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Short communication

The influence of 2,4,6-tribromophenyl isocyanate on the cellular and nuclear uptake of the SV 40 T antigen nuclear localization sequence

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Abstract

The objective of the present study was to evaluate the influence of 2,4,6-tribromophenyl isocyanate (TBPI) on the cellular and nuclear uptake of the fluorescein isothiocyanate (FITC) labeled SV 40 T antigen nuclear localization sequence in human LN18 and U373 glioma cells. Therefore, the FITC-labeled nuclear localization sequence (NLS) of the SV 40 T antigen was coupled to 2,4,6-TBPI. This TBPI–NLS conjugate was taken up by the cell nuclei of more than 90% of human malignant glioma cells. The nuclearly stained cells showed clear signs of cell death. However only up to 10% of the cells were stained after incubation with the TBPI-lacking NLS of the SV 40 T antigen together with free, unbound TBPI. These cells stayed alive. TBPI, when bound to small peptides, may be an important component for future drugs against gliomas.

Keywords: Tribromophenyl isocyanate; NLS; Cancer therapy; Transmembrane transport; Glioma

1. Introduction

Nuclear localization sequences (NLS) are short cationic peptides which act like a nuclear address label on the surface of large cytoplasmic proteins which cannot pass through the small nuclear pores. The NLS will bind strongly to the cytoplasmic receptor importin, and together, the complex will move through the nuclear pore (Kalderon et al., 1984; Görlich et al., 1995).

Fluorescein isothiocyanate (FITC) labeled NLS peptides have not been coupled to 2,4,6-tribromophenyl isocyanate (TBPI) before now, and the therapeutic potential of TBPI as a cell nucleus-directed anti-cancer agent has not yet been evaluated.

Therefore, our conjugate contained both, the FITC-labeled NLS of the SV 40 T antigen and TBPI [*PKKKRKV*K(FITC)GGK (*TBPI*) (conjugate 2)].

In order to determine whether the presence of TBPI in the NLS conjugate influences cytoplasmic and nuclear uptake and cell viability, one further conjugate was synthesized without TBPI [*PKKKRKV*K(FITC)GGK (conjugate 1)].

Mild fixation may lead to artefactual relocation of peptides within the cell (Richard et al., 2003). Therefore, we performed fluorescence live cell microscopy to avoid fixation.

Importance was attached to the questions as to whether cellular and nuclear accumulation of the brominated NLS conjugates can be achieved without further coupling to positively charged transmembrane transport peptides and whether cell viability is influenced.

2. Materials and methods

2.1. Synthesis of conjugates

Synthesis was performed on an ECOSYN P solid phase peptide synthesizer (Eppendorf-Biotronik, Hamburg, Germany) using 9-fluorenylmethyloxycarbonyl (Fmoc) Rink amide TentaGel S RAM (0.25 mM/g) (Rapp Polymere, Tübingen, Germany). All amino

