RSC Advances

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. This *Accepted Manuscript* will be replaced by the edited, formatted and paginated article as soon as this is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Electro catalytic cycle of cytochromes P450: the protective and stimulating roles of antioxidants

V. V. Shumyantseva, [a]* A. A. Makhova,[b] T. V. Bulko,[a] A. V. Kuzikov,[a] E. V. Shich,[b] V. Kukes,[b] and A. I. Archakov[a]

[a]*Institute of Biomedical Chemistry, Pogodinskaya Street, 10, Moscow 119121, Russia*

[b]*I. M. Sechenov First Moscow State Medical University*

[*]Corresponding author:

Victoria V. Shumyantseva, Dr. Sci. Biol.

Tel. +7 499-246 58 20;

Fax: +7 499-245 08 57.

E.mail: viktoria.shumyantseva@ibmc.msk.ru

RSC Advances Accepted Manuscript

Abstract

The electrochemical activity of cytochromes P450 (CYP) 3A4, 2C9 and 2D6 on the screen-printed graphite electrode, nanostructured with gold nanoparticles, stabilized by didodecyldimethylammonium bromide was examined. The analysis of CYP catalytic activity was carried out by a variety of electrochemical techniques, such as cyclic voltammetry, square wave voltammetry, amperometry. A sensitive electrochemical CYP-sensor system was proposed as a reliable candidate for investigation of influence of medicinal preparations - ethoxidol (2-ethyl-6-methyl-3-hydroxypyridine malate), mexidol (2-ethyl-6-methyl-3-hydroxypyridine succinate), cytochrome *c* and L-carnitine - on the CYP redox behavior and electrocatalytic activity. In the presence of antioxidants, the enhancement of electrochemical signal of CYP was registered. It was shown that ethoxidol, in the concentration range with the values of 90–600 μM, stimulates the electrochemical reduction of cytochromes P450, with the maximum stimulating effects for CYP3A4, CYP2C9 and CYP2D6 being estimated, respectively, as 181 ± 15%, 240± 7%, 110± 4% (at 540 μM ethoxidol).

Key words: Cytochrome P450; Direct electron transfer; Antioxidants; Enzyme electrodes, Drug metabolism

RSC Advances Accepted Manuscript

Cytochromes P450 (CYPs) are typical external monooxygenases capable of catalyzing the oxygenation of a variety of organic substances using NAD(P)H and O_2 as cosubstrates. CYPs can act as an electron carrier in reducing compounds during the catalytic cycle and as an oxidase when generating superoxide anions and hydrogen peroxide as reactive oxygen species with organic substrate oxygenation. Water formation is characteristic of CYPs functioning as a monooxygenase, so the enzyme is also called the mixed-function oxidase. CYPs are able to act as four–electron oxidase and while reducing oxygen to two water molecules.[1-6] However, in spite of such multi functionality, the main function of cytochromes P450 is monooxygenation reactions, i.e. hydroxylation of organic molecules towards drugs and foreign chemicals in the body. From this viewpoint, the in vitro biocatalysis with CYPs is a promising approach for drug assay in practical clinical medicine, as well as for analysis of drug-drug interaction, and substrate/inhibitor competence of these enzymes.

Investigation of the catalytic activity of isolated cytochromes from the P450 superfamily requires obligatory presence of redox partners and electron donors (NADPH). However, the availability of redox partners is not obligatory upon electrochemical reduction of P450 family hemoproteins, so the catalytic system is essentially simplified.[7-19] Electrochemical systems execute the dual function: substitute partner proteins and serve as a source of electrons for redox enzymes.

From a chemical viewpoint, this diversity of functions of CYPs is based on oxidative-reductive properties of the P450s' heme iron. From an electrochemical viewpoint, the most characteristic feature of hemoproteins is their ability to perform direct electron transfer from the electrode surface to the heme iron and *vice versa*, - as is reflected on the voltammogram (in the absence of oxygen) in the appearance of a pair of peaks corresponding to the oxidation and reduction processes. The electrode reaction of the heme may be described by the equation:

$$Fe(III) + e^{-} \text{ (from electrode) } + H^{+} \leftrightarrow Fe(II)$$

The relevance of the electrochemical approach is apparent: indeed, cytochromes P450-based electrodes may be used as biosensors in personalized medicine, high-throughput screening and drug interference studies, as well as in therapeutic drug monitoring. Studies aimed at the search of new drugs, estimation of their toxicity and drug-drug interaction have shown that cytochromes P450 are the most significant preparations for use in practical clinical medicine.

RSC Advances Accepted Manuscript

One of the most important methods in personalized medicine is tracing of a dose of clinical drug and correction of drug intake by taking into account specific features of the patient. Pharmacogenetic tests allow to reveal risk of side effects of a number of drugs (for example, anticoagulant warfarin) and to change and/or correct doses of medicinal preparations. Individual reaction to warfarin is caused by single nucleotide polymorphism of the genes coding CYP2C9, and vitamin K-epoxy reductase (VKORC1). Thus, the Roche Company developed genetic tests based on microarray technique (Amplichip CYP450) for genomic predictions.[5, 17]

Despite the importance and broad informational content of the pharmacogenetic tests, their results do not allow exerting influence on activity of the drug metabolizing enzymes. Therefore, it is necessary to develop other approaches, based on regulation of enzymes' activity by means of biologically active compounds or metabolic drug preparations.

The functional significance of cytochrome P450s (CYPs) includes the metabolism of drugs, foreign chemicals and endogenic compounds. Cytochromes P450 play an important role in detoxication of bioactive compounds and hydrophobic xenobiotics both coming from outside (medicines, drugs, food supplements, environment pollutants) and being formed inside cells (cholesterol, saturated and unsaturated fatty acids, steroids, prostaglandins and others) in living organisms. CYP3A4 recognizes and metabolizes a broad range of structurally diverse therapeutic agents.[5, 20] Consequently, many clinically relevant drug-drug interactions are associated with inhibition and/or induction of this enzyme.[21] Antioxidant therapy is used as the addition agent in cellular defense against ROS and in the treatment of different diseases.[22]

In the present study, we have investigated the influence of the metabolic drugs ethoxidol, mexidol and cytochrome *c* possessing antioxidant properties on the electrochemical activity of P450 enzymes. Medicinal preparations ethoxidol and mexidol based on 2-ethyl-6-methyl-3-hydroxypyridine are widely used in clinical practice. They possess antioxidant, nootropic and cerebroprotective effects. We have found that drug-antioxidants: mexidol, ethoxidol and cytochrome *c* stimulated the redox behavior of cytochromes P450 3A4, 2C9 and 2D6 and enhanced electrochemical signal, corresponding to heme reduction. L-carnitine had no effect on CYP electrochemical activity. The data obtained support the availability of regulation of pharmacokinetic parameters and the pronouncement of pharmacodynamic effects due to with the help of the impact of antioxidant medicinal preparations on the activities of P450 3A4, 2C9 and 2D6.

