

5FU Delivery through Biocompatible SF/PEG Nanoshuttles Modulates Colorectal Cancer Cells Migration and Invasion Potential and Alters the Inflammatory Cytokines Expression Profile [†]

Ariana Hudiță ¹, Ionuț Cristian Radu ², Cătălin Zaharia ², Octav Ginghină ^{3,4}, Bianca Gălățeanu ^{1,*} and Marieta Costache ¹

- ¹ Department of Biochemistry and Molecular Biology, University of Bucharest, 91-95 Splaiul Independentei Street, 050095 Bucharest, Romania; arianahudita@yahoo.com (A.H.); marieta.costache@bio.unibuc.ro (M.C.)
- ² Advanced Polymer Materials Group, University Politehnica of Bucharest, 1-7 Gh. Polizu Street, 011061 Bucharest, Romania; radu.ionucristian@gmail.com (I.C.R.); zaharia.catalin@gmail.com (C.Z.)
- ³ Department of Surgery, “Sf. Ioan” Emergency Clinical Hospital, 13 Vitan Barzesti Street, 042122 Bucharest, Romania; octav.ginghina@umfcd.ro
- ⁴ Department II, Faculty of Dental Medicine, “Carol Davila” University of Medicine and Pharmacy Bucharest, 17-21 Calea Plevnei Street, 010232 Bucharest, Romania
- * Correspondence: bianca.galateanu@bio.unibuc.ro; Tel.: +40-21-318-1575
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Abstract: The past few years have witnessed major developments in nanotechnology with great potential in powering new therapeutic tools for cancer management. Our goal in this study was to develop a biocompatible nanoshuttle for the efficient delivery of 5FU in colorectal cancer patients. Silk fibroin/Poly(ethylene glycol) nanoparticles (SF/PEG NPs) were obtained and further loaded with 5FU. These nanoshuttles were characterized in terms of morphological properties, size and size distribution, drug uptake and release potential as well as in vitro cytotoxicity potential screening. The SF/PEG + 5FU NPs cytotoxicity was determined on HT-29 cells after determination of the lethal dose 50 and targeted the evaluation of the cells viability, proliferation potential and migration and invasion potential. The inflammatory profile of RAW 264.7 macrophage cells was also determined by flow cytometry. The basic cytotoxicity screening revealed that the pristine SF/PEG NPs displayed good biocompatibility while the 5FU-loaded NPs induced cytotoxic effects on HT-29 cells. More, the 5FU-loaded SF/PEG NPs significantly reduced the migration and invasion processes as compared with the unloaded NPs. Lastly, we observed that the cytokine inflammatory profile was significantly altered after the treatment with the 5FU-loaded SF/PEG NPs as compared with the unloaded nanoshuttles.

Keywords: colorectal cancer; silk fibroin nanoparticles; drug delivery systems; migration and invasion; 5FU; inflammation

1. Introduction

The past few years have witnessed major developments in nanotechnology with great potential in powering new therapeutic tools for cancer management. Our goal in this study was to develop a biocompatible nanoshuttle for the efficient delivery of 5FU in colorectal cancer patients. Literature reports a wide range of other polymeric nanoparticles (NPs) developed as shuttles for antineoplastic therapy [1–3], however, there is still more need to elucidate safety aspects regarding their in vivo use.

5-fluorouracil (5FU) is a pyrimidine analog that interferes with thymidylate synthesis and belonging to the antimetabolite family of antineoplastic drugs. 5FU is still widely used in the treatment of cancer [3,4] despite its limitations regarding a short biological half-life, toxic side effects and non-selective action against healthy cells. To overcome these disadvantages, numerous researchers have attempted to modify its delivery by the use of polymeric nanoparticles.

Furthermore, polyethylene glycol (PEG) and silk fibroin (SF) have been previously studied as matrix materials for drug delivery systems due to their excellent biodegradability and biocompatibility.

In this context, we propose here the development and in vitro validation of a novel SF/PEG nanosized system for the efficient delivery of 5FU to colorectal cancer patients.

