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DRUG REPURPOSING OF CEFTRIAXONE FOR ITS NEUROPROTECTIVE ACTIVITY IN ALZHEIMER'S DISEASE MODEL

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ABSTRACT

Neurodegenerative diseases are a group of diseases characterized by neuronal degeneration leading to dementia, movement disabilities and disturbing overall quality of life. Alzheimer's disease, AD is one of the neurological disorder majorly effecting the elderly population. The pathophysiological mechanisms include beta amyloid plague and tau protein accumulation causing neurofibrillary tangles. Even though many drugs are available to treat AD there are drawbacks like limited clinical efficacy, adverse drug reactions, decreases blood brain barrier penetration issues associated with the drugs. Multifactorial nature of the disease also is a reason for the failure of existing drugs in some patients. Drug repurposing is a promising tool for drug discovery due to the decreased time required for its safety and efficacy testing rendering it a good option for different targets. Here Ceftriaxone is studied for its neuroprotective activity. MTT assay is performed on neuroblastoma cells treated with beta amyloid. Ceftriaxone in increasing concentrations showed good neuroprotective profile making it a good candidate for anti Alzheimer's drug.

KEYWORDS: Ceftriaxone, Neuroprotective, Alzheimer’s Disease, Beta Amyloid.

INTRODUCTION

Neurodegenerative diseases are characterized by the progressive loss of structure and function of neurons, these loss of structure and function lead to the death of neurons. These disorders primarily affect the central nervous system and result in impairments of movement, cognition, and other neurological functions.[1] Examples include Parkinson's disease, which is a progressive disorder that affects motor function, causing tremors, rigidity, and bradykinesia; Alzheimer’s disease, which leads to memory loss, cognitive decline, and behavioral changes; and motor neuron disease,

they cause weakness, difficulty speaking, swallowing, and eventually paralysis. Each neurodegenerative disease has a distinct pathophysiological mechanism; for instance, Alzheimer's involves amyloid plaques and neurofibrillary tangles.[2]

ALZHEIMER'S DISEASE

Alzheimer's Disease is a chronic illness characterized by Dementia. Dementia is a progressive decline in memory cognition language and behavior that interferes with daily functioning.[3] It has preclinical and prodromal phase (20 years) and average clinical duration of 8-10 years. Age is the main risk factor of Alzheimer's disease. Risk increases sharply after 65 years. Rare in young adults. very common in people over 75-80 years.[4]

PATHOPHYSIOLOCAL MECHANISMS OF ALZHEIMERS DISEASE

1. Amyloid-beta Aggregation

In Alzheimer's disease a protein named Amyloid precursor protein breaks abnormally by the two enzymes β and γ secretases which produce harmful Aβ peptides, especially Aβ42.which forms oligomers, then larger than fiber and at last produce plaques which are toxic to brain cell. They disturb synaptic function, increase oxidative stress and mitochondrial dysfunction and promote neuronal death.

2. NFT formation due to tau protein hyperpolarisation

In brain cells Tua protein is present. Its main function is to support and stabilize the nerve cell. When a person has Alzheimer's disease Tua protein becomes abnormal because it gets too many phosphate groups also called hyperpolarisation. This change cause separation of Tua from the microtubules and sticks each other to form clumps known as neurofibrillary tangles.

3. Synaptic loss, neurodegeneration, selective vulnerability

Memory loss and thinking problems in Alzheimer's disease mainly due to the loss of connection between brain cells. Neurons in some regions are more sensitive which damage more easily than other regions. Damage is due to mitochondrial dysfunction, impaired lysosomes, and excitotoxicity.

4. Activation of glial cells and neuroinflammation.

Long term activation of glial cells causes neuronal damage. Glial cells react with Aβ and tau cause release of reactive oxygens, inflammatory chemical.