Materials and methods.

RSC Advances Accepted Manuscript

Apparatus

Electrochemical studies were performed using an AUTOLAB 12 potentiostat/galvanostat (Metrohm Autolab, the Netherlands) with GPES software (version 4.9.7). All measurements were taken at room temperature. Electrochemical studies on cytochrome P450 3A4, 2C9 and 2D6 were performed in 0.1 M potassium-phosphate buffer containing 0.05 M NaCl, pH 7.4 (PBS). In this work, three-pronged screen-printed electrodes (SPE) were used (AvtoKOM, Russia; http://www.membrans.ru) with working and auxiliary graphite electrodes (graphite paste was from Acheson) and Ag/AgCl reference electrode. The working electrode diameter was 2 mm. All potentials are referred to the Ag/AgCl reference electrode.

Cyclic voltammograms (CV) were recorded at the scan rate 10-100 mV/s. Parameters used in square wave voltammetry (SWV, reduction, aerobic conditions) were as follows: initial potential, 100 mV; final potential, –600mV; step potential, 5 mV; amplitude, 20 mV; frequency, 10 to 100 Hz. For presentation of all electrochemical data, the average values of maximum cathodic peak height of SWV from three independent experiments were used. Electrolysis was conducted at the controlled potential of -0.5 V, for 20 min (erythromycin) and 60 min (testosterone and diclofenac). The apparent Michaelis-Menten (K_m) constant was determined using the electrochemical form of the Michaelis-Menten equation[21] from the amperometry at the controlled reduction potential of -0.5 V in the 1 mL of air-saturated buffer with injection of erythromycin or testosterone. The apparent Michaelis-Menten (K_m) constant for diclofenac was determined previously.[7, 23]

Materials and reagents.

The following reagents were used: didodecyldimethylammonium bromide (DDAB), $HAuCl_4 \cdot 3H_2O$, sodium borohydride, itraconazole (Sigma-Aldrich, USA); diclofenac (2-(2, 6-dichloraniline) phenylacetonic acid) (Novartis, Russia); ethoxidol (2-ethyl-6-methyl-3-hydroxypyridine malate, 50 mg/ml (Sintez, Russia); mexidol (2-ethyl-6-methyl-3-hydroxypyridine succinate, 50 mg/ml (Farmasoft, Russia); erythromycin (Sigma–Aldrich); acetic acid, ammonium acetate, acetylacetone (Spektr-Khim, Russia), testosterone (Sigma–Aldrich).

In electrochemical experiments were used: freshly prepared solutions were used of 10 mM diclofenac in water, 10 mM erythromycin in ethanol–water (7:3), 10 mM testosterone ethanol–water (7:3) and 10 mM itraconazole in dimethylsulfoxide. Recombinant cytochrome P450 3A4 (182 µM) and 2C9 (175 µM) were from the Institute of Bioorganic Chemistry (Minsk,

RSC Advances Accepted Manuscript

Belarus), and 2D6 (30 μM) was from Sigma. Concentration of P450 enzymes was determined by production of a complex of the reduced form with carbon oxide with the absorption coefficient[24] ε_{450}= 91 $mM^{-1}\cdot cm^{-1}$.

P450-electrodes were prepared as earlier described.[25, 26] Briefly, the surface of the working graphite electrode was covered with 2 μl of 5 mM colloid gold solution in 0.1 M DDAB in chloroform, and after the evaporation of chloroform (10 min) 1 μl of 18.2 μM of P450 3A4, 1 μl of 17.5 μM of P450 2C9 or 2 μl of 30 μM of P450 2D6 was placed on the electrode. To prevent the complete drying of the electrodes, they were kept for 12 h at 4°C in a humid chamber.

Determination of the N-demethylase activity of cytochrome P450 3A4.

Formaldehyde is one of the products of the cytochrome P450 3A4-dependent electrocatalytic N-demethylation of erythromycin, and its accumulation was used to assess the activity of the enzyme immobilized on the electrode. The electrode with immobilized cytochrome P450 3A4 was placed into a 1-ml electrochemical cell that contained potassium-phosphate buffer (pH 7.4) and 100 μM erythromycin. Electrolysis was performed at the controlled potential E= -0.5V for 20 min. After the electrolysis, the reaction mixture was mixed with Nash reagent (4 M ammonium acetate, 0.1 M glacial acetic acid, 0.04 M acetyl acetone) at the ratio 1: 1 and incubated at 37°C for 30 min to develop the coloring.[27] The concentration of formaldehyde produced during the cytochrome P450-dependent electrocatalytic N-demethylation of erythromycin, was determined spectrophotometrically using a Cary 100 Scan UV-Vis spectrophotometer (Agilent, the Netherlands), the absorption coefficient being ε_{412}= 4 $mM^{-1}\cdot cm^{-1}$ as in.[28, 29].

HPLC/MS analysis of 6β-hydroxytestosterone and 4-hydroxydiclofenac were conducted as described previously.[30]

Results

The influence of antioxidants on CYP3A4 electrochemical reduction and electrochemical response

RSC Advances Accepted Manuscript

Screen-printed electrodes modified with the membrane-like synthetic surfactant didodecyldimethylammonium bromide (DDAB) and gold nanoparticles (SPE/Au/DDAB) were used for CYP3A4 immobilization of on the electrode surface (SPE/Au/DDAB/CYP3A4). To investigate the electro analytic characteristics of electrochemical systems with cytochrome P450 immobilized on the nanostructured electrode surface, we have resorted to cyclic voltammetry, voltammetric analysis (square wave voltammetry) and amperometry at the controlled reduction potential -0.5 V (vs. Ag/AgCl).

A clear pair of peaks was observed in CV (in argon) with the formal redox potential E^0 = (Ered + Eox)/2) = -0.302 V for CYP3A4 (Fig.1A, inset). As we have shown earlier by use of SPE/Au/DDAB, with the increase of scan rate, the anodic and cathodic peak currents of anaerobic CV were also increased and appeared to be proportional to the scan rate[25, 26], indicating a typical evidence for manifestation of the surface–controlled process.

Figure 1A

Figure 1B

The antioxidant/antihypoxant mexidol 2-ethyl-6-methyl-3-hydroxypyridine succinate (mexidol) and 2-ethyl-6-methyl-3-hydroxypyridine malate (ethoxidol) (both widely used in clinical practice) were tested as inducer/modulators of CYP3A4 activity. Ethoxidol and mexidol stimulated electrochemical reduction of cytochrome P450 3A4 (Fig. 1A). Both these compounds did not produce type I differential binding spectra as are typical for substrates of CYP enzymes.[1-3] In electrochemical experiments, drugs-antioxidants serve as modulating and/or stimulating additives with respect to CYP electrochemical activity due to their free-radical scavenging, antihypoxant properties, and/or electron mediator properties. The increase in the reductive cathodic current in the presence of ethoxidol was also studied by the more sensitive voltammetric analysis method, such as square wave voltammetry (SWV) (see Fig. 1B). Ethoxidol itself did not produce any pronounced peak in the region of potentials +0.1 ÷ -0.7 V.

