ORIGINAL ARTICLE

Preliminary Observations: Neurite Formation in N2a Neuroblastoma Cells by Serum Deprivation and a Retinoic Acid Receptor Agonist (EC23)

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ABSTRACT

Introduction: To induce neurite formation in N2a neuroblastoma cells for a model of neurotoxicity, serum deprivation with and without the addition of 1 µM EC23 were tested. EC23 is a synthetic analogue of retinoic acid that has been used for neuronal differentiation but its use on N2a cells has not been reported. **Methods:** N2a cells (1.3 x 10^3 cells/cm^2) were cultured with serum-deprived media (0.1% or 0.5% foetal bovine serum (FBS)) with or without 1 µM EC23. ImageJ software was used to quantify neurite outgrowth as visualised with anti-beta III tubulin antibody (Tuj1) immunostaining. **Results:** Serum deprivation (SD) to 0.1% FBS ($SD_{0.1}$) caused pronounced cell loss and was not studied further. SD to 0.5% FBS ($SD_{0.5}$) resulted in longer N2a neurite outgrowths (total neurite length = 79.47±57.14 µm) compared to undifferentiated control cells (56.63±26.57 µm; $p<0.001$). Adding 1 µM EC23 ($SD_{0.5+EC23}$) resulted in a marginally lower total neurite length (75.42±61.15 µm; $p<0.01$) compared to $SD_{0.5}$. $SD_{0.5}$ produced the highest number of neurites (725) compared to undifferentiated (125) and $SD_{0.5+EC23}$ (663). **Conclusion:** EC23 (1 µM) does not appear to have remarkable improvement of total neurite length and total neurite number in N2a cells compared to serum deprivation alone. Serum deprivation to 0.5% is suitable for the purpose of inducing neurite outgrowth in N2a cells, without the need for EC23 addition. It must be underscored however, that this work in no way rules out the use of other concentrations of EC23 for N2a differentiation.
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Keywords: Neurite outgrowth, Serum deprivation, EC23, N2a neuroblastoma cells, Differentiation

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INTRODUCTION

Neurite damage is a key feature of neuronal injury. One parameter for assessing neuronal damage is changes to neurite length and complexity [1–3]. To develop a microglia-induced neurotoxicity culture model, the N2a cell line, which originates from mouse brain neuroblastomas will be used alongside BV2 microglia. The model is intended for the study of the inflammatory responses of microglia and how it causes neuronal damage. Many microglia-induced neurotoxicity culture models utilise undifferentiated neural progenitor cells [4], which would be relevant in the context of studying neurogenesis and brain development, but less so for studying the effects of inflammation in the mature brain.

To determine neurite damage in differentiated N2a cells, neurites that are traceable for quantification of length are required. Several protocols exist to induce neurite formation in progenitor cells [5]. One of them is depriving progenitor cells of serum (known as serum deprivation) and adding retinoic acid to switch neural progenitor cells from a proliferative mode to differentiation mode. Serum deprivation differentiates neurons through activation of the PI3K/Akt and Ras/Raf/MEK/ERK1/2 signalling pathways [6]. Retinoic acid (RA) on the other hand is the biologically active form of vitamin A, and is important for many aspects of vertebrate development, including neural development. It binds to Retinoic Acid Receptor/Retinoid X Receptor (RAR/RXR) heterodimers,

which then binds to RA response elements (RAREs) in the DNA. This leads to the synthesis of proteins involved in differentiation [7–10]. Specifically for neuroblastoma cells, RA binds to receptor tyrosine kinase-like orphan receptor 1 (ROR1), which is minimally expressed in other cells but highly expressed in neuroblastoma cells. ROR1 then regulates RAR and promotes differentiation [11].

Serum deprivation together with retinoic acid use has been previously used on N2a cells to differentiate them into neurite-bearing cells. In one study, N2a cells were serum-deprived to 0.1% FBS in combination with 20 µM of retinoic acid [12] and in another study, N2a were serum-deprived to 0.5% FBS in combination with 10 µM of retinoic acid [13]. In the current study, 0.1% and 0.5% FBS serum deprivation on N2a cells were tested, but instead of retinoic acid, a synthetic analogue of retinoic acid, called EC23, was tested.

