



# The NFL-TBS.40-63 peptide targets and kills glioblastoma stem cells derived from human patients and also targets nanocapsules into these cells

Claire Lépinoux-Chambaud, Joël Eyer

## ► To cite this version:

Claire Lépinoux-Chambaud, Joël Eyer. The NFL-TBS.40-63 peptide targets and kills glioblastoma stem cells derived from human patients and also targets nanocapsules into these cells. International Journal of Pharmaceutics, 2019, 566, pp.218-228. 10.1016/j.ijpharm.2019.05.060 . hal-02616081

**HAL Id: hal-02616081**

**<https://univ-angers.hal.science/hal-02616081>**

Submitted on 25 Oct 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

# **The NFL-TBS.40-63 peptide targets and kills glioblastoma stem cells derived from human patients and also targets nanocapsules into these cells.**

Lépinoux-Chambaud C<sup>1</sup> and Eyer J<sup>1§</sup>

5

<sup>1</sup>Micro & Nanomédecines Translationelles-MINT, UNIV Angers, INSERM U1066, CNRS UMR 6021, Angers F-49933 France. joel.eyer@univ-angers.fr

**§Corresponding author:** Eyer Joël

10

Tel: (33) 244-688-488; Fax: (33) 244-688-489; E-mail joel.eyer@univ-angers.fr

**Keywords:** Glioblastoma; stem cells; peptide; targeting; proliferation.

15

20

1

---

<sup>1</sup> Abbreviations : 5-FAM, 5-carboxyfluorescein; BrdU, bromodeoxyuridine; BTIC, brain tumor-initiating cell; DAPI, 4',6-diaminido-2-phenylindole; DiD, 1,10-dioctadecyl-3,3',3'',3'''-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate; GBM, glioblastoma; IC50, half maximal inhibitory concentration; LNC, lipid nanocapsule; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PBS, phosphate-buffered saline; PFA, paraformaldehyde;

## ABSTRACT

25 Glioblastoma stem cells correspond to brain tumor-initiating cells (BTICs) that have been identified in glioblastoma, the most common and aggressive brain tumor, as responsible for tumor initiation, progression and recurrence due to their resistance to current treatments. Targeting these cancer stem cells represents a crucial challenge to develop new therapeutic strategies. Previous works have shown that the NFL-TBS.40-63 peptide, corresponding to a  
30 tubulin-binding site on neurofilaments, targets and reduces *in vitro* and *in vivo* the viability of glioblastoma cells without affecting healthy cells. The objective of this study is to investigate the effect of this peptide on BTICs isolated from human glioblastoma.

The uptake of this peptide alone or coupled to nanocapsules was analyzed by flow cytometry and immunochemistry. Its anti-tumor effect was studied using proliferation, adhesion and  
35 viability assays. Peptide-mediated effects were also evaluated on the BTIC self-renewal ability and by immunocytochemistry to investigate their cell shape and microtubule network. Here we show that the peptide enters massively in BTICs and demonstrates an anti-tumor effect by inhibiting their proliferation and inducing their death through an alteration of their microtubule network and cell-cell adhesion, and a decrease in the self-renewal ability of these  
40 cancer stem cells.

These results indicate that the NFL-TBS.40-63 peptide represents a promising therapeutic drug for glioblastoma treatment by targeting and killing both glioblastoma cells and BTICs to prevent recurrence.

## 1. Introduction

Glioblastoma (GBM) are the most frequent brain tumor with an incidence of 3:100,000 per year in the United States and Europe. They are derived from glial cells, like astrocytes and oligodendrocytes, and classified as grade IV by the World Health Organization, the most aggressive form, with a median survival of 12-15 months (Crocetti et al., 2012; Ostrom et al., 2013). A population of brain tumor-initiating cells (BTICs) with cancer stem cell (CSC) properties and neural stem cell characteristics (sphere formation, self-renewal, and differentiation in neural lineages) has also been described. They are involved in tumor initiation and progression, are resistant to current treatments and are responsible for recurrence. Thus, BTICs represent an important therapeutic target to treat GBM (Bao et al., 2006; Chen et al., 2012; Galli et al., 2004; Singh et al., 2004; Stopschinski et al., 2013).

Previous works showed that the NFL-TBS.40-63 peptide, corresponding to the sequence of a tubulin-binding site (TBS) located on the light subunit of neurofilaments (NFL), targets glioblastoma differentiated cells (GBM cells) and neural stem cells (Barreau et al., 2018a; Berges et al., 2012; Bocquet et al., 2009; Lepinoux-Chambaud et al., 2016). The peptide alone, or nanoparticles functionalized with the peptide, enter massively and preferentially GBM cells *in vitro* and *in vivo*, while the uptake is very low in astrocytes and neurons. This peptide specifically disrupts the microtubule network of GBM cells and reduces the tumor volume in animals bearing glioma, but it does not affect healthy cells *in vitro* and *in vivo* (Balzeau et al., 2013; Berges et al., 2012). This specific anti-glioblastoma peptide binds the C-terminal tail of  $\beta$ III-tubulin, which is overexpressed in GBM cells (Katsetos et al., 2015; Laurin et al., 2015, 2017). Recent works also showed that this peptide targets *in vitro* and *in vivo* neural stem cells from both rats and human. No major effects were observed at low



concentrations, but at high concentrations (over 40  $\mu$ M) it reduces their self-renewal and proliferation, and increases their cell-surface adhesion and differentiation (Barreau et al., 2018a; Lepinoux-Chambaud et al., 2016). Moreover, lipid nanocapsules functionalized with this peptide are targeted similarly to the peptide (Balzeau et al., 2013; Caradori et al., 2018; Barreau et al., 2018). Other studies even showed the beneficial effect of this peptide on oligodendrocytes promoting their differentiation, growth and survival (Fressinaud and Eyer, 2014, 2015).

The differential uptake and effects of the peptide in normal neural cells was already demonstrated in several previous studies through different methods. Several cell lines or primary cells were used including human and rat astrocytes and neurons, human and rat neural stem cells, and rat oligodendrocytes (Barreau et al., 2018a; Berges et al., 2012; Bocquet et al., 2009; Fressinaud and Eyer, 2014, 2015; Lepinoux-Chambaud et al., 2016; Lepinoux-Chambaud and Eyer, 2013). In this study, in order to avoid repeating data already validated several times, we focused our investigations on human BTICs, responsible for glioblastoma initiation, progression and recurrence due to their resistance to current treatments (radiotherapy and chemotherapy). There is a real emergency today to fight this very aggressive, disabling and fatal tumor. Therefore, we investigated the peptide targeting capacity and effects on BTICs isolated from GBM patients. In this study, BTICs are mostly associated with GBM mesenchymal subtype characterized with rapid and aggressive tumor growth (Cusulin et al., 2015). According to Cusulin et al. (2015), BTICs can be divided into two distinct precursor states: the progenitor-like and the stem-like BTICs. Progenitor-like BTICs are related to a low CD133 expression level and a strikingly shorter median survival in mice xenografted ( $67.4 \pm 11.4$  days), compared to stem-like BTICs ( $183.7 \pm 24.5$  days). In this study, the aim was to show the effect of the NFL-TBS.40-63 anti-glioblastoma peptide on

BTICs with the most aggressive status. Moreover, BTICs from each precursor state revealed heterogeneity with differences in stemness features (self-renewal, expression of neural stem cell markers), mutations/deletions typically found in GBM (EGFR, TP53, PTEN, IDH1) and tumorigenicity, as detailed in Table 1 (derived from Cusulin et al., 2015), for the three BTICs used in this study. BTIC12, BTIC25 and BTIC53 are well representative of the heterogeneity that can be observed in the progenitor-like precursor state.

