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HOXB7 siRNA Delivered by Hybrid Nanoparticles and the Co-Therapy with Tamoxifen: Promising Strategy against Hormone Receptor-Positive Breast Cancer †

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Abstract: Breast cancer is the most common type of cancer that affects and kills women annually in the world. It impacts more than two million women and is responsible for the death of approximately 25% of them. Almost 70% of breast cancer diagnosis are positive for hormone receptor and have a good prognosis. However, resistance to drugs used in hormone therapy, such as tamoxifen, is usual and about 40% of recurrences do not respond to it. In some cases, the overexpression of *HOXB7* gene is related to this mechanism and its silencing can reverse the response to Tamoxifen. Here we used copolymer-coated calcium phosphate nanoparticles to deliver *HOXB7* siRNA and restore the sensitization of MCF7 cells to Tamoxifen. Nanoparticle synthesis and characterization were performed and cell viability and gene expression were evaluated. Hybrid nanoparticle presented Z-average diameter of 83 nm and polydispersity index (PdI) of 0.07, while showing good entrapment of siRNA molecules. We also observed a decrease in *HOXB7* gene expression (~65%) promoted by the siRNA molecule delivered by the nanoparticles. The gene silencing has good correlation to the cell viability assay: a reduction in breast cancer viability was observed in 48 (31%) and 72 (38%) hours. As for the co-treatment with tamoxifen, cell viability started dropping after 15 h, which did not occur in the treatment only with Tamoxifen at the same concentration. This result indicates that the biological effect was possibly related to RNAi effect and suggests that *HOXB7* may be promoting cell sensitization to Tamoxifen without reducing cell viability. Overall, these results suggest that the nanostructured system was effective in promoting gene silencing and that the co-therapy can be a promising tool for the treatment of hormone receptor-positive breast cancers.

Keywords: RNAi therapy; calcium phosphate; delivery system

1. Introduction

Cancer is the second worldwide leading cause of fatalities, responsible for one out of six deaths in the world. This corresponds to almost 10 million decease per year due cancer [1]. Breast cancer is

the most common type of cancer that affects and kills women around the world [2]. Hormone receptor-positive breast cancer types Luminal A and B are responsible for 70% of breast cancers diagnoses [3].

Several genes are involved in vital stages of breast cancer. Homeobox genes encode transcription factors that play a crucial role in several processes of embryogenesis [4]. Changes in the expression of members of this family are already known to cause neoplastic anomalies [5]. Among homeobox genes, *HOXB7* is known to be overexpressed in some cancer cell lines (melanoma, ovarian, breast cancer and others) and acts in several metabolic pathways of cancer such as cell proliferation, angiogenesis, invasion, DNA repair, cell survival and drug resistance [6–9]. Studies show the relationship between overexpressed-*HOXB7* and resistance of estrogen receptor-positive tumors to Tamoxifen (TAM) [10,11]. TAM is a selective estrogen receptor modulator that acts as its antagonist in the breast tissue, blocking the signaling cascade of cell proliferation triggered by the binding of estrogen to its receptor [12]. HOXB7 promotes TAM resistance playing a role in two main pathways: EGFR and ER [10,11]. Thus, overexpression of *HOXB7* promotes *HOXB7* is correlated with clinical progression, poor outcome of breast cancer patients [10,13] and shorter relapse-free survival [14]

Targeted therapies such as RNA interference (RNAi) therapy provide new perspectives for the treatment of several diseases, including breast cancer [15]. This became more tangible with the approval of the first RNAi-based medicine ONPATTRO® by the USA Food and Drug Administration, 20 years after the elucidation of the RNAi mechanism by Fire and co-workers [16]. RNAi therapy is based on this mechanism, where small RNA molecules (siRNA) sequence-specifically binds to messenger RNAs (mRNAs), resulting in the cleavage and degradation of the targeted mRNA to inhibit protein synthesis [15].

A combined therapy of subtoxic concentrations of TAM with *HOXB7* silencing is a promising therapy against ER-positive breast cancer [11]. Therefore, in this study hybrid nanoparticles were adapted to carry siRNA molecules for *HOXB7* gene silencing in MCF7 human breast cancer cells. The RNAi effect alone and combined to tamoxifen was evaluated.

