A Novel Polyarginine Containing Smac Peptide Conjugate that Mediates Cell Death in Tumor and Healthy Cells

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Abstract: The seven N-terminal amino acids AVPIAQK (SmacN7) of the mitochondrial protein Smac (second mitochondria-derived activator of caspase) promote caspase activation by binding specifically to inhibitor of apoptosis proteins (IAPs) and blocking their inhibitory activity.

SmacN7 cannot pass through the cell membrane, but to be of therapeutic use it would be essential for it to enter the cell. To achieve transmembrane transport of SmacN7 we coupled it to a novel fluorescein isothiocyanate (FITC)-labelled transmembrane transport peptide RRRRK(FITC)RRRR *via* ß-alanine to produce the conjugate AVPIAQKßA RRRRK(FITC)RRRR.

Because IAPs are much more strongly expressed in the cytoplasm of tumor cells, we expected this conjugate to produce staining of the cytoplasm, and for this to be stronger in tumor cells than in healthy cells.

Surprisingly, we found strong nuclear uptake of the Smac conjugate and of the transport peptide alone without subsequent release in both tumor cells and healthy cells from the bladder, prostate, and brain. This was accompanied by cell death.

In contrast to expectations, it appears that the apoptotic effects observed do not result from the SmacN7 cargo alone.

Key Words: Smac, polyarginine, cell nucleus, cell death, transmembrane transport, glioma, bladder, prostate.

INTRODUCTION

The second mitochondria-derived activator of caspase (Smac)/DIABLO [(direct IAP binding protein with low isoelectric point (pI)], is released from mitochondria in response to apoptotic stimuli. Smac promotes caspase activation by binding to inhibitor of apoptosis proteins (IAPs), which results in the abolishment of their inhibitory activity. Marked expression of IAPs is found predominantly in tumors and is associated with a poor prognosis [1,2]. Therapeutic blocking of cytoplasmic IAPs by Smac has been found to result in reversal of resistance to apoptosis and would thus appear to be highly promising for use in combination with chemotherapeutic agents [3-5] or γ-irradiation [6]. The IAP binding sequence AVPIAQK (SmacN7) cannot pass through the cell membrane [5]. Therefore, established transmembrane transport peptides like Penetratin [3], HIV-tat [4], and polyarginine (hexa- or octaarginine) [5] have been fused to the SmacN7 sequence to achieve cytoplasmic accumulation. Fluorescein isothiocyanate (FITC) was also coupled to these fusion peptides to enable localization *in vitro* and *in vivo* by fluorescence microscopy.

The objective of our study was to couple the SmacN7 peptide to an octaarginine-derived transport peptide in which, as a distinctive feature, an FITC-labelled lysine was flanked on each side by four arginines. This slight modification to the previously successfully used FITC-labelled octaarginine transporter conjugated to Smac [5] was investigated for a potential influence on staining patterns and cell viability before the attachment of the SmacN7 peptide.

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METHODS AND MATERIALS

Synthesis of Conjugates 1-3

The conjugates 1-3 (Table **1**) were synthesized by solid phase peptide synthesis using the Fmoc/tBu technique (Eco-syn P batch synthesizer, Eppendorf Biotronik, Maintal, Germany).

Table 1.

C1	*AVPIAQK*ßAK(FITC)
C2	RRRRK(FITC)RRRR
C3	*AVPIAQK*ßARRRRK(FITC)RRRR

After anchoring the C-terminal arginine as Fmoc Arg (Pbf)-OH on TentaGel S RAM Resin (Rapp Polymere, Tübingen, Germany) the amino acids were incorporated using the Fmoc amino acids (4eq) and TBTU [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate] (4eq) + 8eq diisopropylethylamine for 40 min in DMF as the solvent. The side chains of the amino acids were protected as Pbf (2.2.4.6.7. pentamethyldihydrobenzofuran-5-sulfonyl) for

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arginine, Mmt (N^{ε}- 4 methoxytrityl) for lysine[13] (FITC), Boc (N^{ε}-tert butyloxycabonyl) for lysine[7] and trt (trityl) for glutamine. The deprotection of the Fmoc-groups was performed by shaking the reaction mixture with a 25% piperidine solution in dimethylformamide (DMF) for 12 minutes.

