Cell Nucleus Directed 2,3,5-triiodobenzoic Acid Conjugates

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Abstract: Triiodobenzoic acid (TIBA) represents the core structure of most clinically used contrast agents for computed tomography and other X-ray procedures. To construct an intracellular radiopaque contrast agent, TIBA was coupled to various different positively and negatively charged fluorescein iothiocyanate (FITC)-labelled peptides.

TIBA coupled to the SV40 T Antigen nuclear localization sequence (NLS) stained 80% of human glioma cells and caused cell death. This occurred with C- or N-terminal binding of TIBA and with the correct or mutant NLS.

No cell death and only small numbers of stained cells (below 3 %) were observed after incubation with NLS conjugates lacking TIBA or after incubation with TIBA-conjugates containing a negatively charged polyglutamic acid stretch.

TIBA-conjugates containing the Antennapedia-derived cell-penetrating peptide penetratin were only nuclearly taken up when TIBA and FITC were coupled to lysines outside the 16-amino acid peptide sequence.

The study shows that intracellular TIBA may have potential as a chemotherapeutic agent rather than a contrast agent.

Key Words: 2,3,5-triiodobenzoic acid, NLS, cell nucleus, FITC, transmembrane transport, glioma, penetratin, polyglutamic acid.

INTRODUCTION

Molecular imaging represents a rapidly expanding field in clinical research. Many novel target-specific contrast agents, e.g. against cell membrane receptors, have been designed for use with four main techniques: magnetic resonance imaging, near-infrared optical imaging, positron emission tomography and scintigraphy.

However computed tomography (CT) has not attracted so much interest in this respect, despite its widespread distribution (with access even in smaller hospitals) and the good resolution it offers, especially for investigation of the lungs [1].

In the present work we used triiodobenzoic acid ($C_7H_3O_2I_3$) (TIBA), the core structure of commonly used CT and other X-ray contrast agents (ultravist®, peritrast®, gastrografin®). TIBA has also previously been used in plant growth regulator studies as a synthetic inhibitor of polar auxin transport [2].

We first synthesized fluorescein isothiocyanate (FITC)-labelled conjugates in which TIBA is linked to the C-terminus of the correct or mutant nuclear localization sequence (NLS) of the SV 40 T antigen (Table **1**, Fig. (**1**)). TIBA was also coupled to the N-terminal part of the SV 40 T antigen NLS to elucidate whether nuclear uptake depends on the coupling site (C- or N-terminus).

Additionally, importance was attached to the question as to whether the net charge of the transport peptide is decisive for cytoplasmic uptake of TIBA and whether intracellular accumulation of TIBA results in cell death.

Therefore, both a highly negatively charged polyglutamic acid stretch and the positively charged Antennapedia-derived cell-penetrating peptide penetratin (16 amino acids) were each coupled to TIBA.

Table 1. Formulas of Conjugates 1-8

C1:	**PKKKRKVK(FITC)GGK**
C2:	**PKKTRKVK(FITC)GGK**
C3:	**PKKKRKVK(FITC)GGK(TIBA)**
C4:	**PKKTRKVK(FITC)GGK(TIBA)**
C5:	**P(TIBA)KKKRKVK(FITC)GGK**
C6:	**EEEEEEVK(FITC)GGK(TIBA)**
C7:	**RQIKIWFQNRRMK(FITC)WKK(TIBA)**
C8:	**RQIKIWFQNRRMKWKKK(FITC)GGK(TIBA)**

Single-Letter-Amino Acid Code:

K, lysine; R, arginine; P, proline; V, valine; G, glycine,E, glutamic acid; Q, glutamine; I, isoleucine; W, tryptophan; F, phenylalanine; N, asparagine; M, methionine

FITC, fluorescein isothiocyanate

TIBA, 2,3,5-triiodobenzoic acid

MATERIALS AND METHODS

Conjugate Synthesis

Conjugates C1-C8 were synthesized on a scale of 0.1 mmol/l by solid phase peptide synthesis on an Eppendorf ECOSYN P peptide synthesizer (Eppendorf-Biotronik, Ham-