2. Materials and Methods

2.1. Materials

Calcium chloride and tamoxifen were purchased from Sigma Aldrich. Dibasic sodium phosphate was purchased from Proquimios. Tris (hydroxymethyl) aminomethane (TRIS) and (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) (HEPES) were acquired from Synth. Block copolymer poly(ethylene glycol)-block-poly-L-glutamic acid (PEG-p(Glu)) was purchased from Alamanda Polymers, Inc. Roswell Park Memorial Institute culture medium (RPMI), fetal bovine serum (FBS) and trypsin were acquired from Gibco®. Trypan blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) acquired from Invitrogen. RNeasy® Plus Mini Kit was purchased from Qiagen. High Capacity cDNA Reverse Transcription kit purchased from Applied Biosystems. The qPCR-SYBR-Green mix was acquired from Ludwig Biotecnologia Ltd.a. The siRNA and primer sequences were synthesized by Sigma-Aldrich. HOXB7 siRNA sequences (5′-3′): ACCUACCACUCGCGUGUUC[dT][dT] (sense sequence) and GAACACGCGAGUGGUAGGU[dT][dT] (antisense sequence). Scrambled siRNA sequences (5′-3′): GGAGUCGACGAGCAUAGGU[dT][dT] (sense sequence) and ACCUAUGCUCGUCGACUCC[dT][dT] (antisense sequence). Primer sequences (5′-3′): CCAACCGCGAGAAGATGA (*β-actin* forward), CCAGAGGCGTACAGGGATA (*β-actin* reverse), GCCTACAAATCATCCGGCCA (*HOXB7* forward) and GGTTGGAAGCAAACGCACAA (*HOXB7* reverse).

2.2. Preparation of Hybrid Nanoparticles

A 2.5 M $CaCl_2$ solution was diluted in 10 mM Tris buffer (pH 10) to final concentration of 0.2 M. Another solution containing PEG-PGlu in 50 mM/15 mM Hepes-phosphate buffer (pH 7.2) was

mixed with siRNA solution to obtain 3 µM of siRNA. The former solution was homogenized with the latter solution for around 30 s. *Mock* nanoparticles were prepared by replacing the siRNA for Hepes-phosphate buffer. Each sample solution was used immediately after preparation. All solutions used were previously sterilized.

2.3. Nanoparticle Physicochemical Characterization

2.3.1. Dynamic Light Scattering (DLS)

The determination of average hydrodynamic diameter (Z-average), polydispersity index (PdI) and size distribution of the hybrid nanoparticles were performed by the dynamic light scattering (DLS) technique. DLS measurements were carried out at 25 °C using Zetasizer Nano Z (Malvern Instruments, Malvern, UK) with a He-Ne laser (633) as incident beam and detection angle of 173°.

2.3.2. Zeta Potential

Zeta potential (ZP) values were determined using the electrophoretic mobility technique. These measurements were performed using Zetasizer Nano Z (Malvern Instruments, UK) equipment with an established potential of ±150 mV.

2.3.3. Transmission electron microscopy (TEM)

The morphology analysis was carried out by transmission electron microscopy observations using JEM-1011 (Jeol Ltd., JP, Tokyo, Japan) operated at 80 kV acceleration voltage. Briefly, 2 µL of nanoparticle suspension were placed on amorphous carbon-coated Parlodion® 200 mesh (CF200-Ni EMS) nickel grids and then dried out for 24 h at room temperature. The microscope was operated in bright field mode at a magnification of 50.000×. ImageJ software was used for image processing.

2.3.4. Determination of siRNA Encapsulation in Hybrid Nanoparticles

The estimated amount of siRNA encapsulation in hybrid nanoparticles was evaluated indirectly by the ultrafiltration/centrifugation technique. Briefly, the nanoparticle suspension was transferred to an Amicon ® 10.000 MW device (Millipore, Germany) and centrifuged at 15.000 rpm for 20 min. The filtered sample was collected to determine the non-encapsulated siRNA concentration by measurement of absorbance at 260 nm in NanoDrop Lite Spectrophotometer. The percentage of loaded siRNA was calculated using the following formula:

(1) Encapsulated percentage (%) = total drug content – free drug/total drug content × 100

2.4. Cell Viability Assay

Human breast cell line, MCF7 (ER+, PR+/−, HER2−; ATCC number: HTB-22) were seeded in a 96-well plate (5.000 cells/well) and incubated for 24h at 37 °C under 5% CO_2. Nanoparticle suspensions containing siRNA (10–150 nM siRNA) and controls were added with fresh medium and the cell viability was evaluated after 20 h, 48 h and 72 h incubation by the MTT assay [17]. The absorbance was measured at 540 nm.

