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ISSN 2177-7853

Probucol and Succinobucol display similar lipid-lowering and antioxidant effects: a subacute/subchronic study in mice

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ABSTRACT

Probucol and his monossuccinate, succinobucol, are hypocholesterolemic compounds with antioxidant and anti-inflammatory properties that have been showing promising results in phase III clinical trials. The aim of this study was to investigate the effects of subacute and subchronic treatments with probucol and succinobucol on biochemical parameters (mainly in the plasma lipid levels) in mice, and to compare their antioxidant activities *in vitro*. Animals were treated with probucol or succinobucol (10 mg/kg/day, *oral pathway*) once a day, during 15 (subacute treatment) or 30 (subchronic treatment) days. Subchronic treatments with the two compounds decreased, significantly, plasma total cholesterol (TC) levels. Both treatments decreased the risk of atherosclerosis and the increase of non-HDL cholesterol and no showed signs of hepato- or nephro-toxicity. The free radical scavenger activities of both compounds (evaluated by DPPH *in vitro* assay) were not significantly different, being similar to that of ascorbic acid. In conclusion, both compounds, probucol and succinobucol, represent a good choice in future therapeutic studies on pathological conditions related to hypercholesterolemia and oxidative stress.

Probucol e Succinobucol apresentam efeitos hipolipemiantes e antioxidantes similares: um estudo subagudo/subcrônico em camundongos

RESUMO - O probucol e seu monossuccinato, succinobucol, são compostos hipocolesterolêmicos com propriedades antioxidantes e anti-inflamatórias que tem demonstrado resultados promissores em ensaios clínicos de fase III. O objetivo deste estudo foi investigar os efeitos de tratamentos subagudos e crônicos com probucol e Succinobucol em parâmetros bioquímicos (principalmente os níveis de lipídeos plasmáticos) em camundongos, e comparar suas atividades antioxidantes *in vitro*. Os animais foram tratados com probucol ou succinobucol (10 mg/kg/dia, por via oral) uma vez ao dia, durante 15 (tratamento subagudo) ou 30 dias (tratamento subcrônico). O tratamento subcrônico com os 2 compostos diminuiu significativamente os níveis de colesterol total plasmático. Ambos os tratamentos diminuíram o risco de aterosclerose e aumentaram os níveis de colesterol não HDL e não demonstraram sinais de hepato ou nefrotoxicidade. A atividade sequestradora de radicais livres dos dois compostos (avaliada através do ensaio do DPPH *in vitro*) não foram significativamente diferentes, sendo similar à do ácido ascórbico. Em conclusão, ambos os compostos, probucol e succinobucol, representam uma importante escolha para futuras aplicações terapêuticas em condições patológicas relacionadas à hipercolesterolemia e estresse oxidativo.

Histórico do Artigo

Recebido em: 27/09/2016

Aceito em: 01/11/2016

Keywords:

Oxidative stress

Hypercholesterolemia

Probucol

Succinobucol

Palavras-chave

Estresse oxidativo

Hipercolesterolemia

Probucol

Succinobucol

1. Introduction

Probucol is a phenolic agent first described in 1970 due to its capacity of reducing serum cholesterol in mice, rats, and monkeys (1). A similar effect was observed in humans with serum lipid disorders and in normocholesterolemic subjects (2). A previous report showed that low density lipoprotein (LDL) is the plasma cholesterol fraction whose levels are more affected (reduced) after probucol treatment (3).

Probucol has also shown anti-inflammatory and antioxidant properties (4, 5). In fact, some lines of evidence suggest that the anti-atherosclerotic effect of probucol was independent of its hypocholesterolemic activity, being instead associated with the inhibition of the oxidative modifications of LDL (6) and the reduction of monocyte adherence and infiltration (7). Another study showed that probucol enhanced LDL's resistance to Cu^{++} oxidation and inhibited aortic accumulation of hydroperoxides in hyperlipidemic rabbits (8). Furthermore, probucol has significant add-on anti-atherosclerotic effects when combined with atorvastatin treatment; suggesting that this combination might be beneficial for treatment of atherosclerosis (9).