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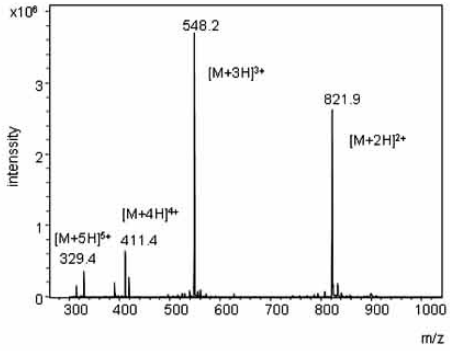
Conjugate 1

PKKKRKVK(FITC)GGK

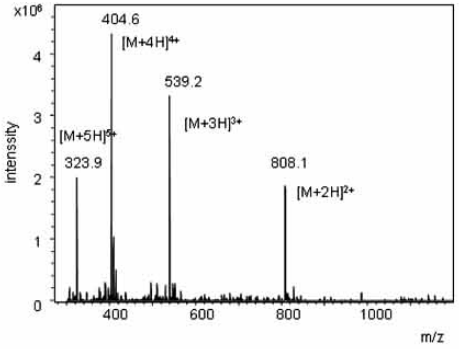
measured mass: 1641.8 Da
calculated mass: 1642.0 Da



Conjugate 2

PKKTRKVK(FITC)GGK

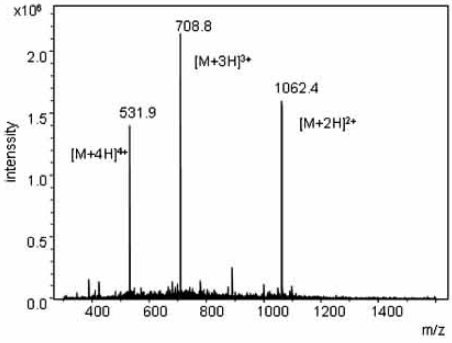
measured mass: 1614.6 Da
calculated mass: 1614.9 Da



Conjugate 3

PKKKRKVK(FITC)GGK(TIBA)

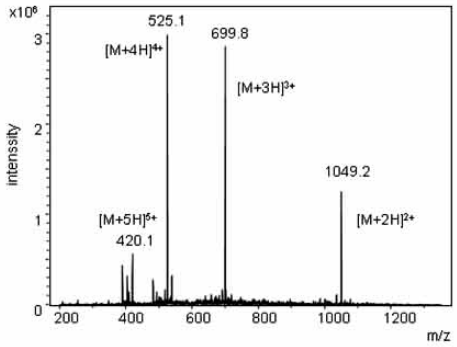
measured mass: 2123.4 Da
calculated mass: 2123.8 Da



Conjugate 4

PKKTRKVK(FITC)GGK(TIBA)

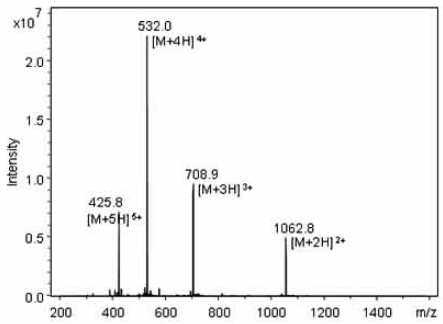
measured mass: 2096.3 Da
calculated mass: 2096.7 Da



Conjugate 5

P(TIBA)KKKRKVK(FITC)-GGK

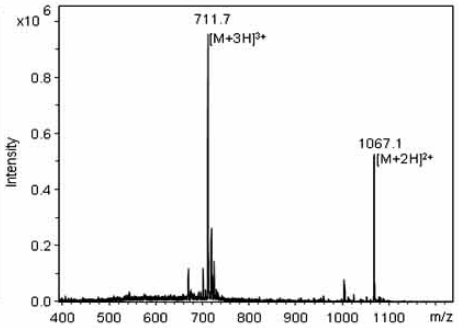
measured mass: 2123.69 Da
calculated mass: 2123.62 Da



