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Biochimica et Biophysica Acta 1758 (2006) 606–610
BIOCHIMICA ET BIOPHYSICA ACTA
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Delivery of substances and their target-specific topical activation

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Received 2 September 2005; received in revised form 17 March 2006; accepted 23 March 2006
Available online 19 April 2006

Abstract

Goal in pharmaceutical research is achievement of necessary drug concentrations in the target organ, effective treatment with safe delivery of genetic agents, while sparing normal tissue and minimizing side effects. A new "BioShuttle"-delivery system harbouring a cathepsin B cutting site, a nuclear address sequence and a functional peptide was developed and tumor cells were treated. Transport and subcellular activation were determined by confocal laser scanning microscopy permitting the conclusion: BioShuttle-conjugates prove as efficient tools for genetic interventions by selective and topical activation of therapeutic peptide precursors by enzymatic cleavage. As shown here for glioma cells and the cathepsin B cleavable site, living cells can be treated with high specificity and selectivity for diagnostic and therapeutic purposes.

Keywords: Cathepsin B; Drug targeting; Glioblastoma multiforme; Intracellular targeting; Solid phase peptide synthesis; SPPS; Transmembrane transport

1. Introduction

The major goal in cancer therapy is to increase the therapeutic ratio by eliminating the disease while minimizing toxicity to normal tissues. Classical drugs enter the cells mainly by diffusion, but the lack of specificity requires high application doses accompanied with increased side effects resulting in a breakdown during therapy. Modern drugs, like DNA-derivatives, are characterized by specificity and sensitivity. However their transport across biological membranes is very poor and needs suitable carrier systems [1]. Delivery of genetic agents into the host cell has been a main strategy, carried out by direct DNA injection, encapsulation into liposomes or other artificial encapsulation strategies have been applied with different success [2]. Drug delivery systems such as lipid-, polymer- and peptide-based particles can be designed to improve the pharmacological and therapeutic properties of drugs administered parenterally [3]. In this context, a further open question is the release of the transported agent at its target site. One possible answer is the use of the modular, peptide-based BioShuttle carrier system which is able to realize an intracellular targeting to subcellular components using cell immanent mechanisms [4]. In neoplastic tissues the content of cathepsin B is increased manifold in contrast to normal cells where it is hardly detectable [5,6]. In order to distinguish between tumor and normal cells we used a BioShuttle transporter for peptide-based drugs which became activated after digestion of a Cathepsin B Cleavable Spacer (BioShuttle-CBCS-conjugate).

2. Material and methods

2.1. Synthesis and purification of the BioShuttle-CBCS-conjugates

For solid phase synthesis of the ^{FITC}VKRKKKP-K$_D$-GFGRK(Dabcyl)-RQIKIWFQNRRM-KWKK [BioShuttle-CBCS$_D$-complex (#5293)] and ^{FITC}VKRKKKP-K$_L$-GFGRK-(Dabcyl)-RQIKIWFQNRRMKWKK [BioShuttle-CBCS$_L$-complex (#5294)] we employed the Fmoc (9-fluorenylmethyl-oxycarbonyl) methodology [7,8] in a fully automated multiple synthesizer (Syro II from MultiSyntech Germany). As coupling agent 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was used. The following side chain protecting groups were employed: Boc (*tert*-butyloxycarbonyl) for Lys and Trp, Trityl (triphenylmethane) for Gln and Asn and Pbf (2,2,4,6,7-Pentamethyldihydrobenzofurane-5-sulfonyl) for Arg. Fmoc-Lys(Dabcyl) was purchased from Merck Biosciences GmbH, Germany. The synthesized peptides were cleaved and deprotected

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doi:10.1016/j.bbamem.2006.03.036

