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# The effect of the structure of branched polypeptide carrier on intracellular delivery of daunomycin

Judit Reményi [a], Gabriella Csík [b], Péter Kovács [c], Francesca Reig [d], Ferenc Hudecz [a,e,*]

[a] Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University, Budapest 112, POB 32 H-1518, Hungary
[b] Department of Biophysics and Radiobiology, Semmelweis Medical University Budapest, Hungary
[c] Department of Genetics, Cell and Immunobiology, Semmelweis University Medical School, 1445 Budapest, Nagyvárad tér 4, Hungary
[d] Department of Peptide and Protein Chemistry, IIQAB, CSIC, Barcelona Spain
[e] Department of Organic Chemistry, Eötvös L. University, Budapest, 112, P. O. Box 32, H-1518, Hungary

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**Abstract**

The conjugate of acid labile *cis*-aconityl-daunomycin (cAD) with branched chain polypeptide, poly[Lys($Glu_i$-DL-$Ala_m$)] (EAK) was very effective against L1210 leukemia in mice. However, Dau attached to a polycationic polypeptide, poly[Lys($Ser_i$-DL-$Ala_m$)] (SAK) exhibited no in vivo antitumor effect. In order to understand this difference we have performed comparative in vitro studies to dissect properties related to interaction with the whole body (e.g., biodistribution) from those present at cellular or even molecular level. We report here (a) the kinetics of acid-induced Dau liberation, (b) interaction with DPPC phospholipid bilayer, (c) in vitro cytotoxic effect on different tumor cells, and (d) intracellular distribution in HL-60 cells of polycationic (cAD-SAK) and amphoteic (cAD-EAK) conjugates. Fluorescence properties of the two conjugates are also reported. Our findings demonstrate that the kinetics of the drug release, intracellular distribution and in vitro cytotoxic effect are rather similar, while the effect on DPPC phospholipid bilayer and fluorescence properties of the two conjugates are not the same. We also found that the in vitro cytotoxicity is cell line dependent. These observations suggest that the structure of the polypeptide carrier could have marked influence on drug uptake related events.


*Keywords:* Polypeptide carrier; Daunomycin; pH-dependent drug liberation; Altered conjugate cytotoxicity; Uptake of daunomycin conjugate

## 1. Introduction

Synthetic linear and branched chain polymers are utilized as macromolecular carriers for "passive" delivery of bioactive molecules. These conjugates could exhibit improved solubility, beneficial pharmacological properties (extended blood survival, specific tissue accumulation), diminished cytotoxicity and increased therapeutic efficacy as compared to the free drugs, radionuclides [1–3]. Among synthetic linear poly-α-amino acids, polyanionic poly(L-glutamic acid), poly(L-aspartic acid) and polycationic poly(L-lysine), poly(L-Orn) have been used for delivery of antitumor agents, enzymes or albumin. Poly (L-lysine) or poly(D-lysine) as lysosomotropic agent was coupled with various antitumor drugs like, methotrexate, (MTX), adriamycin, daunomycin (Dau), mitomycin C or oligonucleotide [2,4,5]. However, very few systematic studies were reported on structural and functional factors required for optimal polypeptide carrier. Therefore we have prepared and investigated structural and biological characteristics of new groups of branched chain polypeptides with the general formula of poly[Lys($X_i$-DL-$Ala_m$)] (XAK), poly[Lys($X_i$-DL-$Ser_m$), (XSK), poly[Lys($X_i$)] ($X_i$K) or poly[Lys(DL-$Ala_m$-$X_i$)] (AXK) where $i=1$, $m=3$, and X represent an additional optically active amino acid residue [6–8]. We have demonstrated that the side chain composition and amino acid sequence of the branches have marked influence on blood clearance, biodistribution [8,9], immunogenicity [6,8] and also on the interaction with

* Corresponding author. Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Pázmány P. sétány 1A, Budapest H-1117, Hungary. Tel.: +36 1 372 2828; fax: +36 1 372 2620.
*E-mail address:* hudecz@szerves.chem.elte.hu (F. Hudecz).

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phospholipid mono- or bilayers [10]. Recently, we have synthesized various conjugates in which antitumor agents (daunomycin [11,12], MTX [13,14], GnRH antagonist [15], amiloride [16], radionuclides [17,18] or epitope peptides [19] have been covalently attached to selected structurally related branched polymeric polypeptides. Pronounced antitumor activity of Ac-[D-Trp$^{1-3}$, D-Cpa$^{2}$, D-Lys$^{6}$, D-Ala$^{10}$]-GnRH-poly[Lys(Ac-Glu$_i$-DL-Ala$_m$)] (Ac-EAK) conjugate has been clearly documented [15]. We have also demonstrated that the effect of peptide conjugates on phospholipid membranes (DPPC, DDPC/PG) is mainly determined by the charge and charge density properties of the carrier polypeptide [20,21]. Carrier-dependent anti-*Leishmania donovani* effect of MTX-polypeptide conjugates was observed in vitro and in vivo [14]. The coupling of acid labile *cis*-aconityl-daunomycin (cAD) to poly[Lys(Glu$_i$-DL-Ala$_m$)] (EAK) resulted in compensation of the immunosuppressive effect of the daunomycin (Dau) and this polymeric conjugate in vivo was very effective against L1210 leukemia producing 66–100% long-term survivors (>60 days) in mice [22]. However, cAD attached to a polycationic polypeptide, poly[Lys(Ser$_i$-DL-Ala$_m$)] (SAK) exhibited no in vivo antitumor effect under similar conditions [12].

In order to understand the differences observed in antitumor activity in vivo, we have performed comparative studies in vitro. Thus, properties related to interaction between the whole body and conjugates (e.g., blood clearance, tissue distribution) could be dissected from those present at cellular or even molecular level. It should be noted that branched polypeptides SAK and EAK are differ only in a single amino acid residue situated at the end of the branches (Fig. 1). However, the presence of different amino acids (Ser in SAK and Glu in EAK) at the side chain terminal position results in altered charge and conformational properties: polypeptide EAK is amphoteric and adopt essentially disordered conformation under nearly physiological conditions (0.2 M NaCl, pH 7.4) [23]. In contrast polypeptide SAK exhibits polycationic characteristics and proved to be markedly ordered in 0.2 M NaCl at pH 7.2, [7]. Under these conditions the calculated α-helix content was 53.1% for SAK, and 6.3% for EAK. Even more pronounced difference was observed in the presence of DPPC (e.g., 71.9% α-helix content for SAK and 7.8% for EAK) or DPPC/PG mixtures [24].