In our experiments, ethoxidol and mexidol enhanced the cathodic reduction current of CYP3A4 in dose–dependent manner (Fig. 2). At 540 μM ethoxidol concentration its stimulating effect on CYP3A4 reduction reached 180±15 %. Mexidol is not as active as ethoxidol in the range of 200÷600 μM and only produces 155±15 % enhancement at 540 μM drug concentration. As we have shown earlier, the electrochemical response of CYP3A4 also increased in the

RSC Advances Accepted Manuscript

presence of antioxidants such as vitamin C, vitamin A, vitamin E [26] and taurine (2-aminoethanesulfonic acid).[29]

Figure 2.

Cytochrome *c* is a metabolic preparation with antihypoxant properties. This drug is used in the therapy of processes dealing with pathologic oxidative reactions. This cytochrome *c* stimulated the electrochemical reduction of CYP3A4 in dose-dependent manner (Fig. 3). After the application of 20-100 μM cytochrome c, the maximum enhancement of reductive peak current of CYP3A4 (140±10%) was observed with 60 μM cytochrome *c*.

Figure 3.

The vitamin-like biologically active compound L-carnitine (3R)-3-hydroxy-4-treemethylammonium butanoate) manifested itself as a strong antioxidant; it acted by reducing and scavenging free radicals formation and by enhancing the activity of antioxidant enzymes.[31] To investigate the influence of L-carnitine on CYP3A4 electrochemical parameters, we used SPE/Au//DDAB/CYP3A4. In the range of 100-600 μM L-carnitine, the voltammetric SWV peak for CYP3A4 in the presence and absence of this compound had the same position and intensity. L-carnitine did not produce any reductive current enhancement or any inhibition of cathodic current (Fig. 4).

Figure 4.

The absence of the stimulating effect of L-carnitine on CYP3A4 reductive peak and its electrochemical response can possibly be explained by the polar structure of this substance and the lack of interaction with the CYP's active site or its' heme. It is known that the main function of CYP enzyme is to catalyze oxidative transformation and to hydroxylate hydrophobic xenobiotics or endogenous compounds, thereby producing more polar products capable of for excretion.[1-3]

Electrocatalysis of CYP in the presence of antioxidants

We have studied the influence of the antioxidant ethoxidol on the catalytic current generated by CYP3A4 in the presence of their typical substrates erythromycin, diclofenac and testosterone. We have conducted the cyclic voltammetry of CYP3A4 on the Au/DDAB modified electrode in the presence of 1 mM erythromycin as substrate, in oxygenated 0.1 M potassium phosphate buffer (pH 7.4). In the case of erythromycin, the catalytic current was registered, indicating the occurrence of the CYP3A4-catalyzed drug metabolism reaction (Fig. 5).

Figure 5.

RSC Advances Accepted Manuscript

With erythromycin as substrate, the ratio of the catalytic current in the presence of oxygen to the catalytic current in the presence of erythromycin corresponded to I_{O2}/I_{er} = 1.30±0.01 (100%), while with ethoxidol plus erythromycin I_{O2}/I_{er+et} = 1.70±0.04 (131%), respectively. The catalytic current of CYP3A4 in the presence of diclofenac and testosterone as measured by SWV, was found to be stimulated by of ethoxidol (Fig. 6).

Figure 6.

The influence of ethoxidol on the erythromycin N-demethylation was studied spectrophotometrically at 412 nm by means of formaldehyde formation using the Nash reagent.[21, 29, 30] The catalytic constant k_{cat} of erythromycin N-demethylation was calculated for CYP3A4+erythromycin and for CYP3A4+ethoxidol+erythromycin, by formaldehyde quantification using a method of Nash.[27] The values obtained in both cases were approximately the same values: 3.1±0.3 min^{-1} and 2.7±0.3 min^{-1}, respectively.

Product formation for erythromycin N-demethylation was measured with Nash reagent as well as by HPLC/MS analysis of 6β-hydroxytestosterone, and 4-hydroxydiclofenac. Analysis of the reaction products and the influence of antioxidants on substrate conversion are summarized in Table 1. Only in the case of taurine was the increase of N-demethylation of erythromycin registered.[29] The apparent Michaelis-Menten (K_m) of CYP3A4 for erythromycin was 70±8 μM and for testosterone, 67±10 μM.

Clinical estimation of cytochrome P450 3A4 activity was carried out using a noninvasive approach by determining of the concentrations ratio of 6β-hydroxycortisol (produced from cortisol only under the catalysis of cytochrome P450 3A4) and cortisol (6β-hydroxycortisol/cortisol). Clinical experiments in ethoxidol-treated patients revealed a statistically significant increase in the concentration ratio of 6β-hydroxycortisol to cortisol before and after a course of ethoxidol intake, which suggesting an increase in the cytochrome P450 3A4 activity towards cortisol as an endogenous substrate of this P450 isoenzyme. Concentrations of 6β-hydroxycortisol and cortisol were determined in urine by using routine high-performance liquid chromatography (HPLC).[32] The determination of the 6β-hydroxycortisol/cortisol ratio in urine showed that the ethoxidol intake enhances this ratio from 3.95±0.88 to 4.92±0.96 at statistically relevant level.[32] The results obtained proved the ability of antioxidants not only to stimulate CYP3A4 electrochemical reduction, but also to influence the clinical substrate metabolism in patients.

Evaluation of ethoxidol influence on CYP2C9 and CYP2D6 redox parameters

RSC Advances Accepted Manuscript

CYP2C9 is recognized as one of the most important drug-metabolizing enzymes in humans, responsible for the hepatic clearance of many non-steroidal and anti-inflammatory agents. CYP2C9 is involved in the metabolism of ~16% of therapeutically important drugs such as the anticoagulant warfarin, hypoglycemic tolbutamide and glipzide. CYP2D6 metabolized basic drugs and other xenobiotics containing a nitrogen atom, such as beta-blockers, tricyclic antidepressants.[2] Both these enzymes have polymorphic human form with different catalytic constants towards widely used drugs.[5, 33-35] The influence of ethoxidol on CYP2C9- and CYP2D6-produced electrochemical response was studied by SWV technique (Fig. 7, 8). It was shown that ethoxidol stimulated the reductive process of these CYP enzymes. Importantly, that the same ethoxidol concentration had different stimulating effects on CYP3A4, 2C9 and 2D6 enzymes (Fig. 9). The most effective stimulation by ethoxidol was registered for CYP2C9.

Figure 7.

We have found that antioxidants could influence direct electron transfer of human CYP3A4, 2C9 and 2D6 enzymes immobilized on electrode surface. In electrochemical experiments, drug-antioxidants served as modulating and/or stimulating additives with respect to P450s electrochemical activity due to their free radical scavenging and antihypoxant properties.