Here we show that the NFL-TBS.40-63 peptide targets **these** BTICs, alone or coupled to nanocapsules. Moreover, it decreases their proliferation and viability, disrupts their microtubule network, cell shape and cell-cell adhesion and affects their self-renewal ability. Together, these results indicate that this peptide represents a promising tool to target and eliminate BTICs by disrupting their tumorigenic properties and thus preventing tumor development and recurrence.

## **2. Material and methods**

### ***2.1. Cell culture and materials***

Human glioblastoma-derived stem cells (GSCs) also known as brain tumor-initiating cells (BTICs) were obtained from Dr. H. Artee Luchman and Pr. Samuel Weiss (Hotchkiss Brain Institute, University of Calgary, Canada). These cell lines were derived from human GBM tissue following patient consent and were established in the BTIC Core Facility. They were cultured as previously described (Cusulin et al., 2015) and used within 25-30 passages of establishment from primary cells. They were grown in Neuro-Cult NS-A Basal Medium (human) supplemented with Neuro-Cult NS-A Supplement (Stem Cell Technologies,

Vancouver, Canada), and were able to form floating spheres. BTIC lines used in this study (BTIC12, BTIC25, BTIC53) are defined as progenitor-like and are associated with a high ability to self-renew (high sphere formation), high proliferation rate, a very striking median survival, and a rapid and aggressive growth (Cusulin et al., 2015).

The NFL-TBS.40-63 peptide (YSSYSAPVSSSLSVRRSYSSSSGS) is biotinylated or coupled to 5-carboxyfluorescein (5-FAM) at the N-terminal domain in order to follow its distribution and with an amidation at the C-terminal domain to protect it from proteolysis. It was synthesized by Eurogentec (Angers, France), Genecust (Ellange, Luxembourg), or Polypeptide (Strasbourg, France). The same sequence was synthesized by the three companies at a purity > 95% with a similar HPLC analysis. The peptide was dissolved in sterile water at 1 mmol/l as stock solutions and showed similar results.

## 2.2. Flow cytometry

200-300 spheres were seeded in 35-mm dishes and pre-incubated for 30 minutes at 4°C or with 10 mmol/l sodium azide in the presence of 6 mmol/l 2-deoxy-D-glucose to deplete cellular ATP. Then increasing concentrations of the 5-FAM labelled peptide were added to the cells during 30 minutes at 37°C.

The lipid nanocapsules (LNCs) were prepared and characterized as previously described in Heurtault et al., 2002, Balzeau et al., 2013 and Carradori et al., 2016-2018 to obtain white LNCs (LNC), LNCs coupled to the biotinylated peptide (LNC-NFL), LNCs containing the lipophilic tracer DiD (1,10-dioctadecyl-3,3,30,30-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate, dissolved at 1 mg/mL in absolute ethanol, Life Technologies, Saint-

Aubin, France) (LNC-DiD), and LNC-NFL-DiD, as illustrated in Fig. 1D. The size of LNCs was between 56 and 60 nm, their polydispersity (PDI) between 0.01 and 0.05, and their zeta potential between -8.5 and -8.7 mV. To produce LNC-NFL, 369  $\mu$ L of 1 mmol/L NFL-TBS.40-63 stock solution was incubated overnight with 1 mL stock LNC under gentle stirring. The size of LNC-NFL was between 58 and 66 nm, their polydispersity (PDI) between 0.09 and 0.2, and their zeta potential between -6.0 and -7.4 mV. Routinely, the LNC characterization, filtration-centrifugation and/or drop tensiometry measurements show that more than 45 % (45.9 to 48.3%) of the peptide interact with LNC, corresponding to approximately 43 to 58 molecules of peptide on nanoparticles (Balzeau et al., 2013; Carradori et al., 2016). To evaluate the uptake of LNCs in BTICs, spheres were cultured during 1 hour at 37°C with the different LNC formulations (LNC, LNC-NFL, LNC-DiD, LNC-NFL-DiD) at 1:100.

Subsequently, after centrifugation (5 minutes at 42 g) cells were dissociated mechanically, centrifuged (5 minutes at 344 g), washed with phosphate-buffered saline (PBS), and finally re-suspended in 50  $\mu$ g/ml propidium iodide (Sigma-Aldrich, Saint Quentin Fallavier, France). The fluorescent positive viable cells that incorporated the 5-FAM labelled peptide or LNCs were analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

### **2.3. Immunocytochemistry**

Spheres were seeded in 24-well plates on 10  $\mu$ g/ml of poly-L-lysine (Sigma-Aldrich, Saint Quentin Fallavier, France) coated coverslips, during 24 hours at 37°C. Then they were treated with the NFL-TBS.40-63 peptide (5-FAM labelled or biotinylated), or with 1  $\mu$ g/ml colchicine (Sigma-Aldrich, Saint Quentin Fallavier, France) during 30 minutes or 5-7 days at

37°C. Several washes with PBS were performed between each step of the protocol. Cells were fixed with 4 % paraformaldehyde (PFA, Euromedex, Souffelweyersheim, France) during 15 minutes. They were then incubated with 0.5 % triton X-100 for 30 minutes before incubation in 1 % bovine serum albumin for 1 hour. BTICs were then incubated overnight at 4°C with mouse anti- $\alpha$ -tubulin, rabbit anti-nestin, or mouse anti- $\beta$ III-tubulin antibodies (Sigma-Aldrich, Saint Quentin Fallavier, France) respectively at 1:1000, 1:100 and 1:200. Primary antibodies were revealed using Alexa Fluor 568 nm labelled anti-mouse or Alexa Fluor 488 nm labelled anti-rabbit antibodies at 1:200 (Life Technologies, Saint-Aubin, France) for 2 hours. Finally 3  $\mu$ mol/l of 4'6-diaminido-2-phenylindole (DAPI, Sigma-Aldrich, Saint Quentin Fallavier, France) were added for 5 minutes before mounting coverslips with a ProLong Gold antifade solution (Thermo Fisher Scientific, Villebon-sur-Yvette, France). BTICs were observed with a confocal microscope (Leica TCS SP8; Leica Biosystems, Nanterre, France).

