

# Value of apoptin's 40-amino-acid C-terminal fragment for the differentiation between human tumor and non-tumor cells

Stefan Heckl · Marc Regenbogen · Alexander Sturzu · Alireza Gharabaghi ·  
Gerhard Feil · Alexander Beck · Hartmut Echner · Thomas Nagele

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**Abstract** Apoptin, a protein of the chicken anemia virus (CAV), consists of 121 amino acids (aa) and represents a novel, potentially tumor-specific therapeutic and diagnostic agent. The C-terminal part of Apoptin (aa 81–121) is believed to contain a bipartite nuclear localization signal (NLS) (NLS1: aa 82–88 and NLS2: aa 111–121), which is only active in tumor cells after phosphorylation of threonine<sup>108</sup> by tumor-specific cytoplasmic phosphokinases. Furthermore, a nuclear export signal (NES) (aa 97–105) seems to enable nuclear export of Apoptin only in healthy cells. The specificity for tumor cell nuclei also applies to the truncated C-terminal part of Apoptin (aa 81–121), which therefore represents a highly attractive peptide sequence for peptide synthesis. Here we describe for the first time the synthesis of fluorescein isothiocyanate (FITC)- and Dansyl-labelled conjugates containing this C-terminal part of Apoptin, with either phosphorylated or

nonphosphorylated threonine<sup>108</sup>. The phosphorylated conjugates were synthesized in an attempt to achieve nuclear accumulation in healthy cells, which lack cytoplasmic tumor-specific phosphokinases. Surprisingly, all the conjugates accumulated rapidly within the cell nuclei of both tumor and non-tumor cells from the bladder, brain and prostate and led to cell death. By coupling Apoptin<sup>81–121</sup> to FITC and DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) at either the C- or N-terminus we could exclude that the coupling site is decisive for tumor cell-specific nuclear localization. The labels FITC, DOTA and Dansyl were not responsible for cell death in healthy cells because cell death was not prevented by using an unlabelled Apoptin<sup>81–121</sup> peptide. Cellular and nuclear uptake of the FITC-labelled Apoptin<sup>81–121</sup> peptide was almost completely abolished after altering the NLS2 (replacement of five arginines with serines).

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S. Heckl (✉) · M. Regenbogen · T. Nagele  
Department of Neuroradiology, University of Tübingen,  
Medical School, Hoppe-Seyler-Str. 3, 72076 Tübingen,  
Germany  
e-mail: stefan.heckl@med.uni-tuebingen.de

A. Gharabaghi  
Department of Neurosurgery, University of Tübingen, Tübingen,  
Germany

G. Feil  
Department of Urology, University of Tübingen, Tübingen,  
Germany

A. Beck  
Center for Clinical Mass Spectrometry, Heilbronn, Germany

A. Sturzu · H. Echner  
Peptide Synthesis Laboratory, Interfaculty Institute of  
Biochemistry, University of Tübingen, Tübingen, Germany

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## Introduction

The chicken anemia virus (CAV) derived viral protein 3 (VP3 or Apoptin) consists of 121 amino acids (13.6 kDa) [1] and has been reported to induce apoptosis in tumor cells but not in healthy cells [2–11]. Apoptin is therefore a highly promising candidate in the development of tumor cell-specific diagnostic and therapeutic agents. Truncation and point mutation studies have shown that the specific nuclear uptake and retention of green fluorescent protein (GFP)-labelled Apoptin by tumor cell nuclei are mediated mainly by a bipartite nuclear localization signal (NLS; NLS1: amino acids 82–88, and NLS2: amino acids

111–121) [6, 8, 9] and a nuclear export signal (NES; amino acids 97–105) [9]. In the tumor-cell cytoplasm, the phosphorylation of threonine<sup>108</sup> by tumor-specific phosphokinases results in activation of the bipartite NLS and inactivation of the NES [4, 9].

Apoptin and its truncated forms, usually fused to GFP, enhanced green fluorescent protein (EGFP), red fluorescent protein from *Discosoma* (dsRed) or hemagglutinine (HA) have previously been produced by plasmids [4–12]. However, to be of wide use in diagnostics and therapy, Apoptin would need to be coupled to many different compounds besides GFP, EGFP, dsRed or HA (e.g. FITC or Dansyl).

Therefore, the objective of the present study was to produce FITC- and Dansyl-labelled peptide conjugates by solid phase peptide synthesis and to evaluate their uptake by the nuclei of tumor cells and healthy cells from the brain, prostate, and bladder. These conjugates contained the above-described tumor-specific key elements of Apoptin (amino acids 81–121: Apoptin<sup>81–121</sup>) with either phosphorylated (conjugates 1 and 2) or nonphosphorylated (conjugates 3 and 4) threonine<sup>108</sup>. Phosphorylation of threonine<sup>108</sup> was undertaken in an attempt to achieve accumulation of the conjugates within the nuclei of healthy cells, which lack the cytoplasmic tumor-specific phosphokinases responsible for activation of the bipartite NLS.

The coupling site (N- or C-terminus) of the label might be responsible for tumor-specific nuclear uptake. Therefore we produced Apoptin<sup>81–121</sup> conjugates which contained fluorescein isothiocyanate (FITC) and DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) at the C- or N-terminus.

DOTA was used with the view to future Apoptin magnetic resonance imaging studies.

The FITC-, DOTA- and Dansyl-labels but not the Apoptin<sup>81–121</sup> peptide itself might induce cell death in healthy cells. Therefore we also synthesized and evaluated the appropriate native Apoptin<sup>81–121</sup> peptide without such labels. In order to elucidate whether the NLS stretches 1 and 2 mediate cellular uptake, we also used FITC-labelled Apoptin<sup>81–121</sup> peptides in which either one or two of the positively charged NLS were altered (NLS1: two lysines and one arginine replaced with serines, NLS2: 5 arginines replaced with serines).

## Materials and methods

### Synthesis of the conjugates

The Apoptin<sup>81–121</sup>  $\beta$ Ala Lys (Dansyl)-OH conjugate 4 and its phospho-analogue (conjugate 2) were produced by solid phase peptide synthesis (Fmoc/tBut strategy, Ecosyn P batch synthesizer, Eppendorf Biotronik, Maintal,

Germany). After the commercially available Fmoc-Lys (Dansyl)-OH moiety (3 eq.) had been anchored on Tentagel R-trityl-chloride resin (1 eq.) (Rapp Polymere, Tübingen, Germany) using diisopropylethylamine (4 eq.) in dichloromethane (1 h, room temperature), the synthesis was performed using Fmoc amino acids, with TBTU [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate] (4 eq.) and diisopropyl ethylamine (DIEA) (8 eq.) as coupling reagents. After each coupling, the Fmoc protective group was cleaved off by a 25% piperidine solution in DMF (dimethylformamide) in an 11 min reaction step.

The side-chain protecting groups were tert-butylether (tbutyl) for thr and ser, tert-butylesters (OBu) for glu and asp, tert-butyloxycarbonyl (tBoc) for lys, trityl (trt) for cysteine, and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (pbf) for arg. The reaction medium was a mixture of DMF, N-methylpyrrolidone, and dichloromethane (1:1:1). After Proline<sup>109</sup> coupling, the peptide resin was divided into two parts. In one, Fmoc Thr (PO (OBzl)OH)-OH was then coupled for the phosphorylated conjugate analogue. In the other, tbutyl-protected threonine was coupled for the nonphosphorylated analogue. The N-terminal prolines were introduced as their Boc-derivatives. A cocktail of trifluoroacetic acid (TFA), anisol, ethanedithiol, water, and triisopropylsilane (12, 0.3, 0.3, 0.3, and 0.1 ml, respectively) was used to deprotect both peptides. After the reaction mixture had been stirred for two hours at room temperature, the peptides were precipitated by filtering the mixtures directly in absolute diethylether, dried, and then purified using a semipreparative high performance liquid chromatography (HPLC) system (column Nucleosil 300, 5  $\mu$ m, C<sub>18</sub> 10  $\times$  250 mm, Buffer A, 0.1% TFA/H<sub>2</sub>O; Buffer B: 80% CH<sub>3</sub>CN/H<sub>2</sub>O, 0.1% TFA 10–90% in 31 min, 214 nm).

The Apoptin<sup>81–121</sup>  $\beta$ Ala N<sup>c</sup> Lys fluorescein thiocarbamoyl peptide (conjugate 3) and its phospho-analogue (conjugate 1) were synthesized by a similar method. Both syntheses started with Fmoc TentaGel R RAM resin (Rapp Polymere, Tübingen, Germany). After removal of the Fmoc group using 25% piperidine in DMF for 12 min, the resins were coupled with Fmoc Lys (Mmt)-OH (3 eq.) using TBTU (3 eq.) + DIEA (6 eq.) for 1 h. The peptide sequences were prepared as described above for the Dansyl conjugates 2 and 4 (Table 1). The N-terminal proline was coupled as Boc-Pro-OH, and the Mmt-groups at the C-terminal lysines were then deprotected using 1% TFA/0.1 ml triisopropylsilane in dichloromethane (1 h, room temperature).

