HT-29 cells *via* macropinocytosis or lipid raft mediated endocytosis. Internalization of Arg_8 peptide was inhibited slightly by cytochalasin D in case of HT-29 cells. We could observe a multiple mechanism in case of Cf-H_iK in case of HepG2 cells, which could explain the effective cellular uptake of this polypeptide. Two other polypeptides, L_iK and RAK enter the cells seemingly by macropinocytosis. Direct translocation of some polylysine based polypeptides cannot be excluded however, especially that of poly[Lys] and the H_iK polypeptide, and especially of Arg_8 peptide, which were detected in the nuclei.

Polypeptide	*HT-29*				*HepG2*			
	colchicine	**EIPA**	**cytochalasin D**	**MβCD**	**colchicine**	**EIPA**	**cytochalasin D**	**MβCD**
poly[Lys]	∅	∅	∅	-	∅	∅	∅	∅
H_iK	∅	∅	-	-	∅	-	-	-
L_iK	∅	∅	-	∅	+	+	-	∅
AK	∅	∅	-	∅	+	+	∅	+
ALK	∅	∅	-	-	∅	∅	∅	∅
RAK	∅	∅	∅	-	∅	∅	-	∅
Arg_8	∅	+	+	∅	∅	∅	-	∅

Table 4 *Effect of endocytosis inhibitors on the uptake of polylysine based cationic polypeptides and Arg_8 by HT-29 human colon carcinoma and HepG2 human hepatocarcinoma cell lines. (∅: no inhibition, -: reduced uptake, +: increased uptake)*

Coupling different cargos to the carrier polypeptides can also influence the rate and presumably the mechanism of the internalization. In our earlier studies we observed a different rate of the uptake of a daunomycin containing conjugate (cAD-SuccEAK, poly[Lys(SuccGlu-DL-Ala$_m$)]) and the carboxyfluorescein-labeled polypeptide (Cf-SuccEAK). [29]

Similarly to the polylysine based polypeptides studied by our group, lipid raft mediated endocytosis and macropinocytosis have been described as the mechanism of the internalization in case of a Qdot-R9 conjugate [39] as well as in case of luciferin-CPP conjugates. [43, 44] Molecular constructs consisting of polymeric, but non-peptidic carrier and cell penetrating peptide have been shown before to bear cell penetrating properties due to their CPP content.[45] Therefore it is important to mention that to the best of our knowledge no systematic studies were reported so far on the uptake characteristics of polymeric polypeptides. Our findings suggest that cationic polypeptides could exhibit cell penetrating properties themselves, without the attachment of CPPs. These cationic polylysine based polymeric polypeptides can be promising candidates of further investigations for example in comparative studies with CPP oligopeptides in order to understand the similarities and differences to be utilized for the development of bioconjugates containing chemotherapeutic agents (e.g. antitumor, antimicrobial) or epitopes (e.g. for synthetic vaccines) and cationic polypeptide carriers that can be able to transport small molecules into the cells.

Experimental Procedures

Reagents

Amino acids, HCl and DMF were purchased from Reanal and Molar Chemicals (Budapest, Hungary). Pentachlorophenol, 1-hydroxybenzotriazole, triethylamine, and DMSO were from Fluka (Buchs, Switzerland), acetonitrile was from Merck (Darmstadt, Germany). FCS was obtained from Gibco Brl. and from Sigma-Aldrich (Budapest, Hungary). MTT, 5(6)-carboxyfluorescein succinimid ester, 0.4% trypan blue solution, RPMI-1640 medium powder, glutamine, gentamycin, chemicals for buffers and eluents were from Sigma-Aldrich (Budapest, Hungary). DiD perchlorate was from R&D Systems (Minneapolis, MN, USA).

Polypeptides

Synthesis of poly[Lys(X_i)],(X_iK), poly[Lys(X_i-DL-Ala$_m$)], (XAK) and poly[Lys(-DL-Ala$_m$-X_i), (AXK) type polypeptides

Poly[Lys(X_i)] (X_iK) where X=His, Leu or Pro, XAK-type polypeptides where X=Arg, Ser or Thr were prepared and characterized according to Mező *et al.* [46, 47] Briefly, in case of X_iK and AXK polypeptides, amino acid X was coupled to the $^{\varepsilon}NH_2$-groups of polylysine as Z-X-OPcp, where X=Pro, His or Leu. The coupling reaction was carried out by *in situ* active ester method using an equivalent amount of 1-hydroxybenzotriazole (HOBt) dissolved in DMF as activating reagent. Poly[Lys(DL-Ala$_m$)], (AK) were synthesized by grafting short oligomeric DL-Ala chains to the $^{\varepsilon}NH_2$ group of lysine residues using *N*-carboxy-DL-Ala-anhydride. Polymerization was continued at room temperature for 2 days. Terminal amino acids (X) were coupled to the $^{\alpha}NH_2$-groups of the terminal alanine residues of the oligo-alanine chains as Z-X-OPcp, where X=Arg, Ser or Thr. The polypeptides were purified by dialysis against distilled water for two days using Visking tube (cutoff = 12000-14000 Da) and isolated by freeze-drying. Amino acid composition of the polymeric polypeptides was determined by amino acid analysis in a Beckman 6300 analyzer

(Fullerton, CA) (poly[Lys], P_iK, H_iK, AK, TAK, SAK and ALK) or in a Sykam Amino Acid S433H analyzer (Eresing, Germany) (L_iK, RAK) Prior to the analysis, the samples were hydrolyzed in 6 M HCl in sealed and evacuated tubes at 105°C for 24 hours.

