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Daunomycin-polypeptide conjugates with antitumor activity

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ABSTRACT

We have developed a group of water-soluble drug conjugates in which daunomycin (Dau) is coupled to cationic, amphoteric or anionic branched polypeptides and a new conjugate containing a cationic polypeptide carrier modified with a cell penetrating octaarginine. We investigated *in vitro* physiological activity of these conjugates in several aspects: *in vitro* cytotoxicity and cytostatic effect, adhesion and cellular uptake were examined on murine (J774 and L1210) and human (MonoMac6 and HL-60) leukemia cell lines and on murine bone marrow derived macrophages. We found that these processes are dependent on the properties of the carrier, on experimental conditions like concentration and incubation time. We found that attachment of polypeptide and cell penetrating peptide to the bioactive agent, depending on the cell line, could significantly improve the antitumor activity of the drug.

1. Introduction

Delivery of daunomycin, a drug of anthracyclin family or its conjugates into tumor cells and into macrophages that infiltrates the tumor tissue could be a promising approach of tumor therapy. Despite its high efficiency, daunomycin has numerous side effects such as vomiting, hair loss and nausea. The drug also causes decreased with blood cell count and has a marked cardiotoxic effect [1]. Coupling the drug to macromolecular or peptide carrier could improve solubility, decrease side effects and can offer the possibility of cell or tissue specific introduction [2,3]. Surface receptors with conservative structure could be targeted with receptor specific carriers. Such receptors (e.g. scavenger receptors) are expressed on macrophages and macrophage like tumor cell lines like J774 [4]. Another approach for intracellular delivery is using cell penetrating peptides like oligoarginines with cationic character. This method is less specific, though also could be highly efficient [5,6]. Polycationic macromolecular polypeptides are also promising candidates for cell penetrating introduction [7]. These polypeptides are proved to be taken up effectively by murine bone marrow derived macrophages, although the mechanism of their uptake is presently not precisely described [8]. Daunomycin-polypeptide conjugates were tested earlier in our research group *in vitro* and *in vivo* on tumor cells and on solid tumors. Daunomycin was coupled to polycationic poly[Lys(Ser$_i$-dl-Ala$_m$)] (SAK) and to amphoteric poly[Lys(Glu$_i$-dl-Ala$_m$)] (EAK) where $m = 2–3$ and $i \sim 1.0$ polypeptides via cis-aconitic acid spacer. The treatment with polycationic poly[Lys(cAD$_j$-Glu$_i$-dl-Ala$_m$)]) (cAD-EAK), where $m = 2–3$ and i and $j \sim 1.0$ conjugate proved to be effective in eliminating of immunosuppressive effect of daunomycin in mice bearing tumor of L1210 origin [9]. Polypeptide-daunomycin conjugates were also effective on multidrug resistant human leukemia (HL-60/MDR1 and HL-60/MRP1) cell lines [10]. In the present work we carried out systematic structure–function studies on the *in vitro* physiological effect of cis-aconityl daunomycin conjugates containing polylysine based polypeptide carrier. Four conjugates with polypeptide carrier of different character were used: poly[Lys-(cAD$_j$-Glu$_i$-dl-Ala$_m$)], (cAD-EAK); its succinylated polyanionic derivative, (poly[Lys-(cAD$_j$-SuccGlu$_i$-dl-Ala$_m$)], (cAD-SuccEAK), poly[Lys-(cAD$_j$-Ser$_i$-dl-Ala$_m$)] (cAD-SAK) and poly[Lys-(cAD$_j$-Ser$_i$-dl-Ala$_m$)]-Cys-Gly-Arg$_8$, (cAD-SAK-Cys-Gly-Arg$_8$), a conjugate that contain a cell penetrating peptide, octaarginine. The schematic structure of the conjugates is presented in Fig. 1. Here we report on our findings concerning the *in vitro* cytotoxicity, adhesion and *in vitro* cytostatic effect of the conjugates on murine bone marrow derived macrophages (BMDM), on murine (J774 and L1210) and human (MonoMac6) tumor cell lines. We also describe the results of a detailed study on the internalization of the conjugates. Data presented indicate the influence of the carrier structure on the uptake of the drug-conjugate and provided guideline for the identification of structural elements essential for the cellular uptake and for improved anti-tumor effect.

2. Materials and methods

2.1. Abbreviations

Abbreviations of amino acids and their derivatives follow the revised recommendation of the IUPAC-IUB Committee on Biochemical

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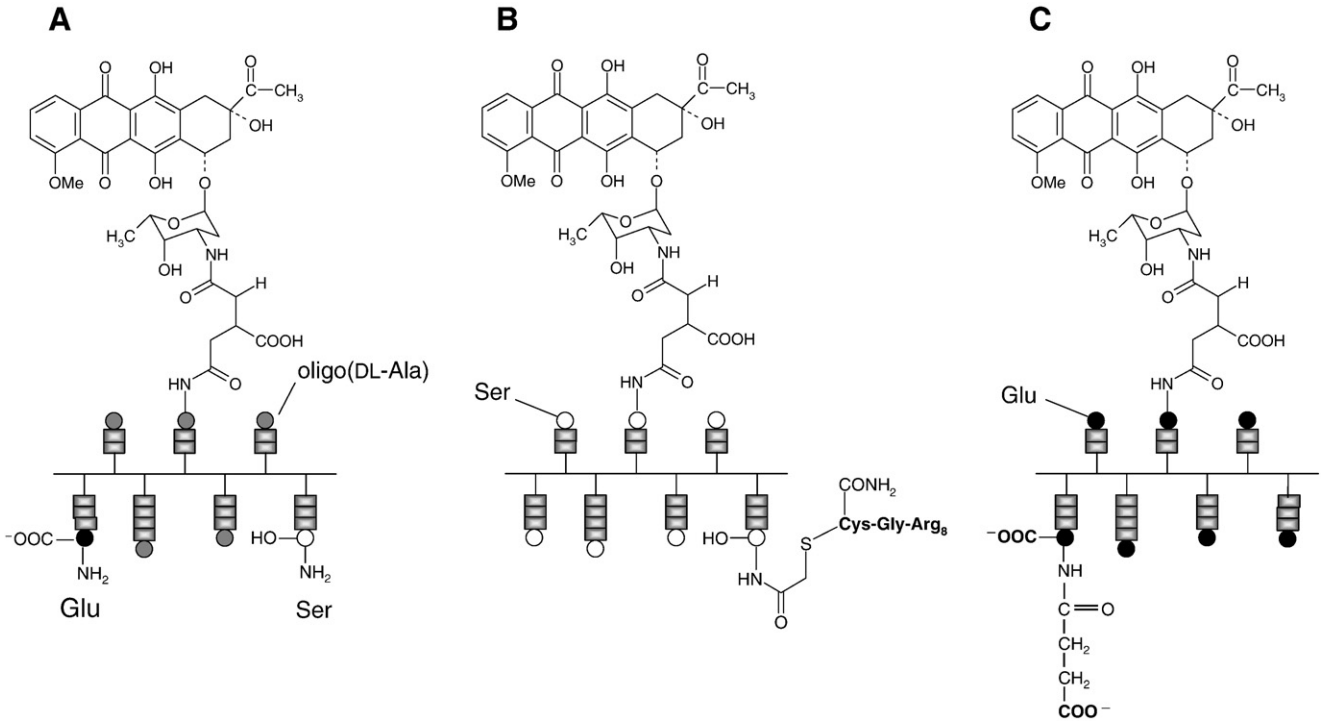