#### **2.4. Proliferation and adhesion investigations**

Spheres were dissociated and plated (1 or  $5 \times 10^4$  cells per well for DNA concentration and Crystal Violet staining, or bromodeoxyuridine (BrdU) analysis respectively) on 10  $\mu$ g/ml of poly-L-lysine (Sigma-Aldrich, Saint Quentin Fallavier, France) on a 96-well microplate (black/clear bottom for DNA concentration or standard for Crystal Violet staining and BrdU analysis). Cells were treated with increasing concentrations of biotinylated peptide or 1  $\mu$ g/ml colchicine during 72 hours at 37°C.

To analyze proliferation, DNA concentration was quantified after PBS washing, freezing cells at -80°C, and using the CyQUANT cell proliferation assay kit (Molecular Probes-Thermo Fisher Scientific, Villebon-sur-Yvette, France). Proliferation was also evaluated using the

BrdU cell proliferation ELISA kit (Abcam, Cambridge, Royaume-Uni). Briefly, BrdU was added to the cells during the last 4 hours of the treatment, and stained after fixation with an anti-BrdU primary detector antibody. BrdU was revealed using a horseradish peroxidase conjugate antibody and addition of the tetramethylbenzidine substrate. BrdU positive BTICs were analyzed by colorimetry.

To analyze cell-surface adhesion, cells were washed with PBS, fixed 10 minutes with 1% glutaraldehyde, and finally stained 10 minutes at room temperature with 1% Crystal Violet solution in 20 % methanol (Sigma-Aldrich, Saint Quentin Fallavier, France). Following abundant washing with water, the relative adhesion of BTICs on the surface was quantified by measuring the absorbance at OD<sub>560nm</sub>.

## **2.5. Sphere formation and viability of BTICs**

Sphere forming properties and BTIC morphology were analyzed by incubating dissociated BTICs with increasing concentrations of biotinylated peptide or 1 µg/ml colchicine. After 6-7 days, BTICs were observed using an inverted microscope (Leica DMI6000) equipped with a CoolSNAP-HQ2 camera and images were taken with the Metamorph 7.1.7.0 software. These BTICs were then collected and dissociated to count viable cell number by trypan blue exclusion.

BTIC viability was also evaluated using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cytotoxicity assay after seeding dissociated BTICs in a 96-well plate (1x10<sup>4</sup> cells per well) and treating them 72 hours at 37°C with increasing concentrations of biotinylated peptide or 1 µg/ml colchicine.

## 2.6. Statistical analysis

Statistical analysis were performed using GraphPad Prism 4 software (GraphPad, San Diego, CA, <http://www.graphpad.com>). All experiments are repeated at least three times. For flow cytometry analysis 20,000 events per sample were analyzed. All data are presented as bar graphs (mean  $\pm$  s.e.m). Significant differences were evaluated by the Student t test to compare the various parameters with control condition.

## 3. Results

### 3.1. The NFL-TBS.40-63 peptide targets BTICs

BTIC lines (BTIC12, BTIC25, BTIC53) are defined as progenitor-like and are associated with a high sphere formation, high proliferation rate, a very striking median survival, and a rapid and aggressive growth (Cusulin et al., 2015). The previously described anti-GBM properties of the peptide (Berges et al., 2012) prompted us to investigate its effects on these cells.

To investigate the peptide targeting capacity, the cells were incubated 30 minutes with increasing concentrations of the 5-FAM labelled peptide, as previously described with other cells (Berges et al., 2012; Lépinoux-Chambaud et al., 2016; Lépinoux-Chambaud and Eyer, 2013). Flow cytometry analysis showed a dose-dependent, massive and rapid uptake of the peptide in all three BTICs (Fig. 1A-B). With increasing concentrations of peptide (5 to 60  $\mu$ mol/l), the percentage of labelled (fluorescent) cells increased respectively from 48.28%  $\pm$

13.25% to 96.55%  $\pm$  1.14% for BTIC12, from 31.89%  $\pm$  4.84% to 96.51%  $\pm$  0.37% for BTIC25, and from 42.24%  $\pm$  4.77% to 95.30%  $\pm$  0.73% for BTIC53. As the peptide uptake on these three cell lines was similar, and to avoid repetitions, all experiments were not performed on each of these three cell lines or were described in supplementary data. To discriminate cell binding from uptake, 0.4% trypan blue was used before FACS reading with BTIC53 as a control to quench the surface-bound fluorescence of the FAM-peptide. Quantification of cell uptake was similar with or without trypan blue, thus confirming the peptide uptake in BTICs (Fig.S1.A). The peptide uptake was also confirmed by immunocytochemistry in BTIC12 (Fig. 1C).

The peptide displays typical characteristics of cell-penetrating peptides, especially internalization in different cell types through passive or active transports, respectively translocation or endocytosis (Alves et al., 2010; Lépinoux-Chambaud and Eyer, 2013; Madani et al., 2011). Here, the uptake mechanism of the peptide in BTIC53 was neither altered after ATP depletion nor at 4°C, indicating an energy and temperature-independent uptake, characteristic of a translocation mechanism (Fig. 1D).

As described in previous works, the peptide can serve as a nanovector to target different cell types, like GBM cells and neural stem cells (Balzeau et al., 2013; Carradori et al., 2016). When the peptide is adsorbed at the surface of LNCs containing a lipophilic tracer (LNC-NFL-DiD), their uptake was significantly increased in BTIC53, with 88.09%  $\pm$  2.91% positive cells, compared to 17.43%  $\pm$  1.95% positive cells with LNC-DiD (without peptide) (Fig. 1E).



### 3.2. *The cancer stem cell properties of BTICs and their cytoskeleton are affected by the NFL-TBS.40-63 peptide*

275

In this study, BTICs were cultured as floating spheres or “tumorspheres”, based on culture methods developed for neural stem cells (Reynolds and Weiss, 1992) and developed for brain tumors (Singh et al., 2004). Floating spheres represent a 3D *in vitro* model often used in cancer research as one of the best intermediate model between the *in vitro* cancer cell line cultures and *in vivo* tumor. This model is relevant to investigate the specific properties of stem cells to form spheres, to self-renew and to differentiate and better reflects the *in vivo* situation, in physiological conditions, mimicking the center and the periphery of a tumor mass.

280

285

The peptide effect was analyzed on the three BTIC lines on their ability to form spheres after 6-7 days (one of the main characteristics of stem cells). In control condition, BTICs formed floating spheres in culture. But this property was dramatically affected with low peptide concentrations, and totally inhibited with high concentrations, similar to colchicine (Fig. 2A).

As the peptide effect on these three cell lines is similar, and to avoid repetitions, the following

290

studies mainly focused on BTIC12, in which the peptide uptake was slightly higher.

The alteration to form spheres with the peptide was associated with a dose-dependent decrease of BTIC12 viability evaluated by MTS assay and trypan blue exclusion when cells were treated with the peptide (Fig. 2B-C). BTIC12 proliferation was also analyzed after 72

295

hours treatment with increasing concentrations of the peptide, using DNA concentration measurement and quantification of BrdU positive cells. The peptide decreased significantly

**BTIC12** proliferation, as observed also after treatment with colchicine, a cytoskeleton drug displaying similar mechanism of action as the peptide (Fig. 2D-E).

