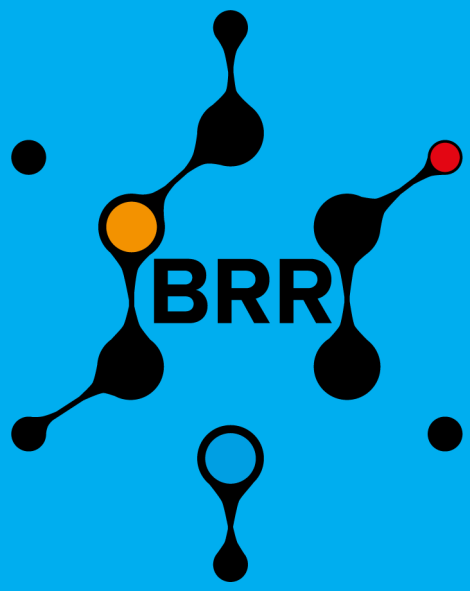

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A novel peptide for microRNA delivery to medulloblastoma cells

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Medulloblastoma is a paediatric brain cancer categorised into various subtypes that have differing prognostic outcomes for patients. As with other cancers, microRNAs have been implicated in medulloblastoma pathogenesis and the loss of specific miRNAs appears to contribute to the disease. There is therefore an urgent need to develop miRNA-replacement therapies for medulloblastoma. However, methods for targeted delivery of small RNAs to medulloblastoma cells have not been fully established. As a step towards tackling this challenge, we have developed self-assembling peptide nanoparticles for small RNA delivery to medulloblastoma cells. We generated an amphiphilic peptide, TY-28, using solid-phase peptide synthesis and combined TY-28 with miR-124-3p. Analysis of the resulting complexes by electron microscopy and dynamic light scattering confirmed the formation of nanoparticles. The ability of the NPs to penetrate cells was monitored by labelling the miRNA with a fluorescent dye. The NP:miRNA complexes were readily internalised by group 3 medulloblastoma cells, and the accumulation of the complexes increased over time. Levels of uptake were 6-fold higher at 24 hours compared to 4 hours in serum-free medium. The uptake of the NPs complexes by the cells did not differ in the presence and absence of serum, suggesting the presence of serum did not affect complex stability. Our findings point to the translational potential of self-assembled NPs to delivery miRNA mimics to medulloblastoma cells.

Key words: Medulloblastoma; MicroRNA; Peptides; Nanoparticles

INTRODUCTION

Primary tumours of the central nervous system (CNS) are the most common cancer sites in children [1]. Medulloblastoma (MB) stands out among these cancers as it accounts for ~10% of malignant brain tumours in children from birth up to 14 years old [2]. Molecular profiling has enabled four main medulloblastoma subgroups to be differentiated on the basis of transcriptional and epigenetic landscapes: Wingless (WNT)-activated, Sonic Hedgehog (SHH)-activated, Group 3 and Group 4 [3-6]. Further disease heterogeneity has also been recognised, with four subtypes of SHH and eight subtypes (I-VIII) spanning Group 3 and 4 [7, 8]. Treatment outcomes depend on the disease subtype and highly aggressive tumours metastasise through cerebrospinal fluid to other regions of the CNS, ultimately leading to mortality [9].

Mature miRNAs are small ~22 nucleotide non-coding RNAs that repress gene expression upon binding to the 3′ untranslated region (UTR) of target mRNA transcripts [10]. Importantly, mature miRNAs are structurally and functionally similar to small-interfering RNA (siRNA) and both types of small RNA are loaded into Argonaute (AGO) proteins to form the RNA-induced silencing complex (RISC) [11]. The miRNA/siRNA guides the RISC to target mRNA transcripts in order to silence target genes through AGO-dependent mechanisms [12].

These miRNAs are only partially complementary to their targets, and a given miRNA can downregulate hundreds of targets, first through acute translational repression but subsequently by steady-state mRNA destabilisation [13]. Changes in miRNA expression are associated with the hallmarks of cancer and therefore represent important targets for the development of nucleic acid therapies [14-17].

