Engineering Macaca fascicularis cytochrome P450 2C20 to reduce animal testing for new drugs

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1. Introduction

In order to obtain Food and Drug Administration (FDA) approval of new drugs numerous preclinical studies must be conducted on animals particularly primates. In view of the large number of animals used in drug safety testing (Report from the European Commission and European Parliament, 2010; Statistics of Scientific Procedures on Living Animals, Great Britain, 2010), there is an ever-increasing demand to reduce their use through the development of in vitro tests [1,2].

Cytochrome P450 enzymes (CYP or P450) are central to the study of toxicology due to their fundamental role as the primary drug me- tabolizing enzymes found in the human liver [3] and macaques are widely used in preclinical testing of new drugs due to more pharmaco- kinetic similarities to humans than any other non-human primate models. However, metabolic patterns different to those seen in humans can be detected and they are normally related to the presence of differ- ent P450 isoforms. The lack of information on cytochrome P450 genes has hampered the understanding of drug metabolism in macaque spe- cies. Prior to 2011 only ten Macaca fascicularis (M. fascicularis) P450

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abstract

In order to develop in vitro methods as an alternative to P450 animal testing in the drug discovery process, two main requisites are necessary: 1) gathering of data on animal homologues of the human P450 enzymes, currently very limited, and 2) bypassing the requirement for both the P450 reductase and the expensive co- factor NADPH. In this work, P450 2C20 from Macaca fascicularis, homologue of the human P450 2C8 has been taken as a model system to develop such an alternative in vitro method by two different approaches. In the first approach called “molecular Lego”, a soluble self-sufficient chimera was generated by fusing the P450 2C20 domain with the reductase domain of cytochrome P450 BM3 from Bacillus megaterium (P450 2C20/BMR). In the second approach, the need for the redox partner and also NADPH were both obviated by the direct immobi- lization of the P450 2C20 on glassy carbon and gold electrodes. Both systems were then compared to those obtained from the reconstituted P450 2C20 monooxygenase in presence of the human P450 reductase and NADPH using paclitaxel and amodiaquine, two typical drug substrates of the human P450 2C8. The K_M values cal- culated for the 2C20 and 2C20/BMR in solution and for 2C20 immobilized on electrodes modified with gold nanoparticles were 1.9±0.2, 5.9±2.3, 3.0±0.5μM for paclitaxel and 1.2±0.2, 1.6±0.2 and 1.4±0.2μM for amodiaquine, respectively. The data obtained not only show that the engineering of M. fascicularis did not affect its catalytic properties but also are consistent with K_M values measured for the microsomal human P450 2C8 and therefore show the feasibility of developing alternative in vitro animal tests.

proteins were annotated on the SwissProt database, but recently its entire genome has been determined [4], leading to the discovery of many novel cytochromes P450.

M. fascicularis P450 2C20 has been shown to correspond in terms of homology and function to the human P450 2C8 [5] that belongs to the important 2C family responsible for the metabolism of more than 10% of all prescribed drugs. The high sequence homology between human P450 2C8 and M. fascicularis P450 2C20 together with the catalytic data available in literature [6] indicate that macaque P450 2C20 is a good candidate for the development of preclinical in vitro tests.

Studies on microsomes are hindered by the fact that P450 proteins are expressed at low levels in some organisms and isolation of fully ac- tive P450 from animal extracts is difficult [7]. Although, in vitro methods can be developed, the need for the NADPH cofactor and the reductase are making their application labour intensive and expensive. Fortunate- ly, different research groups have already overcome these problems for the of human P450 enzymes that are studied in more details than their animal counterparts. The lessons learnt from these studies can therefore be extended to the in vitro applications of the animal P450 enzymes. With regards to the first obstacle i.e. redox partner, our laboratory has pioneered the generation of the chimeric proteins between P450 2E1 and a bacterial reductase (P450 BM3 reductase domain) [8] and extend- ed it to all major human P450 isoforms [9] resulting in self-sufficient human P450 enzymes. Regarding the second obstacle i.e. requirement for NADPH, bioelectrochemical approaches can be adopted. The immo- bilization of P450 enzymes on electrode surfaces to achieve direct electron transfer between the protein and the electrode can give rapid information on the P450 enzymatic activity on potential drugs and/or inhibitors bypassing the need for both redox partner and NADPH (electron donor). Although the electrochemical activity of P450 enzymes on electrodes is a very challenging task, human P450 enzymes such as P450 2E1 [10], 2C9 [11] and 3A4 [12] have been successfully entrapped or covalently linked in an electro-active form to different surfaces. More recently, drug–drug interactions of immobilized human P450 3A4 have also been measured [13].

