ESTIMATION OF MUTAGENIC POTENTIAL OF THE VALPROIC ACID DERIVATIVE CONTAINING A TERTIARY AMINO GROUP

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The model of severe poisoning with acetylcholinesterase inhibitors has shown the possibility of drug treatment of toxic effects with valproic acid containing a tertiary amino group. The study was aimed to assess potential mutagenic effects of the valproic acid derivative containing a tertiary amino group when studing its safety. Testing for toxicophores and assessment of the mutagenic effect probability were perfomed using the QSAR Toolbox offline software (v4.5 SP1). The Ames test with and without metabolic activation was used to estimate mutagenic potential of valproic acid containing a tertiary amino group *in vitro*. The computer prediction results predicted that the test substance would show no mutagenic effects in the Ames test. These data were confirmed by the *in vitro* Ames test for a broad range of concentrations of valproic acid containing a tertiary amino group (0.02–5.0 mg/mL). The concentrations of valproic acid containing a tertiary amino group exceeding 1.58 mg/mL have a bacteriostatic effect on the TA 100 *S. typhimurium* strain and the WP2 uvr A pKM 101c *E. coli* strain. Thus, the valproic acid derivative containing a tertiary amino group possesses no mutagenic effect, it can be recommended for further preclinical trials of therapeutic efficacy and safety.

Keywords: valproic acid, acetylcholinesterase inhibitors, computer prediction, mutagenicity, Ames test, anticholinergics

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Compliance with ethical standards: the study was performed *in silico* and *in vitro*, no approval by the Ethics Committee was required.

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ОЦЕНКА МУТАГЕННОГО ПОТЕНЦИАЛА ПРОИЗВОДНОГО ВАЛЬПРОЕВОЙ КИСЛОТЫ С ТРЕТИЧНОЙ АМИНОГРУППОЙ

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Моделирование тяжелого отравления ингибиторами ацетилхолинэстеразы показало возможность фармакологической терапии токсических проявлений препаратом вальпроевой кислоты с третичной аминогруппой. Целью работы было исследовать потенциальную мутагенную активность вальпроевой кислоты с третичной аминогруппой в рамках изучения ее безопасности. Анализ наличия токсикофоров и оценку вероятности проявления мутагенности выполняли с использованием автономного программного обеспечения QSAR Toolbox (v4.5 SP1). Для оценки мутагенного потенциала вальпроевой кислоты с третичной аминогруппой *in vitro* использовали тест Эймса с метаболической активацией и без. Результаты компьютерного прогнозирования предсказали отсутствие мутагенного действия изучаемой субстанции в тесте Эймса. Данные были подтверждены в тесте Эймса *in vitro* для широкого диапазона концентраций вальпроевой кислоты с третичной аминогруппой (0,02–5,0 мг/мл). В концентрации выше 1,58 мг/мл вальпроевая кислота с третичной аминогруппой обладает бактериостатическим действием на штаммы *S. typhimurium* TA 100 и *E. coli* WP2 uvr A pKM 101. Таким образом, производное вальпроевой кислоты с третичной аминогруппой не обладает потенциальным мутагенным действием, его можно рекомендовать для дальнейшего исследования терапевтической эффективности и безопасности в доклинических исследованиях.

Ключевые слова: вальпроевая кислота, ингибиторы ацетилхолинэстеразы, компьютерное прогнозирование, мутагенность, тест Эймса, холиноблокаторы

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Organophosphorus compounds and carbamates are the commonly used insecticides that inhibit cholinesterase activity, causing acute muscarinic toxicity symptoms and some symptoms of nicotine-like toxicity [1]. Furthermore, reversible acetylcholinesterase inhibitors are more and more often used for pharmacological support of patients with neurodegenerative diseases [2, 3]. The increasing adoption of acetylcholinesterase inhibitors as insecticides and pharmacological agents increases the risk of household and industrial poisoning requiring immediate medical intervention. When the levels of exposure are high, cholinesterase inhibition quickly leads to accumulation of acetylcholine neurotransmitter, the endogenous ligand of muscarinic and nicotinic receptors [4]. The sudden and rapid increase in acetylcholine levels in synapses results in hyperstimulation of cholinergic receptors and the symptoms of cholinergic crisis. Atropine is most commonly

used as an antidote to poisoning, including poisoning with acetylcholinesterase inhibitors [5]. Insufficient protective effect of atropine associated with the lack of nicotinolytic effect, along with the risk of excessive atropinization during care provision, necessitates the need to develop drugs with minimum toxicity possessing central anticholinergic activity.