The N-terminal amino acids were incorporated as their Boc derivatives.

After completion of the synthesis the Mmt-side chain protecting group was removed by addition of 1% TFA/0.1 ml triisopropylsilane for 1h at room temperature.

The introduction of the fluorescein isothiocarbamyl moiety was performed using fluorescein 5(6) isothiocyanate (5eq) in the presence of 5 equiv. diisopropylethylamine in DMSO as solvent by shaking the reaction mixture overnight.

The deprotection from the resin and from the remaining protecting groups was reached by using a cocktail of 12 ml trifluoroacetic acid, 0.3 ml ethanethidiol, 0.3 ml anisole, 0.3 ml water, 0.1 ml triisopropylsilane for 2 h at room temperature. After filtering the reaction mixture directly in abs. diethylether the precipitated peptides were filtered, washed with diethylether and dried. The conjugates were purified by semipreparative HPLC [column: Nucleosil 100, 5 µm, C_{18} 10 x 250 mm Machery & Nagel (Düren, Germany); solvent system: buffer A: 0.1% TFA/H_20; buffer B: 80% CH_3CN/ 0.1% TFA/H_20 gradient 10-90%; buffer B in 31 minutes, detection 214 nm, flow 4ml/min]. The lyophilized peptides were shown to be ≥ 97% pure by analytical HPLC, and their molecular weights were determined by electrospray ionization mass spectrometry (ESI-MS).

Electrospray Ionisation Mass Spectrometry (ESI-MS)

Conjugates 1-3 were analyzed by ESI-MS on an Esquire3000+ ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany). Conjugates were dissolved in 40% ACN, 0.1% formic acid (Sigma-Aldrich, Tauffkirchen, Germany) in water (v/v/v) (20 pmol/µl) and constantly infused using a syringe pump (5 µl/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 -3700 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 [7].

Normal human astrocytes CC-2565 (NHA) (two different clones) were also purchased from Cambrex and cultured in astrocyte basal medium CC-3187 (ABM) with supplements (CC-4123, AGM Single Quots) as recommended by the supplier and previously described [8].

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 as described elsewhere [9].

Human malignant LN 18 and U373 glioma cells, the human urothelial carcinoma cell line HT 1197, 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)-Gold (PAA laboratories, Pasching, Austria).

Incubation with Conjugates 1-3

All cells were grown to 80% confluency in 75 cm^2 culture flasks (Corning Costar, Bodenheim, Germany) at 37°C in an atmosphere of 5% CO_2 (v/v).

Accutase™ (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_2 (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 1-3 in D-PBS for 20 minutes. After this, the cells were rinsed three times with buffer and then incubated with Ready Mix Medium again.

Detection of phosphatidylserine in the outer membrane leaflet of apoptotic cells was performed with the Annexin-V-Alexa™ 568 Reagent according to the manufacturer's protocol (Roche Molecular Biochemicals, Indianapolis, USA).

The FITC conjugates 1-3 were investigated by Confocal laser scanning microscopy. Confocal laser scanning microscopy was performed on an inverted LSM510 laser scanning microscope (Carl Zeiss, Jena, Germany) (objectives: LD Achroplan 40x0.6, Plan Neofluar 20x0.50, 40x0.75). For fluorescence excitation, the 488 nm line of an argon ion laser and the 543 nm line of a helium-neon laser with appropriate beam splitters and barrier filters were used for FITC and Alexa, respectively. Superimposed images of FITC- and Alexa-stained samples were created by overlaying coincident views.

All measurements were performed on living, non-fixed cells.

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

Alexa-Annexin-staining ratios were acquired by counting Alexa-Annexin-stained and non-stained cells of image sections containing approximately 300 cells each. Three independent incubations were evaluated for all staining ratios.

Fluorescence Activated Cell Sorting (FACS)

For FACS, all cell types were grown under the same conditions in 75 cm^2 culture flasks (Corning Costar, Bodenheim Germany) (80% confluency). Accutase™ (PAA laboratories, Pasching, Austria) was added to achieve detachment of the cells. Fluorescence activated cell sorting (FACS) was performed using a Becton Dickinson FACSCalibur. 1 x 10^6 cells were incubated in 100 µl of conjugate solutions 1-3 (26 µM and 260 µM) or PBS alone for 20 minutes at 37°C in an atmosphere of 5% CO_2. Afterwards the cells were washed three times in PBS and centrifuged at 800 rpm (rounds per minute) for 5 min. 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.

