International Journal of Pharmaceutics 454 (2013) 738–747

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

The NFL-TBS.40-63 anti-glioblastoma peptide enters selectively in glioma cells by endocytosis

Claire Lépinoux-Chambaud, Joël Eyer*

Laboratoire Neurobiologie & Transgenese, LUNAM, UPRES EA-3143, Université d'Angers, Centre Hospitalier Universitaire, Bâtiment IBS-IRIS, 49033 Angers, France

ARTICLE INFO

Article history:
Received 21 January 2013
Received in revised form 29 March 2013
Accepted 2 April 2013
Available online 17 April 2013

Keywords:
Peptide
glioblastoma
Endocytosis
Uptake
Tyrosine kinase receptor

ABSTRACT

Glioblastoma are the most frequent and aggressive tumour of the nervous system despite surgical resection associated with chemotherapy and radiotherapy. Recently, we showed that the NFL-TBS.40-63 peptide corresponding to the sequence of a tubulin-binding site of neurofilaments, enters selectively in glioblastoma cells where it blocks microtubule polymerization, inhibits their proliferation, and reduces tumour development in rats bearing glioblastoma (Bocquet et al., 2009; Berges et al., 2012a). Here, we characterized the molecular mechanism responsible for the uptake of NFL-TBS.40-63 peptide by glioblastoma cells. Unlike other cell penetrating peptides (CPPs), which use a balance between endocytosis and direct translocation, the NFL-TBS.40-63 peptide is unable to translocate directly through the membrane when incubated with giant plasma membrane vesicles. Then, using a panel of markers and inhibitors, flow cytometry and confocal microscopy investigations showed that the uptake occurs mainly through endocytosis. Moreover, glycosaminoglycans and αVβ3 integrins are not involved in the NFL-TBS.40-63 peptide recognition and internalization by glioblastoma cells. Finally, the signalling of tyrosine kinase receptors is involved in the peptide uptake, especially via EGFR overexpressed in tumour cells, indicating that the uptake of NFL-TBS.40-63 peptide by glioblastoma cells is related to their abnormally high proliferative activity.

1. Introduction

Intermediate filament proteins, like vimentin, keratin, desmin, GFAP, and the three neurofilament subunits, are capable to bind free unpolymerized tubulin on specific sites named tubulin-binding sites (TBS), and thus can affect microtubule polymerization in vitro and in vivo (Bocquet et al., 2009). One of these peptides, derived from the light neurofilament subunit (NFL-TBS.40-63), is able to enter massively glioblastoma cells, and only poorly in healthy cells from the nervous system, like neurons and astrocytes. This peptide is internalized in the cytoplasm of glioblastoma cells where it disrupts microtubule network, and thus inhibits their cellular proliferation and induces cell death by apoptosis. When the peptide is injected in rats bearing glioma, it is localized mainly in and around the tumour, and inhibits its development (Berges et al., 2012a). This peptide can also improve the in vitro and in vivo targeted uptake of lipid nanocapsules by glioblastoma cells (Balzeau et al., 2013). These data indicate that this NFL-TBS.40-63 peptide behaves like a cell penetrating peptide and show a selective entry in glioblastoma cells. Thus, it could represent a promising therapeutic candidate to selectively treat glioblastoma.

Glioblastoma are brain tumours derived from glial cells, and represent the most frequent (2500 new cases/year in France) and aggressive tumours of the nervous system (grade IV according to World Health Organization and with a median survival around one year). Despite surgical resection associated with chemotherapy and radiotherapy, no specific treatment exists to eradicate such tumours. The therapeutic drugs currently used, like alkylating agents (Temozolomide) (Stupp et al., 2009), also affect healthy cells

Abbreviations: Antp, antennapedia-homeodomain-derived antennapedia; ATP, adenosine triphosphate; BSA, bovine serum albumin; chlorpr., chlorpromazine hydrochloride; CPP, cell penetrating peptide; cytoch. D, cytochalasin D; DAPI, 4′,6′-diamidino-2-phenylindole; DMA, 5-(N,N-dimethyl) amiloride hydrochloride; DMEM, Dubelcco's modified Eagles medium; DTT, dithiothreitol; EGFR, epidermal growth factor receptor; FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyanate; GPMV, giant plasma membrane vesicles; HSPGs, heparan sulfate proteoglycans; MAPK, mitogen-activated protein kinase; MβCD, methyl-β-cyclodextrin; NFL, neurofilament light subunit; $NaClO_3$, sodium chlorate; PBS, phosphate buffered saline; PDGFR, platelet-derived growth factor receptor; PFA, paraformaldehyde; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; PMA, phorbol 12-myristate 13-acetate; PTEN, phosphatase and TENsin homolog; RGD, arginine–glycine–aspartic acid; RNase, ribonuclease; RTK, tyrosine kinase receptor; Tat, trans-activator of transcription; TP10, transportan 10; TBS, tubulin-binding site; VEGFR, vascular endothelial growth factor receptor.

* Corresponding author. Tel.: +33 244 688 488; fax: +33 244 688 489.
E-mail addresses: joel.eyer@univ-angers.fr, joel.eyer@inserm.fr (J. Eyer).

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http://dx.doi.org/10.1016/j.ijpharm.2013.04.004

particularly in the nervous system. The lack of specificity of current treatments and their side effects urgently needs the development of new therapeutic strategies targeting tumour cells.

The aim of this study is to elucidate the molecular mechanisms involved in the internalization of the NFL-TBS.40-63 peptide by glioblastoma cells. Previous work showed that the NFL-TBS.40-63 peptide is internalized massively in glioblastoma cells and poorly in other cells from the nervous system, like astrocytes and neurons. This entry is inhibited by low temperature (4 °C) and is energy-dependent, characteristics of endocytosis (Berges et al., 2012a). Here, we further investigate the entry mechanism, and compare it to the known uptake mechanisms of cell penetrating peptides (CPPs). According to their extracellular concentrations, the CPPs (e.g. penetratin, Tat, or TP10) enter cells following a balanced equilibrium between direct translocation through the membrane and endocytosis through intracellular vesicles (Alves et al., 2010; Madani et al., 2011). A well-known CPP, the Tat.48-60 peptide, derived from the trans-activator of transcription (Tat) of human immunodeficiency virus, can translocate through the membrane and accumulate in the cell nucleus (Vives et al., 1997). Recently, the Vim-TBS.58-81 peptide, derived from intermediate filament protein vimentin, was described as a novel CPP that enters glioblastoma cells via endocytosis pathways (Balzeau et al., 2012).

Here, we show that unlike the other CPPs, the NFL-TBS.40-63 peptide is unable to translocate across the plasma membrane of giant plasma membrane vesicles (GPMVs) derived from glioblastoma cells, indicating that it cannot translocate directly in cells. Moreover, using flow cytometry with different inhibitors of endocytosis and confocal imaging analysis with markers of the different endocytic pathways, we also show the involvement of this active transport for the NFL-TBS.40-63 peptide uptake by glioblastoma cells. Finally, we analyzed the possible participation of different cell surface molecules, like heparan sulfate proteoglycans (HSPGs) and αVβ3 integrins that are both over-expressed in glioblastoma. The αVβ3 integrin was identified in glioblastoma tumours, but was not expressed in normal adult brain (Gladson and Cheresh, 1991). The glioma cells also express increased quantities of heparan sulfate compared to normal brain (Steck et al., 1989; Sallinen et al., 2000). These two types of cell surface receptors were known to participate in the CPP and RGD peptide uptake respectively (Alves et al., 2010; Letoha et al., 2010; Shi et al., 2011). However, glycosaminoglycans and αVβ3 integrins are not involved in the NFL-TBS.40-63 peptide recognition and internalization by glioblastoma cells.