Considering the potential differences of conjugates in acid-induced release of Dau, in their interaction with phospholipid bilayer and in biological activity, the aim of present study was to compare the (a) liberation kinetics of the drug, (b) effect of conjugates on DPPC phospholipid bilayer, (c) in vitro cytotoxic effect of conjugates on four different tumor cell lines, and (d) cellular distribution after uptake by HL-60 cells using confocal laser microscopy. To exclude artefacts fluorescence properties of daunomycin as well as of the two conjugates (cAD-EAK and cAD-SAK) are also reported. Our findings demonstrate that the kinetics of the drug release, the in vitro cytotoxic effect and intracellular distribution are rather similar, while the effect on DPPC phospholipid bilayer and fluorescence properties of the two conjugates are not the same. We found also that the in vitro cytotoxicity of conjugates is cell line dependent. These observations suggest that the structure of the polypeptide carrier could have marked influence on drug uptake related events.

## 2. Materials and methods

### 2.1. Materials

Daunomycin hydrochloride was a gift from the Institute of Drug Research Ltd. Budapest, Hungary; *cis*-aconitic-anhydride, *N*-ethyl-*N*′-[3-(dimethylamino)

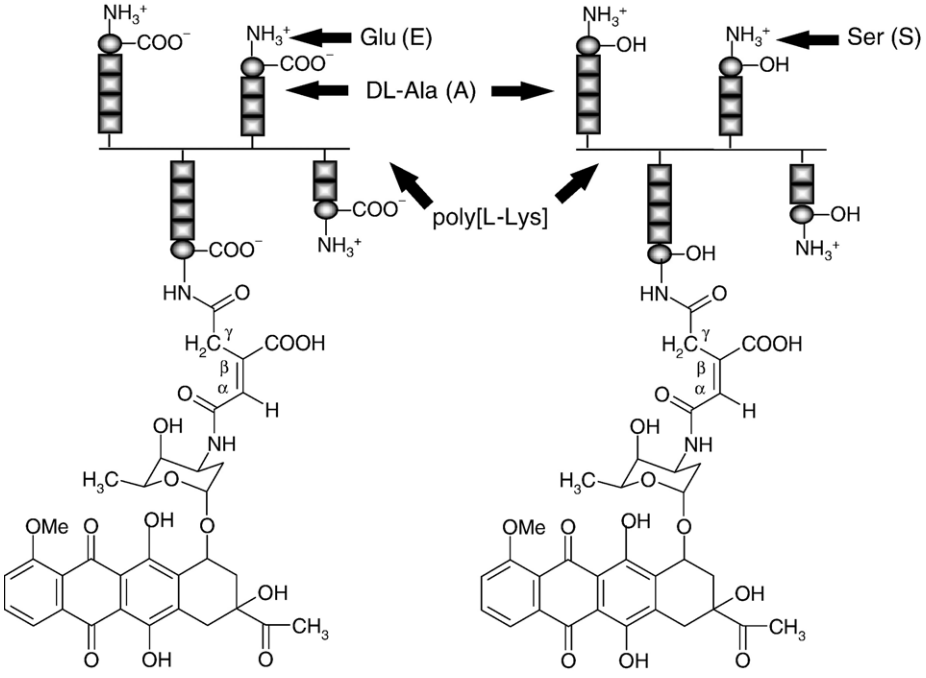


Fig. 1. Schematic structure of daunomycin-branched chain polypeptide conjugates, cAD-EAK and cAD-SAK.

propyl]carbodiimide hydrochloride (EDAC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DME:F12 medium powder, RPMI-1640 medium powder, glutamine, gentamycin, DPPC (dipalmitoyl phosphatidyl choline) were from Sigma-Aldrich (Budapest, Hungary). Fetal bovine serum (FBS) was obtained from Gibco Brl. Solvents (dioxane, methanol, acetonitrile, dimethyl sulfoxide (DMSO)), trifluoroacetic acid (TFA) and other chemicals for buffers and eluents were obtained from Reanal, Budapest, Hungary. Solvents (chloroform, tetrahydrofuran) used were Merck analytical grade products. Fluorescent probes DHP (1, 6-diphenyl-1, 3, 5 hexatriene) and TMA-DPH [1-(trimethylammoniumphenyl)-phenyl-1, 3, 5 hexatriene *p*-toluene-sulfonate were from Molecular Probes (Eugene, Oregon, USA).

*cis*-Aconityl daunomycin (cAD), poly[Lys-($Glu_{0.9}$-DL-$Ala_{3.0}$)], (EAK), poly[Lys-($Ser_{1.0}$-DL-$Ala_{3.5}$)], (SAK), poly[Lys-($cAD_i$-$Glu_i$-DL-$Ala_m$)], (cAD-EAK) and poly[Lys-($cAD_i$-$Ser_i$-DL-$Ala_m$)], (cAD-SAK) were synthesized in our laboratory [11,12] as described in detail for cAD-EAK previously [11]. Briefly, the coupling of cAD to branched polypeptide with $\overline{M}_w = 36500$ ($\overline{DP}_n = 80$) for EAK and with $\overline{M}_w = 34000$ ($\overline{DP}_n = 80$) for SAK was achieved by a carbodiimide method. The carboxyl groups of cAD were activated by water-soluble carbodiimide using 1.5 times molar excess of EDAC. The cAD-conjugate preparations were purified by gel-filtration and characterized by reversed-phase HPLC, cAD content, average degree of substitution ($\overline{DS}$) and ($\overline{M}_w$) determination. 1 mg of cAD-EAK conjugate sample ($\overline{DS} = 5.4\%$) used in this study contained 0.08 μmol (0.042 mg) daunomycin. 1 mg of cAD-SAK conjugate sample ($\overline{DS} = 3.3\%$) used in this study contained 0.05 μmol (0.025 mg) daunomycin.