Fig. 1. Schematic presentation of daunomycin conjugates containing poly[L-Lys] based polypeptide carriers. A, poly[Lys-(cAD$_i$-Ser$_i$/Glu$_i$-dl-Ala$_m$)] (cAD-SAK/cAD-EAK). B, poly[Lys-(cAD$_j$-Glu$_i$-dl-Ala$_m$)]-Cys-Gly-Arg$_8$ (cAD-SAK-Cys-Gly-Arg$_8$). C, poly[Lys-(cAD$_j$- SuccGlu$_i$-dl-Ala$_m$)] (cAD-SuccEAK).

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 [11]. For the sake of brevity codes of branched chain polypeptides were constructed by us using the one-letter codes of amino acids. All amino acids are l configuration unless otherwise stated. The other abbreviations in this paper are the following: EDC: *N*-ethyl-*N*′-[3-(dimethylaminopropyl)carbodiimide hydrochloride, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, TFA: trifluoroacetic acid, FBS: fetal bovine serum, DMSO: dimethyl sulfoxide, TEA: triethylamine, DCM: dichloromethane, NMM: *N*-methylmorpholine, PBS: phosphate-buffered saline, Succ: succinyl, CF: 5(6)-carboxyfluorescein, rMu M-CSF: recombinant murine macrophage colony stimulating factor.

2.2. Materials

Daunomycin hydrochloride was a gift from the Institute of Drug Research Ltd. (Budapest, Hungary); *cis*-aconitic anhydride, EDC, MTT, RPMI-1640 medium powder, glutamine, gentamycin, TFA and other chemicals for buffers and eluents were from Sigma-Aldrich (Budapest, Hungary). FBS was obtained from Gibco Brl. and from Sigma-Aldrich (Budapest, Hungary). Dioxane, methanol, acetonitrile, DMSO, chloroform, and tetrahydrofuran were obtained from Molar Chemicals (Budapest, Hungary). rMu M-CSF was from R&D Systems (Minneapolis, MN, USA). Poly[Lys-(Ser$_{0.9}$-dl-Ala$_{2.5}$)], (SAK); poly[Lys-(Glu$_{1.0}$-dl-Ala$_{2.3}$)], (EAK), were synthesized in our laboratory as described in detail previously [12,13].

2.3. Synthesis of cAD-polypeptide conjugates

Cis aconityl daunomycin (cAD) was synthesized according to Ryser and Shen [14] with modifications [15]. cAD was coupled to the polypeptides using water-soluble carbodiimide EDC (1.5 eq.) as coupling reagent. The conjugates were purified by gel permeation chromatography. The conjugates were then freeze-dried and they were dissolved in distilled water for the experiments. Daunomycin content of the conjugates was determined by UV spectrophotometry.

2.4. Synthesis of cAD-SAK-Cys-Gly-Arg$_8$

Cys-Gly-Arg$_8$ peptide was synthesized by manual solid phase synthesis on Rink-amide resin (0.335 g, 0.73 mmol/g) using Fmoc/tBu strategy [16]. Coupling the Cys-Gly-Arg$_8$ peptide to the cAD-SAK conjugate was performed according to Bánóczi et al. [17]. First, the free amino groups of the side chain terminating Ser was chloroacetylated [18], then 1.2 eq. of Cys-Gly-Arg$_8$ peptide was added to the chloroacetylated conjugate in 0.1 M Tris–HCl (pH 8.2) buffer. The conjugation was continued for 24 h at RT and finally terminated by addition of an excess of Cys. The conjugate was purified by dialysis against distilled water and then freeze-dried. The conjugate was characterized by amino acid analysis. Daunomycin content was determined by UV spectrophotometry.

2.5. Succinylation of cAD-EAK

cAD-EAK (10 mg) was dissolved in carbonate buffer (0.5 M, pH 9.2) then 220 μL (220 μmol) succinic anhydride dissolved in DMSO (c = 100 μg/mL) was added with continuous stirring in 30 min at room temperature; pH was adjusted between 9.0 and 9.2 with 0.1 M NaOH. The reaction was continued for 4 h at RT. The product was purified by gel permeation chromatography on PD10 column filled with Sephadex G25 using distilled water as eluent and the column was washed with 1% acetic acid (v/v).

2.6. Labeling of polymeric polypeptides with 5(6)-carboxyfluorescein

Polypeptides (10 mg) (6×10^{-7} to 1.9×10^{-7} mol) were dissolved in 2 mL sodium-carbonate buffer (0.1 M; pH 9.4). 5(6)-carboxyfluorescein succinimid ester was dissolved in DMF (c = 10 mg/mL, 0.21 mM) and was added to the polypeptide solution in 20 μL aliquots. The reaction was continued for 1 h at room temperature with continuous stirring.