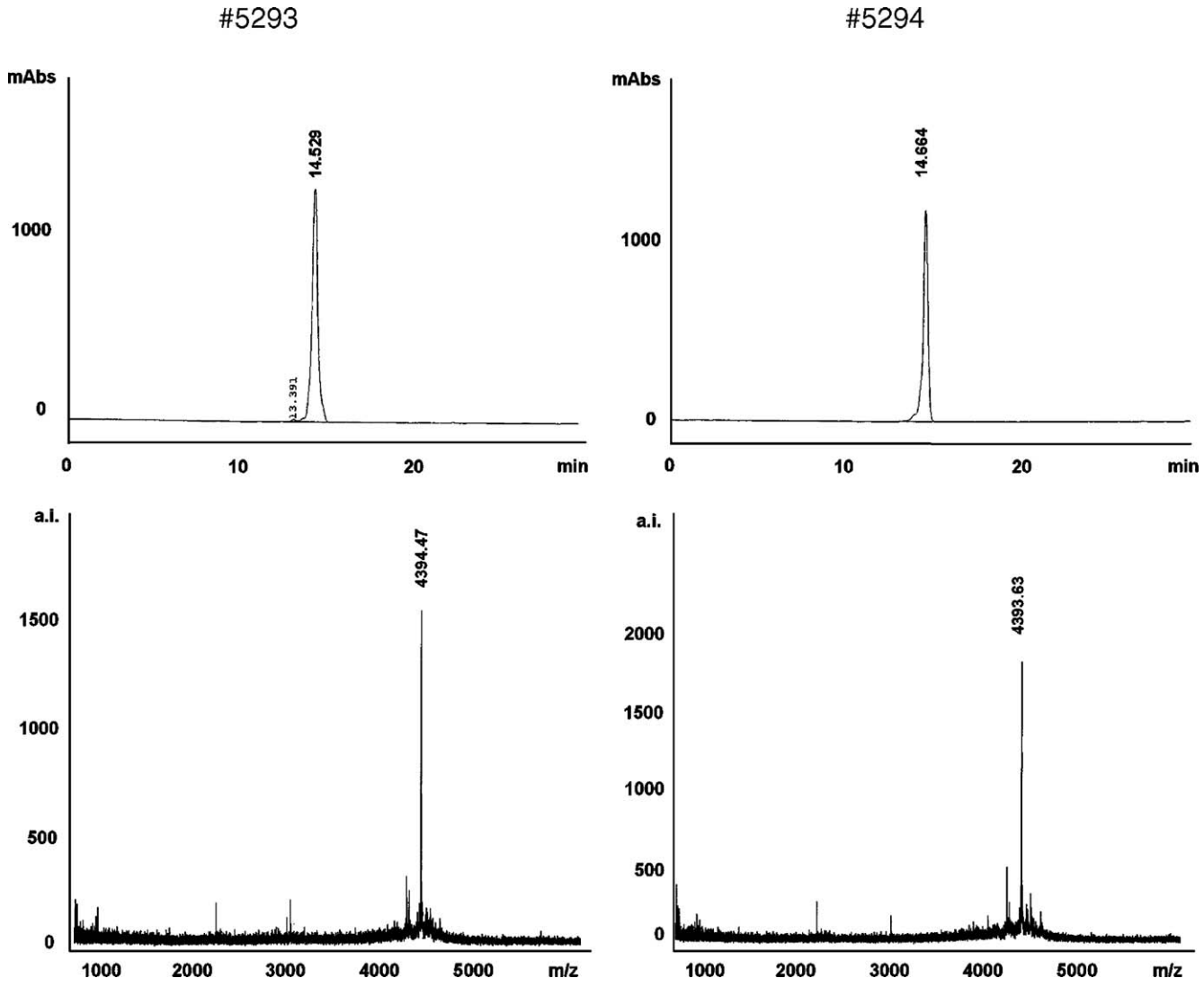


Fig. 1. The HPLC peak reports the *D*-form of BioShuttle–$CBCS_D$-complex (left site) and the *L*-form of the BioShuttle–$CBCS_L$-complex (right site). The estimated substance purity is 99.3% with the retention time of 14.6 min (#5293) and 100% and with 15.5 min (#5294) (upper part). The lower part represents the corresponding mass spectra. Abscissa: HPLC: Minutes, Ordinate: HPLC: milliVolt Absorption Unit, MS: ratio masse charge (*m*/*z*), Ion Intensity (a. I.).

from the solid support by treatment with 90% trifluoroacetic acid, 8% tri-isopropylsilane, and 2% water (v/v/v/) for 2.5 h at room temperature. The products were precipitated in ether. The crude material was purified by preparative HPLC on a Kromasil 100–10C18 μm reverse phase column (30 × 250 mm) using an eluent of 0.1% trifluoroacetic acid in water (A) and 80% acetonitrile in water (B) The peptide was eluted with a successive linear gradient of 10% B to 80% B in 30 min at a flow rate of 23 ml/min. The fractions corresponding to the purified protein were lyophilized. The purified material was characterized with analytical HPLC and matrix assisted laser desorption mass spectrometry (MALDI-MS) (Fig. 1).