### 2.2. Release of the drug from the cAD-branched chain polypeptide conjugates

Liberation of daunomycin from its *cis*-aconityl-daunomycin conjugates was studied by analytical RP-HPLC on a Delta Pak $C_{18}$ column (3.9×300 mm, packed with 15 μm silica, 300 Å pore size). The isocratic elution was developed using $(NH_4)_2CO_3$(3 w/w%):MeOH=50:50, v/v as mobile phase using a flow rate of 1 ml/min at room temperature. Peaks were detected at $\lambda$=490 nm. The samples were dissolved in 0.1 M citrate-phosphate buffer at various pH (3, 4, 5, 6 or 7.4). The concentration of samples was 0.5 mg/ml for Dau content. The solutions were kept at 25 °C or 37 °C for various time periods up to 72 h. The amount of free daunomycin released from cAD-conjugates was determined by using calibration curves. Calibration curves for daunomycin, *cis*-aconityl-daunomycin were established as follows. The compounds were dissolved in 0.1 M citrate-phosphate buffer (pH 7.4) at concentration of 0.5 mg/ml for daunomycin content and 20 μl of solutions were injected into RP-HPLC column. Calibration curves derived from the area under the curve (AUC) and drug concentration was calculated from 2 to 5 parallel measurements.

### 2.3. Preparation of labeled DPPC liposomes

Small unilamellar liposomes of DPPC were prepared by sonication as described before [25]. Briefly, stock solution of DPPC in chloroform was dried to a film using a rotary evaporator. Lipids were hydrated with sodium-acetate buffer (pH 7.4). Sonication was performed using an MSE Ultrasonic Desintegrator. The power output was approx. 150 W. Clear solutions were obtained after 15–20 min sonication. The size distribution of liposome preparation was checked by light scattering measurement. The final phospholipid concentration in the stock solution was 1.5 mM. Liposomes were incubated with either DHP or TMA-DPH (at $c = 10^{-5}$ M) [26] at 55 °C (higher than the phase transition temperature of DPPC) at the saturation concentration of vesicles.

### 2.4. Analysis of interaction between DPPC liposome and cAD-conjugates

Daunomycin or cAD-conjugates were added to the DHP/TMA-DPH labeled liposomes to result in a $2.4\times10^{-5}$ M final daunomycin concentration and 300:1=lipid : daunomycin ratio and fluorescence measurements were performed after 1 h incubation. Steady-State fluorescence measurements were carried out using an Aminco-Bowman spectrofluorimeter provided with a thermostated four positions cuvette holder. The temperature was controlled using a circulatory bath (Techne), and samples were continuously stirred. Fluorescence anisotropy of DHP and TMA-DPH in the temperature range 25–50 °C at 2 degrees intervals, was measured at excitation wavelength of $\lambda$=355 nm and monitoring wavelength at $\lambda$=430 nm for TMA-DPH and $\lambda$=426 nm for DHP. Anisotropy-temperature curves were recorded. From the first derivative of each curve the respective transition from gel to liquid crystalline state ($T_c$ value) was determined.

The fluorescence anisotropy was defined as:

$$A = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

where $I_{VV}$ and $I_{VH}$ are observed intensities measured with emission polarizers parallel and perpendicular to the vertically polarized exciting beam, respectively. $G$ is a wavelength dependent factor used to correct for the inability of the instrument to transmit differently polarized light equally. Values were corrected for background light-scattering and samples containing unlabeled suspension.

### 2.5. Fluorescence spectroscopy

Daunomycin and its polypeptide conjugates (cAD-EAK or cAD-SAK) were dissolved in 0.1 M citrate-phosphate buffer at various pH (3, 4, 5, 6 and 7.4) directly before spectral recordings. The daunomycin concentration of samples was determined by absorption spectroscopy using Cary 4 E spectrophotometer and adjusted to $3.276\times10^{-5}$ M.

Corrected steady-state emission and excitation spectra of daunomycin and its polypeptide conjugates were obtained using a FS900CD spectrofluorimeter (Edinburgh Analytical Instruments, UK) with a Xe lamp excitation and a Hammamatsu photomultiplier (R955) detection. Emitted fluorescence intensity was measured at an angle 90° relative to the exciting light. Resolution of monochromators was 0.5 nm. The excitation wavelength was $\lambda$=488 nm, and the emission spectra were recorded between $\lambda$=475–800 nm.

### 2.6. Cell culture

The c26 mouse colon carcinoma and MDA-MB 435 P human breast carcinoma adherent cell lines were cultured at 37 °C and 5% $CO_2$ in DME:

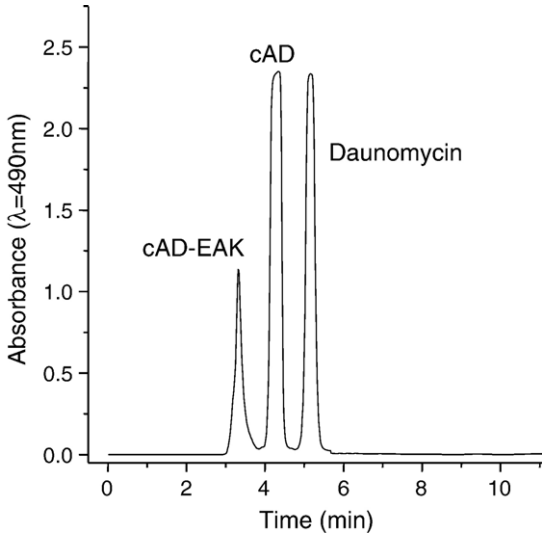


Fig. 2. Analytical RP-HPLC profile of the mixture of daunomycin, *cis*-aconityl-daunomycin and cAD-EAK conjugate. The isocratic elution was developed using eluent: $(NH_4)_2CO_3$(3 w/w%):MeOH=50:50 v/v. Column: C18 Delta Pak column (3.9 mm×300 mm, 15 μm silica, 300 Å pore size). Flow rate was 1 ml/min at room temperature. 20 μl of the samples were injected, and peaks were detected at $\lambda$=490 nm.

F12=1:1 (v/v) medium containing 10% FBS, 2 mM glutamine. The L1210 mouse and HL-60 human lymphoid suspension cell lines were cultured at 37 °C and 5% $CO_2$ in RPMI-1640 medium containing 10% FBS, 2 mM glutamine and 160 μg/ml gentamycin.

### 2.7. In vitro cytotoxicity of cAD-conjugates

The cells were placed in 96-well plate with each well containing $5\times10^3$ cells. After incubation at 37 °C for 24 h, the cultured cells were treated with daunomycin, or with cAD-EAK, cAD-SAK conjugates dissolved in serum-free DME-F12 or RPMI-1640 medium. For the treatment, compounds were used in the $10^{-9}$–$10^{-3}$ M concentration range for daunomycin content. In control experiments, cells were treated with serum-free medium only at 37 °C. After incubation for 3 h, cells were washed with serum-free medium three times and serum-containing medium was added to the cells. After 4 days at 37 °C, the 3-(4,5-dimehyltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay was carried out. The yellow solution of MTT at concentration of 2 mg/ml was added to each well. The purple crystal obtained was solved in 100 μl DMSO and the optical density (OD) of the samples was measured at $\lambda$=540 nm using ELISA Reader (Labsystems, Finland). We have calculated the % of cytotoxicity using the following equation:

Cytotoxicity $\%=(1-OD_{treated}/OD_{control})\times100$, where $OD_{treated}$ and $OD_{control}$ correspond to the optical density of the samples from treated cells and the control cells at $\lambda$=540 nm, respectively.