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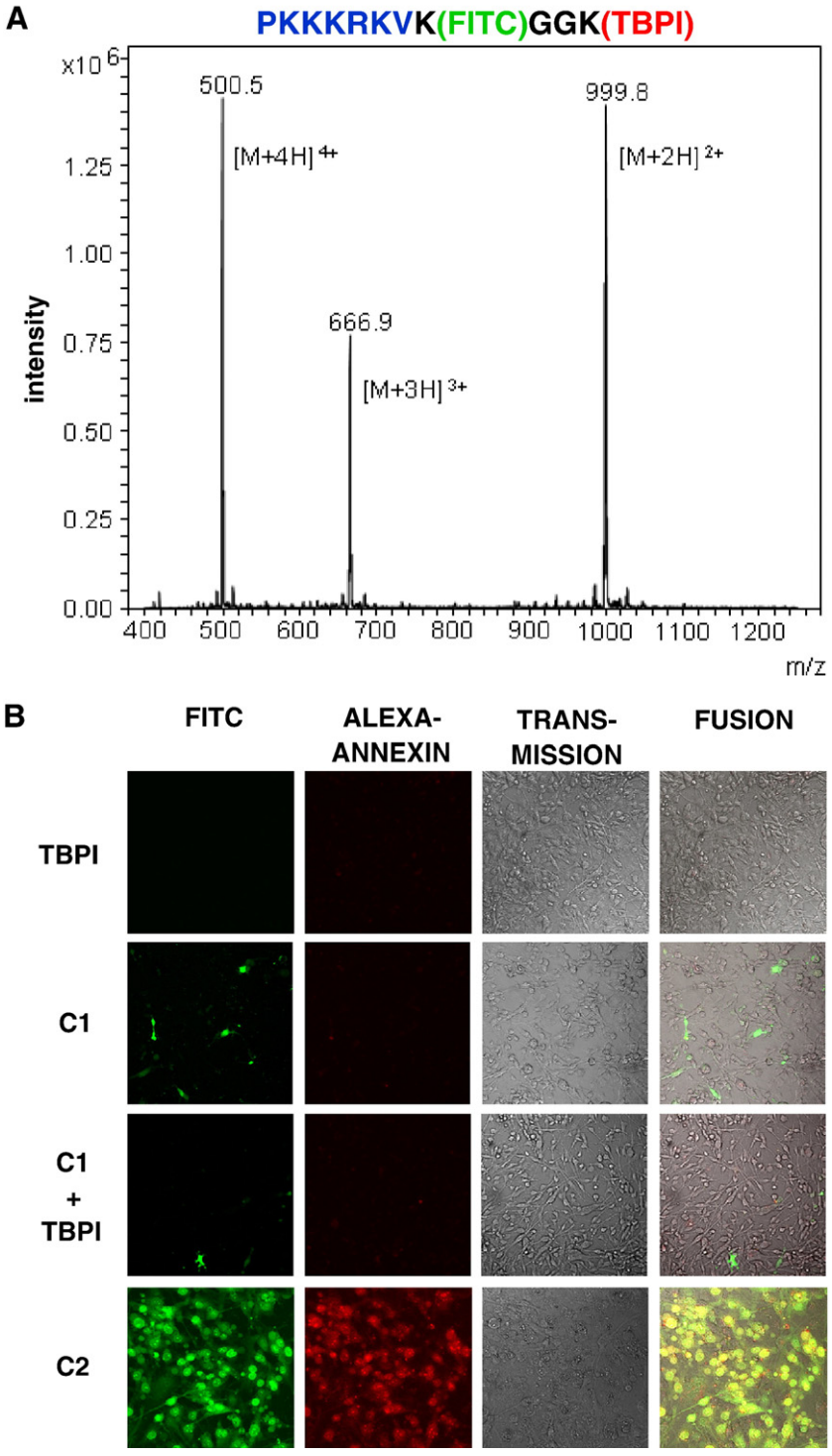
acids (0.1 mM per 0.4 g resin) except the N-terminal proline were incorporated with aminofunctions protected by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. The side chain functions were protected as *tert*-butyl (tBu) ethers (threonine), 2.2.4.6.7. pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) (arginine), *tert*-butyloxycarbonyl (Boc) (lysine, except lysine 8) or 4-methyltrityl (Mtt) (lysine-8) (Merck, Darmstadt, Germany).

Fmoc Lys (N^{ε}-2,4,6-tribromophenyl-ureido)-OH was prepared by coupling of N^{ε}-Fmoc Lys-OH with 2,4,6-tribromophenyl isocyanate (TBPI) (Sigma Aldrich, Taufkirchen, Germany) by activation with isobutylchloroformiate (iBuOCOCI) (1 eq.) and *N*-methylmorpholin (NMM) (1 eq.) (Fluka, Buchs, Switzerland) (mixed anhydride coupling). The substance was recrystalized from dimethylformamide (DMF)/diethylether (DEE) (Merck). All couplings were performed using a fourfold excess of amino acids and the coupling reagent 2-(1-*H* benzotriazol-1-yl)-1.1.3.3.-tetramethyluronium tetrafluoroborate (TBTU) (Merck)+diisopropylethylamine (DIEA) (Merck) (2 eq.) over the amount of