EC23 (4-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-ylethynyl)benzoic acid (para-isomer)) is an analogue of retinoic acid that has also been used for neuronal differentiation, including for human embryonal carcinoma stem cell line, TERA2.cl.SP12 [14], human neuroblastoma cells, SH-SY5Y [15, 16] and primary mouse neural stem cells [17]. It was synthesised as a photostable analogue of RA and has a similar chemical structure and mechanism of action as retinoic acid. It binds to RAR and phosphorylates ERK1/2 to induce a genomic response for differentiation [18]. Previously, within the research group, EC23 was used to differentiate NE-4C, a mouse neuroectodermal cell line [19].

MATERIALS AND METHODS

N2a neuroblastoma cultures

N2a cells (Neuro-2a (ATCC CCL-131)) were gifted by Prof. Dr. S. Vikineswary Sabaratnam of the Institute of Biological Sciences, Universiti Malaya, Malaysia. Cells were cultured in Dulbecco's Minimal Essential Medium – High Glucose (DMEM-HG, Cat. No. 12100046, GIBCO, Thermo-Fisher Scientific, Paisley, Scotland, UK), supplemented with 10% foetal bovine serum (FBS, Cat. No. 10270098, GIBCO, Thermo-Fisher Scientific, Paisley, Scotland, UK), 1% non-essential amino acids (NEAA, Cat. No. 11140050, GIBCO, Grand Island, New York), 1% penicillin-streptomycin (Pen-Strep, GIBCO, Cat. No. 15140122, Grand Island, New York), 0.5% amphotericin B/fungizone (GIBCO, Cat. No. 15290026, Grand Island, New York) and sodium bicarbonate powder (Sigma-Aldrich, Cat. No. S5761, USA) to balance the pH to 7.42-7.43.

Differentiation of N2a cells with serum deprivation (SD) in the absence and presence of EC23

N2a cells were seeded at a density of 1.3 x 10^3 cells/cm^2 onto poly-L-lysine (0.15 µg/ml for 2 hours at room temperature)-coated coverslips, in 24-well plates and incubated overnight at 37°C prior to differentiation. N2a cells were differentiated with serum deprivation (0.1% or 0.5% FBS), with or without 1 µM EC23 for 7 days. Undifferentiated N2a cells were cultured in 10% FBS-containing media as control. Media was changed every 48 hours. The treatment groups are depicted in Figure 1.

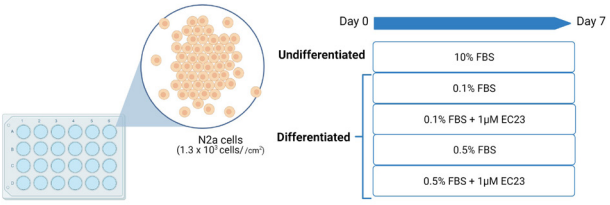

Figure 1: The N2a differentiation protocol. N2a cells (1.3 x 103 cells/cm2) were seeded into 24-well plates and the following day treated with serum deprivation -/+ 1 µM EC23 for a total of 7 days, with a media change every 48 hours (diagram created with BioRender.com).

Anti-beta III tubulin antibody (Tuj1) immunostaining

After induction of differentiation, cells were incubated with 20 µg/ml propidium iodide (PI) (Molecular Probes, Oregon USA) for 10 minutes in a 37°C incubator, washed with PBS and fixed with 4% paraformaldehyde for 1 hour at 4oC, followed by permeabilisation with 0.2% Triton-X in 1% BSA in PBS for 30 minutes. For PI-positive controls, the cells were permeabilised with 0.1% Triton-X prior to PI staining. The cells were then incubated with the primary antibody, an anti-beta III tubulin (neuronal marker) rabbit polyclonal antibody (Tuj1, Abcam, Cat. No. ab18207) at 1:1000 dilution overnight at 4°C. Following that, cells were rinsed with PBS and incubated with donkey anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor™ 488 (Invitrogen, Thermo-Fisher Scientific, Cat. No. a21206) diluted to 1:500 with 0.2% Triton-X in 1% BSA in PBS at room temperature and in the dark for 1 hour. Counterstaining with DAPI was done for 10 minutes at room temperature in the dark. Coverslips were then mounted on glass slides using DPX mounting media (CellPath, Cat. No. SEA-1304-00A, UK) or Prolong Gold Anti-fade Mountant (Thermofisher Scientific, Cat. No. P36930, USA) and left overnight at room temperature before being stored at 4°C. Slides were viewed with a fluorescence microscope (Olympus BX51 Tokyo, Japan) and images of representative regions were taken with 20x objective lens. Qualitative microscope observations were performed with the following stringency: Two observers (N.N.A.P. and S.V.), one of whom was blinded (S.V.). The blinded observer viewed the slides in a random manner (not per sequence of experimental groups). A total of three coverslips were evaluated per group from two independent experiments (n=6).

