Modulation of cytochrome P450 enzyme system by selected flavonoids

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Abstract

OBJECTIVES: The aim of this study was to assess the effect of various flavonoids on the NADPH:cytochrome P450 oxidoreductase (CYPOR) activity in respect of the reduction of different electron acceptors as well as to study an impact of flavonoids on monooxygenation of a model substrate of cytochrome P450 (CYP).
DESIGN: The modulation of CYPOR activity was determined spectrophotometrically based on the time course of the reduction of different electron acceptors. The CYP reduction was monitored via its complex formation with CO, having pronounced the absorption maximum at 450 nm. Finally, effect of CYPOR stimulation by 7,8-benzoflavone (ANF) on 7-pentoxyresorufin O-dependentylation was assayed in the microsomal monooxygenation system using the fluorimetric detection of formed resorufin.
RESULTS: The stimulation of CYPOR activity via ANF was found to be associated with following electron acceptors: cytochrome c, potassium ferricyanide, cytochrome b_5, but not with CYP. Surprisingly, 5,6-benzoflavone, a position isomer of ANF, was ineffective in the CYPOR stimulation as well as the other flavonoids tested. In microsomal preparations, ANF did not markedly enhance the reaction rate of monooxygenation of CYP2B4 model substrate.
CONCLUSION: Our results document that among all of the tested flavonoids only ANF is able to stimulate CYPOR activity, however, the ANF-mediated stimulation of CYPOR has no impact on the oxidative metabolism catalyzed by CYP system.

INTRODUCTION

Flavonoids are dietary phytochemicals, which belong to popular chemopreventive compounds exerting a great variety of beneficial effects on the human health. However, in some flavonoids have been observed mutagenic (e.g. quercetin) and/or prooxidant effects and some of them interfere with essential biochemical pathways. Among proteins interacting with flavonoids, cytochromes P450 (CYPs) – monooxygenases metabolizing xenobiotics (e.g. drugs, carcinogens) and endogenous substrates (e.g. steroids) - play the most prominent role (Hodek *et al.* 2002). Flavonoids might both inhibit or stimulate the activity of several CYPs, and/or induce an expression of certain CYPs (Moon *et al.* 2006). Owing to the structure similarity with an estrogen skeleton, some flavonoids show an estrogenic or anti-estrogenic activity (for review see: Adams & Chen, 2009). Flavonoids are known to be able to inhibit activation of certain carcinogens while on the other hand they can at the same time induce CYPs activating another group of carcinogens. Hence, a detailed study on the flavonoid interactions with the CYP multi-en-

ORIGINAL ARTICLE

Abbreviations & units

ANF – α-Naphthoflavone (7,8-benzoflavone)
AAI – aristolochic acid I (8-methoxy-6-nitrophenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid)
BNF – β-Naphthoflavone (5,6-benzoflavone)
CYP – cytochrome P450
DIA – diamantane
CYPOR – NADPH:cytochrome P450 reductase

zyme system, namely from the view of the modulation of CYP activity, is important to prevent carcinogen activation and changes in drug and endogenic compound metabolism.

Depending on the structure, flavonoids inhibit various CYPs. 7,8-Benzoflavone (ANF) is known to be a potent inhibitor of CYP1A1/2, however, it has no effect on the other CYPs, e.g. CYP2B4. In our recent experiments this flavonoid stimulated the metabolic activation of aristolochic acid I, 8-methoxy-6-nitrophenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid (AAI) to intermediates covalently binding DNA. ^{32}P-Postlabeling technique proved an 8-fold increase in DNA adducts formation in the presence of 10 μM ANF (Stiborova *et al.* 2005). This increase in the reductive activation of AAI was suggested to be caused by the stimulation of NADPH:CYP reductase (CYPOR) activity. As the CYP multi-enzyme system is involved in both the reductive and oxidative carcinogen activation (Dracinska *et al.* 2006), our data raised several questions on the possible impact of the CYPOR stimulation on cancer risk for humans.

The aim of the presented study was to reveal the CYPOR stimulatory effect in respect of the involvement of other electron acceptors (natural, artificial) and the role of other flavonoids, and moreover, to find the effect of CYPOR stimulation on CYP-mediated metabolic reactions.