2. Experiments

2.1. FU-Loaded PEGylated Silk Fibroin Nanoparticle Synthesis and Characterization

The drug delivery systems were obtained via nanoprecipitation method based on *Bombyx mori* silk fibroin (SF) and chemical modification with polyethylene glycol (PEG). Based on the good solubility of the 5-fluorouracil (5FU) in the SF solution, the drug was loaded within the nanoparticles via direct dissolution in the polymer solution. The drug content (DC) was determined by the ratio of the mass of the 5FU loaded in nanoparticles to the total mass of the 5FU-loaded nanoparticles. The encapsulated amount of the drug was considered as the total amount added to the polymer solution. After evaporation of the solvent/non-solvent, the total amount of drug is supposed to remain in the nanoparticles.

2.2. Cell Cultures and Experimental Design

Human adenocarcinoma HT-29 colorectal cells (ATCCs) and mouse macrophages RAW 264.7 cells (ATCCs) were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin under humidified air, in standard cell culture conditions (37 °C, 5% CO₂). The culture medium was renewed every other day. When the confluence reached 80%, cells were routinely subcultured using enzymatic treatment with trypsin/EDTA solution for cellular detachment of HT-29 from culture vessels or by mechanical detachment in the case of RAW 264.7 macrophages.

2.3. Lethal Dose 50 Calculation (LD₅₀)

In order to investigate the cytotoxic potential of the 5FU PEGylated SF NPs and to determine the optimal concentration for further biological experiments, the MTT viability assay was performed. Briefly, HT-29 cells were seeded at a density of 5×10^3 cells/well in 96-well culture plates and incubated overnight to allow cellular attachment. The next day, the culture medium was discarded and replaced with the following dilutions of the 5FU PEGylated SF NP stock solution, prepared freshly: 20 mg/mL, 15 mg/mL, 12 mg/mL, 10 mg/mL, 8 mg/mL, 6 mg/mL, 4 mg/mL and 2 mg/mL. For the experimental controls, the culture media were refreshed. After 24 h of treatment, all the culture media were discarded and replaced with 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT solution (Sigma Aldrich), freshly prepared in FBS free culture medium. After 3 h of incubation at 37 °C, the MTT solution was removed and the resulting formazan crystals were solubilized in DMSO. The optical density (OD) of the resulting solution was measured at 550 nm using the FlexStation III microplate multimodal reader (Molecular Devices). The LD₅₀ was calculated considering the experimental control as 100% cell viability. Therefore, the LD₅₀ was the concentration corresponding to the mean optical density equal to half the mean optical density of the control.

2.4. Cell Viability

2.4.1. MitoTracker Assay

To evaluate the effect of simple and 5FU-loaded SF/PEG NP treatment on the cellular viability of HT-29 tumor cells in time, the MitoTracker assay was employed. In this view, HT-29 cells were seeded at a density of 1×10^5 cells/well in 12-well culture plates and treated after confirming cellular attachment with 12 mg/mL simple and 5FU-loaded SF/PEG NPs. After 24 h, 48 h and 72 h of treatment, the HT-29 cells were stained with MitoTracker™ Red CMXRos (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendation. At each experimental time, the culture medium and treatment solutions were removed and replaced with a prewarmed staining solution containing 100 nM MitoTracker. After 40 min of incubation in standard cell culture conditions, the staining solution was removed and cells were fixed with 4% paraformaldehyde (PFA) solution and permeabilized using a 2% bovine serum albumin (BSA)/0.1% Triton X-100 solution. Before the imagistic investigation of the HT-29 tumor cells, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The HT-29 monolayers were analyzed using the IX73 Olympus fluorescence microscope and CellSenseF software.

2.4.2. Live and Dead Assay

The effect of 5FU-loaded SF/PEG NPs on HT-29 cell survival was evaluated using the Live/Dead (Invitrogen). Briefly, HT-29 cells were seeded in 12-well culture plates at a density of 1×10^5 cells/well and treated the next day with simple and 5FU SF/PEG NPs. After 24 h, 48 h and 72 h of culture, the HT-29 monolayers were washed with PBS and stained with a fresh solution containing 2 μ M calcein AM and 4 μ M EthD-1 available in the staining kit. After 15 min of incubation at room temperature in the dark, the staining solution was replaced with PBS and the probes were investigated using the IX73 Olympus fluorescence microscope. Data were acquired in the CellSenseF software.