The co-treatment NP-siHOXB7 and TAM followed similar proceedings. NP-si*HOXB7* (final concentration 100 nM) and TAM at different concentrations (0.3–30 µM) were added at the same time to the plate and analyzed after 15 h.

2.5. Real-Time PCR (qPCR)

MCF7 cells were cultured on a 6-well plate at a density of 1×10^6 cells/well. Fresh medium with hybrid nanoparticles containing siRNA (si*HOXB7* or *siScr*) or free si*HOXB7* were applied to each well to a final siRNA concentration of 150 nM. After 20 h, the cells were harvested and RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The extracted RNA was quantified and standardized for cDNA synthesis using High Capacity cDNA

Reverse Transcription kit (Applied Biosystems, Foster City, California, FL, USA). Real-time PCR was carried out using StepOne Plus Real-Time (Applied Biosystems, USA) and qPCR-SYBR-Green mix (Ludwig Biotecnologia Ltd.a, Alvorada, Brasil). *Beta actin* was used as reference gene and the obtained data were normalized before statistical analysis.

2.6. Statistical Analysis

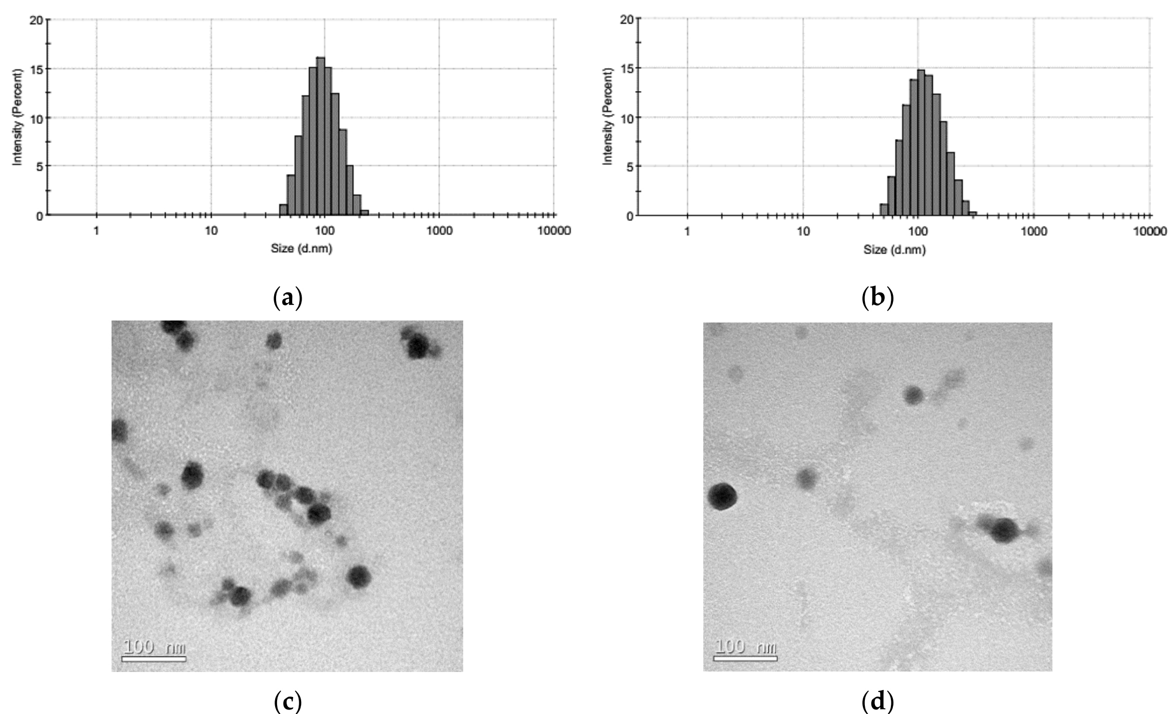
Analysis of variance (ANOVA) followed by Tukey post-hoc test was performed to test the treatment effects and compare individual treatment groups, respectively, using the software GraphPad Prisma 5.0 (GraphPad Software, Inc., San Diego, CA, US). Statistical significance is represented as * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$. The results reported were expressed as mean values (±SEM).