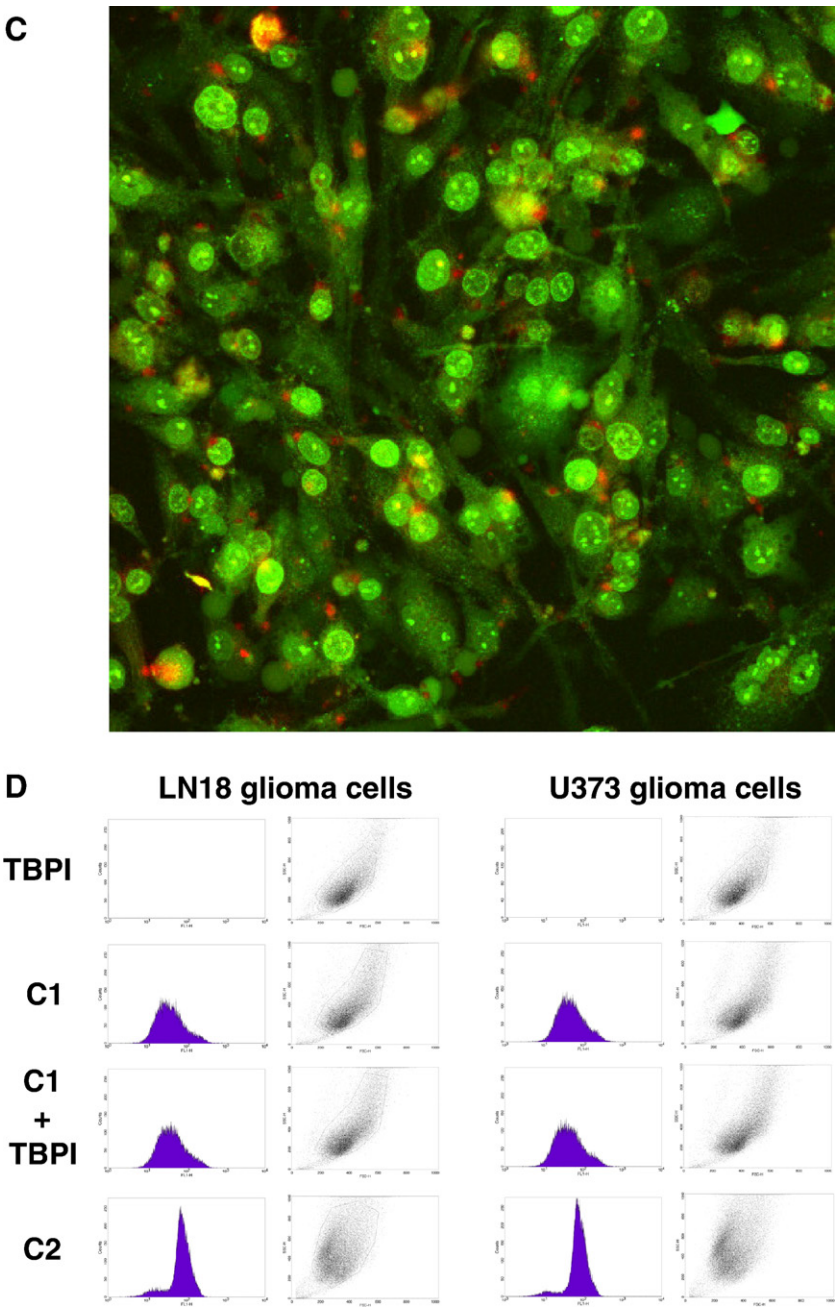


Fig. 1. A. Positive ion mode electrospray ionisation (ESI) mass spectra acquired from conjugate 2 [PKKKRKVK(FITC)GGK(TBPI)] with an average molecular mass of 1997.75 Da (calculated average mass: 1997.82 Da). Single-letter amino acid code: K, lysine; R, arginine; P, proline; V, valine; G, glycine. FITC, fluorescein isothiocyanate. TBPI, 2,4,6-tribromophenyl isocyanate ($Br_3C_6H_2NCO$). B. Confocal laser scanning microscopy (CLSM) images of human malignant LN18 glioma cells. The Annexin-V-Alexa™ 568 Reagent was used to detect phosphatidylserine in the outer membrane leaflet of necrotic or apoptotic cells. Incubation with either TBPI alone or the non-TBPI-containing conjugate 1 alone did not result in a marked binding of the Annexin-V-Alexa™ 568 Reagent to the surface of the LN18 glioma cells. Coincubation of TBPI with conjugate 1 also failed to result in binding of the Annexin-V-Alexa™ 568 Reagent to the surface of the LN18 glioma cells and was not associated with a higher cellular staining rate. However, a high percentage of nuclearly stained cells and binding of Annexin-V-Alexa™ 568 Reagent was found after incubation with the TBPI-containing conjugate 2. C. Low power CLSM image of human malignant LN18 glioma cells demonstrating that a very large number of cell nuclei has been stained by the FITC-labeled TBPI-containing conjugate 2 (260 μM). Most of these cells show expression of phosphatidylserine in the outer membrane leaflet and are therefore labeled by the Annexin-V-Alexa™ 568 Reagent (red dots). D. FACS (fluorescence activated cell sorting) analysis. An obvious increase in strongly stained cells to more than 90% (right-hand shift of histogram peak) is seen only after incubation with the TBPI-containing conjugate 2 (260 μM) (LN18 glioma cell line: C1: 9%; C1 + TBPI: 10%; C2: 90% and U373 glioma cell line: C1: 8%; C1 + TBPI: 9%; C2: 94%). Then, two morphologically distinct cell populations could be distinguished by their forward and side scatter characteristics. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resin. Before the coupling of the protected amino acids, the Fmoc groups were removed from the amino end of the growing fragment using 25% piperidine (PP) (Merck) in dimethylformamide (DMF). The FITC moieties were introduced in the lysine-8 residue with fluorescein-5(6)-isothiocyanate in dimethylsulfoxid (DMSO) (Sigma Aldrich) + *N*-methylmorpholin (NMM) (1 eq.)