2.5. Migration and Invasion

The migration and invasion of tumor HT-29 cells exposed to simple or 5FU-loaded SF/PEG NPs were assessed using transwell chambers (8 μ m, Corning). For the migration assay, HT-29 cells were plated at a density of 5×10^4 cells/well into the upper chamber with FBS-free medium or with FBS-free medium with 12 mg/mL NPs. The lower chamber was filled with 20% FBS-supplemented serum. After incubation with the NPs for 48 h, the upper surface of the inserts was removed using a cotton swab, and then the inserts were fixed with 4% PFA and stained with 0.5% crystal violet solution. After the imagistic evaluation by contrast phase microscopy of the inserts, the migrative HT-29 cells were destained on a shaker with a 10% acetic acid solution for 20 min, and finally the optical density of the resulting solutions was measured at 590 nm with a microplate reader. For the invasion assay, the same experimental protocol was used, the only difference being that before HT-29 cell seeding, the upper chamber was precoated with Matrigel.

2.6. Inflammation Assay

The proinflammatory potential of the simple or 5FU-loaded SF/PEG NPs was investigated using the RAW 264.7 cell line. Therefore, the RAW 264.7 cells were seeded at a density of 5×10^3 cells/well in 96-well culture plates, cultured 24 h and then deprived of FBS medium for an additional day. Afterward, the macrophages were treated with simple and 5FU-loaded SF/PEG NPs and LPS (20 mg/mL) for 24 h, the time point where the culture media were collected. The culture media samples were investigated by flow cytometry using a bead-based multiplex assay (BD CBA Inflammation Kit) designed for the analysis of the following cytokines' protein levels: Interleukin-6 (IL-6), Interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), Interferon- γ (IFN- γ), tumor necrosis factor (TNF) and Interleukin-12p70 (IL-12p70). Briefly, 50 μ L sample was incubated for 2 h at room temperature and darkness with 50 μ L of IL-6, MCP-1, IFN- γ , TNF- α and IL-12p70

mixed capture beads and 50 μ L inflammation PE detection reagent. Each sample was prepared in triplicate for statistical significance data analysis. A standard curve was prepared at the same time by adding in the control tubes 50 μ L of inflammation standard dilutions instead of the samples. After a wash step, all tubes were analyzed in a Cytoflex (Beckman Coulter) flow cytometer using CytExpert Software (Beckman Coulter) for sample acquisition and data analysis.

3. Results

3.1. PEGylated Silk Fibroin Samples Loaded with 5FU

SF/PEG NPs were obtained and further loaded with 5FU. The encapsulation efficiency for these NPs was determined to be 88%, while 89% of the incorporated 5FU is released in 4 h in simulated biological fluids.

3.2. Determination of LD₅₀

In order to determine the working dose, an MTT assay was employed to screen a range of eight concentrations in terms of cell viability after 24 h of treatment. As shown in Figure 1, half of the cells' viability was displayed by the HT-29 cells exposed to 12 mg/mL SF/PEG NPs + 5FU.

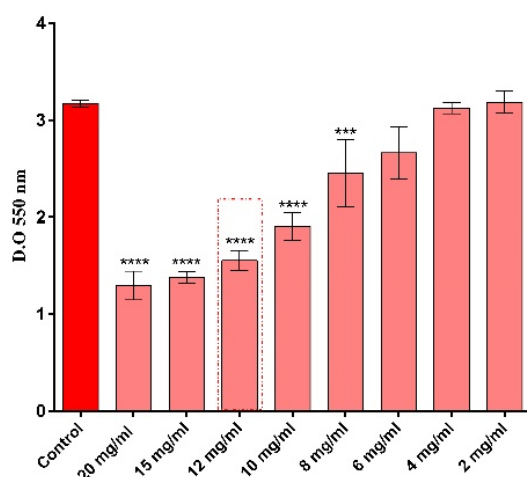


Figure 1. Graphical representation of cell viability after 24 h of treatment with 20 mg/mL, 15 mg/mL, 12 mg/mL, 10 mg/mL, 8 mg/mL, 6 mg/mL, 4 mg/mL and 2 mg/mL silk fibroin/poly(ethylene glycol) nanoparticles (SF/PEG NPs) + 5-fluorouracil (5FU) vs. an untreated sample, as revealed by the MTT assay. (***p* < 0.001, *****p* < 0.0001).

