

## Block Copolymer Carrier Systems for Translymphatic Chemotherapy of Lymph Node Metastases

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**Abstract.** *The presence of lymph node metastases relevantly and significantly impairs disease-specific survival in patients suffering from squamous cell carcinoma of the upper aerodigestive tract. In a VX2 animal tumor model, we present an interstitial translymphatic therapeutic approach using cis-diaminedichloro-platinum(II) (CDDP) conjugated to a poly(ethylene oxide)-block-poly(lysine) (PEO-b-PLys) block copolymer tracking systems for the successful treatment of lymph node metastases. Most effective was the application of a high cargo-load CDDP tracking system (48 wt.% CDDP) curing 90% of the animals and causing only minor local side-effects. Systems containing 1 or 10 wt.% of CDDP were less effective but still cured 50% of the animals. Moreover, the administration of 1 or 10 wt.% of CDDP consistently limited tumor growth to the draining lymph nodes (50%) and prevented systemic distribution of the metastasis even with 1 wt.% CDDP load. The systems contained 0.25-0.003 mg/kg per body weight CDDP compared to 1 ml/kg per body weight as usually used for intravenous administration. This approach encourages further and more detailed research of a CDDP-based interstitial translymphatic administration of chemotherapy for lymphogenic metastasizing carcinomas in different body regions.*

Despite significant progress in the fields of drug delivery, surgery and radiochemotherapy, no significant increase in disease-specific survival for head and neck cancer has been accomplished (1). It is a generic problem that tumors often reappear after a completed therapy due to residual malignant cells in the lymphatic system (2, 3). Successful

treatment of squamous cell carcinoma of the upper head and neck region (HNSCC), which dominantly spreads via the lymphatic system, requires an as yet unavailable effective systemic cancer therapy acting primarily within the lymphatic system (4-7). The metastatic cascade first leads to regional lymph node disease. In the further course distant metastases may occur. Statistics indicate an increase in HNSCC, with a rate of survival of about 40-50% for all sites (8, 9). In up to 85% of cases, lymph node metastases are present (10-12) and 20% or more of patients are already suffering from metastatic spread via the lymphatic system at first presentation (13). Lymph node metastases significantly and negatively affect disease-specific survival in HNSCC. The overall disease-specific survival for patients with N0 neck (no lymph node disease) is 67.9% compared to 39.9% for patients with N+ neck (histologically proven lymph node disease) (8-13).

Here, we present a novel therapeutic approach utilizing translymphatic administration of cis-diaminedichloro-platinum(II) (CDDP) conjugated to a poly(ethylene oxide)-block-poly(lysine) (PEO-b-PLys) block copolymer for the treatment of lymph node metastases after surgical resection of the primary tumor.

### Materials and Methods

*Materials.* All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received or as specified in the referenced publications. CDDP (Aldrich 99.999%) was used as received from Sigma-Aldrich.

#### *Synthesis of block copolymer carriers*

*Solution-phase synthesis of carrier system (PI).* Poly(ethylene oxide)-block-poly(Z-L-lysine) (PEO-b-PZLYs) was synthesized by ring-opening polymerization of Z-L-lysine-N-carboxyanhydride using  $\alpha$ -methoxy- $\omega$ -amino-PEO<sub>114</sub>(PEO<sub>114</sub>-NH<sub>2</sub>) as the macroinitiator (28). A solution of 2.50 g PEO<sub>114</sub>-NH<sub>2</sub> and 4.97 g Z-L-lysine-NCA in dry (dimethylformamide, DMF) was stirred for 48 h at 40 °C under a dry argon atmosphere. After evaporation of the solvent, the residual solid was re-dissolved in CHCl<sub>3</sub> and precipitated in petroleonon-ether. The Z-protecting groups were removed by treatment of the

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PEO<sub>114</sub>-*b*-PZLLys<sub>40</sub> with 30% HBr in glacial acid (w/w). The reaction mixture was diluted with water, neutralized with NaOH, dialyzed against water (MWCO 1000 Da), and freeze-dried. <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>) and size-exclusion chromatography (SEC) confirmed the chemical structure of the copolymer: PEO<sub>114</sub>-*b*-PZLLys<sub>40</sub> with a polydispersity index of 1.2.