The product was purified by gel filtration on a PD10 column filled with Sephadex G25 using distilled water as eluent. The samples were then freeze-dried and they were resolved in PBS (0.1 M, pH 7.4) before use. Carboxyfluorescein content of the samples was determined by RP-HPLC as described earlier [8].

2.7. Cell lines

Cell lines cells were maintained in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine and 0.16 mg/mL gentamycin in 5% CO_2 atmosphere.

2.8. Isolation and culturing of bone marrow culture-derived macrophages (BMDM)

Bone marrow cells were isolated from the femur and tibia of female Balb/c mice at 6–8 weeks of age. Cells were maintained in 10 cm bacteriological Petri dishes in RPMI 1640 medium supplemented with 10%FCS, 10 mM HEPES, 0.16 mg/mL gentamycin and 10 ng/mL rMu M-CSF. The macrophages were harvested with PBS (0.1 M, pH 7.4) + 10 mM EDTA and plated on 24-well suspension culture plates 24 h prior to the uptake assay or on 96 well tissue culture plate 24 h before the cytotoxicity assay.

2.9. Measurement of cellular uptake by flow cytometry

Twenty-four hours prior to the experiment cells were plated on 24 well plates for suspension cells. Cells were washed twice with FCS-free RPMI medium before the treatment. The compounds to be tested were added to the cells at 0.1, 1, 10, 50 and 100 μg/mL concentration, and then incubated with the compounds for 0–60 min. After washing twice with PBS (0.1 M; pH 7.4), cells were examined by flow cytometry (BD LSR II). Fluorescence intensity of coupled daunomycin was recorded in case of cAD conjugates ($\lambda_{ex} = 488$ nm and $\lambda_{em} = 571$ nm) and fluorescence intensity of CF was measured in case of CF-SuccEAK ($\lambda_{ex} = 488$ nm and $\lambda_{em} = 519$ nm) from 5000 to 10000 cells and fluorescence mean was calculated. Assays were performed in duplicates with two or three parallels in each assay. Statistical analysis of data was performed by Student's *t* test at the 95% confidence level.

2.10. Measurement of cytotoxicity and in vitro antitumor effect by MTT assay

Cells were plated into 96 well tissue culture plate in culture medium with initial cell number of 10^4 or 5×10^3 cells/well, 24 h prior to the treatment. The compounds were added to the wells in serum free medium in 200 μL final volume. After a 1.5 h incubation cells were washed twice with serum free medium and in case of the cytostasis assay fresh culture medium was added to each well and cells were maintained at 37 °C for further 72 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well at 0.36 mg/mL final concentration. After 3.5 h incubation the absorbance was measured with ELISA-reader (Labsystems MS Reader) at 540 and 620 nm as reference wavelength. Assays were performed in duplicates with two or four to six parallels in each assay. Statistical analysis of data was performed by Student's *t* test at the 95% confidence level. Cytotoxic/cytostatic effect was calculated with the following Eq. (1):

$$\text{Cytotoxicity / cytostasis}[\%] = (1 - \text{OD}_{\text{treated}} / \text{OD}_{\text{control}}) \times 100 \quad (1)$$

2.11. Measurement of the adhesion of murine BMDM

Adhesion of murine bone marrow derived macrophages was studied in xCELLigence instrument (Roche, Basel, Switzerland) that measures electrical impedance by micro-electrodes integrated at the bottom of each well of tissue culture E-Plates coated with fibronectin. First, baseline were recorded in culture medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine and 0.16 mg/mL gentamycin and 10 ng/mL M-CSF) as control and in separate wells in the solution of the conjugates dissolved in culture medium ($c = 100$ μg/mL). After 1 h pre-incubation, 10^4 macrophages were plated into each well. Adhesion was monitored for 48 h at 37 °C in 5% CO_2 atmosphere. Cell index was calculated by the software of xCELLigence instrument from electrical impedance data (Eq. (2))

$$\text{Cell index} = (Z_i - Z_0) / 15, \quad (2)$$

Where Z_0 is electrical impedance at 0 time point and Z_i is electrical impedance at *i* time point.

3. Results

3.1. Daunomycin conjugates

Three daunomycin conjugates containing cis-aconityl spacer (cA) and polylysine based polycationic (poly[Lys-(cAD$_i$-Ser$_i$-dl-Ala$_m$)], (cAD-SAK), amphoteric poly[Lys-(cAD$_j$-Glu$_i$-DL-Ala$_m$)], (cAD-EAK) or anionic poly[Lys-(cAD$_j$-SuccGlu$_i$-DL-Ala$_m$)], (cAD-SuccEAK)) polypeptide carrier as well as a conjugate with additional Arg_8 moiety (cAD-SAK-Cys-Gly-Arg_8) were used in these studies. The schematic structure of these compounds is presented in Fig. 1, while their chemical characteristics are summarized in Table 1.

3.2. In vitro cytotoxicity

Cytotoxicity of the cAD conjugates was tested at the concentration range of 0.5–500 μg/mL calculated on the basis of its daunomycin content by MTT assay. Results are expressed as a percentage of untreated cells. Data are presented in Fig. 2. We found that cAD conjugates were not toxic on BMDM, MonoMac6 and L1210 cells up to $c = 500$ μg/mL after 1.5 h treatment. J774 cells proved to be slightly more sensitive to the treatment with the conjugates, but only at high concentration ($c > 50$ μg/mL) after 1.5 h incubation (37%).

3.3. Cellular uptake: Concentration dependence

Internalization of conjugates was examined under different conditions using flow cytometry. The influence of conjugate concentration and temperature dependence was investigated on the uptake. We also studied the involvement of scavenger receptor in the internalization process using inhibition assays. The murine leukemia cell lines (J774, MonoMac6 and L1210) as well as BMDM internalized the cAD conjugates (Fig. 3) as well as HL-60 human leukemia cells (data not shown). The uptake was concentration dependent. All the conjugates were taken up the most efficiently by BMDM (from

Table 1
Chemical characteristics of daunomycin conjugates.