A neuromodulator drug, (1-methylpiperidin-4-yl)-2-propylpentanoate hydrochloride (valproic acid derivative containing a tertiary amino group), was synthesized in the Golikov Research Clinical Center of Toxicology of FMBA of Russia [6]. The rat models of severe carbamate poisoning showed diverse pharmacological effects of this drug, it had both anticholinergic and anticonvulsant effects [7].

At the initial stage of the study of the pharmacologically active compound toxic effects, the computer prediction methods are used in silico before conducting in vitro and in vivo experiments. Potential mutagenic activity should be assessed when studying the test substance safety. In case mutagenic potential of the new compound has been revealed, it is necessary to consider the presence of functional groups determining the toxic effects, along with the available experimental data on the compounds with similar structure [8]. For that the specially developed software is used that makes it possible to assess genotoxic potential of promising pharmacologically active compounds based on the analysis of structural similarity and the presence of toxicophores [9]. The Ames test is used for primary screening aimed to assess the new drugs' mutagenic potential in vitro [10].

The study was aimed to assess potential mutagenic effects of the valproic acid derivative containing a tertiary amino group using the in silico and in vitro tests.

METHODS

Research object

The research object was represented by (1-methylpiperidin-4-yl)-2-propylpentanoate hydrochloride (valproic acid derivative containing a tertiary amino group), synthesized in the Drug Synthesis Laboratory, Golikov Research Clinical Center of Toxicology of FMBA of Russia. An in silico study was performed using the following SMILES formula of the test compound: CCCC(CCC)C(=O)OC1CCN(C)CC1.

In silico mutagenicity assessment

Analysis of the structural fragments indicative of potential genotoxicity of the tertiary amino group-containing valproic acid derivative, identification of probable mechanisms underlying mutagenic effects, and estimation of the probability of mutagenic effects in the Ames test based on the available experimental data on the compounds with similar structure were performed using the QSAR Toolbox offline software (v4.5 SP1).

Bacterial reverse mutations in S. typhimurium TA 1535, TA 1537, TA 98, TA 100 with or without metabolic activation and E. coli WP2 uvr A pKM 101 with or without metabolic activation were considered to be the study endpoints.

The following profilers available for the selected endpoints were chosen as algorithms for identification of the studied compound specific features, i.e. for profiling: "DNA alert for Ames assay, chromosomal aberrations, and micronucleus test according to the protocol developed by the Laboratory of Mathematical Chemistry, Burgas, Bulgaria" ("DNA alert for AMES, CA, and MNT by OASIS"), "in vitro mutagenicity alert (for Ames test) according to the protocol developed by Istituto Superiore di Sanità (Rome, Italy)" ("*in vitro* mutagenicity alert (Ames) by ISS"), "DNA binding according to the protocol developed by the Organization for Economic Co-operation and Development" ("DNA binding by OECD"), and "DNA binding according to the protocol developed by the Laboratory of Mathematical Chemistry, Burgas, Bulgaria" ("DNA binding by OASIS").

The primary sample of chemical substances similar to the valproic acid derivative containing a tertiary amino group, which was based on structure, was compiled based on the presence of the following functional groups: branched alkane containing a tertiary carbon or tertiary amine or an ester derived from a carboxylic acid or tertiary aliphatic amine.

The categories were clarified based on the specific DNA binding mechanism determined for the valproic acid derivative containing a tertiary amino group in accordance with the algorithm "DNA alert for Ames assay, chromosomal aberrations, and micronucleus test according to the protocol developed by the Laboratory of Mathematical Chemistry, Burgas, Bulgaria (OASIS)" ("DNA alert for AMES, CA, and MNT by OASIS"). This algorithm considers probable genotoxicity (for example, genetic mutations in *in vivo* and *in vitro* tests, DNA damage and/or reparation, DNA and/or protein damage in the liver, chromosomal aberrations, transgenic rodent mutations) and carcinogenicity. Categorization by the substance structure was performed using the organic functional group profiler developed by the U.S. Environmental Protection Agency (US EPA).