Discussion

Our earlier electrochemical studies were performed to examine the influence of vitamins B group on the catalytic activity of cytochrome P450 3A4 towards diclofenac.[25] Diclofenac (voltaren) is a non-steroidal anti-inflammatory drug used for treatment of arthritis, ankylosing spondylitis and acute muscle pain. [25,31] We have shown that thiamine (vitamin B1) and riboflavin (vitamin B2) blocked the catalytic activity of cytochrome P450 3A4 towards diclofenac. Diclofenac produced 137% increase of catalytic current without thiamine, but in the presence of thiamine, only 67% of catalytic current can be measured. Thiamine acts as a noncompetitive inhibitor against organic substrate (diclofenac) with the electrochemical inhibition constant K_i = 0.45±0.15 mM.[25] Therefore, thiamine prevents the electrochemical reduction of P450 3A4 and reduces the catalytic activity of cytochrome P450 3A4 towards diclofenac. Riboflavin (vitamin B2) also exhibits the inhibitory properties with respect to P450 3A4 electrocatalytic activity on the electrochemical SPE/DDAB/Au/CYP3A4+diclofenac system. Our results demonstrate that, based on the electrochemical behavior of the electrode/P450 3A4 system, it is possible to assess the influence of vitamins B group on the catalytic activity of cytochrome P450 alone and to evaluate the substrate/inhibitor competence of this enzyme. As have been shown in

RSC Advances Accepted Manuscript

pharmacokinetics experiments with volunteers, application of vitamins B group permits to shorten the longitude of diclofenac therapy and to reduce the daytime dose of this drug.[25]

Vitamins with antioxidant properties, such as vitamin C, vitamin A, and vitamin E, stimulate electrochemical reduction of cytochrome P450 3A4.[26] Vitamin C (in the range 0.03-1 mM), vitamins A and E (in the range 10-100 μM) stimulated the dose-dependent growth of the cathodic peak current of cytochrome P450 3A4, corresponding to heme reduction according to the equation Fe (III) +1 e → Fe (II). Electrochemically-driven P450 catalysis is accompanied by ROS generation;[31] therefore the influence of free radical-scavenging substances (ROS “traps” or antioxidants) on electrocatalysis may be reasonably expected (Scheme 1).

Scheme 1.

RH is the substrate for cytochrome P450; ROH is the product of P450-catalyzed monooxygenase reaction.

Reactive oxygen species interact with cytochrome P450; causing an inactivation of this protein.[37-40] Antioxidants can diminish the level of ROS by radical-scavenging effect, modulating the activities of CYPs, which are known to generate reactive intermediates. Scavenging substances are essential in the antioxidant defense against ROS, and can influence the catalytic functions of this hemoprotein. In CYP catalysis, the antioxidants may play a role of antihypoxant substances increasing the local oxygen level (Scheme 1).

The modulation of CYP catalytic activity is a very important problem in the pharmacokinetic and therapeutic areas. CYP2C9 enzyme, due to single nucleotide polymorphism, has two allelic variants (CYP2C9*2 and CYP2C9*3). These allelic variants of drug-metabolizing enzymes possess different catalytic parameters (K_m and k_{cat}) towards the substrates S-warfarin and tolbutamide.[33,34] This phenomenon must be taken into account during drug intake.

From this viewpoint, the search for substances capable of modulating the catalytic activity of CYP enzymes is a very promising task.

It was shown previously that vitamin C and cytochrome *c* can enhance the electron transfer in reactions mediating redox processes by serving as nonspecific redox activity facilitators for heme peroxidases such as chloroperoxidase and horseradish peroxidase.[41] It was shown also that vitamin

RSC Advances Accepted Manuscript

C, being a strong antioxidant, is capable of scavenging ROS in the low concentration ranges, and poses prooxidant capacity in high concentration.[42] Our experimental data are also revealed the active role of antioxidants in CYP electrocatalysis.

The electrochemically driven cytochromes P450 catalysis is an alternative model system for pharmacological research and drug-drug interaction studies. The increase in reductive cathodic current might be attributed to the Fe^{+3} heme reduction reflecting the stimulating effect of antioxidants. Based on the analysis of electrochemical parameters of P450, the algorithm, allowing elucidating the influence of antioxidants on CYP electrochemical activity, was developed. Electrochemical investigations encompassed CYP3A4, CYP2C9 and CYP2D6 isoenzymes. The electrochemical experiments have elucidated the possible mechanism of dose-dependent interaction of antioxidants with clinical drugs. These findings provide data for future clinical risk prediction studies – especially for those devoted to the interaction of drugs with antioxidants. Regulation and modulation of cytochrome P450 3A4 activity through the action of vitamins-antioxidants, upon their appointment in a combination with clinical drugs metabolized by P450, will probably become an essential requirement in clinical routine practice. Antioxidants intake can lead to alteration in pharmacodynamic efficiency - which demands special attention from the doctor since the prescribed medical product can bring about changes in an efficiency/safety profile.

It is necessary to keep in mind and to consider the interplay and balance between antioxidant properties of drugs, dealing, on the one hand, with (i) the ability of antioxidants to destroy hydrogen peroxide (a participant in CYP peroxide shunt reactions), and (ii) with the stabilizing effect of antioxidants on hemeproteins, realizing itself through the lowering of the ROS level; on the other hand, the account must be taken upon drug intake, of the antihypoxant properties of antioxidants, supplying CYPs by with additional oxygen. Electrochemically driven CYP reactions may have practically relevant providing a useful tool for drug assay studies.

Acknowledgments

The work is done in the framework of the State Academies of Sciences fundamental research program for 2013-2020.

Disclosure

The authors report no conflict of interest in this work.

RSC Advances Accepted Manuscript

Figure legends

Figure 1A. Cyclic voltammograms (CV) of SPE/Au/DDAB/CYP3A4 (——) and SPE/Au/DDAB/CYP3A4 after mexidol addition (90 μM) (· · ·). Inset. CV of CYP3A4 in argon, at scan rate of 50 mV/s in 0.1 M PBS, pH 7.4.

Figure 1B. Square-wave voltammetry (SWV) (reductive curves) of SPE/Au/DDAB/CYP3A4 before (——) and after the addition of the ethoxidol (90 μM) (· · ·).

Figure 2. The dependence of cathodic current intensity of reductive peak of SWV (%) on the concentration of ethoxidol and mexidol.

Figure 3. The dependence of cathodic current intensity (%) on the concentration of the cytochrome *c* (0-100 μM).

Figure 4. Square-wave voltammograms (SWV) (reductive curves) of SPE/Au/DDAB/CYP3A4 (——); SPE/Au/DDAB/CYP3A4+L-carnitine (186 μM) (- - -); SPE/Au/DDAB/CYP3A4+L-carnitine (372 μM) (· · ·).