Conjugate[a]	Polypeptide carrier[b]	Dau [S%][c]
cAD-SAK	poly[Lys(Ser$_{0.9}$-DL-Ala$_{2.5}$)]	12.0
cAD-SAK-Cys-Gly-Arg_8	poly[Lys(Ser$_{0.8}$-DL-Ala$_{2.4}$)]	11.0
cAD-EAK	poly[Lys(Glu$_{1.0}$-DL-Ala$_{2.3}$)]	7.0
cAD-SuccEAK	poly[Lys(Glu$_{1.0}$-DL-Ala$_{2.3}$)]	7.0

[a] Code of each branched chain polymeric polypeptide carrier is based on one-letter symbol of amino acids.
[b] Amino acid composition was determined by amino acid analysis as after hydrolysis in 6 M HCl at 105 °C for 24 h.
[c] Average degree of substitution for daunomycin was determined by UV spectrophotometry.

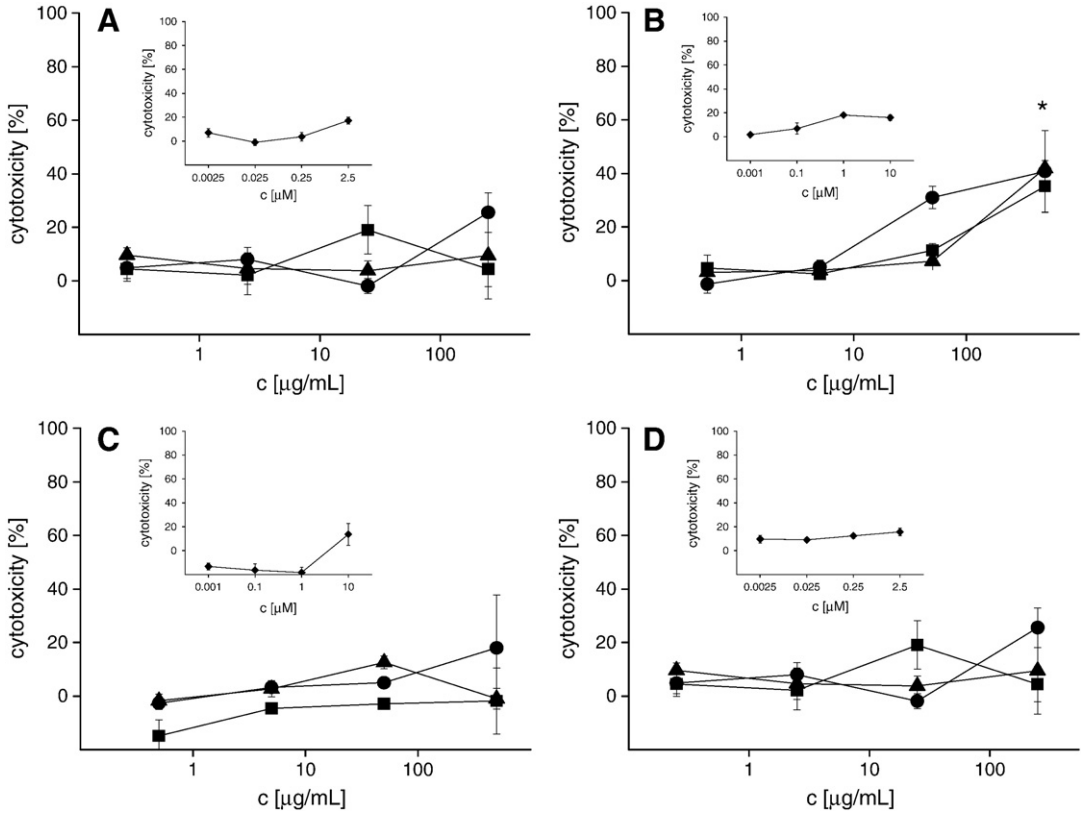

Fig. 2. The effect of cAD conjugates on the viability of murine bone marrow derived macrophages (BMDM), murine and human leukemia cell lines. Diagrams represent the average cytotoxicity of the conjugates as a percentage of untreated control ± SD. Statistical analysis of data was performed by Student's *t* test. A, BMDM. B, J774. C, MonoMac6. D, L1210. ■, cAD-SAK; ●, cAD-EAK; ▲, cAD-SuccEAK; inserts: ◆, daunomycin.

$c \leq 10$ μg/mL). Results show that the uptake was also cell specific: J774 cells internalized cAD-SuccEAK the most effectively, while L1210 cells and BMDM favored cAD-EAK (Fig. 3). The uptake of cAD-SAK-Cys-Gly-Arg_8 conjugate was investigated on BMDM and HL-60 human leukemia cells (Fig. 4). Results indicate that the internalization of cAD-SAK-Cys-Gly-Arg_8 by BMDM as well as by HL-60 cells was