RESULTS

Three Conjugates were Synthesized

AVPIAQKßAK(FITC) [C1: calculated mass: 1313.63 Da, measured mass:1313.64 Da], RRRRK(FITC)RRRR [C2: calculated mass: 1783.97 Da, measured mass: 1783.85 Da], and AVPIAQKßARRRRK(FITC)RRRR [C3: calculated mass: 2562.44 Da, measured mass: 2562.90 Da] (Table **1**).

Significant autofluorescence of the tumor cells and their healthy counterparts was excluded by CLSM.

After incubation of the adherent growing cell lines with conjugate 1 at concentrations of 26 and 260 µM most of the healthy and tumor cells showed no nuclear or cytoplasmic staining (Fig. **1**). None of the cell types showed signs of cell death, as demonstrated by the lack of Annexin-V-Alexa™ 568 Reagent binding to phosphatidylserine in the outer membrane leaflet and the normal morphological appearances, with intact cell turgor (Fig. **1**).

However, strong staining, which was predominantly nuclear, was found in both tumor and healthy cells after incubation with conjugates 2 and 3 (Figs. **2A-D**, **3** and **4**). These cells showed clear signs of cell death, as demonstrated by the binding of Annexin-V-Alexa™ 568 Reagent to phosphatidylserine in the outer membrane leaflet and the loss of cell turgor (Figs. **2A-D**).

FACS analysis and CLSM revealed a slightly larger number of cells with strong nuclear staining after incubation with the transport peptide alone (conjugate 2) at 26 and 260 µM (Figs. **3** and **4**), the number of stained cells being lower at the lower concentrations of both conjugates 2 and 3.

Even at the lower concentration the nuclei were clearly stained, and cytoplasmic staining was not predominant after incubation with conjugates 2 or 3. No release of the conjugates from the nuclei of any of the cell types was found.

After incubation with conjugates 2 and 3 two cell populations could be distinguished on the basis of their morphology (side scatter versus forward scatter analysis) (Fig. **3**).

Cells with no uptake of conjugates 2 or 3 remained intact, and Annexin-V-Alexa™ 568 Reagent was not bound by the cell surface (Fig. **2A,D**).

DISCUSSION

The seven N-terminal amino acids of the Smac peptide (SmacN7), which specifically reverse the inhibition of apoptosis by binding to IAPs in the cytoplasm of tumor cells, cannot pass through the outer cell membrane when administered systemically or *in vitro*. However, tumor cells could be made sensitive to the apoptosis-inducing effects of anticancer drugs or γ-irradiation if the SmacN7 peptide could enter the cell and bind to the IAPs within the cytoplasm [3-5].

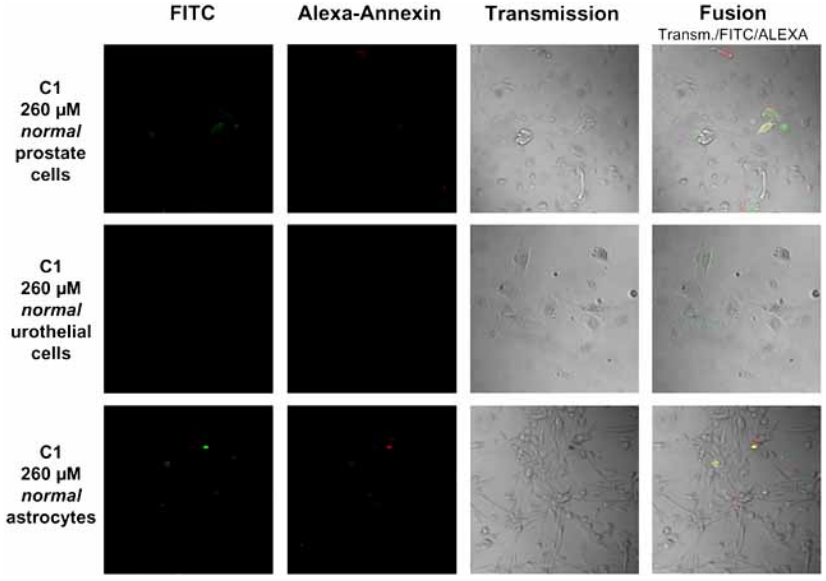

Fig. (1). Confocal laser scanning microscopy (CLSM) images of healthy cells from the bladder, brain and prostate after incubation with conjugate C1.