Finally, as several cellular properties can influence the uptake efficacy of CPPs, we focused our study on cell cycle phase and signalling pathways that can regulate internalization. It is known that the activation of tyrosine kinase receptors (RTKs) regulates numerous components of the endocytic machinery, like the phosphoinositides involved in several signal-transduction and membrane-trafficking systems (Le Roy and Wrana, 2005; Doherty and McMahon, 2009; Czech, 2000). Moreover, the tumour genesis can be related to the overexpression or the up-activation of different components of RTK signalling pathways, including EGFR, VEGFR or PDGFR that are over-expressed in tumours compared to healthy cells, especially in the U87-MG glioblastoma cell line (Sallinen et al., 2000; Dimitropoulos et al., 2010). Here we show that the uptake of the NFL-TBS.40-63 peptide in glioblastoma cells depends on the cell cycle, and that the peptide penetrates selectively in active proliferative cells depending on signalling pathways over-expressed in tumour cells.

Together, these data indicate that the NFL-TBS.40-63 peptide uptake occurs only by endocytic routes. The regulation of this active transport by intracellular signalling pathways over-expressed in glioblastoma cells promotes the massive uptake of NFL-TBS.40-63 peptide. The abnormally high proliferative activity of glioblastoma cells facilitates the NFL-TBS.40-63 peptide uptake, while a small amount of peptide is internalized in lower proliferative cells, like human astrocytes.

2. Materials and methods

2.1. Cell culture and materials

Human U87-MG and T98G glioblastoma cells, as well as rat 9L and F98 glioma cells (obtained from ATCC, Manassas, VA, USA) were cultured in T75 flasks at 37 °C under 5% CO_2, in DMEM with 1 g/L L-glucose (Lonza, France), supplemented with 10% foetal calf serum, 5% L-glutamine, and 5% penicillin/streptomycin (Lonza, France) and passaged every 2–3 days. Normal human astrocytes (obtained from Lonza) were cultured in T75 flasks at 37 °C under 5% CO_2 in AGM™ Astrocyte Growth Medium (Lonza).

Peptides were synthesized by Millegen (Toulouse). NFL-TBS.40-63 (YSSYSAPVSSSLSVRRSYSSSSGS) and Tat.48-60 (GRKKRRQRRRPPQ) peptides are biotinylated or coupled to carboxy-fluorescein isothiocyanate (FITC), and dissolved in sterile water.

Methyl-β-cyclodextrin (MβCD, 10 mg/mL), chlorpromazine hydrochloride (chlorpr., 50 μmol/L), nystatin (25 μg/mL), phorbol 12-myristate 13-acetate (PMA, 10 μg/mL), cytochalasin D (cytoch. D, 4 μmol/L), 5-(N,N-dimethyl) amiloride hydrochloride (DAM, 1 mmol/L), sodium chlorate ($NaClO_3$, 60 mmol/L), wortmaninn (100 nmol/L), ara-c (5 μg/mL), colchicine (1 μg/mL), U 0126 (40 μmol/L), PD 98059 (50 μmol/L), and sunitinib (1 μmol/L) were obtained from Sigma. PI-103 (50 μmol/L), genistein (400 μmol/L), H-7 (100 μmol/L) were obtained from Merck. Gefitinib (50 μmol/L) was obtained from Santa Cruz Biotechnology. The anti-human CD51/CD61 (αVβ3 integrin) biotin was obtained from eBioscience. The avidin–alexafluor 488 nm (avidin-AF488) and the transferrin from human serum conjugated to alexafluor 568 nm (transferrin AF568) were obtained from Molecular Probes. The cholera toxin subunit B conjugated to alexafluor 555 nm (cholera toxin B-AF555) and dextran conjugated to alexafluor 568 nm (dextran-AF568) were obtained from Invitrogen.

2.2. Flow cytometry

The uptake of the FITC labelled NFL-TBS.40-63 peptide was measured with a FACSscan (Becton Dickinson). Cells were seeded in 6 well plates (4–5 × 10^5 cells/well) during 24 h, and then treated with the different inhibitors during 30 min at 37 °C. To investigate the role of heparan sulphates, cells were treated for 48 h with 60 mmol/L $NaClO_3$. The involvement of the αVβ3 integrins was analyzed using a biotinylated anti-human αVβ3 integrin antibody (5 μg/mL anti-human CD51/CD61) during 1 h at 37 °C. Then 20 μmol/L FITC labelled peptides used at the indicated concentrations were added to the cells for 30 min at 37 °C. Subsequently, cells were washed twice with PBS-1× and then detached with trypsin–EDTA-1× (Sigma) incubation during 5–10 min. After centrifugation (5 min at 2000 rpm), cells were washed twice with PBS-1× and re-suspended in 50 μg/mL propidium iodide (PI, Sigma). The fluorescent positive cells that incorporated the FITC labelled peptides were analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

To analyze the possible effect of the cell cycle on the NFL-TBS.40-63 peptide uptake, cells were seeded in 6 well plates (4–5 × 10^5 cells/well) and after 24 h they were treated with ara-c or colchicine during 24 h, or without serum during several days at 37 °C. To analyze the effect of the different treatments, following trypsinization and centrifugation (5 min at 2000 rpm), cells were permeabilized with PBS-1×–Tween 0.5% and fixed with ethanol 70% before adding RNase 1 mg/mL during 30 min at 37 °C. The cell suspension was diluted in 1 μg/mL PI, before determination of the

cell phase by flow cytometry. To measure the NFL-TBS.40-63 peptide uptake, the cells were pretreated with ara-c, colchicine or without serum, as mentioned previously. After these treatments, cells were incubated with 20 μmol/L FITC-peptide and analyzed by flow cytometry as previously described.

2.3. Confocal microscopy

To study the molecular mechanism of the internalization of NFL-TBS.40-63 peptide we used endocytosis markers, transferrin AF-568 internalized in clathrin-coated pits, cholera toxin B-AF555 internalized by caveolin-coated endocytosis vesicles, and dextran-AF568 to stain macropinocytosis vesicles. Cells were seeded in 24 well plates (3×10^4 cells/well) containing coverslips. After 48 h, cells were co-incubated with 25 μg/mL transferrin-AF568, 10 μg/mL cholera toxin B-AF555 or 10 mg/mL dextran-AF568 and 20 μmol/L FITC labelled NFL-TBS.40-63 peptide during 1 h at 37 °C. After washing with PBS-1×, cells were fixed in 2% paraformaldehyde for 10 min. Cells were then washed three times in PBS-1× before adding 3 μmol/L 4′,6′-diamidino-2-phenylindole (DAPI, Sigma) for 5 min. Finally, cells were washed twice in PBS-1× and the coverslips were mounted with an anti-fading mounting medium. Stained cells were observed with a LSM 700 Zeiss confocal microscope, and images were analyzed with Zen 2009 software.