In this paper, M. fascicularis P450 2C20 was cloned and expressed in E. coli and purified in a soluble form that was subsequently used to develop two alternative approaches for the P450 in vitro animal tests. Firstly, the M. fascicularis P450 2C20 gene is fused with the gene of the reductase domain of Bacillus megaterium P450 BM3 resulting in P450 2C20/BMR. The solubility and the catalytic self-sufficiency of this chimera greatly simplify the in vitro studies of cytochrome P450 2C20 bypassing the need of a redox partner in solution. In the past, due to the high sequence similarity between the BMR and the mammalian cytochrome P450 reductase, chimeric P450 enzymes with BMR have been successfully constructed by our group and others [14].

Secondly, the purified recombinant P450 2C20 is immobilized by different methods on electrode surfaces. These include the entrapment within a cationic polymer, in a co-cross-linked gel, a cationic polymer and gold nanoparticles and the covalent linkage on gold electrode via a self-assembled monolayer.

The activity of the M. fascicularis P450 2C20 tested under the condi- tions of the two approaches with two know marker substrates of its human homologue P450 2C8 is measured and compared to that obtained with the reconstituted system in the presence of human reduc- tase and NADPH. The marker substrates used were paclitaxel (TaxolTM), known to be one of the most effective drugs in the treatment of breast, lung and ovarian cancer [15], and amodiaquine, an anti-malarial drug [16].

2. Materials and methods

2.1. Reagents

Kits for plasmid and gene purification were purchased from Sigma Aldrich (Italy). Restriction enzymes, T4 DNA ligase, Vent Polymerase and dNTPs were from New England Biolabs (UK). Chromatographic resins were purchased from GE healthcare (Italy).

Paclitaxel (2α,4α,5β,7β,10β,13α)-4,10-bis(acetyloxy)-13-{[(2R, 3 S)-3-(benzoylamino)-2-hydroxy-3-phenylpropanoyl]oxy}-1,7- dihydroxy-9-oxo-5,20-epoxytax-11-en-2-yl benzoate), amodiaquine (4 - [(7 - chloroquinolin-4-yl)amino]-2-[(diethylamino)methyl]phenol), acetonitrile, methanol, and NADPH were purchased from Sigma-Aldrich (Italy).

2.2. Cloning of recombinant P450 2C20 and P450 2C20/BMR

The gene coding for the M. fascicularis P450 2C20 was amplified from the liver cDNA pool of M. fascicularis (Biochain, UK). The primers used were 2C20_FW: 5′-GCA GGG AGT GTT ATA AAA GCC TTG GAG-3′ and 2C20_RV: 5′-GCT CAC GGG ATA TTG AGT GCA TGG-3′. The complete gene was blunt end ligated in pBS SKII + using the EcoRV restriction enzyme. Modifications were introduced by PCR that in- cluded the truncation of the N-terminus by removing the first 20 amino acids and replacing them with methionine and alanine and ad- dition of a tag of four histidine residues at the C-terminus before the STOP codon. Furthermore, the NdeI and HindIII restriction sites were introduced at the 5′ and 3′ of the CYP2C20 gene, respectively. These two restriction sites are compatible with the pCW ori(+) expression vector. The amplicon obtained was digested with NdeI and HindIII

restriction enzymes and inserted into the cloning vector pCW ori(+) pre-digested with the same restriction enzymes. The presence of the correct insert was confirmed by DNA sequencing.