The Annexin-V-Alexa™ 568 Reagent was used to detect phosphatidylserine in the outer membrane leaflet of necrotic or apoptotic cells.

Most of the cells showed no nuclear or cytoplasmic staining. No marked binding of the Annexin-V-Alexa™ 568 Reagent to the surface of the cells was found.

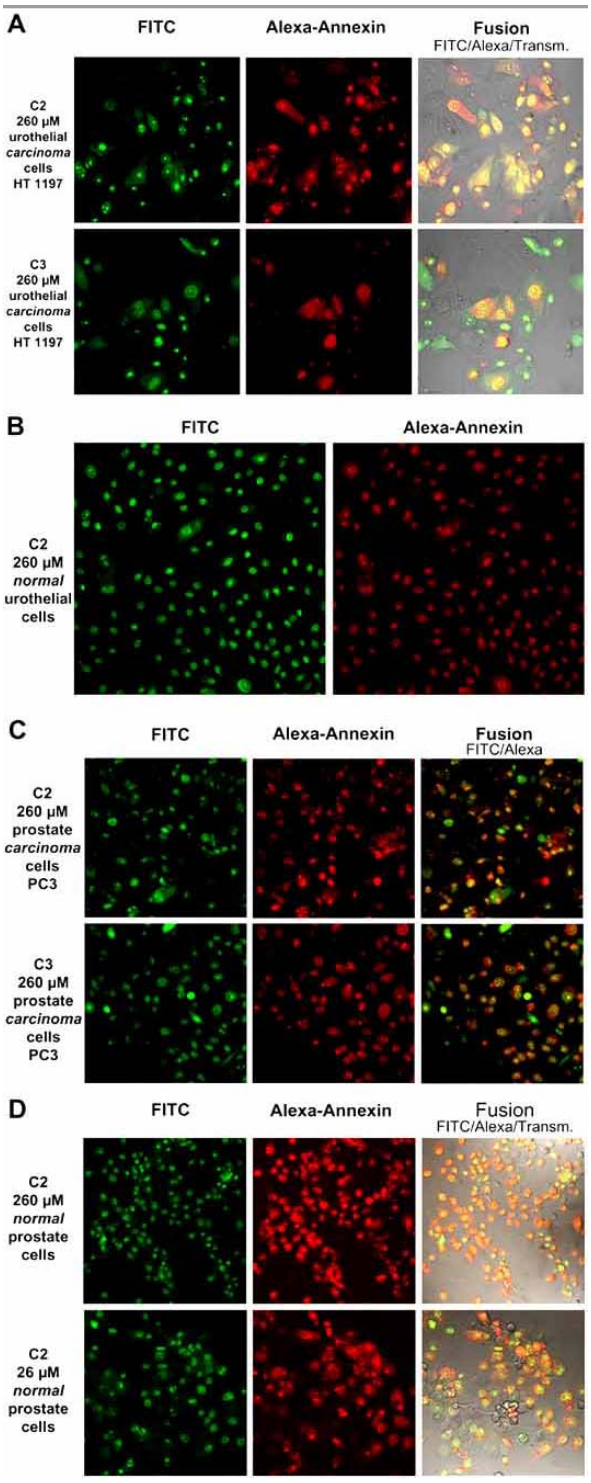

Fig. (2). Confocal laser scanning microscopy (CLSM) images of healthy and tumorous cells from the bladder and prostate.

A high percentage of nuclearly stained cells and binding of Annexin-V-Alexa^{TM} 568 Reagent was found after incubation with FITC-labelled conjugates 2 and 3 (26 or 260 µM).