2.4. Preparation of giant plasma membrane vesicles (GPMVs) treated with FITC labelled peptides

To evaluate the passive transport of the NFL-TBS.40-63 peptide in a natural membrane model without endocytosis processes, we used a protocol previously described for the direct translocation of CPPs (Tat peptide, penetratin, TP10) (Amand et al., 2011; Saalik et al., 2011). The preparation of the GPMVs was performed according to Bauer et al. (2009). Cells were cultured during 48 h and washed twice with GPMV buffer (2 mmol/L CaCl2, 150 mmol/L, NaCl, 10 mmol/L HEPES, pH 7.4). Then, they were incubated with GPMV buffer supplemented with 25 mmol/L formaldehyde (FA, Sigma) and 2 mmol/L dithiothreitol (DTT, Euromedex) for 4 h. After incubation, the supernatant containing the detached GPMVs was collected and centrifuged at 19,000 rpm for 45 min at 4 °C. The pellet was dissociated in GPMV buffer. The GPMV suspension was used the same day of their preparation. GPMVs were treated with 20 μmol/L FITC labelled peptides during 1 h. Then, 50 μL of GPMV suspension were deposited onto glass slides and covered with a glass coverslip. Cells with GPMVs were imaged with a Leica DMI6000 inverted microscope and analyzed with Metamorph 7.1.7.0 software. The GPMVs treated with the peptides were visualized with a Leica fluorescent microscope and analyzed with Image J 1.43 software.

2.5. Statistical analysis

All experiments were repeated at least three times. For FACS analysis, twenty thousand events per sample were analyzed and dead cells stained with PI were excluded (representing for each samples less than 10% of dead cells excluded). Results were presented as mean percentage of fluorescent cells that have incorporated the FITC labelled peptide and data were represented as bar graphs with the standard error of the mean (SEM). Statistical analysis was performed with *t* of Student test by using Prism 3.00 (GraphPad software, San Diego, CA). Asterisks indicate significant level versus the control condition: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

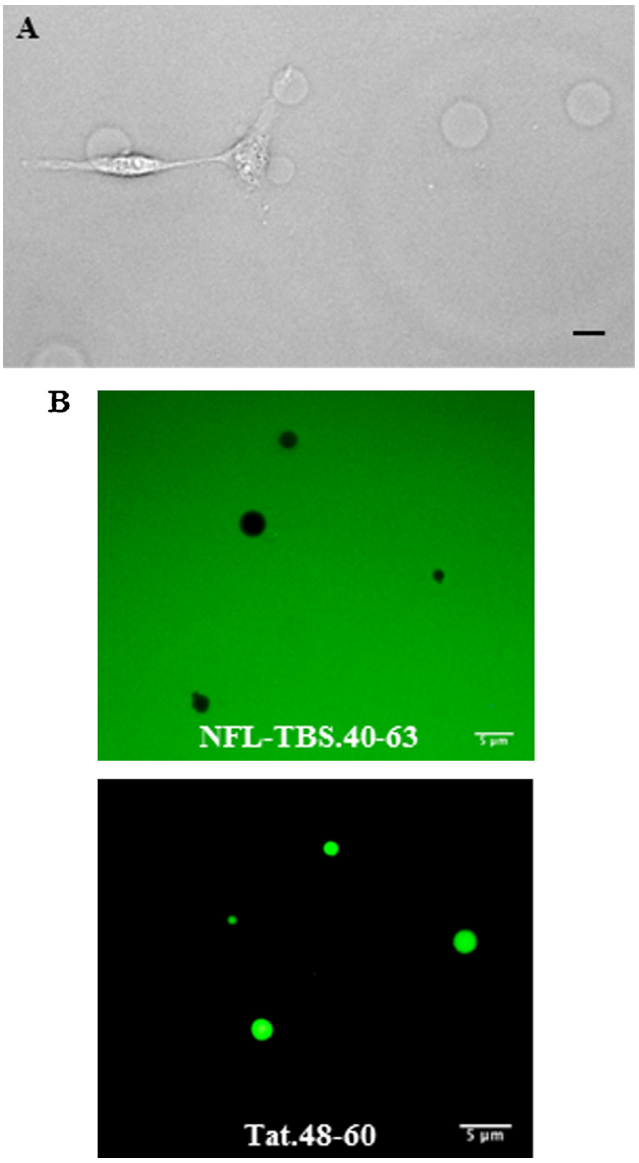

Fig. 1. The uptake of NFL-TBS.40-63 and Tat.48-60 peptides in GPMVs derived from U87-MG cells. (A) U87-MG cells treated with vesiculation chemicals produce GPMVs. (B) GPMVs were isolated and incubated with 20 μmol/L FITC labelled NFL-TBS.40-63 and Tat.48-60 peptides during 60 min. The images of the fluorescent signal of FITC labelled peptides were obtained by fluorescent microscopy. Scale bar: 5 μm.

3. Results

3.1. Unlike the CPPs in general, the NFL-TBS.40-63 peptide is unable to translocate directly across the plasma membrane (Fig. 1)

The CPPs can translocate directly through the membrane and they can use different endocytic pathways as well as nonendocytic uptake routes (Duchardt et al., 2007). A balance between the passive or active entry of such peptides depends on their concentration (Alves et al., 2010; Jiao et al., 2009). Here, to determine if the NFL-TBS.40-63 peptide can passively translocate through the membrane, we used giant plasma membrane vesicles (GPMVs) obtained following the treatment of U87-MG cells with GPMV buffer supplemented with formaldehyde and DTT (Fig. 1A). These GPMVs were then incubated during 60 min with 20 μmol/L NFL-TBS.40-63 and Tat.48-60 peptides labelled with FITC. The peptide penetration was analyzed by fluorescent microscopy. We observed a strong

accumulation of fluorescently labelled Tat.48-60 peptide in GPMVs, confirming the direct and passive translocation of this CPP (Fig. 1B). On the opposite, when the GPMVs were incubated with the FITC labelled NFL-TBS.40-63 peptide, the fluorescence was observed outside the vesicles but no vesicle was labelled (Fig. 1B). Similar results were also obtained when GPMVs were treated with lower concentration of peptides (5 μmol/L), at 4 °C, or following different times of incubation (10 min and 30 min) (data not shown). Taken together, these results demonstrate that the NFL-TBS.40-63 peptide cannot penetrate in GPMVs through a passive direct translocation, like other CPPs.

3.2. Several endocytosis pathways are involved in the uptake of the NFL-TBS.40-63 peptide (Fig. 2)

Previous work showed that the internalization of the NFL-TBS.40-63 peptide is temperature and energy-dependent. Following depletion of adenosine triphosphate (ATP) or incubation at 4 °C, the peptide uptake was strongly reduced in U87-MG cells (Berges et al., 2012a). Such inhibition of the uptake indicates that an endocytosis process is involved. To further confirm this possibility and to define whether a particular pathway (clathrin and caveolin-coated pits, and macropinocytosis) could be used by the peptide to penetrate in glioblastoma cells, we used different inhibitors and markers of the three major endocytosis pathways.

Flow cytometry analysis reveals that the presence of MβCD significantly inhibits the peptide uptake by 41.55 ± 3.668%. This inhibitor disrupts plasma membrane lipid rafts and perturbs the formation of both clathrin-coated pits and caveolin-coated endocytic vesicles (Rodal et al., 1999; Duchardt et al., 2007).