2.2. Cathepsin B in vitro assay

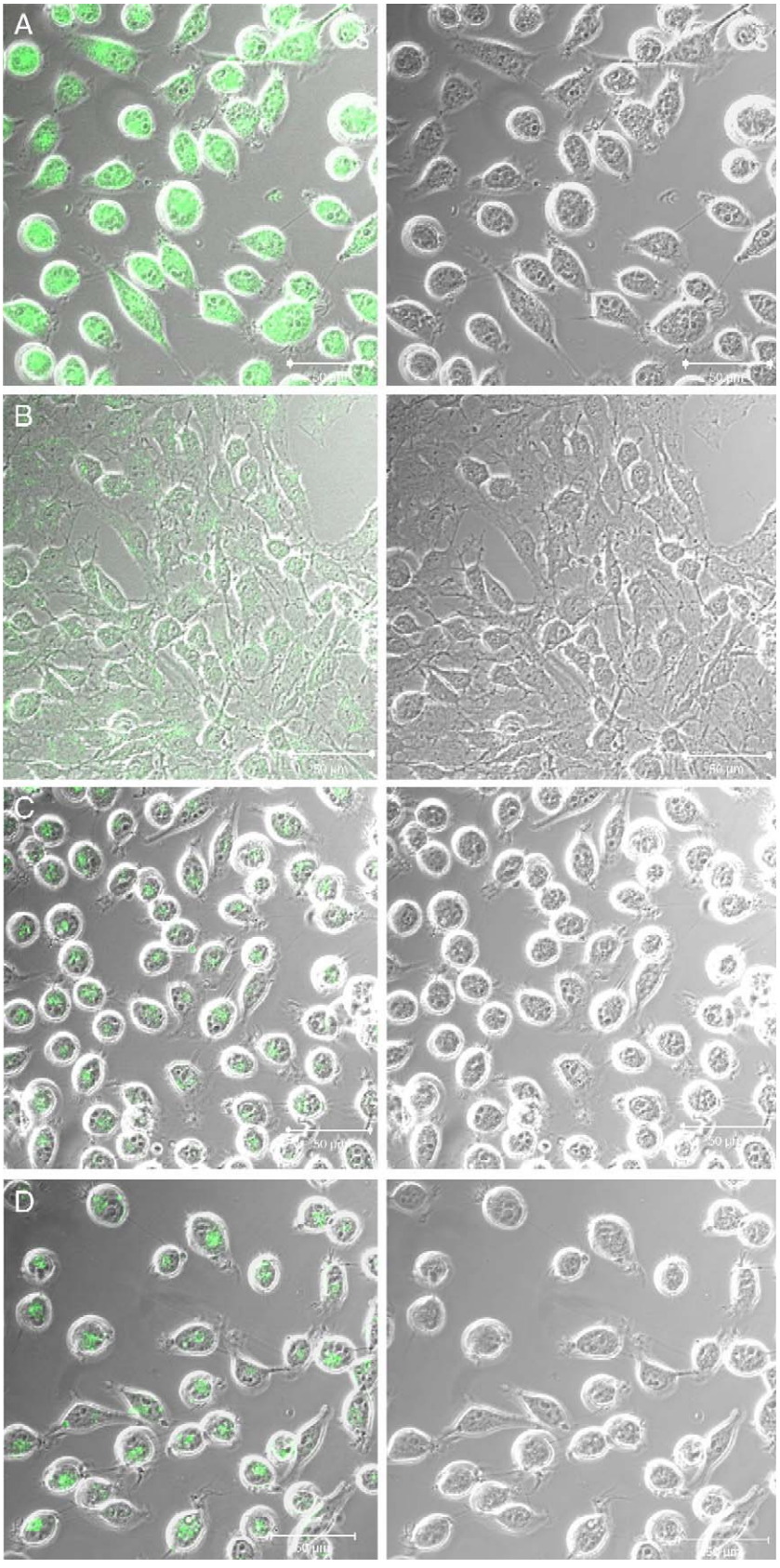
In order to proof the cathepsin B specific cleavage we incubated 10 Units (5 μg enzyme, Sigma-Aldrich, Germany) with 100 μg peptide for 60 min 40 °C in 20 mM sodium acetate buffer at pH 6 in a total volume of 500 μl. The BioShuttle–$CBCS_L$-complex was enzymatically digested and furthermore separated by HPLC. The chromatography was carried out with a Kromasil KR 100–10C18 column, the sample was eluted with a gradient consisting of buffer A 0.1% TFA in water and eluent B 0.1% TFA in water/acetonitrile (20/80). The gradient was applied for 30 min from 5% to 80% B. The probe was detected at 210 nm wavelength. Using the MALDI-MS, the molecular identification of the metabolites was achieved.