The P450 2C20 construct cloned in pCW ori(+) described above was also fused in frame with the P450 BM3 reductase domain of the B. megaterium (BMR). The BMR domain had previously been cloned in pCW ori(+) [9]. Again for ease of purification of the P450 2C20/ BMR, a tag of six histidine residues was added at the C-terminus of the BMR domain by PCR. The His tag and the restriction site for HindIII present at the C-terminus of P450 2C20 sequence were removed and substituted with the AvrII restriction enzyme site which is compatible with the N-terminus of BMR domain [9]. The entire chimeric gene was sequenced prior to expression and purification studies.

2.3. Expression and purification of P450 2C20 and P450 2C20/BMR

Large-scale expression (4 liters) of the P450 2C20 and P450 2C20/ BMR was achieved starting from an overnight culture of a colony of E. coli DH5a transformed with the appropriate plasmid grown over- night in 5 mL of LB with 100 μg/mL of ampicillin at 37 °C. This was used as the inoculum of 500 mL of Terrific broth (TB), containing 100 μg/mL of ampicillin. The culture was grown at 37 °C, until an optical density at 600 nm of 0.4–0.6 was achieved. At this point protein produc- tion was induced by the addition of IPTG (1 mM), δ-aminolevulinic acid (heme precursor) was also added to a final concentration of 0.5 mM. The induced cells were then grown for 48 h at 28 °C. After this time the cells were harvested by centrifugation at 4000 rpm for 20 min at 4 °C. The cell pellet was stored at −20 °C.

Purification of P450 2C20 and P450 2C20/BMR was carried out starting from the isolated

cytosolic fraction using an anion exchange DEAE sepharose column (GE-healthcare, Italy) as described previously [9] followed by a nickel ion affinity chromatography step (GE-healthcare, Italy). Both proteins remained bound to the Nickel column through the engineered His-tag and were eluted using a 0–40 mM linear histidine gradient. Presence and purity of the proteins were detected at the spec- trophotometer by measuring the peak absorbance at 418 nm and calcu- lating the 417 nm:280 nm ratio, respectively.

Prior to storage a SDS-Page gel of the purified protein was run and the UV-visible spectra of the oxidized, reduced and reduced-carbon monoxide bound forms of the protein were recorded on a Hewlett- Packard 8453 diode array spectrophotometer. The P450 concentration was calculated by using the method described by Omura and Sato [17].

2.4. Metabolism of selected drugs by P450 2C20 and P450 2C20/BMR

The activity of the P450 2C20 and its chimera P450 2C20/BMR was measured by monitoring the 6α-hydroxylation of paclitaxel and N-demethylation of amodiaquine in 200 μl of 50 mM potassium phos- phate buffer pH 7.4 containing 5 mM $MgCl_2$ at 37 °C and initiated by the addition of 1 mM NADPH. In the case of P450 2C20 the system was reconstituted with equimolar amounts of human P450 reductase (Invitrogen, Italy). The P450 2C20/BMR chimera did not require the addition of other redox partners. Both proteins were used at the final concentration of 0.3 μM. All reactions were terminated by the ad- dition of 20 μL of methanol. The metabolites were separated by HPLC (Agilent Technologies-1200 series, Italy) coupled with diode array detec- tor using a 4.6 × 150 mm 5 μm Eclipse XDB-C18 column (Agilent Tech- nologies, USA).

Paclitaxel and its metabolite were detected at 230 nm and separated by a linear gradient of 10–100% methanol as reported previously [18]. Amodiaquine and N-desethylamodiaquine were detected at 340 nm and separated as described by previously [19] by an isocratic gradient in a mobile phase composed of: 750 mL of 0.1 M KH_2PO_4, 250 mL of 100% methanol and 2.5 mL of perchloric acid. All experiments were car- ried out in triplicates.

The amount of 6α-hydroxy paclitaxel and N-desethylamodiaquine was calculated from calibration curves of the standard products pur- chased from Sigma (Italy). K_M values were calculated by measuring the amount of product formed at increasing concentrations of paclitaxel and amodiaquine.