To further confirm this result and distinguish between these two endocytic pathways, we used a panel of inhibitors for each pathway. Chlorpromazine and potassium depletion inhibit clathrin-dependent endocytosis (Larkin et al., 1983; Duchardt et al., 2007). Nystatin and PMA inhibit caveolin-dependent endocytosis (Anderson et al., 1996). When human glioblastoma cells were treated with these inhibitors, the NFL-TBS.40-63 peptide uptake was affected. Fig. 2A indicates that 29.45 ± 2.806% and 38.50 ± 3.492% of peptide uptake occurs via respectively clathrin and caveolin-dependent endocytosis. Inhibitors of macropinocytosis were also tested. Cytochalasin D is known to inhibit F-actin polymerization and DAM inhibits Na^+/H^+ exchanger (Nakase et al., 2004; Koivusalo et al., 2010). Here we show that the macropinocytosis is also involved, with approximately 31.58 ± 6.562% of peptide uptake through this pathway (Fig. 2A).

Finally, we used confocal microscopy to determine whether the NFL-TBS.40-63 peptide is co-localized with endocytosis markers. Transferrin, cholera toxin B and dextran were used to stain respectively clathrin-coated endosomes, caveolin-coated endosomes, and vesicles of macropinocytosis. The majority of internalized peptide was observed as endosome-like punctate signals (Fig. 2B). As a part of the internalized peptide co-localizes with each endocytosis marker, it indicates that the peptide uses these three endocytic pathways to enter cells.

3.3. Involvement of endocytosis pathways in the NFL-TBS.40-63 peptide uptake depends on the cell line and on extracellular peptide concentration (Table 1 and Fig. 3)

The involvement of endocytosis was also evaluated in other glioma cell lines, originating from two different species, and in a human astrocyte cell line. The results presented in Table 1 confirm that the peptide preferentially penetrates in glioma cells when compared to healthy cells, as previously demonstrated (Berges et al., 2012a).

We also investigated the endocytosis mechanism in these different cell lines by pre-treating cells with endocytosis inhibitors, and then incubating them with the FITC labelled peptide. The results and the statistical analysis showed that the three major endocytic pathways were used by the peptide to penetrate the human glioblastoma cells (U87-MG and T98G) whereas in the rat glioma cells (F98 and 9L) the peptide uptake occurred by the clathrin-dependent endocytosis and the macropinocytosis. Moreover, lower proportions of the peptide internalized in human astrocytes were achieved only through macropinocytosis (Table 1).

It has been shown that endocytosis plays an important role for the internalization of CPPs (Richard et al., 2003). In U87-MG cells, we showed that the Tat.48-60 peptide can translocate passively through the membrane as previously reported (Fig. 1). The uptake of Tat.48-60 peptide by U87-MG cells is not inhibited by cytochalasin D, but is inhibited by DAM, suggesting a partial internalization through macropinocytosis (Supplementary Fig. S1). This endocytic pathway was also described for the Tat.48-60 peptide uptake (Wadia et al., 2004; Gump et al., 2010; Yukawa et al., 2010). The Antennapedia-homeodomain-derived antennapedia (Antp), Tat and R9 peptides use the three endocytic pathways (macropinocytosis, clathrin and caveolae-mediated endocytosis), and the mechanism of their uptake depends on peptide concentration (Duchardt et al., 2007). Therefore, we also analyzed the involvement of these endocytic pathways in the NFL-TBS.40-63 peptide uptake, depending on the extracellular peptide concentration. At low concentrations the peptide uptake is only sensitive to PMA, strongly indicative of an internalization through caveolae-dependent endocytosis. However, at higher concentrations, the uptake decreases in the presence of chlopromazin, PMA or DAM, which indicates that the peptide uptake occurs through these three endocytic pathways (Fig. 3).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2013.04.004.

3.4. No classical cell surface recognition of the NFL-TBS.40-63 peptide by glioblastoma cells is required for its internalization (Figs. 4 and 5)

Several studies indicated the importance of heparan sulfate proteoglycan (HSPG) receptors for the accumulation and uptake of Tat peptide in CHO cells (Richard et al., 2005), as well as penetratin and Tat peptides in K562 cells (Letoha et al., 2010), and R9 peptide in HeLa cells (Duchardt et al., 2007). Moreover, a marked decrease of CPP uptake occurs following undersulfation of heparan sulfate (Letoha et al., 2010).

We analyzed the role of HSPGs on the uptake of the NFL-TBS.40-63 peptide in U87-MG cells, by pre-treating these cells with sodium chlorate ($NaClO_3$), an agent known to reduce the sulfation of HSPGs (Safaiyan et al., 1999). At a low peptide concentration, the sodium chlorate treatment had no effect. But at higher peptide concentration, different effects on the peptide uptake were observed. At 20 μmol/L of peptide, the decreased sulfation of HSPGs affected the uptake of Tat peptide but had no effect on the uptake of the NFL-TBS.40-63 peptide (Fig. 4). However, the HSPG receptors do not seem to be the major or only way for Tat internalization, and controversies still exist in the literature concerning the involvement of this uptake mechanism of Tat peptide (Subrizi et al., 2011).

We also analyzed the involvement of the αVβ3 integrins, known to be involved in the cell surface recognition of RGD peptides. Moreover, RGD peptides were used for imaging the expression of αVβ3 integrin in tumours (Dijkgraaf et al., 2011). This binding affinity of RGD conjugates for αVβ3 integrins has been shown in U87-MG cells (Shi et al., 2011). Another group demonstrated that peptide derived from α3 chain of type IV collagen interacts with