### 2.8. Curve fitting

Results obtained from the daunomycin release studies and from in vitro cytotoxicity measurements were analysed by Origin 5.0 software using exponential and sigmoid curve fitting, respectively.

### 2.9. Uptake of the conjugates by HL-60 human lymphoid leukemia cells

The cells were placed in 24-well plate with each well containing $5\times10^5$ cells/1 ml medium. Cells were treated with daunomycin, cAD-EAK, or cAD-SAK conjugate at the 2 μM concentration for Dau content. The control cells were treated with EAK or SAK polypeptide, or incubated with medium only. Compounds were dissolved in serum-free DME-F12 or RPMI-1640 medium and incubation was carried out for 15 min, 1, 3, 8 or 24 h. Then cells were washed with PBS (0.1 M; pH 7.4) twice, stored at 4 °C for overnight. The living cells were mounted onto microscopic slides and analysed using confocal scanning laser microscope with $\lambda_{ex}$=480 nm using MRC 1024 (Bio-Rad, California, USA).

## 3. Results

In cAD-branched polypeptide conjugates one carboxyl group of the cAD was linked to the α-amino group of the side chain terminal Glu (EAK) or Ser (SAK) amino acid to provide covalent α-amide bonding between branched polypeptide SAK or EAK and cAD (Fig. 1).

### 3.1. Release of the drug from the cis-aconityl-daunomycin (cAD)-branched chain polypeptide conjugates (cAD-EAK and cAD-SAK)

In order to mimic conditions of the endocytotic uptake pH dependence of the liberation of the drug from daunomycin-

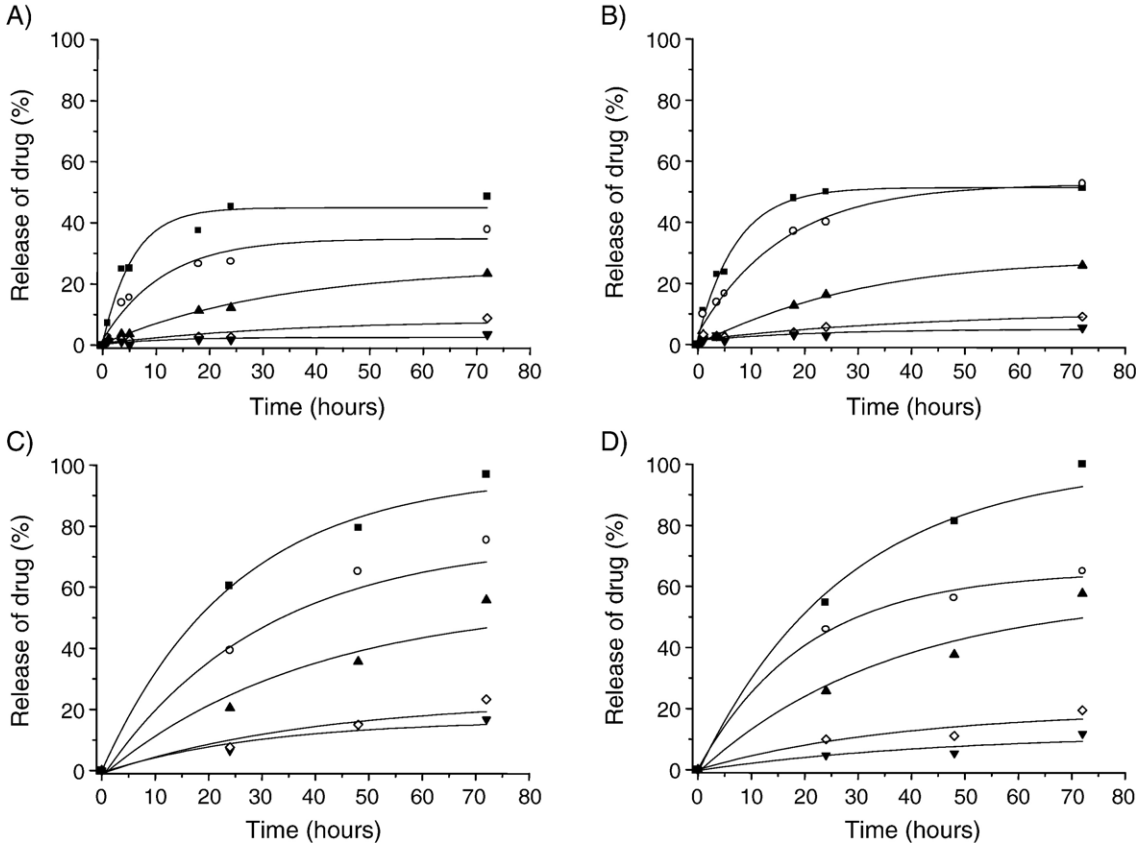


Fig. 3. pH and temperature dependence of the drug release from cAD-EAK (A and C, 25 °C and 37 °C, respectively) and cAD-SAK (B and D, 25 °C and 37 °C, respectively) conjugates detected by analytical RP-HPLC. Elution was carried out as described in Fig. 2. Samples were dissolved in 0.1 M citrate-phosphate buffer (c=0.5 mg/ml for daunomycin content) at pH 7.4 (▼), pH 6 (◇), pH 5 (▲), pH 4 (○) and pH 3 (■).

polypeptide conjugates containing polycationic (cAD-SAK) or amphoteric (cAD-EAK) polypeptide was studied as function of pH (3, 4, 5, 6, 7), of time (up to 72 h) and of temperature (25 °C and 37 °C). For these measurements, a reversed-phase HPLC based methodology was developed and used for the determination of the free drug released (Fig. 2). Fig. 3 shows the drug release from the cAD-EAK (Fig. 3A, C) and cAD-SAK (Fig. 3B, D) conjugates. We found that not only daunomycin but also *cis*-aconityl-daunomycin are released from both conjugates under conditions studied (data not shown). At nearly physiological pH, mimicking extracellular and intracellular circumstances, only 1–2% of the drug (daunomycin plus cAD) was released even after 72 h from cAD-EAK conjugate at 25 °C and about 6–8% at 37 °C (Fig. 3C). Under acidic circumstances, the drug release significantly increased to 8% at pH 6.0; 23% at pH 5.0; 34% at pH 4.0 and about 50% at pH 3.0 after 72 h incubation period at 25 °C. Interestingly, about 100% of the drug was released after 72-h incubation at pH 3.0 at 37 °C. At physiological pH again only 3–4% at 25 °C and 10–12% at 37 °C (Fig. 3B) of the drug was released from the polycationic cAD-SAK conjugate. Lowering the pH resulted in increased drug liberation to yield 8% at pH 6.0, 55% at pH 3.0 after 72 h at 25 °C. However, almost the total amount of daunomycin was liberated at pH 3.0 after 72 h incubation at 37 °C (Fig. 3D).