2.3. Cell culture and experimental accomplishment

C6 glioblastoma and HeLa cells (DKFZ tumor bank) were cultivated in minimal essential medium supplemented with 10% Fetal Bovine Serum, 2 mM glutamine, 100 $U \times ml^{-1}$ penicillin, 100 $\mu g \times ml^{-1}$ streptomycin (Sigma-Aldrich).

Fig. 2. CLSM exposures of C6 glioblastoma cells (lines A, C and D) and HeLa cervix carcinoma cells (line B) are shown. The left column reveals the emitted fluorescence light, whereas the right column represents the corresponding DIC-pictures of the cells (differential interference contrast). Line A shows C6 glioma cells treated with BioShuttle harbouring the cleavable *L*-form of the cathepsin recognition site [BioShuttle–$CBCS_L$-complex]. Line B shows HeLa cells treated with BioShuttle with the *L*-form of the cathepsin B cleavage site (KGFGRK). Line C shows C6 glioblastoma cells treated with BioShuttle containing the uncleavable *D*-form of the cathepsin B recognition site [BioShuttle–$CBCS_D$-complex]. The lower row (line D) demonstrates a slight fluorescence signal in C6 cells treated with die cathepsin B proinhibitor CA-074 methyl ester after 24 h of incubation. The bars represent 50 μm.

To perform fluorescence microscopic studies, the cathepsin B expressing C6 glioblastoma cell line and HeLa cells (5×10^5) were incubated for 24 h in quadriperm® plus (Heraeus) containing sterile glass coverslips. After two wash-cycles with MEM, the cells were incubated with BioShuttle–CBCS$_{\mathbf{L}}$-complex (10 μM). To investigate the stereochemical specificity of cathepsin B, the corresponding *D*-form of the cathepsin cleavable spacer was incubated as a further control with



BioShuttle–$CBCS_D$-complex (10 μM) at 37 °C in a 5% CO_2 atmosphere for 30 min. The culture medium was removed to enable microscopic studies. Cells were washed twice and were finally embedded in Moviol®.

2.4. Confocal imaging

The intracellular distribution of the BioShuttle–$CBCS_L$-complex and BioShuttle–$CBCS_D$-complex in non fixed C6 and HeLa cells was verified by a Zeiss confocal laser scanning microscope (LSM 510-Meta). For excitation, we used the laser lines at 488 nm and 543 nm wavelengths and the corresponding barrier filters for emission. Simultaneously with the fluorescence images, also images in differential interference contrast (DIC) were taken. Parameters of the image acquisition were adapted to show the signal intensities in accordance with the visual microscopic image aspect.