Conjugate 6

EEEEEEVK(FITC)GGK(TIBA)

measured mass: 2132.20 Da
calculated mass: 2132.34 Da



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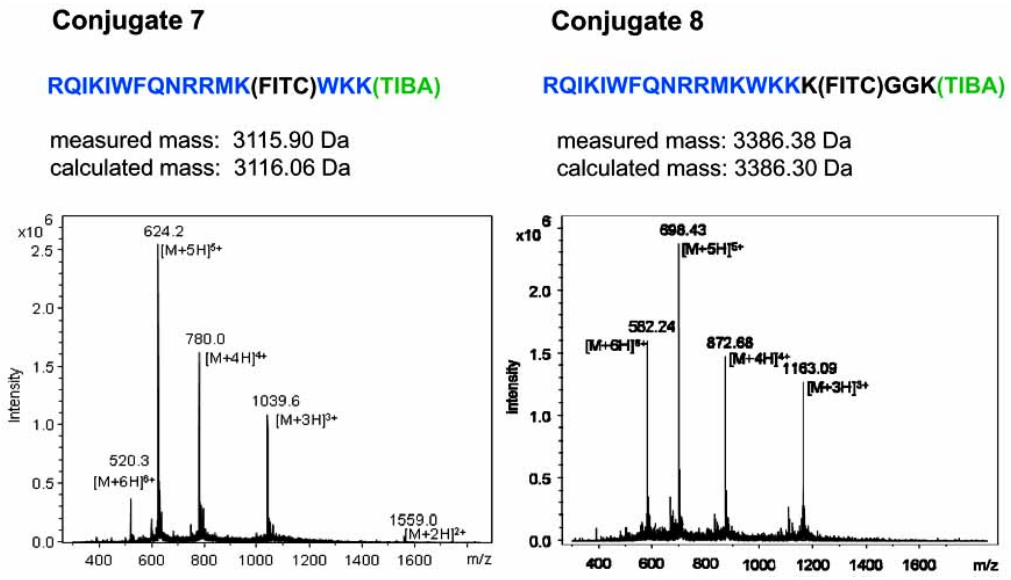


Fig. (1). Positive ion mode electrospray ionization (ESI) mass spectra of conjugates 1-8.

burg, Germany) employing the Fmoc strategy on Tentagel S rink amid resin (Rapp-Polymere, Tübingen, Germany). Triiodobenzoic acid was introduced as Fmoc Lys-N^{ε}-2,3,5-triiodobenzoyl during peptide synthesis. In the case of conjugate C5 the N-terminal proline was incorporated as its Fmoc-derivative. After removal of the N-terminal Fmoc group, the TIBA-moiety was coupled with 2-(1-H benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate as coupling agent.

Fmoc-Lys(2,3,5-triiodobenzoyl)-OH was prepared by activation of the 2,3,5,-triidobenzoic acid (TIBA) (Sigma-Aldrich, Taufkirchen, Germany) with the mixed anhydride method using isobutylchloroformiate (iBuOCOCI) (1 eq.) (Merck) and N-methylmorpholin (NMM) (1 eq.) (Fluka, Buchs, Switzerland).

Lysine side chains carrying a fluorescein were protected with Dde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-ethyl. After specific deprotection, the fluorescein isothiocyanate was coupled to the peptide using 0.5 mM with equal amounts of diisopropylethylamine (DIPEA) in DMSO (dimethylsulfoxide) at room temperature over night.

Substance purity (at least 97%) was verified by high performance liquid chromatography (HPLC). Molecular masses were confirmed by Electrospray ionisation mass spectrometry (ESI-MS) on an Esquire3000+ ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany).

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy was performed on an inverted LSM 510 laser scanning microscope (Carl Zeiss). Cell culture, conjugate incubation (260µM), Annexin-V-Alexa^{TM} 568- and propidium iodide-counterstaining and image evaluation (Image J, Wayne Rasband, NIH, Bethesda, MD, USA) were performed as previously described [3,4].

Flow Cytometry

Fluorescence activated cell sorting (FACS) was performed using a Becton Dickinson FACSCalibur. Sample preparation, measurement and evaluation were performed in accordance with Sturzu *et al*., 2008 [3].