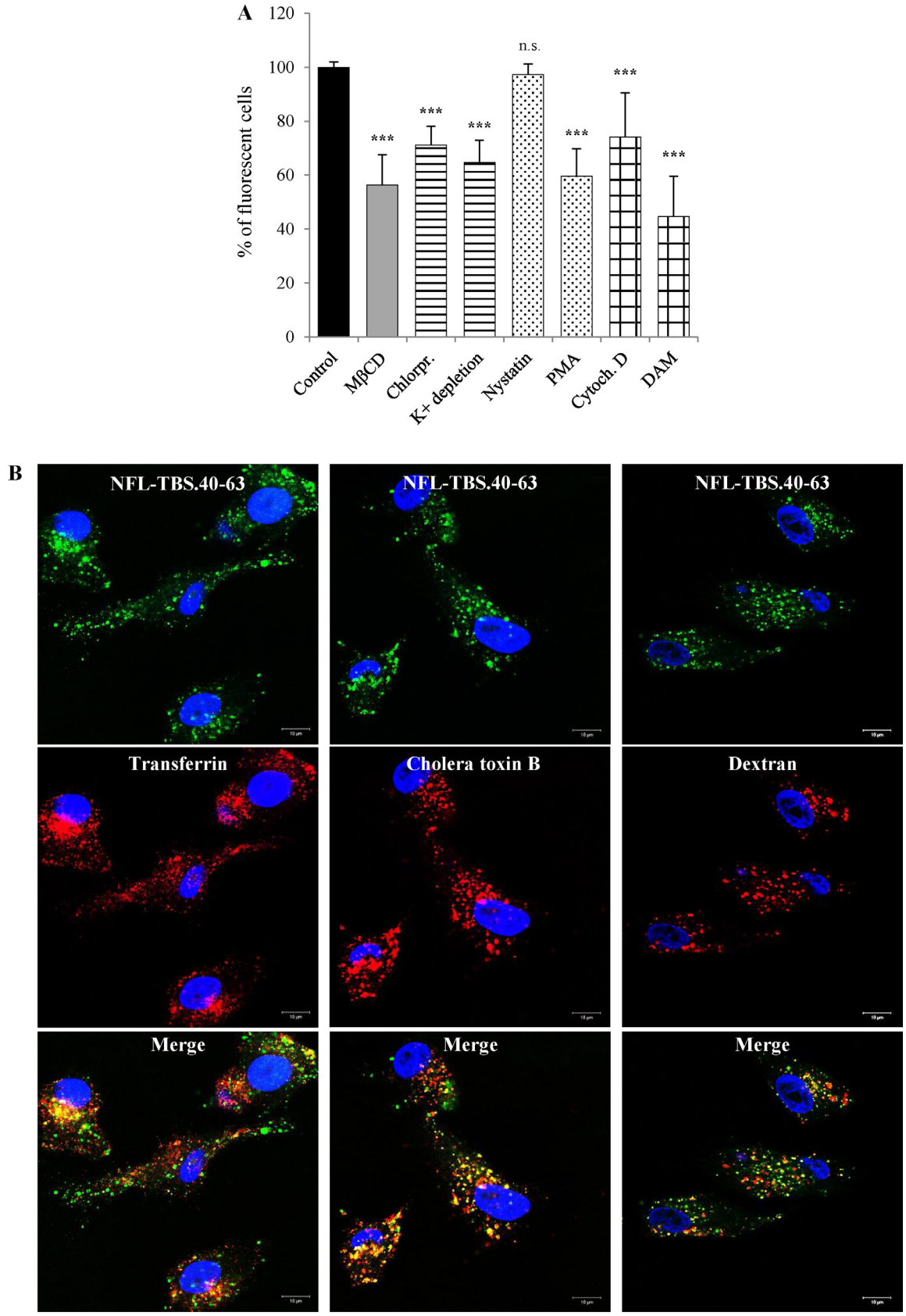

Fig. 2. The NFL-TBS.40-63 peptide internalization in U87MG cells. (A) Cells were pre-treated with 10 mg/mL methyl-β-cyclodextrin (MβCD), 50 μmol/L chlorpromazine (chlorpr.), 25 μg/mL nystatin, 10 μg/mL PMA, 4 μmol/L cytochalasin D (cytoch. D) and 1 mmol/L DAM, during 30 min. Then they were incubated with 20 μmol/L FITC labelled NFL-TBS.40-63 peptide during 30 min. Cellular uptake was assessed by FACS analysis and the percentage of fluorescent cells was determined comparatively with untreated sample (control). n.s.: not significant; $^{**}P < 0.01$; $^{***}P < 0.001$. (B) Human U87-MG cells were grown with 25 μg/mL transferrin-AF568, 10 μg/mL cholera toxin B-AF555, or 1 mg/mL dextran-AF568 in the presence of 20 μmol/L FITC labelled NFL-TBS.40-63 peptide during 1 h. Nuclei were labelled with DAPI (blue). Fluorescent labelling was analyzed by confocal microscopy. Multiple regions show co-localization of the peptide with the endocytosis markers fas revealed by yellow punctate signals. Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
The NFL-TBS.40-63 peptide uptake and the involvement of the three endocytosis pathways depend on cell lines. The uptake of NFL-TBS.40-63 peptide was compared in human cell lines (U87-MG and T98G), in rat glioma cell lines (F98 and 9L) and in human astrocytes. The cells were pre-treated during 30 min with the inhibitors of the endocytic pathways used in Fig. 1A, and then incubated with 20 μmol/L FITC labelled NFL-TBS.40-63 peptide during 30 min. Cellular uptake was assessed by FACS analysis and the percentage of fluorescent cells was determined. Significant results versus control are presented as mean ± SEM.

	Cell line	NFL-TBS.40-63 uptake (%)			
		Total uptake	Clathrin-dependent endocytosis	Caveolin-dependent endocytosis	Macro-pinocytosis
Human GBM cell lines	U87-MG	95.17 ± 0.628	29.45 ± 2.806	38.5 ± 3.492	31.58 ± 6.562
	T98G	85.47 ± 0.983	16.48 ± 3.363	6.38 ± 2.981	16.19 ± 3.714
Rat glioma cell lines	F98	89.48 ± 1.586	19.37 ± 2.589	–	29.98 ± 3.730
	9L	89.59 ± 0.692	12.38 ± 2.789	–	22.99 ± 3.279
Human astrocytes	h. astro	47.13 ± 2.645	–	–	29.58 ± 6.582

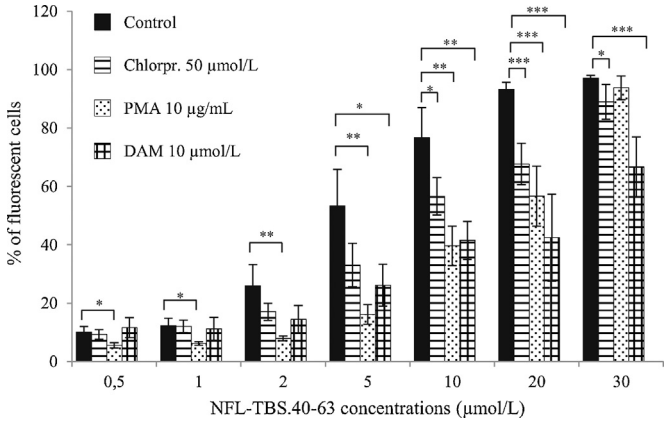

Fig. 3. The involvement of the three endocytosis pathways depends on the extracellular NFL-TBS.40-63 peptide concentration. Human U87-MG cells were pre-treated with 50 μmol/L chlorpromazine (chlorpr.), 10 μg/mL PMA or 1 mmol/L DAM, during 30 min and then incubated with 20 μmol/L FITC labelled NFL-TBS.40-63 peptide during 30 min. Cellular uptake was evaluated by FACS analysis and the percentage of fluorescent cells was determined comparatively with untreated samples (control). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

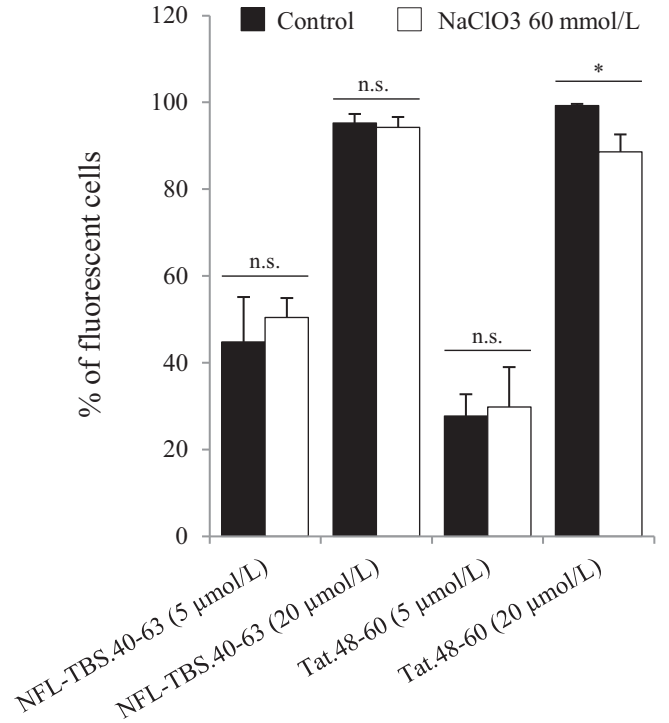

Fig. 4. Evaluation of glycosaminoglycans on the uptake of NFL-TBS.40-63 and Tat.48-60 peptides in U87-MG cells. Cells were pre-treated with 60 mmol/L $NaClO_3$ during 48 h. Then cells were incubated with 20 μmol/L FITC labelled peptides during 30 min. Cellular uptake was assessed by FACS analysis and the percentage of fluorescent cells was determined comparatively with untreated samples (control). n.s.: not significant; $^{*}P < 0.05$.

