Electrochemical behavior and determination of tumor inhibiting or promoting activities of flavonoids*

K. KOZICS, Z. KRAJČOVIČOVÁ, A. VACHÁLKOVÁ

Laboratory of Mutagenesis and Carcinogenesis, e-mail: katarina.horvathova@savba.sk, Cancer Research Institute, Slovak Academy of Sciences, 833 91 Bratislava, Slovak Republic

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This paper deals with determination of the tumor inhibiting or promoting activities of 11 flavonoids performed by DC polarography. Flavonoids were tested in the presence of polyaromatic carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) or in combination with DMBA and 12-O-tetradecanoylphorbol-13-acetate (TPA), which is known as a specific tumor promoter for epidermal carcinogenesis.

We found that in this experimental system the promotory or inhibitory activities of studied flavonoids depend on the number and position of hydroxyl groups in their chemical structures and are related to the polarographic behavior of these compounds. Flavonoids, which are hydroxylated at the ring B, are reduced in anhydrous DMF on a mercury dropping electrode in two one-electron steps. Absence of the hydroxyl groups in these positions caused their reduction in three one-electron steps. Similarly, flavonoids with hydroxyl groups at the ring B have been shown to inhibit the activities of DMBA (2.7–45.9%) and of DMBA + TPA (47.2–78.2%). Missing hydroxyl groups caused weaker inhibitory activity against DMBA + TPA (19.01–38.74%) and the enhancement of DMBA activity (31.08–66.21%).

Presented data demonstrated that the electrochemical method – DC polarography is very sensitive, simple technique for determination of the tumor inhibiting or promoting activities of the studied compounds.

Key words: DC polarography, tumor promoting activity, tumor inhibiting activity, flavonoids, 7,12-dimethylbenz(a)anthracene, 12-O-tetradecanoylphorbol-13-acetate

Several polyphenolic compounds are known as cancer chemopreventive agents. In particular, flavonoids are a class of natural polyphenolic compounds, widely distributed in the plant kingdom, that display a variety of biological activities, including tumor growth inhibition and chemoprevention [1].

Structurally diverse flavonoids were reported to inhibit tumorigenesis induced by polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines [2]. PAHs are converted metabolically to deploy their bioactivity by P450 through aryl hydrocarbon receptor (AhR) pathway. AhR is a ligand-activated transcription factor that mediates PAH-induced toxicity, teratogenicity and carcinogenicity [3, 4]. Upon binding of PAH, AhR transcriptionally induces multiple isoforms of P450 including CYP1A1, CYP1A2 and CYP1B1. These P450 isozymes catalyze the two-step oxidation of PAHs, such as 7,12-dimethylbenz(a)anthracene (DMBA), to their ultimative carcinogenic metabolites [5]. DMBA, a more potent skin tumor initiator, binds to a greater extent to epidermal DNA and generates more complex DNA adduct profile than benzo[a]pyrene (B[a]P) [6]. DMBA bioactivation to mutagenic and carcinogenic metabolites leads to formation of both syn- and anti-DMBA-3,4-diol-1,2-epoxides [7]. Both the metabolites of DMBA are tumor initiators in mouse epidermis [8]. BALASUBRAMANIAN and GOVINDASAMY [9] investigated the inhibitory effect of quercetin on DMBA-induced hamster buccal pouch carcinogenesis. Dietary quercetin inhibited the incidence of both papillomas and tumors induced by DMBA [9].

12-O-tetradecanoylphorbol-13-acetate (TPA) is known to act as a specific promoter of epidermal tumorigenesis iniciated by DMBA, originally detected in oil prepared from seeds of *Croton tiglium* L. TPA has highly pleiotropic effects

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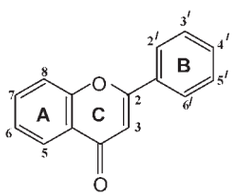
on cells in culture and on tissues *in vivo* [10]. Cellular prooxidant state appears to play an important role at critical steps of skin carcinogenesis. Tumor promoters like TPA increase the generation of reactive oxygen species (ROS), and several molecular events linked to tumor promotion may be mediated through these oxygen-centred free radicals. ROS may be formed in the TPA-treated epidermal cells themselves and/or released by the activated phagocytic cells infiltrating the skin during inflammation [11]. Fisetin and kaempferol potently inhibited TPA-caused epidermal ornitine decarboxylase induction and epidermal lipoxygenase activity in CD-1 mice [12]. Quercetin markedly suppressed the effect of TPA on skin tumor formation in the CD-1 mice initiated by DMBA [13].

