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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

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Abstract

We synthesized several novel compounds to evaluate the different effects of non-iodinated and mono- or diiodinated benzoic acid on the cellular and nuclear uptake of the SV 40 T antigen nuclear localization sequence (NLS) in human LN18 and U373 glioma cells. The skeletal structure of all the conjugates contained the fluorescein isothiocyanate (FITC)-labeled NLS of the SV 40 T antigen, to which either benzoic acid, mono- or diiodobenzoic acid was coupled.

As shown by confocal laser scanning microscopy (CLSM) and fluorescence-activated cell sorting (FACS), the basic FITC-labeled NLS alone was taken up by the nuclei of only a few glioma cells which remained intact.

The coupling of non-iodinated benzoic acid (BA) did not result in a markedly larger number of nuclearly stained cells.

A very marked increase in cells with nuclear staining was found with the conjugate containing monoiodobenzoic acid (MIBA). This was also associated with a high cell death rate. Similar results were obtained with the conjugate containing diiodobenzoic acid (DIBA). However, coincubation with free mono- or diiodobenzoic acid and the basic FITC-labeled NLS did not result in a marked change in the number of strongly stained cells or cell viability compared to the results of incubation with the FITC-labeled NLS alone.

Surprisingly, FITC-labeled MIBA- and DIBA-conjugates containing a scrambled SV 40 T antigen NLS were also taken up by the cell nuclei of LN18 and U373 glioma cells and led to cell death.

Such mono- or diiodobenzoic acid conjugates may therefore have potential in the development of new non-radioactive drugs against malignant glioma cells.

Keywords: Benzoic acid; 4-Monoiodobenzoic acid; 2,5-Diiodobenzoic acid; Cell nucleus; Cancer therapy; Transmembrane transport

1. Introduction

Radiolabeled iodobenzoic acids, coupled to antibodies or receptor peptide ligands, are widely used to image and to destruct targeted cells (Vaidyanathan et al., 2004, 2006; Collingridge et al., 2003).

However, the therapeutic potential of non-radioactive mono- and diiodobenzoic acids as cell nucleus-directed anti-cancer agents has not yet been evaluated.

Until now, non-iodinated benzoic acid (BA), monoiodobenzoic acid (MIBA), or diiodobenzoic acid (DIBA) have not been coupled to nuclear localization sequence (NLS) peptides.

We therefore designed conjugates comprised of non-iodinated BA, MIBA, or DIBA and the NLS of the SV 40 T antigen.

NLS are short cationic peptides which mediate nuclear translocation of large cytoplasmic proteins which cannot pass

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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 (Nakielny and Dreyfuss, 1999).

To determine intracellular localization by confocal laser scanning microscopy (CLSM), fluorescein isothiocyanate (FITC) was coupled to all the conjugates.

Investigations were undertaken to determine whether cellular and nuclear accumulation of the MIBA– or DIBA–NLS conjugates can be achieved and whether the iodine atoms within the conjugate structure are decisive for the cellular and nuclear uptake process and for cell death. Sequence-specific nuclear uptake was examined by using MIBA- and DIBA-conjugates containing a scrambled SV 40 T antigen NLS.

2. Materials and methods

2.1. Peptide synthesis

Seven conjugates (Fig. 1) were synthesized on an Eppendorf ECOSYN P solid-phase peptide synthesizer (Eppendorf-Biotronik, Hamburg, Germany) employing 9-fluorenylmethyloxycarbonyl (Fmoc) Rink amide TentaGel S RAM (0.25 mM/g) (Rapp Polymere, Tübingen, Germany). All amino acids (0.1 mM per 0.4 g resin) except the N-terminal pro-