3. Results and Discussion

3.1. Preparation and Characterization of Hybrid Nanoparticles

Hybrid nanoparticles carrying siRNA sequences complementary to HOXB7 gene (NP-siHOXB7) and empty hybrid nanoparticles (NP-mock) were prepared by self-assembly of inorganic and organic components. According to the size distribution histogram weighted by intensity (Figure 1a,b), the formulations NP-siHOXB7 and NP-mock presented Z-average of 87.9 ± 0.54 and 104.96 ± 0.23 nm and PdI of 0.100 ± 0.005 and 0.123 ± 0.004, respectively. Also, TEM images were obtained and showed a homogeneous and spherical morphology (Figure 1c,d).

In addition, the histogram showed an unique narrow pick with hight intensity distribution, implying a monodispersed suspension. This assumption was confirmed by the low number of PdI of approximatley 0.1 for both formulations [18]. Similar results were obtained by other authors that used PEG-polyanion calcium phosphate nanoparticles [19–21].



(**a**) (**b**) (**c**) (**d**)

Figure 1. Size distribution histogram of hybrid nanoparticles and TEM images. (**a**) Histogram of hybrid nanoparticles carrying siRNA; (**b**) Histogram of empty hybrid nanoparticles; (**c**,**d**) TEM images of hybrid nanoparticles.

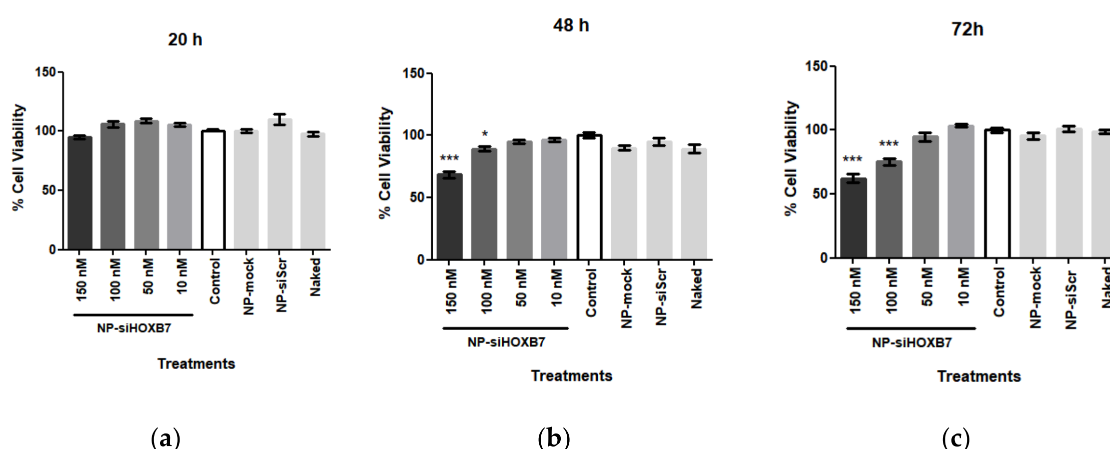
In addition to the size, PdI and morphology, Zeta potential (ZP) measurements revealed a neutral-like charged suspension of 0.015 ± 0.04 and −1.28 ± 0.1 for NP-siHOXB7 and NP-mock, respectivly. This neutral ZP was expected due to the presence of the PEG corona on the surface of the nanoparticle and it is considered an advantageous feature since it prevents non-specific interactions between nanoparticles and culture medium components [25]. Even more, the hydrophlic PEG corona promotes steric stabillization which prevents nanoparticle aglomeration and cacium phosphate crystals growth [19].