2.5. Electrochemical measurements

A glass three-electrode cell, equipped with a platinum wire counter electrode, an Ag/AgCl reference electrode (3 M NaCl — BASi, UK) and a 3 mm diameter glassy carbon working electrode (BASi, UK), was used throughout. All electrochemical experiments were carried out in 50 mM potassium phosphate buffer pH 7.4, containing 100 mM KCl as supporting electrolyte using an Autolab PGSTAT12 potentiostat (Ecochemie, The Netherlands) controlled by GPES3 software. Cyclic voltammetry experi- ments were carried out under anaerobic conditions in a glove box (Belle Technologies, UK). Cyclic voltammograms were collected between 0 and – 750 mV (vs Ag/AgCl) at a scan rate range of 30–110 mV s^{-1} in the supporting electrolyte solution. Electrocatalysis experiments were carried out using chronoamperometry or square wave voltammetry. For chronoamperometric measurements a potential bias of –650 mV (vs Ag/AgCl) was applied for 30 min at 37 °C.

Bioelectrocatalysis experiments in square wave voltammetry were carried using the following

working parameters: vertex potential 0.1 and –0.7 V; amplitude 0.09 V; step potential 0.02 V; frequency 230 Hz. In all cases, the products formed were separated and analyzed by HPLC.

All bioelectrocatalysis experiments were carried out in triplicates (three different electrodes).

2.6. Immobilization of P450 2C20 on glassy carbon electrode

The glassy carbon (GC) working electrode was manually polished with alumina and subsequently rinsed and sonicated in ultra-pure deionized water. The N-terminally modified P450 2C20 enzyme was immobilized on GC electrodes using different immobilization tech- niques described below.

P450 2C20 entrapped in polydiallyldimethylammonium chloride (PDDA) film was obtained by mixing equal volumes of 30 μM protein and surfactant solutions before drop-coating onto the electrode surface. P450 2C20 immobilization on GC electrode with bovine serum albumin (BSA) and glutaraldehyde (GLUT) was obtained by mixing 6 μL of 30 μM 2C20 and 6 μL of 3 mM BSA solutions with 2 μL of 25% glutaraldehyde solution as reported previously [20].

P450 2C20 immobilization on GC electrodes using didodecyldimethy- lammonium bromide (DDAB) stabilized gold nanoparticles (AuNPs) was obtained by the following procedure: 2 μl of 5 mM colloidal gold in 0.1 M DDAB/chloroform was entrapped on the electrode sur- face. After evaporation of the chloroform (10 min), 5 μl of the P450 2C20 solution was added onto the electrode surface. The electrodes were kept 12 h at 4 °C in a humid chamber to prevent their total drying.

2.7. Immobilization of P450 2C20 on gold electrode

Gold electrodes (11 mm × 5 mm, custom made by Arrandee, Germany) consisting of a vacuum evaporated thin gold film (thickness 200 nm) on borosilicate glass were annealed with a butane flame to ob- tain flat re-crystallized Au(111) terraces and were functionalized with dithio-bismaleimidoethane (DTME) as reported previously [12,21–23]. This functionalization led to the formation of maleimide-terminated groups that subsequently interacted with exposed cysteine residues present on the surface of the M. fascicularis P450 2C20 (30 μM) resulting in covalent linkage of the protein to the Au surface.

2.8. Homology modelling of M. fascicularis P450 2C20

Homology modelling was used to build a 3D model of P450 2C20. M. fascicularis P450 2C20 and human P450 2C8 show a sequence iden- tity of 92% and therefore the crystal structure of P450 2C8, PDB ID: 2NNJ [24] was used as the template for the 2C20 model. The structure was then refined with YASARA using the “md refine” macro [25]. The quality of the refined P450 2C20 model was validated with PROCHECK [26]. The P450 2C20 model was subsequently used to calculate the sol- vent accessible area of the twelve cysteine residues present in its prima- ry amino acid sequence.