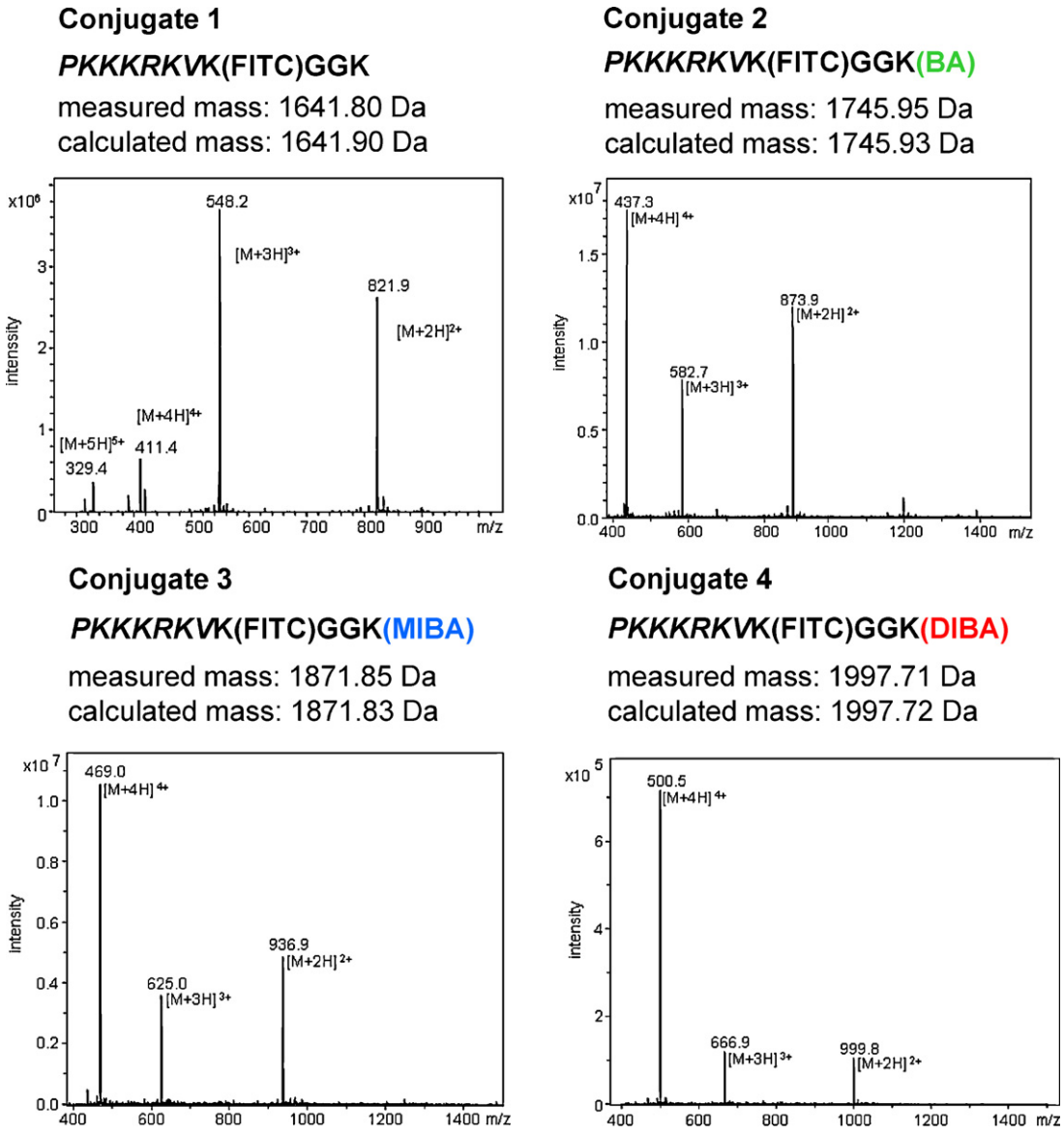

Fig. 1. Electrospray ionisation (ESI) mass spectra acquired from conjugates 1–7 (positive ion mode). Monoisotopic molecular masses of 1641.80 (C1), 1745.95 (C2), 1871.85 (C3), 1997.71 (C4), 1642.00 (C5), 1871.81 (C6) and 1997.75 (C7) Da could be detected by the formation of: double-charged molecular ions ($[M+2H]^{2+}$, *m/z* 821.9 (C1), 873.9 (C2), 936.9 (C3), 999.8 (C4), 821.6 (C5), 936.4 (C6), 999.9 (C7)), triple-charged molecular ions ($[M+3H]^{3+}$, *m/z* 548.2 (C1), 582.7 (C2), 625.0 (C3), 666.9 (C4), 548.1 (C5), 624.9 (C6), 666.9 (C7)), quadruple-charged molecular ions ($[M+4H]^{4+}$, *m/z* 411.4 (C1), 437.3 (C2), 469.0 (C3), 500.5 (C4), 411.5 (C5), 469.2 (C6), 500.6 (C7)) and quintuple-charged molecular ions ($[M+5H]^{5+}$, *m/z* 329.4 (C1)). Single letter amino acid code: K, lysine; R, arginine; P, proline; V, valine; G, glycine; FITC, fluorescein isothiocyanate; BA, benzoic acid; MIBA, 4-monoiodobenzoic acid; DIBA, 2,5-diiodobenzoic acid.