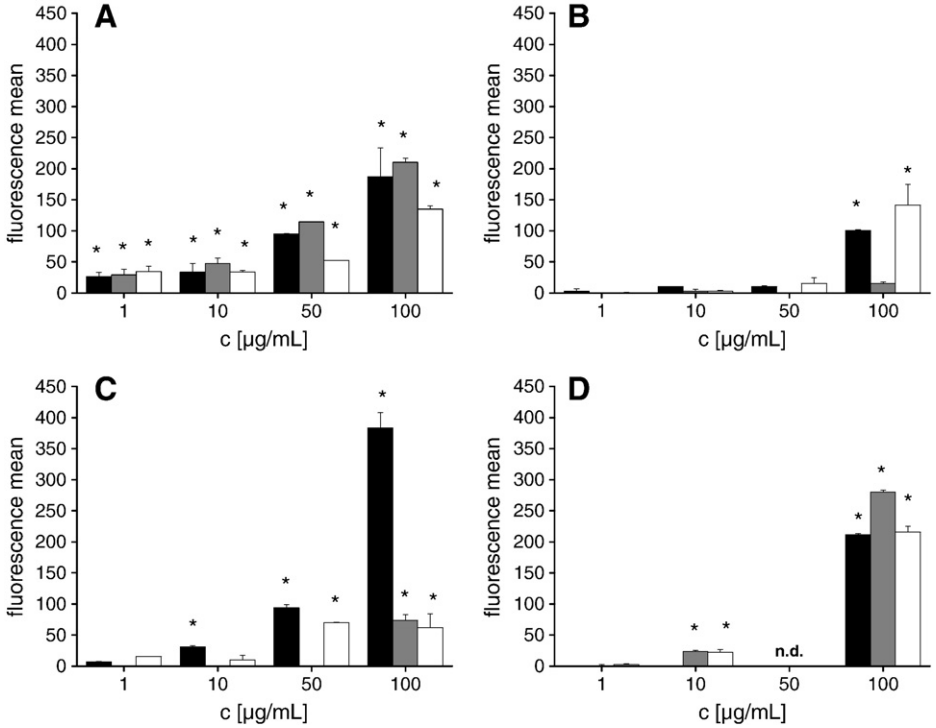

Fig. 3. Concentration dependence of the uptake of cAD conjugates after 60 min incubation. Statistical analysis of data was performed by Student's *t* test; *: $p<0.05$. A, BMDM. B, J774. C, MonoMac6. D, L1210. ■, cAD-SAK; ■, cAD-EAK; □, cAD-SuccEAK.

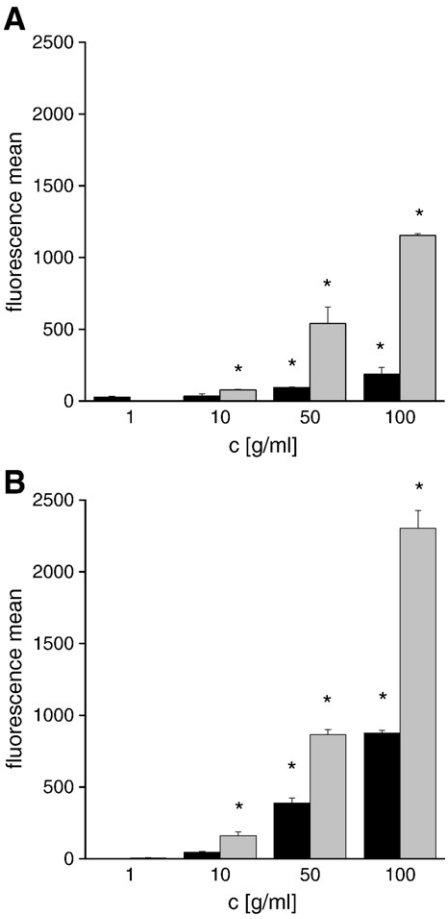

Fig. 4. Concentration dependence of the uptake of the cAD-SAK (■) and cAD-SAK-Cys-Gly-Arg_8 (■) conjugates by BMDM (A) and HL-60 (B) cells after 60 min incubation. Statistical analysis of data was performed by Student's *t* test; *: $p<0.05$.

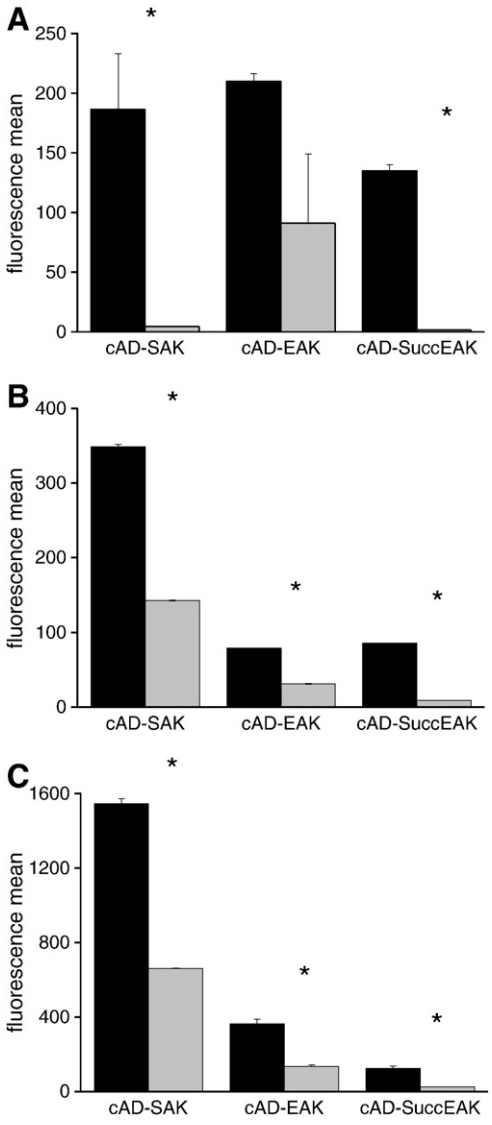

Fig. 5. Uptake of cAD conjugates at 4 and 37 °C at c = 100 μg/mL after 1 h. Columns represent the average of mean fluorescence values of a representative experiment of two independent assays ± SD after subtracting the control. A, BMDM. B, J774. C, MonoMac6. ■, 37 °C; ■, 4 °C. Statistical analysis of data was performed by Student's *t* test; *: $p<0.05$.

concentration dependent. HL-60 human leukemia cells internalized the conjugate more effectively over 10 μg/mL concentration than murine bone marrow derived macrophages. The presence of the cell penetrating peptide in the conjugate resulted in a marked increase in the internalization: conjugate containing Arg_8 was ingested even at lower concentration ($c = 10$ μg/mL) and more efficiently by both cell types compared to the conjugate containing only the polycationic polypeptide carrier.

3.4. Cellular uptake: Temperature dependence

The uptake of cAD-SAK, cAD-EAK and cAD-SuccEAK conjugates was examined also at 4 °C versus 37 °C on BMDM, J774 and MonoMac6 cells by flow cytometry. In the case of both tumor cell lines (J774 and MonoMac6) a substantial extracellular binding of cAD-SAK could be observed (Fig. 5B and C). Nevertheless, a significant difference could be detected between the uptake at 37 °C and extracellular binding at 4 °C of all the three conjugates studied.