Unfortunately, probucol is no longer available in many countries due to concerns of efficacy and adverse and undesirable effects, including lowering of HDL cholesterol and prolongation of cardiac repolarization with concomitant risks of arrhythmias (10, 11). On the other hand, recent studies have shown that probucol prevented secondary cardiovascular attack in a population with heterozygous familial hypercholesterolemia in Japan, without showing adverse effects like prolongation of cardiac repolarization (12). In another study with familial hypercholesterolemia patients, the combination of probucol, LDL-apheresis, statin and coronary angioplasty reduced the rates of major coronary events, such as cardiac death and nonfatal myocardial infarction (13). More recently, Guo et al. (14) showed that the combined use of atorvastatin and probucol in patients diagnosed with acute coronary syndrome, reduce oxidized LDL and increase paraoxonase-1 expression more effectively than atorvastatin alone.

Besides the research on the cardiovascular properties, our research group has been demonstrating the neuroprotective effects of probucol in different experimental models (15-17). We reported that Probucol is able to counteract the behavioral and biochemical impairments in two different experimental models of Alzheimer disease in mice (18, 19). This effect could be attributed to its antioxidant properties, although the involvement of cholesterol metabolism modulation in this benefit cannot be ruled out. In fact, although studies on the potential relationship between the neuroprotective and lipid-lowering effects of probucol are sparse, the possibility is worthy of consideration.

In the search for lipid-lowering compounds with anti-inflammatory and antioxidant properties, but without the adverse effects displayed by probucol, succinobucol was developed (11). Succinobucol (AGI-1067), the monosuccinic acid ester of probucol, is a metabolically stable compound that retains antioxidant and anti-inflammatory properties equipotent to those of probucol, but did not cause the mentioned collateral cardiovascular effects (20). Succinobucol inhibits atherosclerosis in hypercholesterolemic monkeys, LDL receptor and apoE knockout mice, and this has led to its clinical evaluation as an anti-atherosclerotic drug (11, 21). In hypercholesterolemic monkeys, succinobucol displayed dose-dependent lowering of LDL cholesterol and elevation of HDL cholesterol. It has also shown to reduce restenosis after percutaneous coronary interventions to an extent similar to probucol and to prevent atherosclerosis, but without significantly prolongation of the QT interval and excessively lowering HDL (22).

We also studied the neuroprotective effects of succinobucol in different experimental models (23-25). We demonstrated that the treatment with succinobucol reduced early non-

motor symptoms and neurodegeneration/neuroinflammation induced by intranasal MPTP administration in mice, an experimental model of Parkinson disease (24). When compared to probucol, succinobucol was more effective in mitigating brain mitochondrial dysfunction and oxidative stress *in vitro* (26).

Based on the aforementioned evidences, this study was designed to investigate the effects of subacute and subchronic treatments with probucol and succinobucol on biochemical parameters (mainly those related to plasma lipid levels) in mice, as well as to compare their antioxidant activities under *in vitro* conditions. This comparative evaluation on probucol vs. succinobucol was performed because, in addition to adverse effects, pharmacological efficacies should also be taken into consideration when selecting compounds for future experimental or clinical researches.

2. Material and methods

Chemicals

Probucol and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). Succinobucol was synthesized according to previous literature (27). In brief, 1,8-Diazabicyclo[5.4.0]undec-7-ene (2.1 mmol) was added dropwise to a solution of probucol (1.0 mmol) and anhydride succinic (2.1 mmol) in dry tetrahydrofuran (15 mL) under an argon atmosphere. The reaction was stirred at room temperature for 1 h. After this time the reaction was quenched with 1 M HCl solution and the aqueous layer was extracted with ethyl acetate.

The organic phase was dried over MgSO₄, filtered, and the volatiles were completely removed under vacuum to give the crude residue. Purification by flash chromatography with a mixture of hexane/ethyl acetate (80:20) afforded the desired Succinobucol (Yield: 37%).

¹H NMR (CDCl₃, 200 MHz): δ = 7.63 (s, 2H); 7.45 (s, 2H); 5.37(s, 2H); 2.99 (t, *J* = 6.97 Hz, 2H); 2.78 (t, *J* = 6.97 Hz, 2H); 1.46 (s, 6H); 1.44 (s, 18H); 1.34 (s, 18H). ¹³C NMR (CDCl₃, 100 MHz): δ = 177.21; 171.87; 155.00; 148.56; 142.63; 135.99; 134.69; 134.11; 129.55; 122.02; 59.49; 35.47; 34.32; 31.44; 30.57; 30.40; 30.28; 28.44.