Moreover, we observed that these effects were also associated with an increase of adherent

300 **BTIC12** when cells were incubated with the peptide but, this was not observed with colchicine (Fig. 3A-B).

These peptide effects were also associated with an alteration of the **BTIC12** cytoskeleton as already observed for GBM cell lines (Berges et al., 2012). When **BTIC12** were incubated

305 during 6-7 days in control condition or with 10  $\mu\text{mol/l}$  of the peptide, their shape, microtubule network (revealed with anti- $\alpha$  tubulin and anti- $\beta$ III tubulin antibodies) and nestin expression were similar. Following colchicine treatment cells became round and  $\alpha$ -tubulin and nestin expression was unchanged, but  $\beta$ III tubulin expression was altered. With 40  $\mu\text{mol/l}$  of peptide, or higher, the cytoskeleton of some **BTIC12** was profoundly disorganized with an abnormal  
310 cell shape and the disappearance of nestin and tubulin (Fig. 4A-B).

## 4. Discussion

315 The medical need for effective and specific therapeutic tools for GBM treatment represents an important challenge in cancer research. Current treatments only increase median survival by a few months, and they cannot eradicate this aggressive and invasive brain tumor. Recently, it has been established the existence of BTICs with cancer stem cell properties and neural stem cell characteristics that are involved in tumor initiation and progression, that are resistant to  
320 current anti-cancer treatments, and thus are responsible for recurrence. Consequently, it is critical to develop new therapeutic drugs able to target and kill both GBM cells and BTICs. In

particular, targeting microtubules in cancers represent one of the core strategies used in cancer therapy to block their cell division and invasion, and induce their death (Katsetos et al., 2015).

Such a strategy requires the development of specific tubulin-binder agents with no

325 cytotoxicity against healthy cells to prevent side effects, and with a preferential GBM targeting capacity.

Here, we describe the NFL-TBS.40-63 peptide, which corresponds to the sequence of a tubulin-binding site located **on the low subunit of** neurofilaments, as a potential effective anti-

330 GBM agent. As previously shown, this peptide is selectively and massively internalized in GBM cells derived from rat, mouse and human through temperature and energy-dependent mechanism (Berges et al., 2012; Lépinoux-Chambaud and Eyer, 2013). **On the opposite, its**

**uptake by BTICs derived from GBM patients occurs through temperature and energy-**

**independent mechanism like for normal neural stem cells (Lépinoux-Chambaud et al. 2016;**

335 **Carradori et al. 2016; Barreau et al. 2018; this study).** Previous works showed a much lower uptake of the peptide in other neural cells, including astrocytes and neurons (Berges et al.,

2012), with no major toxicity against healthy cells. **Further investigations could consist in**

**evaluating the NFL-TBS.40-63 peptide uptake in BTICs at increasing concentrations,**

**compared to other well-known CPP, by using different endocytic inhibitors and/or markers**

340 **and giant plasma membrane vesicles which are deprived of energy. To further elucidate the molecular mechanisms involved in the uptake of the peptide, we could also investigate the**

**cellular signals required for activating different endocytic pathways, as already performed for**

**GBM cells (Lépinoux-Chambaud and Eyer, 2013).**

345 Different pathways are involved **for this peptide uptake depending on the cell type.** The use of active or passive transports was described for other cell-penetrating peptides depending on

several parameters, including the nature and structure of the peptide, the carried molecule, the cell type, or the membrane lipid composition (Falanga et al., 2015; Carradori et al. 2018). The membrane interaction of the RW16 cell-penetrating peptide was enhanced in cancer cells

350 whose membranes are composed with more anionic lipids than those of healthy cells (Jobin et al., 2013). Recently, it has been established that the amount of lipid droplets containing excessive lipids and cholesterol is higher in colon cancer stem cells than in cancer cells (Beloribi-Djefafli et al., 2016). Lipidomic analyzes also showed that shingomyeline and ceramide concentrations in cell membranes play a crucial role in the uptake mechanism of the

355 nona-arginine peptide (Wallbrecher et al., 2017). Similarly, the difference of lipid composition between neural stem cells from the brain and the spinal cord profoundly affects the uptake of the NFL peptide (Carradori et al. 2018). Further investigations are necessary to compare the lipid membrane composition of GBM cells versus BTICs and other cells in order to better understand the molecular mechanism of the peptide uptake (active in differentiated

360 cells or passive in stem cells), and why its uptake is more important in cancer cells. **For example, it would be interesting to study in GBM cells and BTICs the differences in plasma membrane lipid composition, in fluidity and in permeability by using respectively LC-MS analysis, generalized polarization of Laurdan and fluorescent dextran penetration, as already performed between neural stem cells isolated from sub-ventricular zone and from central**

365 **canal (Carradori et al., 2018).**

Interestingly, the peptide disrupts selectively the microtubule networks from both GBM cells and BTICs, but has no major effect on microtubules from healthy cells, such as neurons, astrocytes or neural stem cells (Barreau et al., 2018; Berges et al., 2012; Lépinoux-Chambaud

370 et al., 2016; Lépinoux-Chambaud and Eyer, 2013). Indeed, this peptide-specific effect can be linked to the specific binding of the peptide to the C-terminal domain of the  $\beta$ III-tubulin

isotype (Laurin et al., 2015, 2017), which is over-expressed in GBM cells. While the  $\beta$ III-tubulin isotype is also expressed in neurons where the peptide has no detectable effect on microtubules, its expression is significantly upregulated in tumorigenesis, particularly in  
375 GBM, and is associated with an aberrant or a “dedifferentiation” phenomenon correlated with the acquisition of stem cell-like phenotypes (Katsetos et al., 2015). One possibility to explain the differential effect of the peptide on microtubules could be that the C-terminal domain of  $\beta$ III-tubulin is differently modified in these different cells.

380 Moreover, we showed that the microtubule disruption of human BTICs by the NFL-TBS.40-63 peptide is associated with an alteration of their cell integrity and properties. As already described for GBM cells (Berges et al., 2012), this peptide decreased the growth of human BTICs and induced their death in a dose-dependent manner, even if these cells are defined by a rapid and aggressive growth mostly characteristic of GBM mesenchymal subtype (Cusulin  
385 et al., 2015). Another tubulin binder, the BAL101553 that is the prodrug of BAL27862, was described as the first tubulin-binder with activity against cancer stem cells isolated from GBM patients and characterized by a high tumorigenicity (Berges et al., 2016). This compound binds tubulin on the colchicine site (Prota et al., 2014), whereas the NFL-TBS.40-63 peptide binds selectively  $\beta$ III-tubulin on its C-terminal domain. To the best of our knowledge, the  
390 binding site of the peptide is different from all other known tubulin-binders (Laurin et al., 2017; Liu et al., 2014).