After neutralization with diisopropylethylamine, the fluoresceinthiocarbamoyl moieties were introduced using fluorescein-5,6-isothiocyanate in DMSO (dimethylsulfoxide) by shaking the resins over night in the presence of 1 equivalent diisopropylethylamine.

**Table 1** Formulas of Apoptin<sup>81–121</sup> conjugates 1–9

Conj. 1:	<b>PKPPSKKRSCDPSEYRVSELKESLITTP</b> <u><b>TPSRPRTAKRRIRL</b></u> $\beta$ AlaK(FITC)
Conj. 2:	<b>PKPPSKKRSCDPSEYRVSELKESLITTP</b> <u><b>TPSRPRTAKRRIRL</b></u> $\beta$ AlaK(Dansyl)
Conj. 3:	<b>PKPPSKKRSCDPSEYRVSELKESLITTPSRPRTAKRRIRL</b> $\beta$ AlaK(FITC)
Conj. 4:	<b>PKPPSKKRSCDPSEYRVSELKESLITTPSRPRTAKRRIRL</b> $\beta$ AlaK(Dansyl)
Conj. 5:	<b>PKPPSKKRSCDPSEYRVSELKESLITTP</b> <u><b>SPSTAKSSISL</b></u> $\beta$ AlaK(FITC)
Conj. 6:	<b>PSPPSKSS</b> SCDPSEYRVSELKESLITTP <u><b>SPSTAKSSISL</b></u> $\beta$ AlaK(FITC)
Conj. 7:	<b>PKPPSKKRSCDPSEYRVSELKESLITTPSRPRTAKRRIRL</b> K(DOTA)GGK(FITC)
Conj. 8:	(DOTA)K(FITC) <b>PKPPSKKRSCDPSEYRVSELKESLITTPSRPRTAKRRIRL</b> $\beta$ AK
Conj. 9:	<b>PKPPSKKRSCDPSEYRVSELKESLITTPSRPRTAKRRIRL</b> $\beta$ AK

Single-Letter Amino Acid Code: K, Lysine; R, Arginine; P, Proline; V, Valine; G, Glycine; S, Serine; C, Cysteine; D, Aspartic Acid; E, Glutamic Acid; Y, Tyrosine; L, Leucine; I, Isoleucine; T, Threonine, A, Alanine

$\beta$ -Ala:  $\beta$ -Alanine

pT: phosphorylated Threonine

FITC: fluorescein isothiocyanate

DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10 tetraacetic acid

Correct bipartite nuclear localization signal (NLS1 and NLS2) (amino acids 82–88 and 111–121) (bold)

Mutant NLS1: two lysines and one arginine replaced with serines (amino acids 82–88, bold italic and underlined)

Mutant NLS2: 5 arginines replaced with serines (amino acids 111–121, bold italic and underlined)

Nuclear export signal (NES) (amino acids 97–105, double underlined)

For the synthesis of FITC-labelled conjugates 5 and 6 with either one or two mutated NLS, the peptide resin was divided into two parts after Serine<sup>89</sup> coupling.

Peptide deprotection and purification procedures were carried out as described for conjugates 2 and 4.

For the synthesis of conjugates 7 and 8 Tentagel R rink amid resin (Rapp-Polymere, Tübingen, Germany) was used as carrier material.

The lysine side chain designated for DOTA was protected with Mmt (4-methoxytrityl), the lysine side chain carrying FITC was protected with Dde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-ethyl) (conjugate 7). For the synthesis of conjugate 8 Fmoc-Lys(Mmt)-OH was used.

For introduction of DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), the Mmt protective group was cleaved off by repeated treatment with a solution of 1% TFA (trifluoroacetic acid) and 1% TIPS (triisopropylsilane) in DCM during one hour. After several washing steps with DMF and neutralising of the resulting TFA-salt with DIPEA the deprotected side chain was accessible for DOTA coupling. Coupling was carried out with three equivalents of 1,4,7,10-tetraazacyclododecane-1,4,7-tris-tert-butyl acetate-10-acetic acid (Macrocyclics, Dallas, Texas, USA), 3 equivalents of TBTU and 6 equivalents of DIPEA during 1.5 h at room temperature. Then the Dde side chain protective group was cleaved off by repeatedly treating the resin with a solution of 2.5% hydrazinhydrate in DMF over the course of one hour.

After several washing steps with DMF, FITC was coupled to the peptide using 0.5 mM fluorescein-5-

isothiocyanate with an equal amount of DIPEA in DMSO at room temperature over night. The peptide resin was then washed with DMF, methanol and DCM.

Cleavage of the remaining side chain protective groups was carried out simultaneously with cleavage of the peptide from the resin in one final step. The dry resin was therefore stirred at room temperature for 3 h in a mixture of 12 ml TFA, 0.3 ml ethanedithiol (EDT), 0.3 ml anisole, 0.3 ml water and 0.1 ml TIPS.

The peptide was then precipitated with cold pure diethyl ether, filtered, washed again with ether and dried in vacuum. The resulting raw peptide was purified via semipreparative HPLC. Conjugate 9 without labels was also synthesized by solid phase peptide synthesis (Fmoc-strategy, Tentagel R rink amid resin).

Peptide deprotection and purification procedures (conjugates 7–9) were carried out as described for conjugates 2 and 4. The conjugates 1–9 were shown to be pure by analytical HPLC ( $\geq 92\%$ ), and their molecular weights were determined by electrospray ionization mass spectrometry (ESI-MS).

#### Electrospray ionization mass spectrometry (ESI-MS)

The conjugates 1–9 (Table 1) were analyzed by ESI-MS on an Esquire3000+ ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany). They were dissolved in 40% ACN, 0.1% formic acid in water (v/v/v) (20 pmol/ $\mu$ l) and constantly infused using a syringe pump (5  $\mu$ l/min flow rate). Mass spectra were acquired in the positive ion mode.

Dry gas (6 l/min) temperature was set to 325°C, the nebulizer to 20.0 psi, and the electrospray voltage to –3,700 V.

## Cell cultures

Human prostate epithelial cells CC-2555 (PrEC) (two different clones) were purchased from Cambrex (Verviers, Belgium) and cultured in prostate epithelial basal medium CC-3165 (PrEBM) with supplements (CC-4177, PrEGM Single Quots) as recommended by the supplier and previously described [13].

Normal human astrocytes CC-2565 (NHA) (two different clones) were also purchased from Cambrex and cultured in astrocyte basal medium (ABM) with supplements (AGM Single Quots CC-4123) as recommended by the supplier and previously described [14].

Surgical specimens were obtained from the urinary bladder in two patients with no histological evidence of urothelial dysplasia or neoplasia. This was approved by the Ethics Committee of Tübingen University Hospital and had full patient consent. The procedures were performed in accordance with ethical standards as formulated in the Helsinki Declaration of 1975 (revised 1983). Urothelial cell cultures were established and characterized as described elsewhere [15].

Human malignant LN18 and U373 glioma cells, the human urothelial carcinoma cell line HT1197, and the human prostate cancer cell lines DU-145 and PC-3 were cultured in RPMI-1640 Ready Mix Medium containing L-glutamine and 10% fetal bovine serum (FBS) (PAA laboratories, Pasching, Austria).

## Incubation with conjugates

All cells were grown to 80% confluency in 75 cm<sup>2</sup> culture flasks (Corning Costar, Bodenheim, Germany) at 37°C in an atmosphere of 5% CO<sub>2</sub> (v/v).

Accutase<sup>TM</sup> (PAA laboratories) was added to detach the cells which were harvested and subsequently transferred to 16-well plates (NUNC, Wiesbaden, Germany) [37°C, 5% CO<sub>2</sub> (v/v)]. Cells (80% confluency) were incubated with Dulbecco's phosphate buffered saline (D-PBS; GIBCO; Invitrogen, Germany) alone (negative controls) and with 26 μM and 260 μM solutions of conjugates 1–4 in D-PBS for 20 min. After this, the cells were rinsed three times with buffer and then incubated with Ready Mix Medium again.

The detection of phosphatidylserine in the outer membrane leaflet of apoptotic cells was performed with the Annexin-V-Alexa<sup>TM</sup> 568 Reagent according to the

manufacturer's protocol (Roche Molecular Biochemicals, Indianapolis, USA).

The FITC conjugates (1 and 3) were detected by confocal laser scanning microscopy (CLSM) and the Dansyl conjugates (2 and 4) by fluorescence microscopy.

Astrocytes and human U373 glioma cells were also incubated with conjugates 1–9 (260 μM) for twenty minutes. After this, the cells were rinsed three times with buffer and then incubated with Ready Mix Medium again. Propidium iodide (PI) was added to the medium (1–5 μmol l<sup>-1</sup> PI; Molecular Probes, Eugene, OR, USA) to detect cells with damaged cell membranes. Confocal laser scanning microscopy was performed on an inverted LSM 510 laser scanning microscope (Carl Zeiss, Jena, Germany) (objectives: LD Achromplan 40 × 0.6, Plan Neofluar 20 × 0.50, 40 × 0.75). For fluorescence excitation, the 488 nm line of an argon laser and the 543 nm line of helium neon laser with appropriate beam splitters and barrier filters were used for FITC and Alexa/PI, respectively. Superimposed images of FITC- and Alexa/PI-stained samples were created by overlaying coincident views.