Neurite measurements with NeuronJ

NeuronJ [20], a plugin for Image J image analysis

software was used for neurite measurements. For this, a total of 2 independent differentiation experiments were performed, each in triplicate. Ten random fields from each coverslip were captured at 100x magnification. The images were traced for neurite length by a blind observer (M.M.). Length and number of primary, secondary, tertiary, and quaternary neurites were measured. Only neurite outgrowths with lengths the same or longer than the diameter of the soma was traced [21–23]. The types of neurite were labelled according to their origin: originating from the soma (primary), from the primary neurites (secondary), from the secondary neurites (tertiary) and finally from the tertiary neurites (quaternary) [22].

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6.0.7 (San Diego, CA, USA). Significance was assessed using the Kruskal-Wallis test followed by Dunn's multiple comparison test.

RESULTS

N2a neuroblastomas were differentiated using serum-deprived media with the addition of 1 µM EC23 for 7 days. The 1 µM EC23 concentration has previously been used by other groups on neural progenitor cells [14, 17, 24] and also in our laboratory for successfully differentiating the mouse neural progenitor cell line, NE-4C [19]. During optimisation of the N2a differentiation protocol, morphological observations of N2a cells revealed elements of cytotoxicity for higher EC23 concentrations, albeit in a different experimental set-up that utilised a 7+1-day approach (7 days serum deprivation, followed by addition of EC23 for 24 hours. Although this is a different experimental set-up compared to the differentiation technique reported in the methodology, it offers some insight into the range of EC23 concentrations and their effects on N2a cells. A more thorough dose-response testing of EC23 on N2a cytotoxicity and neurite formation is needed for future studies. The possibility of EC23 enhancing neurite outgrowths at other concentrations, optimised specifically for the N2a cells, is also not ruled out. This is a gap in the literature that must be filled before any concrete conclusion on the usefulness of EC23 on N2a cell differentiation can be made.