3. Results

3.1. P450 2C20 and 2C20/BMR cloning, expression and purification

The gene coding for P450 2C20 was amplified from M. fascicularis liver cDNA and the constructs coding for the P450 2C20 and 2C20/ BMR were engineered and cloned in the

expression vector pCW. After expression and purification described in Materials and methods (Section 2.3.), yields of 11 and 8 mg of pure protein per litre of culture for P450 2C20 and 2C20/BMR were obtained, respectively (Fig. 1A and C). Typical UV–vis spectra of the purified P450 2C20 and 2C20/ BMR chimera in the oxidized state show a Soret peak at 417 nm, the α and β bands at 570 nm and 535 nm, respectively. In addition, the pu- rified 2C20/BMR chimera also displays an extra shoulder in the 450–500 nm region due to the presence of flavin cofactors of the BMR domain which is similar to those reported for other cytochrome P450/ reductase fusion proteins [8,9]. As expected, the 450–500 nm shoulder disappears after reduction with dithionite due to the conversion of the flavin cofactors to their fully reduced hydroquinone species. The Soret peaks show the characteristic shift to 450 nm upon reduction and bub- bling of carbon monoxide due to the formation of the reduced and

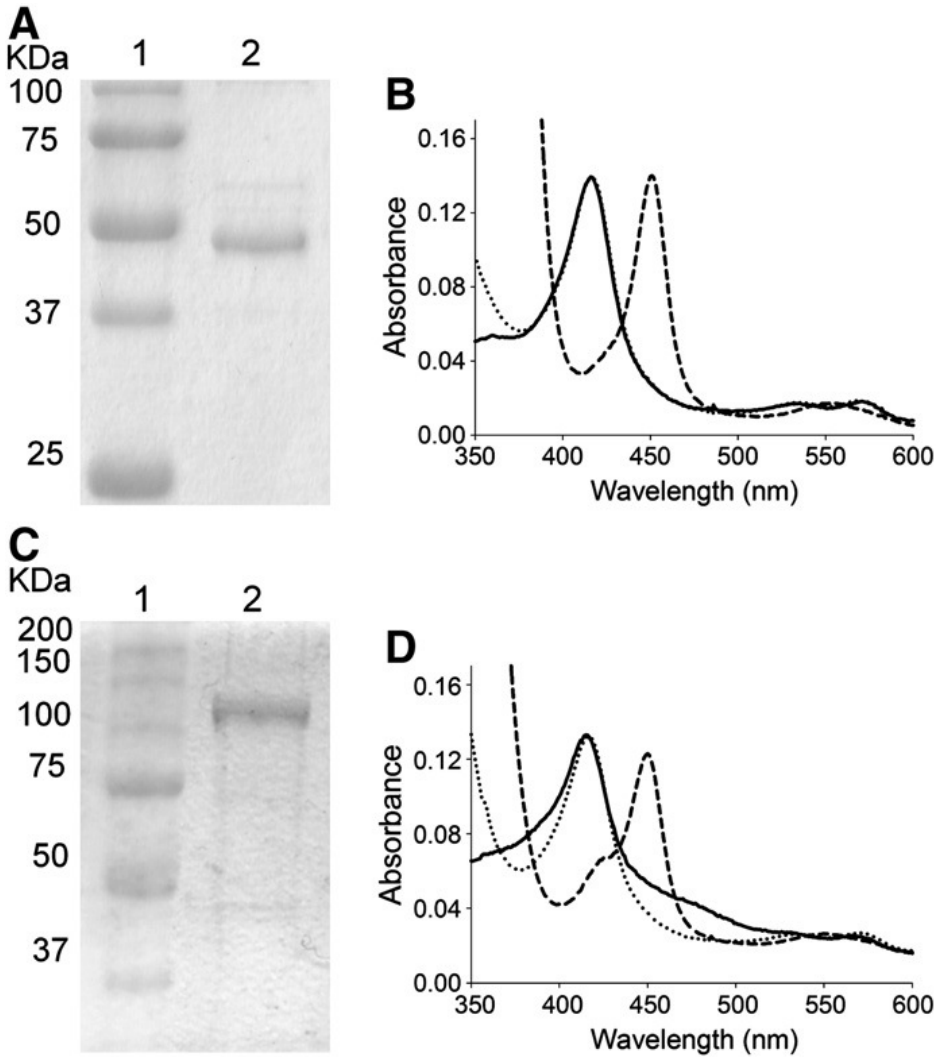

Fig. 1. Purification and spectral characterization of P450 2C20 and P450 2C20/BMR chimera. 10% SDS-PAGE of the purified P450 2C20 (A) and P450 2C20/BMR (C). Lane 1: high molecular weight markers, lane 2: the pure proteins. The absorbance spectra of the purified P450 2C20 (B) and P450 2C20/BMR (D) in the oxidized (solid black line), reduced by the addition of dithionite (dotted line), and the reduced carbon monoxide-bound form (dashed line).