MATERIAL AND METHODS

Chemicals. 5,6-Benzoflavone, 7,8-benzoflavone, bicinchoninic acid, quercetin dihydrate, alpha-lipoic acid, morin hydrate, NADPH + H$^+$, NADP$^+$, 7-penthoxyresorufin, resorufin were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were purchased from standard commercial sources and were of the highest quality available.

Animal treatment and preparation of microsomes. All studies with rabbits were conducted in accordance with the Decree on breeding and use of experimental animals (No. 311/1997 Coll., Ministry of Agriculture, Czech Republic), which is in compliance with the Declaration of Helsinki. Male rabbits (2.5 kg) obtained from AnLab, Czech Republic, were housed in cages at 22°C with a 12 hr light/dark period and *ad libitum* standardised diet and water access. To increase amounts of CYPs (namely CYP2B4 and 1A2), CYPOR and cytochrome b_5, rabbits were treated with phenobarbital in drinking water for ten days (0.1% (w/v) solution). The animal, fasted overnight, was sacrificed and microsomal fraction from the whole liver of each rabbit was prepared as described previously by Krizkova *et al.* (2008). The total CYP content was measured based on the complex of reduced CYP with CO (Omura & Sato, 1964), and the total CYPOR activity was determined as NADPH:cytochrome c reductase activity (Williams & Kamin, 1962). Protein concentrations were assessed using bicinchoninic acid and bovine serum albumin as the standard (Smith *et al.* 1985). From individual liver microsomal fractions CYP1A2, CYP2B4, CYPOR or cytochrome b_5 were isolated as described by Stiborova *et al.* (2001) and Strittmatter *et al.* (1978). Microsomal fractions and enzyme preparations were stored at -80°C before use.

Cytochrome c and ferricyanide reduction. Incubation mixtures prepared in spectrophotometric cuvettes (1 cm pathlength) consisted of 0.4 μM CYPOR in 300 mM potassium phosphate buffer (pH 7.5), 0.5 mg/ml cytochrome c or 0.1 mM $K_3[Fe(CN)_6]$, and 0-50 μM lipoic acid or flavonoid (methanol stock solution) in a final volume of 1 ml. After iniciation with 10 μl of 10 mM NADPH, the reduction of an electron acceptor was recorded at 550 nm and 420 nm, for cytochrome c and $K_3[Fe(CN)_6]$, respectively, on Hewlett Packard 8453 E spectrophotometer for 2 min.

Cytochrome b_5 reduction. Incubation mixtures were prepared in spectrophotometric cuvettes (1 cm pathlength). The mixtures consisted of 0.4 μM CYPOR, 4 μM cytochrome b_5 and NADPH regenerating system (1 mM NADP$^+$, 10 mM D-glucose 6-phosphate, 1 unit/ml D-glucose 6-phosphate dehydrogenase, 10 mM $MgCl_2$) in a final volume of 1 ml of 100 mM potassium phosphate buffer (pH 7.5) (Sistkova *et al.* 2008).

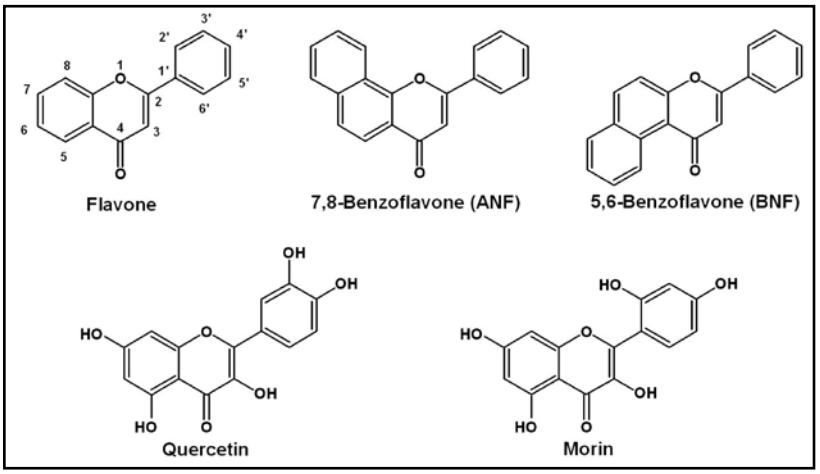

Figure 1. Chemical structures of tested flavonoids.