3. Results and discussion

The degradation of the extracellular matrix protein, a prerequisite for the invasive phenotype in glioma cells, is associated with the increased expression levels of proteases like cathepsins which are associated with increased motility and the potential to hydrolyse macromolecular components of the extracellular matrix [9]. Increased expression and activity of the lysosomal cysteine protease cathepsin B have been observed in different tumors, especially including prostate, breast, and gastrointestinal cancers [10]. Increased cathepsin B expression and high activity of the corresponding gene product are associated with tumor malignancy in brain tumors [11]. The correlation between the amounts of cathepsin B mRNA, protein, enzymatic activity and tumor progression could serves as an evidence for the involvement of cathepsin B in the tumor pathology [6].

To proof the elevated levels of cathepsin B in the C6 glioma tumor cells, they were treated with BioShuttle–$CBCS_L$-complex showing a fluorescence signal in the nuclei (Fig. 2, line A, left picture). This demonstrates the enzymatic cleavage in the cytoplasm liberating the nuclear localization signal (NLS) and movement of the separated sequence into the nuclei. By that we were able to distinguish between cathepsin B high level expression and background expression in HeLa cells. In HeLa cells, the NLS peptide could not be liberated by cathepsin B cleavage and therefore did not show a significant nuclear fluorescence (line B). In order to determine the enzymatic substrate stereospecificity (of cathepsin B), we constructed a BioShuttle–CBCS-conjugate with one single amino acid (lysine) in the enzyme cleavage site in D-conformation as opposed to the L-compound. With the construct we could demonstrate that this sterical property protects the BioShuttle–$CBCS_D$-complex against enzymatic digest (Fig. 2, line C).

Fig. 2D shows that the inactivation of cathepsin B could be achieved by the highly selective cathepsin B proinhibitor CA-074 methyl ester [12]. To test whether CA-074 methyl ester has the capacity to inactivate cathepsin B, C6 cells were incubated for 24 h with the proinhibitor (10 μm).

This membrane-permeable compound was applied to the cell culture medium where its intracellularly converted to CA-074 by endogenous esterases [13]. The picture shows that the cathepsin B activity was strongly reduced and cleavable BioShuttle–$CBCS_L$-complex remained extensively undigested by the enzyme (Fig. 2, line D). There is only a slight fluorescence signal in the cytosol of C6 cells treated with the cathepsin B