5. Genetic and molecular factors

Studies conducted show that genes increase the risk of Alzheimer's disease. Genes are working together, that's why it's known as genetically complex disease. most important genes are APOE, TREM2, ABCA7.[5]

CLINICAL FEATURES AND DIAGNOSIS

In Alzheimer's disease initially it occurs with memory loss a subtle one and later it affects the other cognitive domains. When the disease moves into the chronic effect it can also affect behavioral and psychological symptoms like agitation and apathy may occur.[6]

Nowadays, along with clinical assessment they start to use biomarkers for example cerebrospinal fluid, PET Imaging. With the biomarkers they are also using neuroimaging. The definitive diagnosis which is based on neuropathological still depends upon post-mortem examination.[7]

THERAPEUTIC CHALLENGES AND CURRENT STATUS

Even after decades of research in this filed there is still not proper cure for the Alzheimer disease. There are some sort of available therapies like cholinesterase inhibitor and NMDA receptor antagonist. Even though these therapies are available they only provide with the initial relief, but these can't halt disease progression. In the late stage of clinical trials there are lot of failed therapies targeting Aβ or tau, these failures raise the question like the heterogenicity of the AD pathology and the complexes in disease mechanism.[8]

In early detection it states that the biomarkers can be used in this disease including immunotherapy and modulation in inflammation. Currently there is new approaches like next generation amyloid and tau antibodies, drugs targeting neuroinflammation, metabolism, vascular health and synaptic function.[9]

Drawbacks of present Anti-Alzheimer's drugs

1. **Clinical efficacy is limited**

Drugs only show symptomatic benefits do not prevent the disease progression.

2. **Lack of disease-modifying action**

Does not any action in underlying pathology .it only target on neurotransmitter levels.[10]

3. **Safety and adverse reaction**.

Some of the currently approved drug have adverse effect for example cholinesterase inhibitors have adverse effect of gastrointestinal effects, bradycardia, insomnia.

4. **Blood brain barrier BBB penetration issues**

Many of the drug have low penetration in blood brain barrier. This will reduce the efficacy of the drug due to low brain bioavailability.[11]

5. **Poor Accessibility and high cost**

Approved monoclonal antibodies are expensive, require IV infusion, MRI monitoring which are not widely accessible.[12]

Ceftriaxone- Drug profile

Ceftriaxone is a semisynthetic cephalosporin third generation drug with long half-life. Recommended once daily administration. Administrated in both intravenously and intramuscularly. Has broad spectrum activity against both gram positive and gram-negative bacteria. It has a lot of activity than first and second cephalosporins against gram negative bacteria. Ceftriaxone administrated in intravenous and intramuscular are generally tolerated by adults and children. Mostly reported adverse effects are diarrhea, rash or pruritus recommended dose for adults is 1-2g once daily.[13] in case of severe infections and pathogens are only moderately sensitive to ceftriaxone dosage may be increased. recommended dose for infants and young children is 20-80 mg\kg. For the study of neuroprotective activity ceftriaxone is chosen because it is proven for disease modifying potential in neurodegenerative disorders and neuroprotectivity Beyond its

antibacterial action, ceftriaxone exhibits significant neuroprotective properties.[14] It upregulates the glutamate transporter EAAT2 (GLT-1), thereby reducing glutamate excitotoxicity, which plays a major role in neuronal damage in Alzheimer's disease. In addition, ceftriaxone decreases oxidative stress and neuroinflammation and enhances neuronal survival.

Studies by Yimer et al. (2019) and Tikhonova et al. (2021) have demonstrated that ceftriaxone reduces amyloid-β–induced neuroinflammation and improves GLT-1 expression, further supporting its neuroprotective role. Owing to its safety, tolerability, and favorable pharmacokinetic profile, ceftriaxone is considered a promising repurposed drug candidate for the treatment of Alzheimer's disease.[15]

Drugs repurposing

Drug repurposing is a promising tool for novel drug discovery. it is relatively rapid, less costly, and possesses minimal risk of adverse effects to study participants. These advantages significantly affect the major challenges of conventional de novo drug discovery.[16] Drug repurposing for central nervous system (CNS) disorders represents an attractive and dynamic strategy that significantly reduces drug development time and cost.[17]

Ceftriaxone is third generation cephalosporin belonging to the group of β-lactam antibiotics mostly used for local and systemic infections. Recent evidence from preclinical studies highlighted the therapeutic efficacy of ceftriaxone against many of the neurological disorders, drug dependency, as well as neuroprotective activity against various neurotoxic chemicals.[18]

MATERIALS AND METHODS

SHSY-5Y (Neuroblastoma cells) cell line was purchased from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's Modified Eagles medium (DMEM) (Sigma Aldrich, USA) in 25 cm^2 tissue culture flask. 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml) were supplemented with the culture medium. The cells are kept in a humidified 5% CO_2 incubator at 37 degrees Celsius. Using an inverted microscope the viability of cells was evaluated by direct observation.