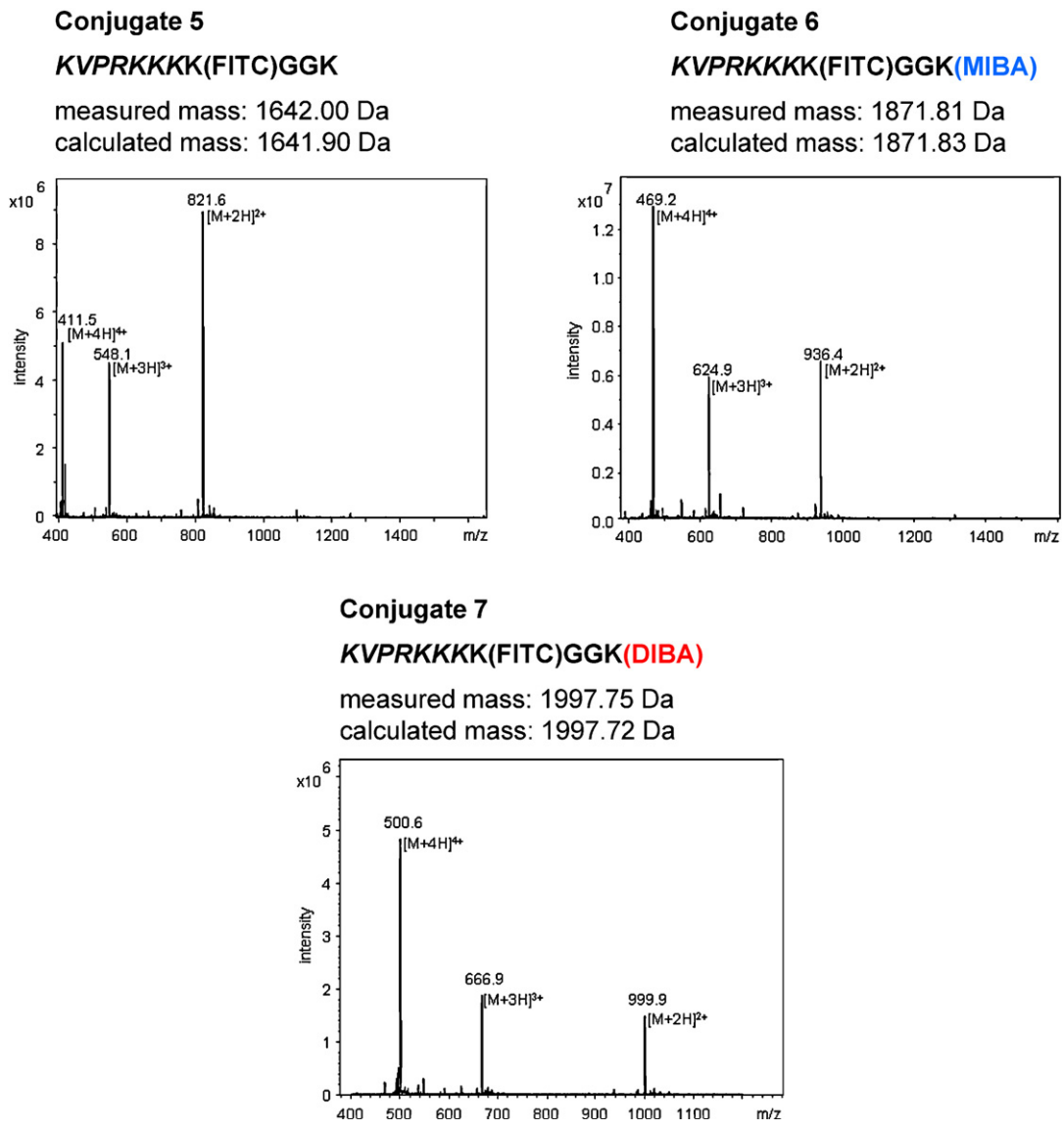

Fig. 1. (*Continued*)

line were incorporated with aminofunctions protected by the 9-fluorenylmethyloxycarbonyl group. The side-chain functions were protected as 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}-benzoyl), Fmoc Lys (N^{ε}-4-monoiodobenzoyl) and Fmoc Lys (N^{ε}-2,5-diiodobenzoyl) were prepared by coupling of N^{ε}-Fmoc Lys–OH with benzoic acid, 4-monoiodobenzoic acid and 2,5-diiodobenzoic acid (Sigma–Aldrich, Taufkirchen, Germany) by activation with isobutylchloroformiate (iBuOCOCl) (1 equiv.) (Merck) and *N*-methylmorpholin (NMM) (1 equiv.) (Fluka, Buchs, Switzerland) (mixed anhydride coupling). The substance was recrystallized 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 equiv.) over the amount of 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. The FITC moieties were introduced in the lysine-8 residue with fluorescein-5(6)-isothiocyanate in dimethylsulfoxide (DMSO) (Sigma–Aldrich) + *N*-methylmorpholin (1 equiv.) (Merck) after removal of the 4-methyltrityl 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 (Sigma–Aldrich), 0.3 ml ethandithiol (EDT) (Sigma–Aldrich), 0.3 ml anisole (Merck), 0.3 ml water and 0.1 ml triisopropylsilane (Sigma–Aldrich) for 2 h. The mixture was filtered and washed with TFA and

the combined filtrates were precipitated with anhydrous diethylether.

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_20; buffer B: 80% acetonitrile (ACN) (Sigma–Aldrich)/0.058% TFA/H_20; 4 ml/min).

The conjugates were assayed for purity by analytical high performance liquid chromatography and electrospray ionisation mass spectrometry (ESI/MS). Substance purity was at least 97%.

2.2. Electrospray ionisation mass spectrometry (ESI–MS)

Conjugates 1–7 were analyzed by ESI–MS on an Esquire3000+ ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany) (Fig. 1).

Conjugates were dissolved in 40% ACN, 0.1% formic acid (Sigma–Aldrich) 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.

2.3. Confocal laser scanning microscopy and annexin V binding assay

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 (v/v), 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 1–7 dissolved in Dulbecco's phosphate-buffered saline (PBS) (D-PBS; Gibco; Invitrogen, Karlsruhe, Germany) at 260 μM.

Cells were also coincubated with either BA, MIBA or DIBA and conjugates 1 (correct NLS) or 5 (scrambled NLS) at 260 μM. For controls, cells were incubated with PBS or either BA, MIBA or DIBA (260 μM in PBS) alone.

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^{TM} 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 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 at least three times 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, 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 staining ratios were acquired by counting PI stained and non-stained cells of image sections containing approximately 300 cells each.

Three independent incubations were evaluated for all staining ratios.

2.4. Flow cytometry

For FACS, human LN18 and U373 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^{TM} (PAA laboratories, Pasching, Austria) was added to achieve detachment of the cells, which were harvested and subsequently aliquoted into Eppendorf tubes (Eppendorf, Hamburg, Germany) (1×10^6 cells per tube). The cells in the first tube served as a control (PBS only). The cells in the other tubes were incubated for 20 min with BA, MIBA and DIBA alone (260 μM), conjugates 1 and 5 alone (260 μM), conjugate 1 plus either BA, MIBA or DIBA (260 μM), conjugate 5 plus either MIBA or DIBA (260 μM) and conjugates 2, 3, 4, 6 and 7 alone (260 μM).

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. For FACS evaluation the mean FITC fluorescence intensity for each conjugate was acquired using the WinMDI software (Joseph Trotter, Scripps Research Institute, USA). All investigations were performed in triplicate and statistically evaluated.

3. Results

Seven FITC-labeled conjugates were synthesized: the correct NLS of the SV 40 T antigen alone (conjugate 1), the correct NLS of the SV 40 T antigen with non-iodinated benzoic acid (conjugate 2), 4-monoiodobenzoic acid (conjugate 3), or 2,5-diiodobenzoic acid (conjugate 4), the scrambled NLS of the SV 40 T antigen alone (conjugate 5) and the scrambled NLS of the SV 40 T antigen with 4-monoiodobenzoic acid (conjugate 6), or 2,5-diiodobenzoic acid (conjugate 7) (Fig. 1).