Figure 5. Cyclic voltammograms of SPE/Au/DDAB/CYP3A4 (——); SPE/Au/DDAB/CYP3A4 + erythromycin (1 mM) (· · ·); SPE/Au/DDAB/CYP3A4 + erythromycin (1 mM) + ethoxidol (180 μM) (- - -).

Figure 6. Square-wave voltammograms (SWV) (reductive curves) of SPE/Au/DDAB/CYP3A4 (——); SPE/Au/DDAB/CYP3A4 + ethoxidol (90 μM) (· · ·); SPE/Au/DDAB/CYP3A4 + ethoxidol (90 μM) + diclofenac (100 μM) (- - -).

Figure 7. Square-wave voltammograms (SWV) (reductive curves) of SPE/Au/DDAB/CYP2C9 (——); SPE/Au/DDAB/CYP2C9 + ethoxidol (90 μM) (· · ·); SPE/Au/DDAB/CYP2C9 + ethoxidol (90 μM) + diclofenac (100 μM) (- - -).

Figure 8. Square-wave voltammograms (SWV) (reductive curves) of SPE/Au/DDAB/CYP2D6 (——); SPE/Au/DDAB/CYP2D6 + ethoxidol (90 μM) (- - -).

Figure 9. Normalized peak intensity, (%) of reductive SWV of screen-printed electrodes in aerobic buffer (with baseline correction): Au/DDAB/CYP3A4 + ethoxidol (540 μM); Au/DDAB/CYP2D6 + ethoxidol (540 μM); Au/DDAB/CYP2D6 + ethoxidol (540 μM).

RSC Advances Accepted Manuscript

Table 1. Catalytic parameters for SPE/Au/DDAB/CYP3A4 electrocatalytic reactions

Enzyme + substrate	**Antioxidant**	**K_m, μM**	**k_{cat}, min^{-1}**
CYP3A4+testosterone	-	67±10	**0.003±0.0005***
CYP3A4+ testosterone	cytochrome *c*		0.002±0.0005*↓↑
CYP3A4+ testosterone	ethoxidol		n.d
CYP3A4+diclofenac	-	40±8 [7]	**0.01±0.006***
CYP3A4+ diclofenac	cytochrome *c*		0.004±0.0003*↓
CYP3A4+ diclofenac	ethoxidol		0.003±0.0006*↓
CYP3A4+erythromycin	-	70±8	**3.1±0.3****
CYP3A4+ erythromycin	taurine		**↑3.6 ±0.3**** [29]
CYP3A4+ erythromycin	ethoxidol		2.7±0.3**↓
CYP2C9 + diclofenac	-	4.3±2.4 [18]	0.005±0.0005*
CYP2C9 + diclofenac	cytochrome *c*		0.004±0.0003*↓↑
CYP2C9 + diclofenac	ethoxidol		0.002±0.0001*↓

n.d. - not determined

* Product formation was determined by mass-spectrometry.

** Product formation was determined with Nash reagent.

RSC Advances Accepted Manuscript

References

1. A. A. Archakov and G. I. Bachmanova, *Cytochrome P450 and Active Oxygen*, Taylor & Francis, London, 1990.
2. D. F. V. Lewis, *Guide to Cytochromes P450: Structure and Function,* Taylor & Francis, London, 2001.
3. P. R. Ortiz de Montellano, *Cytochrome P450: Structure, Mechanism, and Biochemistry*, Kluwer Academic/Plenum Publishers, New York, 2005.
4. E. G. Hrycay and S. M. Bandiera, *Arch. Biochem. Biophys.*, 2012, **522**, 71–89.
5. U. Zanger and M. Schwab, *Pharmacol. Ther.*, 2013, **138**, 103–141.
6. D. W. Nebert and D. W. Russel, *The Lancet*, 2002, **360**, 1155-1162.
7. V. Shumyantseva, T. Bulko, E. Suprun, Y. Chalenko, M. Vagin, Y. Rudakov, M. Shatskaya and A. Archakov, *Biochem. Biophys. Acta, Proteins and Proteomics*, 2011, **1814**, 94-101.
8. V. Shumyantseva, E. Suprun, T. Bulko, Y. Chalenko, A. Archakov, in *Nanomedicine in Diagnostics*, ed. N. Rozlosnik, CRC Press, Taylor & Francis Group, New York, 1st ed., 2012, ch.4, pp. 68-95.
9. E. Schneider and D. Clark, *Biosens. Bioelectron.*, 2013, **39**, 1-13.
10. A. Yarman, U. Wollenberger and F. W. Scheller, *Electrochim. Acta*., 2013, **110**, 63-72.
11. H. Colas, K. Ewen, F. Hannemann, N. Bistolas, U. Wollenberger, R. Bernhardt and P. de Oliveira, *Bioelectrochemistry*, 2012, **87**, 71-77.
12. R. W. Estabrook, K. M Faulkner, M. S. Shet and C. W. Fisher, *Method Enzymol*., 1996, **272**, 44-51.
13. N. Bistolas, U. Wollenberger, C. Jung and F. W. Scheller, *Biosens. Bioelectron.*, 2005, **20**, 2408-2423.
14. V. Shumyantseva, T. Bulko, and A. Archakov, *J. Inorg. Biochem.*, 2005, **99**, 1051-1063.

RSC Advances Accepted Manuscript

15. A. K. Udit and H. B. Gray, *Biochem. Biophys Res. Commun.*, 2005, **338**, 470-476.

16. C. Baj-Rossi, J. T. Rezzonico, A. Cavallini, F. Grassi, G. De Michelli and S. Carrara, *Biosens. Bioelectron.*, 2014, **53**, 283-287.

17. C. Baj-Rossi, G. De Micheli and S. Carrara, in Biosensors for Health, Environment and Biosecurity, ed. A. Serra, InTech Publisher, Vienna, 2011, 448-482.

18. A. Fantuzzi, L.H. Mak, E. Capria, V. Dodhia, P. Panicco, S. Collins and G. Gilardi, *Anal. Chem.*2011, **83**, 3831–3839.

19. P. Sun and Y. Wu, *Sensors and Actuators B*, 2013, **178**, 113– 118.

20. S. Zhou, C. Xue, X .Yu, C. Li and G. Wang, *Ther. Drug Monit.*, 2007, **29**, 687-708.

21. A. Sucheta, R. Cammack, J. Weiner and F. Armstrong, *Biochemistry*, 1993, **32**, 5455-5465.

22. G. S. Celep and F. Marotta, *Oxid. Antioxid. Med. Sci.*, 2014, **3**, 5-8.

23. S. Sadeghi, S. Ferrero, G. Di Nardo and G. Gilardi, *Bioelectrochemistry*, 2012, **86**, 87-91.

24. T. Omura and R. Sato, *J. Biol. Chem.*, 1964, **239**, 2379-2385.

25. A. A. Makhova, V. V. Shumyantseva, E. V. Shich, T. V. Bulko, V. G. Kukes, O. S. Sizova, G. V. Ramenskaya, S. A. Usanov and A. I. Archakov, *BioNanoScience*, 2011, **1**, 46-52.