Early studies revealed that the expression of miR-124-3p was significantly reduced in medulloblastoma cells compared to normal cerebellum, with overexpression of miR-124-3p inhibiting the proliferation of medulloblastoma cells in vitro and in a xenograft mouse model of the disease [18-21]. However, mechanisms for targeted effective delivery of miR-124-3p mimics to patient cells have not been established and no clinical trials for miR-124-3p replacement therapy in MB have been registered on the https://clinicaltrials.gov/ database.

Self-assembling peptide nanoparticles (NPs) have emerged as novel drug delivery systems for cancer [22, 23]. Multiple efforts have been made to deploy such peptide-based drug delivery systems (DDS) for delivery of miRNA [24-29], miRNA inhibitors [30] and siRNA [25, 31-43]. However, the potential of self-assembling peptide NPs for miRNA/siRNA delivery to medulloblastoma cells has received little attention.

Recently, we introduced amphiphilic cell penetrating peptides (CPP) comprised of a peptide inhibitor of matrix metalloproteinase 9 (MMP-9) and an N-terminal cholesterol moiety to drive self-assembly in aqueous media [44, 45]. The peptides, with and without encapsulation of siRNA cargo, traversed an in vitro model of the blood-brain barrier (BBB) model [44, 45]. We re-engineered the peptides using hydrophobic amino acids to generate TY-28 (sequence GGGWGPIACDIFTNSRGKRA) with a view to simplifying the production of peptide NPs by obviating the incorporation of cholesterol.

In this study, we have evaluated the translational potential of TY-28-based NPs by investigating their ability to deliver miRNA into medulloblastoma cells. We generated and characterised self-assembled TY-28 NPs loaded with a miR-124-3p mimic and used HD-MB03 group 3 medulloblastoma cells as a disease model. The miRNA-loaded NPs were characterised using electron microscopy and dynamic light scattering. Uptake of the miRNA-loaded NPs by medulloblastoma cells was tested in the presence and absence of serum. Our findings suggest that TY-28 associates with miR-124-3p mimic to form NPs that are readily internalised by medulloblastoma cells and lay the foundation for further work to develop miRNA-based nucleic acid therapies for childhood brain cancer.

MATERIAL AND METHODS

Peptide Synthesis

Fmoc N-protected amino acids, Rink Amide ProTide resin and Oxyma Pure™ were purchased from CEM Microwave Technology Ltd, dimethylformamide (DMF) from Acros Organics and other reagents were from Merck. Solid-phase peptide synthesis (SPPS) was performed on a CEM Liberty Blue® automated microwave peptide synthesizer. Briefly, 14 g of Oxyma in 100 mL of dimethylformamide (DMF) was used as the activator base and 15 mL of N,N′-diisopropylcarbodiimide (DIC) in 100 mL of DMF as the activator. Wash steps were performed with DMF and deprotection with 20% piperidine in DMF, then 179 mg of Rink amide resin was added to the reaction vessel. Upon completion of the peptide synthesis reaction, the resin was filtered using a 10 mL syringe and a silicone-based porous bed while washing with dichloromethane. Diethyl ether was added to the syringe and the resin shrunk by vacuum suction. A cleavage cocktail of 400 µL triisopropyl silane (TIPS), 400 µL distilled water and 3.2 mL trifluoroacetic acid (TFA) was used to detach the peptide from the resin, rotating the syringe for 3-4 hours at room temperature. The peptide was precipitated in cold diethyl ether and centrifuged (Hermle Z400 centrifuge) at 3500 rpm for 5 minutes. The previous step was repeated twice to remove impurities and residues of TIPS and TFA. The diethyl ether was evaporated in a fridge at 4ºC before dissolving the pellet in 7.5 mL pyrogen-free water by sonication in an ultrasonic cleaning tank. Afterwards, the solution was split into seven 2 mL vials and freeze-dried overnight at -60°C.

Dynamic Light Scattering (DLS)

A Malvern Zetasizer Nano ZS® (Malvern, Worcestershire, UK) was employed to quantify the zeta potential, polydispersity index (PdI) and particle size using the dynamic light scattering (DLS) method. For analysis of particle size and PdI, a 4 clear-sided cuvette (DTS0012) from Malvern was used. TY-28 powder (12 mg) was dispersed in 3 mL of DNase/RNase-free water, and the diameters of the peptide NPs were measured after a delay of two minutes.