Significant autofluorescence of human malignant LN18 and U373 glioma cells was excluded by confocal laser scanning microscopy.

Incubation with either benzoic acid, 4-monoiodobenzoic acid, or 2,5-diiodobenzoic acid alone did not appear to pro-

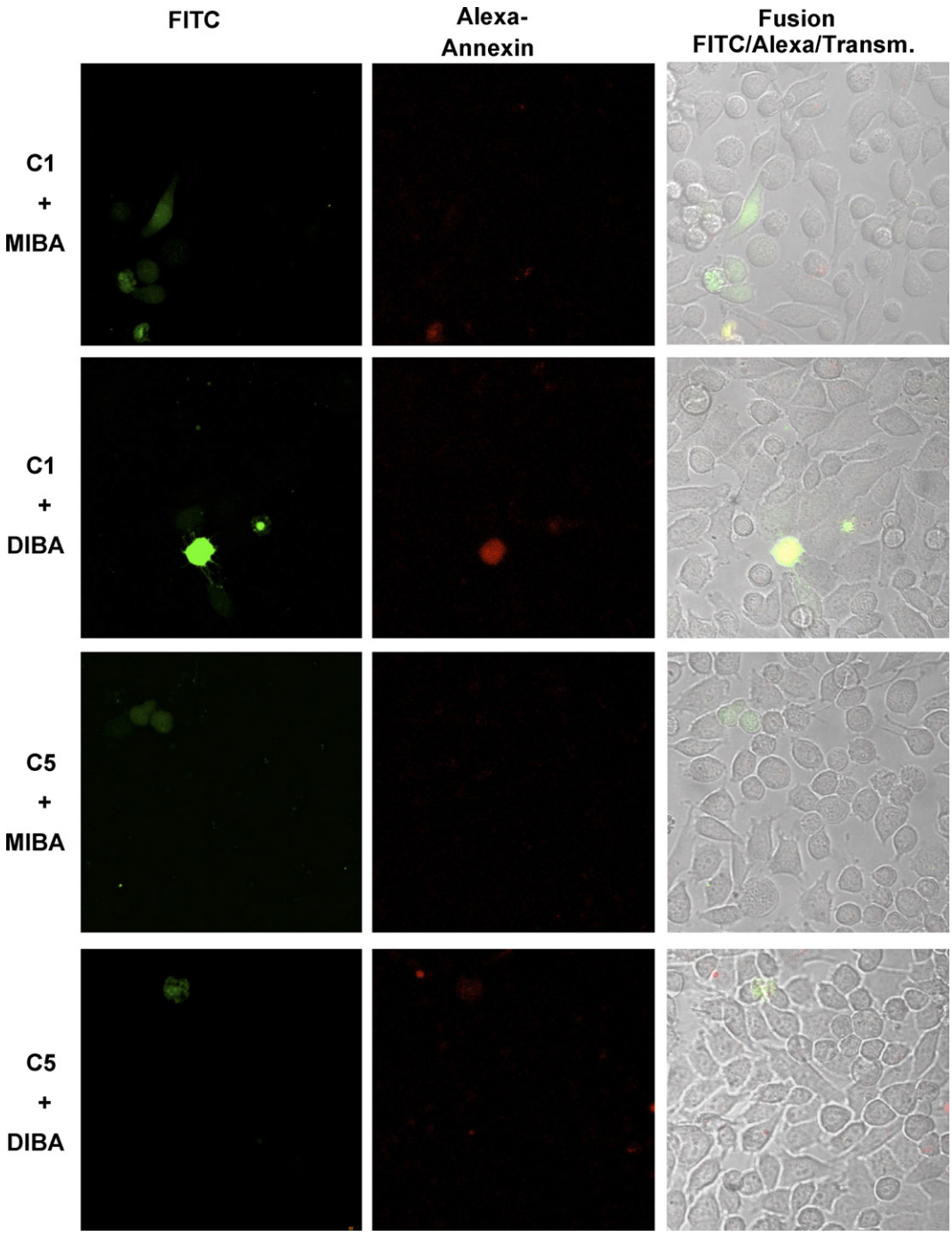

Fig. 2. Confocal laser scanning microscopy images of human malignant LN18 glioma cells. After coincubation of either MIBA or DIBA with the FITC-labeled MIBA- and DIBA-free conjugates C1 or C5 only a small number of FITC-stained cells was observed. Only a few cells were dead [binding of the Annexin-V-Alexa^{TM} 568 reagent (red) to the surface]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

duce any cytotoxic effects at a concentration of 260 μM. No cell death, and only a small number of FITC-stained cells was observed after incubation with conjugate 1, which lacked BA, MIBA and DIBA (Figs. 3A and 4).

Coincubation with conjugate 1 and either BA, MIBA or DIBA (260 μM) did not result in a marked change in the number of strongly FITC-stained cells or cell viability compared to the results of incubation with the FITC-labeled NLS alone (Figs. 2 and 4).