26. V. V. Shumyantseva, A. A. Makhova, T. V. Bulko, A. V. Kuzikov, E. V. Shich, E. V. Suprun, V. Kukes, S. Usanov and A. Archakov, *Oxidants and Antioxidants in Medical Science* 2013, **2**, 113-117.

27. T. Nash, *Biochem. J.*, 1953, **55**, 416–421.

28. V. V. Shumyantseva, T. V. Bulko, G. P. Kuznetsova, N. F. Samenkova and A. I. Archakov, *Biochemistry (Moscow)*, 2009, **74**, 438-444.

29. V. V. Shumyantseva, A. A. Makhova, T. V. Bulko, R. Bernhardt, A. V. Kuzikov, E. V. Shich, V. G. Kukes and A. I. Archakov, *Biochemistry (Moscow)*, 2015, **80**, 366-373.

RSC Advances Accepted Manuscript

30. O. V. Gnedenko, E. O. Yablokov, S. A. Usanov, D. V. Mukha, G. V. Sergeev, T. V. Bulko, A. V. Kuzikov, N. E. Moskaleva, V. V. Shumyantseva, A. S. Ivanov and A. I. Archakov, *Chemical Physics Letters*, 2014, **593**, 40-44.

31. G. Guerreiro, C. P. Mescka, A. Sitta, B. Donida, D. Marchetti, T. Hammerschmidt, J. Faverzani, D. Coelho, M. Wajner, C. S. Dutra-Filho and C. R. Vargas, *International Journal of Developmental Neuroscience*, 2015, **42**, 10–14.

32. V. A. Otdelenov, V. V. Smirnov, A. V. Dmitriev, V. V. Poroikov, V. V. Shumyntseva, L. M. Krasnykh, D. A. Sychev and V. G. Kukes, *Lekarstv. Prep. Ratsional. Farmakoterap. (in Russian)*, 2013, **3**, 30-36.

33. D. L. Johson, B. C. Lewis, D. J. Elliot, J. O. Miners and L. L. Martin, *Biochem. Pharmacol.*, 2005, **69**, 1533-1541.

34. Y. Mie, E. Tateyama and Y. Komatsu, *Electrochimica Acta,* 2014, **115**, 364-369.

35. A. Bogni, M. Monshouwer, A. Moscone, M. Hidestrand, M. Ingelman-Sundberg, T. Hartung and S. Coecke, *Toxicology in Vitro*, 2005, **19**, 621-629.

36. S. Shen , M. R. Marchick , M. R. Davis , G. A. Doss and L. R. Pohl, *Chem. Res. Toxicol.* 1999, **12**, 214-222.

37. Yu. O. Rudakov, V. V Shumyantseva, T. V. Bulko, E. V. Suprun, G. P. Kuznetsova, N. F. Samenkova and A. I. Archakov, *J. Inorg. Biochem.*, 2008, **102**, 2020-2025.

38. H. Yasui, S. Hayashi and H. Sakurai, *Drug Metab. Pharmacokinet.,* 2005, **20**, 1-13

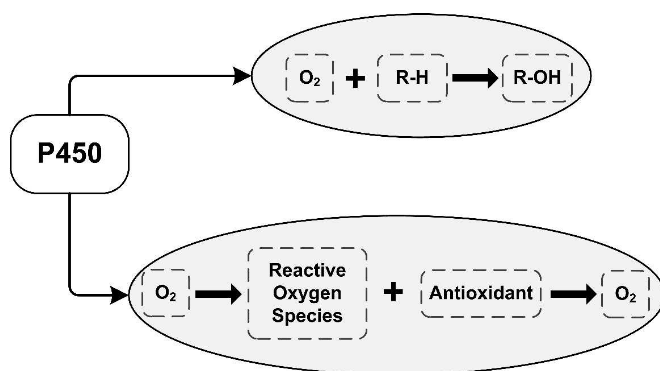
39. F. P. Guengerich, *Biochemistry*, 1978, **17**, 3633-3639.

40. S. Bondy and S. Naderi, *Biochem. Pharmacol.*, 1994, **48**, 155–159.

41. S. Gade, S. Bhattacharya and K. Manoj, *Biochem. Biophys. Res. Commun.*, 2012, **419**, 211-214.

42. C. Bian, H. Xiong, X. Zhang, Y. Ye, H. Gu and S. Wang, *Sensors and Actuators B: Chemical* 2012, **169**, 368-373.

RSC Advances Accepted Manuscript



Scheme 1.

RSC Advances Accepted Manuscript

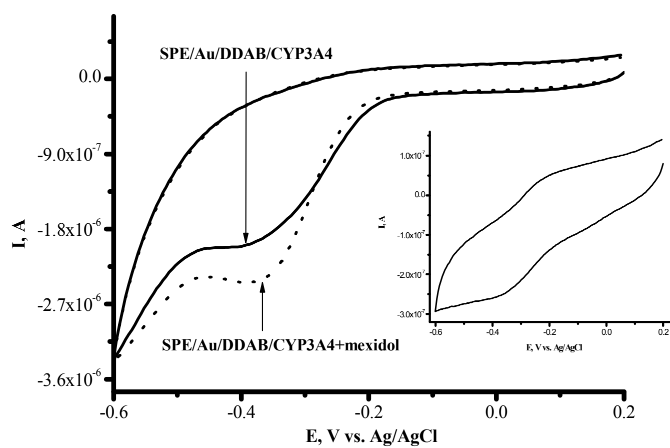


Figure 1A. Cyclic voltammograms (CV) of SPE/Au/DDAB/CYP3A4 (—) and SPE/Au/DDAB/CYP3A4 after mexidol addition (90 μM) (· · ·). Inset. CV of CYP3A4 in argon, at scan rate of 50 mV/s in 0.1 M PBS, pH 7.4.

RSC Advances Accepted Manuscript

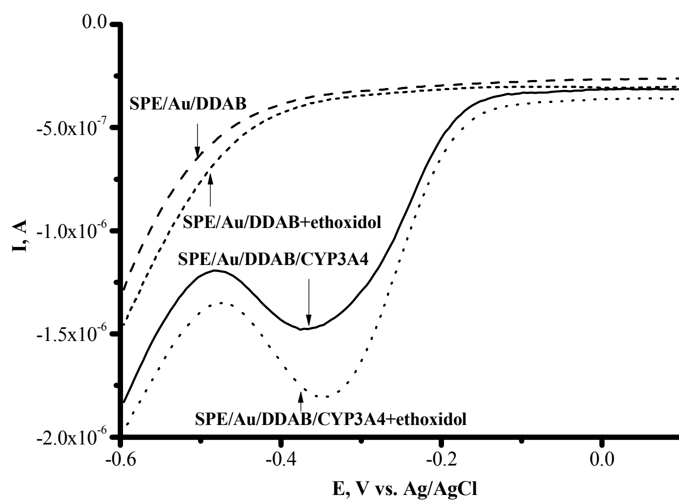


Figure 1B. Square-wave voltammetry (SWV) (reductive curves) of SPE/Au/DDAB/CYP3A4 before (——) and after the addition of the ethoxidol (90 μM) (· · ·).