### 3.2. Interaction between DPPC liposomes and cAD-conjugates

The interaction between cAD-conjugates and phospholipid membranes was investigated using a very simple phospholipid bilayer, DPPC. A fluorescent probe with positive electrical charge TMA-DPH as well as a hydrophobic one, 1,6-diphenyl-1,3,5-hexatriene (DPH), were used to analyse the effect of cAD-polypeptide conjugates and daunomycin (data not shown) on the outer surface and on hydrophobic core of bilayers [26]. For these studies small unilamellar vesicles (SUVs) were used, so that light scattering was not important. Changes in fluorescence intensity and/or polarization of probes in bilayers were determined as a function of temperature. Transition from gel to liquid crystalline state ($T_c$ ~ 40–41.5 °C for DPPC) was characterized by the analysis of $T_c$ values in the presence or absence of conjugates. The optimal probe/phospholipid molar ratios were determined by incubation of plain liposomes with increasing volumes of concentrated solutions of each label followed by fluorescence intensity measurements. Up to a certain label/lipid relationship, there was no increase in the

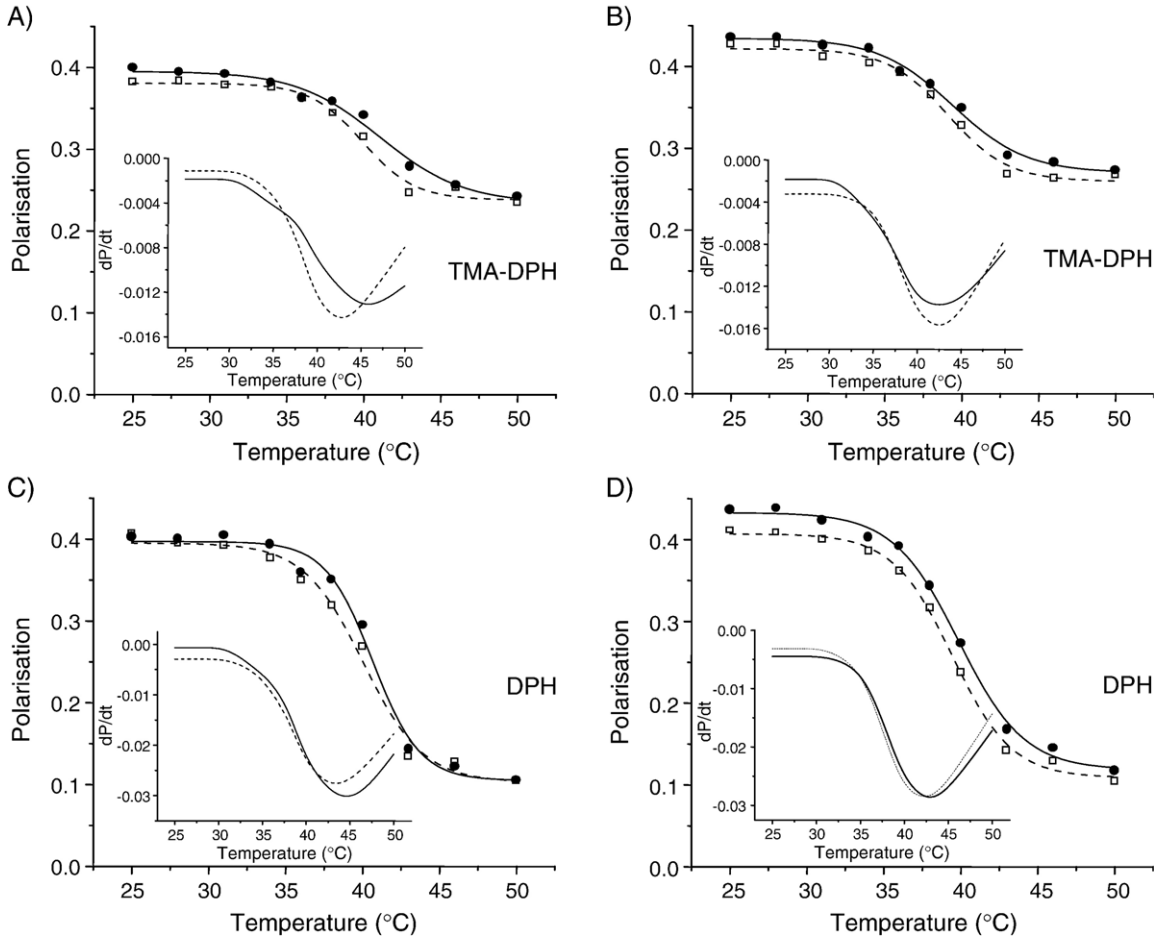


Fig. 4. Interaction between cAD-EAK (A and C) or cAD-SAK (B and D) and liposome of DPPC 100% composition using DPH or TMA-DPH marker (buffer □, conjugate ●). The study was performed by fluorescence anisotropy method as described in Materials and methods.

fluorescence of the samples thus indicating that the system was already saturated and stable. Under conditions applied (neutral pH, low conjugate concentration, probe/phospholipid ratio), liposomes composed of DPPC could be considered neutral.

Our experiments suggest that cAD-EAK had significant effect on the anisotropy of the TMA-DPH and also of DPH in membranes with 100% DPPC ($\Delta T_c$=4 °C and $\Delta T_c$=3 °C) (Figs. 4A and C). cAD-SAK exhibited essentially no interaction under identical conditions (Figs. 4B and D). The data show that cAD-EAK conjugate has pronounced destabilising effect not only on the surface ($\Delta T_c$=4 °C), but also on the inner part of the bilayer ($\Delta T_c$=3 °C) of the DPPC liposomes. In contrast, the cAD-SAK conjugate caused only negligible effect ($\Delta T_c$=0–0,5 °C) on the phospholipid bilayers. We also observed that Dau markedly interacted with the surface and also with inner part of the DPPC phospholipid bilayer (data not shown).