Conjugate 2 also stained only a small number of cells (comparable to the number stained by conjugate 1). The amount of dead cells increased slightly (binding of Annexin-V-Alexa^{TM} 568 reagent to phosphatidylserine in the outer membrane leaflet) (Figs. 3A and 4). A very marked increase in the proportion of strongly FITC-stained cells was observed after incubation with conjugate 3 containing MIBA (Figs. 3A and 4). This was associated with a high cell death rate (Figs. 3A and 4). The addition of a further iodine atom to the conjugate structure (conjugate 4)

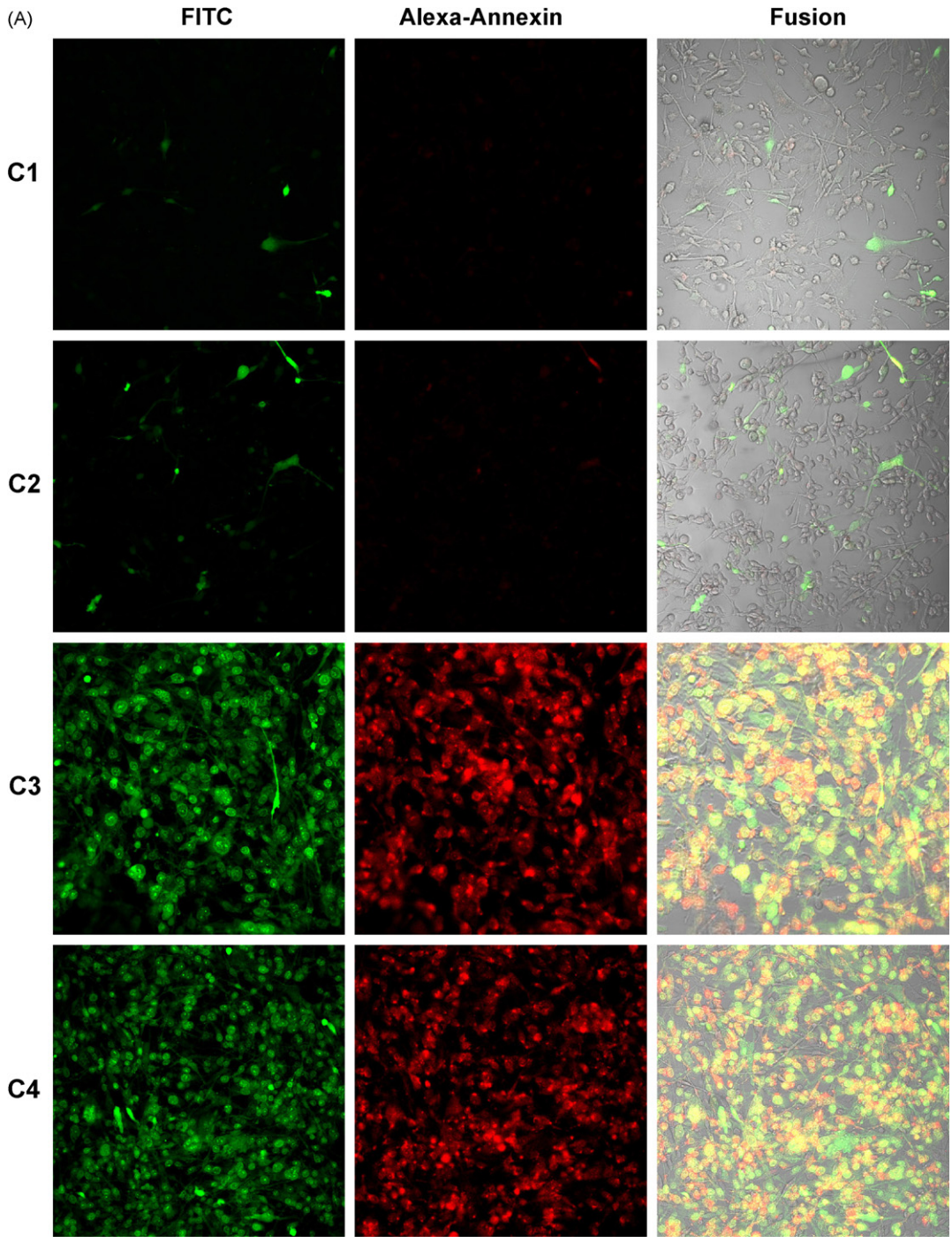

Fig. 3. Confocal laser scanning microscopy images of human malignant LN18 glioma cells. (A) Only a few cells were nuclearly stained after incubation with the iodine-free conjugates 1 and 2. Most of the cells remained intact and showed no marked binding of the Annexin-V-Alexa^{TM} 568 reagent (red) to the surface of the LN18 glioma cells. By contrast, a high percentage of nuclearly stained cells and binding of Annexin-V-Alexa^{TM} 568 reagent was found after incubation with the MIBA- or DIBA-containing conjugates 3 and 4. (B) The MIBA- and DIBA-containing scrambled NLS conjugates 6 and 7 stained a higher amount of cell nuclei compared to the MIBA and DIBA lacking scrambled NLS conjugate 5. Nuclear staining was associated with cell death [binding of the Annexin-V-Alexa^{TM} 568 reagent (red) to the surface of the LN18 glioma cells]. (C) Close-up images of single cells showing the cytoplasmic and nuclear localization of conjugates C3, C4, C6 and C7. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

did not increase the number of stained cells over that stained by conjugate 3, containing only one iodine atom (Figs. 3A and 4).

Only a small number of FITC- and Annexin-stained cells was observed after incubation with the scrambled NLS conjugate 5, which lacked MIBA and DIBA (Figs. 3B and 4).

Coincubation with the FITC-labeled scrambled NLS (conjugate 5) and either MIBA or DIBA (260 μM) did not result in a marked change in the number of FITC- and Annexin-stained cells compared to the results of incubation with the FITC-labeled scrambled NLS alone (Figs. 2, 3B and 4).