RSC Advances Accepted Manuscript

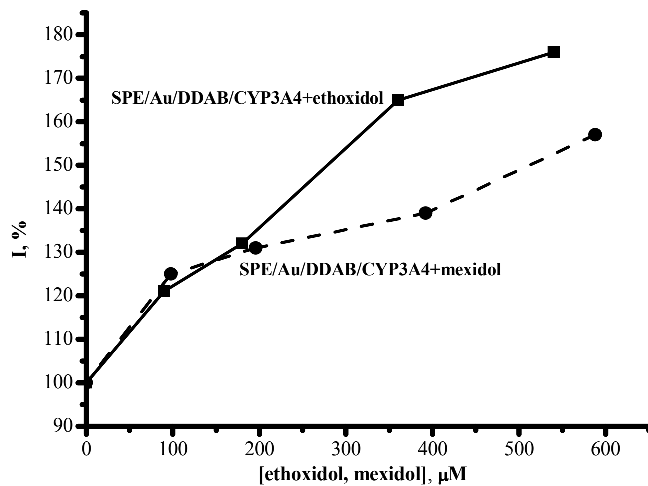


Figure 2. The dependence of cathodic current intensity of reductive peak of SWV (%) on the concentration of ethoxidol and mexidol.

RSC Advances Accepted Manuscript

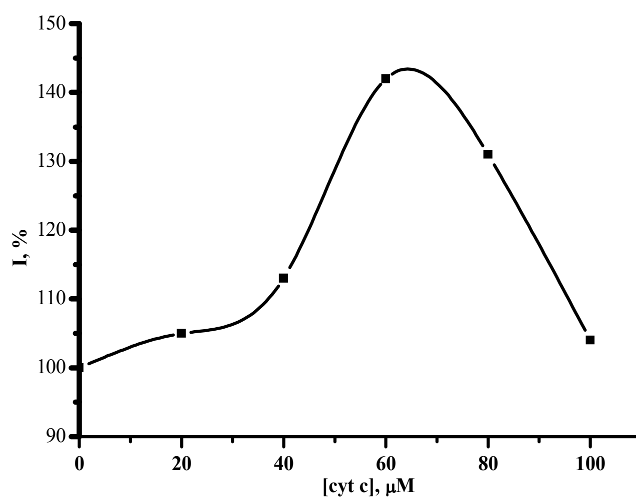


Figure 3. The dependence of cathodic current intensity (%) on the concentration of the cytochrome *c* (0-100 μM).

RSC Advances Accepted Manuscript

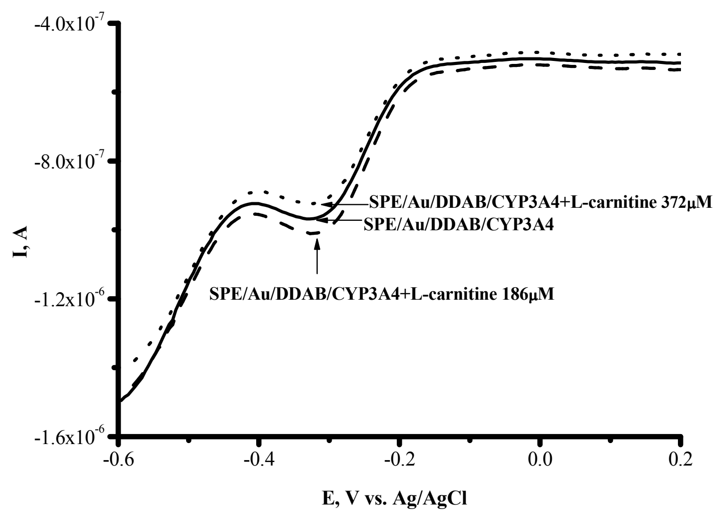


Figure 4. Square-wave voltammograms (SWV) (reductive curves) of SPE/Au/DDAB/CYP3A4 (—); SPE/Au/DDAB/CYP3A4+L-carnitine (186 μM) (- - -); SPE/Au/DDAB/CYP3A4+L-carnitine (372 μM) (· · ·).

RSC Advances Accepted Manuscript

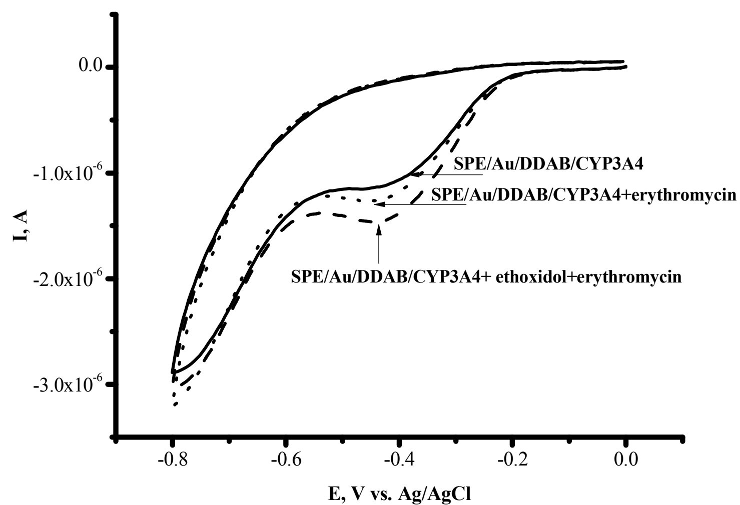


Figure 5. Cyclic voltammograms of SPE/Au/DDAB/CYP3A4 (——); SPE/Au/DDAB/CYP3A4 + erythromycin (1 mM) (· · ·); SPE/Au/DDAB/CYP3A4 + erythromycin (1 mM) + ethoxidol (180 μM) (- - -).

RSC Advances Accepted Manuscript

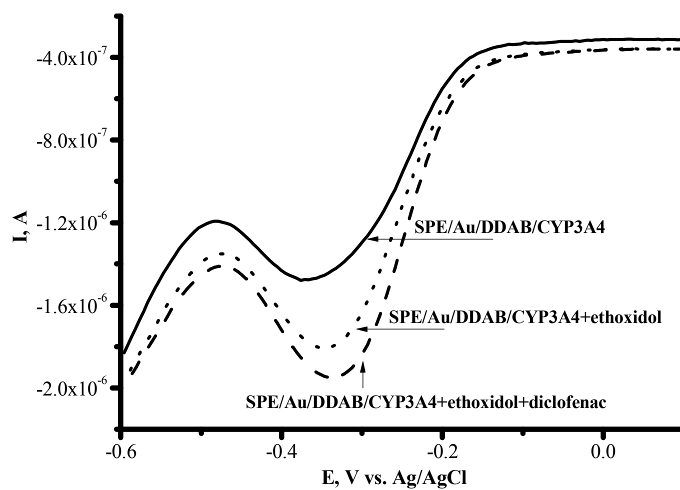


Figure 6. Square-wave voltammograms (SWV) (reductive curves) of SPE/Au/DDAB/CYP3A4 (——); SPE/Au/DDAB/CYP3A4 + ethoxidol (90 μM) (· · ·); SPE/Au/DDAB/CYP3A4 + ethoxidol (90 μM) + diclofenac (100 μM) (- - -).