(Merck) after removal of the 4-methyltrityl (Mtt) group from lysine-8 with trifluoroacetic acid (TFA) in dichloromethane (DCM) (1%) (Merck)+triisopropylsilane (TIS) (1%) (Fluka) for 1 h at room temperature. The N-terminal proline was incorporated as its Boc derivative. Simultaneous cleavage of the amino acid side chain protecting groups was performed by incubating the resin in a mixture of 12 ml trifluoroacetic acid (TFA) (Sigma Aldrich), 0.3 ml ethandithiol (EDT) (Sigma Aldrich), 0.3 ml anisole (Merck), 0.3 ml water and 0.1 ml triisopropylsilane (TIS) (Sigma Aldrich) for 2 h. The mixture was filtered and washed with TFA and the combined filtrates were precipitated with anhydrous diethylether (DEE).

The crude products were further purified by high performance liquid chromatography (HPLC) on a Nucleosil 100 C18 (7 μm) 250×10 column (Macherey & Nagel, Düren, Germany), elution being monitored at 214 nm (buffer A: 0.07% TFA/H_2O, buffer B: 80% acetonitrile (ACN) (Sigma Aldrich)/0.058% TFA/H_2O; 4 ml/min). The conjugates were assayed for purity (at least 98%) by analytical high performance liquid chromatography (HPLC) and electrospray ionisation mass spectrometry (ESI/MS).

2.2. Electrospray ionisation mass spectrometry (ESI-MS)

ESI-MS was acquired on an Esquire3000+ ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany) (positive ion mode) (Fig. 1A) [Dissolution of conjugates in 40% ACN, 0.1% formic acid (Sigma Aldrich) in water (v/v/v) (20 pmol/μl), infusion via a syringe pump (5 μl/min flow rate), dry gas (6 l/min) temperature: 325 °C, nebulizer: 20.0 psi, electrospray voltage: −3700 V].

2.3. Confocal laser scanning microscopy (CLSM) and viability test

Human malignant LN18 and U373 glioma cells were grown to 70% confluency in RPMI-1640 Ready Mix Medium containing L-glutamine and 10% fetal bovine serum (FBS)-Gold (PAA laboratories, Pasching, Austria) at 37 °C, 5% CO_2 (vol/vol), in 4-well plates (NUNC, Wiesbaden, Germany) with about 300,000 cells per well.

Cells were incubated at 37 °C in an atmosphere of 5% CO_2 for 20 min with each of the conjugates dissolved in Dulbecco's phosphate buffered saline (PBS) (D-PBS; Gibco Invitrogen, Karlsruhe, Germany) at 260 μM. Cells were also coincubated with the non-TBPI-containing conjugate 1 and free TBPI (260 μM). For controls, cells were incubated with PBS or TBPI (260 μM in PBS) alone.

After this, the cells were rinsed three times with buffer and then incubated with Ready Mix Medium again. Then, phosphatidylserine was detected in the outer membrane leaflet of apoptotic cells by using the Annexin-V-Alexa™ 568 Reagent according to the manufacturer's protocol (Roche Molecular Biochemicals, Indianapolis, USA). Confocal laser scanning microscopy was performed on an inverted LSM510 laser scanning microscope (Carl Zeiss, Jena, Germany) (objectives: LD Achroplan 40×0.6, Plan Neofluar 20×0.50, 40×0.75). For fluorescence excitation, the 488 nm line of an argon ion laser and the 534 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.

2.4. Flow cytometry

For FACS, human U373 and LN18 glioma cells were grown in 75 cm^2 culture flasks (Corning Costar, Bodenheim Germany) (70% confluency) under the same conditions as described under confocal laser scanning microscopy. Accutase™ (PAA laboratories, Pasching, Austria) was added to achieve detachment of the cells, which were harvested and subsequently aliquoted into Eppendorf tubes (Eppendorf, Hamburg, Germany) (6×10^6 cells per tube). The cells in the first tube served as a control (PBS only). The cells in the other four tubes were incubated with TBPI alone (260 μM), conjugate 1 alone (260 μM), conjugate 1 plus TBPI (260 μM), and conjugate 2 alone (260 μM) (20 min).