The aim of this study was to assess tumor promoting or inhibiting activities of flavonoids (Fig. 1) – galangin (GA), chrysin (CH), baicalein (BA), 7-hydroxyflavone (7-OH-FL), 7,8-dihydroxyflavone (7,8-OH-FL), quercetin (QU), rutin (RU), luteolin (LU), apigenin (AP), fisetin (FI) and kaempferol (KA) performed by electrochemical method DC polarography. Electrochemical methods have contributed much to our understanding of anticancer agents and they have been used in cancer pharmacology in a variety of ways [14]. The use of DC polarography as a pre-screening method for identifying potential inhibitors of chemical carcinogens and tumor promoters is convenient as compared to the conventional *in vivo* tests [15], which are all expensive and time consuming. The method which is based on the increase or decrease of current values observed with the chemical carcinogen DMBA in the absence or presence of the proved tumor promoter TPA is well suited to the identification of substances that might pose as chemopreventive agents. The technique should not replace the conventional *in vivo* tests, but it can supplement these tests as an indicator of substances to which attention should by paid in this respect. Moreover, data of the reduction potential ($E_{1/2}$), reversibility or irreversibility of the reduction process, and the numbers of electrons accepted by flavonoids during the reduction were examined. All the data were analyzed in relation to the structure of the compounds.



Flavonoids	3	5	6	7	8	3′	4′
Apigenin		OH		OH			OH
Baicalein		OH	OH	OH			
Chrysin		OH		OH			
7,8-Dihydroxyflavone				OH	OH		
Fisetin	OH			OH		OH	OH
Galangin	OH	OH		OH			
7-Hydroxyflavone				OH			
Kaempferol	OH	OH		OH			OH
Luteolin		OH		OH		OH	OH
Quercetin	OH	OH		OH		OH	OH
Rutin	Rham*	OH		OH		OH	OH

Figure 1. The chemical structures of flavonoids tested. *Rham – rhamnosyl-glucosyl.

Material and methods

Chemicals. Flavonoids and TPA were obtained from Sigma-Aldrich Chemie, Steinheim, Germany. DMBA [16] was a commercial origin from Fluka Chemie AG, Switzerland. N,N-dimethylformamide (DMF), extra dry was obtained from Acros Organics, USA. Tetrabutylammonium perchlorate (TBAP), used as the supporting electrolyte at a concentration of 0.15 $mol.l^{-1}$, was a product of Fluka Chemie AG, Switzerland.

Polarographic conditions. All compounds were investigated using the polarographic analyzer PA4 equipped with the two-line recorder XY 4106 from Laboratorní přístroje, Prague, Czech Republic. Polarographic experiments were performed in a polarographic cell adapted for the work in anhydrous system. As the indicating electrode, a mercury dropping electrode was used with a drop time of 3 s and mercury flow rate of 2.27 $mg.s^{-1}$ at a mercury column high 81 cm. As the reference electrode, a saturated calomel electrode (SCE) modified for anhydrous conditions was used. As the auxiliary electrode, a platinum electrode (Radelkis, Budapest, Hungary) was used.

All polarographic measurements were carried out at room temperature in a stream of dry nitrogen in order to exclude atmospheric oxygen from the polarographic cell. Reversibility of reducing polarographic waves, exact values of their particular half-wave potentials and the number of electrons participating in a reduction process were determined by logarithmic analysis as a relationship between $\log I_d/(I_l-I_d)$ vs. potential E, where I_d represents a value of diffuse current related to particular potential E and I_l is a value of limit current.

Tumor promoting or inhibiting activities of compounds were determined based on the decrease or increase of current values observed with the chemical carcinogen DMBA ($3.98.10^{-5}$–$4.94.10^{-4}$ $mol.l^{-1}$) in the absence or presence of the proved tumor promoter TPA ($5.4.10^{-4}$ $mol.l^{-1}$). This activity is expressed in percents as the difference of the I_d values measured for the carcinogen alone or in the presence of tumor promoter and of the I_d values measured after addition of the substance under evaluation. The concentration of all flavonoids tested during their polarographic reduction was 5.10^{-4} $mol.l^{-1}$ (Fig. 2).