*Solid-phase supported synthesis of carrier systems (P2).* The synthesis was performed according to procedures reported elsewhere (29). Briefly, the PEO-*block*-polypeptide carrier was synthesized *via* solid-phase supported peptide synthesis techniques following standard Fmoc protocols (31). After stepwise polypeptide assembly on a PAP resin (Rapp, Tübingen, Germany) using HBTU/NMP/piperidine protocols, liberation of the conjugate was achieved by treatment with 99% TFA (trifluoroacetic acid), 1% TMSBr (trimethylsilyl bromide), 2-6 h. The cleavage mixture was concentrated, diluted with methanol and dialyzed subsequently against methanol and water (MWCO 3500 Da), followed by freeze-drying. The chemical identity of **P2**: PEO<sub>73</sub>-*b*-PLys<sub>35</sub>, was confirmed by matrix-assisted laser desorption-ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) and <sup>1</sup>H-NMR.

*Preparation of the CDDP-loaded block copolymer tracking systems.* Exemplary procedure for **T1**: 93.6 mg CDDP were incubated with 120 mg **P1** in 24 mL of a physiological NaCl solution at 23 °C. CDDP was solubilized within 24-48 h. The clear, slightly yellowish solution was stored at 4 °C in the dark for the duration of the therapy. Occasionally formed precipitate could rapidly be redissolved by heating the sample for a few minutes to 37 °C. Prior to administration, the solution was sterilized by filtration through a sterilized 0.2-µm syringe filter (Millipore, Billerica, USA).

#### Instrumentation:

(i) *Proton nuclear magnetic resonance spectroscopy (<sup>1</sup>H-NMR):* Spectra were recorded at room temperature on a Bruker DPX-400 Spectrometer (Bruker, Ettlingen, Germany) operating at 400.1 MHz.

(ii) *MALDI-TOF MS:* Measurements were performed on a Voyager-DE STR BioSpectrometry Workstation MALDI-TOF mass spectrometer (PerSeptive Biosystems, Inc., Framingham, MA, USA). The samples were dissolved in 0.1% TFA in acetonitrile-water (1:1 v/v) at a concentration of 0.1 mg/mL. One µL of the analyte solution was mixed with 1 µL of alpha-cyano-4-hydroxycinnamic acid matrix solution consisting of 10 mg of matrix dissolved in 1 mL of 0.3% TFA in acetonitrile-water (1:1 v/v). From the resulting mixture, 1 µL was applied to the sample plate. Samples were air-dried at ambient temperature. Measurements were performed at an acceleration voltage of 20 kV. Each spectrum obtained was the mean of 250 laser shots.

(iii) *Size-exclusion chromatography (SEC):* Measurements were performed on a set-up from Thermo Separation Products (Abbott, Waukegan, USA) equipped with UV ( $\lambda=270$  nm) and RI detectors. The column set consisted of four 300x8 mm columns filled with a PSS-GRAM spherical polyester gel having an average particle size of 10 µm and a pore size of 30, 30, 100, and 3000 Å, respectively. The eluent used was *N,N*-dimethylacetamide + 0.5% (w/w) LiBr at a temperature of 70 °C and a flow rate of 0.8 mL/min; 100 µL of 0.15 wt % polymer solutions were injected. Calibration of the columns was carried out with polystyrene standards.

Table I. *Experimental groups.*

Control I	No therapy	7 animals
Control II	P1 block polymer without CDDP	7 animals
Treatment group I	<b>T1; P1</b> , 48 wt.% CDDP	10 animals
Treatment group II	<b>T2; P2</b> , 10 wt.% CDDP	7 animals
Treatment group III	<b>T3; P3</b> , 1 wt % CDDP	7 animals

(iv) *Elemental analysis:* Palladium analysis was carried out at Terracon Service Laboratories (Germany). Pt determination was performed utilizing inductively coupled plasma optical emission spectroscopy (ICP-OES) according to a DIN 38406-E22 procedure.

(v) *Analytical ultracentrifugation (AUC):* Sedimentation velocity experiments (1200 rpm up to 60000 rpm) were performed on an Optima XLI (Beckman Coulter, Palo Alto, USA) equipped with interference and absorption optics. The sedimentation of the platinum complex was followed independently from that of the polymeric carrier at a wavelength of 300 nm.