3.3. HT-29 Cells' Viability

3.3.1. MitoTracker Assay

The HT-29 tumor cells were stained with MitoTracker dye in order to label mitochondria within live cells after 24 h, 48 h and 72 h of treatment with 12 mg/mL SF/PEG NPs \pm 5FU. The obtained results are presented in Figure 2a and show that both control HT-29 cells and pristine SF/PEG NP-treated HT-29 are viable, showing a strong positive signal for the mitochondrial staining. In HT-29 cell cultures treated with 5FU-loaded SF/PEG NPs, a decrease of the mitochondrial membrane potential is observed even after 24 h of treatment. The 5FU-loaded drug delivery system treatment triggered a reduction of the mitochondrial density as compared with the control HT-29 cells and simple SF/PEG treatment. After 72 h of treatment, only a small number of cells were positive for the MitoTracker staining, showing that the viability of the HT-29 cells decreases after SF/PEG NPs in a time-dependent manner.

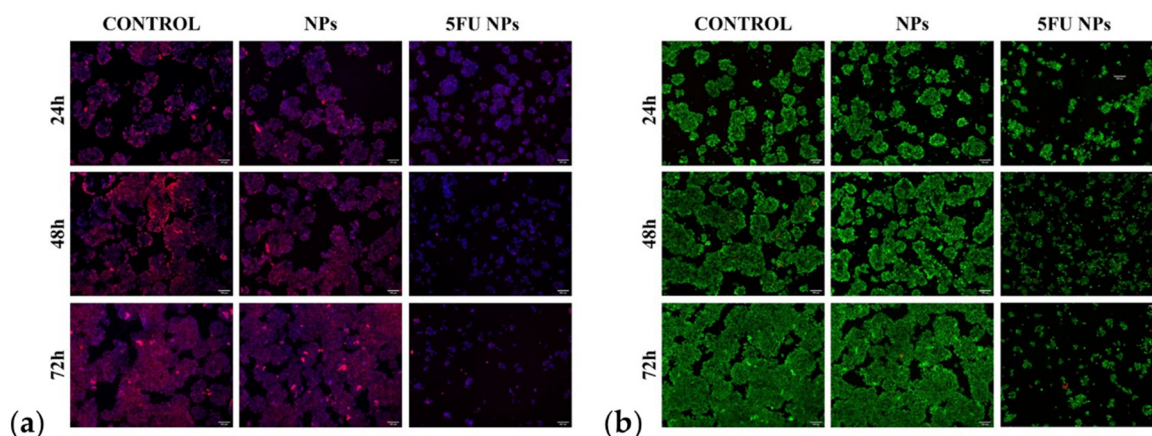


Figure 2. Fluorescence microscopy images of HT-29 adenocarcinoma cells treated for 24 h, 48 h and 72 h with SF/PEG NPs ± 5FU and stained with (a) MitoTracker™ Red CMXRos for MitoTracker assay and (b) Calcein AM (green fluorescence) and EthD-1 (red fluorescence) for Live/Dead assay.

3.3.2. Live/Dead Assay

Furthermore, the HT-29 monolayers were stained with Calcein AM and EthD-1 for Live/Dead assay after 24 h, 48 h and 72 h of treatment with 12 mg/mL SF/PEG NPs ± 5FU. The fluorescence microscopy images obtained are presented in Figure 2b and show that cells did proliferate both in the control as well as in the sample treated with pristine SF/PEG NPs. More, cell viability between these two samples was similar during the experimental time frame. In contrast, when treated with 5FU-loaded SF/PEG NPs, HT-29 cells' viability and proliferation potential dramatically decreased. The fluorescence microscopy images show that the culture did not display specific clusters as shown in the untreated control.