The structures of both probucol and succinobucol are illustrated in Figure 1.

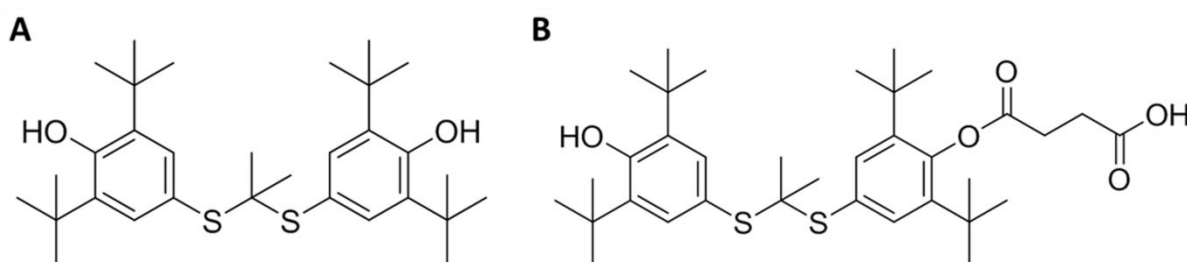


Figure 1. Chemical structure of probucol (A) and succinobucol (B).

Animals and experimental protocol

Adult Swiss male mice (4 month old), from our own breeding colony, were maintained at 22 °C ± 1, on a 12 h light: 12 h dark cycle, with free access to food and water. All experiments were approved by our Ethics Committee for Animal Use at the Universidade Federal de Santa Catarina, Brazil (PP00326/CEUA 23080.013800/2009-90/UFSC).

Normolipidemic mice were randomly divided into 3 experimental groups (n = 7-10) and were treated with probucol or succinobucol (10 mg/kg/day, oral pathway/gavage, once a

day) (24, 28) or with vehicle (2% DMSO in water). After 15 or 30 days of treatment, the mice were deprived of food for one night and anesthetized with isoflurane (1 mL/mL; Abbot Laboratórios do Brasil Ltda., RJ, Brazil) using a vaporizer system (SurgiVet Inc., WI, USA).

The blood was collected by cardiac puncture in heparinized tubes and mice were killed by decapitation. Plasma was separated by centrifugation of the whole blood at 1,000 x g for 10 min and stored at -80°C for biochemical analyzes.

Biochemical analyses

Plasma glucose, urea, creatinine, aspartate transaminase (AST), alanine transaminase (ALT), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL) and triglycerides (TG) levels were determined by enzymatic assays, using diagnostic kits following the manufacturers' instructions (Labtest Diagnostica®, Lagoa Santa-MG, Brazil).

The lipoproteins LDL, IDL and VLDL (non HDL-cholesterol) were calculated as (TC - HDL). In exploitation of lipid metabolism, we evaluated the cardiovascular risk factors TC/HDL ratio, TG/HDL and the atherogenic index (AI), calculated as (TC-HDL)/HDL (29).

DPPH radical scavenging

The antioxidant activities of probucol and succinobucol were determined by the scavenging of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), assayed *in vitro*. This method is a chemical assay used to evaluate the antioxidant activity of specific compounds or extracts, that are allowed to react with the stable radical DPPH in a ethanol solution (30). Briefly, the compounds were mixed with a 2,2-diphenyl-1-picrylhydrazylhydrate ethanol solution (0.25 mM), to give final concentrations of 3 μM , 10 μM , 25 μM , 50 μM , 75 μM , and 100 μM . After 30 minutes at room temperature, the absorbance was measured at 540 nm in a spectrophotometer. Ascorbic acid was used as a positive control and a comparative antioxidant compound at the same concentrations. The IC_{50} (50% inhibitory concentration) values and their respective 95% confident intervals were calculated.