Ames assay

The test was performed using the MPF™ Penta 1 kit (Xenometrix; Switzerland) containing all necessary microbiological media and supplements, appropriate bacterial strain, positive controls, components of the S9 rat liver microsomal fraction. Three repetitions were carried out for each concentration of the valproic acid derivative containing a tertiary amino group. The test variants involving metabolic activation of the system by the Aroclor 1254 induced rat liver homogenate S9 microsomal fraction with the NADP cofactor and glucose-6-phosphate or no activation by the S9 microsomal fraction were used.

The concentration range was selected using an overnight S. typhimurium TA98 culture to choose the test compound maximum concentration, at which no cytotoxic effects would be observed, as well as to estimate drug solubility in experimental conditions. Sterile water for injection was used as a solvent. The test concentrations used for preliminary testing were as follows: 0.01 mg/mL, 0.02 mg/mL, 0.05 mg/mL, 0.16 mg/mL, 0.50 mg/mL, 1.58 mg/mL, and 5.00 mg/mL. The signs of cytotoxicity were determined based on no bacterial growth at certain concentration of the test compound.

Data analysis

Significance of differences between binomial distributions in the Ames test was determined using the cumulative binomial [11, 12]. The cumulative binomial probability (B-value) exceeding 0.99 indicated that the study result was associated with mutagenic effects of the drug with the probability ≥ 99%. In addition to probability, we assessed the factor by which the number of revertant colonies exceeded the baseline. The baseline was calculated as a sum of the average number of spontaneous reversions (revertants in the negative control sample) and the standard deviation. When the number exceeded the baseline less than twice, the result was considered to be non-significant and was not considered as positive. When the concentration-dependent effect or the baseline value exceeded more than

Table 1. Results of testing the valproic acid amino ester containing a tertiary amino group using the Ames assay involving no activation by microsomal fraction (M ± SD)

Test substance	Concentration	Strains				
		TA98	TA100	TA1535	TA1537	*E. coli* Combo
Valproic acid amino ether	0.02 mg/mL	0.7 ± 0.6	5.7 ± 1.2	2.7 ± 1.5	0.0 ± 0.0	5.7 ± 2.0
	0.05 mg/mL	0.7 ± 0.6	5.3 ± 2.1	0.0 ± 0.0	1.7 ± 0.6	7.3 ± 0.6
	0.16 mg/mL	0.7 ± 0.6	7.7 ± 2.1	0.3 ± 0.6	1.0 ± 0.0	6.0 ± 1.0
	0.50 mg/mL	1.0 ± 0.0	5.3 ± 3.2	1.0 ± 1.0	1.0 ± 0.0	8.0 ± 2.0
	1.58 mg/mL	0.3 ± 0.6	4.3 ± 0.6	0.7 ± 1.2	1.7 ± 2.1	3.3 ± 1.2
	5.00 mg/mL	1.3 ± 2.3	1.7 ± 2.1#	0.0 ± 0.0	1.0 ± 1.0	0.0 ± 0.0#
Negative control	0	1.2 ± 1.2	7.6 ± 3.2	1.3 ± 1.2	1.6 ± 2.6	6.2 ± 3.9
Negative control baseline	–	2.4	10.8	2.5	4.2	10.1
Positive control for TA 98 strain	2.0 μg/mL	47.6 ± 1.1	–	–	–	–
Positive control for TA 100 strain	0.1 μg/mL	–	46.3 ± 2.5	–	–	–
Positive control for TA1535 strain	100 μg/mL	–	–	48.0 ± 0.0	–	–
Positive control for TA1537 strain	15 μg/mL	–	–	–	48.0 ± 0.0	–
Positive control for *E. coli* Combo strains	2.0 μg/mL	-	-	-	-	34.0 ± 3.7

Note: # — decrease in the level of spontaneous reversions (B-value ≤ 0.01)

twice was revealed, the test drug was classified as mutagen. The data considerably lower than the level of spontaneous reversions (B-value ≤ 0.01) can be indicative of the drug citotoxic effect.