Transmission Electron Microscopy (TEM)

TY-28 NPs were visualized and characterized with morphological descriptions using electron microscopy as previously reported [44, 45]. Copper grids with carbon film were employed and a droplet of TY-28 NPs (3 mg/mL) in DNase/RNase-free water was applied to the grid and air dried in a fume hood. The NPs were observed at a 100 kV accelerating voltage on a FEI Morgagni 268D transmission electron microscope (Oxford Instruments).

Cell Culture and Transfection for Cell Imaging

HD-MB03 medulloblastoma cells were a kind gift from Gianpiero Di Leva (Keele University, United Kingdom) and have been described in detail elsewhere [46]. Cells were maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with foetal calf serum and antibiotics. For NP uptake studies, cells were seeded at 30,000 cells per well of an 8-well chambered coverglass (Nunc™ Lab-Tek™ II Chamber Slide™ System, ThermoFisher) the day prior to transfection. Fluorescently labelled miR-124-3p was obtained from Horizon Discovery (Cambridge, UK), with TAMRA attached to the 3' end of the passenger strand. Briefly, 7.5 µl of fluorescent miRNA (20 µM stock) was added to 100 µl nuclease free water in a 1.5 ml microcentrifuge tube. Separately, 3 mg TY-28 peptide was dissolved in 650 µl nuclease free water. The peptide was then added dropwise to the miRNA solution, and mixed gently by pipetting up and down 4 times, giving a final miRNA concentration of 200 nM and final peptide final concentration of 4 mg/ml. The culture medium on the wells was replaced with 180 µl of complete or serum-free medium, then 20 µl of the peptide:miRNA mixture added to give a final miRNA concentration of 20 nM (4 pmol per well).

Confocal Microscopy and Image Analysis

Cells were fixed in ice-cold methanol for 10 min at -20°C and mounted in Fluoromount G with DAPI for nuclear counter-staining. Cells were visualised on a Zeiss LSM 900 confocal microscopy using an excitation wavelength of 561 nm and an emission window of 545-700nm for TAMRA, while an excitation wavelength of 405 nm and an emission window of 400-535 nm was used for DAPI.. Fluorescence was quantified as integrated densities in ImageJ [47]. The corrected total cell fluorescence (CTCF) was calculated using the following formula: CTCF = Integrated density – (area of selected cell x mean fluorescence of background readings).

Statistical Analysis

Analysis of variance (ANOVA) and Student's t tests were performed where indicated, using Microsoft Excel. P values <0.05 were considered significant.

RESULTS AND ISCUSSION

Characterisation of miR-124-3-loaded peptide nanoparticles

To gain insight into the ultrastructure of NPs formed upon combining TY-28 peptide with miRNA, samples were analysed by transmission electron microscopy. Visual assessment of the electron micrographs indicated the NPs were largely ovoid in shape, but the sizes appeared to vary considerably (Fig. 1A). Consistent with this, a broad size distribution was observed by dynamic light scattering in the range of 20-300 nm , with peaks of 38.9 nm and 136.6 nm (Fig 1B). Typical ghost-peaks are also visible in Fig. 1B, which are not related to particle size. In addition, the polydispersity index of the NPs was 0.843, which reflects a degree of heterogeneity given that homogenous NPs are expected to give PDI of <0.2 [48]. The zeta potential of the NPs was 0.963 mV, reflecting a positive surface charge to facilitate electrostatic interactions with the negatively charged miRNA. Together, these findings indicate that the combination of TY-28 and miRNA results in formation of NPs in a size range suitable for drug delivery to the brain [49].