To achieve this, the IAP-targeting SmacN7 peptide has been coupled to several different cationic peptides. First, the FITC-labelled HIV-tat transduction domain was used to accumulate Smac within the cytoplasm of U87 MG gliomas xenografted in the brains of nude mice [4]. Such HIV-tat-SmacN7 conjugates were found to enhance the antitumor activity of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) after local administration into the tumor. These conjugates were not toxic to healthy brain tissue. The coupling of Smac to the FITC-labelled Drosophila Antennapedia Penetratin sequence clearly enhanced the induction of apoptosis by several anticancer agents, including paclitaxel, etoposide, 7-ethyl-10-hydroxy-camptothecin (SN-38), and doxorubicin [3]. Finally, hexa- and octaarginines have also been used to transfer the Smac peptide into the cytoplasm, leading to reversal of the apoptosis resistance of human non-small cell lung cancer (NSCLC) *in vitro* and *in vivo* [5].

Octaarginines have been reported to be highly efficient in transporting a number of different compounds into the cytoplasm without influencing cell viability [10]. In our study, we also used eight arginines to transport the Smac peptide across the outer cell membrane. However, in contrast to Yang *et al*. [5], who used an FITC-labelled transport peptide containing 8 consecutive arginines flanked by three glycines at the N-terminus and glycine and cysteine at the C-terminus, we placed an FITC-labelled lysine residue at the center of an eight-arginine transport peptide, which led to an unexpected change in nuclear uptake and induction of cell death.

Surprisingly, the conjugate containing the transport peptide alone, without the Smac peptide, was specifically taken up by the nuclei of both tumor and healthy cells from the brain, bladder, and prostate and led to cell death.

Because of the marked potential of the transport peptide itself to induce cell death, even after an incubation period of just twenty minutes, it was not possible to discriminate between the effects of Smac coupled to the transport peptide (conjugate 3) and those of the transport peptide alone (conjugate 2).

A specific interaction of Smac with cytoplasmic IAPs seems to be rather unlikely because conjugate 3 (transport peptide coupled to Smac) accumulated rapidly within the cell nucleus but not the cytoplasm. Signs of cell death (loss of cell turgor and annexin staining) were already apparent after 20 minutes. By contrast, the octaarginine-containing Smac conjugate described by Yang *et al*. [5] did not lead to caspase activation in NSCLC until 36-48 hours after incubation.

It remains unclear why our transport peptide was taken up predominantly by the cell nuclei. One possible explanation might be that the two stretches of 4 arginines separated by the FITC-labelled lysine residue strongly resemble the ATK point mutagenesis of the bipartite nuclear localization sequence of the human T-Cell lymphotrophic virus type 1 Tof protein [11]. Passive diffusion could also be expected in the case of molecules of less than 10 kDa [12], such as our transport peptide. However, it seems that small conjugates do not automatically accumulate within the nuclear compartment (e.g. by diffusion), as exemplified by the small cytoplasmic transduction peptide fused to Smac described by