Statistical analysis

Data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Differences among the 3 groups were analyzed by one-way ANOVA followed by the Tukey *post hoc* test. Results are expressed as mean \pm SEM. The differences were considered significant when $p < 0.05$.

3. Results

The plasma total cholesterol (TC) levels were significantly decreased by administration of both probucol and succinobucol when mice were treated for 30 days (Figure 2). No differences were found between groups when mice were treated for only 15 days, clearly indicating a time-dependent effect of treatment. However, significant decreases in the Atherogenic Index (AI) were observed after both probucol and succinobucol treatments (including after subacute treatment). After 30 days of treatment, probucol and succinobucol did not change the plasma HDL-cholesterol and triglycerides, while non-HDL cholesterol levels were significantly decreased (at the same extent for both compounds) after subchronic treatment when compared to control mice (Table 1).

No significant differences between groups were observed in the body weight (data not shown). Both compounds reduced the cardiovascular risk factor TC/HDL after 15 and 30

days of treatment in a similar manner, but no effect was promoted in TG/HDL ratio. There was a significant decrease in the AI of mice treated with both probucol and succinobucol when compared to control group, indicating a diminished risk of atherosclerosis (Table 1). However, no differences between probucol- and succinobucol-treated mice were observed.

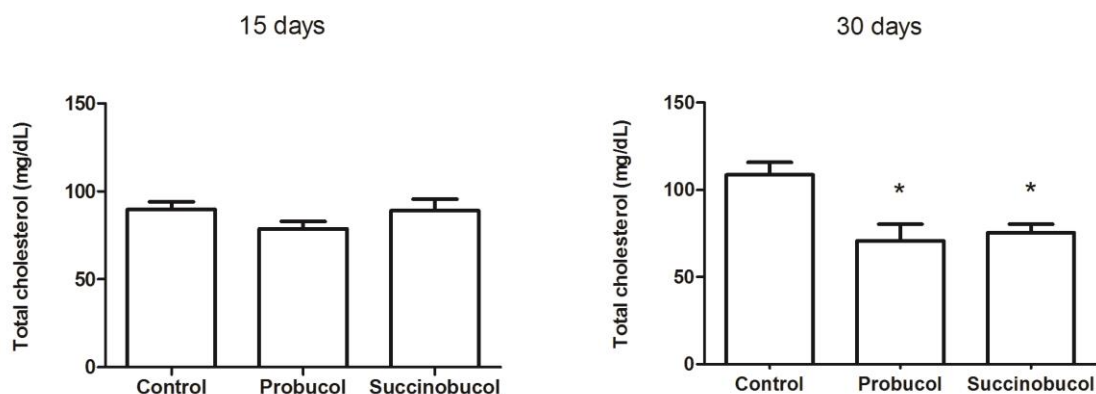


Figure 2. Effects of probucol and succinobucol on plasma total cholesterol level after subacute (15 days) and subchronic (30 days) treatments. Swiss mice were treated with probucol or succinobucol (10 mg/kg/day; gavage) or vehicle during 15 or 30 days. Data are expressed as mean \pm SEM (n=3-5 animals per group). *p<0.05 indicates the difference when compared to control group (one way ANOVA, followed by Tukey *post-hoc* test).

No significant signs of systemic toxicity (weight loss and mortality) were observed in probucol or succinobucol-treated mice until the end of experiment. The levels of aspartate transaminase (AST) and alanine transaminase (ALT), and the levels of creatinine and urea were analyzed as markers of liver and kidney injury, respectively. No significant changes on these parameters were observed after treatments (Table 2). In addition, both compounds did not alter plasma glucose levels.

In an attempt to compare the antioxidant/scavenging activities of both compounds, an *in vitro* (DPPH radical scavenging) assay was performed, using ascorbic acid as a positive control (Table 3). IC₅₀ (50% inhibitory concentration) values were calculated by measuring the compound capacity of reducing 50% of added DPPH. There was no significant difference between the scavenger activities of probucol and succinobucol. Moreover, the scavenger activities of both compounds were not significant different of that of ascorbic acid, a standard antioxidant/scavenger compound.