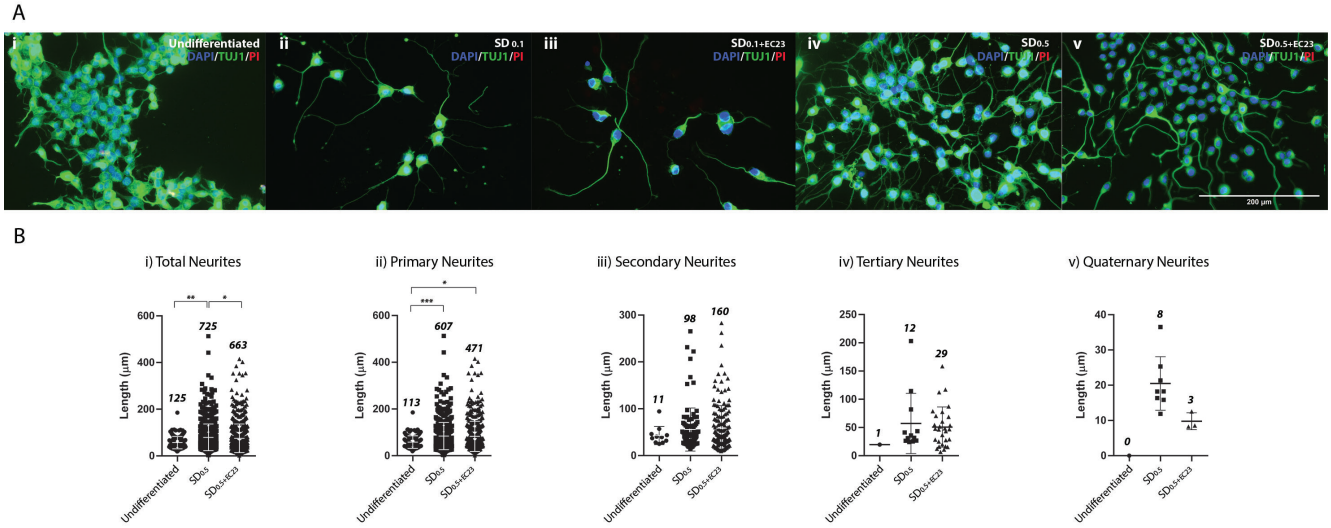

Figure 2: Tuj1 immunostaining of N2a cells and scatter plots of lengths of neurites and neurite number. (A) N2a cells (seeding density:1.3 x 103 cells/cm2) (i), treated with 0.1% FBS (ii), 0.1% FBS + 1 µM EC23 (iii), 0.5% FBS (iv) and 0.5% FBS + 1 µM EC23 (v) for 7 days, stained with Tuj1 followed by a secondary antibody conjugated with Alexa Fluor 488 (green), DAPI (blue) and PI (red). Scale bar: 200 µm. (B) Scatter plots display total neurite number and their lengths (i), primary neurites (ii), secondary neurites (iii), tertiary neurites (iv), and quaternary neurites (v) obtained by semiautomatic tracing of Tuj1 immuno-stained N2a cells from 10 random fields using an ImageJ plugin, Neuron J. Numbers written at the top of each column are the neurite number for each treatment group. Data are mean ± SD of triplicates from two independent experiments. *p<0.01, **p<0.001 and ***p<0.0001 (Kruskal-Wallis with Dunn's multiple comparison).

To induce neurite formation in N2a cells, N2a neuroblastomas were serum-deprived (SD) to 0.1% FBS ($SD_{0.1}$) or 0.5% FBS ($SD_{0.5}$) or with the addition of 1 µM EC23 ($SD_{0.1+EC23}$ or $SD_{0.5+EC23}$). Tuj1 immunostaining revealed cell and neurite morphology whilst DAPI demonstrated cell number and distribution (Figure 2A). Cells were also stained for propidium iodide (PI) to determine cell death of differentiated N2a cells, and the cells stained negative for PI (Figure 2A). None of the cells, regardless of treatment showed PI positivity (Figure 2A). This however does not indicate that there was no cell death in the treated wells, as many floating cells were observed at the end of the time point (data not shown) which would have been washed away during the immunostaining process.

N2a cells were considered as morphologically differentiated if they bore neurites equivalent or longer than 1× its cell body diameter [21–23]. In terms of morphology, undifferentiated N2a cells showed typical amoeboidal morphology, with a few cells demonstrating neurites (Figure 2Ai). $SD_{0.1}$ resulted in profound cell loss, with many of the randomly selected fields intended for neurite tracing measurements containing no cells (Figure 2Aii). Remaining cells exhibited neurites (Figure 2Aii), however, the $SD_{0.1}$ groups were not further assessed as it would not address the need of producing sufficient neurite-bearing N2a cells for a model of neurotoxicity. For $SD_{0.5}$ and $SD_{0.5+EC23}$, many cells were neurite-bearing and there was no overt cell loss (Figure 2Aiv and 2Av). There appears to be slightly less cells in EC23-treated cultures (Figure 2Av compared to serum-deprived cultures (Figure 2Aiv), however this seems to be a very subtle effect and was not quantified.