Cytochrome b_5 reduction in the absence or presence of 50 μM 7,8-benzoflavone was monitored at 426 nm on Hewlett Packard 8453 E spectrophotometer for 2 hrs.

Formation of cytochrome P450–CO complex. Reaction mixtures in a final volume of 2 ml contained 100 mM potassium phosphate buffer (pH 7.5), microsomal fractions or CYPOR (0.4 μM) and CYP preparations (2 μM CYP in final concentration) and 50 μM 7,8-benzoflavone or 10 μM diamantane. After saturating with CO gas (45 sec) the sample was divided into two cuvettes (1 cm pathlength) and the reduction started by the addition of NADPH regeneration system (10 μl of the stock) into a sample cuvette. The formation of cytochrome P450–CO complex was recorded in capped cuvettes at 450 nm on spectrophotometer Specord M40 for 60 min.

Assay of CYP metabolic activity. 7-Pentoxyresorufin-O-depenthylase activity was determined in phenobarbital induced microsomes according to Burke & Mayer, (1974). Reaction mixture contained in a final volume of 0.5 ml: 5 μM 7-pentoxyresorufin, microsomal fraction (protein concentration 1 mg/ml) in 100 mM potassium phosphate buffer (pH 7.5) and 100 μM 7,8-benzoflavone or methanol (flavonoid solvent). The reaction was started by the addition of NADPH regeneration system (10 μl of the stock). Formation of resorufin was continuously measured for 10 minutes at room temperature by monitoring its fluorescence (excitation and emission wavelengths of 530 and 585 nm, respectively) using fluorescence spectrometer (LS 55 Perkin Elmer). The rate of dealkylation was estimated on the basis of a resorufin standard curve.

RESULTS

Cytochrome c was used as an electron accepting protein to study the reduction of this haemoprotein by CYPOR in the presence of ANF, 5,6-benzoflavon (BNF), flavone, quercetin, morin and alpha-lipoic acid (CYPOR inhibitor) (for structures see ***Fig. 1***). Data in ***Figures 2*** and ***3*** show that only ANF was able to stimulate the reduction of cytochrome c. When potassium ferricyanide was used to accept electrons from CYPOR, the results (see ***Fig. 4***) were almost identical to cytochrome c experiments. ***Figure 5*** depicts the stimulatory effect of ANF on the reduction of cytochrome b_5, which can be slowly reduced also by CYPOR. Finally, the stimulatory effect of ANF on CYPOR was tested with CYPs (direct reaction partners of CYPOR) using the reduced CYP-CO complex. In these experiments, a gradual transfer of the first electron (fast process) and the second electron (slow process) from NADPH via CYPOR to haem iron of CYP was monitored. Curves in ***Figure 6*** show that ANF enhanced the speed of reduction of CYP in microsomal samples. Since the reaction rate of CYP reduction is affected by binding of CYP substrate/inhibitor (e.g. ANF) into the enzyme active centre, further experiments were carried out in recon-

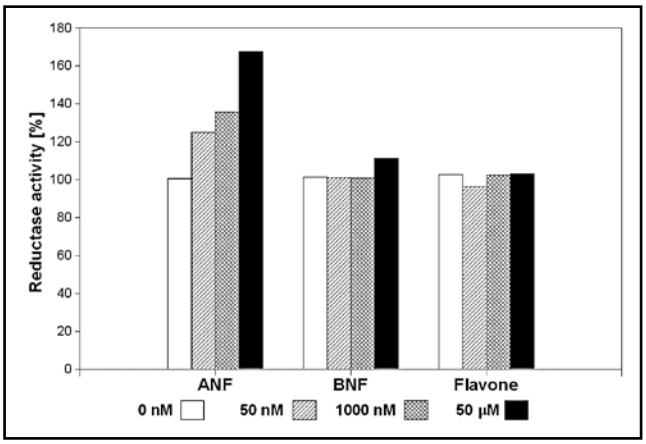

Figure 2. Effect of flavonoids on cytochrome c reduction. Tested compounds were added from methanol stocks to reach 0-50 μM final concentrations. Results are shown in values relative to untreated control (100%). Plotted data are averages from two measurements which did not differ more than 10%.