4. Discussion

Probucol is a phenolic agent with hypocholesterolemic, anti-inflammatory and antioxidant properties (1, 2, 4, 5). It was used in clinical practice in many countries until the discovery of adverse QT-interval prolonging effects, limiting its application only to a few countries (10). However, recent studies with subjects with familial hypercholesterolemia showed the benefits of probucol without its undesirable side effects (12). On the other hand, succinobucol, a phenolic agent which shares the sulfur moiety from probucol, keeps its hypocholesterolemic, anti-inflammatory and antioxidant activities without showing undesirable side effects, unlike probucol (20-22).

Table.1. Effects of probucol and succinobucol on lipid status, cardiovascular index and atherogenic index after subacute (15 days) and subchronic (30 days) treatments. Swiss mice were treated with probucol or succinobucol (10 mg/kg/day; gavage) or vehicle during 15 or 30 days. Data are expressed as mean \pm SEM (n=3-5 animals per group).

Lipid status	15 days			30 days		
	Control	Probucol	Succinobucol	Control	Probucol	Succinobucol
<i>Triglycerides (mg/dL)</i>	85.88 \pm 3.92	73.39 \pm 7.50	66.68 \pm 9.75	80.08 \pm 8.25	73.39 \pm 7.50	78.19 \pm 5.57
<i>HDL-cholesterol (mg/dL)</i>	20.31 \pm 1.14	24.14 \pm 2.19	24.97 \pm 2.78	21.65 \pm 1.01	24.21 \pm 0.87	24.07 \pm 1.60
<i>non-HDL-cholesterol (mg/dL)</i>	69.35 \pm 3.59	54.52 \pm 2.60 *	61.06 \pm 5.00	84.89 \pm 8.02	55.89 \pm 3.05 **	52.79 \pm 2.66 **
<i>TG/HDL</i>	4.04 \pm 0.19	3.24 \pm 0.60	2.44 \pm 0.12	4.10 \pm 0.29	2.93 \pm 0.29	3.18 \pm 0.36
<i>TC/HDL</i>	4.43 \pm 0.16	3.31 \pm 0.17 **	3.35 \pm 0.16 **	5.07 \pm 0.64	3.33 \pm 0.14 *	3.22 \pm 0.21 *
<i>Atherogenic index</i>	3.43 \pm 0.16	2.31 \pm 0.17 **	2.35 \pm 0.16 **	4.07 \pm 0.64	2.33 \pm 0.14 *	2.45 \pm 0.28 *

*p<0.05, **p<0.01, indicates the difference when compared to control treated group (one way ANOVA, followed by Tukey *post-hoc* test).

Table. 2. Effects of probucol and succinobucol on plasma biochemical parameters after subacute (15 days) and subchronic (30 days) treatments. Swiss mice were treated with probucol or succinobucol (10 mg/kg/day; gavage) or vehicle during 15 or 30 days. Data are expressed as mean \pm SEM (n=3-5 animals per group).

Biochemical parameters	15 days			30 days		
	Control	Probucol	Succinobucol	Control	Probucol	Succinobucol
<i>Urea (mg/dL)</i>	43.14 \pm 5.38	51.21 \pm 2.64	53.65 \pm 2.86	37.25 \pm 4.46	46.34 \pm 2.10	42.23 \pm 5.49
<i>Creatinine (mg/dL)</i>	0.30 \pm 0.02	0.31 \pm 0.03	0.34 \pm 0.08	0.41 \pm 0.10	0.43 \pm 0.05	0.35 \pm 0.10
<i>Glicose (mg/dL)</i>	92.00 \pm 7.76	89.09 \pm 3.71	91.58 \pm 12.3	86.47 \pm 24.42	80.67 \pm 26.37	79.04 \pm 18.72
<i>AST (U/dL)</i>	69.83 \pm 13.02	61.15 \pm 4.07	70.95 \pm 3.58	81.60 \pm 14.55	75.30 \pm 12.53	84.84 \pm 2.78
<i>ALT (U/dL)</i>	55.69 \pm 4.29	54.04 \pm 2.52	56.76 \pm 3.15	49.82 \pm 4.02	49.29 \pm 2.27	51.53 \pm 4.88

Table. 3. *In vitro* antioxidant activities of probucol and succinobucol. Scavenging properties of probucol and succinobucol were analyzed by DPPH radical assay. Standard ascorbic acid was used as a positive control. Values were expressed as mean IC₅₀ and their respective confidence intervals (CI 95%).