Neurite measurements were performed to quantify primary, secondary, tertiary, quaternary and total neurite length and counts using NeuronJ [20]. Cells in the $SD_{0.5}$ group had significantly longer total neurite length (79.47±57.14 µm) compared to the undifferentiated group (56.63±26.57 µm; Figure 2Bi) ($p<0.001$) and the $SD_{0.5+EC23}$ group (75.42±61.15 µm; Figure 2Bi)) ($p<0.01$). $SD_{0.5}$ and $SD_{0.5+EC23}$ also had significantly longer primary neurites (84.15±58.04 µm, $p<0.0001$ and 83.07±64.75 µm, $p<0.01$ respectively) compared to the undifferentiated group (58.31±26.72 µm; Figure 2Bii). Both treatments also showed a trend of longer tertiary neurites (57.16 ± 53.37 µm and 66 ± 35.62 µm, respectively) and quaternary neurites (20.47 ± 7.60 µm and 9.79 ± 2.41 µm), although significance could not be tested due to insufficient readouts for undifferentiated cells; undifferentiated N2a only had 1 tertiary neurite and no quaternary neurites (Figure 2Biv and 2Bv. The scatterplots also show that $SD_{0.5}$ produced the highest number of neurites in total (725 neurites), highest primary and quaternary neurites (607 and 8 neurites, respectively), while $SD_{0.5+EC23}$ induced more secondary and tertiary neurites formation (160 and 29 neurites, respectively). Undifferentiated N2a cells (Figure 2Ai) did produce neurites spontaneously, but in lower numbers (125 neurites in total) than the treated groups (725 for $SD_{0.5}$ and 471 for $SD_{0.5+EC23}$) and with no quaternary neurites formed.

DISCUSSION

Undifferentiated N2a cells are a useful tool in neurodevelopmental studies. To study neurotoxicity in mature neurons however, N2a cells require differentiation. An important parameter to measure in neurotoxicity models are neurite length, branching and number. All this contribute to the complexity of neurite outgrowths from the soma, which is a feature that is reduced in neurotoxic/neuro-inflammatory disease such as Alzheimer's disease [25] and in chemical-induced neural perturbation [26]. Here, serum deprivation and addition of 1 µM of the retinoic acid analogue EC23 in forming neurites in N2a cells were investigated. Comparing serum deprivation and 1 µM EC23 on N2a neurite formation within the same study allows for a controlled assessment of the two techniques.

Several papers have described various protocols for N2a differentiation into neurite-bearing cells [12, 13, 27–32]. However, these publications typically do not describe the reasons for their choice of seeding density, serum concentration, RA concentration and total duration of differentiation. The current protocol in this study is a combination of decisions made upon observations of how the cells were in culture and also the available details of published protocols. Kumar & Katyal (2018) and Tremblay et al. (2010) were able to differentiate N2a cells with serum deprivation to 0.1% FBS and 0.5% FBS, respectively, without and in combination with retinoic acid [12, 13]. In another study, Namsi et al. (2018) even acquired differentiated N2a with a media completely deprived of serum (0% FBS) [28]. In this study, N2a cells were tested for differentiation with 0.5% FBS, 0.1% FBS and with the RA analogue, EC23 at 1 µM. Although these studies were able to acquire neurite-bearing N2a cells with extremely low serum concentration and even 0% serum, N2a cells in the current study were unable to survive in 0.1% FBS during the 7 days of differentiation period. It is noteworthy that a lower seeding density (1×10^3 cells/cm^2 compared to 1.25×10^4/cm^2 and 2×10^4 cells/cm^2 used in the cited studies) and a longer differentiation period (7 days compared to their 24 hours and 48 hours in the cited studies) was used here. A lower seeding density was selected in this study as the measure of differentiation is morphology of the cells - a lower seeding density with longer differentiation period allowed visualization and discernment of the features of the neurites clearly for neurite tracing. Whilst for Namsi et al. (2018) and Kumar and Katyal (2018), a requirement for higher cell seeding density and a shorter differentiation period was preferable as flow cytometric cell cycle arrest and PCR-based gene expression analyses were used as the measure of differentiation [12, 28]. These assays require large number of cells for a sufficient readout. It is likely that lower seeding densities and longer induction periods do not favour such a low concentration of FBS (0.1%) for N2a cells.

In the current study, differentiation with serum deprivation to 0.5% FBS with and without addition of 1 µM EC23 induced neurite formation. Interestingly, addition of 1 µM EC23 in the 0.5% FBS groups resulted in a marginally lower total neurite length. A plausible reason for no additive effect of EC23 could be that the EC23 concentration of 1 µM does not have any synergistic effect on serum-deprived N2a cells. Therefore, for a microglia-induced neurotoxicity model utilising N2a cells, a simpler and cost-saving approach of only serum deprivation to 0.5% FBS without the need

for 1 µM EC23 was more advantageous.