Cells seeding in 96 well plate

Cells have grown for two days. Cells are counted using hemocytometers. The monolayers of cells were trypsinzed and these were suspended in 10% DMEM. In 96 well plate, 100µl cell suspension ($5x10^3$ cells/well) was seeded. It is then incubated at 37 degree Celsius in a CO_2 incubator.

Preparation of compound stock

1mg of the Ceftriaxone was weighed and completely dissolved in 1mL DMEM using a cyclomixer. To ensure the sterility extract solution was filtered through o.22 µM Millipore syringe. To induce toxicity Beta Amyloid 10 µM was added.[19]

Cytotoxicity Evaluation

When the cells attained sufficient growth, Beta Amyloid (10µM) was added to induce neurotoxicity. Beta amyloid will cause neurodegeneration. The cells are incubated for one hour after the addition of beta amyloid protein. Then Ceftriaxone is added to the amyloid treated cells in the concentrations of compound were added at concentrations of

100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml and 6.25 µg/ml of DMEM. These are done in triplicates to reduce errors. These are incubated at 37 degrees Celsius for 24 hours in humidified 5% CO2 incubator. Untreated control cells and Beta Amyloid alone treated wells were also maintained.

Cytotoxicity Assay by Direct Microscopic observation

After 24 hours of treatment 96 well plates were observed in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera). The cell microscopy is recorded as images. It is thoroughly checked for any cytotoxic indicators like cell shrinking, granulation and vacuolization in the cytoplasm.

Cytotoxicity Assay by MTT Method

In 3 ml PBS 15 mg of MTT (Sigma, M-5655) was dissolved and then it is filtered using membrane filter for sterility. The cells were already incubated for 24 hours and after that period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells. Gentle shaking is done to the plates for the solution to completely reach the cells. These are then incubated for 4 hours in incubator at 37 degrees. The supernatant was removed after incubation. 100µl of MTT Solubilization Solution was added and cells were pipetted for gentle mixing for solubilizing formazan crystals. At a wavelength of 540 nm absorbance values were measured by using microplate readers.[20]

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples x } 100}{\text{Mean OD of control group}}$$

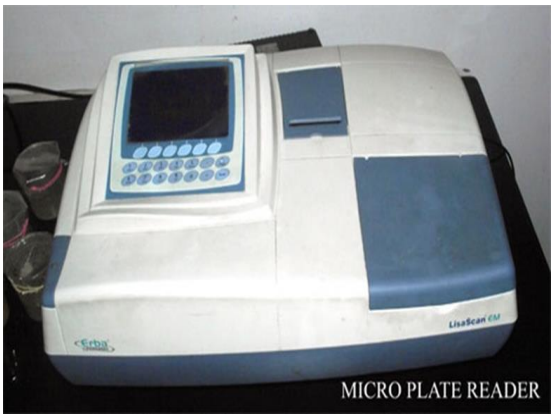


Figure 1: Microplate Reader.

Figure 2: Phase Contrast Microscope.