3.4. Migration and Invasion

In order to evaluate the motility of the HT-29 tumor cells exposed to SF/PEG NPs ± 5FU treatment, the transwell assay was employed. As shown in Figure 3a, the Matrigel cell invasion assays revealed that the simple SF/PEG NPs had no effect on HT-29 invasive potential, while the 5FU-loaded SF/PEG NP treatment significantly inhibited the invasion potential of the HT-29 tumor cells. Moreover, the same pattern was also observed in the migration assay, where the HT-29 tumor cells' capacity to migrate was severely affected by the 5FU SF/PEG NP treatment.

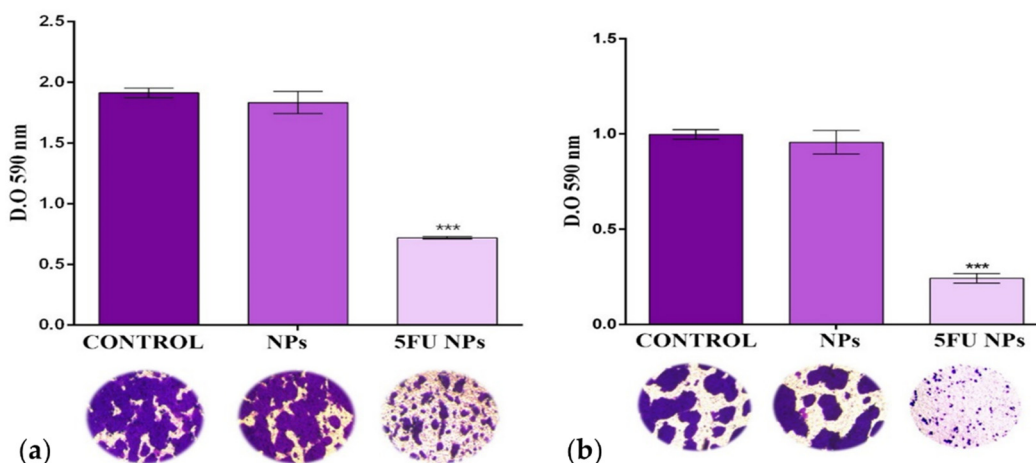


Figure 3. Graphical representation of the crystal violet staining of the (a) invasive HT-29 cells and (b) migrative HT-29 cells after 48 h of treatment with SF/PEG NPs ± 5FU. (***) $p < 0.001$.

3.5. Inflammation Assay

To evaluate the inflammatory potential of the SF/PEG NPs, RAW 264.7 cells were used as the *in vitro* model. The cells were treated both with pristine and 5FU-loaded SF/PEG NPs, and the results were compared with those obtained by analyzing an untreated sample and an LPS-stimulated culture. All the data obtained are represented in Figure 4 and show that the treatment with LPS induced an efficient stimulation of the cells, with elevated levels of IL-6, IL-10 and TNF α . The treatment with unloaded SF/PEG NPs did not induce any modification in the expression of these cytokines as compared with the untreated control. In contrast, the SF/PEG NPs + 5FU significantly increased the expression of IL-6 and TNF α as compared with the control. No treatment altered the expression of IL-10, except the LPS stimulation.