Dansyl fluorescence microscopy was performed using a Zeiss Axioplan 2 Microscope with a Zeiss NEOFLUAR 20x/0.50 air objective. A HBO50 mercury vapour lamp was used for fluorescence excitation. The set of filters for dansyl detection consisted of a 365 nm excitation filter, a 395 nm beam splitter and a LP420 emission filter. The Zeiss Axiovision 4.5 software was used for acquisition and evaluation of cell pictures.

All measurements were performed at least three times on living, non-fixed cells.

Fluorescence activated cell sorting (FACS) was performed using a Becton Dickinson FACSCalibur. 1 × 10<sup>6</sup> cells (all cell types described under cell cultures) were incubated in 100 μl of conjugate solutions 1 and 3 (26 μM and 260 μM) or PBS alone for 20 min. Healthy astrocytes and U373 glioma cells were also incubated in 100 μl of conjugate solutions 5–8 (260 μM). Then 300 μl FACS buffer (D-PBS containing 1% paraformaldehyde) was added. The samples were measured immediately. Approximately 25,000 events were recorded per sample. Fluorescence excitation was achieved by an Argon laser (488 nm). Fluorescence was detected using a 540–565 nm bandpass filter. All investigations were performed in triplicate.

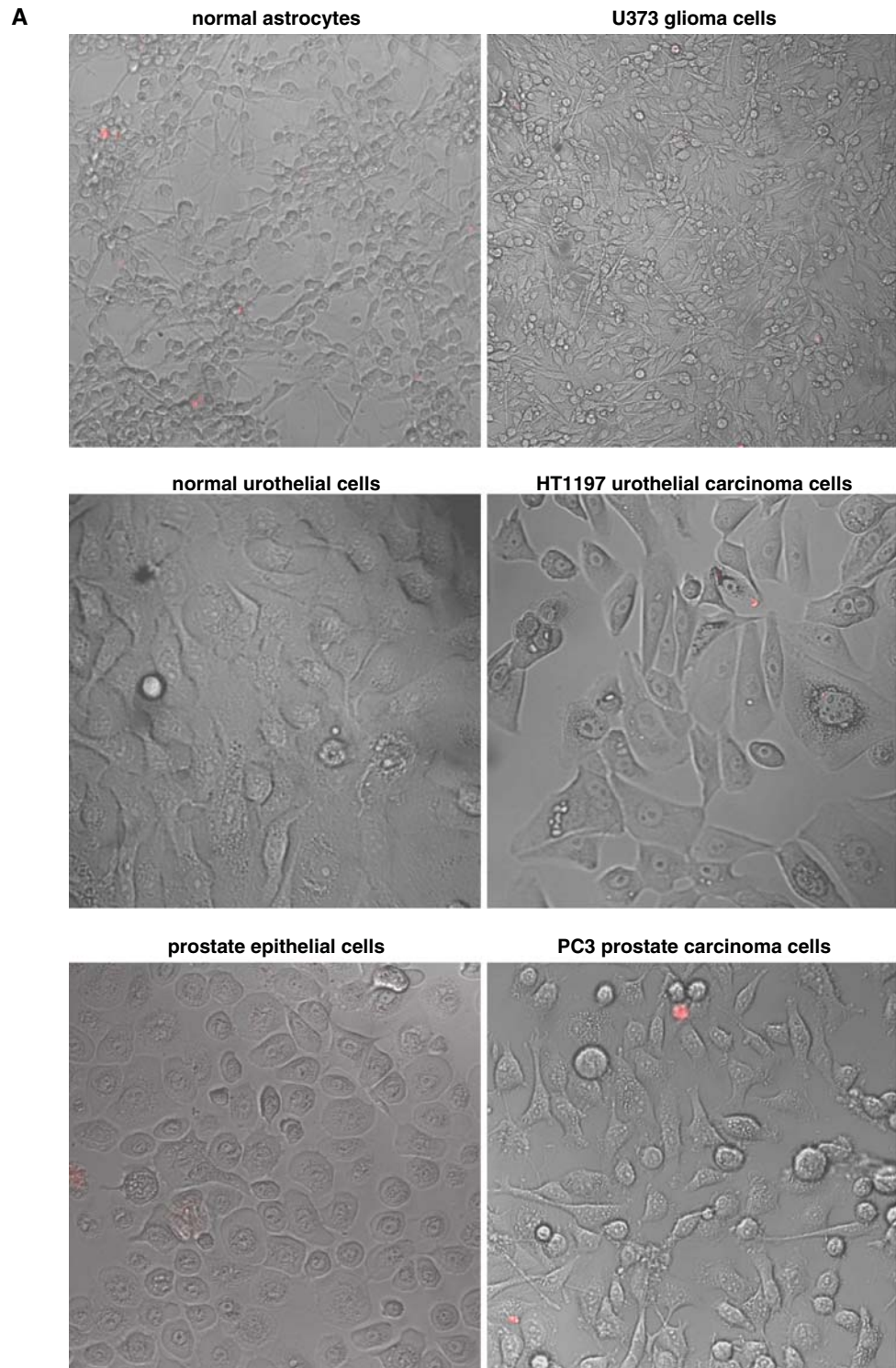
For evaluation of FITC and Dansyl staining ratios images of adherent cells were converted to jpg format using the LSM Image Browser software (Carl Zeiss, Germany).

Using the Image J software (Wayne Rasband, National Institute of Health, USA) the mean brightness values of stained and non-stained cells (about 150 cells per incubation), as well as the mean brightness of the background

were acquired. The threshold for cell staining was observed at a brightness value equal to the mean background value +10%.

PI and Alexa-Annexin-staining ratios were acquired by counting PI or Alexa-Annexin-stained and non-stained cells of image sections containing approximately 300 cells

**Fig. 1** Confocal laser scanning microscopy (CLSM) images of healthy and tumorous cells after incubation with PBS alone (**a**) and after incubation with FITC-labelled threonine<sup>108</sup> phosphorylated Apoptin<sup>81–121</sup> (conjugate 1) (**b**) and non-phosphorylated Apoptin<sup>81–121</sup> (conjugate 3) (**c–e**). In Fig. 1A Fusion images of FITC, Alexa-Annexin and Transmission are depicted. No signs of cell death [no binding of Annexin-V-Alexa<sup>TM</sup> 568 Reagent] were observed after incubation with PBS alone (**a**). After incubation with the conjugates most of the cells show expression of phosphatidylserine in the outer membrane leaflet and are therefore labelled by the Annexin-V-Alexa<sup>TM</sup> 568 Reagent (**b–e**)



each. Three independent incubations were evaluated for all staining ratios.