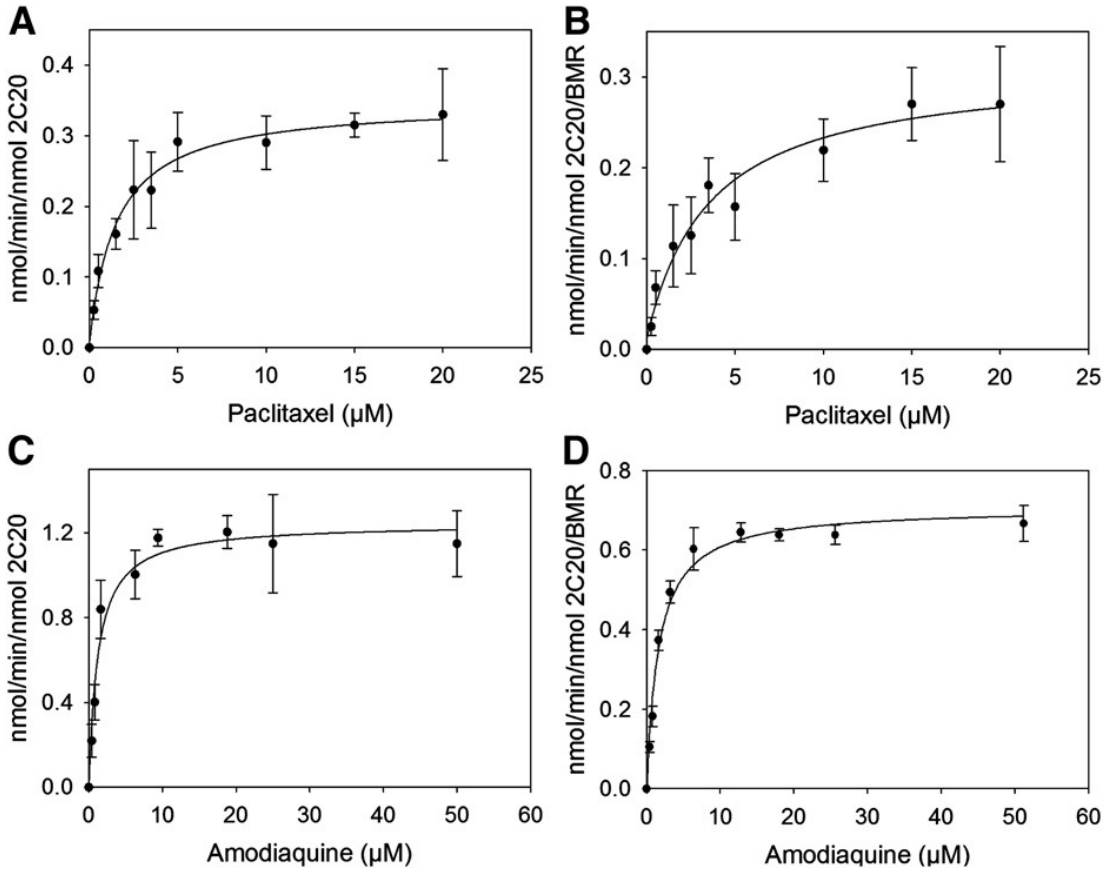

Fig. 2. Michaelis–Menten plots of the turnover of paclitaxel and amodiaquine by P450 2C20 (A and C) and P450 2C20/BMR (B and D), respectively.

carbon monoxide-bound adduct and the α and β bands are replaced by a single broad peak centered at 550 nm as shown in Fig. 1B and D.