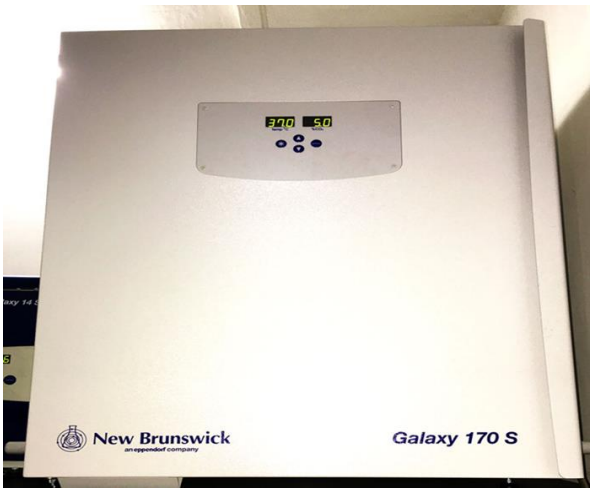
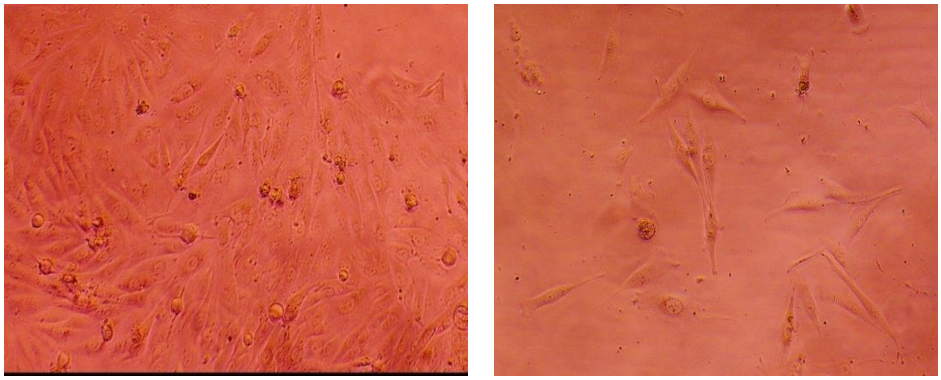


Figure 3: CO2 Incubator.

RESULTS AND DISCUSSION

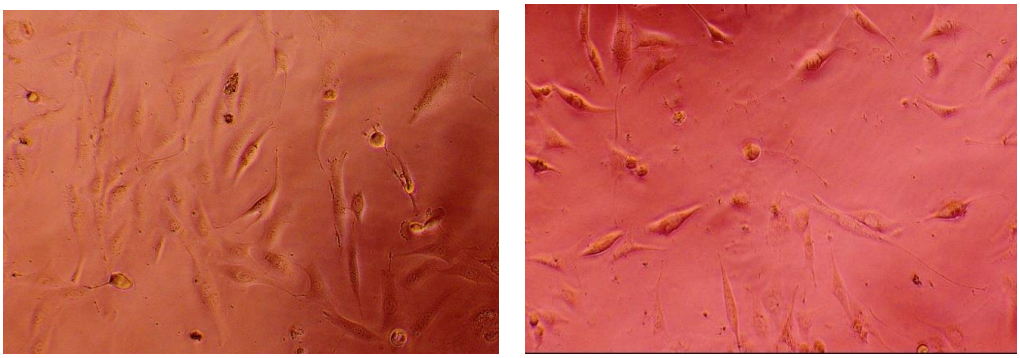
MTT Assay

When the SHSY5Y human neuroblastoma cell line was treated with beta amyloid to conduct the MTT assay. By using 570 nm a calibrated microplate reader cell viability can obtained through the optical density OD for specifically each condition like OD1, OD2, OD3. The control group viability is calculated as the percentage viability; the reference will be set as 100% viability. To confirm the minimal experimental variability and high procedure consistency, the control reading consistently shows the identical OD values across the three replicates.

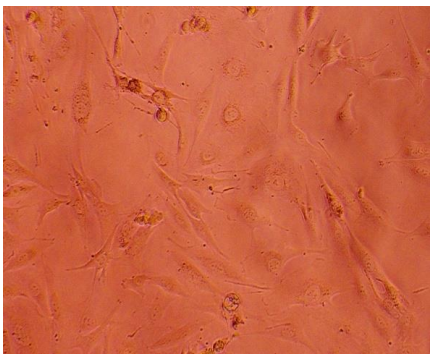


a **b**

Figure 6: a: Normal SHSY5Y human neuroblastoma cell line b: beta amyloid treated cell.