The differences between defined groups were tested for significance using the Student's t-test. For the purpose of comparing dose-response effects linear regression analysis

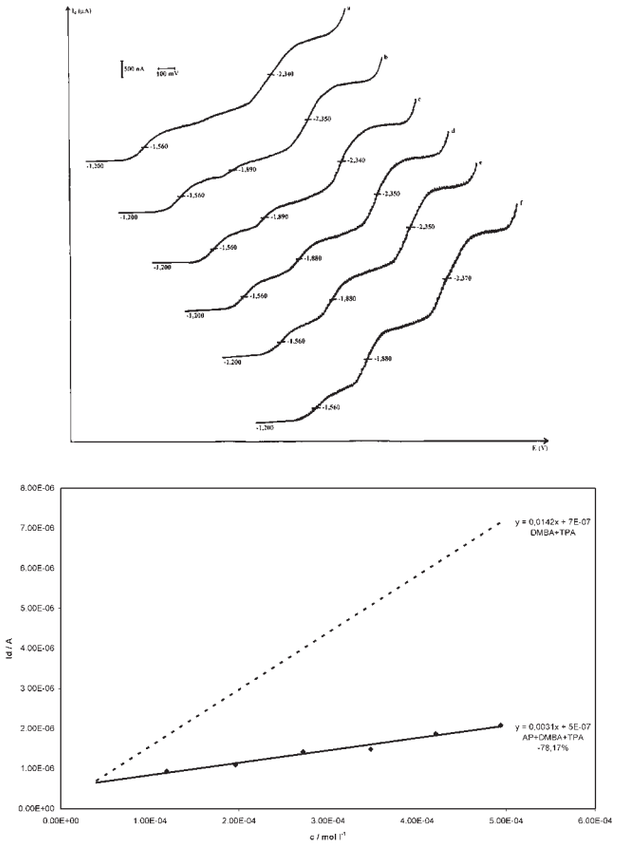


Figure 2. Determination of potential inhibitory activity of apigenin in the presence of DMBA+TPA. Polarografic reduction of apigenin in anhydrous DMF in the presence of DMBA+TPA. c_{AP}=5.10^{-4} mol.l^{-1}; c_{TPA}=5.4.10^{-4} mol.l^{-1}; c_{DMBA}: a) 0.00 mol.l^{-1}; b) 3.98.10^{-5} mol.l^{-1}; c) 1.19.10^{-4} mol.l^{-1}; d) 1.96.10^{-4} mol.l^{-1}; e) 2.72.10^{-4} mol.l^{-1}; f) 4.21.10^{-4} mol.l^{-1}; z.e. 0.15 mol.l^{-1} TBAP; scan rate 10 mV.s^{-1}; reg. from –1.200 V vs. SCE.

was used. Data are means of 3 independently performed experiments.

Results and discussion

Polarographic reduction of studied flavonoids in anhydrous conditions. Studied flavonoids, which are presented in Figure 1, are stable in anhydrous DMF at room temperature and generally yield well developed polarographic waves in non-aqueous DMF, with use of 0.15 mol.l^{-1} TBAP as the supporting electrolyte. The resulting polarographic catod curves are suitable for determining of reducing half-wave potentials and other electrochemical magnitudes involved in the reduction mechanism of a mercury dropping electrode. The diffuse character of polarographic waves has been verified through a linear dependence of these waves on the square root of the hight of the mercury column. To study the mechanism of polarographic reduction of the flavonoids investigated, logarithmic analysis has been performed [17–19]. The measured values for all the eleven flavonoids are listed in Table 1. Generally, all flavonoids tested are reduced in anhydrous DMF in the presence of 0.15 mol.l^{-1} TBAP on a mercury dropping electrode in two or three well defined steps.