Additionally, serum deprivation to 0.5% FBS produced a higher total number of neurites compared to SD0.5 with the addition of EC23. Since the aim is to acquire neurite-bearing N2a cells is for use in a microglia-induced neurotoxicity model, the neurites in this model must be robust enough in number and length to detect amelioration of neurotoxicity in the presence of a therapeutic substance. Therefore, the number of differentiated cells available is as important as the neurite lengths as the neurotoxicity will cause a further reduction in the number of cells. Furthermore, to assess the neurite profiles by image analysis and to detect differences, a substantial number of neurite-bearing cells will be needed.

The comparison of serum deprivation to serum deprivation with EC23 was performed in a manner of convenience and suitability for incorporation into a neurotoxicity model. Only one concentration of EC23 was tested and not more, especially when serum deprivation already revealed sufficient neurite-bearing cells for the context of the downstream study. Although dose response experiments were performed with EC23, they were performed to gauge any potential EC23 toxicity on N2a cells, not for its differentiation effect. Hence, the possible usefulness of other concentrations of EC23 on N2a differentiation is not excluded. For a fair judgement on EC23's ability to differentiate N2a cells, a more thorough optimisation process must be done on EC23 concentrations in the non-cytotoxic range. Based on the number of neurite outgrowths and length, serum deprivation to 0.5% FBS appears to provide neuronal differentiation for a microglia-induced neurotoxicity model, with induction of a total of 725 neurites and highest total neurite length of the 3 groups at 79.47±57.14 µm. For definite assessment of neuron maturity, use of synaptic markers such as synaptophysin and PSD95, and functionality testing of neurons via electrophysiology would be essential. However, the preliminary needs for the culture model are for neurons with neurite outgrowths and the differentiation protocols were evaluated for neurite morphology using Tuj1 immunostaining for beta III tubulin.

Neurites are small processes on developing neurons that ultimately develop into axons or dendrites and beta III tubulin is a cytoskeleton protein that allows visualisation of neuron cell bodies, dendrites, and axons [33]. Neurite number, branching order and neurite length provided a three-pronged approach to looking at neurite arborization and complexity. This requires meticulous manual tracing of each neurite, discerning which originate from which soma and excluding those in which their origin cannot be discerned confidently by the person analysing, including overlapping and clumped cells. Hence, a lower seeding density was used in an effort to reduce cell clumping and overlapping and to allow for clearer visualisation of each neurite's origin and pathway. For neurite tracing, the ImageJ plugin called NeuronJ, a semi-automatic tracing plugin, was employed in this study [20, 34]. Another commonly used plugin is Simple Neurite Tracer , which gives values from the Sholl Analysis which, analysing individual cell neurite arborization and the cumulative area that the cells extend to [35]. Yet another one would be NeurphologyJ, which sums up overall neurite length irrespective of their origin [36]. These are all valuable neurite analysing plugins in ImageJ. For the purpose of acquiring differentiated N2a cells, significantly different enough from undifferentiated cells, for incorporation into the microglia-induced neurotoxicity model model, NeuronJ was selected. Prospectively, for a study solely focusing on detailed features of N2a differentiation, a more informative neurite tracing plugin can be employed. Although only two independent experiments were conducted for the neurite analysis using NeuronJ, since then, 0.5% FBS serum deprivation has been used to successfully differentiate N2a in a model of microglia-induced neurotoxicity and neurite length analyses were subsequently performed.

CONCLUSION

At the seeding density that we used ($1.3 \times 10^3/cm^2$), (i) N2a cells were unable to survive in 0.1% FBS, (ii) able to survive, adhere to culture surface and differentiate into traceable, measurable neurite-bearing cells in 0.5% FBS, but (iii) did not improve in their neurite features upon addition of 1 µM EC23. It must be underscored however, that other concentrations of EC23 were not tested in this study for their differentiating ability on N2a cells. Therefore, the possible synergistic differentiation effect of other concentrations of EC23 with serum deprivation on the N2a cells are not excluded. For the purpose of building a microglia-induced neurotoxicity model using differentiated N2a cells, serum deprivation to 0.5% FBS is sufficient for neurite tracing measurements without the need for addition of 1 µM of EC23.

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