RESULTS

In silico mutagenicity assessment

No experimental data of the studies of the valproic acid derivative containing a tertiary amino group were found in the databases used by the QSAR Toolbox (v4.5 SP1), the drug was assigned no CAS number.

Profiling based on nonspecific endpoints revealed no "in vitro mutagenicity alert (Ames) by ISS" and "DNA alert for AMES, CA, and MNT by OASIS" for the systems with and without metabolic activation. The use of general mechanistic approach based on the "DNA binding by OASIS" algorithm revealed no alerts, while the "DNA binding by OECD" algorithm alerted to probable mono-nucleophilic substitution reaction yielding the reactive iminium ion.

Initial sample of chemical substances similar to the test valproic acid derivative based on the "organic functional groups" criterion that were taken from the European Chemicals Agency database included 12,963 compounds. Among them 2,300 compounds had the data for the endpoint "assessment of bacterial reverse mutations in *S. typhimurium* TA 1535, TA 1537, TA 98, TA 100 showing or not showing metabolic activation" and 299 had the data for the endpoint "assessment of bacterial reverse mutations in *E. coli* WP2 uvr A pKM 101 showing or not showing metabolic activation".

Subsequent selection of analogues was based on the specific DNA binding mechanisms identified for the valproic acid derivative. Among analogues, for which experimental data were available, chemical substances were found showing both positive and negative results of the Ames assay involving *S. typhimurium* TA 1535, TA 1537, TA 98, TA 100 and *E. coli* WP2 uvr A pKM 101. Furthermore, the program warned that there were chemical substances different from the test substance in the database. In this regard the "DNA alert for AMES, CA, and MNT by OASIS", "organic functional groups" developed by the U.S. Environmental Protection Agency (US EPA) and "structural similarity" profilers were used to refine the database. As a result, 80 chemical compounds with similar structure and DNA binding type, for which experimental data of the Ames assay involving S. typhimurium TA 1535, TA 1537, TA 98, TA 100 were available, were selected, along with 33 chemical compounds with similar structure and DNA binding type, for which experimental data of the Ames assay involving *E. coli* WP2 uvr A pKM 101 with and without metabolic activation were available. None of the analogues showed mutagenic effects in the Ames test. In silico prediction based on the test results of five most close analogues with the significance level of 0.00412 predicted no mutagenic effects exerted by the valproic acid amino ester in the Ames test involving *S. typhimurium* TA 1535, TA 1537, TA 98, TA 100 and E. coli WP2 uvr A pKM 101 with and without metabolic activation.

Determining the substance concentration range of interest

The valproic acid derivative containing a tertiary amino group exerted no cytotoxic effects in the studied concentration range. All the studied concentrations remained soluble under the conditions of preliminary testing. That is why the Ames assay was performed in the concentration range of 0.02–5 mg/mL with a half an order increment (0.02 mg/mL, 0.05 mg/mL, 0.16 mg/mL, 0.50 mg/mL, 1.58 mg/mL, 5.00 mg/mL).

Results of the test without metabolic activation of the system

Table 1 provides the mean and standard deviation (M ± SD) for the number of wells with revertant colonies in a series of three iterations of 48 wells per each studied concentration of the valproic acid amino ester substance, positive and negative controls in the system without microsomal fraction activation.

Standard mutagens were used as positive controls: 2-nitrofluorene (2.0 μg/mL for the TA98 strain), 4-nitroquinoline-N-oxide (0.1 μg/mL for the TA100 strain), N4-aminocytidine (100 μg/mL for the TA1535 strain), 9-aminoacridine (15 μg/mL

Table 2. Results of testing the valproic acid amino ester containing a tertiary amino group using the Ames assay involving activation by S9 microsomal fraction (M ± SD)