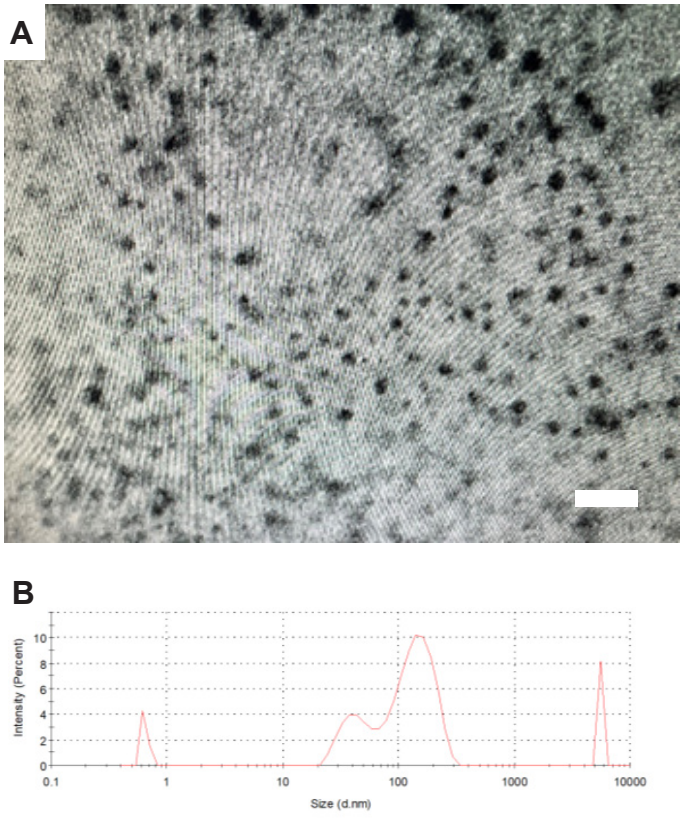

Figure 1. *Structural properties of peptide-miRNA complexes (A) Transmission electron micrograph of TY-28 peptide NPs loaded with miR-124-3p. Scale bar = 2000 nm. (B) Representative size distribution of the miR-124-3p-loaded TY-28 peptide NPs assessed by dynamic light scattering. The main particle size is in the range of 20-300 nm. The artefact peaks (ghost peaks) are also visible at both extreme ends of the x-axis, which do not represent particles.*

Uptake of miR-124-3p-loaded peptide nanoparticles

For evaluation of the ability of TY-28 to deliver miRNA into medulloblastoma cells, fluorescently labelled miR-124-3p was complexed with TY-28 and the complexes applied to cells in the presence or absence of serum. Fluorescence was observed distributed uniformly throughout the cytoplasm and nucleus of the cells after 24 h (Fig. 2A). Levels of fluorescence appeared similar regardless of whether serum was maintained in or excluded from the culture media (Fig. 2A). However, quantification of the fluorescence indicated a slight reduction in uptake by cells maintained in complete media compared to those maintained in serum-free medium during the transfection (Fig.2B). Together, these observations suggest that the ability of the peptide-NPs