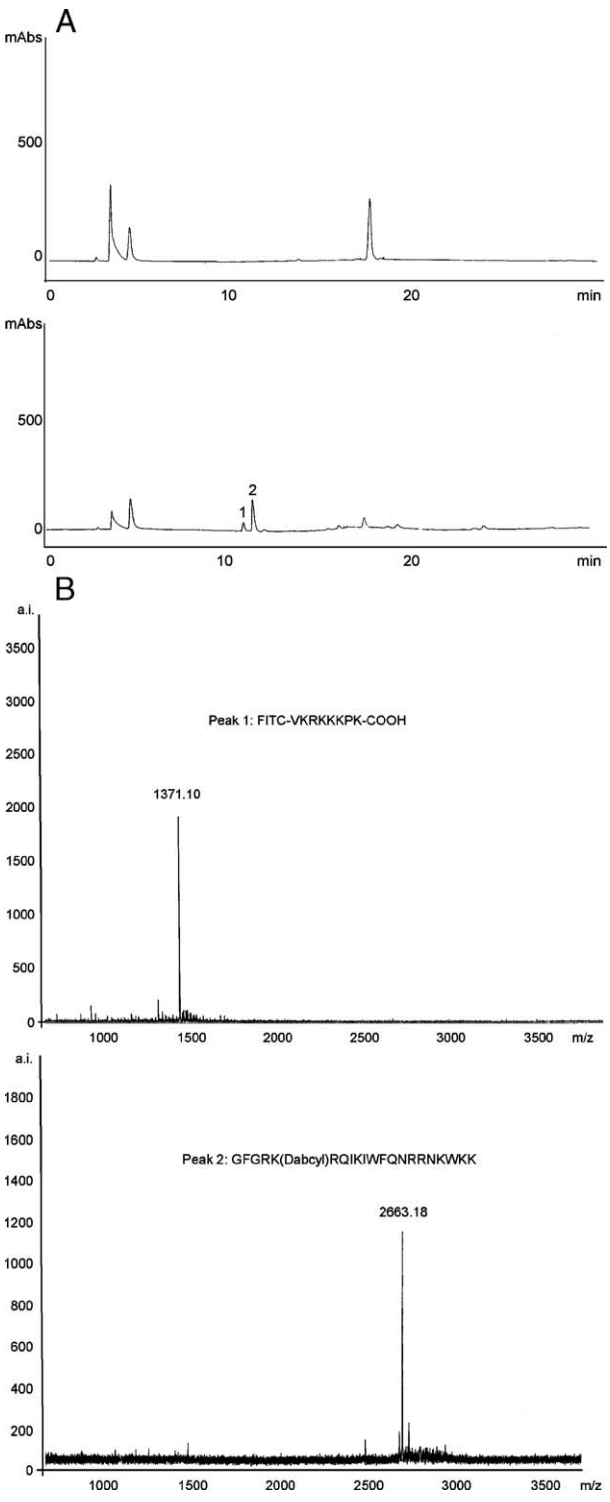


Fig. 3. (A) Shows the HPLC diagrams of the cathepsin B in vitro assay. The upper part represents the graphic of the HPLC procedure without enzyme. The peak (19 min) corresponds to the undigested BioShuttle–$CBCS_L$-complex. The lower part shows the resulting peaks 1 and 2 (11 and 12 min) after 60 min enzymatic digest. The peak of the BioShuttle–$CBCS_L$-complex nearly disappeared. Both diagrams exhibit two identical injection peaks (left, in front). Abscissa: HPLC: Minutes, Ordinate: HPLC: milliVolt Absorption Unit. (B) The right part of panel B represents the corresponding MS spectra of the peaks 1 and 2 containing both metabolites. The MWs 1371.10 and 2663.18 according to the peaks 1 and 2 respectively are demonstrated. MS: ratio masse charge (*m*/*z*), Ion Intensity (a. I.).

proinhibitor CA-074 methyl ester (Sigma-Aldrich), presumably because the NLS was inaccessible for the importin-mediated nuclear transport machinery. An in vitro assay using the purified cathepsin B enzyme and the substrate with the enzymatic recognition site could demonstrate the effect as shown in Fig. 3.

Fig. 3A (upper part) represents the peak of the undigested BioShuttle–$CBCS_L$-complex. After enzyme treatment, the lower part of the Fig. 3A exhibits two additional peaks which indicate the enzymatic cleavage products of the BioShuttle–$CBCS_L$-complex after 60 min digest. The substantial content of the peaks was determined by mass spectrometry methods which resulted in the relevant molecular weights. The peak 1 corresponds to the metabolite 1 (FITC-VKRKKKPK-COOH, MW calculated: 1371.71) and the peak 2 correlates to metabolite 2 (GFGRK(Dabcyl)RQIKIWFQNRRMKWKK, MW calculated: 2663.50) (Fig. 3B).

The presented delivery platform has the potential to transport highly efficient, highly specific high molecular weight drugs. These will be liberated and activated at their targets by the intrinsic mechanisms of cells.

The stable residence time of several hours of the BioShuttle–$CBCS_L$-complex in the cytoplasm allows administration without fear for unwanted agent-activation in the blood circuit and the surrounding tissue.

The use of the BioShuttle-mediated delivery systems for agents with high molar mass like peptide- or nucleic acid-molecules is an efficient new two-step approach in the field of diagnostic and therapy of genetically based diseases like cancer. It uses the cell's own mechanisms which are inactive in normal cells and tissues but highly activated in neoplastic target cells.

As future aspects, we plan to use such specific peptide spacers, designed to be biorecognizable by intracellular enzymes, as site-specific activators. As an auspicious approach to increase the cell-specific release of diagnostics for intravital imaging as well as for therapy, we can apply highly reactive chemotherapeutics and peptide- or nucleic acid-based molecules. This study proves the concept of the BioShuttle–$CBCS_L$-complex strategy but further optimization is necessary.

Acknowledgements

We would like to thank Gabriele Müller and Ditmar Greulich for taking care of our cell lines, Mario Koch for technical help in chromatography and Stephanie E. Combs for critical reading of the manuscript.

This work was supported by Deutsche Krebshilfe, D-53004 Bonn; Grant Number: 106335.

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