Compounds	IC ₅₀ μ M (95% CI)
<i>Ascorbic acid</i>	53.30 (47.82-59.42)
<i>Probucol</i>	51.37 (46.14-57.20)
<i>Succinobucol</i>	54.51 (47.18-62.97)

Because no comparative data on the efficacies of probucol and succinobucol have been experimentally investigated so far, this study investigated the lipid-lowering effects of probucol and succinobucol administration in mice after subacute (15 days) and subchronic (30 days) treatments.

Probucol and succinobucol showed a significant reduction of plasma total cholesterol at 30 days of treatment, supporting the findings of earlier studies (1, 21). Furthermore, the two compounds did not cause alterations on total triglycerides levels on both treatments, corroborating previous studies developed in mice and humans (1, 3, 31, 32). The mechanisms by which probucol reduces cholesterol are still unclear, although it was suggested that probucol enhances catabolic excretion of cholesterol into bile (33).

Probucol, unlike previous reports, did not show significant reduction on the HDL cholesterol on both treatments (31). Although this may be due to the reduced time of the study, these findings are worth noticing, since the reduction of HDL cholesterol levels

increases the possibility of developing atherosclerosis (34). Succinobucol also did not reduce HDL cholesterol at both times of treatment, being in agreement with previous studies, when succinobucol showed, in fact, an increase on the HDL cholesterol levels (21).

High levels of LDL correlate with the risk of cardiovascular events in human populations, and augment individual susceptibility to atherosclerosis and its complications. Several interventions that lower LDL levels by independent mechanisms diminish the likelihood of atherosclerotic events (35, 36). In our study, probucol and succinobucol showed a significant similar decrease in non-HDL levels. Early studies suggested that the decrease in LDL cholesterol may be related to probucol's ability to increase LDL particles turnover by increasing LDL clearance without altering synthesis (37, 38). On the other hand, the mechanisms of the hypolipidemic effects of succinobucol remains unclear. Moreover, the AI, defined as the ratio of TC-HDL/HDL, is believed to be an important risk factor for atherosclerosis. Our data clearly demonstrate that the compounds significantly decrease this ratio (at the same extent), indicating the beneficial effects on cardiovascular system.

In this study, we used the alteration of aspartate transaminase (AST) and alanine transaminase (ALT) levels as markers of liver injury and the increase in creatinine and urea levels as indicators of kidney damage. Probucol and succinobucol did not caused significant change of AST, ALT, creatinine and urea levels on both evaluated times, indicating no significant signs of hepato- and nephrotoxicity.

The free radical scavenging activities of probucol and succinobucol were determined by the DPPH assay. The DPPH is a stable free radical frequently used to determine the free-radical scavenging activities of antioxidant compounds (39). In this assay we measured the capacities of probucol and succinobucol in scavenging the free radical DPPH, comparing them with ascorbic acid. Both compounds showed similar free radical scavenging activities when compared to each other and to the standard antioxidant compound (ascorbic acid). Our results showed that probucol and succinobucol have similar scavenging capacities when compared to ascorbic acid. This is in line with the fact that the antioxidant effects of probucol and its analogue could prevent the LDL oxidation, a crucial event to atherosclerotic lesion formation (21, 40).

The most relevant findings of this study are that probucol and succinobucol displayed *similar* lipid-lowering (*in vivo*) and antioxidant (*in vitro*) effects. This comparative evaluation on probucol vs. succinobucol is important because no comparative data on both compounds have been experimentally addressed so far. Both compounds presents similar lipid-lowering and antioxidant effects and might represent a good choice in future studies on pathological conditions related to hypercholesterolemia and oxidative stress.

Acknowledgement: The authors are thankful to the financial supports by (i) Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), (ii) IBN.NET/CNPq, (iii) PRONEX-CNPq/FAPESC (NENASC project), (iv) Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and (v) INCT-CNPq-Excitotoxicity and Neuroprotection are gratefully acknowledged. M.F. and A.L.B. are recipients of CNPq fellowship.

Conflict of interest: The authors declare no conflict of interest.

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