3.2. In vitro drug metabolism by P450 2C20 and 2C20/BMR

The activity of P450 2C20 and the self-sufficient chimera P450 2C20/BMR was assayed in vitro with paclitaxel and amodiaquine, drugs that are typical substrates of the human P450 2C8 orthologue. To date paclitaxel (TaxolTM) has been demonstrated to be one of the most effective drugs in the treatment of breast, lung and ovarian cancer due to its ability to bind polymerized tubulin, stabilize microtubules and block eukaryotic cell division (mitosis) arresting the growth of tumors [15]. Paclitaxel is the prototypical substrate of human P450 2C8 [5], and its 6α-hydroxylation has been the most commonly used in vitro marker for this enzyme [27,28].

Amodiaquine on the other hand, is an anti-malarial drug and its N-demethylation has gained attention as an alternative marker for human P450 2C8 because of its high affinity and high rate of turnover [16,29].

The specific products obtained from the oxidation of paclitaxel and amodiaquine by P450 2C20 and its chimera were analyzed by HPLC and the retention times of the chromatograms were compared to those of their specific standards. Reactions were carried out with in- creasing concentrations of paclitaxel and amodiaquine and the amount of product(s) formed was calculated from the peak areas and their corresponding standard curves. Control reactions for each drug in the absence of NADPH were also carried out. The purified 2C20 and 2C20/ BMR were not only

active toward the human P450 2C8 maker sub- strates but also showed K_M values comparable to those of the human counterpart. The Michaelis–Menten curves obtained are reported in Fig. 2 and the data resulting from the fitting are summarized in Table 1. K_M values of 1.9 ± 0.2 and 3.0 ± 0.7 μM were calculated for the turnover of paclitaxel by 2C20 and 2C20/BMR, respectively. These values are in the same range as that of 2.3 ± 0.7 μM reported by Hanioka and co-workers for the turnover of paclitaxel by the human 2C8 enzyme [30]. In the case of amodiaquine turnover by 2C20 and 2C20/BMR, K_M values of 1.2±0.2 and 1.6±0.2μM were obtained, respectively.

Also in this case, the values obtained are consistent with literature values for the human 2C8 enzyme i.e. 0.72 ± 0.04 μM for purified and 1.89 ± 0.06 μM for microsomal 2C8 [29].

These data are very encouraging in that they indicate that the engi- neering of M. fascicularis P450 2C20 has not affected its catalytic activity. Moreover, the rates obtained for the 2C20/BMR chimera reveal that the bacterial BMR reductase domain is capable of forming a functional com- plex with the M. fascicularis P450 2C20 resulting in a self-sufficient en- zyme which bypasses the need for the addition of external redox partners and therefore is an even better in vitro model system than that of the P450 2C20 alone. The small differences found in the K_M values are well within the error of the data generally found for the different P450 systems. Taken together these results show that the catalytically self-sufficient P450 2C20/BMR is a promising system in the substitution of the scarification of M. fascicularis.

3.3. P450 2C20 immobilization on electrodes

The second approach for developing an in vitro system for bypassing animal testing is the direct immobilization of M. fascicularis P450 2C20 on electrode surfaces and therefore obviating both the requirement for a redox partner and the addition of NADPH cofactor. Bioelectrochemical methods lead not only to the acquisition of fundamental knowledge on the redox properties of P450 enzymes [31] but also open opportunities for technological and commercial applications [32,33]. To this end, the purified P450 2C20 was initially immobilized on both carbon and gold

Table 1.

Comparison of KM values of paclitaxel and amodiaquine for human P450 2C8 and M. fascicularis P450 2C20 and P450 2C20/BMR.

	H. sapiens P450 2C8	*M. fascicularis* P450 2C20	Chimera P450 2C20 /BMR
Paclitaxel (μM)	2.3 ± 0.7[a]	1.9 ± 0.2	3.0 ± 0.7
Amodiaquine (μM)	0.72 ± 0.04[b] 1.89 ± 0.06[b]	1.2 ± 0.2	1.6 ± 0.2

a [30]

b [29]

surfaces and its redox properties acquired and compared. Subsequently, the turnover of the two selected drugs, paclitaxel and amodiaquine, by the immobilized enzyme were measured and the products analyzed by HPLC and compared to the data obtained in the first in vitro approach, i.e. the oxidation of drugs by the chimeric 2C20/BMR in solution.