On human GBM cell lines the anti-tumor activity of the peptide showed a half maximal inhibitory concentration (IC<sub>50</sub>) close to 100-200  $\mu$ mol/L. These previous works also showed  
395 a significant decrease of the tumor volume in rats with GBM after a unique local treatment with 5.10<sup>3</sup>  $\mu$ mol/L of the peptide. Moreover, no particular toxicity was observed in healthy

rats that received the same administration of the peptide (Berges et al., 2012). According to our data, the IC<sub>50</sub> of the peptide on BTICs (40-60 µmol/L) is three times lower than that on human GBM cell lines. To complete these results, it would be interesting to study the peptide effect in a co-culture model mixing GBM cells with BTICs to confirm the anti-tumor activity of the peptide on both cancer cells. Finally, further investigations could consist in studying the peptide efficiency in patient-derived xenograft models to determine the *in vivo* effective dose of the peptide in animals for clinical use in human.

The peptide also induced a loss of stem cell property by preventing the gliomasphere formation from human BTICs and by disrupting their cell-cell adhesion in favor of an increase in their cell-surface adhesion that could be related to their increased differentiation. The treatment response and adaptation of cancer cells (and cancer stem cells) are important topics in the development of anti-cancer therapies to avoid any resistance and recurrence.

Previous studies showed that the peptide can target human neural stem cells and increase their adhesion and their differentiation in the neuronal and astrocytic pathways at low concentrations (Barreau et al., 2018). In this study, we showed that the peptide also increased the adhesion of BTICs at low concentrations and kill them at higher. This result suggest that the peptide could also induce differentiation of BTICs in GBM cells for BTICs that would be exposed to low concentrations of peptide, thus making these cells always sensitive to the peptide with no possibility to escape the treatment, and making them sensitive to current treatment (radiotherapy and chemotherapy with temozolomide). Further investigations could explore the effect of the peptide on BTIC differentiation at increasing concentrations and their response to treatment during several successive passages *in vitro*.

In addition, the peptide behaves like a cell-penetrating peptide with a GBM targeting selectivity. Indeed, this peptide exhibits two important different characteristics with its targeting selectivity to GBM cells, BTICs and neural stem cells, and with its specific anti-tumor activity on GBM cells and BTICs disrupting their microtubule networks, but not on healthy cells. *In vivo* previous studies already demonstrated that after a single intra-tumoral injection, this peptide (used at 5 mmol/L) induces an improvement of the general state of the animals and significantly reduces the tumor volume, without lesion in healthy tissue. The animals were treated with 5 mmol/L of peptide (Berges et al., 2012), corresponding approximately to 5.25 mg/kg in an adult rat. The Human Equivalent Dose (HED) of the peptide would be 0.85 mg/kg (considering a typical adult with body weight of 60 kg and Km 37 and the following formula:  $\text{HED (mg/kg)} = \text{Animal Dose (mg/kg)} \times \text{Animal Km} / \text{Human Km}$  (Nair and Jacob, 2016)). For the standard local treatment, the Gliadel Wafers (carmustine) used by neurosurgeons contain 7.7 mg of carmustine per wafer, and a total dose of 61.6 mg can be used when 8 wafers are implanted in the brain resection. In a typical adult, this dose of carmustine corresponds to 0.13 mg/kg for one wafer and 1.03 mg/kg for 8 wafers (Panigrahi et al., 2011). Thus, the amount of peptide (0.85 mg/kg) should be relevant for clinical translation in the case of its single local administration. If it is necessary to administrate the peptide several times, or on a long period of time, this dose and/or the delivery system of the peptide should be investigated to evaluate the best therapeutic efficacy and the most appropriate dosage. In particular, *in vivo* models should be used to characterize the possible toxicological effect of this peptide on healthy animals following increased and/or repetitive intravenous administration of the peptide, and its efficacy on animals bearing intracranial glioblastoma. Alternatively, another solution to explore could be the long-term infusion of peptide using an Alzet pump for *in vivo* experiments on animals bearing BTICs induced tumor.

Therapeutic peptides represent a promising avenue to treat several diseases including cancer.

As mentioned by Marqus et al., 2017, peptides offer many important advantages, including their small size, rapid synthesis, ability to penetrate cell membranes, high activity, and

especially their limited toxic side effects. These peptides can be used alone or coupled to

450 different cargos to serve as drug-delivery system (Marqus et al., 2017). When LNCs are functionalized with the NFL-TBS.40-63 peptide their penetration in BTICs is significantly increased, as already described for GBM cells or in neural stem cells (Balzeau et al., 2013; Carradori et al., 2016). Such nanocapsules coupled to the peptide represent therefore a powerful drug-delivery system.

455 All together our data indicate that the NFL-TBS.40-63, represents a promising and innovative therapeutic tool to treat GBM and prevent recurrence by destroying both GBM cancer cells and BTICs (Fig. 5). Glioblastoma represent a rare disease with different malignant cell populations. There are many studied therapeutic drugs with chemoresistance or with

460 specificity for a signaling pathway (Auffinger et al., 2015; Phuphanich et al., 2013; Schuster et al., 2015), that failed because they are confronted with the heterogeneity of the different malignant cell populations within GBM which represents an obstacle to their therapeutic success. GBM behavior not only depends on genetic identity but also and mostly on differentiation status. Glioblastoma belong to tumors whose differentiated cells are able to

465 dedifferentiate, which is a major mechanism of therapeutic resistance (Schneider et al., 2016). A therapeutic drug, like the NFL-TBS.40-63 peptide, that can target and kill all GBM cell populations represents an innovative and efficient anti-tumor treatment in this severe indication.

470



## 5. Conclusion

In summary, this study reports the capacity of the NFL-TBS.40-63 peptide, a tubulin-binder agent derived from neurofilaments, to target selectively both glioblastoma cancer and  
475 glioblastoma stem cells (BTICs) in which it destroys their microtubule network. By inhibiting the proliferation and viability of these cancer stem cells and preventing their ability to self-renew, this peptide reduces their tumorigenicity. Moreover, according to previous studies, the anti-tumor effect of this peptide was already demonstrated *in vitro* on GBM cell lines and *in vivo* in bearing a GBM tumor, without cytotoxic effect on healthy tissue (Berges et al., 2012).  
480 All these key features of the NFL-TBS.40-63 peptide make it a promising therapeutic candidate against GBM by targeting and killing both cancer cells and BTICs that are resistant to current treatments (Fig. 5).

### 485 Conflict of interest

The authors declare that they have no conflict of interest, with respect to the research, authorship, and/or publication of this article.

### 490 Funding

This work has been supported by AFM (Association Française contre les Myopathies), ARC (Association pour la Recherche sur le Cancer), Ligue contre le Cancer 49 and 85, MATWIN (Maturation & Accelerating Translation With Industry), UNAM (Université Nantes, Angers, Le Mans), and "Région des Pays de la Loire" to Eyer Joël.

495 Lépinoux-Chambaud Claire was supported by the “Société d’Accélération du Transfert de  
Technologie (SATT) Ouest Valorisation“.