Test substance	Concentration	Strains				
		TA98	TA100	TA1535	TA1537	*E. coli* Combo
Valproic acid amino ether	0.02 mg/mL	0.7 ± 0.6	8.7 ± 3.2	1.0 ± 0.0	1.3 ± 0.6	10.0 ± 1.0
	0.05 mg/mL	0.7 ± 1.2	8.0 ± 0.0	1.3 ± 1.2	1.7 ± 1.5	8.3 ± 1.5
	0.16 mg/mL	1.0 ± 1.0	10.0 ± 3.6	2.3 ± 2.3	0.7 ± 0.6	8.0 ± 3.0
	0.50 mg/mL	1.3 ± 1.2	13.0 ± 1.0	1.3 ± 0.6	2.7 ± 0.6	8.0 ± 0.0
	1.58 mg/mL	3.0 ± 2.0	17.3 ± 1.5	1.3 ± 0.6	0.7 ± 0.6	0.7 ± 0.6#
	5.00 mg/mL	0.0 ± 0.0	1.3 ± 0.6#	0.3 ± 0.6	0.7 ± 0.6	0.0 ± 0.0#
Negative control	0	1.0 ± 1.2	8.4 ± 2.6	1.5 ± 1.2	0.8 ± 0.8	7.2 ± 4.2
Negative control baseline	–	2.2	11.0	2.7	1.6	11.4
2-aminoanthracene	1.0 μg/mL	47.9 ± 0.5	–	–	–	–
2-aminoanthracene	2.5 μg/mL	–	48.0 ± 0.2	43.8 ± 3.1	41.3 ± 7.1	–
2-aminoanthracene	400 μg/mL	–	–	–	–	30.0 ± 8.1

Note: # — decrease in the level of spontaneous reversions (B-value ≤ 0.01)

for the TA1537 strain), 4-nitroquinoline-N-oxide (2.0 μg/mL for the wp2 uvrA and wp2 [pKM101] strains). These mutagens effectively induced reverse mutations in bacterial cells. The average number of the negative control revertant colonies for all strains did not exceed the maximum permissible value.

The findings showed that the valproic acid amino ester concentrations of 0.02 mg/mL, 0.05 mg/mL, 0.16 mg/mL, 0.5 mg/mL, 1.58 mg/mL, and 5.00 mg/mL did not induce mutations in the system without metabolic activation.

The decrease in the number of revertant colonies relative to the level of spontaneous reversions in the negative control sample of this strain and the number of revertant colonies at lower test substance concentrations was reported for the TA100 strain of *S. typhimurium* and the mixture of *E. coli* strains wp2 uvrA and wp2 [pKM101] (*E. coli* Combo) at the valproic acid amino ester concentration of 5.0 mg/mL in the system without metabolic activation. B-value was below 0.01, which could indicate that the valproic acid amino ester concentrations exceeding 5.0 had a bacteriostatic effect on these strains.

Results of the test with metabolic activation of the system by microsomal fraction (+S9)

Table 2 provides the mean for the number of revertant colonies and standard deviation (M ± SD) of three iterations per strain in the system with activation by the S9 microsomal fraction.

Various 2-aminoanthracene concentrations were used as positive controls for all strains. Testing of substances in the presence of S9 microsomal fraction showed that the average number of mutant colonies in the sections containing a positive control exceeded the minimum permissible value. The average number of colonies with reverse mutations in the sections containing a negative control did not exceed the maximum permissible value in the presence of S9 microsomal fraction.

The valproic acid amino ester did not induce mutations in the studied concentration range in the system with metabolic activation.

The decrease in the number of revertant colonies relative to the level of spontaneous reversions in the negative control sample of this strain and the number of revertant colonies at lower test substance concentrations was reported for the TA100 strain of S. typhimurium at the valproic acid amino ester concentration of 5.0 mg/mL and for the *E. coli* Combo mixture of strains at the valproic acid derivative concentrations of 1.58 mg/mL and 5.0 mg/mL in the system with metabolic activation. B-value was below 0.01, which could confirm the hypothesis that the valproic acid derivative concentration exceeding 1.58 mg/mL had a cytotoxic effect on these strains.

DISCUSSION

The QSAR Toolbox (v4.5 SP1) uses more than 50 databases of chemical substances and contains information on approximately 100,000 compounds. The studied valproic acid containing a tertiary amino group was not found in the databases used, which meant that there were no results of open-label trials of this substance.

Metabolic activation of the relatively inert functional groups into electrophilic reactive intermediates is considered to be an essential event in etiology of many side effects caused by drug intake. That is why assessment of biochemical reactivity of functional groups and structural motifs of potential pharmacological substances is important from a safety standpoint. And the alerts obtained by profiling should be considered when planning further preclinical and clinical trials [13].