RESULTS AND DISCUSSION

The TIBA-NLS-conjugates 3 and 4 containing the correct or mutant SV 40 T antigen NLS (Table **1**) both stained the nuclei of approximately 80% of human glioma cells and showed a comparably high cell death rate. By contrast, TIBA-free conjugates 1 and 2 also containing the correct and mutant NLS showed only minimal nuclear staining of fewer than 3%. Also, no necrotizing effect was observed after incubation with either TIBA-free conjugates 1 and 2 or coincubation with conjugates 1 and 2 and free unbound TIBA (Figs. (**2a**, **3**)).

The cellular and nuclear uptake rate and the cytotoxic potential of the triiodobenzoic acid conjugates was not higher than that of mono- and diiodobenzoic acid SV 40 T antigen NLS conjugates [4] and tribromphenyl isocyanate SV 40 T antigen NLS conjugates [5] used in previous studies.

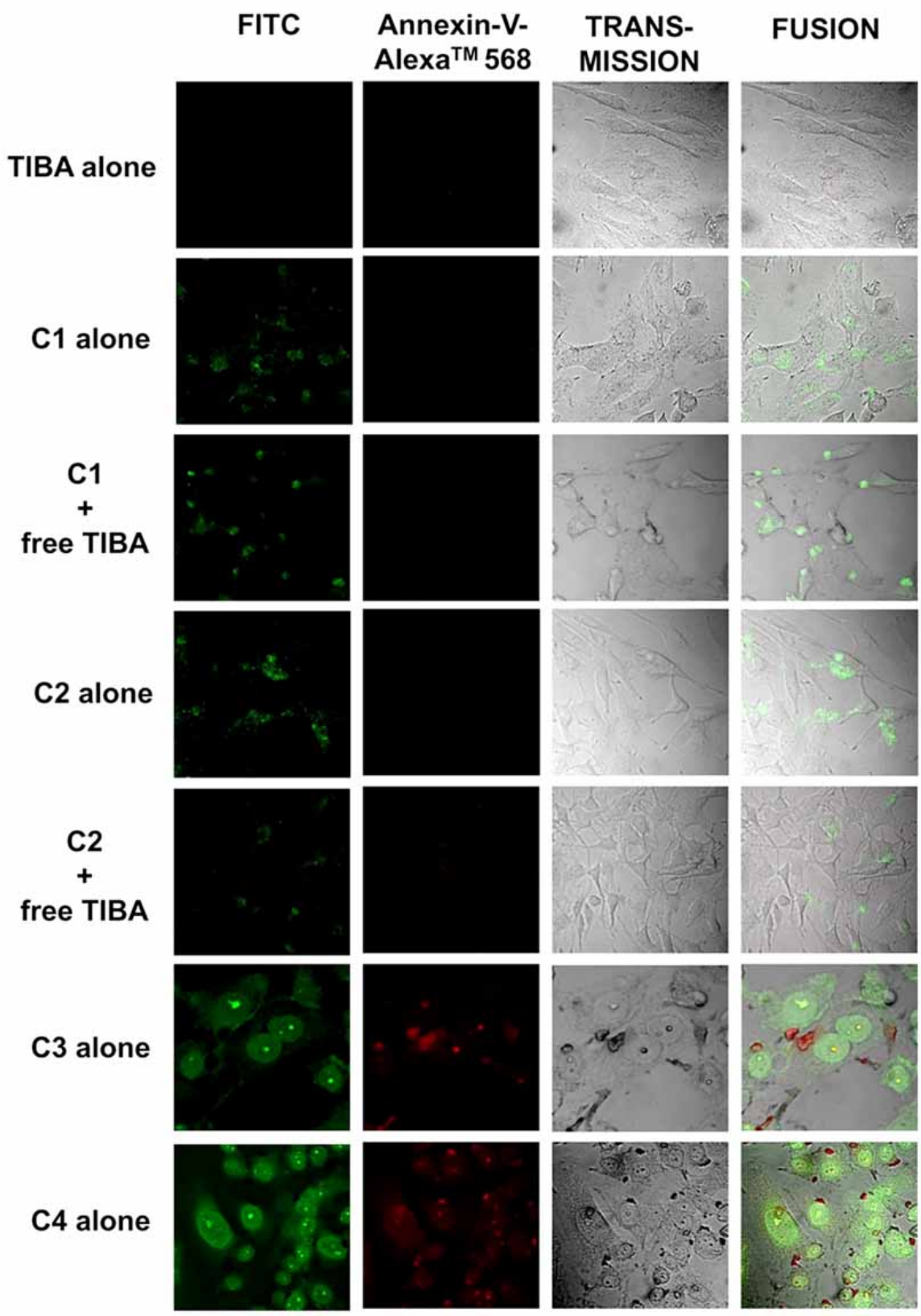
Nuclear staining resulting from nuclear receptor-independent passive diffusion could be expected in the case of molecules of less than 60 kDa [6], such as our TIBA-conjugate 4 containing a mutant nuclear localization sequence (PKKTRKV).