### *3.3. In vitro antitumor effect of the conjugates in mouse and human tumor cells*

The antitumor activity of daunomycin-polypeptide conjugates (cAD-EAK, cAD-SAK) was studied and compared at $10^{-9}$–$10^{-3}$ M concentration range (for daunomycin content) on the following four tumor cell lines: L1210 mouse lymphoid leukemia, c26 murine colon carcinoma, MDA-MB 435P human breast carcinoma and HL-60 human lymphoid leukemia cell line using MTT-assay. The level of the cytotoxicity (%) caused by conjugate treatment was studied as a function of concentration. Based on these curves the $IC_{50}$ values were determined. Results summarizing inhibitory effect of conjugates are presented in Fig. 5. The lowest $IC_{50}$ values for the both conjugates were measured in the case of HL-60 human leukemia cells (Fig. 5A), indicating the most pronounced antitumor effect was observed against these cells. The $IC_{50}$ values for cAD-EAK (5.0 μM) and for cAD-SAK (3.5 μM) conjugates obtained on HL-60 cells are very similar. Higher $IC_{50}$ values were calculated from curves obtained with L1210 murine leukemia cells treated with these conjugates (Fig. 5B). The $IC_{50}$ value for cAD-SAK conjugate was 30.8 μM and for cAD-EAK it was 51.3 μM. Fig. 5C shows the effect of cAD-conjugates on c26 murine colon carcinoma cells. The $IC_{50}$ value of cAD-SAK conjugate ($IC_{50}$=117.4 μM) was much lower than that of the cAD-EAK conjugate ($IC_{50}$=629.9 μM), indicating that the cAD-SAK conjugate has more potent inhibitory effect on c26 cell proliferation than that of cAD-EAK conjugate. Almost identical $IC_{50}$ values were obtained from experiments with MDA-MB 435 P human breast carcinoma cells ($IC_{50}$=195.2 μM) for both conjugates (Fig. 5D). In our experiments, EAK or SAK carrier molecules had no effect against all cell lines studied at the concentration range examined (data not shown).

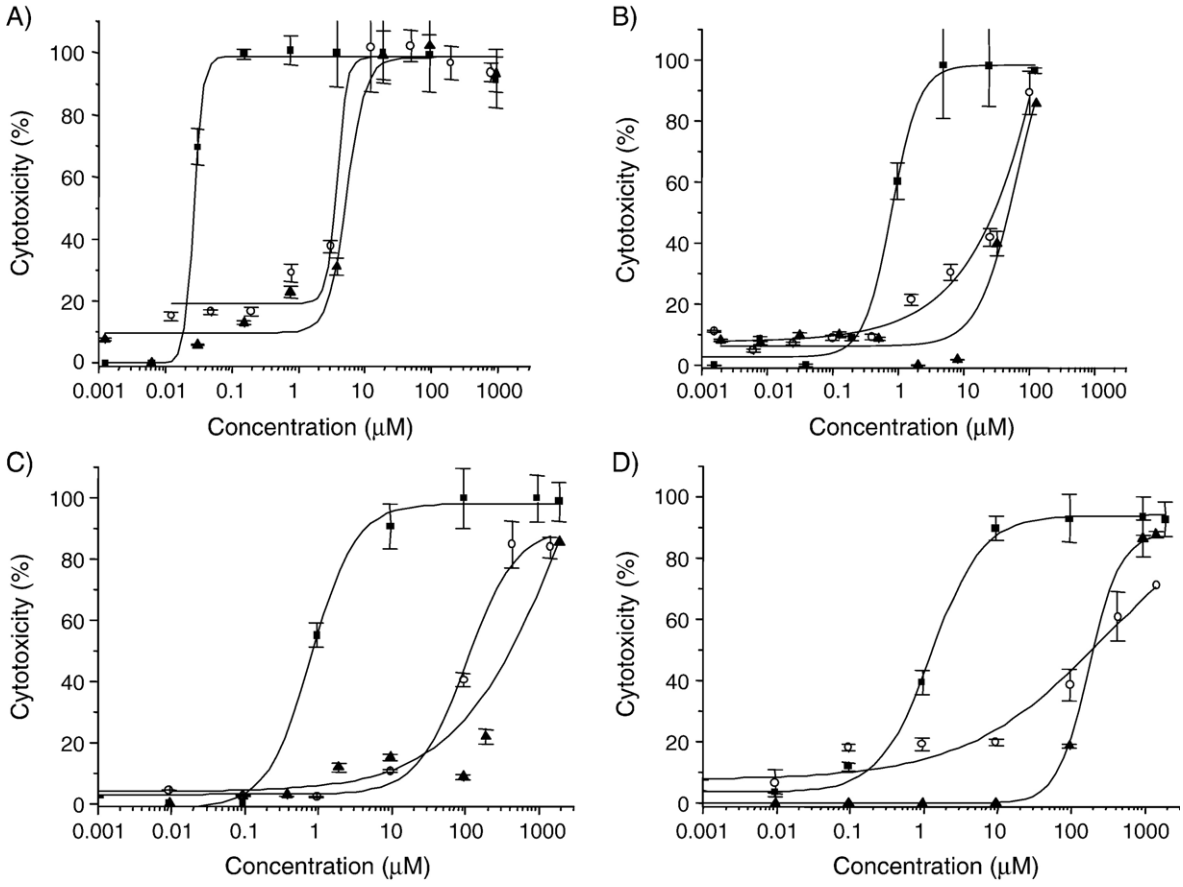


Fig. 5. The in vitro antitumor effect of cAD-EAK (▲), cAD-SAK (○) conjugates and daunomycin (■) on HL-60 human lymphoid leukemia cells (A), L1210 murine lymphoid leukemia cells (B), c26 murine colon carcinoma cells (C) and on MDA-MB 435 P human breast carcinoma cells (D). The cells were treated with samples at a range of $10^{-9}$–$10^{-3}$ M for daunomycin content and cytotoxicity was determined by MTT-assay as described in the Materials and methods.