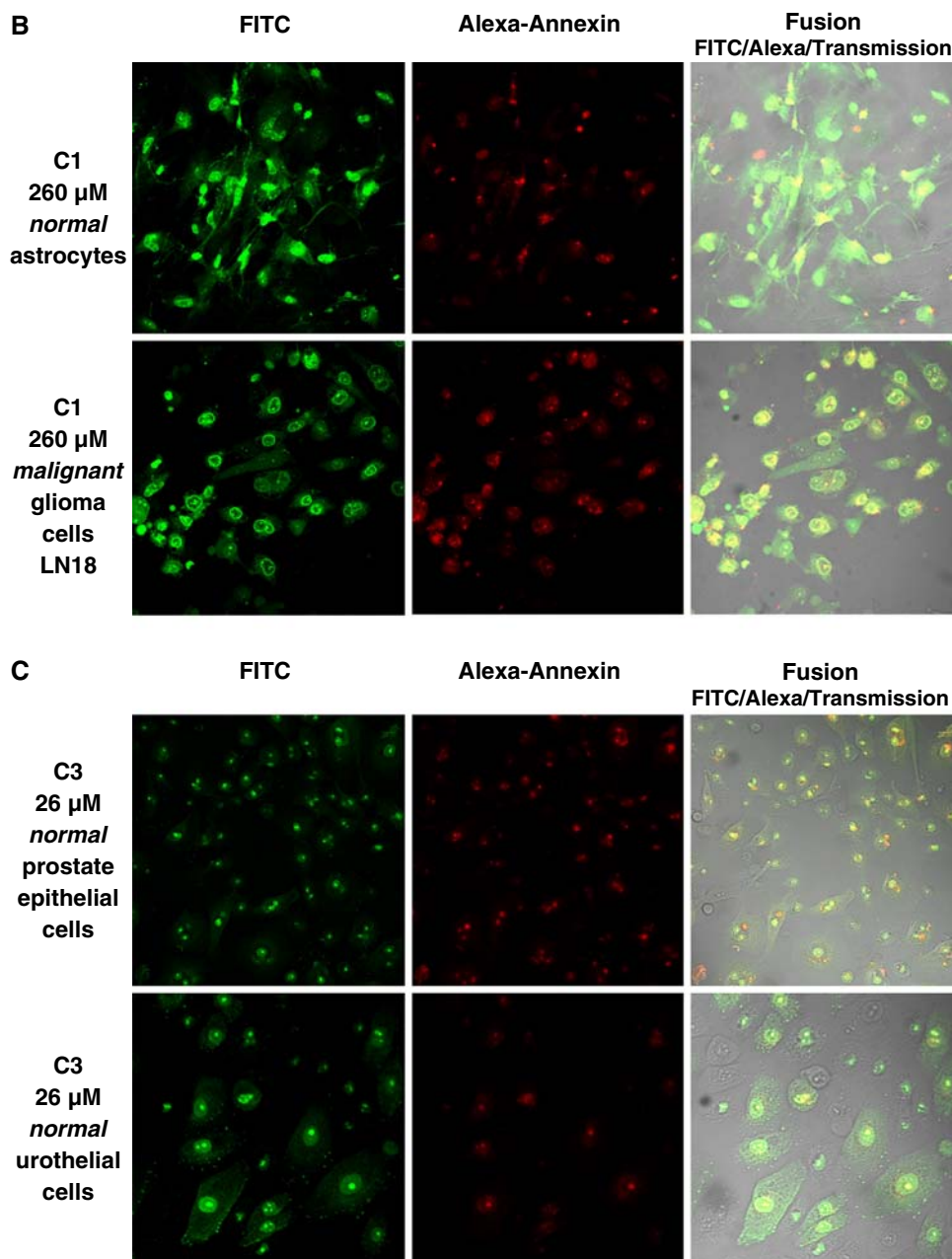
## Results

Nine conjugates were synthesized

C-terminally FITC labelled Apoptin<sup>81–121</sup> with phosphorylated or non-phosphorylated threonine<sup>108</sup> (conjugates 1 and 3), C-terminally Dansyl labelled Apoptin<sup>81–121</sup> with phosphorylated or non-phosphorylated threonine<sup>108</sup> (conjugates 2 and 4), C-terminally FITC labelled

Apoptin<sup>81–121</sup> containing either one or two altered NLS stretches (conjugates 5 and 6), Apoptin<sup>81–121</sup> dual labelled with FITC and DOTA at the C- or N-terminus (conjugates 7 and 8) and an appropriate Apoptin<sup>81–121</sup> peptide without FITC, Dansyl or DOTA (conjugate 9). The molecular weights were determined by ESI-MS [calculated average molecular weights: C1: 5,435.2 Da; C2: 5,279.1 Da; C3: 5,355.2 Da; C4: 5,199.1 Da; C5: 5,009.6 Da; C6: 4,858.3 Da; C7: 5,914.8 Da; C8: 5,871.8 Da; C9: 4,965.8 and measured average molecular weights: C1: 5,433.8 Da; C2: 5,277.6 Da; C3: 5,355.2 Da; C4: 5,198.3 Da; C5: 5,008.0 Da; C6: 4,856.6 Da; C7: 5,912.1 Da; C8: 5,868.9 Da; C9: 4,966.4 Da].

Fig. 1 continued



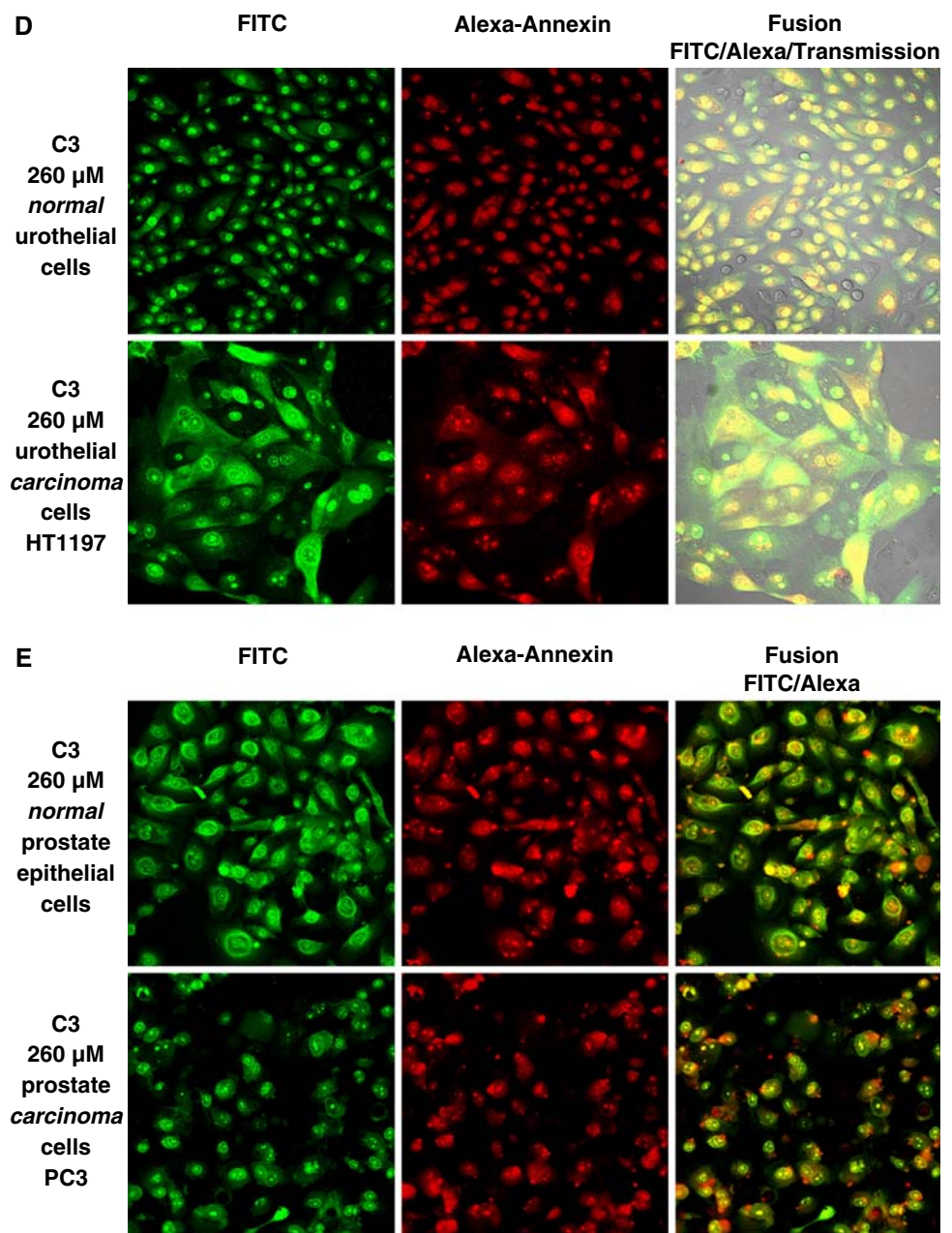
Significant autofluorescence of the tumor cells and their healthy counterparts was excluded by CLSM and fluorescence microscopy. Annexin-V-Alexa<sup>TM</sup> 568 Reagent was not bound by the cell surface (Fig. 1a).

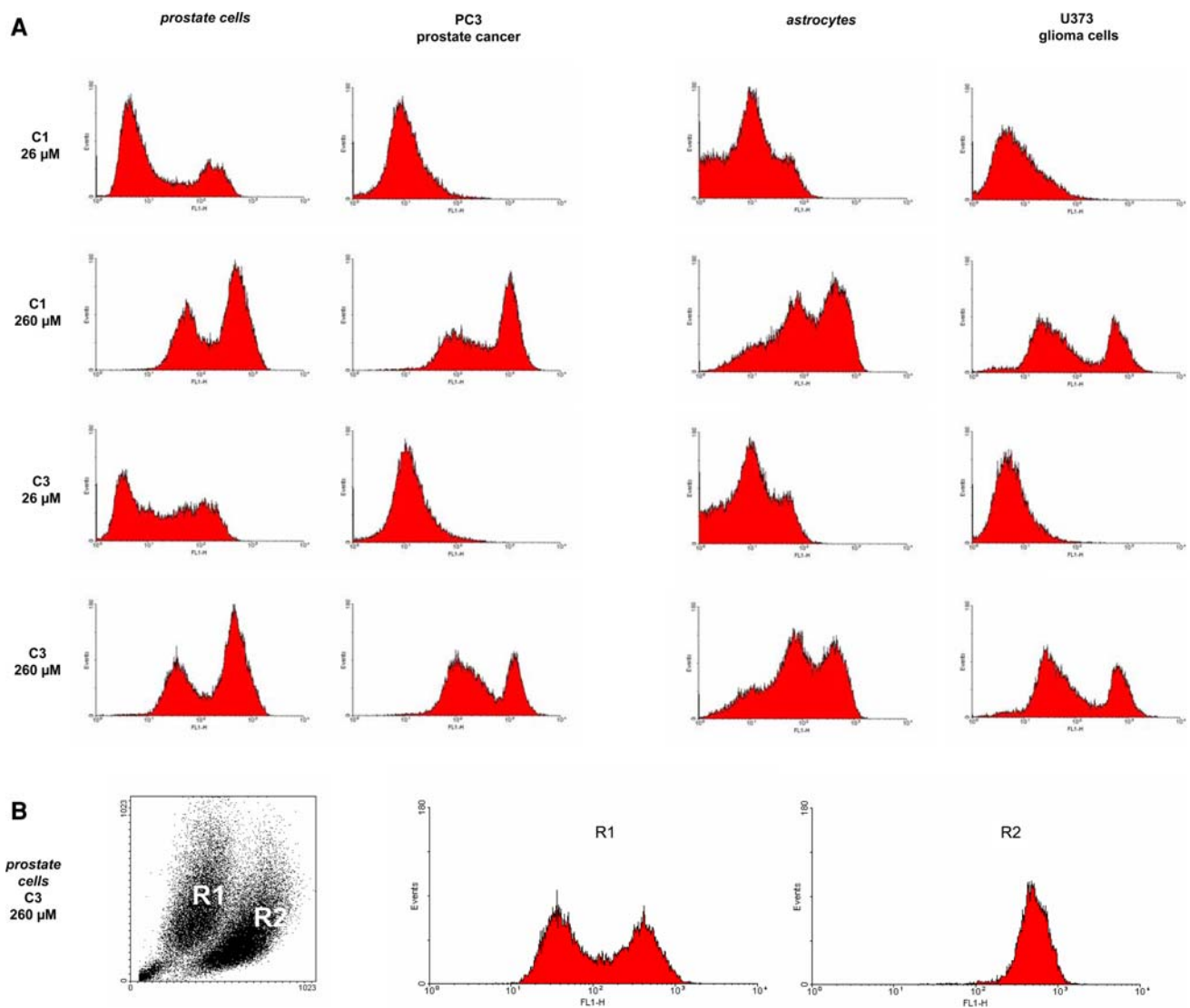
After incubation of the adherent growing cell lines with the phosphorylated FITC and Dansyl conjugates (1 and 2) for 20 min at 26  $\mu$ M and 260  $\mu$ M concentrations, CLSM, fluorescence microscopy and FACS revealed nuclear staining in both normal and tumor cells (Figs. 1b, 2 and 3). Both conjugates stained more healthy cells compared to their tumorous counterparts (Fig. 4a and b). The nuclei of more cells (especially amongst the healthy cells) were