(vi) *Dynamic light scattering (DLS):* Solutions of CDDP loaded carrier polymers containing 5 mg/mL polymer were filtered through 5 µm filters into cylindrical scattering cells (Hellma, Müllheim, Germany). Measurements were performed at 25 °C on a commercial goniometer with a digital correlator (both ALV, Langen, Germany) and a He-Ne laser (Polytec, Waldbronn, Germany) at a wavelength  $\lambda=633$  nm. The data evaluation of the correlation functions was based on the computer program CONTIN (Provencher, 1982).

*Animals and animal treatment.* The study was performed according to the PHS Policy on Humane Care and Use Laboratory Animals, the NIH Guide for Care and Use of Laboratory Animals, and the Animal Welfare ACT; the animal use protocols (VI 63 – 19 c 20-15 (1) MR 20/3 – Nr. 33/2003) were approved by the Institutional Animal Care and Use Committee (IACUC) of the Local Government of Giessen, Germany. The study used a total of 38 healthy adult, 0.5- to 1-year-old female, specific pathogen-free, Iffa Credo New Zealand White outbred rabbits weighing 2.5-3.5 kg (Behring Institute, Marburg, Germany).

*Tumor model and cold steel resection, general trial protocol.* For tumor implantation 0.1-0.25 mL of a suspension, containing 1-2x10<sup>7</sup> vital VX2 carcinoma cells, was injected concentrically between the lateral auricle edge and the central auricular artery into the cranial section of the upper third of the right auricle as described elsewhere (30). On the 17th day after tumor induction, all animals underwent 2/3 ablation of the right auricle. Tumor resection was followed by observation (control I), daily interstitial, translymphatic injections of P1 block polymer without CDDP (control II) or CDDP-loaded systems (0.15 mL) (treatment group I-III) *via* a butterfly canula (Venofix®, 21 Gauge, Braun, Melsungen, Germany) into the auricular stump for 21 days. Treatment groups are shown in Table I. After a follow-up time of 28 days all animals were sacrificed. For evaluation of distant metastases, both lungs and the liver were extirpated, sliced in 3 to 4 mm-thick slices and examined for macroscopic changes. Histological work-up included hemalain and eosin (H&E) staining of 3-µm serial sections.

Table II. Characteristics of CDDP-loaded tracking systems.

Tracking system	Carrier	[Carrier] <sub>0</sub> (mg/mL)	[CDDP] <sub>0</sub> (mg/mL)	[CDDP] <sub>0</sub> /[Lys] <sub>0</sub>	CDDP load (wt. %)
<b>T1</b>	<b>P1</b>	5.0	4.68	0.80	48
<b>T2</b>	<b>P2</b>	5.0	0.55	0.09	10
<b>T3</b>	<b>P2</b>	5.0	0.05	0.01	1

## Results

Two polymer carriers of different molecular weight distribution and composition were utilized. PEO<sub>114</sub>-*b*-PLys<sub>40</sub> (**P1**; the subscripts denoting the average numbers of repeat units) was prepared by anionic ring-opening polymerization of lysine based *N*-carboxyanhydrides (NCA) using an PEO<sub>114</sub>-NH<sub>2</sub> macroinitiator (28). The polydispersity index of the sample was 1.2, corresponding to approximately 40 ± 15 L-Lys repeat units. PEO<sub>73</sub>-*b*-PLys<sub>35</sub> (**P2**), was synthesized by solid phase-supported peptide synthesis (SPPS) from a PEO<sub>73</sub>-NH<sub>2</sub> functionalized resin (29). This sample, unlike **P1**, exhibits a definite number of exactly 35 Lys units. However, the aim of this first study was not to compare the performance of the two different carriers but to investigate different CDDP loads.

Three CDDP-loaded tracking systems (**T1-T3**, Table II) were prepared by incubating the desired amount of CDDP with the polymeric carriers in physiological saline solution. Conjugation of CDDP to the peptide segment of the carrier should occur, as suggested for congener systems (21, 24, 28), *via* a ligand exchange reaction of a CDDP chloro-ligand by an *ε*-amino group of lysine. The exact structure of the complex is not known yet. However, it is noteworthy that the straightforward mixing of CDDP and PEO-*b*-PLys results in pharmacologically active platinates.