Compounds, which are not hydroxylated at the ring B (GA, CH, BA, 7-OH-FL, 7,8-OH-FL) are reduced in anhydrous DMF on a mercury dropping electrode in three diffuse one-electron steps, the first and the second step being reversible and the third irreversible. The $E_{1/2}$ values of these compounds ranged from –1.410 V to –1.550 V vs. SCE for the first polarographic wave, from –1.810 V to –1.950 V vs. SCE for the second polarographic wave and the $E_{1/2}$ values of the third polarographic wave ranged from –2.270 V to –2.420 V vs. SCE (Tab. 1). GA, which has two hydroxyl groups at the ring A and is hydroxylated in the position 3 of the ring C, is reduced at $E_{1/2}$ values that were more negative than the other compounds studied. 7-OH-FL, CH and 7,8-OH-FL which have only one or two hydroxyl groups at the ring A are reduced at $E_{1/2}$ values that were more positive than $E_{1/2}$ values of GA. Addition of other hydroxyl group to the ring A (BA) resulted in a shift of the $E_{1/2}$ values towards more positive values.

QU, LU, FI, KA and AP are reduced in strictly anhydrous DMF on a mercury dropping electrode in two diffuse well defined steps. The first step is one-electron reversible reduction, the second step is one-electron irreversible reduction. The $E_{1/2}$ values of these compounds ranged from –1.560 V to –1.680 V vs. SCE for the first polarographic wave and from –2.360 V to –2.390 V vs. SCE for the second polarographic wave indicating the shift of the $E_{1/2}$ values to more negative values with increasing number of hydroxyl groups (Tab. 1).

RU, which is a glycoside of QU, is reduced under the same experimental conditions on a mercury dropping electrode in two diffuse one-electron steps. Substitution of hydroxyl group of QU in the position 3 of the ring C by the sugar moi-

Table 1. Values of half wave potentials ($E_{1/2}$) of tested flavonoids

Compound	Hydroxylation at the ring B	$E_{1/2\,I.}$/V	$E_{1/2\,II.}$/V	$E_{1/2\,III.}$/V
GA	–	–1.550	–1.950	–2.420
7,8–OH–FL	–	–1.540	–1.900	–2.340
CH	–	–1.460	–1.880	–2.400
7–OH–FL	–	–1.510	–1.920	–2.290
BA	–	–1.410	–1.810	–2.270
QU	+	–1.680	–2.390	–
LU	+	–1.590	–2.380	–
KA	+	–1.600	–2.380	–
FI	+	–1.610	–2.390	–
AP	+	–1.560	–2.360	–
RU	+	–1.560	–2.160	–

$E_{1/2I.}$ – the half wave potential of the first polarographic wave, $E_{1/2II.}$ – the half wave potential of the second polarographic wave, $E_{1/2III.}$ – the half wave potential of the third polarographic wave

ety rutinose caused the shift of the $E_{1/2}$ values towards more positive values (Tab. 1).

Our results are in good agreement with results published by NOVOTNÝ et al [20] and VACHÁLKOVÁ et al [21] describing polarographic behavior of GA, CH and KA in anhydrous DMF.

Effects of flavonoids on the activity of DMBA. Plant phenols are known to inhibit the mutagenicity of several bay-region diol-epoxides of PAHs. The binding of bay-region diol-epoxides of PAHs to target tissue DNA is thought to be essential for the initiation of cancer by the compounds. DAS et al [22] investigated the effect of polyphenols on PAH-DNA adduct formation in the epidermis and lung of SENCAR mice. They found that plant phenols e.g. QU are potent inhibitors of carcinogen binding to epidermal and lung DNA and suggest that these plant phenols could prove useful in modifying the risk of tumor induction by PAHs such DMBA and B[a]P in these two tissues. Dietary administration of QU (2 or 5%) in Sprague–Dawley rats reduced the incidence of mammary tumor induction by the carcinogens DMBA [23]. Chemopreventive effect of BA on DMBA-induced DNA damage was evaluated in the breast cancer cell line MCF-7 [24].

DMBA is the best studied PAH widely used in experimental carcinogenesis [16, 25]. The polarographic behavior of DMBA on the mercury dropping electrode in anhydrous-dimethyl sulfoxide has been already studied [26] but in anhydrous DMF in the presence of TBAP, as a supporting electrolyte, it has not been studied yet. DMBA is reduced in anhydrous DMF on a mercury dropping electrode in two diffuse one-electron steps with the values of $E_{1/2}$ –2.010 V and –2.480 V vs. SCE.