3.5. The role of scavenger receptor A (SR-A) in the uptake

We examined the role of the scavenger receptor in the internalization of cAD-EAK and cAD-SuccEAK conjugates by J774, (Fig. 6A and B) and by BMDM (Fig. 6C and D). Uptake was inhibited either with the SR ligand, sulphated polysaccharide fucoidan or poly(I) (Fig. 6) or a SR-A specific monoclonal antibody 2F8. Inhibition of the uptake of cAD-SuccEAK with poly(I) was compared to that of CF-SuccEAK. No statistically significant inhibition of the uptake of cAD-SuccEAK could be observed by fucoidan ($c = 100$ μg/mL) or poly(I) ($c = 50$ μg/mL) in case of J774 cells. When blocking scavenger receptor with fucoidan in case of bone marrow derived macrophages, we could observe only a slight, though statistically significant decrease in the uptake of cAD-SuccEAK and a slight more pronounced effect when using poly(I) as inhibitor (Fig. 6B and C). SR-A specific monoclonal antibody also inhibited the internalization of cAD-SuccEAK (Fig. 7A) by BMDM. The cargo (cAD vs. CF) coupled to the polypeptide carrier influenced markedly the internalization via scavenger receptor by the macrophages (Fig. 7B). In case of CF-SuccEAK a more pronounced inhibition of the uptake could be observed compared to cAD-SuccEAK (inhibition = 63% and 35%, respectively) when using poly(I) as inhibitor (Fig. 7C–E).

3.6. Adhesion of BMDM

Adhesion of murine bone marrow derived macrophages was studied on fibronectin coated surface treated with all cAD conjugates and daunomycin (as control) prior to the experiment. Results show

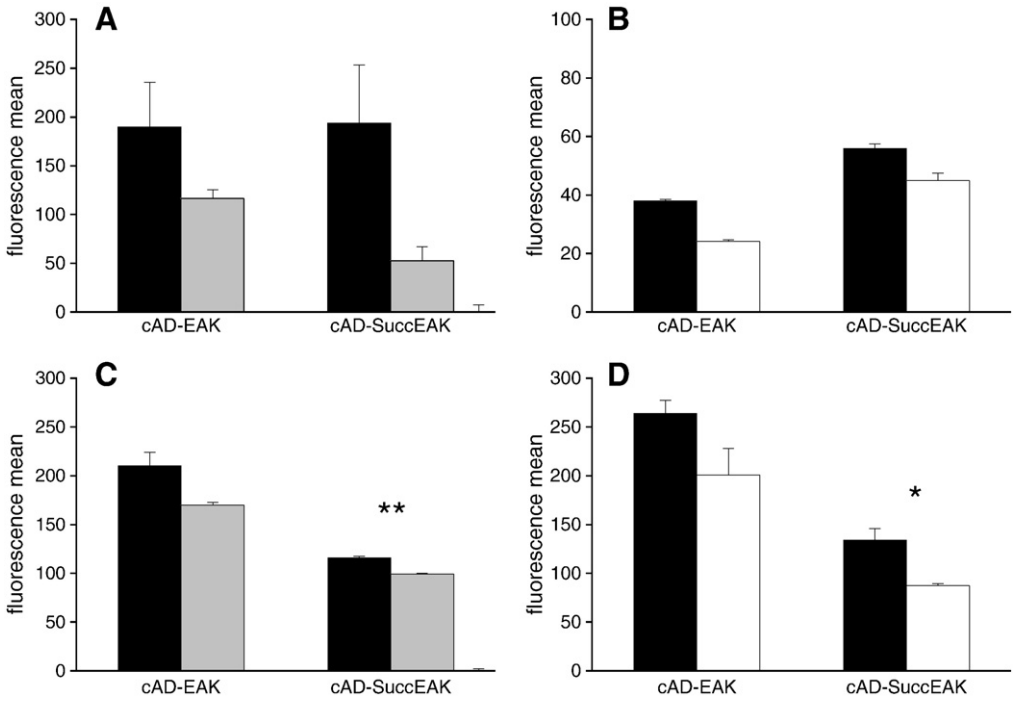

Fig. 6. Uptake of the conjugates after blocking scavenger receptor. Columns represent the average of mean fluorescence values of a representative experiment of two independent assays ± SD after subtracting the control. A and B, J774. C and D, BMDM. ■, uptake of the conjugates ($c = 100$ μg/mL) without inhibitor; ▒, uptake of the conjugates after pretreatment with fucoidan ($c = 100$ μg/mL); □, uptake of the conjugates after pretreatment with poly(I) ($c = 50$ μg/mL). Statistical analysis of data was performed by Student's t test; *: $p < 0.05$.

that free daunomycin inhibited the adhesion of the surface, which could be explained with the cytotoxic effect of the drug on BMBM cells. All four cAD-SAK, cAD-SAK-Cys-Gly-Arg_8, cAD-EAK and cAD-SuccEAK conjugates increased the adhesion of the macrophages. The most adhesive compounds were cAD-EAK and cAD-SuccEAK (Fig. 8A). In case of cAD-SAK a long-term cytotoxicity could be detected, which resulted in the detachment of the macrophages after 30 h. By contrast, cAD-SAK-Cys-Gly-Arg_8 was not significantly toxic to the macrophages even after 50 h incubation (Fig. 8B).