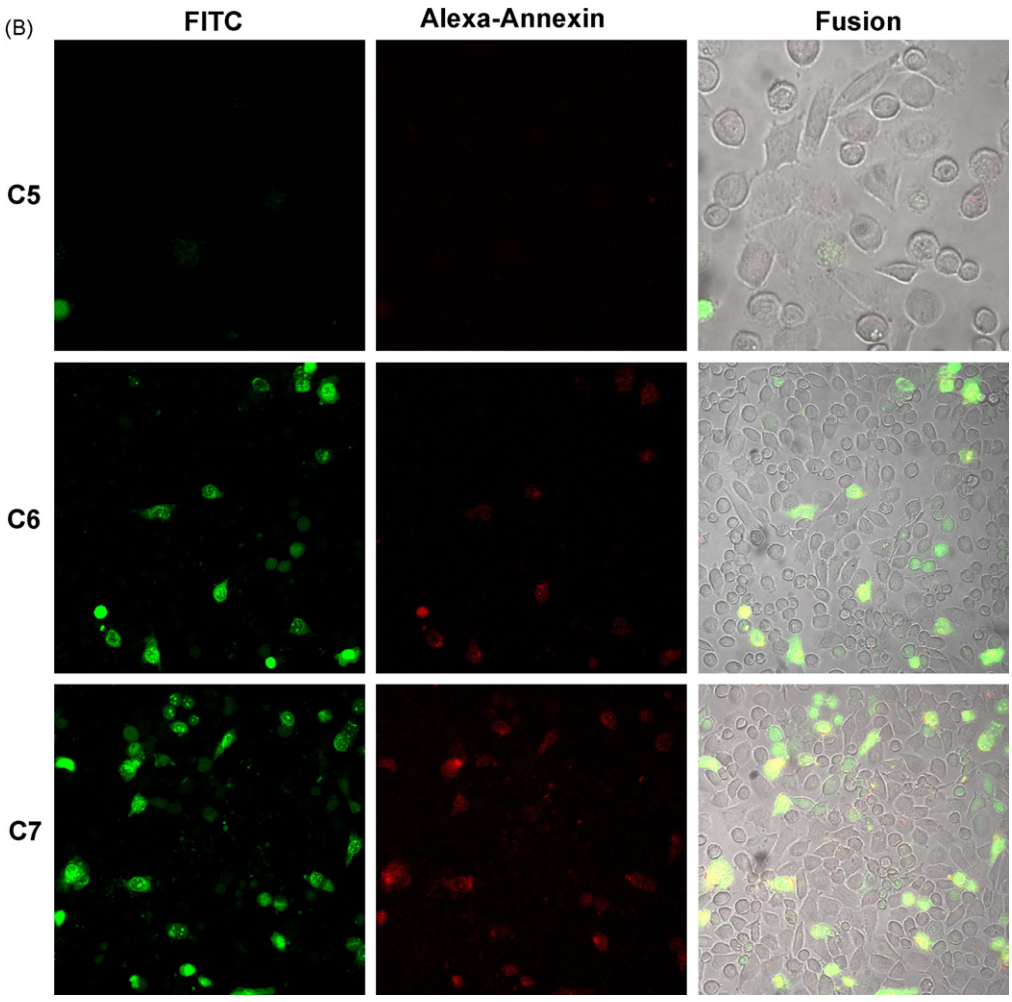

Fig. 3. (*Continued*)

By contrast, MIBA- and DIBA-conjugates 6 and 7 with the scrambled NLS were nuclearly taken up by a clearly higher amount of cells (approximately half of the amount of cells which were nuclearly stained by the MIBA- and DIBA-conjugates 3 and 4 with the correct SV 40 T antigen NLS) (Fig. 4). Conjugates 6 and 7 also led to cell death (Figs. 3B and 4).

4. Discussion

The cellular uptake of radioactive iodine (e.g. I131) is strongly dependent on the human sodium iodine symporter (hNIS), which is mainly expressed on the surface of thyroid cells. Treatment with this substance is therefore restricted to thyroid carcinoma. In an attempt to extend the use of radioiodine therapy to other tumors, the hNIS gene has even been transferred into prostate cancer and glioma cells via an adenovirus (La Perle et al., 2002; Cho et al., 2002).

We chose the NLS of the SV 40 T antigen as a vehicle to transport non-iodinated and mono- or di-iodinated BA from the cell culture medium into the cell nucleus independent of the hNIS, peptide receptor ligands, and antibodies. Several other compounds [e.g. 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 successfully across the nuclear membrane using the NLS of the SV 40 T antigen. 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 this NLS.

In this study we found that conjugates comprised of this NLS and non-radioactive mono- or di-iodobenzoic acids were taken up by the nuclei of a very large portion of LN18 and U373 glioma cells (Figs. 3 and 4). By contrast, few cells were stained by the FITC-labeled NLS alone (conjugate 1) or coupled to non-iodinated benzoic acid (conjugate 2) (Figs. 2–4). The iodine atoms within the conjugate structure therefore seem to play a decisive role in the marked augmentation of the cellular and nuclear uptake process, which is followed by cell death. The replacement of a second water atom in the NLS monoiodobenzoyl-conjugate with an iodine atom (conjugate 4) did not result in a further increase in the number of stained cells (Figs. 3 and 4). It remains unclear how the iodine atom within the conjugate structure interacts with the cellular and nuclear membranes. However, coincubation with uncoupled BA, MIBA, or