The goal of our work was to evaluate the effects of studied flavonoids on the activity of DMBA. We found that in this experimental system the promotory or inhibitory activity of studied flavonoids depends on the number and position of hydroxyl groups in the chemical structure and is related to the polarographic behavior of these compounds.

Flavonoids, which are not hydroxylated at the ring B and are reduced in three one-electron steps (GA, CH, BA, 7-OH-FL, 7,8-OH-FL) have been shown to enhance the activity of DMBA (Fig. 3A, Tab. 2). The highest promotory activity against DMBA was found for BA, which possesses hydroxyl groups in the positions 5, 6 and 7 of the ring A. BA enhaced the activity of DMBA by 66.21%. GA and CH are hydroxylated only in positions 5 and 7 of the ring A. Their promotory activity decreased to 55.4 %, 56.8%, respectively. 7-OH-FL possesses only one hydroxyl group in the position 7 of the ring A and has been shown to enhance the activity of DMBA by 43.24%. Promotory activity of flavonoids is probably related to hydroxylation of the ring A in the positions 5, 6 and 7. Increase of the number of hydroxyl groups in these positions enhanced the promotory activity of flavonoids against DMBA. On the other hand, the presence of the hydroxyl group in the position 8 of the ring A (7,8-OH-FL)

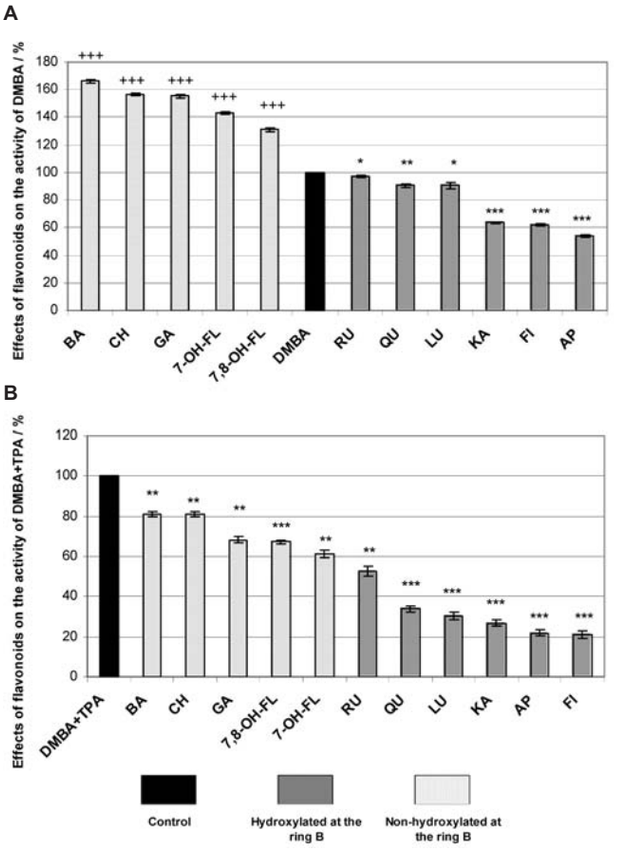


Figure 3. Determination of tumor inhibiting or promoting effects of tested flavonoids against the chemical carcinogen DMBA (A) and against the combination of tumor promoter TPA and DMBA (B) by the DC polarographic method. *Significantly different decrease from the control value ($0.01<p<0.05$); **Significantly different decrease from the control value ($0.001<p<0.01$); ***Significantly different decrease from the control value ($p<0.001$); $^{+++}$Significantly different increase from the control value ($p<0.001$). (A) control is DMBA; (B) control is DMBA+TPA.

Table 2. Effects of tested flavonoids against DMBA and DMBA+TPA assessed by the DC polarographic method

Flavonoids	Hydroxylation at the ring B	Activity DMBA / %	Activity DMBA+TPA / %
BA	–	+66.21	–19.01
CH	–	+56.80	–18.90
GA	–	+55.40	–31.70
7-OH-FL	–	+43.24	–38.74
7,8-OH-FL	–	+31.08	–32.80
RU	+	–2.70	–47.20
QU	+	–9.50	–66.20
LU	+	–9.50	–69.70
KA	+	–36.51	–73.30
FI	+	–37.84	–78.87
AP	+	–45.90	–78.17

decreased the promotory activity of this flavonoid against DMBA (Fig. 3A, Tab. 2).