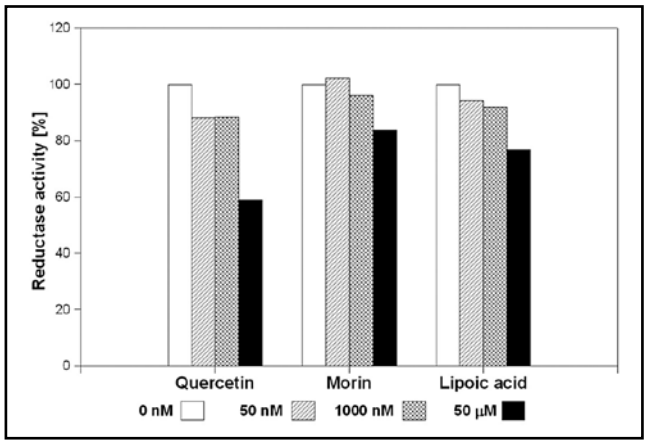

Figure 3. Effect of flavonoids and lipoic acid on cytochrome c reduction. Tested compounds were added from methanol stocks to reach 0-50 μM final concentrations. Results are shown in values relative to untreated control (100%). Plotted data are averages from two measurements which did not differ more than 10%.

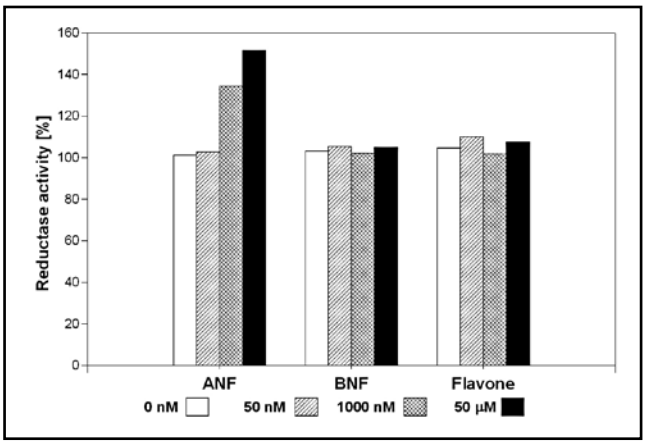

Figure 4. Effect of flavonoids on potassium ferricyanide reduction. Tested compounds were added from methanol stocks to reach 0-50 μM final concentrations. Results are shown in values relative to untreated control (100%). Plotted data are averages from two measurements which did not differ more than 10%.

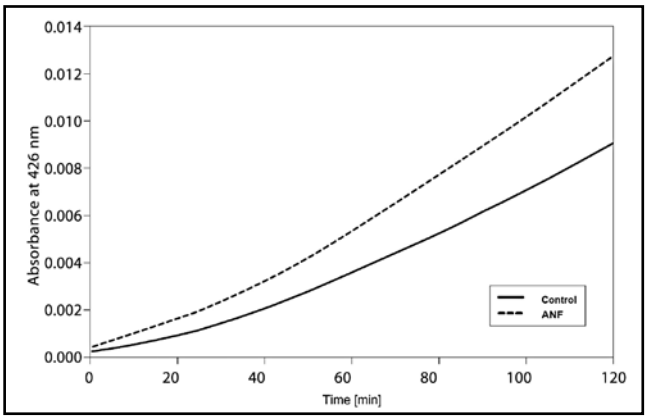

Figure 5. Time course of cytochrome b_5 reduction in the absence or presence of 50 μM ANF.

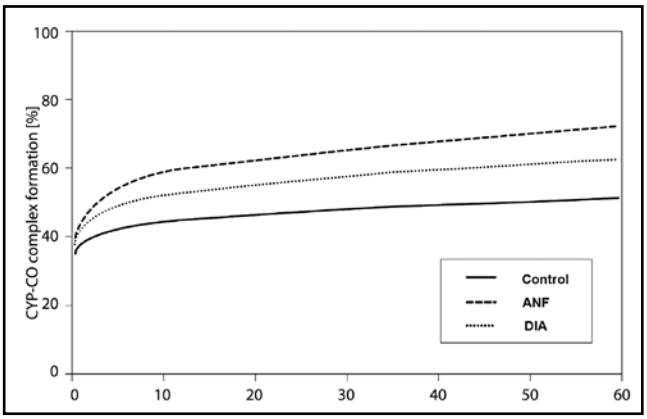

Figure 6. Formation of CYP–CO complex in the microsomal system in the presence of either 50 μM ANF, 10 μM DIA or methanol (solvent control). A 100% complex formation was achieved by CYP reduction using sodium dithionite.