stained at the higher concentration (Fig. 2). Predominant cytoplasmic staining, with dark cell nuclei, was not seen.

In all the cell types investigated, clear signs of cell death were seen in the cells with nuclear staining, as demonstrated by binding of Annexin-V-Alexa<sup>TM</sup> 568 Reagent to phosphatidylserine in the outer membrane leaflet and morphologic changes, with loss of cell turgor (Fig. 1b). No release of phosphorylated conjugates 1 or 2 from the cell nuclei of any cell types was found (Figs. 1 b and 3). Cells that exhibited no uptake of conjugates 1 and 2 remained intact and the Annexin-V-Alexa<sup>TM</sup> 568 Reagent was not bound by the cell surface.

Fig. 1 continued





**Fig. 2** (a) Fluorescence activated cell sorting (FACS) analysis of healthy and malignant cells. A small percentage of the cells were strongly labelled after incubation with FITC-labelled threonine<sup>108</sup> phosphorylated (C1) and nonphosphorylated (C3) Apoptin<sup>81–121</sup> conjugates at 26  $\mu$ M. An obvious increase in cells with strong staining was observed after incubation with the same conjugates at 260  $\mu$ M (right-hand shift). The rate of cellular uptake of conjugate 1 was not reduced by the phosphate group. Two morphologically

distinct cell populations could be distinguished by their forward and side scatter characteristics (C1 and C3, 260  $\mu$ M) (astrocytes, prostate cells). (b) It is exemplarily shown that two morphologically distinct cell populations were only found after incubation of healthy cells with conjugate 1 and 3 at the higher concentration (forward and side scatter characteristics). The second cloud [Region (R) 2] represents the FL1high population which contains the strongly stained and dead cells

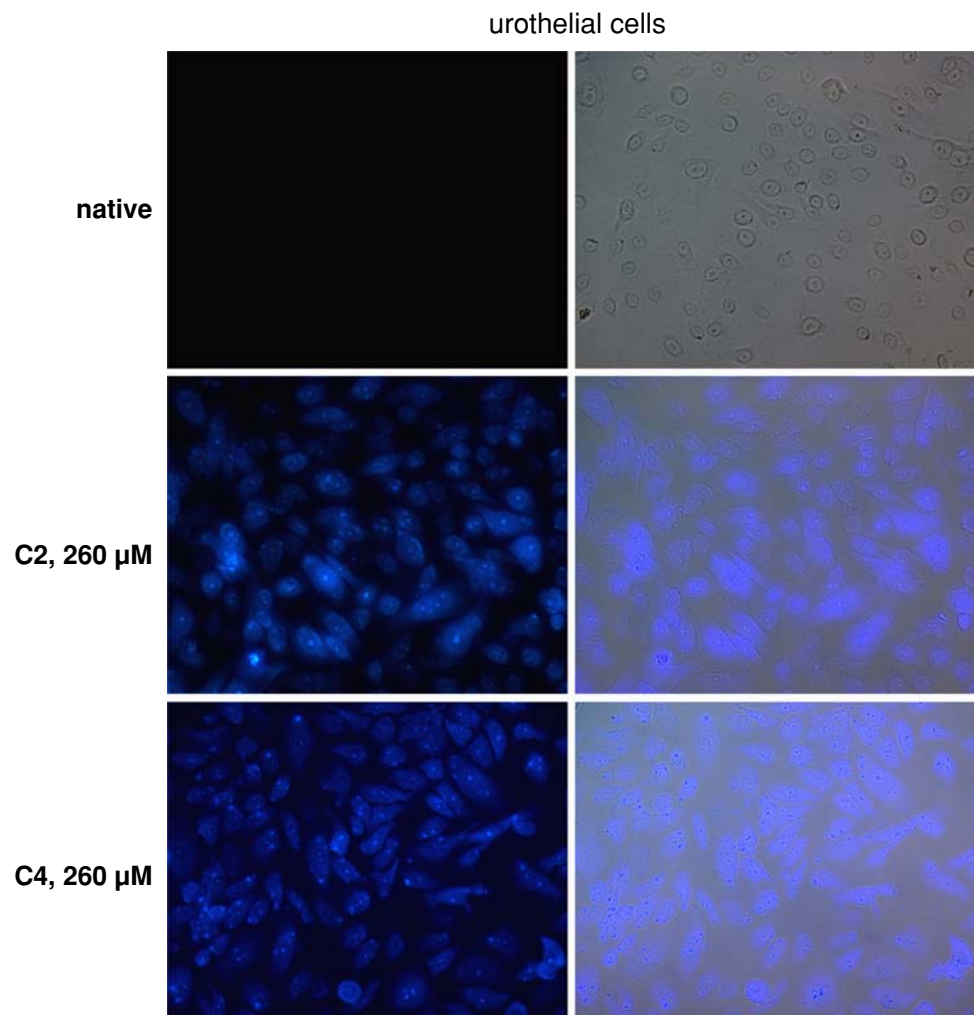
Similar nuclear uptake by tumor and healthy cells was found after incubation with the nonphosphorylated FITC and Dansyl conjugates 3 and 4 (Figs. 1 c–e, 2 and 3). Both conjugates stained a higher percentage of healthy cells compared to their tumorous counterparts (Fig. 4a and b). FACS analysis, CLSM and fluorescence microscopy revealed staining of almost the same number of tumor or healthy cells after incubation with these nonphosphorylated conjugates (Figs. 1 c–e, 2, 3 and 4a, b). Even the lower concentration produced clear nuclear staining, and

predominant staining of the cytoplasm was not characteristic (Fig. 1c). As with conjugates 1 and 2, the number of cells with nuclear staining also increased when conjugates 3 and 4 were applied at the higher concentration (Figs. 1d, e and 2). These cells also showed signs of cell death (Annexin staining) and no nuclear efflux.

After replacement of five arginines with serines within the second NLS stretch of the C-terminally FITC-labelled Apoptin<sup>81–121</sup> (conjugate 5) cellular uptake was almost completely abolished in healthy astrocytes and U373



**Fig. 3** Fluorescence and transmission light microscopy images of healthy urothelial cells after incubation with Dansyl-labelled, threonine<sup>108</sup> phosphorylated (conjugate 2) and nonphosphorylated (conjugate 4) Apoptin<sup>81–121</sup> at 260  $\mu$ M. Both conjugates stained the cell nuclei



glioma cells (Figs. 5a and 6). No cell death was found (no increase of PI uptake compared to the native control) (Figs. 5a and 6). Similar results were obtained after incubation with conjugate 6 containing two mutated NLS (NLS1: two lysines and one arginine replaced with serines; NLS2: five arginines replaced with serines) (Figs. 5a and 6). The C-terminally DOTA- and FITC-labelled Apoptin<sup>81–121</sup> conjugate 7 was equally nuclearly taken up by healthy astrocytes and human U373 glioma cells, however in a minor degree as compared to the C-terminally FITC labelled Apoptin<sup>81–121</sup> lacking DOTA conjugate 3 (Figs. 5a, b and 6).