c **d**



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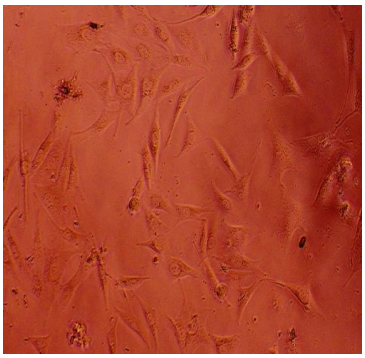

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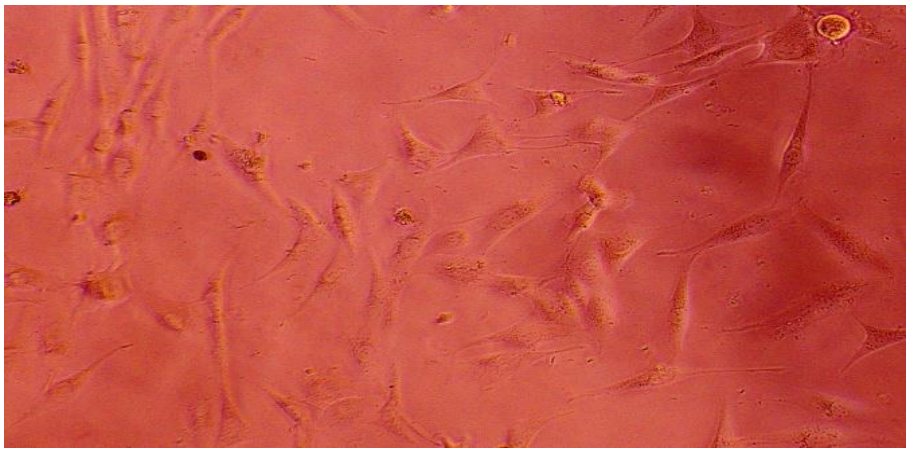

g

Figure 7: c: 6.25 ug/ml Ceftriaxone treated cell. d: 12.5 ug/ml Ceftriaxone treated cell. e: 25 ug/ml Ceftriaxone treated cell. f: 50 ug/ml Ceftriaxone treated cell. g: 100 ug/ml Ceftriaxone treated cell.

Table 1: Statistical analysis of MTT assay done on SHSY5Y human neuroblastoma cell line using different concentrations of Ceftriaxone.

Cell line - SHSY5Y									
Sample Code- SNRSS4									
	OD1	**OD2**	**OD3**	**Percentage viability 1**	**Percentage viability 2**	**Percentage viability 3**	**Average**	**Stdev**	**Std error**
Control	0.3484	0.3398	0.3471	100	100	100	100	0	0
Beta amyloid	0.1673	0.1756	0.1694	48.0195	51.67746	48.8044	49.5005	1.92575	1.11183
6.25	0.1829	0.1951	0.1936	52.4971	57.41613	55.7764	55.2299	2.50463	1.44605
12.5	0.2006	0.2018	0.2141	57.5775	59.38788	61.6825	59.5493	2.05726	1.18776
25	0.2289	0.2374	0.2241	65.7003	69.86463	64.5635	66.7095	2.79092	1.61134
50	0.2471	0.2416	0.2471	70.9242	71.10065	71.1899	71.0716	0.13518	0.07805
100	0.2641	0.2513	0.2571	75.8037	73.95527	74.0709	74.6099	1.03542	0.5978

When it is treating with the beta- amyloid peptide (Aβ1-42) shows the decreases in cell viability with an average of 49.50% ± 1.11 (standard error of the mean, SEM), illustrating pronounced cytotoxic effects. Comparing to control the OD reading has been decreased which show the evidence that reduction in beta amyloid is compromised cell health. When they are co treated with **Ceftriaxone**, the concentration increase **(**ranging from 6.25 to 100 µM), cell viability was observed due to increase in dose dependent. At 6.25 µM, viability recovered to an average of 55.23% ± 1.45, with further improvement to 59.55% ± 1.19 at 12.5 µM. The most notable enhancement occurred at 25 µM, with viability reaching 66.71% ± 1.61. Higher concentrations exhibited a stronger protective effect, with 50 µM treatment yielding 71.07% ± 0.08, and the highest concentration tested (100 µM) achieving a maximum viability of 74.61% ± 0.60.