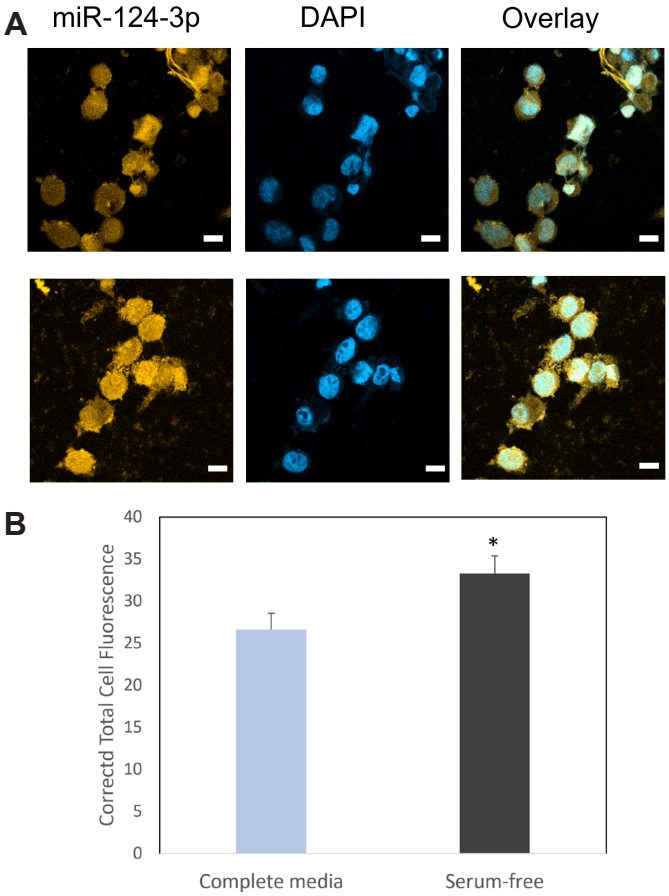

Figure 2. *Peptide nanoparticle-mediated uptake of miRNA by medulloblastoma cells. Representative image of HD-MB03 cells transfected with fluorescently labelled miR-124-3p using TY-28 peptide NPs. Upper panel, cells were maintained in complete medium during transfection. Lower panel, cells were maintained in serum-free medium during transfection. Nuclei were stained with DAPI. Scale bar = 10 µm. (B) Comparison of fluorescence intensities of cells transfected with fluorescently labelled miR-124-3p in the presence (blue bars) and absence (dark bars) of serum. The cells were fixed after 24 h and the corrected fluorescence intensity determined from n = 30 cells from 5 fields of view in each case. *, p <0.05*

to be internalised was not affected by the presence of serum. Importantly, no uptake was observed in cells incubated with the labelled miR-123-3p alone (data not shown). This indicates that the miRNA is not internalised in the absence of the peptide NP.

To determine whether TY-28:miRNA complexes were equally accessible to other cell types, uptake by epidermal keratinocytes was also examined. Few fluorescent cells were observed after exposing HaCaT keratinocytes to TY28:miR-184 complexes for 24 h, and the NPs that were taken up appeared to form a single large cytosolic aggregate or diffuse complexes that were confined to different parts of cytoplasm (data not shown). This suggests that TY-28 NPs are not indiscriminately internalised in a non-specific manner that enables penetration of any cell type. This is an important consideration in terms of the safety of TY-28 NPs, but systematic evaluation of the safety of TY-28 NPs on all major organs and tissues is required to support clinical translation.

Kinetics of uptake

Early studies suggested the uptake of CPPs peaks between 1 and 3 hours after application to cells [50]. We therefore examined the uptake of the peptide NPs after short exposure to the cells. Limited uptake was observed at 0.5–4 h application, and there was no statistically significant difference in fluorescence between cells in complete media and those in serum-free media (Fig 3A). Corrected fluorescence intensities ranged from 3.6 to 5.8 in complete media and 2.8 to 5.2 to under serum-free conditions (Fig. 3A). This contrasts with the fluorescence intensities of 27 and 33 in complete and serum-free media, respectively, after 24 h of incubation with the peptide NP complexes (Fig 2B). Thus comparison of fluorescence intensities at 4 and 24 h indicated ~6-fold higher accumulation of TY-28:miR-124-3p complexes in HD-MB03 cells maintained in complete and serum free medium, respectively (Fig. 3B). These findings suggest the internalisation of the peptide:miRNA complexes was time-dependent, with substantially higher levels of the complexes by 24 h compared to 4 hours or less. In this work, we focussed on miRNA-loaded TY-28 uptake. However, we cannot rule of the possibility that accumulation kinetics of TY-28:miRNA complexes may differ from those of unloaded TY-28 nanoparticles. Experiments are thus required using fluorescently labelled TY-28, generated either using fluorescently labelled natural amino acids or fluorescent amino acid analogues, to determine how the uptake dynamics compares to those reported here for the miRNA-loaded TY-28. The latter approach may obviate some of the concerns that have been raised about the effects of fluorescent dyes on the behaviour of CPPs [51]

Together these findings indicate that TY-28-based peptide NP can deliver miRNA into medulloblastoma cells, and the NP:miRNA complexes accumulate throughout the cell in a diffuse pattern. We found that a 72-h treatment with 20 nM miRNA reduced cell viability to 20% compared to untreated cells when miR-124-3p was delivered using TY-28. However, the effects of miR-124-3p were not specific, as a similar decrease in cell viability was observed using a non-targeting control oligonucleotide. This observation was not limited to TY-28 given that no difference in viability was detected when cells were transfected with miR-124-3p or control oligonucleotide using a commercial transfection reagent (nanocin RNA, from Tecrea Ltd). Thus, the ability of TY-28 to promote accumulation of miR-124-3p in MB03 cells does not appear sufficient to evoke miRNA-specific effects under our experimental conditions. Notably, studies from the Khvorova group suggest intracellular accumulation of exogenous small RNA (siRNA in their studies) does not of itself adequately reflect efficacy [52]. Hence, there may be factors linked to intracellular localisation, trafficking and endosomal escape, or lack thereof, that preclude detection of differential miR-124-3p activity compared to non-targeting oligonucleotides under our conditions.