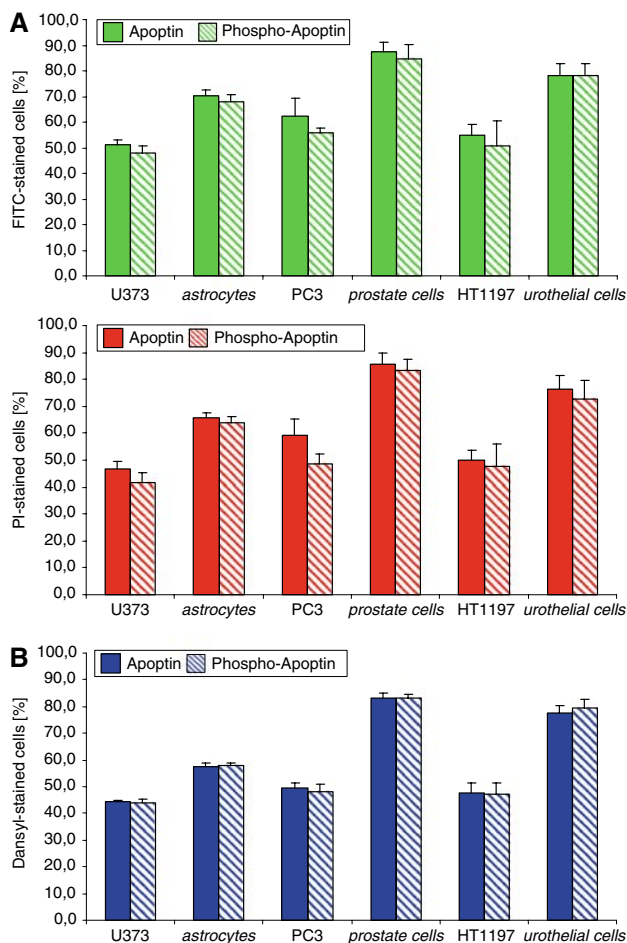
The coupling of DOTA and FITC to the N-terminal part of Apoptin<sup>81–121</sup> did not prevent Apoptin from entering the cell nuclei of healthy astrocytes (more than twice as much of the cell nuclei of healthy astrocytes were stained after coupling FITC and DOTA to the N-terminal but not C-terminal part) (Figs. 5b and 6). No nuclear efflux of conjugates 7 and 8 was found. The Apoptin<sup>81–121</sup> peptide without labels led to cell death in an equal amount of

healthy astrocytes and human U373 glioma cells (Figs. 5c and 6).

## Discussion

To achieve tumor cell-nucleus specific accumulation with a relatively short peptide sequence, we at first synthesized FITC and Dansyl conjugates containing the amino acids 81–121 of Apoptin, which include the bipartite NLS (amino acids 82–88 and 111–121) and the NES (amino acids 97–105) (Table 1).

After incubation with conjugates 1 (FITC) and 2 (Dansyl), both with phosphorylated threonine<sup>108</sup>, at two different concentrations, the nuclei of both tumor and healthy cells from the brain, bladder and prostate were clearly stained (Figs. 1b, 2, 3, 4 and 6). This finding was expected, because it is the phosphorylation of threonine<sup>108</sup> by tumor-specific cytoplasmic phosphokinases that is thought to be responsible for the active status of the



**Fig. 4** Percentage of FITC and PI stained human healthy and tumorous cells after incubation with the C-terminally FITC (a) and Dansyl (b) labelled conjugates 1–4 (260  $\mu$ M). The healthy cells and their tumorous counterparts were equally stained by both conjugate types. The highest staining rate was found in healthy urothelial and prostate cells. FITC staining closely correlated with cell death (PI-staining). The examinations were performed three times. The standard deviation of the mean is depicted

bipartite NLS [4, 9]. These two conjugates did not leave the nuclei of the tumor cells or healthy cells, which is consistent with the theory postulated by Poon et al. [9] that the NES would have been inactivated by phosphorylation, resulting in nuclear retention.

Surprisingly, cell death was already found in both the tumor cells and healthy cells after only 20 min (Figs. 1b and 6b). This stands in contrast to the findings of a previous study, which demonstrated that the enforced nuclear uptake of Apoptin fused with the SV 40 T antigen NLS did not lead to cell death in healthy cells [6].

Contrary to expectations, conjugates 3 and 4, which contained nonphosphorylated threonine<sup>108</sup>, were also taken up by the cell nuclei of both healthy and tumor cells, showing that cytoplasmic phosphorylation was not a prerequisite for the nuclear uptake of these conjugates

(Figs. 1c–e, 3, 4 and 6). The conjugates did not leave the cell nuclei, and were associated with cell death in both healthy and tumor cells.

It is possible that the staining of larger numbers of cells at even lower concentrations could have been achieved by the coupling of potent transmembrane transport peptides like the HIV-tat (transactivator of transcription) protein transduction domain to the Apoptin<sup>81–121</sup> conjugates 1–4, as was done in another study using a plasmid that expressed the Apoptin<sup>1–121</sup>-HIV-tat fusion protein [7]. Incubation with this protein led to cytoplasmic accumulation in 1BR3 human primary fibroblasts and nuclear accumulation in human squamous cell carcinoma (HSC-3) cells.

We used the transmembrane transport activity of the Apoptin<sup>81–121</sup> peptide itself, which appears to be associated with the cationic peptide stretches (NLS1 and NLS2) (Table 1). Almost complete abolishment of cellular and nuclear uptake was found after replacement of the 5 arginines with serines within the NLS2 of the C-terminally FITC labelled Apoptin<sup>81–121</sup> (conjugate 5). Similar results were obtained when using the C-terminally FITC labelled Apoptin<sup>81–121</sup> with two mutated NLS [NLS1: two lysines and one arginine replaced with serines; NLS2: 5 arginines replaced with serines].

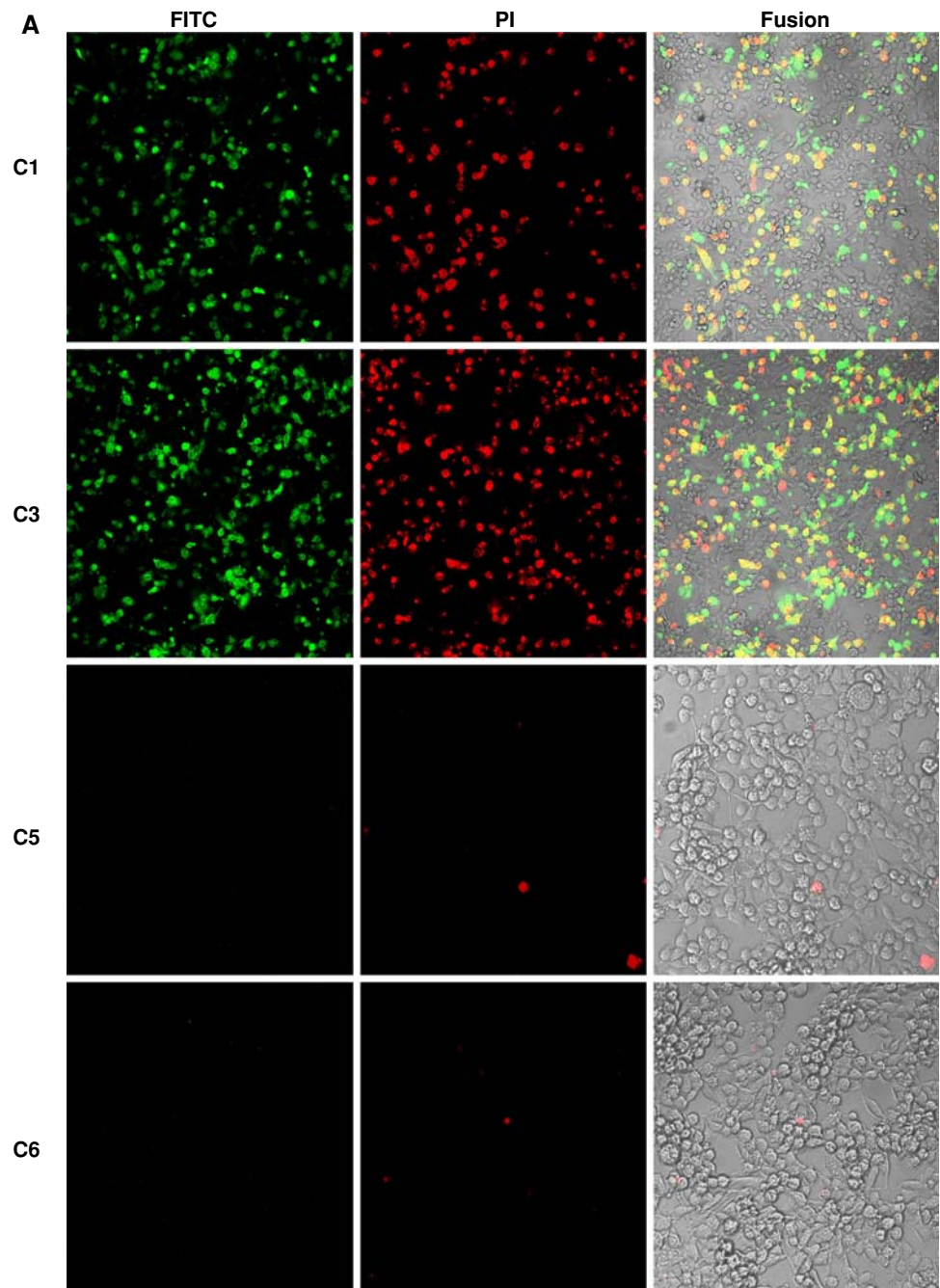
Fluorescence live cell microscopy was used in this investigation, in contrast to previous Apoptin studies [2–4, 6, 7, 9–11]. This method was chosen to avoid the necessity for fixation because, according to Richard et al. [16], even mild fixation may lead to artefactual relocation of peptides like HIV-tat within the cell.