These results demonstrate that the Ceftriaxone mitigates β-amyloid-induced cytotoxicity in a concentration-dependent manner. The data collectively suggest that increasing doses confer greater cellular protection, significantly improving survival rates and maintaining cell integrity under toxic conditions.

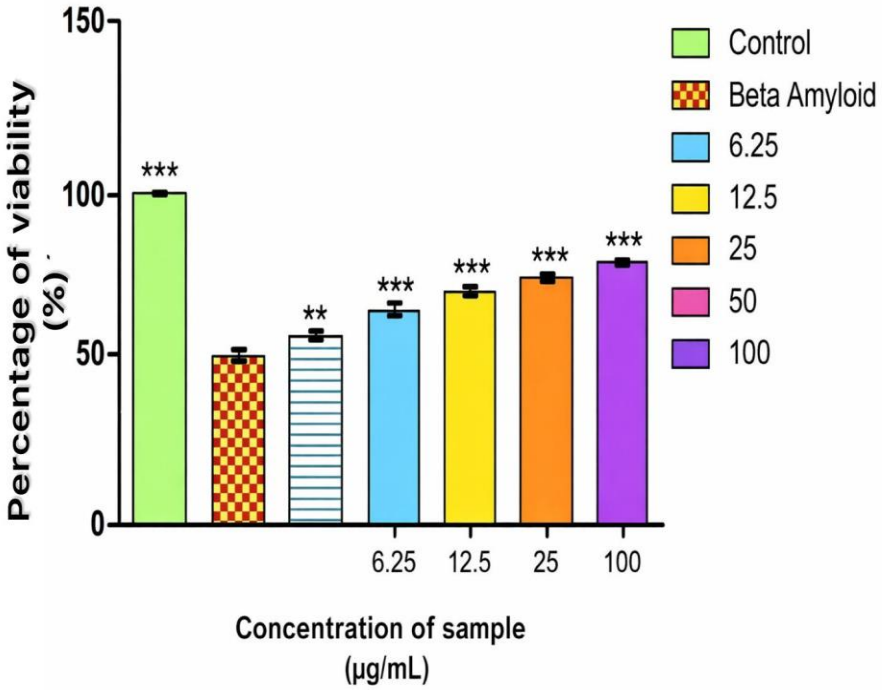

Figure 4: Graphical representation depicting the neuroprotective effect of Ceftriaxone by MTT assay.

Along Y axis Percentage viability, Along X axis varied concentration of Ceftriaxone is plotted. All experiments were done in triplicates and results represented as Mean+/- SE. One-way ANOVA and Dunnett test were performed to analyze data. ***p< 0.0001 compared to β amyloid exposed group indicates that Ceftriaxone has got significant neuroprotective activity

SUMMARY AND CONCLUSION

Neurodegenerative diseases are characterized by the progressive loss of neurons in the central nervous system, leading to impairment of cognitive, motor, and behavioural functions. Common examples include Alzheimer's disease, Parkinson's disease, Huntington's disease, and Amyotrophic Lateral Sclerosis (ALS).

Drug repurpose is considered a promising tool for novel drug discovery as it is relatively rapid, less costly, and poses a minimal risk of adverse outcomes to study participants.

The results of this study indicate that ceftriaxone possesses significant neuroprotective activity against β-amyloid-induced toxicity in SHSY-5Y human neuroblastoma cells. The increase in cell viability with increasing concentrations of ceftriaxone suggests that it can reduce neuronal damage caused by amyloid toxicity. The highest concentration tested (100 μM) achieving a maximum viability of 74.61% ± 0.60.These results demonstrate that the Ceftriaxone mitigates β-amyloid-induced cytotoxicity in a concentration-dependent manner. The data collectively suggest that increasing doses confer greater cellular protection, significantly improving survival rates and maintaining cell integrity under toxic conditions

Based on these findings, ceftriaxone may be considered a promising repurposed drug candidate for Alzheimer's disease. Overall, the study supports the potential role of drug repurposing as an effective strategy in developing better therapeutic options for Alzheimer's disease.

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