Overall, the presence of serum in the culture medium had limited effects on the uptake of miRNA-loaded TY-

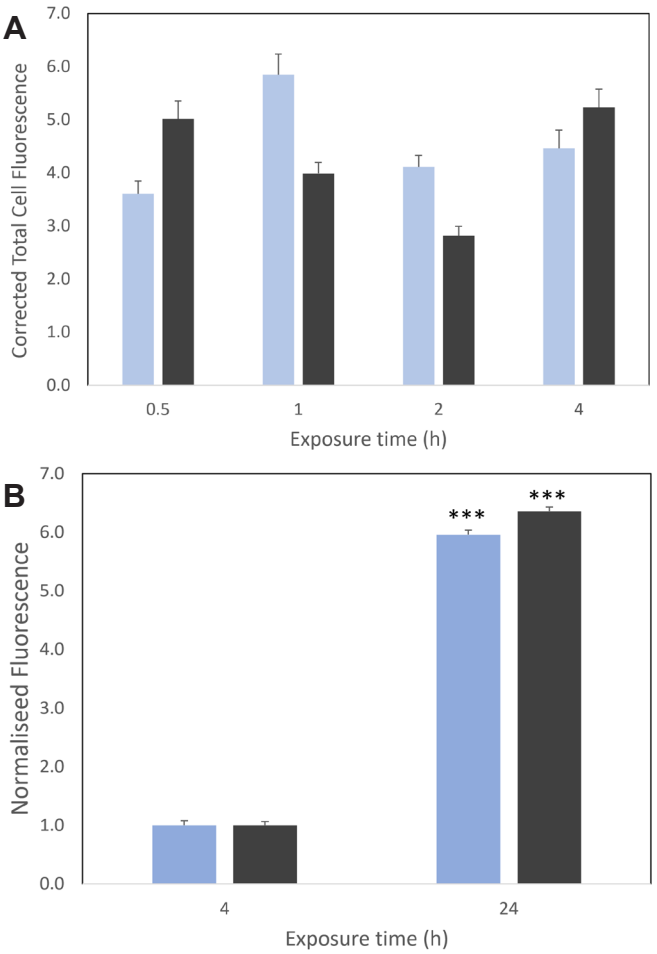

Figure 3. *Temporal analysis of peptide-miR-124-3p uptake. (A) Cells transfected with TY-28 peptide NPs-loaded with fluorescently labelled miR-124-3p in the presence (blue bars) or absence (dark bars) of serum were fixed at the indicated time points. The corrected fluorescence intensity was determined from n = 30 cells from 5 fields of view in each case. (B) The corrected fluorescence intensities at 24 h from Fig. 2B were normalised to those of the 4 h time point from Fig. 3A above. *** p <0.001*

28 nanoparticles. Temporal analysis suggest somewhat slow uptake kinetics that seems limited internalisation over short time frames (<4 hours) given way to substantial accumulation within the medulloblastoma cells over 24 h. One limitation of the study, however, is that only HD-MB03 medulloblastoma cells have been examined. Additional studies are required on other medulloblastoma cells to establish the broad translational potential of TY-28 NPs for delivery of miRNA and other small RNAs in medulloblastoma.

The study also lacks functional evaluation of the miR-124-3p loaded NPs. Our initial tests suggested the TY28:miR-124-3p complexes were as effective as a commercial agent at reducing the viability of HD-MB03 cells. However, at the concentration used, the non-targeting control miRNA mimic reduced viability to the same extent. Hence further studies are required to establish the potential of miR-124-3p-loaded peptide NPs to inhibit the viability of medulloblastoma cells.

In conclusion, we show for the first time to our knowledge effective internalisation of miRNA-loaded peptide NPs by medulloblastoma cells. We envisage the application of these peptide NPs in the context of miRNA-replacement therapy for medulloblastoma. Further testing of the miR-124-3p loaded NPs in preclinical models of medulloblastoma will assist in finding its ultimate potential as a medulloblastoma therapy for the 21st century.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

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