The nuclear uptake of conjugate 5 containing TIBA at the N-terminal end (TIBA coupled to proline) (Fig. **2c**) possibly also functions independently of the cytoplasmic nuclear receptors importin alfa and beta. It is known, that the recognition of the NLS of the SV 40 T antigen is hampered after modification of its amino acids [7].

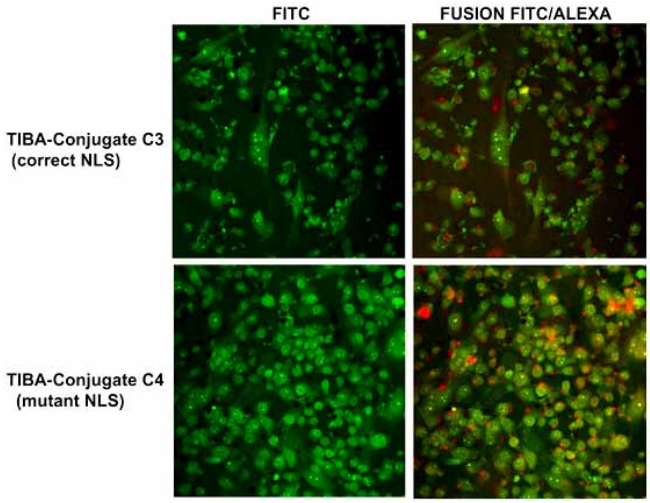
As expected, the hexaglutamic acid stretch could not mediate cellular uptake of TIBA (conjugate 6) (Fig. **2c**).

This is in accordance with a previously synthesized polyglutamic acid conjugate containing FITC and fullerene, which was not taken up by the cell [8].

a)


FITC
Annexin-V-Alexa™ 568
TRANS-MISSION
FUSION
TIBA alone
C1 alone
C1 + free TIBA
C2 alone
C2 + free TIBA
C3 alone
C4 alone

b)



c)