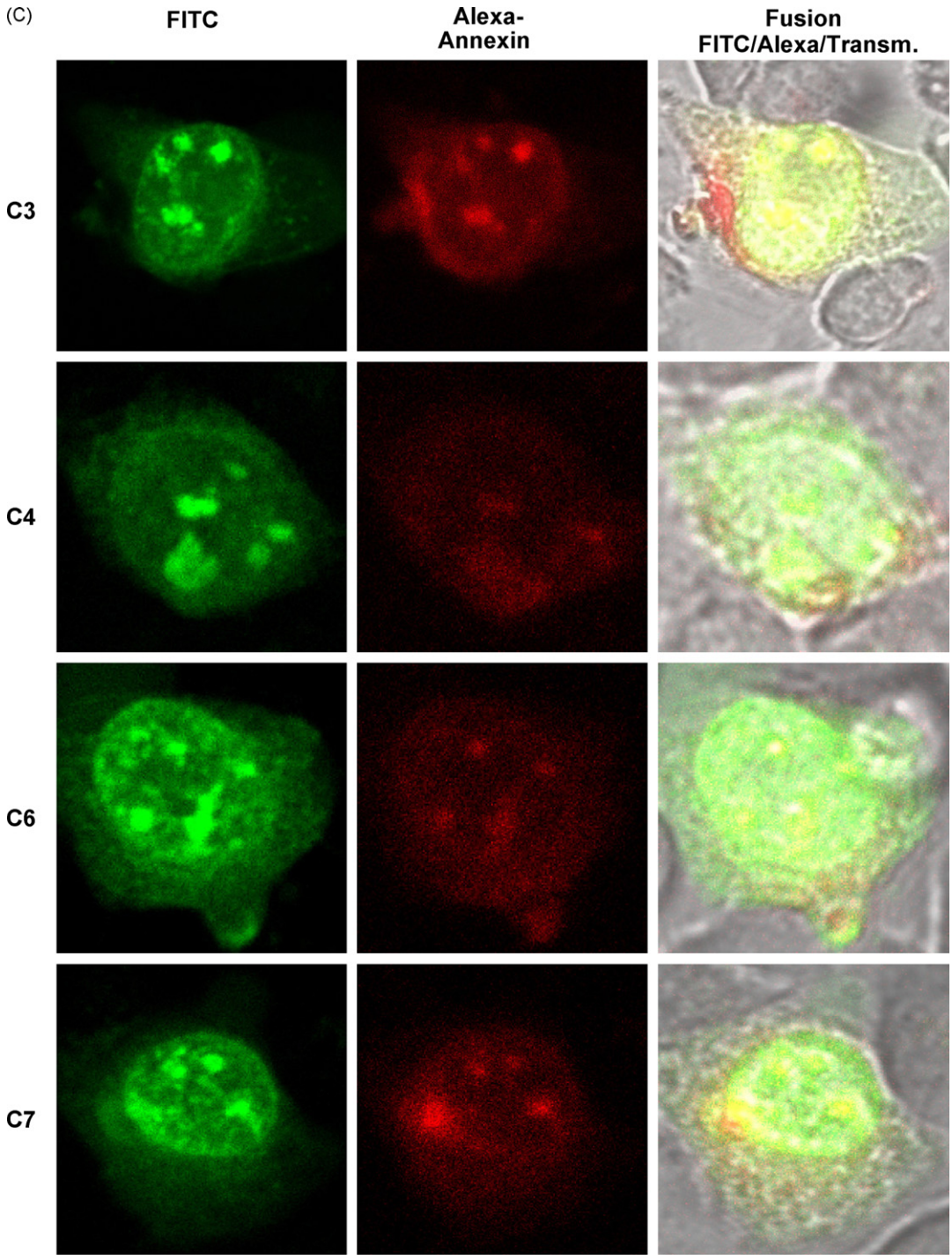

Fig. 3. (*Continued*)

DIBA and the non-iodinated NLS conjugate 1 did not result in an increase in stained cells compared to incubation with conjugate 1 alone (Fig. 2). Additionally, the few stained cells showed no signs of cell death (Fig. 2), indicating that the coupling of MIBA or DIBA to the NLS peptide is mainly responsible for the observed effects. An unpredictable effect of the FITC-labeled NLS peptide on the electron system of the coupled but not uncoupled MIBA or DIBA could be responsible for the better cellular and nuclear uptake.

In our experiments, we were also able to achieve cytoplasmic and nuclear staining with the MIBA– and DIBA–NLS conjugates containing the scrambled NLS of the SV 40 T antigen (conjugates 6 and 7) (Fig. 3B) at a concentration of 260 μM. However, the scrambled MIBA– and DIBA–NLS conjugates stained only about half of the amount of glioma cells that were stained by the correct MIBA– and DIBA–NLS conjugates indicating that the glioma cells react differently towards the correct and scrambled NLS conjugates (Figs. 3B and 4).