αVβ3 integrins (Fawzi et al., 2000; Thevenard et al., 2006). These studies and the over-expression of these integrins in glioblastoma (Gladson and Cheresh, 1991) led us to analyze possible involvement of the integrin binding, by blocking integrins with an anti-αVβ3 integrin antibody. Staining of cells with biotinylated anti-human αVβ3 integrin followed by avidin-AF488 confirms the presence of these integrins in U87-MG cells. The mean percentage of fluorescent positive cells was 93.41 ± 2.914% compared to 12.47 ± 1.655% of fluorescent positive cells only treated with avidin-AF488. When cells were pre-incubated with the anti-αVβ3 integrin antibody the uptake of NFL-TBS.40-63 peptide was not altered. More than 95% of cells incorporated the peptide in the presence or absence of this antibody (Fig. 5). Taken together these data indicate that the NFL-TBS.40-63 peptide is not recognized at the cell surface by the HSPGs or the αVβ3 integrins.

To confirm these results, we also analyzed the NFL-TBS.40-63 peptide uptake by quenching the surface-bound fluorescence of the peptide by adding 0.4% trypan blue (Sigma) before FACS analysis to measure only the intracellular fluorescence. Results confirm that there is no classical cell surface recognition of the NFL-TBS.40-63 peptide by the glioblastoma cells as there is no difference with or without quenching (Supplementary Fig. S2).

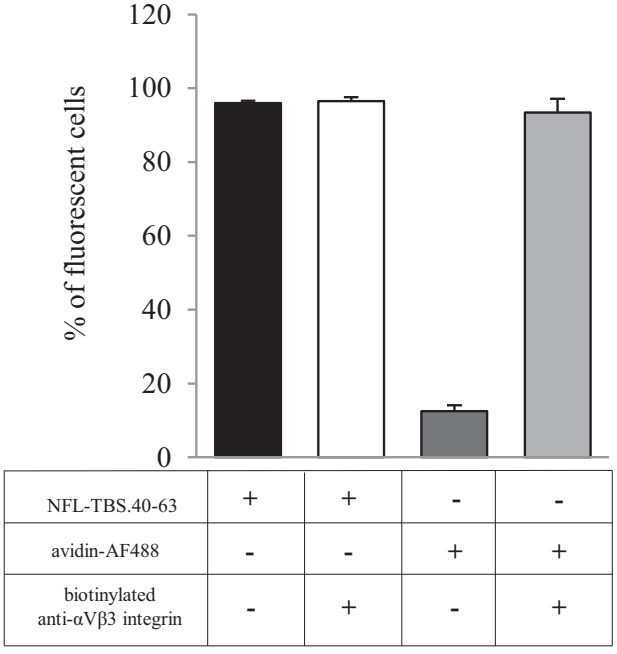

Fig. 5. Evaluation of αVβ3 integrins on the uptake of NFL-TBS.40-63 peptide in U87-MG cells. Cells were treated with 20 μmol/L FITC labelled NFL-TBS.40-63 peptide or Avidin-AF488 (at 1/200) during 30 min before FACS analysis. Cells were also pre-treated with 5 μg/mL biotinylated anti-αVβ3 integrin antibody during 1 h and then incubated with avidin-AF488 or 20 μmol/L FITC labelled NFL-TBS.40-63 peptide during 30 min. Cellular uptake was assessed by FACS analysis.