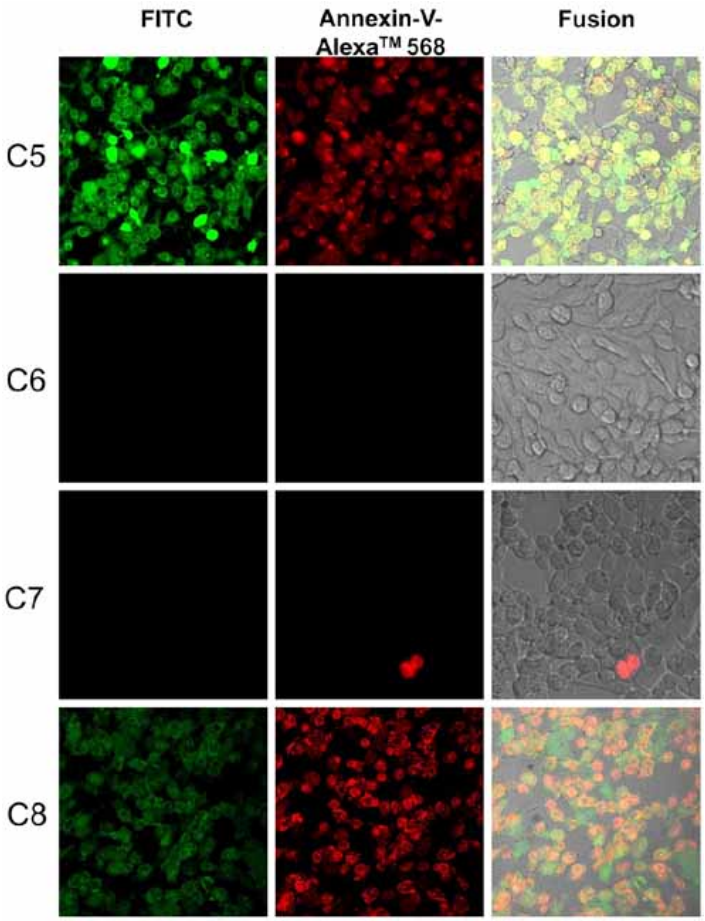


Fig. (2). a) Confocal laser scanning microscopy (CLSM) images of human malignant U373 glioma cells.

The Annexin-V-Alexa^{TM} 568 Reagent was used to detect nonviable cells.

Incubation with either TIBA alone or the non-TIBA-containing conjugates 1 [PKKKRKVK(FITC)GGK] and 2 [PKKTRKVK(FITC)GGK] alone (260 μM) did not result in Annexin-V-Alexa^{TM} 568 Reagent staining. Coincubation of TIBA with conjugates 1 and 2 (260 μM) also failed to result in Annexin-V-Alexa^{TM} 568 Reagent staining and was not associated with a higher cellular staining rate. However,

(Fig. 2 Contd….)

staining with Annexin-V-Alexa^{TM} 568 Reagent was found after incubation with the TIBA-containing conjugates 3 [PKKKRKVK(FITC)GGK(TIBA)] and 4 [PKKTRKVK(FITC)GGK(TIBA)] (260 μM).

No signs of cell death were observed after incubation with either PBS or 0.1% DMSO/PBS alone (CLSM images not shown).

b) Low power CLSM images of human malignant U373 glioma cells demonstrating that a very large number of cell nuclei has been stained by the FITC-labelled TIBA-containing conjugates 3 (row 1) and 4 (row 2) (260μM) (left column) (260 μM). Most of these cells were stained with Annexin-V-Alexa^{TM} 568 Reagent (red) (right column).

c) Confocal laser scanning microscopy (CLSM) images of human malignant U373 glioma cells.

A high percentage of cells were stained with FITC and PI after incubation with the FITC-labelled conjugates 5 (TIBA coupled to the N-terminus of the SV 40 T antigen NLS [P(TIBA)KKKRKVK(FITC)GGK] and 8 (TIBA coupled to lysines outside the penetratin sequence [RQIKIWFQNRRMKWKKK (FITC)GGK(TIBA)]) (260 μM). Incubation with either conjugate 6 [EEEEEEVK(FITC)GGK(TIBA)] or 7 [RQIKIWFQNRRMK(FITC)WKK(TIBA)] did not result in a marked PI or FITC staining (260 μM).