Analysis of **T1** and **T2** by AUC and dynamic light scattering indicated that these are molecular carriers, rather than colloidal ones. AUC results further suggested the absence of non-conjugated CDDP, and thus a lymphatic transport of tracking systems should not be disturbed by any diffusive outflow of CDDP. Moreover, agglomeration could not be observed even after one year's storage at 4 °C, demonstrating the extraordinary long-term stability of dissolved tracking systems.

All seven animals of control group I suffered from metastatic spread (Table III), leading to necrotic, vital lymph node metastases in the primary and secondary lymph node stations. In six of seven animals far distant metastases were observed. Application of the carrier **P1** without CDDP (control group II) did not have any impact on the development or metastatic spread of the tumor, indicating the absence of sufficient immune stimulation by the carrier alone.

Table III. Therapeutic results of control experiments (control I: no therapy, control II: application of **P1** without CDDP) and of application of different tracking systems.

	Control I	Control II	<b>T1</b>	<b>T2</b>	<b>T3</b>
Tumor-free	0/7	0/7	9/10	5/7	2/7
Primary lymph node metastases	7/7	7/7	1/10	2/7	5/7
Secondary lymph node metastases	7/7	6/7	1/10	0/7	1/7
Distant pulmonary metastases	6/7	5/7	0/10	0/7	0/7

Entries *x/n* denote the number of animals *x* in a group of *n* animals showing a certain indication.

In nine of ten animals, treated by the CDDP-loaded tracking system **T1** (48 wt.% CDDP), the histological work-up of the primary lymph node (Figure 1c) showed no vital tumor cells, but scarring and secondary follicles. One remaining animal exhibited remaining vital tumor cells in the first two draining lymph node stations. None of these animals suffered from far distant spread, either in the lymphatic system or to the lungs.

Five of seven animals treated with **T2** and two of seven animals treated with **T3** did not show any vital cancer cells in the draining lymph nodes. None of the 14 animals in these groups exhibited far distant metastases after the follow-up survey and only one developed metastases in the second draining lymph node.

No severe systemic or local side-effects were recognized during therapy or the follow-up phase. Mild adverse effects, closely limited to local reactions at the locus of injection, were observed in all 31 animals (therapy groups) such as local hair loss after 7-12 days accompanied by mild, local inflammation after 10-14 days. Bacterial superinfections or systemic reactions (fever, elevated liver enzymes, decreased kidney function, weight loss, significant hair loss) were not observed at any time.

## Discussion

The strategy applied utilizes a translymphatic administration of CDDP conjugated to a PEO-*b*-PLys block copolymer. The method of administration in combination with size and structure of the polymer-drug complex assures distribution of the drug within the lymphatic system, starting from a region close to the primary tumor and, most probably, following similar passages as the lymphatic metastasizing cells. The drug-carrier conjugates are therefore referred to as tracking systems.

The highly toxic CDDP (14) is one of the most effective chemotherapeutics used for treatment of squamous cell cancer (15). However, several negative aspects have been