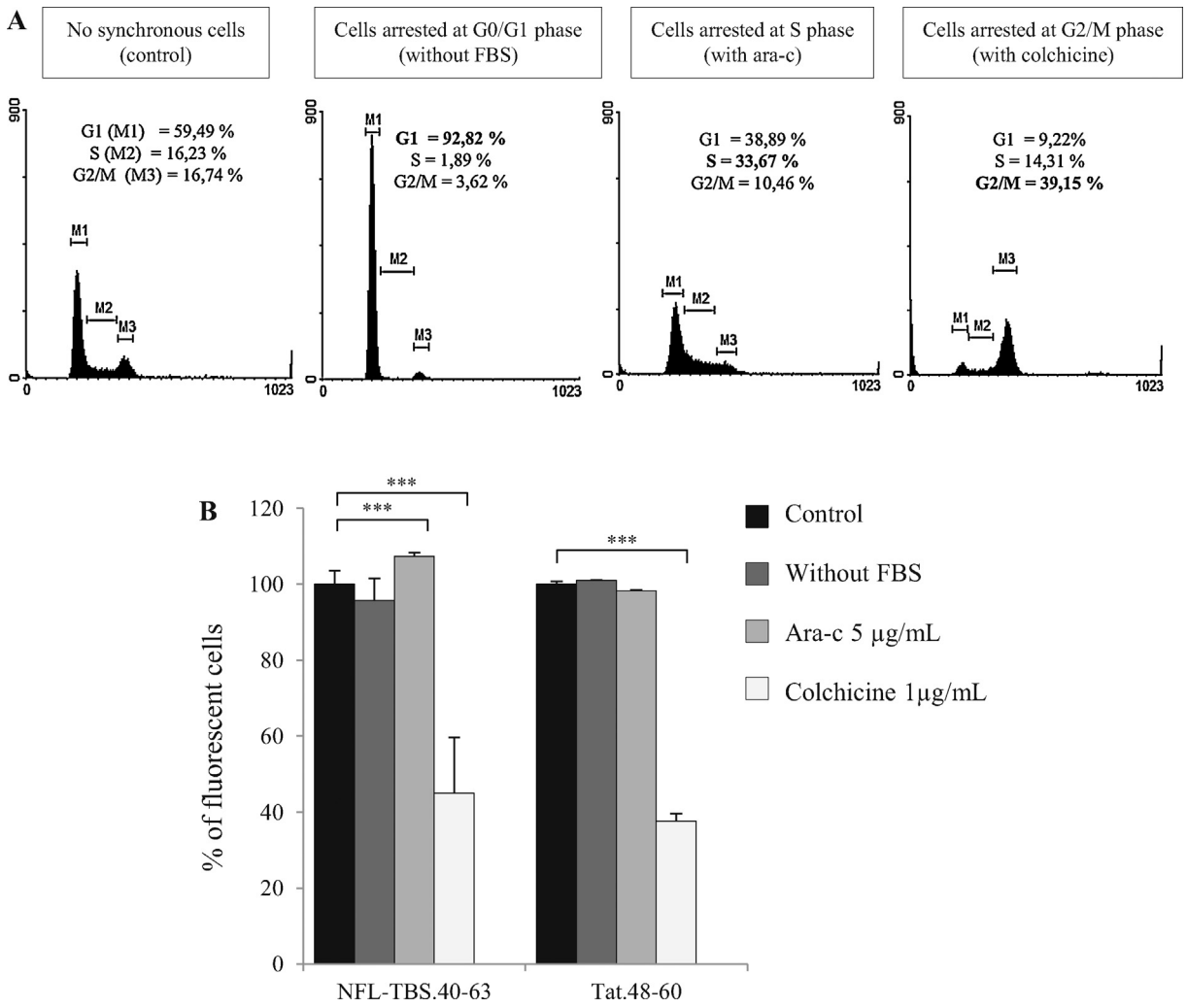

Fig. 6. The uptake of NFL-TBS.40-63 and Tat.48-60 peptides is more active during the G1 and S phases. (A) U87-MG cells were not synchronized (control), arrested at G0/G1 phase (without FBS), S phase (with 5 µg/mL ara-c), and G2/M phase (with 1 µg/mL colchicine). (B) Then 20 µmol/L FITC labelled NFL-TBS.40-63 and Tat.48-60 peptides were added to the U87-MG cells during 30 min. Cellular uptake was assessed by FACS analysis and the percentage of fluorescent cells was determined comparatively with untreated samples (control). ***$P < 0.001$.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2013.04.004.

3.5. *The NFL-TBS.40-63 peptide is selectively internalized in active proliferative cells (Figs. 6 and 7)*

Previous studies indicated the selective uptake of the NFL-TBS.40-63 peptide in glioblastoma cells, whereas its uptake is limited in normal healthy cells (astrocytes and neurons) (Berges et al., 2012a). To investigate whether the uptake of Tat.48-60 and NFL-TBS.40-63 peptides might be related to a special cell cycle phase, we cultured cells under conventional asynchronous conditions or arrested them at specific stages of their cell cycle. The cell cycle was arrested at G0/G1 phase by serum deprivation, at S phase by ara-c treatment (equivalent to thymidine), and at G2/M phases by colchicine treatment (equivalent to nocodazole) (Radis-Baptista et al., 2012) (Fig. 6A).

The uptake of Tat.48-60 peptide occurred mostly during the G1/S phase of U87-MG cell cycle (Fig. 6B), as previously described for crotamine in CHO cells (Nascimento et al., 2007). Similarly, the NFL-TBS.40-63 peptide was selectively internalized during the G1 and S phases of U87-MG cell cycle. A slight but systematic increased uptake of the peptide was found following ara-c treatment (when compared to un-arrested cells), indicating that the peptide enters in U87-MG preferently during the S phase. It is important to note that when incubation in serum deprivation condition was prolonged (after 3 days), the peptide uptake was significantly decreased in U87-MG cells (Supplementary Fig. S3). FACS analysis showed that more than 65% of cells survived despite the 11 days incubation without serum.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2013.04.004.

To further elucidate the molecular mechanisms involved in the uptake of the NFL-TBS.40-63 peptide, we investigated the cellular signals required for activating different endocytic pathways, in particular the role of RTKs and signalling pathways associated to PI3K/Akt and MAPK (Fan and Weiss, 2010; Nikiforova and Hamilton, 2011; Seger and Krebs, 1995). Fig. 7B shows no major effect for the Tat.48-60 peptide uptake, while inhibition of RTKs with genistein decreased markedly the internalization of NFL-TBS.40-63 peptide, and inhibition of serine–threonine receptors with H-7 had no effect. Moreover, the inhibitors of PI3K (wortmannin and PI-103) and MAPK (PD 98059 and U 0126) respectively inhibit approximately 28% and 26% of the peptide uptake. Interestingly, only the inhibition of EGFR by gefitinib altered the uptake of NFL-TBS.40-63 peptide decreasing to 53.38 ± 2.301% the

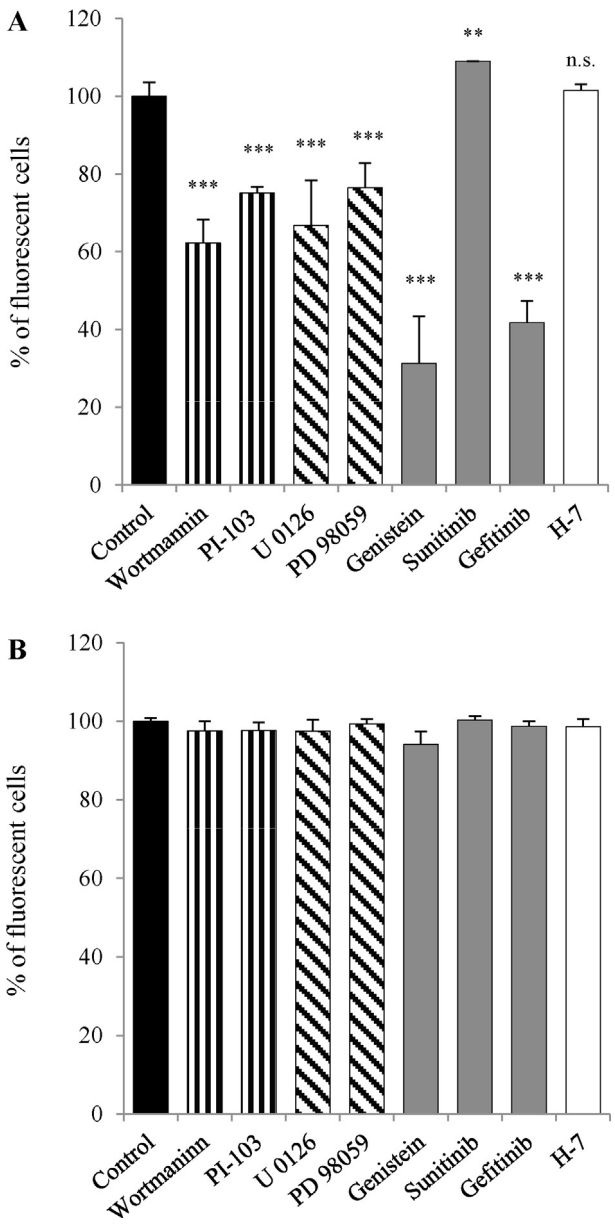

Fig. 7. The NFL-TBS.40-63 peptide internalization in U87-MG cells pre-treated with inhibitors of RTK signalling pathways. U87-MG cells were pre-treated during 30 min with inhibitors of PI3K/Akt signalling pathway (100 nmol/L Wortmaninn and μmol/L PI-103), with inhibitors of MAPK signalling pathway (40 μmol/L U 0126 and 50 μmol/L PD 98059), with inhibitors of RTK (400 μmol/L genistein, 1 μmol/L sunitinib and 50 μmol/L gefitinib), and with inhibitor of serine–threonine receptor (100 μmol/L H-7). Then, 20 μmol/L FITC labelled NFL-TBS.40-63 (A) and Tat.48-60 (B) peptides were added to the cells during 30 min. Cellular uptake was assessed by FACS analysis and the percentage of fluorescent cells was determined comparatively with untreated samples (control). n.s.: not significant, $^{**}P < 0.01$; $^{***}P < 0.001$.

peptide incorporation in U87-MG. In contrast, the peptide uptake was slightly increased when VEGFR and PDGFR were inhibited by sunitinib. These results indicate the importance of the signalling cascade including EGFR, PI3K/Akt and MAPK in the endocytic internalization of the NFL-TBS.40-63 peptide (Fig. 7A).