### **Ethical approval**

500 The authors declare that there is no study with human participants, or with animals.

### **Contributorship**

Informed consent was obtained from all individual participants included in the study.

505

### **CRedit author statement**

**Claire Lépinoux-Chambaud:** Conceptualization, Methodology, Validation, Formal  
Analysis, Investigation, Writing – Original Draft, Visualization, Project Administration.

510 **Joël Eyer:** Conceptualization, Resources, Writing – Review & Editing, Supervision, Funding  
Acquisition.

### **Acknowledgement**

515 The authors warmly acknowledge Dr. H. Artee Luchman and Pr. Samuel Weiss from the  
Hotchkiss Brain Institute (University of Calgary) for their collaboration and their advices. The  
authors also acknowledge Rodolphe Perrot from SCIAM (Service Commun d’Imageries et  
d’Analyzes Microscopiques), and Catherine Guillet from PACeM (Plateforme d’Analyzes  
Cellulaire et Moléculaire).

## 520 **References**

- Alves, I.D., Jiao, C.-Y., Aubry, S., Aussedat, B., Burlina, F., Chassaing, G., Sagan, S., 2010. Cell biology meets biophysics to unveil the different mechanisms of penetratin internalization in cells. *Deliv. Ther. Mol. Bench Bedside* 1798, 2231–2239. <https://doi.org/10.1016/j.bbamem.2010.02.009>
- 525 Auffinger, B., Spencer, D., Pytel, P., Ahmed, A.U., Lesniak, M.S., 2015. The role of glioma stem cells in chemotherapy resistance and glioblastoma multiforme recurrence. *Expert Rev. Neurother.* 15, 741–752. <https://doi.org/10.1586/14737175.2015.1051968>
- Balzeau, J., Pinier, M., Berges, R., Saulnier, P., Benoit, J.-P., Eyer, J., 2013. The effect of functionalizing lipid nanocapsules with NFL-TBS.40-63 peptide on their uptake by  
530 glioblastoma cells. *Biomaterials* 34, 3381–3389. <https://doi.org/10.1016/j.biomaterials.2013.01.068>
- Bao, S., Wu, Q., McLendon, R.E., Hao, Y., Shi, Q., Hjelmeland, A.B., Dewhirst, M.W., Bigner, D.D., Rich, J.N., 2006. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 444, 756–60.
- 535 Barreau, K., Montero-Menei, C., Eyer, J., 2018a. The neurofilament derived-peptide NFL-TBS.40-63 enters in-vitro in human neural stem cells and increases their differentiation. *PloS One* 13, e0201578. <https://doi.org/10.1371/journal.pone.0201578>
- Beloribi-Djefafli, S., Vasseur, S., Guillaumond, F., 2016. Lipid metabolic reprogramming in cancer cells. *Oncogenesis* 5, e189.
- 540 Berges, R., Balzeau, J., Peterson, A.C., Eyer, J., 2012. A tubulin binding peptide targets glioma cells disrupting their microtubules, blocking migration, and inducing apoptosis. *Mol Ther* 20, 1367–77.
- Berges, R., Tchoghandjian, A., Honore, S., Esteve, M.A., Figarella-Branger, D., Bachmann, F., Lane, H.A., Braguer, D., 2016. The Novel Tubulin-Binding Checkpoint Activator

545 BAL101553 Inhibits EB1-Dependent Migration and Invasion and Promotes  
Differentiation of Glioblastoma Stem-like Cells. *Mol Cancer Ther* 15, 2740–2749.

Bocquet, A., Berges, R., Frank, R., Robert, P., Peterson, A.C., Eyer, J., 2009. Neurofilaments  
bind tubulin and modulate its polymerization. *J Neurosci* 29, 11043–54.

Carradori, D., Dos Santos, A.G., Masquelier, J., Paquot, A., Saulnier, P., Eyer, J., Preat, V.,  
550 Muccioli, G.G., Mingeot-Leclercq, M.-P., des Rieux, A., 2018. The origin of neural  
stem cells impacts their interactions with targeted-lipid nanocapsules: Potential role of  
plasma membrane lipid composition and fluidity. *J. Control. Release Off. J. Control.  
Release Soc.* 292, 248–255. <https://doi.org/10.1016/j.jconrel.2018.11.005>

Carradori, D., Saulnier, P., Preat, V., des Rieux, A., Eyer, J., 2016. NFL-lipid nanocapsules  
555 for brain neural stem cell targeting in vitro and in vivo. *J Control Release* 238, 253–  
62.

Chen, J., Li, Y., Yu, T.S., McKay, R.M., Burns, D.K., Kernie, S.G., Parada, L.F., 2012. A  
restricted cell population propagates glioblastoma growth after chemotherapy. *Nature*  
488, 522–6.

560 Crocetti, E., Trama, A., Stiller, C., Caldarella, A., Soffietti, R., Jaal, J., Weber, D.C., Ricardi,  
U., Slowinski, J., Brandes, A., 2012. Epidemiology of glial and non-glial brain  
tumours in Europe. *Eur J Cancer* 48, 1532–42.

Cusulin, C., Chesnelong, C., Bose, P., Bilenky, M., Kopciuk, K., Chan, J.A., Cairncross, J.G.,  
Jones, S.J., Marra, M.A., Luchman, H.A., Weiss, S., 2015. Precursor States of Brain  
565 Tumor Initiating Cell Lines Are Predictive of Survival in Xenografts and Associated  
with Glioblastoma Subtypes. *Stem Cell Rep.* 5, 1–9.

Falanga, A., Galdiero, M., Galdiero, S., 2015. Membranotropic Cell Penetrating Peptides: The  
Outstanding Journey. *Int J Mol Sci* 16, 25323–37.

- Fressinaud, C., Eyer, J., 2015. Neurofilaments and NFL-TBS.40-63 peptide penetrate  
570 oligodendrocytes through clathrin-dependent endocytosis to promote their growth and  
survival in vitro. *Neuroscience* 298, 42–51.  
<https://doi.org/10.1016/j.neuroscience.2015.04.003>
- Fressinaud, C., Eyer, J., 2014. Neurofilament-tubulin binding site peptide NFL-TBS.40-63  
increases the differentiation of oligodendrocytes in vitro and partially prevents them  
575 from lysophosphatidyl choline toxicity. *J. Neurosci. Res.* 92, 243–253.  
<https://doi.org/10.1002/jnr.23308>
- Galli, R., Binda, E., Orfanelli, U., Cipelletti, B., Gritti, A., De Vitis, S., Fiocco, R., Foroni, C.,  
Dimeco, F., Vescovi, A., 2004. Isolation and characterization of tumorigenic, stem-  
like neural precursors from human glioblastoma. *Cancer Res* 64, 7011–21.
- 580 Heurtault, B., Saulnier, P., Pech, B., Proust, J.-E., Benoit, J.-P., 2002. A novel phase  
inversion-based process for the preparation of lipid nanocarriers. *Pharm. Res.* 19, 875–  
880.
- Jobin, M.L., Bonnafeous, P., Temsamani, H., Dole, F., Grelard, A., Dufourc, E.J., Alves, I.D.,  
2013. The enhanced membrane interaction and perturbation of a cell penetrating  
585 peptide in the presence of anionic lipids: toward an understanding of its selectivity for  
cancer cells. *Biochim Biophys Acta* 1828, 1457–70.
- Katsetos, C.D., Reginato, M.J., Baas, P.W., D’Agostino, L., Legido, A., Tuszynski, J.A.,  
Draberova, E., Draber, P., 2015. Emerging microtubule targets in glioma therapy.  
*Semin Pediatr Neurol* 22, 49–72.
- 590 Laurin, Y., Eyer, J., Robert, C.H., Prevost, C., Sacquin-Mora, S., 2017. Mobility and Core-  
Protein Binding Patterns of Disordered C-Terminal Tails in beta-Tubulin Isoforms.  
*Biochemistry (Mosc.)* 56, 1746–1756.