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 20,000 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.

2.5. Semi-thin sections

A portion of the cells stained for FACS analysis was fixed in paraformaldehyde with 2% agar, dehydrated in ethanol, embedded in Lowicryl K4M (Polysciences, Eppelheim, Germany) and UV-polymerized at room temperature according to the manufacturer's instructions. Semi-thin sections (about 0.4 μm) were cut and evaluated with the fluorescence microscope.

3. Results

We produced two FITC-labeled NLS conjugates: the NLS of the SV 40 T antigen without TBPI [conjugate 1: PKKKRKVK (FITC)GGK] and with TBPI [conjugate 2: PKKKRKVK(FITC) GGK(TBPI)]. Molecular weights were determined by electrospray ionisation mass spectrometry (ESI-MS) [conjugate 1 (calculated monoisotopic mass: 1641.90 Da, measured monoisotopic mass: 1641.80 Da); conjugate 2 (calculated average mass: 1997.82 Da, measured average mass: 1997.75 Da)] (Fig. 1A).

Prior to the incubation with the probes, LN18 and U373 glioma cells showed no significant autofluorescence (confocal laser scanning microscopy).

After incubation with unbound TBPI both cell lines showed no signs of cell death and no increase of fluorescence compared to the control.

Only a few cells were nuclearly stained after incubation with the FITC-labeled NLS alone (conjugate 1) or after coincubation

with the FITC-labeled NLS conjugate and the unbound TBPI (260 μM) (no more than 10%) (Fig. 1B,D). These cells stayed alive (Fig. 1B). It was not possible to distinguish stained and non-stained cells based on their morphology (Fig. 1D).

A very marked increase in the proportion of strongly stained cells to more than 90% was observed after incubation with the TBPI-containing conjugate (Fig. 1B–D). This was associated with a 90% cell death rate (binding of Annexin-V-Alexa™ 568 Reagent to phosphatidylserine in the outer membrane leaflet) (Fig. 1B,C) and two morphologically distinct cell populations could be distinguished by their forward and side scatter characteristics (Fig. 1D). Intracellular staining (especially nucleoli) was confirmed by the examination of semi-thin sections (about 0.4 μm) of the incubated cells.

4. Discussion

NLS peptides are used mainly as a vehicle to transport non-peptide substances from the cytoplasm into the cell nucleus. For example the DNA intercalating pyrene (Haefliger et al., 2005), cobaltocenium (Noor et al., 2005), carboplatin analogues (Aronov et al., 2004), gold particles (Feldherr et al., 1992), chlorin e_6 (Akhlynina et al., 1997), trastuzumab (anti-HER2/neu monoclonal antibody) (Costantini et al., 2007) and deoxyribonucleic acid (DNA) (Zanta et al., 1999) have been transported across the nuclear membrane using the NLS of the SV 40 T antigen.

By using a caged NLS of the SV 40 T antigen it was even possible to regulate nuclear translocation of an NLS–albumin conjugate in dependence of ultraviolet light (Watai et al., 2001).

However little interest seems to have been attached to the question as to whether there are any compounds that could optimize the cellular and nuclear staining rate of the SV 40 T antigen NLS.

After incubation with the TBPI-lacking NLS-peptide (conjugate 1) only a few cells (up to 9%) were nuclearly stained (Fig. 1B,D). These cells showed no signs of cell death (Fig. 1B). After coincubation with the FITC-labeled NLS of the SV 40 T antigen (conjugate 1) and free, unbound TBPI no further increase of nuclearly stained cells could be achieved (Fig. 1B,D).

However, the coupling of TBPI to the NLS resulted in a marked increase in the proportion of strongly stained cells to more than 90% (Fig. 1B–D), and was associated with a high cell death rate (90%), as indicated by the binding of Annexin-V-Alexa™ 568 Reagent (Fig. 1B,C).

Incubation with free, unbound TBPI did not result in cell death in both glioma cell lines, either alone or in conjunction with the non-TBPI-containing conjugate. Therefore cell death cannot be attributed to a destructive effect of TBPI on the outer cellular membrane.

Further studies will show whether the same effects can be obtained by using similar conjugates which contain only one or two bromo atoms. Additionally it should be evaluated whether cellular and nuclear uptake of TBPI can also be achieved by using mutant nuclear localization sequences or other small peptides which do not contain any NLS.

In summary, the TBPI–NLS conjugate with its cell death inducing properties may serve as an important building block for future non-radioactive anti-cancer drugs.

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