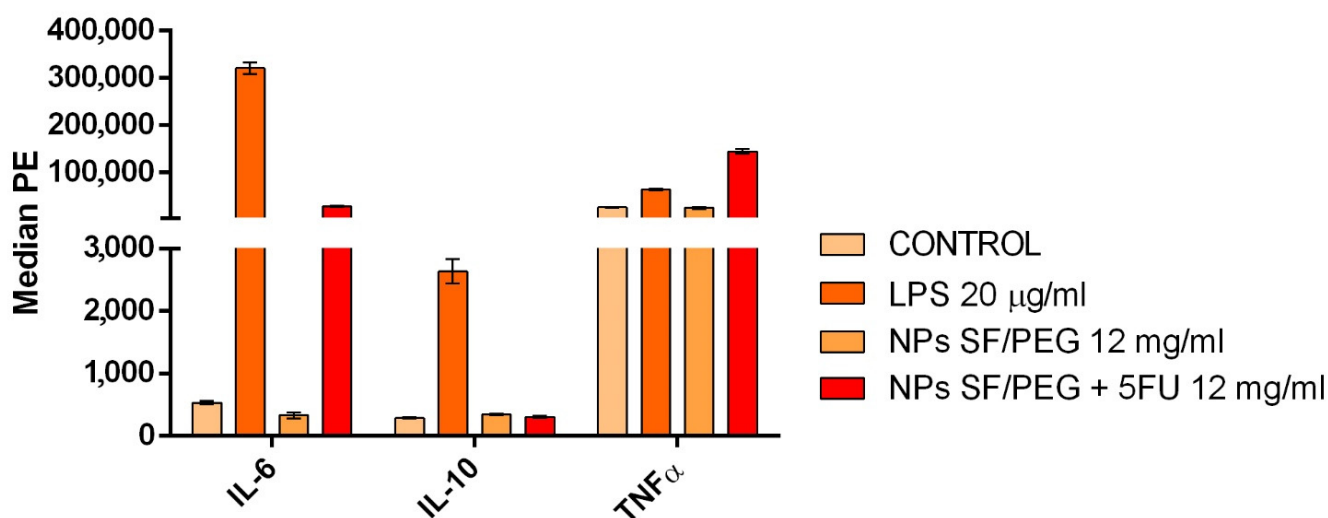


Figure 4. Graphical representation of the protein expression levels of IL-6, IL-10 and TNF α after the treatment of RAW 264.7 cells with SF/PEG NPs \pm 5FU as compared with a reference sample and an LPS-stimulated control.

4. Discussion

In this study, we aimed to investigate the cytotoxic potential of the developed 5FU-loaded SF/PEG NPs on HT-29 adenocarcinoma cells for validating a potential efficient delivery of the antineoplastic drug to colorectal cancer patients. After confirmation of the drug encapsulation efficiency (88%) and release potential (89% in 4 h), the LD₅₀ was determined at 12 mg/mL and further used in the study. Both cell viability MitoTracker and Live/Dead assays performed at 24 h, 48 h and 72 h of treatment showed that the pristine SF/PEG NPs display good biocompatibility on HT-29 cells: they do not alter cells' viability or proliferation potential. In contrast, when loaded with 5FU, the treatment becomes cytotoxic with visible effects as soon as 24 h of treatment. The 5FU-loaded SF/PEG NP treatment inhibits the colorectal cancer cell migration and invasion by decreasing severely the cell motility after 48 h of exposure to the treatment. More, we investigated the inflammatory cytokine modulation potential in a macrophage cell line. After confirming the stimulation of the cells, we observed that only the 5FU-loaded SF/PEG NPs were able to increase the expression on IL-6 proinflammatory cytokine and tumor necrosis factor (TNF α) as compared with an untreated control. These results indicate that the treatment could be able to stimulate macrophage cells to produce an efficient inflammatory response against tumor cells and act directly on the colorectal adenocarcinoma cells by decreasing their viability, proliferation, invasion and migration potential.

5. Conclusions

In conclusion, our results show that the proposed formulation of 5FU displays significant efficiency on the HT-29 cell line and might be a promising delivery system for the targeted delivery of the antineoplastic drug in vivo.

Author Contributions: M.C. and C.Z. conceived and designed the experiments; A.H. and I.C.R. performed the experiments; B.G. and O.G. analyzed the data; B.G. and A.H. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

SF	silk fibroin
PEG	polyethylene glycol
5FU	5-fluorouracil
DC	drug content
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum
NPs	nanoparticles
SF/PEG NPs	PEGylated silk fibroin nanoparticles
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	optical density
LD50	lethal dose 50
PFA	paraformaldehyde
BSA	bovine serum albumin
DAPI	4',6-diamidino-2-phenylindole
IL-6	Interleukin-6
IL-10	Interleukin-10
MCP-1	monocyte chemoattractant protein-1
IFN- γ	Interferon- γ
TNF	tumor necrosis factor
IL-12p70	Interleukin-12p70

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