- Laurin, Y., Savarin, P., Robert, C.H., Takahashi, M., Eyer, J., Prevost, C., Sacquin-Mora, S.,  
2015. Investigating the Structural Variability and Binding Modes of the Glioma  
595 Targeting NFL-TBS.40-63 Peptide on Tubulin. *Biochemistry (Mosc.)* 54, 3660–9.
- Lepinoux-Chambaud, C., Barreau, K., Eyer, J., 2016. The Neurofilament-Derived Peptide  
NFL-TBS.40-63 Targets Neural Stem Cells and Affects Their Properties. *Stem Cells*  
*Transl Med* 5, 901–13.
- Lepinoux-Chambaud, C., Eyer, J., 2013. The NFL-TBS.40-63 anti-glioblastoma peptide  
600 enters selectively in glioma cells by endocytosis. *Int J Pharm* 454, 738–47.
- Liu, Y.M., Chen, H.L., Lee, H.Y., Liou, J.P., 2014. Tubulin inhibitors: a patent review. *Expert*  
*Opin Ther Pat* 24, 69–88.
- Madani, F., Lindberg, S., Langel, Ü., Futaki, S., Gräslund, A., 2011. Mechanisms of Cellular  
Uptake of Cell-Penetrating Peptides. *J. Biophys.* 2011.  
605 <https://doi.org/10.1155/2011/414729>
- Marqus, S., Pirogova, E., Piva, T.J., 2017. Evaluation of the use of therapeutic peptides for  
cancer treatment. *J Biomed Sci* 24, 21.
- Nair, A.B., Jacob, S., 2016. A simple practice guide for dose conversion between animals and  
human. *J. Basic Clin. Pharm.* 7, 27–31. <https://doi.org/10.4103/0976-0105.177703>
- 610 Ostrom, Q.T., Gittleman, H., Farah, P., Ondracek, A., Chen, Y., Wolinsky, Y., Stroup, N.E.,  
Kruchko, C., Barnholtz-Sloan, J.S., 2013. CBTRUS statistical report: Primary brain  
and central nervous system tumors diagnosed in the United States in 2006-2010.  
*Neuro Oncol* 15 Suppl 2, ii1-56.
- Panigrahi, M., Das, P.K., Parikh, P.M., 2011. Brain tumor and Gliadel wafer treatment. *Indian*  
615 *J. Cancer* 48, 11–17. <https://doi.org/10.4103/0019-509X.76623>
- Phuphanich, S., Wheeler, C.J., Rudnick, J.D., Mazer, M., Wang, H., Nuño, M.A., Richardson,  
J.E., Fan, X., Ji, J., Chu, R.M., Bender, J.G., Hawkins, E.S., Patil, C.G., Black, K.L.,

Yu, J.S., 2013. Phase I trial of a multi-epitope-pulsed dendritic cell vaccine for patients with newly diagnosed glioblastoma. *Cancer Immunol. Immunother.* 62, 125–135. <https://doi.org/10.1007/s00262-012-1319-0>

Prota, A.E., Danel, F., Bachmann, F., Bargsten, K., Buey, R.M., Pohlmann, J., Reinelt, S., Lane, H., Steinmetz, M.O., 2014. The novel microtubule-destabilizing drug BAL27862 binds to the colchicine site of tubulin with distinct effects on microtubule organization. *J Mol Biol* 426, 1848–60.

Reynolds, B.A., Weiss, S., 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255, 1707–1710.

Schneider, M., Strobele, S., Nonnenmacher, L., Siegelin, M.D., Tepper, M., Stroh, S., Hasslacher, S., Enzenmuller, S., Strauss, G., Baumann, B., Karpel-Massler, G., Westhoff, M.-A., Debatin, K.-M., Halatsch, M.-E., 2016. A paired comparison between glioblastoma “stem cells” and differentiated cells. *Int. J. Cancer* 138, 1709–1718. <https://doi.org/10.1002/ijc.29908>

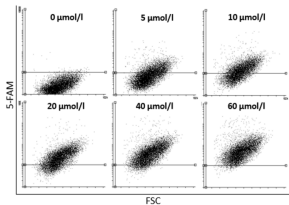
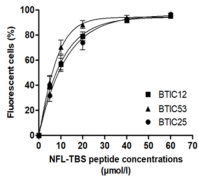
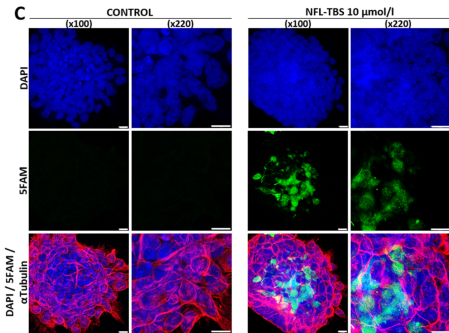
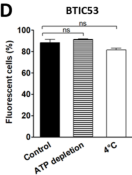
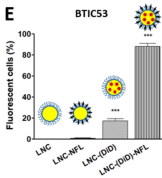
Schuster, J., Lai, R.K., Recht, L.D., Reardon, D.A., Paleologos, N.A., Groves, M.D., Mrugala, M.M., Jensen, R., Baehring, J.M., Sloan, A., Archer, G.E., Bigner, D.D., Cruickshank, S., Green, J.A., Keler, T., Davis, T.A., Heimberger, A.B., Sampson, J.H., 2015. A phase II, multicenter trial of rindopepimut (CDX-110) in newly diagnosed glioblastoma: the ACT III study. *Neuro-Oncol.* 17, 854–861. <https://doi.org/10.1093/neuonc/nou348>