Labeling of polymeric polypeptides with 5(6)-carboxyfluorescein

10 mg (6×10^{-7} – 1.9×10^{-7} mol) polypeptides were dissolved in 2 mL sodium-carbonate buffer (0.1M; pH 9.4). 5(6-)carboxyfluorescein succinimid ester was added to the polypeptide solution dissolved in DMSO (c=10 mg/mL, 0.21 mM) in aliquots with continuous stirring. The reaction was continued for 1 hour at room temperature. The labeled polypeptide was purified on PD10 column filled with Sephadex G25 with distilled water as eluent, then freeze-dried.[28] Carboxyfluorescein content of the samples was determined by a UV spectroscopy using a Perkin Elmer Lambda 2S UV/VIS Spectrometer at λ=492 nm.

Functional studies

Cell culturing

HT-29 human colon carcinoma (ATCC® HB-8065) and HepG2 (ATCC® HTB-38) human hepatocellular carcinoma cell-lines were maintained in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, and 0.16 mg/mL gentamycin in at 37°C in 5% CO_2 atmosphere. Cells were harvested at confluent state by treating with 0.5 g/L trypsin for 10 minutes.

In vitro *cytotoxicity assay*

Cells were plated into 96 well tissue-culture plate in 200 µl culture medium (RPMI 1640, 10% FCS, 2 mM/mL L-glutamine, and 0.16 mg/mL gentamycin) with initial cell number of 10^4 cells/well. Cells were treated with the unlabeled polypeptides dissolved in fresh culture medium at final concentrations of 6.25, 12.5, 25, 50 and 100 µg/mL concentrations. After 60 minutes incubation at 37°C cell viability was determined by MTT-assay. MTT at a final concentration of 0.36 mg/mL was added to each well. After 3 hours incubation formazan crystals were dissolved in DMSO, and then absorbance was measured with ELISA-reader (Labsystems iEMS Reader) at λ=540 nm and 620 nm as reference wavelength. Cytotoxicity was expressed in the percentage of untreated control. Cytotoxic effect was calculated with the following equation:

$$\text{Cytotoxicity } [\%] = (1-OD_{treated}/OD_{control})\times 100$$

50 percent lethal concentration (LC_{50}) was determined by performing a logistic fit on the data points using Microcal Origin 9.2 and the calculating X values at Y=50.

Uptake of carboxyfluorescein-labeled polypeptides

Cells were plated into 24-well tissue culturing plate (10^5 cells/well) 24 hours before the experiments. Cells were washed with serum free medium and then incubated with the solution of the Cf-labeled polypeptides dissolved in serum free RPMI 1640 at 0.1 µg/mL, 1 µg/mL, 10 µg/mL, 50 µg/mL and 100 µg/mL concentration for 1 hour at 37°C or 4°C. In case of inhibition, cells were pre-treated with colchicine (10 µM) inhibitor of pinocytosis [31], MβCD (2.5 mM), inhibitor of caveole/lipid raft mediated endocytosis [32], EIPA (75 µM) [33] and cytochalasin D (5 µg/mL) for inhibiting macropinocytosis [34], for 30 minutes followed by 1 hour incubation with the

Cf-polypeptides. Cells were then washed twice with PBS (0.1M, pH 7.4) and harvested with 0.5 g/L trypsin (treated for 10 minutes). Cells were examined by flow cytometry on a BD LSR II flow cytometer. Data were recorded measuring 5000-10000 cells and fluorescence mean values were calculated. Statistical analysis of data was performed using Student's *t*-test at the 95% confidence level.

Study of the uptake by confocal laser scanning microscopy

For the microscopic studies cells were cells were seeded onto Lab-Tek II 8-chamber Slide (Thermo Fisher Scientific, Waltham, MA, USA) with initial cell number of 3×10^4 cells/well 24 hours prior to the experiment. Cells were treated with the Cf-labeled polypeptides in serum free RPMI 1640 medium for 1 hour. Extracellular fluorescence was quenched using 0.01 % trypan blue solution for 15 minutes at room temperature in the dark. Cells were washed twice with PBS (0.1 M pH 7.4) and then fixed with 4% paraformaldehyde in PBS (0.1 M pH 7.4) for 15 minutes at room temperature. Membrane structures were labeled with DiD perchlorate dissolved in DMSO at c= 40 μg/ml. Precipitated crystals of DiD were removed by washing with PBS (0.1 M pH 7.4). Cells were studied with a Zeiss LSM 710 confocal microscope (Carl Zeiss Microscopy, Jena, Germany), using its 63x oil immersion objective. Carboxyfluorescein was excited at λ=488 nm and DiD at λ=530 nm. Pictures were edited with ImageJ software.

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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 is used in accordance with the recommended nomenclature of graft polypeptides [48]. 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 abbreviation in this paper are the following - Cf, 5(6)-carboxyfluorescein; CPP, cell penetrating peptide; DiD, 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; EIPA, 5-(*N*-Ethyl-*N*-isopropyl)amiloride; FCS, fetal calf serum; HOBt, 1-hydroxy-bezotriazole; LC_{50}, 50% lethal concentration; MβCD, methyl-β-cyclodextrin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OPcp, pentachlorophenyl ester; PBS, phosphate-buffered saline; Pcp, pentachlorophenyl; Z: benzyloxycarbonyl; X_iK, poly[Lys(X_i)]; AK, poly[Lys(DL-Ala_m)]; XAK, poly[Lys(X_i-DL-Ala_m)], AXK, poly[Lys(DL-Ala_m-X_i)]

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