3.7. In vitro antitumor effect of cAD conjugates

In vitro antitumor effect of the conjugates (cAD-SAK, cAD-EAK, and cAD-SuccEAK) was studied on J774, MonoMac6 and L1210 cells by

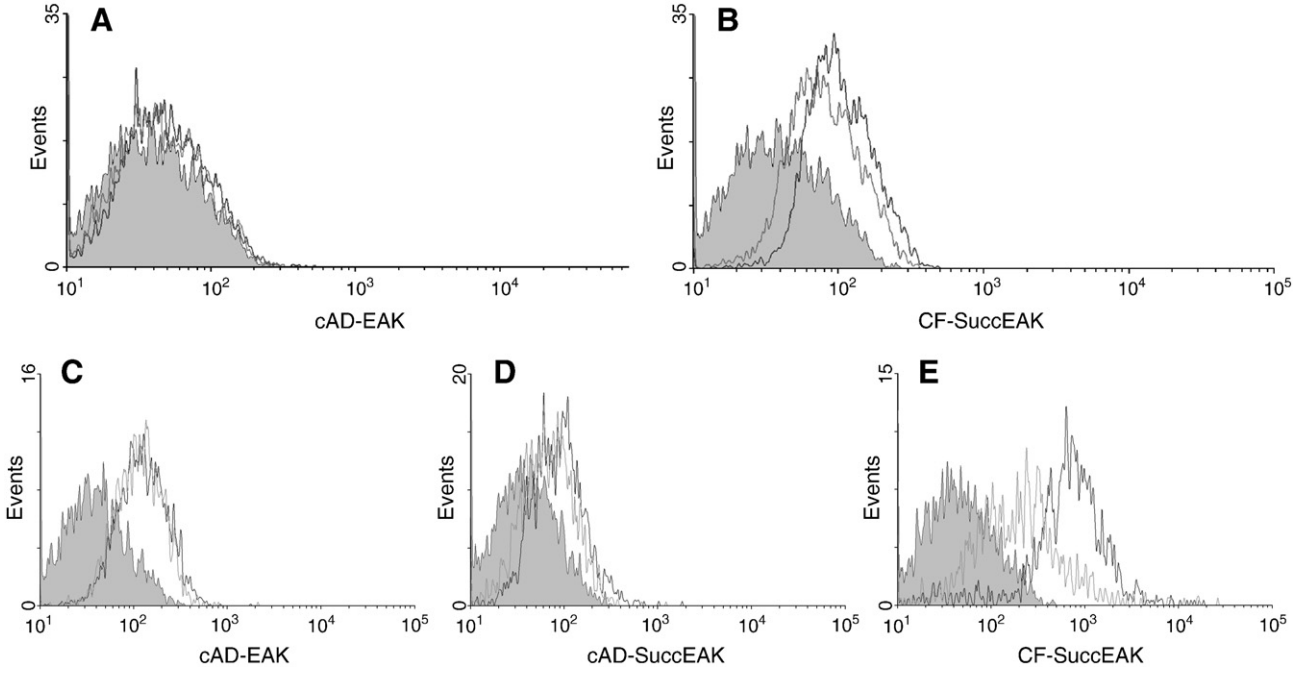

Fig. 7. Uptake of cAD-EAK and cAD-SuccEAK conjugates by BMDM after blocking class A scavenger receptor with mAb 2F8. A, cAD-EAK. B, cAD-SuccEAK and with poly(I). C, cAD-EAK. D, cAD-SuccEAK. E, CF-SuccEAK. Data were recorded by measuring 10000 cells and were analyzed by FACS DiVa software. Filled diagrams represent untreated control macrophages, black lines represent uptake without inhibitor and grey lines represent uptake of the conjugate by BMDM in the presence of mAb 2F8.

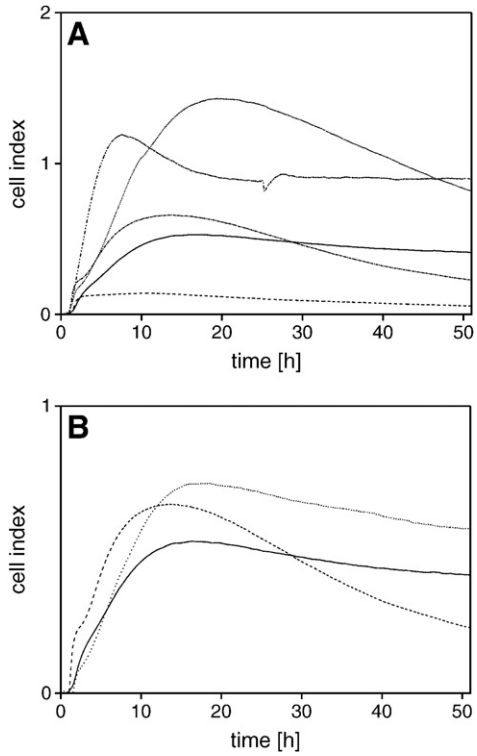

Fig. 8. Adhesion of murine bone marrow derived macrophages on fibronectin coated (—) surface treated with daunomycin (---), cAD-SAK (-·-), cAD-EAK (···) and cAD-SuccEAK (-···-) (A) and cAD-SAK-Cys-Gly-Arg_8 (···) (B), where cAD-SAK (---) was used as control. Conjugate concentration was 100 μg/mL.

MTT assay. Results indicate that the conjugates inhibited only the growth of J774 cells with similar efficacy (cAD-SAK $IC_{50} = 80.77 \pm 0.57$ μg/mL and cAD-SuccEAK: $IC_{50} = 81.52 \pm 1.73$ μg/mL, cAD-EAK: $IC_{50} = 62.38 \pm 13.5$ μg/mL). In case of MonoMac6 cells, cAD-EAK elicited only a slight inhibitory effect, but no IC_{50} values could be calculated (Fig. 9).

4. Discussion

Anthracyclin type drug, daunomycin is applied mainly for therapy of leukemia (e.g. acute myeloid leukemia, acute lymphocytic leukemia and chronic myelogenous leukemia), neuroblastoma and other types of cancer. Daunomycin can inhibit division of tumor cells via intercalating DNA, stabilizing topoisomerase II–DNA complex or enhancing the production of free radicals [19,20].

For the present studies, daunomycin was coupled to polylysine based branched polypeptide carriers with different charge properties. Biological properties of polymeric polypeptides based on poly[L-lysine] were thoroughly investigated in our research group. *In vitro* and *in vivo* cytotoxicity, immunogenicity, immunomodulatory effect, biodistribution and blood survival have been studied [21]. It was ascertained that the composition of the side chain and particularly the character of the terminal amino acid significantly influences the biological properties including cellular uptake of the polypeptides and even that of their conjugates with methotrexate [22], daunomycin [2,23,24] and a GnRH antagonist peptide [25]. We have also demonstrated that polylysine based branched polypeptides with polyanionic character (SuccEAK and MalEAK) enter macrophages via SR-A mediated endocytosis [26].