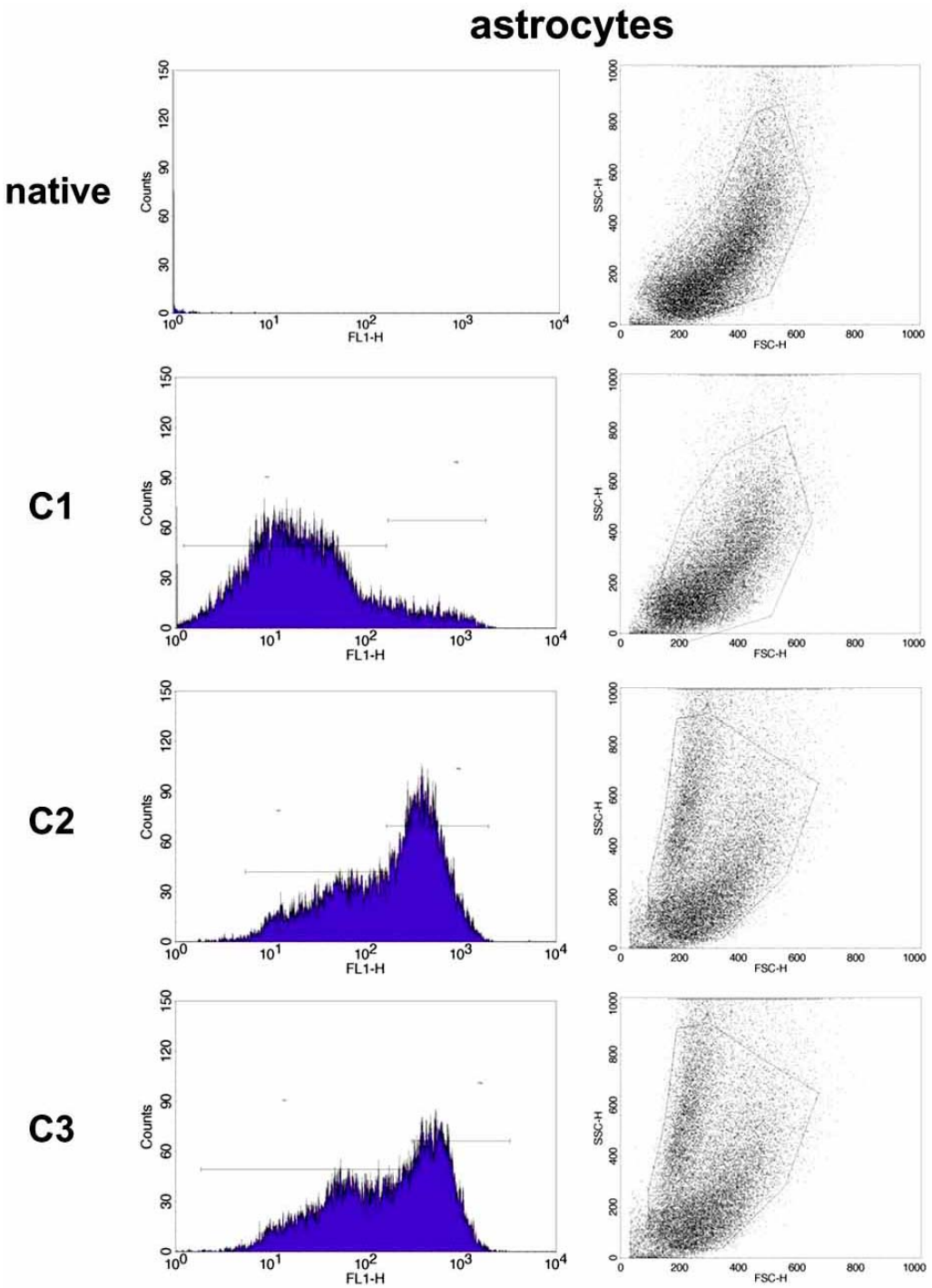

Fig. (3). FACS (fluorescence activated cell sorting) analysis of normal astrocytes after incubation with FITC-labelled conjugates 1-3. An obvious increase in strongly stained cells is seen after incubation with conjugates 2 or 3 (right-shifted histogram peak, left column). A slightly higher number of strongly cells was found after incubation with the transport peptide alone (conjugate 2) (260 μM). After incubation with conjugates 2 and 3 two cell populations could be distinguished on the basis of their morphology (two clouds of dots in the side scatter versus forward scatter analysis, right column).

Kim *et al*. [13] and the nonaarginine peptide described by Fuchs *et al*. [14].

We used fluorescence live cell microscopy in order to avoid fixation because, according to Richard *et al*. [15] and Fuchs *et al*. [10], even mild fixation may lead to an artefactual relocation of peptides like HIV-tat or nonaarginine within the cell.