The fact that all our FITC- and Dansyl-labelled conjugates 1–4 were found to accumulate within the nuclei of both tumor and non-tumor cells could have several reasons. Passive diffusion could be expected in the case of molecules of less than 60 kDa [17], such as our Apoptin conjugates. However, it seems that small conjugates do not automatically accumulate within the nuclear compartment (e.g. by diffusion), as exemplified by a small SV 40 T antigen NLS conjugate containing cobaltocenium [18]. This conjugate was not found to be taken up by the cell nuclei when a mutant NLS was used.

Apoptin fusion proteins containing GFP or HIV-tat are not taken up by the nuclei of healthy cells although their molecular masses are well below the cut-off mass for diffusion through the nuclear pore complex (60 kDa). This could be explained by the fact, that apoptin forms functional complexes of 30–40 subunits, which are far much larger than 60 kDa. The structural motifs responsible for multimerization were mapped to the N-terminal half of Apoptin<sup>1–121</sup> [19]. Our conjugates 1–9 did not contain this N-terminal half and multimerization was rather unlikely.

The N-terminal but not C-terminal coupling of imaging labels might be responsible for the tumor-specific nuclear

**Fig. 5** Confocal laser scanning microscopy (CLSM) images of human malignant U373 glioma cells (**a**) and healthy astrocytes (**b, c**). (**a**) A high percentage of nuclear stained cells was found after incubation with the non-phosphorylated and threonine<sup>108</sup> phosphorylated Apoptin<sup>81–121</sup> conjugates 1 and 3. This was associated with cell death (Propidium Iodide uptake) (260  $\mu$ M). By contrast conjugate 5 (mutant NLS2) and conjugate 6 (mutant NLS 1 and 2) were not taken up by the cells which remained intact (no Propidium Iodide uptake) (260  $\mu$ M). (**b**) A higher number of astrocyte nuclei were stained after incubation with the N-terminally dual labelled FITC-DOTA-Apoptin<sup>81–121</sup> conjugate 8 compared to the C-terminally dual labelled FITC-DOTA-Apoptin<sup>81–121</sup> conjugate 7 (260  $\mu$ M). This was also associated with a higher cell death rate (Propidium Iodide uptake). (**c**) Propidium Iodide uptake in human astrocytes and human U373 glioma cells after incubation with the unlabelled Apoptin<sup>81–121</sup> conjugate 9 (260  $\mu$ M). Both cell types showed a comparable high number of dead cells



uptake of Apoptin<sup>81–121</sup> conjugates. With the aim to create a novel tumor-specific dual labelled contrast agent for magnetic resonance imaging we coupled both DOTA (chelator of gadolinium) and FITC to either the N- or C-terminal part of Apoptin<sup>81–121</sup> (conjugates 7 and 8, Table 1). The N-terminal labeling led to a better cellular uptake, especially in healthy astrocytes (Figs. 5b and 6). However no tumor cell-specific nuclear accumulation was found after incubation with both conjugates. Both conjugates led to cell death (Figs. 5b and 6).

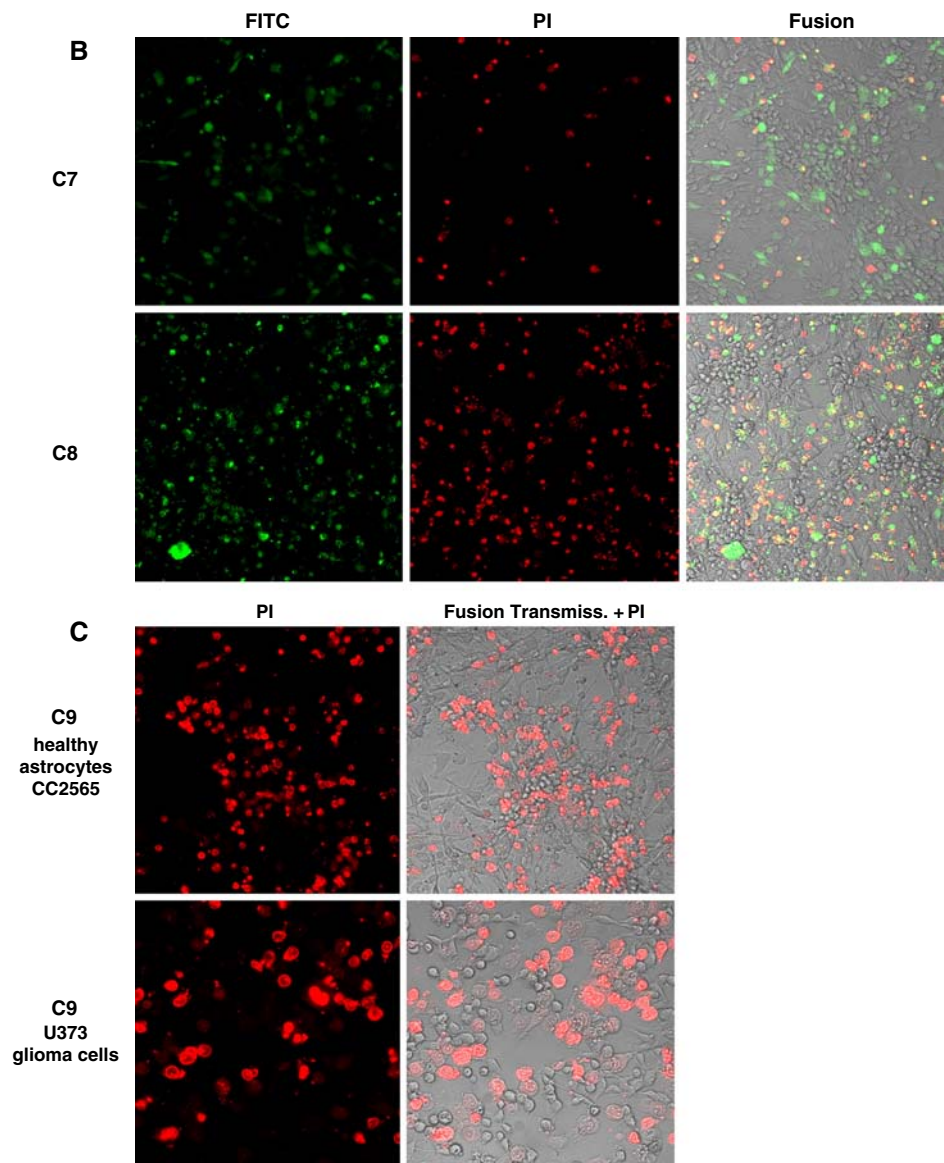
It is possible that FITC or DOTA, which are much smaller than GFP, EGFP, HA or dsRed, could exert some

previously unrecognized specific effects on the nuclear uptake and export behavior of the C-terminal part of Apoptin (amino acids 81–121) by influencing its three-dimensional structure. For instance, GFP influences the function of native Apoptin [5, 9, 11]. GFP-Apoptin but not hemagglutinin (HA)-Apoptin localizes in the cell nuclei of healthy cells [11].

In our study we obtained comparable results when Dansyl, another fluorophore, was coupled to the same peptide sequence (Table 1, Figs. 3, 4b and 6).

Cell death in healthy cells might be induced by the FITC-, DOTA- and Dansyl-labels but not the Apoptin<sup>81–121</sup>

Fig. 5 continued



peptide itself. However a comparable high cell death rate in healthy cells was also found after incubation with the unlabelled Apoptin<sup>81–121</sup> peptide (conjugate 9) (Figs. 5c and 6).

Wadia et al. [12] and Guelen et al. [7] postulated that nuclear uptake of Apoptin<sup>1–121</sup> could function in a concentration-dependent manner. After transfection of tumor and healthy cells higher expression levels of Apoptin could be obtained in tumor cells. Thus, Wadia et al. [12] concluded that Apoptin contains a concentration-dependent NLS rather than tumorigenic selective NLS. However, Poon et al. [8] found no relationship between the cytoplasmic concentration of Apoptin and the extent of nuclear accumulation.