Encapsulation efficiency (EE) was analyzed by espectrophotometry by an indirect method using the ultrafiltration/centrifugation technique. The percentag of EE was approximatley 65%. The percentage of encapsulated siRNA is inversely proportional to the PEG-polyanion concentration [23]. This relationship is due to the competition between Ca^{2+} ions and the polyanionic block and siRNA, both negatively charged [19,24].

3.2. Cell Viability and Gene Knockdown

MCF7 human breast cancer cell line incubated with NP-siHOXB7 were analyzed by MTT assay to evaluate citotoxic effect with and without TAM co-treatment. Figure 2 shows cell viability 20, 48 and 72 h after application of NP-siHOXB7 in four different siRNA concentrations: 10, 50, 100 and 150 nM. Treatments emplying hybrid nanoparticle with siSramble (NP-siScr), empty nanoparticle (NP-mock) and free siHOXB7 (naked siRNA) were used as controls.

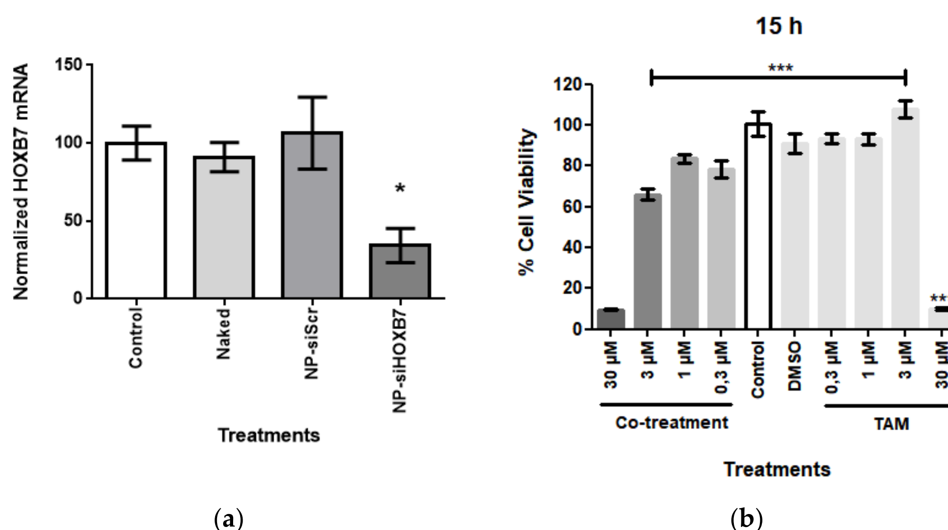
Cell vability was dependent of incubation time and concentration. None of the mentioned treatments and controls showed cytotoxicity after 20 h of incubation. However, after 48 h a reduction of 31% ($p < 0.001$) in cell viability was observed at 150 nM siRNA. After 72 h, the reduction increased to 38% ($p < 0.001$) at the same concentration. The same pattern was observed for the treatment with 100 nM siHOXB7. After 48h, viability reduced 12.8%, while in 72h the reduction reached 20%. Ma and co-worker [18] also observed reduced cell viability after siRNA (400 nM) treatment in 48h.



(**a**) (**b**) (**c**)

Figure 2. Cell viability evaluation after NP-si*HOXB7* treatment on MCF7 breast cancer cells at (**a**) 20 h ; (**b**) 48 h; (**c**) 72 h. One-way ANOVA followed by Tukey test, n = 6, * $p < 0.05$, *** $p < 0.001$, compared with control. NP-si*HOXB7* = hybrid nanoparticle carrying si*HOXB7*; NP-si*Scr* = hybrid nanoparticle carrying scrambled siRNA; NP-mock = empty hybrid nanoparticle; Naked = free si*HOXB7*.

HOXB7 siRNA sequences were loaded into hybrid nanoparticles and the *HOXB7* expression was evaluated after 20 h of incubation by quantitative PCR. The result showed a reduction of approximately 65% ($p < 0.05$) of the gene expression (Figure 3a). The expressive silencing rate can be related to a high encapsulation rate of siRNA molecules by the nanoparticles and its effectiveness in acting as transfetion agent to MCF7 cells. This result also shows that the designed siRNA sequence was effective in promoting gene silencing.