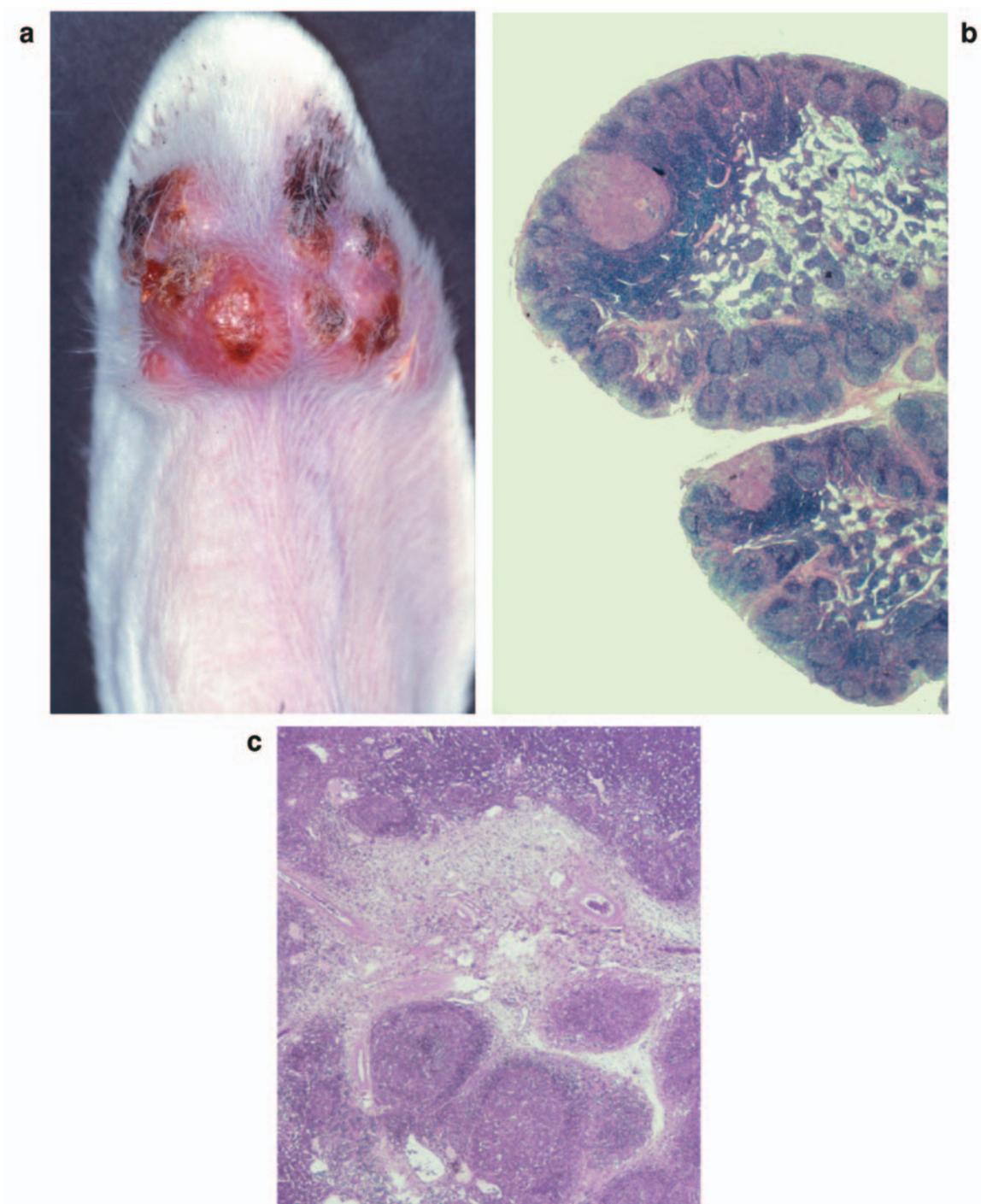


Figure 1. Image of the ear of a New Zealand white rabbit after incubation of a VX2 carcinoma (a) and representative histological cuts showing a primary lymph node before (b) and after treatment with **TI** (c).

described, such as rapid blood clearance and occurrence of nephrotoxicity (16, 17), thus limiting the clinical use of intravenous administration of CDDP. Therapeutic indices of platinum-based cytostatics can be dramatically improved by using polymeric carriers (18-24). However, blood system

administration of CDDP-polymer conjugates suffers from slow cross-distribution to the lymphatic system. This and the frequent appearance of early release of the drug prevents a therapeutically effective dose from being attained at the locus of a lymph node metastasis (7).

The interstitial drug administration applied in this study allows the solution to enter into the narrow spaces between the tissues. This mimics the physiological pathway of lymph fluid since increased interstitial pressure activates lymphogenic absorption and drainage (7). The slow process of lymphogenic absorption (25) requires a strong conjugation of the CDDP to the polymeric carrier. PLys-based copolymers such as PEO-*b*-PLys exhibit the desired high complexation strength toward platinum (21). To date, however, these were not applied for the delivery of CDDP due to the inherent toxicity of PLys (26) and the expected low therapeutic effectiveness.