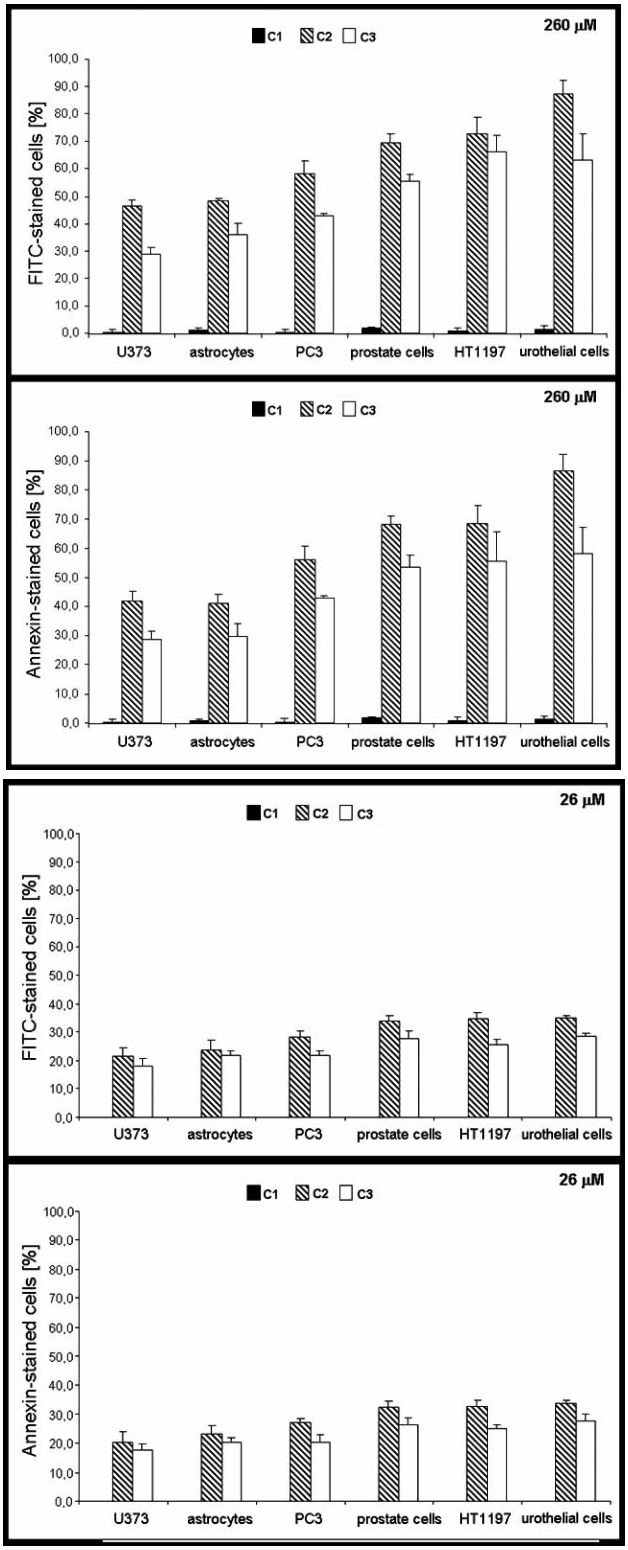

Fig. (4). Percentage of FITC- and Alexa-Annexin-stained human healthy and tumorous cells after incubation with conjugates C1-3 at two different concentrations (**A**, 260 µM; **B**, 26 µM). The highest amount of cells was stained after incubation with C2, followed by C 3 (**A,B**). C1 was only slightly taken up by the cells (**A,B**). The highest staining rate (C2, 260 µM) was found in healthy urothelial cells. FITC staining closely correlated with cell death (Annexin-staining).

The number of FITC and Alexa-Annexin-stained cells decreased when conjugates C1-C3 were applied at the lower concentration (**B**).

The examinations were performed three times. The standard deviation of the mean is depicted.

Surprisingly, despite the strong transmembrane and nuclear transport capacity of our transport peptide, some of the cells remained unstained, even at the higher concentration, indicating that these cells react differently towards the FITC-labelled conjugate. It is unclear whether the cellular uptake of such conjugates depends on the cell cycle stage, as has been shown for nuclear uptake [16], or on some other kind of cell heterogeneity. The composition of the cell membrane probably changes according to the cell cycle stage, or may even differ amongst the individual cells of a specific cell line. However, in our study, several different tumorous and healthy cell types were found to display the same uptake behavior.

The exact mechanism by which the FITC-labelled transport peptide C2 induces cell death remains to be further investigated.

In summary, we demonstrated that slight alterations within FITC-labelled arginine-containing transmembrane transport peptides may result in a completely different uptake pattern, which, with the transport peptide we used, was predominantly nuclear rather than cytoplasmic and was accompanied by cell death. Such transport peptides therefore need to be evaluated carefully before they are coupled to small apoptosis-inducing cargoes, such as Smac.

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FORMULAS OF CONJUGATES 1-3

Single-Letter Amino Acid Code

A = Alanine

V = Valine

P = Proline

I = Isoleucine

Q = Glutamine

K = Lysine

R = Arginine

β-A = ß-Alanine

FITC = Fluorescein isothiocyanate

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