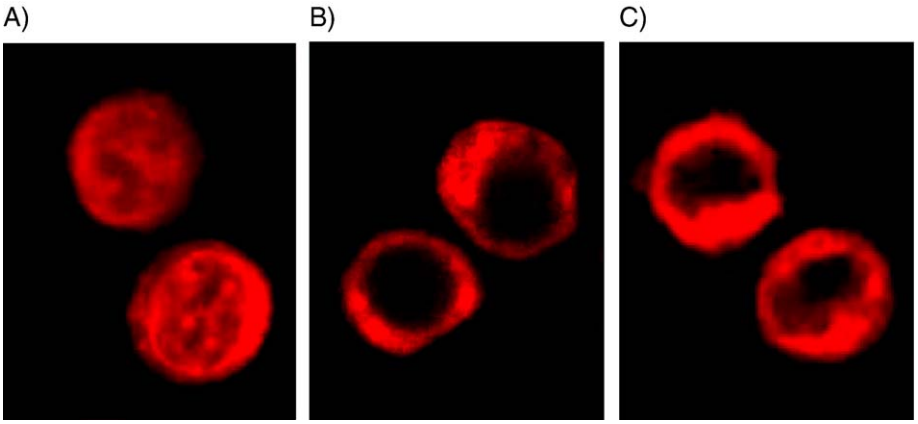


Fig. 6. Intracellular distribution of daunomycin (A), cAD-EAK (B) and cAD-SAK (C) conjugates by living HL-60 human lymphoid leukemia cells. The cells were incubated with daunomycin or cAD-polypeptide conjugates at concentration of 2 μM for daunomycin for 3 h. Cells were analysed by a confocal scanning laser microscope at $\lambda_{ex}$=480 nm using MRC 1024 (Bio-Rad).

### *3.4. Uptake of the conjugates by HL-60 human lymphoid leukemia cells*

Considering that conjugates exhibited the most pronounced antitumor effect against HL-60 cells cytotoxicity, we have selected this cell line for uptake experiments. In order to determine the localization of daunomycin, cAD-EAK and cAD-SAK conjugates within HL-60 cells, confocal laser scanning microscopy study was performed. The cells were treated with daunomycin, cAD-EAK or cAD-SAK conjugates and also with EAK or SAK polypeptide for 15 min, 1, 3, 8 or 24 h. Fig. 6 shows that daunomycin, cAD-EAK or cAD-SAK conjugates accumulated in cells after 3 h incubation. After treatment with daunomycin, we found that the nucleus had red fluorescence, indicating that daunomycin could be mainly detected in the nucleus (Fig. 6A). Opposite to the hypothesis for conjugates of the literature, in the case of cAD-EAK or cAD-SAK conjugates treatments we observed no red fluorescence in the nucleus, while the cytoplasm exhibited red fluorescence, meaning that the conjugates are present in the cytoplasm (Figs. 6B and C) even after 8 or 24 h incubation (data not shown). It should be noted that there are differences between the two confocal microscopy images. In the case of cAD-EAK conjugate the laser amplifier was in 100%, while in the case of cAD-SAK conjugate this laser amplifier was only 30%. The untreated cells or the cells treated with EAK or SAK polypeptide (data not shown) alone had no autofluorescence behavior. To exclude artefact deriving from the altered fluorescence properties of conjugates fluorescence properties of the conjugates and Dau, as control were investigated.

### *3.5. Fluorescence emission spectra of the daunomycin and its peptide conjugates*

For the better understanding of the results of confocal laser scanning microscopy we compared the spectral characteristics of the free Dau and that of cAD-EAK, cAD-SAK conjugates. Fig. 7 shows the fluorescence emission spectra ($\lambda_{ex}$=488 nm) of solutions containing equimolar concentration of daunomycin. The absorbance of the solutions at the absorption maximum of daunomycin was similar in the range of experimental error.

As one can see, there is no difference between the shape of the emission curves of the conjugates and daunomycin. However, the fluorescence intensity of the conjugates decreased markedly as compared to free daunomycin. In this respect, there is also significant between the two conjugates cAD-EAK and cAD-SAK, i.e., the fluorescence intensity of cAD-EAK conjugate is lower than that of cAD-SAK conjugate.

There are two possible reasons for this decrease in fluorescent intensity of daunomycin in its conjugated form. Alteration of electronic structure may involve the excitable electrons of daunomycin and results a consequent change in its fluorescence lifetime. This is not very probable since the spectral distributions of the light emitted by the free and conjugated are the same. The other possibility is that the daunomycin fluorescence is quenched by the peptide part of the conjugate. This concept can be supported also by the strong influence of peptide structure on the fluorescent intensity and not on the fluorescence spectrum. However, further experiments-lifetime measurements and

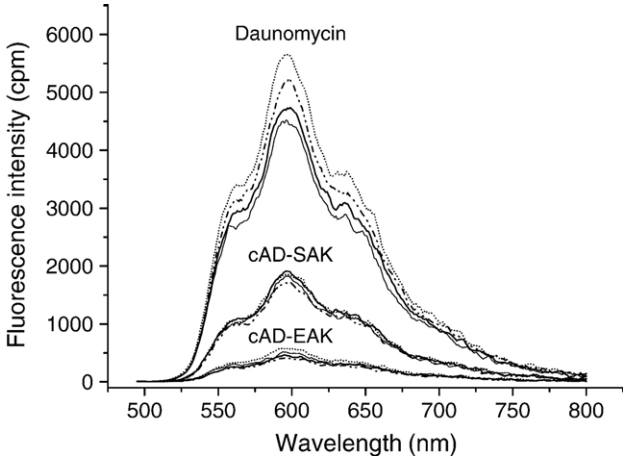


Fig. 7. The effect of pH on the emission spectrum of daunomycin, cAD-EAK and cAD-SAK conjugates. The daunomycin content of the sample was $3.276\times10^{-5}$ M. pH 7.4 ▬▬, pH 5.0 ------, pH 4.0 – – –, pH 3.0.

fluorescence quenching studies are necessary to support one or other possibility.

In addition, the pH dependence of the emission spectra of the sample was recorded. The spectrum of daunomycin was pH dependent, decreasing the pH the fluorescence maximum of the spectra slightly increased. But no essential changes in the spectra of conjugates were observed as a function of pH.

## 4. Discussion

The aim of this study was to analyse the influence of the carrier structure, predominantly charge properties, on in vitro antitumor effect and on the cellular uptake of branched chain polypeptide conjugates with Dau. Previously, we have demonstrated that amphoteric cAD-EAK conjugate has pronounced in vivo antitumor effect on L1210 leukemia bearing mice [22]. However, similar conjugate with polycationic carrier did not inhibit the tumor growth [12]. In order to understand the difference observed, we have dissected biodistribution related events (blood clearance, tissue distribution) from those occurring at cellular level. It should be noted that acid labile *cis*-aconityl spacer [12] was used in both conjugates to connect daunomycin and polypeptide carrier.