RSC Advances Accepted Manuscript

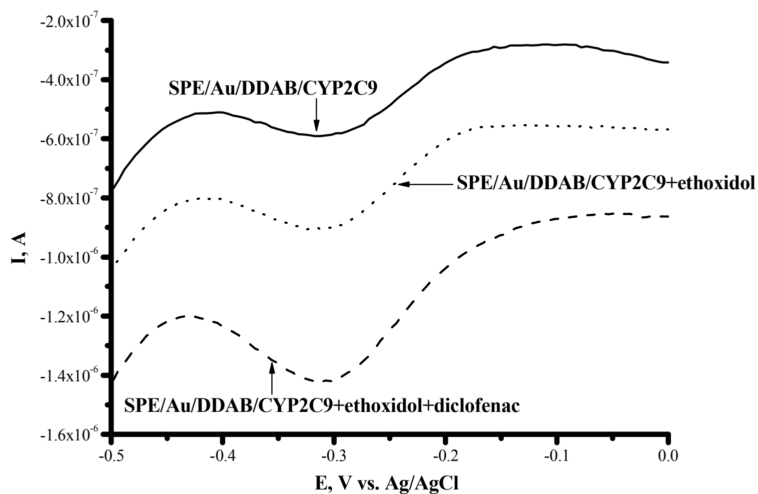


Figure 7. Square-wave voltammograms (SWV) (reductive curves) of SPE/Au/DDAB/CYP2C9 (—); SPE/Au/DDAB/CYP2C9 + ethoxidol (90 μM) (· · ·); SPE/Au/DDAB/CYP2C9 + ethoxidol (90 μM) + diclofenac (100 μM) (- - -).

RSC Advances Accepted Manuscript

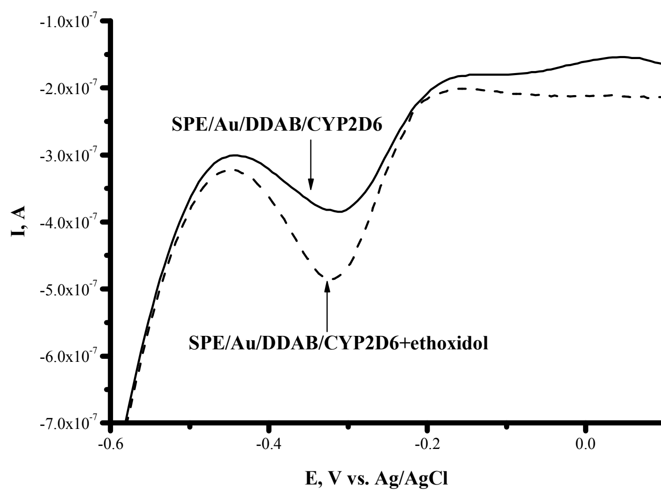


Figure 8. Square-wave voltammograms (SWV) (reductive curves) of SPE/Au/DDAB/CYP2D6 (—); SPE/Au/DDAB/CYP2D6 + ethoxidol (90 μM) (- - -).

RSC Advances Accepted Manuscript

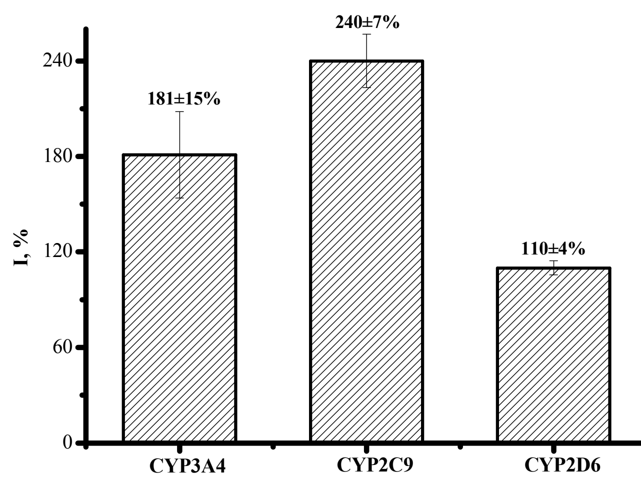
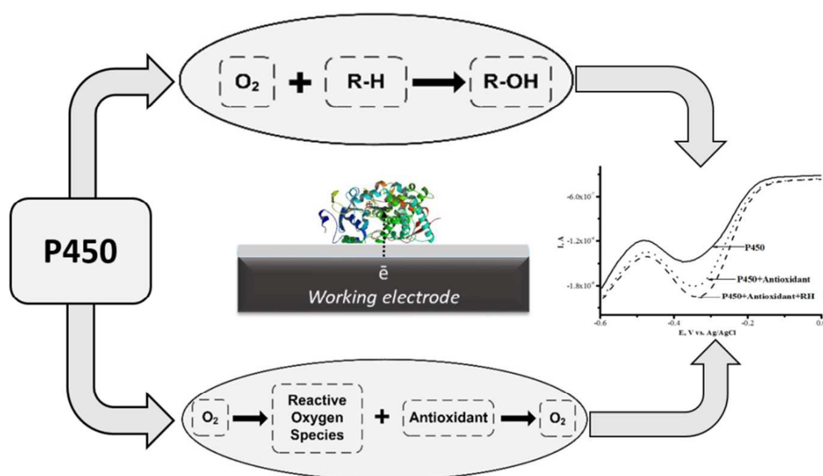


Figure 9. Normalized peak intensity, (%) of reductive SWV of screen-printed electrodes in aerobic buffer (with baseline correction): Au/DDAB/CYP3A4 + ethoxidol (540 μM); Au/DDAB/CYP2D6 + ethoxidol (540 μM); Au/DDAB/CYP2D6 + ethoxidol (540 μM).

RSC Advances Accepted Manuscript

Graphical Abstract

Investigation of catalytic activity of isolated cytochromes from the cytochromes P450 superfamily requires obligatory presence of oxidation-reduction (redox) partners and electron donors such as NAD(P)H. However, redox partners are not obligatory upon electrochemical reduction of P450 family hemeproteins, and the catalytic system is essentially simplified. P450-based enzyme electrodes may be used as biosensors in personalized medicine, high-throughput screening and drug interference studies. Cytochromes P450 catalysis is accompanied by reactive oxygen species (ROS) generation; therefore the influence of free radical-scavenging substances (ROS "traps" or antioxidants) on electrocatalysis may be reasonably expected.



RSC Advances Accepted Manuscript