Flavonoids, which are hydroxylated at the ring B and are reduced in anhydrous DMF on a mercury dropping electrode in two one-electron steps (QU, RU, LU, AP, FI, KA) have been shown to inhibit the activity of DMBA (Fig. 3A, Tab. 2). Similary to promotory activity, the number of hydroxyl groups is important for the inhibitory activity against the chemical carcinogen DMBA. Decrease of the number of hydroxyl groups at the rings A and B caused enhancement of inhibitory activity of studied flavonoids. AP, FI and KA, which possess together three hydroxyl groups at the rings A and B, decreased the activity of DMBA in the range from 36.51% to 45.9%. QU, RU and LU, which possess another one hydroxyl group, had only weak inhibitory activity against DMBA (Fig. 3A, Tab. 2).

Effects of flavonoids on the simultaneous reduction of DMBA and TPA. Polyphenols exhibited antitumorigenic activity in two-stage mouse skin carcinogenesis using DMBA and TPA in specific pathogen-free female ICR mice [27]. A series of 14 flavonoids were examined for their ability to inhibit the Epstein-Barr virus early antigen activation by TPA, and were found to inhibit tumor promotion in DMBA-initiated, TPA promoted model of skin carcinogenesis [28]. Initiation and promotion of two-stage skin carcinogenesis in mice treated with the promoter TPA was blocked when QU was administered 30 min before promoter application [29]. By blocking skin tumor promotion QU could be considered an effective agent in preventing skin cancer [30]. The BIRT laboratory [31,32] conducted a series investigation into the inhibition of chemically induced (DMBA/TPA-treated) and UV-light-induced skin carcinogenesis by AP in mice. Parallel studies in the PELLING laboratory with cultured epidermal cells [33] suggested that the inhibition of skin carcinogenesis might be due to the inhibition of cell cycle arrest by AP.

The polarographic behavior of TPA which is a specific promoter of epidermal tumorigenesis iniciated by DMBA, has been studied by several authors [21]. TPA is reduced in anhydrous DMF on a mercury dropping electrode in two diffuse one-electron steps with the values of $E_{1/2}$ –2.090 V and –2.480 V vs. SCE [21].

It has been shown that in experimens with DMBA+TPA all flavonoids investigated possess inhibitory activity. The value of inhibitory activity depends on the number and position of hydroxyl groups at the ring B.

Similary to experiments with DMBA flavonoids QU, RU, LU, AP, FI and KA, which are reduced in two one-electron steps and are hydroxylated at the ring B, have been shown to possess high inhibitory activity against DMBA + TPA (Fig. 3B, Tab. 2). Another important factor was the number of hydroxyl groups at the rings A and B. FI, KA and AP (Fig. 2) have been shown to be the most potent compounds. They decreased the activity of DMBA+TPA in the range from 73.3% to 78.87%. LU and QU, which have together four hydroxyl groups at rings A and B, decreased the activity of DMBA + TPA in the range from 66.2% to 69.7% (Fig. 3B, Tab. 2).

7-OH-FL, 7,8-OH-FL, GA, BA and CH, which are not hydroxylated at the ring B and are reduced in three one-electron steps, have been shown to inhibit the activity of DMBA + TPA (Fig. 3B, Tab. 2). Their inhibitory activity is much more lower than of flavonoids, which possess hydroxyl groups at the ring B. The value of the inhibitory activity of DMBA+TPA of these compounds was in the range from 18.9% to 38.74% (Fig. 3B, Tab. 2).

Conclusions

The presented data demonstrated that electrochemical method – DC polarography is very sensitive, simple technique for preliminary determining of tumor inhibiting or promoting activities of studied compounds. Use of electrochemical methods could therefore contribute much to our understanding of anticancer agents. The obtained results are correlated with their structural arrangement and organization of the hydroxyl groups. In our work we wanted to demonstrate the possibility of using DC polarography as an analytical method in the search for compounds with presumed biological activity. Flavonoids are possible cancer chemopreventive agents and, further investigations to explore the mechanisms of action of these promising compounds are warranted.

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