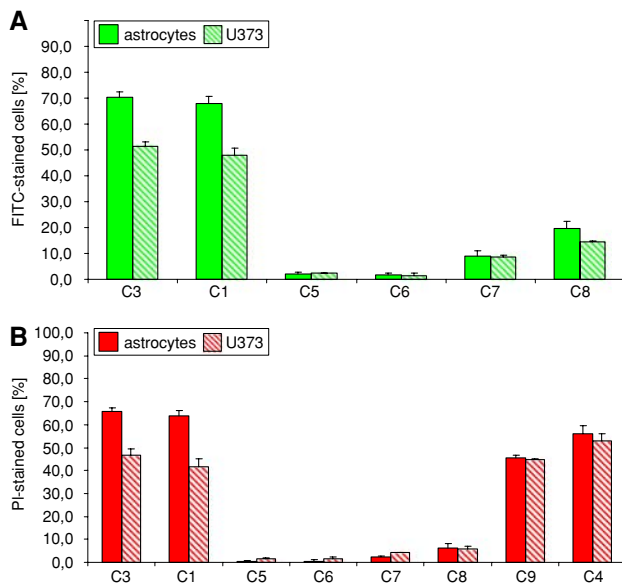
In our study the tumor and healthy cells were not transfected with Apoptin but were incubated with the

synthetic product. Surprisingly, the conjugates were preferentially taken up by the cell nuclei of healthy cells (Figs. 4a, b and 6).

Nuclear staining by the conjugates closely correlated with cell death (Figs. 4a and 6). Cells which were incubated at the higher concentration showed a higher amount of nuclear staining and dead cells (Fig. 2).

Thus, our findings suggested a concentration-dependent transport of Apoptin<sup>81–121</sup> across the outer cellular membrane.

It was previously shown that non-phosphorylatable mutants still induce apoptosis of cancer cells [4, 7, 10, 11]. This was explained by the existence of two different apoptosis domains at the N- and C-terminus [6]. The apoptosis domain at the N-terminus functions independently of the tumor-specific phosphokinases. Our



**Fig. 6** Percentage of FITC (a) and PI (b) stained human healthy astrocytes and human U373 glioma cells after incubation with conjugates C1, 3, 4, 5, 6, 7, 8 and 9 (260  $\mu$ M). Both cell types were equally stained by the C-terminally FITC-labelled non-phosphorylated and phosphorylated Apoptin<sup>81–121</sup> (C3, C1). A remarkable decrease in nucleary stained cells was observed after incubation with the C-terminally FITC labelled Apoptin<sup>81–121</sup> conjugates 5 and 6 with one or two mutated NLS. The N-terminally dual labelled FITC-DOTA-Apoptin<sup>81–121</sup> conjugate 8 stained a higher amount of astrocyte nuclei compared to the corresponding C-terminally labelled conjugate 7. For conjugates 1, 3, 5, 6, 7 and 8 the FITC-staining rate closely correlated with cell death rate (Propidium Iodide uptake). The unlabelled and Dansyl labelled Apoptin<sup>81–121</sup> conjugates 9 and 4 also led to high numbers of PI-stained dead cells (comparable to the C-terminally FITC labelled conjugates 1 and 3). The examinations were performed three times. The standard deviation of the mean is depicted

conjugates lack the N-terminal apoptosis domain. This might explain their high nuclear uptake rate in healthy cells.

It is possible that the tumor-specific effects of Apoptin noted in many cell lines (heterogenic or isogenic) may not depend exclusively on the above mentioned C- and N-terminal apoptosis domains, but may also be related to some other previously unknown cell-line specific factors (e.g. composition of the cellular and nuclear membrane).

The tumor/healthy cell pairs from the brain, prostate, and bladder that were used for the nuclear uptake studies have not previously been used in Apoptin research.

This aspect might be very important when comparing the effects of GFP-Apoptin and its truncated forms on tumor and healthy cells from different origins, such as SAOS-2 human osteosarcoma cells and VH10 normal human skin fibroblasts [3–7]. However, differences in the composition of the cell membrane might even exist between the tumor and non-tumor cell type pairs used in our study.

Finally, the N-terminal part of Apoptin with a leucine-rich element (amino acids 33–46) could have more influence on tumor-specific nuclear uptake than has been suspected. This notion was put forward by Danen-Van Oorschot et al. [6] and Heilmann et al. [10] who, unlike Poon et al. [9], suspect that the leucine-rich element contains a cytoplasmic retention signal, which could be the main factor responsible for the lack of nuclear accumulation in healthy cells. However, inclusion of the N-terminal part would result in larger conjugates of about 14 kDa, and a proportionate increase in peptide compared to the labels (FITC, Dansyl, DOTA) which are responsible for the imaging signal.

## Conclusion

The C-terminal coupling of FITC or Dansyl to phosphorylated or nonphosphorylated Apoptin<sup>81–121</sup> revealed nuclear uptake without subsequent release in three healthy/tumor cell pairs from the bladder, brain, and prostate. Cell death was not restricted to tumor cells. Similar results were obtained in human astrocytes and glioma cells with Apoptin<sup>81–121</sup> which was C- or N-terminally dual labelled with FITC and DOTA or with unlabelled native Apoptin<sup>81–121</sup>.

Cellular and nuclear uptake of the C-terminally labelled Apoptin<sup>81–121</sup> conjugate was almost completely abolished after replacement of 5 arginines within the second nuclear localization signal (NLS2) (amino acids 111–121).

Future studies will be needed to understand the mechanism by which conjugates 1–4 and 7–9 induce cell death.

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## References

- Noteborn MH, de Boer GF, van Roozelaar DJ et al (1991) Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. *J Virol* 65:3131–3139
- Zhuang SM, Shvarts A, van Ormondt H, Jochemsen AG, van der Eb AJ, Noteborn MH (1995) Apoptin, a protein derived from chicken anemia virus, induces p53-independent apoptosis in human osteosarcoma cells. *Cancer Res* 55:486–489
- Danen-Van Oorschot AA, Fischer DF, Grimbergen JM et al (1997) Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells. *Proc Natl Acad Sci USA* 94:5843–5847
- Rohn JL, Zhang YH, Aalbers RI et al (2002) A tumor-specific kinase activity regulates the viral death protein Apoptin. *J Biol Chem* 277:50820–50827
- Rohn JL, Zhang YH, Leliveld SR et al (2005) Relevance of apoptin's integrity for its functional behavior. *J Virol* 79:1337–1338
- Danen-Van Oorschot AA, Zhang YH, Leliveld SR et al (2003) Importance of nuclear localization of apoptin for tumor-specific induction of apoptosis. *J Biol Chem* 278:27729–27736

7. Guelen L, Paterson H, Gaken J, Meyers M, Farzaneh F, Tavassoli M (2004) TAT-apoptin is efficiently delivered and induces apoptosis in cancer cells. *Oncogene* 23:1153–1165
8. Poon IK, Oro C, Dias MM, Zhang JP, Jans DA (2005) A tumor cell-specific nuclear targeting signal within chicken anemia virus VP3/apoptin. *J Virol* 79:1339–1341
9. Poon IK, Oro C, Dias MM, Zhang J, Jans DA (2005) Apoptin nuclear accumulation is modulated by a CRM1-recognized nuclear export signal that is active in normal but not in tumor cells. *Cancer Res* 65:7059–7064
10. Heilman DW, Teodoro JG, Green MR (2006) Apoptin nucleocytoplasmic shuttling is required for cell type-specific localization, apoptosis, and recruitment of the anaphase-promoting complex/cyclosome to PML bodies. *J Virol* 80:7535–7545
11. Lee YH, Cheng CM, Chang YF, Wang TY, Yuo CY (2007) Apoptin T108 phosphorylation is not required for its tumor-specific nuclear localization but partially affects its apoptotic activity. *Biochem Biophys Res Commun* 354:391–395
12. Wadia JS, Wagner MV, Ezhevsky SA, Dowdy SE (2004) Apoptin/VP3 contains a concentration-dependent nuclear localization signal (NLS), not a tumorigenic selective NLS. *J Virol* 78:6077–6078
13. Subbarayan V, Sabichi AL, Llansa N, Lippman SM, Menter DG (2001) Differential expression of cyclooxygenase-2 and its regulation by tumor necrosis factor- $\alpha$  in normal and malignant prostate cells. *Cancer Res* 61:2720–2726
14. Mahajan SD, Schwartz SA, Shanahan TC, Chawda RP, Nair MPN (2002) Morphine regulates gene expression of  $\alpha$ - and  $\beta$ -chemokines and their receptors on astroglial cells via the opioid  $\mu$  receptor. *J Immunol* 169:3589–3599
15. Eder IE, Corvin S, Maneschg C et al (2000) Selective culture conditions for different types of primary human bladder cells. *World J Urol* 18:371–375
16. Richard JP, Melikov K, Vives E et al (2003) Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J Biol Chem* 278:585–590
17. Gerace L, Burke B (1988) Functional organization of the nuclear envelope. *Annu Rev Cell Biol* 4:335–374
18. Noor F, Wüstholtz A, Kinscherf R, Metzler-Nolte N (2005) A cobaltocenium-peptide bioconjugate shows enhanced cellular uptake and directed nuclear delivery. *Angew Chem Int Ed* 44:2429–2432
19. Leliveld SR, Zhang YH, Rohn JL, Noteborn MH, Abrahams JP (2003) Apoptin induces tumor-specific apoptosis as a globular multimer. *J Biol Chem* 278:9042–9051