While the PLys segment of the block copolymer binds to the CDDP, the solvating PEO block acts as a compatilizer to the lymphogenous environment. It seems likely that after administration and lymph adsorption, the drug complex will not be homogeneously distributed within the lymphatic system. Instead, the highest concentration might occur close to the locus of injection. This would be of advantage, since its gradual distribution would mirror that of the metastasizing cells, decreasing with distance from the primary tumor. The cellular uptake of the drug tracking systems into the cancer cells is believed to follow an endocytotic pathway. Cell membrane translocation might be promoted by the cationic nature of the PLys segment.

Without therapeutic intervention, all animals of the control group suffered from a strong metastatic spread, leading to massive necrotic, vital lymph node metastases in the primary and secondary lymph node stations, while in nine out of ten animals, treated by the CDDP-loaded tracking system **T1** (48 wt.% CDDP), no lymph node disease remained. The appearance of visual scarring and secondary follicles confirmed the tumor destruction process in the lymph node. Only one animal exhibited remaining vital tumor cells in the first two draining lymph node stations. None of these ten animals suffered from far distant spread. Based on these results, the tracking system succeeded in inhibiting further progress of lymphogenic and hematogenic metastatic spread, as well as fully destroying the metastases in 90% of the animals.

Lowering the CDDP load in the tracking systems **T2** (10 wt.% CDDP) and **T3** (1 wt.% CDDP) reduced the therapeutic effectiveness. However, a significant impact on malignant disease could be still observed. Five out of seven animals (71%) treated with **T2** and two out of seven animals (29%) treated with **T3** did not show any vital cancer cells in their system. It is noteworthy that none of the 14 animals (0%) in these groups exhibited far distant metastases after the follow-up survey and only one (7%) developed metastases in the second draining lymph node. These results underline the remarkable regional control of the disease, preventing the systemic lymphogenous and hematogenous extension of the cancer.

The metastatic cascade of the untreated squamous cell carcinoma of the head and neck region was successfully

inhibited by an interstitial translymphatic application of CDDP-loaded PEO-*b*-PLys tracking systems. The results show significant impact on the lymphatic disease in all cases. Most effective was the application of a high cargo-load CDDP tracking system (**T1**, 48 wt.% CDDP) curing 90% of the animals and causing only minor local side-effects. Systems containing 1 or 10 wt.% of CDDP (**T2** and **T3**) were less effective but still cured 50% of the animals. Moreover, the administration of **T2** and **T3** always limited tumor growth to the draining lymph nodes (50%) and prevented systemic distribution of the metastasis even with 1 wt.% CDDP load. The systems contained 0.25-0.003 mg/kg/body weight CDDP compared to 1 ml/kg/body weight as usually used for intravenous administration. When no CDDP chemotherapy was applied, all 14 animals examined showed advanced local lymph node disease. In 78% of the control group this was accompanied with systemic tumor distribution.

One might discuss a passive targeting mechanism on the basis of an enhanced uptake of the tracker by highly active, fast dividing tumor cells. Due to variations of the membrane permeability during the different cell cycles a tracker translocation might be enhanced in mitosis and the late S-phase (27). The trafficking of the tracing system in the cells and the specific pathway of cell damaging that triggers apoptosis is currently unknown. Endosomal or lysosomal escape of the tracker or the drug seems likely and might be facilitated by the molecular binding of the cationic segment to the endo/lysosomal membrane that potentially leads to a weakening of the membrane structure. The damage of the DNA by the platinum complex might occur by divalent 1,2-intrastrand cross linking or generation of monovalent lesions.

The approach described here encourages further and more detailed research of a CDDP-based interstitial translymphatic administration of chemotherapy of lymphogenic metastasizing carcinoma in different body regions. Further study will include the exploration of solid-phase peptide synthesis that allows precise, sequence controlled synthesis of the peptide segment. Thus, the design of PEO-*b*-polypeptides with advanced functions such as programmed carrier degradation or specific liberation of reporter molecules after delivery of the drug load might be possible. Furthermore, strategic advantages are expected during drug approval procedures by *e.g.* the Food and Drug Administration (FDA) since the functional block is well defined.

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