4. Discussion

In this study, we investigated the molecular mechanism involved in the selective uptake of the NFL-TBS.40-63 peptide by glioblastoma cells. This peptide corresponds to the sequence of the tubulin-binding site located on the intermediate filament protein neurofilament light subunit (Bocquet et al., 2009). In glioma cells, but not in healthy cells, this peptide inhibits microtubule formation and leads to a decreased cell viability, proliferation and migration. This peptide shows a dose-dependent uptake by both glioblastoma cells and astrocytes, with a limited internalization in healthy cells. The uptake was shown to be temperature and energy-dependent, which suggests an endocytosis uptake (Berges et al., 2012a).

Here we confirmed that the NFL-TBS.40-63 peptide is incorporated through clathrin-dependent endocytosis and macropinocytosis in rat glioma cell lines. In human glioma cells, the peptide penetrates cells through these two mechanisms and also by caveolin-dependent endocytosis, whereas only macropinocytosis is responsible for the peptide uptake in human astrocytes. Confocal microscopy analysis confirms these results in U87-MG cells where the NFL-TBS.40-63 peptide appears as punctate signals, characteristic of endosomes, and co-localized with markers of these uptake processes. A similar energy-dependent uptake was described for Vim-TBS.58-81, another TBS peptide derived from the intermediate filament vimentin, which enters glioblastoma cells through the caveolar endocytic pathway (Balzeau et al., 2012). However, it is important to note that the involvement of the different endocytic pathways depend on the extracellular peptide concentration.

To elucidate the NFL-TBS.40-63 peptide internalization, and as the NFL-TBS.40-63 peptide shares some common characteristics with the CPPs, we comparatively analyzed the molecular mechanisms involved in CPP uptake, in particular the Tat.48-60 peptide. These peptides are defined as short and water-soluble, with positive charges at physiological pH, and they can be internalized in different cell types (Madani et al., 2011; Lindgren et al., 2000; Mueller et al., 2008). Here, we show that the Tat.48-60 peptide uptake in U87-MG cells occurs through macropinocytosis, whereas the NFL-TBS.40-63 peptide uses the three endocytosis pathways. Importantly, CPPs are also able to directly translocate through the membrane, by an energy-independent process (Richard et al., 2003; Duchardt et al., 2007), as shown here for Tat.48-60 translocation. However, the NFL-TBS.40-63 peptide is unable to directly translocate in giant plasma membrane vesicles derived from U87-MG cells. Thus, unlike the CPPs uptake that occurs following a balance between direct translocation and endocytosis (Alves et al., 2010; Madani et al., 2011), the NFL-TBS.40-63 peptide uptake occurs only through endocytic pathways.

Another important difference shown between the uptake of the NFL-TBS.40-63 peptide and CPPs is the involvement of HSPGs expressed at the cell surface that play key roles in the accumulation and internalization of CPPs (Richard et al., 2005; Letoha et al., 2010). Inhibition of heparan sulfates in U87-MG cells does not alter the uptake of NFL-TBS.40-63 peptide, even if HSPGs are overexpressed in these cells (Steck et al., 1989; Sallinen et al., 2000). As these results alone cannot explain the selectivity of the peptide for glioblastoma cells, we also investigated the role of other overexpressed receptors, like αVβ3 integrins (Gladson and Cheresh, 1991; Shi et al., 2011), but these integrins do not affect the NFL-TBS.40-63 peptide uptake. Together, these data clearly indicate that the selective entry of the NFL-TBS.40-63 peptide does not involve the recognition of the peptide by these cell surface molecules.

Interestingly, we observed that NFL-TBS.40-63 uptake is more active during the G1 and S phases of cell cycle, as demonstrated for Tat.48-60 in U87-MG cells and for crotamine peptide in CHO cells. It seems that this selective uptake occurs in active proliferative cells, as already described for crotamine uptake (Nascimento et al., 2007). Moreover, several studies showed that the positive charge of NFL-TBS.40-63 and CPPs sequence alone is not sufficient for their internalization but that the amino acid sequence is important for peptide uptake (Futaki et al., 2001; Berges et al., 2012b).

Finally, we showed that signalling pathways involved in endocytic mechanism play a key role for the internalization of

NFL-TBS.40-63 peptide in glioblastoma cells. Our results provide evidence that activation of EGFR and signalling pathways associated, PI3K/Akt and MAPK, is crucial to allow NFL-TBS.40-63 peptide uptake in cells. It is known that the activation of tyrosine kinase receptors (RTKs) regulates numerous components of the endocytic machinery, like the phosphoinositides. For example, PdtIns(4,5)P2 is distributed at the plasma membrane and has a role in endocytosis. PdtIns3P is activated by PI3K and is enriched in early endosomes (Le Roy and Wrana, 2005; Doherty and McMahon, 2009; Czech, 2000; Raiborg et al., 2013). The involvement of specific cellular signals has already been described for the endocytosis of the human papillomavirus type 16 (HPV-16) (Schelhaas et al., 2012). In many human cancers, including glioblastoma, various mutations are found in signalling molecules, as gain-of-function mutations in PI3K catalytic subunit or loss of PTEN (an oncogene that down-regulate the PI3K/Akt pathway in normal condition) (Cheng et al., 2009). The Cancer Genome Atlas (TCGA) provides a network view of the pathways altered in GBM tumours. TCGA pinpoints deregulation of RTK/PI3K pathways in most of these tumours. In human glioblastoma, TCGA reports that EGFR is the RTK the most frequently mutated and/or amplified (TCGA, 2008). For these reasons, one possibility is that the selective uptake of NFL-TBS.40-63 peptide in glioblastoma cells is related to the over-activation of EGFR and deregulation of signalling molecules involved in the endocytic mechanism. Eierhoff et al. (2010) demonstrated that variation of expression or activity of RTKs, especially EGFR, altered the uptake of influenza A viruses (IAV) into cells. Recent studies showed that less than 10% of NFL-TBS.40-63 peptide can penetrate the human prostate carcinoma cells (LNCaP) that expressed low levels of EGFR (Berges et al., 2012a; Bonaccorsi et al., 2007). Finally, our data suggest that the abnormally high proliferative activity and EGFR up-activation in glioblastoma cells promote NFL-TBS.40-63 peptide uptake, while a small amount of peptide is internalized in lower proliferative cells, like human astrocytes. These results indicate that the NFL-TBS.40-63 peptide selectivity and specific effect on glioma cells (Berges et al., 2012a) could offer new therapeutic strategies of targeted delivery, improving efficacy and reducing side effects.

Acknowledgements

This work was supported by grants from ARC (Association pour la Recherche sur le Cancer), AFM (Association Française contre les Myopathies), and La Région des Pays de la Loire (CIMATH programme) to J. Eyer. C. Lepinoux-Chambaud was supported by a fellowship from le Ministère de la Recherche et des Technologies. We thank Emilie Lauret for her help with confocal microscopy, and Catherine Guillet (SCCAN platform) for her help with FACS measures.

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