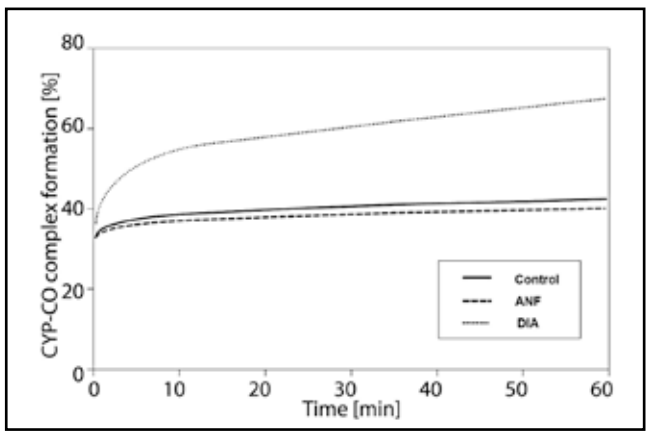

Figure 7. Formation of CYP–CO complex in CYP2B4 reconstituted system in the presence of either 50 μM ANF, 10 μM DIA or methanol (solvent control). A 100% complex formation was achieved by CYP reduction using sodium dithionite.

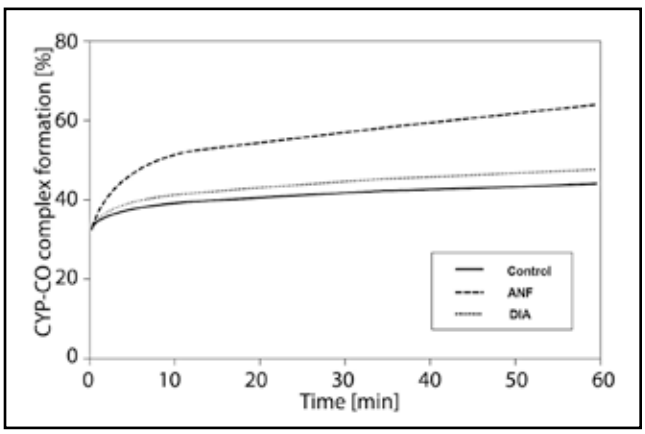

Figure 8. Formation of CYP–CO complex in CYP1A2 reconstituted system in the presence of either 50 μM ANF, 10 μM DIA or methanol (solvent control). A 100% complex formation was achieved by CYP reduction using sodium dithionite.

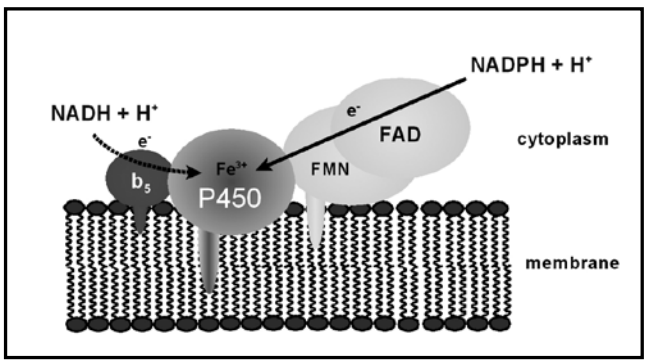

Figure 9. Scheme of CYP multienzyme system in a microsomal membrane. Arrows show the electron flow from donors via FAD-FMN reductase or cytochrome b_5 to haem iron of CYP.

stituted system, composed of purified CYPOR and CYP1A2 or CYP2B4, which are the major CYPs present in the microsomal samples used. In the reconstituted system containing CYP2B4 (binds DIA, but not ANF), DIA stimulated the CYP reduction, but ANF was ineffective (see ***Fig. 7***). For comparison, the same experiments were carried out with CYP1A2 (binds ANF, but not DIA) instead of CYP2B4. ***Figure 8*** shows that the ANF stimulatory effect on CYP reduction occurred, while DIA did not affect the CYP1A2 reduction. To reveal further the impact of ANF-mediated stimulation of CYPOR on the CYP reduction, the metabolic activity of CYP2B4 (CYP not affected by ANF binding) was examined. The reaction rate of O-dependentylation of 7-pentoxyresorufin (specific substrate of CYP2B4) was not changed by 100 μM ANF present in the reaction mixture (data not shown).