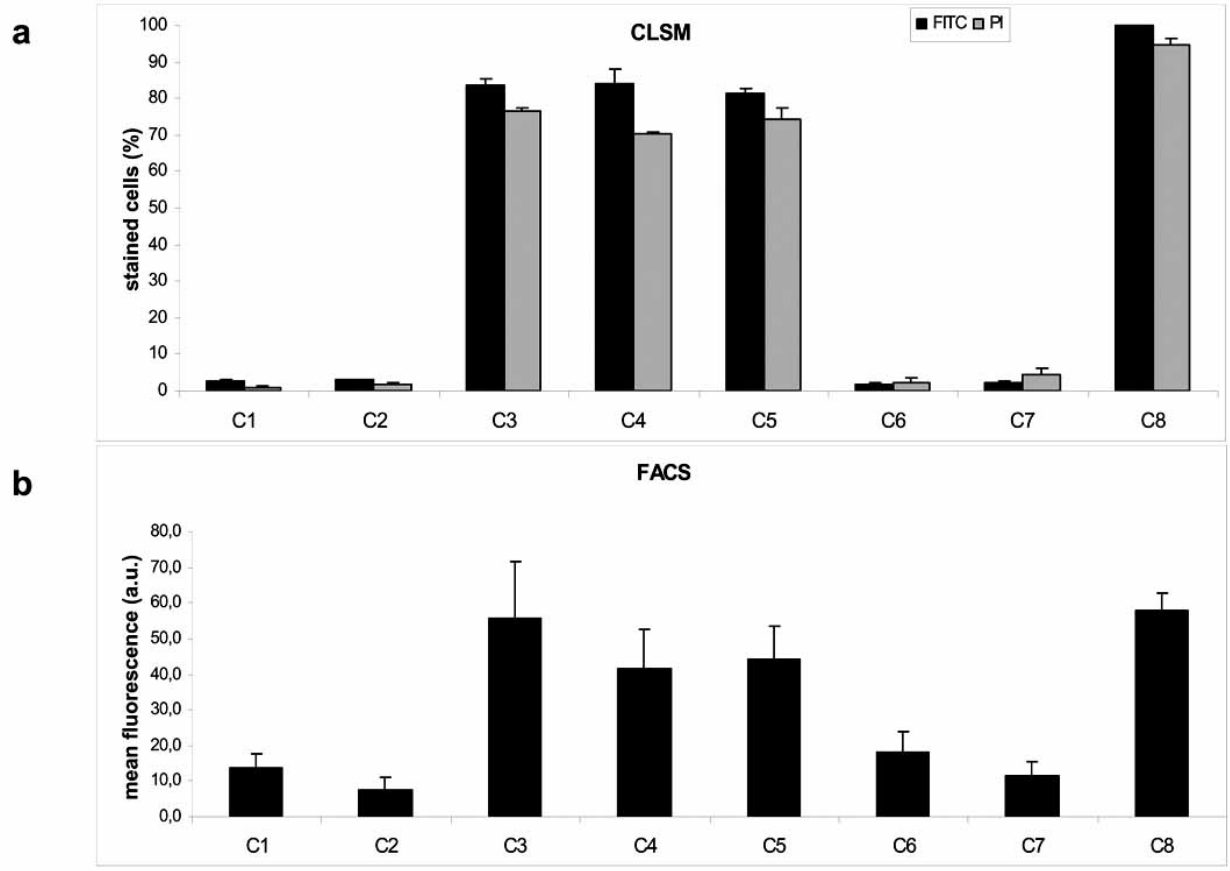


Fig. (3). a) Percentage of FITC- and PI-stained cells after incubation with the non-TIBA-containing NLS conjugates (1 and 2), the TIBA containing NLS conjugates (3-5) and the TIBA-containing hexaglutamic acid and penetratin conjugates (6-8) (260 μM).

Compared to the conjugates 1, 2, 6 and 7, the conjugates 3, 4, 5 and 8 were associated with a clearly higher number of FITC- and Propidium-Iodide (PI)-stained cells.

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

b) FACS (fluorescence activated cell sorting) analysis showing a low mean fluorescence intensity of cells after incubation with the non-TIBA-containing NLS conjugates (1 and 2) and the TIBA-containing hexaglutamic acid and penetratin conjugates (6 and 7) (260μM).

An up to fivefold increase in mean fluorescence intensity was observed after incubation with the TIBA-containing conjugates 3-5 and 8.

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

Cellular uptake of TIBA might also be mediated by another positively charged peptide lacking the NLS of the SV 40 T antigen with a comparable number of positively charged amino acids. Therefore, we synthesized conjugate 7 containing TIBA and penetratin, a 16-residue peptide derived from the Antennapedia homeodomain of Drosophila (residues 43-58) which previously successfully transported various substances through the outer cellular membrane [9].

It is thought that the positively charged amino acids of antennapedia interact with the negatively charged phosphol-

ipids of the cell membrane resulting in cellular uptake of the peptide [9].