The mechanistic profilers used in our study involve alerts that are based on the chemistry of the reactions associated with genotoxicity and on the hypothesis that electrophilic potential of a chemical is associated with genotoxic properties [14]. According to the “DNA binding by OASIS” algorithm, chemical structure of valproic acid containing a tertiary amino group is not associated with genotoxicity, however, the “DNA binding by OECD” algorithm alerts to probable mono-nucleophilic substitution reaction yielding the reactive iminium ion as a potential DNA adduct formation pathway [15].

DNA adduct formation can weaken a bond between the nitrogenous base and deoxyribose and result in the base loss (depurination or depyrimidination). Such DNA modification results in generation of the unstable apurinic/apyrimidinic site (AP site). The lack of appropriate base in the DNA matrix may result in blocking of DNA and RNA polymerases, as well as in single nucleotide substitutions and deletions/insertions. Chemical reactivity of AP sites causes DNA breaks, as well as DNA–protein and DNA–DNA crosslinks, thereby contributing to high mutagenicity and cytotoxicity of such damage [16].

In addition to probable genotoxicity, metabolic reactions yielding the reactive iminium ions can result in organ-specific toxicity. Neurotoxic effects of haloperidol, which, like the valproic acid derivative, has 4-piperidinyl in its molecular structure, are considered to be associated with the pyridine derivative formation, while the iminium ion is an intermediate of this process. However, loperamide that also has 4-piperidinyl in

its structure and forms a pyridine derivative via metabolization involving cytochrome CYP3A4 possesses no neurotoxic effects [15]. The differences between safety profiles of haloperidol and loperamide support the view that not all compounds involved in the same bioactivation patterns cause similar toxic effects. The fact that the valproic acid derivative is through bioactivation yielding DNA adducts and organ-specific toxic metabolites, including neurotoxic ones, should be considered when studying the substance pharmacokinetics.

Assessment of the probability of mutagenic effects in the Ames test using the QSAR Toolbox (v4.5 SP1) software with the significance level set at 0.00412 makes it possible to predict that the valproic acid derivative containing a tertiary amino group would show no mutagenic effects in the Ames test involving *S. typhimurium* TA 1535, TA 1537, TA 98, TA 100 and *E. coli* WP2 uvr A pKM 101 with or without metabolic activation.

Such assessment is consistent with the results of our *in vitro* study. According to the results, the concentrations of valproic acid containing a tertiary amino group of 0.02 mg/mL, 0.05 mg/mL, 0.16 mg/mL, 0.5 mg/mL, 1.58 mg/mL, and 5.00 mg/mL did not indice frameshift mutations (*S. typhimurium* strains TA98 and TA1537) and base-pair substitutions (S. typhimurium strains TA100, TA1535 and *E. coli* strains wp2 uvrA and wp2 [pKM101]) in the Ames assay without metabolic activation. The test system supplementation with the metabolic fraction of the liver did not affect the test substance genotoxic effects.

It is interesting to note cytotoxic effects of the concentration of valproic acid containing a tertiary amino group exceeding 1.58 mg/mL on the TA 100 *S. typhimurium* strain and WP2 uvr A pKM 101 *E. coli* strain in the tests both with and without metabolic activation. On the one hand, such an effect can mask mutagenic effects of the test substance high concentrations. On the other hand, cytotoxic effects can be associated with generation of DNA adducts resulting from metabolic activation, along with generation of AP sites and interstrand cross-links in the DNA molecule. However, cytotoxic effects of the valproic acid derivative containing a tertiary amino group have been also shown in the Ames test with metabolic activation, which contradicts this statement.

CONCLUSIONS

The results of the in silico and in vitro Ames test show that the valproic acid derivative containing a tertiary amino group possesses no mutagenicity. This pharmacologically active compound can be recommended for further preclinical trials of therapeutic efficacy and safety. However, it is important to note, that cytotoxic effects of valproic acid containing a tertiary amino group on some bacterial strains can mask its mutagenic effects when the concentration is high. Considering cytotoxic effects and the possibility of DNA adduct formation, it is necessary to study probable carcinogenic and cytotoxic effects using the tests involving mammalian cells and the experiments involving animal models.

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