DISCUSSION

The cytochrome P450 multi-enzyme system plays a key role in the process of chemical carcinogenesis (for molecular organization see ***Fig. 9***). Although there is accumulating evidence on oxidative activation of carcinogens by CYPs, much less is known about the reductive pathway. To prevent carcinogen activation,

the so-called chemopreventive compounds are frequently used in order to inhibit CYP-mediated reactions. However, among adverse effects on the human health (Hodek *et al.* 2009), the stimulation of carcinogen activating enzymes should be considered, too. The stimulation of CYPOR by ANF, resulting in the reductive activation of carcinogenic AAI, might serve as an example of unexpected behaviour of a chemopreventive flavonoid (Stiborova *et al.* 2005). This finding raised a question whether the found stimulatory effect of ANF on CYPOR is a common feature of flavonoids or whether it is associated with this particular compound. In order to elucidate this unusual effect of ANF on CYPOR, several different acceptors of the electron flow from the CYPOR were employed and their reduction was monitored in the presence of BNF (isomer of ANF), flavone, and its penta-hydroxylated derivatives, quercetin and morin. Surprisingly, except for ANF, none of the tested flavonoids (see ***Fig. 2*** and ***3***) was a CYPOR stimulator. Moreover, hydroxylated flavonoids exerted even an inhibitory effect, similar to alpha-lipoic acid that is a known CYPOR inhibitor (Slepneva *et al.* 1995). Furthermore, the involvement of electron acceptors in the CYPOR stimulation was examined. Ferricyanide and haemoproteins, cytochrome c and cytochrome b_5, were effective electron acceptors in this process. The role of CYP is much more complex. In the microsomal preparation, containing several CYPs, the ANF-mediated stimulation of CYP reduction might be attributed either to the binding of ANF in the CYP active centre, which facilitates its reduction, or to the interaction of ANF with CYPOR, resulting in an enhanced flow rate of electrons. To distinguish between these two effects of ANF, reconstituted systems, containing but one CYP, were used. In the case of CYP1A2 system (binding ANF, but not DIA), both effects of ANF could occur simultaneously (see ***Fig. 8***) and thus, it would be impossible to distinguish between them. Because of that the system containing CYP2B4 (binding DIA, but not ANF) was employed. No ANF-mediated stimulation of CYP reduction was detectable in this system (see ***Fig. 7***). The gradual and rather slow reduction of the haem iron of CYPs most likely explains why the stimulated CYPOR providing an enhanced flow rate of electrons did not influence CYP containing systems. Thus, we can conclude that the ANF stimulation of CYPOR is associated with the CYPOR protein macromolecule, since it is independent of the electron acceptor used. As shown in ***Figure 9***, also electrons from cytochrome b_5 can participate in the CYP reduction. Hence, it was interesting to know, whether the ANF-stimulated reduction of cytochrome b_5 has any impact on the CYP-catalyzed metabolism. These experiments were carried out with microsomal fractions of phenobarbital-pretreated rabbits, which contained cytochrome b_5, CYPOR, and both studied CYPs. As CYP2B4 do not interact with ANF, the specific metabolic activity of this CYP, O-dependentylation of 7-pentoxyresorufin, was selected to determine the impact of ANF on CYP-mediated metabolism. Results showed that the reaction rate of O-dependentylation of 7-pentoxyresorufin was not affected by the presence of ANF. Thus, even with the whole CYP multi-enzyme system (microsomal fraction) an expected stimulatory effect of ANF on CYP-mediated metabolism was not proved. Finally, we can summarize that the CYP reduction is the rate-limiting step of the monooxygenation of CYP substrates and thus, the stimulation of CYPOR does not increase the rate of the oxidative pathway catalyzed by the whole CYP multi-enzyme system.

Acknowledgements

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