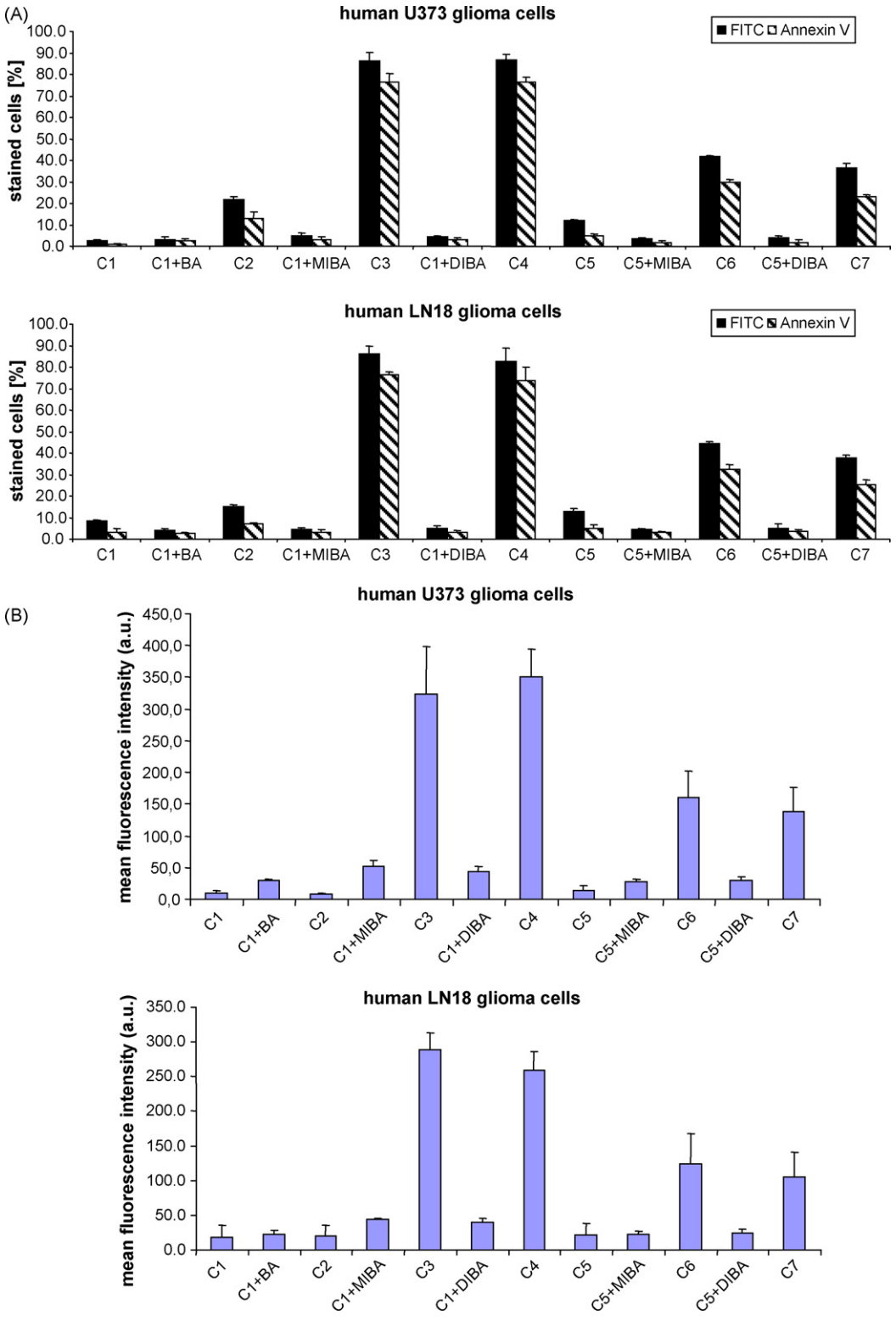

Fig. 4. Percentage of FITC- and PI-stained human U373 and LN18 glioma cells after single incubation with conjugates C1-7 and after coincubation (C1 with BA, MIBA and DIBA; C5 with MIBA and DIBA). A remarkable increase in nuclearly stained cells was only observed after incubation with the MIBA and DIBA containing correct (C3 and C4) and scrambled (C6 and C7) NLS conjugates. This was associated with cell death. The MIBA and DIBA containing scrambled NLS conjugates C6 and C7 resulted in fewer nuclearly stained cells than the MIBA and DIBA conjugates with the correct NLS (C3 and C4). The examinations were performed three times. The standard deviation of the mean is depicted. (B) FACS analyses of human LN18 and U373 glioma cells showing low mean fluorescence signal intensity after single incubation with conjugates C1, C2 and C5. Coincubation of C1 (with BA, MIBA, DIBA) and C5 (with MIBA, DIBA) did not result in a remarkable increase in mean fluorescence signal intensity. An obvious increase in mean fluorescence signal intensity was only observed after incubation with the MIBA and DIBA containing correct (C3 and C4) and scrambled (C6 and C7) NLS conjugates. Among these, the MIBA and DIBA conjugates with the correct NLS led to a higher increase of mean fluorescence intensity than those with the scrambled NLS. Fluorescence intensity was depicted in arbitrary units. The absolute fluorescence intensity values depend from measurement settings and can vary. This led to different scales for the two glioma cell lines shown (U373: 450 a.u; LN18: 350 a.u.).

Nuclear staining resulting from nuclear receptor-independent passive diffusion could be expected in the case of molecules of less than 60 kDa (Wei et al., 2003), such as our conjugates 6 and 7 with the scrambled NLS. However, it seems that small conjugates do not automatically accumulate within the nuclear compartment (e.g. by diffusion) as exemplified by small SV 40 T antigen NLS conjugates containing the DNA intercalating pyrene (Haefliger et al., 2005), cobaltocenium (Noor et al., 2005), deoxyribonucleic acid (Zanta et al., 1999) or carboplatin analogues (Aronov et al., 2004). No nuclear uptake was found when a mutant NLS was used or when the correct NLS was omitted.

Further studies will be needed to determine whether comparable cellular and nuclear uptake results can be obtained by coupling MIBA or DIBA to other peptides (e.g. polyarginine) or whether the effect is most apparent with the correct and scrambled SV 40 T antigen NLS conjugates.

In summary, the small, non-radioactive MIBA– and DIBA–NLS conjugates may, in the future, serve as important building blocks in the development of small, cell-death-inducing iodine conjugates that are able to enter the cell cytoplasm and nucleus without the need for the hNIS gene or large transmembrane transport peptides. Future improvements will need to include modification of these conjugates such that they are taken up only by tumor cell nuclei after systemic administration. However, local administration into the tumor cavity after brain tumor surgery might be feasible for the MIBA– and DIBA–NLS conjugates presented here.

Acknowledgment

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