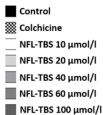
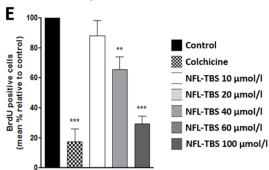
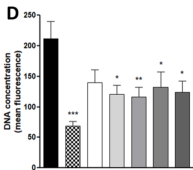
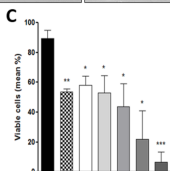
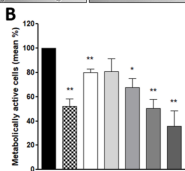
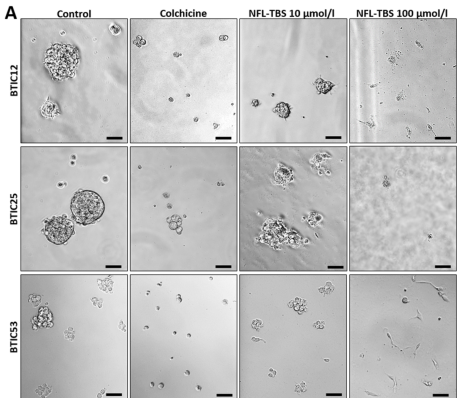
Singh, S.K., Hawkins, C., Clarke, I.D., Squire, J.A., Bayani, J., Hide, T., Henkelman, R.M., Cusimano, M.D., Dirks, P.B., 2004. Identification of human brain tumour initiating cells. *Nature* 432, 396–401.

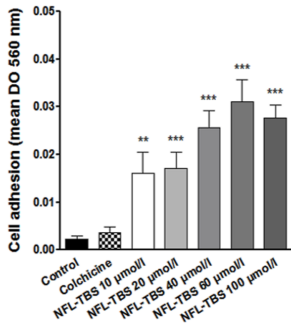
Stopschinski, B.E., Beier, C.P., Beier, D., 2013. Glioblastoma cancer stem cells--from concept to clinical application. *Cancer Lett* 338, 32–40.

Wallbrecher, R., Ackels, T., Alis Olea, R., Klein, M.J., Caillon, L., Schiller, J., Bovee-Geurts,  
P.H., van Kuppevelt, T.H., Ulrich, A.S., Spehr, M., Adjobo-Hermans, M.J.W., Brock,  
645 R., 2017. Membrane permeation of arginine-rich cell-penetrating peptides independent  
of transmembrane potential as a function of lipid composition and membrane fluidity.  
J Control Release.

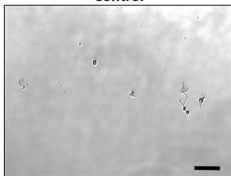


**A****B****C****D****E**

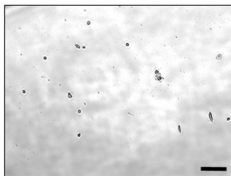


**A****B**

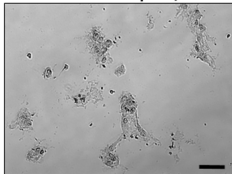
Control



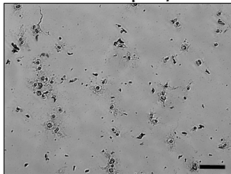
Colchicine



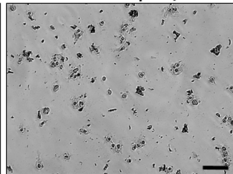
NFL-TBS 10 µmol/l

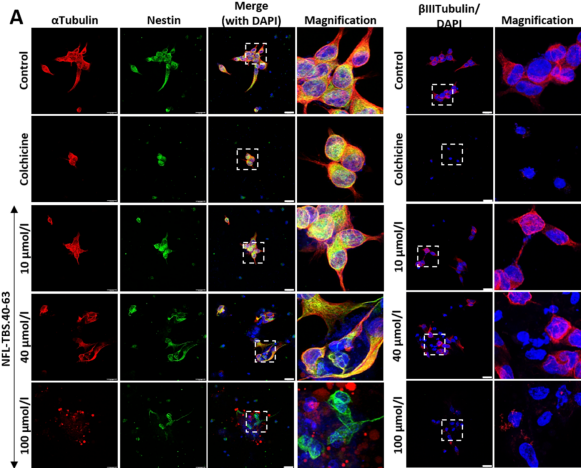


NFL-TBS 40 µmol/l

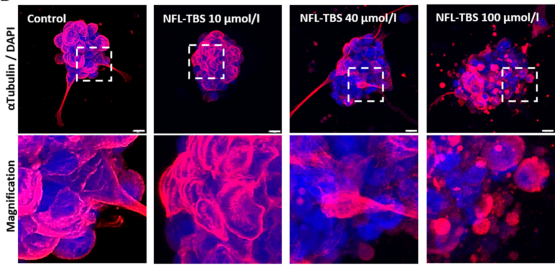


NFL-TBS 100 µmol/l

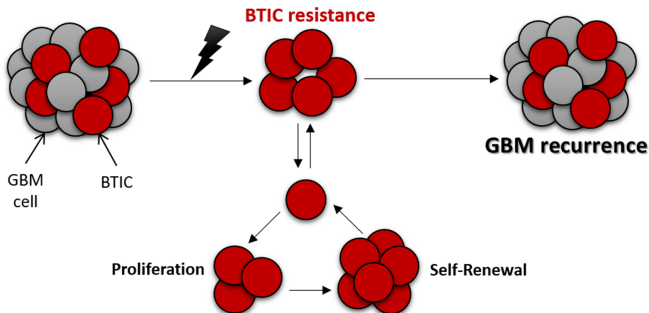




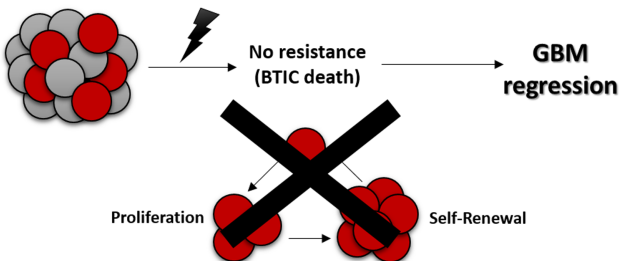
**B**



## STANDARD THERAPIES:



## NFL-TBS.40-63 TREATMENT:



**Table:**

**Table 1: Heterogeneity between the three BTICs lines according to Cusulin et al. (2015).**

BTIC lines	Stemness characteristics (approximate %)			Mutational status				Tumorigenicity
	sphere forming cells	EGFR+ cells	CD15+ cells	<i>EGFR</i>	<i>TP53</i>	<i>PTEN</i>	<i>IDH1</i>	Median survival of xenografted animals
BTIC12	11%	< 10%	> 30%	wt	mut	mut	wt	96 days (N = 6)
BTIC25	17%	< 10%	< 2%	wt	mut	mut	wt	48.5 days (N = 6)
BTIC53	15%	> 50%	> 50%	mut	mut	wt	wt	60 days (N = 5)

mut: mutated; wt: wildtype.

# Glioblastoma (GBM)



Standard  
therapies



**BTIC  
resistance**



**GBM  
Recurrence**

Anti-GBM  
peptide



**No resistance  
(BTIC death)**



**GBM  
Regression**

○ Differentiated GBM cell    ● Brain-tumour initiating cell (BTIC)