Surprisingly, our FITC-labelled Penetratin-TIBA-conjugate 7 was not taken up by the U373 glioma cells (Fig. **2c**) even though its positive net charge is comparable to that of conjugate 3.

The three-dimensional structure of penetratin might have been altered by coupling FITC and TIBA to lysines within its C-terminal part.

By contrast conjugate 8 (TIBA and FITC coupled to lysines outside the penetratin sequence) was nuclearly taken up by a clearly higher amount of glioma cells which showed signs of cell death (Fig. **2c**).

The relatively high concentration (260µM) was chosen with respect to future X-ray studies (e.g. computed tomography). Furthermore, the TIBA-free controls (1 and 2) and the TIBA-conjugates 6 and 7 (no cellular uptake) did not cause cell death when used in the same concentrations. So cell death is clearly caused by conjugate uptake and not by the high conjugate concentration during the incubation.

In summary, we have shown that TIBA, when linked to the correct or mutant NLS of the SV 40 T antigen, can be imported into the cell nucleus. The coupling site of TIBA to the NLS (C- or N-terminal) is not decisive for cytoplasmic or nuclear uptake. Positively charged peptides, when coupled to TIBA, do not guarantee its cellular uptake as was shown by the TIBA-penetratin-conjugate. Due to their high cytotoxicity these intracellular TIBA conjugates may be more suitable for chemotherapy than for imaging applications.

Local administration into the tumor cavity after brain tumor surgery might be feasible with some of the TIBA conjugates described here. For systemic application, the TIBA conjugates have to be modified such that they are only taken up by tumor cells (e.g. *via* tumor-specific receptors or after activation by tumor specific enzymes).

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REFERENCES

[1] Walter, D.; De Man, B.; Iatrou, M.; Edic, P.M. Future generation CT imaging. *Thorac. Surg. Clin*., **2004**, *14*, 135-149.

[2] Geldner, N.; Friml, J.; Stierhof, Y.D.; Jurgens, G.; Palme, K. Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature*, **2001**, *413*, 425-428.

[3] Sturzu, A.; Regenbogen, M.; Klose, U.; Echner, H.; Gharabaghi, A.; Heckl, S. Novel dual labelled nucleus-directed conjugates containing correct and mutant nuclear localisation sequences. *Eur. J. Pharm. Sci*., **2008**, *33*, 207-216.

[4] Heckl, S.; Sturzu, A.; Regenbogen, M.; Beck, A.; Gharabaghi, A.; Echner, H. The differential influence of non-iodinated and mono- or diiodinated benzoic acids on cellular and nuclear uptake of the nuclear localization sequence of the SV 40 T antigen. *Int. J. Pharm*., **2008**, *355*, 131-140.

[5] Heckl, S.; Sturzu, A.; Gharabaghi, A.; Echner, H.; Nagele, T. The influence of 2,4,6-tribromophenyl isocyanate on the cellular and nuclear uptake of the SV 40 T antigen nuclear localization sequence. *Eur. J. Pharmacol*., **2008**, *583*, 32-36.

[6] Wei, X.; Henke, V.G.; Strübing, C.; Brown, E.B.; Clapham, D.E. Real-time imaging of nuclear permeation by EGFP in single intact cells. *Biophys. J.,* **2003**, *84*, 1317-1327.

[7] Watai, Y.; Sase, I.; Shiono, H.; Nakano, Y. Regulation of nuclear import by light-induced activation of caged nuclear localization signal in living cells. *FEBS Lett*., **2001**, *488*, 39-44.

[8] Yang, J.; Wang, K.; Driver, J.; Yang, J.; Barron, A.R. The use of fullerene substituted phenylalanine amino acid as a passport for peptides through cell membranes. *Org. Biomol. Chem*., **2007**, *5*, 260-266.

[9] Christiaens, B.; Grooten, J.; Reusens, M.; Joliot, A.; Goethals, M.; Vandekerckhove, J.; Prochiantz, A.; Rosseneu, M. Membrane interaction and cellular internalization of penetratin peptides. *Eur. J. Biochem*., **2004**, *271*, 1187-1197.