In this study four conjugates were used: a conjugate containing amphoteric (cAD-EAK); anionic (cAD-SuccEAK) polypeptide carrier and two polycationic conjugates without (cAD-SAK) or with cell penetrating octaarginine (cAD-SAK-Cys-Gly-Arg_8). Cytotoxicity and cellular uptake were examined on murine bone marrow derived macrophages and also on murine (J774 and L1210) and human (MonoMac6) tumor cell lines. Two of these were macrophage like cell lines J774 and MonoMac6). After 1.5 h incubation neither the conjugates nor daunomycin were toxic on bone marrow derived macrophages or on murine and human tumor cell lines.

The influence of the conjugates on the adhesion of bone marrow derived macrophages was also investigated. The macrophages attached to the surface coated with the conjugates the highest adhesion was observes in case of the amphoteric cAD-EAK.

Two approaches of intracellular drug delivery of daunomycin are discussed in this paper. We studied the influence of the polypeptide carrier structure. First we analyzed the cell specific delivery of a cAD-conjugate via scavenger receptor A, in which daunomycin was coupled to a polypeptide carrier of polyanionic character. Inhibition experiments were carried out in order to clarify if scavenger receptor plays a role in the internalization of the polyanionic daunomycin conjugate. SR inhibitors like the sulphated polysaccharide fucoidan and a four-stranded nucleic acid, poly(I) were used. Class A scavenger receptor was also blocked with a receptor specific monoclonal antibody, 2F8 [27]. Our observations indicated that pretreatment with the inhibitors resulted in a slightly reduced internalization of succinylated cAD-conjugate, cAD-SuccEAK with polyanionic character

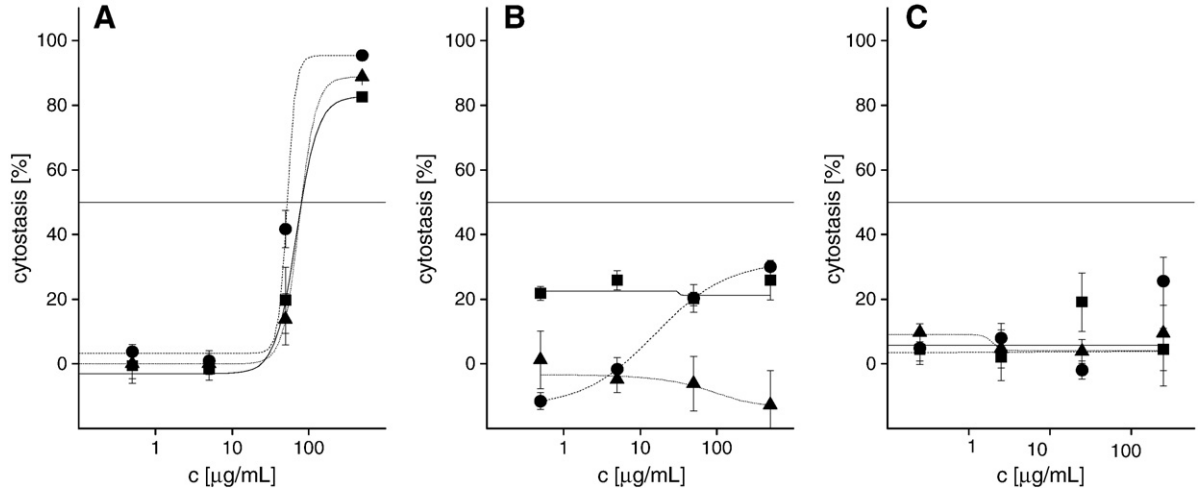

Fig. 9. *In vitro* antitumor effect of cAD conjugates on J774 (A), MonoMac6 (B) and L1210 (C) cells. Diagrams represent the average cytostatic effect of the conjugates as a percentage of untreated control ± SD. ■, cAD-SAK; ●, cAD-EAK; ▲, cAD-SuccEAK.

by murine bone marrow derived macrophages, but no significant inhibition could be detected in case of J774 tumor cell line. As the internalization of amphoteric cAD-EAK conjugate used as control was essentially not altered by the SR inhibitors, we suppose that the charge of the polypeptide carrier influences the mechanism of internalization of the cAD conjugates. We can establish that SR-A could play a role in the uptake of the polyanionic conjugate. Compared the level of internalization of 5(6)-carboxyfluorescein labeled SuccEAK (CF-SuccEAK) and cAD-SuccEAK we found CF-SuccEAK more effective than the daunomycin containing conjugate. Based on these results we can suppose that the cargo has a crucial influence on the internalization process. The low effect of the scavenger receptor inhibitors also points out that other mechanisms are also involved in the internalization process of the polyanionic cAD-SuccEAK conjugate. The second way of intracellular delivery is cell penetration. It is known that cationic oligopeptides like arginine rich peptides and 4- to 9-mer oligoarginines enter the cells with high efficiency and they are able to carry even a protein cargo into the cell [28–30]. Results showed that cells internalized cAD-SAK-Cys-Gly-Arg_8 at higher level than that of conjugate cAD-SAK. This indicates that the presence of oligoarginine in the conjugate has a marked influence on the uptake properties.

We also studied *in vitro* antitumor effect of cAD conjugates and we found that their cytostatic effect was cell specific. Three conjugates studied (cAD-SAK, cAD-EAK and cAD-SuccEAK) inhibited the growth of J774 cells but had no antitumor effect on the other two cell lines (MonoMac6 and L1210). *In vitro* antitumor effect of the conjugates proved to be independent on the charge of the *N*-terminal amino acid of the side chains of the carrier polypeptides.

Taken together, the cAD conjugates depending on their structure are promising candidates for eliminating certain types of tumor cells e.g. murine macrophage like cells.

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