(**a**) (**b**)

Figure 3. (**a**) Knockdown of *HOXB7* gene expression in human ER-positive breast cancer cells after 20 h incubation with NP-si*HOXB7* (one-way ANOVA followed by Tukey test, n = 3, * $p < 0.05$, compared with control). (**b**) Cell viability evaluation after NP-si*HOXB7* and tamoxifen co-treatment on MCF7 breast cancer cells at 15 h. One-way ANOVA followed by Tukey test, n = 6, ** $p < 0.01$, *** $p < 0.001$, compared with free TAM. NP-si*HOXB7* = hybrid nanoparticle carrying si*HOXB7*; NP-si*Scr* = hybrid nanoparticle carrying scrambled siRNA; Naked = free si*HOXB7*; TAM = tamoxifen.

HOXB7 is a gene that encodes a transcriptor factor that acts on several methabolic pathways in cancer cells proliferation and suvival, which makes the silencing of this gene relevant as a RNAi treatment option. The qPCR result explains what was observed in cell viability experiments. At 20 h, there was *HOXB7* gene silencing (Figure 3a), without affecting cell viability as observed in the 20 h MTT assay with NP-siHOXB7 treatment (Figure 2a). Over the time, we could observe the reduction in viability, possibly due to the consumation of already produced *HOXB7* protein associated to the gene knockdown.

Noteworthy, NP-*mock* and NP-*siScr* treatments did not presented cytotoxic effects in any incubation period, assuring that the used hybrid nanosystem *per se* is biocompatible and do not present cell toxicity, as also shown by other authors [20–22]. Therefore, we can infer that the cell viability reduction is entirely due to sequence-specific silencing of HOXB7 gene by siRNA molecule.

Figure 3b shows the results of co-treatment of NP-*siHOXB7* at 100 nM combined with different TAM concentrations (0.3–30 µM). In 15 h incubation time there was significat difference of cell viability between TAM treatment and co-treatment with NP-*siHOXB7* at TAM concentration of 3 µM. The difference between both treatments was a reduction of about 40% ($p < 0.001$) in cell viability. Noteworthy *HOXB7* gene silencing would be sensitizing MCF7 cells to TAM without reducing cell viability as observed in NP-*siHOXB7* treatment alone (Figure 2a). The decreased gene expression effect is also observed in co-therapy cell viability assay at 15 h (Figure 3b). Therefore, this result also supports a sensibilization of MCF7 cells to tamoxifen due to *HOXB7* silencing.

Some authors have showed HOXB7 gene knockdown in order to better understand its action on cancer pathways. Gene silencing was observed by Ma et al. [18] at a siRNA final concentration of 200 and 400 nM and even higher silencing rates (>80%) were calculated by Wu et al. [7]. However, transfection agents used by these and other studies [7–8,10,18] are toxic and demands change of culture medium periodically [22]. Other reports used retroviral vectors as transfection agents to carry a shRNA encoder plasmid as gene silencing strategy, which demands a different intracellular pathway since it has to be delivered inside cell nucleus [23]. Here, we succesfully tested a non-toxic effective transfection agent based on biocompatible components for *HOXB7* siRNA delivery to breast cancer cells.

Together, these findings demonstrates the relationship between *HOXB7* gene and TAM resistance in ER-positive breast cancer that has yet to be better elucidated. However, they reassure

that combined treatments are a promissing strategy for anticancer therapy, especially related to tamoxifen resistence.

4. Conclusions

In this work, the nanoparticle formulation used presented suitable physicochemical characteristics, showed to be an effective transfection agent and a key tool to siRNA deliver in breast cancer cells and gene silencing success. In this way, *HOXB7* silencing promoted a reduction in cell viability. Even more, *HOXB7* silencing enhanced the efficacy of tamoxifen treatment by promoting breast cancer cells sensitization in subtoxic concentrations. Here, we demonstrated that the in vitro knockdown of *HOXB7* gene by siRNA delivered by PEG-polyanion-coated hybrid nanoparticles combined with tamoxifen is a promising tool for ER-positive breast cancer treatment and should be analyzed in vivo in future research.

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

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