First, we have analysed the effects of carrier polypeptide on the liberation of daunomycin under conditions mimicking the pH of biological fluids. In this study, we have used buffer with neutral (pH 7.4) or acidic (pH 3.0–5.0) characteristics modelling circumstances in human serum and lysosome compartment, respectively. Our data show that the rate of the drug release was time-, pH- and temperature-dependent in the case of both conjugates. Lowering the pH resulted in higher drug liberation. The rate of the drug release was higher at 37 °C than at 25 °C at every pH. While only about 50% of the conjugated drug was liberated from both conjugates at 25 °C at pH 3, almost the total amount of carrier- bound daunomycin was released at 37 °C. It should be noted that in the secondary lysosome the pH is between 5.0 [27] and 4.0 [28]. At pH 4.0, we found that 70% of the covalently attached daunomycin was released at 37 °C from these conjugates. From this, one can conclude that no complete liberation could occur within the cell even in acid labile spacer in the construct. Similar findings were reported by Etrych et al. in a comparative study, the authors studied HPMA-doxorubicin conjugates with different linkages (*cis*-aconityl, hydrazone or catepsin sensitive GFLG) and found 100% drug release from conjugates containing the hydrazone bond [29]. It is important to note that elevation of temperature from 25 °C to 37 °C resulted in a dramatic increase in the release of the drug. This finding – for the first in the literature – suggests the marked role of temperature on the drug liberation in conjugates with *cis*-aconityl linkage. Based on our data, we could conclude that the chemical structure and charge characteristics of polypeptide carrier (amphoteric EAK or polycationic SAK) had negligible effect on daunomycin liberation.

In order to study the interaction between of cAD-conjugates and simple, artificial phospholipid bilayer composed of neutral and saturated DPPC, fluorescence anisotropy experiments were carried out. Interaction of daunomycin with model membranes has been already studied by several authors [30]. It has been found that Dau perturbs the lipid phase transition associated with thermodynamic parameters: it decreases the enthalpy change, lowers the transition temperature. The main phase transition of saturated phospholipid bilayer was significantly broadened in the presence of daunomycin indicating a reduced co-operative behavior of the phospholipid molecules [31]. It has been proposed that daunomycin is preferentially, but not exclusively could be localized at membrane 'surface' domains. A smaller population of the membrane-associated Dau can diffuse into deeper hydrophobic regions [32]. The analysis of data derived from DPPC studies in the presence of cAD-EAK showed higher values as compared to the references. The $T_c$ values derived from the respective curves indicate that cAD-EAK increases the microviscosity of the DPPC bilayer. Similarly, the anisotropy profiles of both probes in the presence of cAD-SAK conjugate suggest almost no interaction with pure DPPC. Our data show that the conjugates are able to interact with model membranes applied, but this effect is proved to be dependent on the carrier part of the constructs. It is attractive to speculate that the cellular uptake of conjugates could be related to the level of membrane destabilisation induced by cAD-conjugates with amphoteric or polycationic polypeptide.

For comparing the antitumor effect of amphoteric cAD-EAK and polycationic cAD-SAK, four different cell lines (L1210 murine leukemia, c26 murine colon carcinoma, HL-60 human leukemia and MDA-MB 435 P human leukemia) were used in our experiments. Data suggest that both conjugates were more effective against leukemia cells (murine and human leukemia cells) than on adherent cells of different origin (murine colon carcinoma or human breast carcinoma). The most pronounced cytotoxic effect of both cAD-conjugates as well as of free daunomycin was detected on HL-60 human leukemia cells. It should be noted that in the clinical application, daunomycin alone or in combination therapy has been usually used for the treatment of leukemia [33]. On the basis of the carrier charge, significant difference of the antitumor effect of the conjugates containing amphoteric or polycationic polypeptide was found only against the c26 murine colon carcinoma cells. In contrast, the cAD-EAK and cAD-SAK conjugates had almost identical antitumor effect on human breast carcinoma, murine and human leukemia cell lines, so the cytotoxic effect was independent of the structural (e.g., charge) properties of the carrier polypeptide. However, on c26 cells we observed pronounced carrier effect. Taken together, our data suggest that the antitumor effect of conjugates on three cell lines was almost independent from the polypeptide carrier used, but showed marked cell line dependency.

Considering that both conjugates were highly cytotoxic on HL-60 cells, we have studied their uptake. These preliminary experiments to compare the intracellular distribution of the Dau with that of the conjugates showed significant differences. The free daunomycin localized both in the cytosol and in the nucleus, but both of the conjugates was visible only in the cytoplasm of the HL-60 cells even after 24 h incubation under the circumstances studied. This observation is in opposite with

the speculations in the literature [34], but is in good agreement with experimental data of Hovorka et al., Kratz et al. and Rodrigues et al. Using laser scanning confocal microscopy Kratz et al. showed that free doxorubicin accumulated in the cell nucleus, whereas its transferrin conjugate localized primarily in the cytoplasm of MDA-MB-468 breast cancer and of LXFL 529 cells after a 24 h incubation [35]. Similar findings were reported by Rodrigues et al. by fluorescence microscopy. The authors demonstrated the presence of PEG-doxorubicin conjugate also predominantly in the cytoplasm of LXFL 529 lung cancer cells and the free drug appeared in the nucleus [36]. Hovorka et al. compared the accumulation of *N*-(2-hydroxypropyl)methacrylamide-(pHPMA)-GFLG-bound doxorubicin and of free drug [37]. They were unable to detect doxorubicin-related signal in the nucleus after 72 h incubation in conjugate treated cells, while the free doxorubicin accumulated in a relatively high quantity after 90 min incubation.

Interestingly, Omelyanenko et al. observed doxorubicin related fluorescence in fixed HepG2 and OVCAR-3 cells in the nucleus after the uptake of the HPMA conjugate [38]. The authors assumed that doxorubicin released from the conjugate was responsible for the red colour in the confocal microscopy images. These data together with our own findings suggest that the mechanism of action of the conjugate is probably different from that of the free drug. To verify this proposition and to understand better the mechanism of action (e.g., the route of uptake) of cAD-EAK or cAD-SAK conjugates further experiments are in progress.

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