3.3.1. Immobilization on glassy carbon (GC) electrodesPurified M. fascicularis P450 2C20 was immobilized on GC elec-

trode surfaces using three different methods: (A) entrapment within the cationic polymer polydiallyldimethylammonium chloride (PDDA) (GC/2C20/PDDA); (B) entrapment in a gel co-cross-linked with bovine serum albumin (BSA) and glutaraldehyde (GC/2C20/BSA-GLUT); and (C) entrapment within cationic surfactant didodecyldimethylammonium bromide (DDAB) modified with gold nanoparticles (AuNPs) (GC/2C20/ AuNPs).

In the first two methods (A and B) the P450 2C20 was immobilized on the GC electrode in a non-oriented fashion by entrapment either within polydiallyldimethylammonium chloride (GC/2C20/PDDA) or in a gel co-cross-linked with BSA by glutaraldehyde (GC/2C20/BSA-GLUT). Electrochemical characterization was performed at room temper- ature (22 °C) under anaerobic conditions (b 2 ppm oxygen) to prevent the formation of the Fe^{2+}–dioxygen complex of the second electron transfer. A typical series of cyclic voltammograms of GC/2C20/PDDA and GC/2C20/BSA-GLUT at different scan rates are shown in Fig. 3A-B. Although the experiments were carried out in a glove box under anaero- bic conditions, trace amounts of oxygen were not completely eliminated and result in the observed higher cathodic currents compared to the anodic ones. Cyclic voltammograms were taken at different scan rates ranging from 30 to 110 mV s^{-1} and cathodic and anodic peak currents for both these immobilization strategies were linearly dependent on the scan rate, suggesting that the quasi-reversible reaction is a surface- controlled process, as expected for an immobilized electroactive species.

In the first immobilization method, GC/2C20/PDDA, anodic and ca- thodic peak currents were detected at – 317 ± 5 mV and – 182 ± 7 mV,

respectively, resulting in the calculated midpoint potential (Em) of – 249 ± 4 mV (vs Ag/AgCl) which is in the range of electrochemically determined values reported in the literature for different human P450 enzymes [31]. The peak-to-peak separation (ΔE) calculated in this case was 135 ± 5 mV. The increased ΔE value suggests a slow electron transfer to-and-from the electrode to the protein and has been reported for other immobilized P450 enzymes on different electrodes [31].

In the second method where the protein was immobilized with BSA and glutaraldehyde a ΔE of around 139 mV was also obtained however, the anodic and cathodic peaks were detected at more positive values of – 287 ± 15 mV and – 148 ± 5 mV, respectively, with a resulting midpoint potential of – 217 ± 8 mV (vs Ag/AgCl).

The integration of the reduction peak from the baseline corrected cyclic voltammogram allowed for the calculation of the charge transferred upon reduction of the protein and the determination of the quantity of the electroactive immobilized protein using the Laviron equation [34]. The surface coverage on GC/PDDA and GC/ BSA were calculated to be 1.3×10^{12} and 1.9×10^{12} molecules/cm^2, respectively.

In the third immobilization strategy on GC electrodes, gold nano- particles (AuNPs) were used. Achieving high sensitivity is one of the major goals in bioelectrochemistry and one successful strategy is the use of nanomaterials such as AuNPs [35] or carbon nanotubes. AuNPs stabilized by the cationic surfactant DDAB, may provide effective elec- tron transfer to-and-from the GC electrode. To this end, the P450 2C20 was immobilized on GC electrodes using AuNPs to enhance the sensitivity. Electrochemical detection of P450 2C20 protein was possi- ble when 5–100 pmol of the enzyme was adsorbed on the surface of AuNPs. Once the protein was successfully immobilized, electrochemical characterization was performed at room temperature (22 °C) under anaerobic conditions as mentioned above. A series of cyclic voltammo- grams at different scan rates for GC/2C20/AuNPs are shown in Fig. 3C. The anodic and cathodic peak currents were detected at − 202 ± 2 mV and − 122 ± 2 mV, respectively, with a calculated midpoint potential of

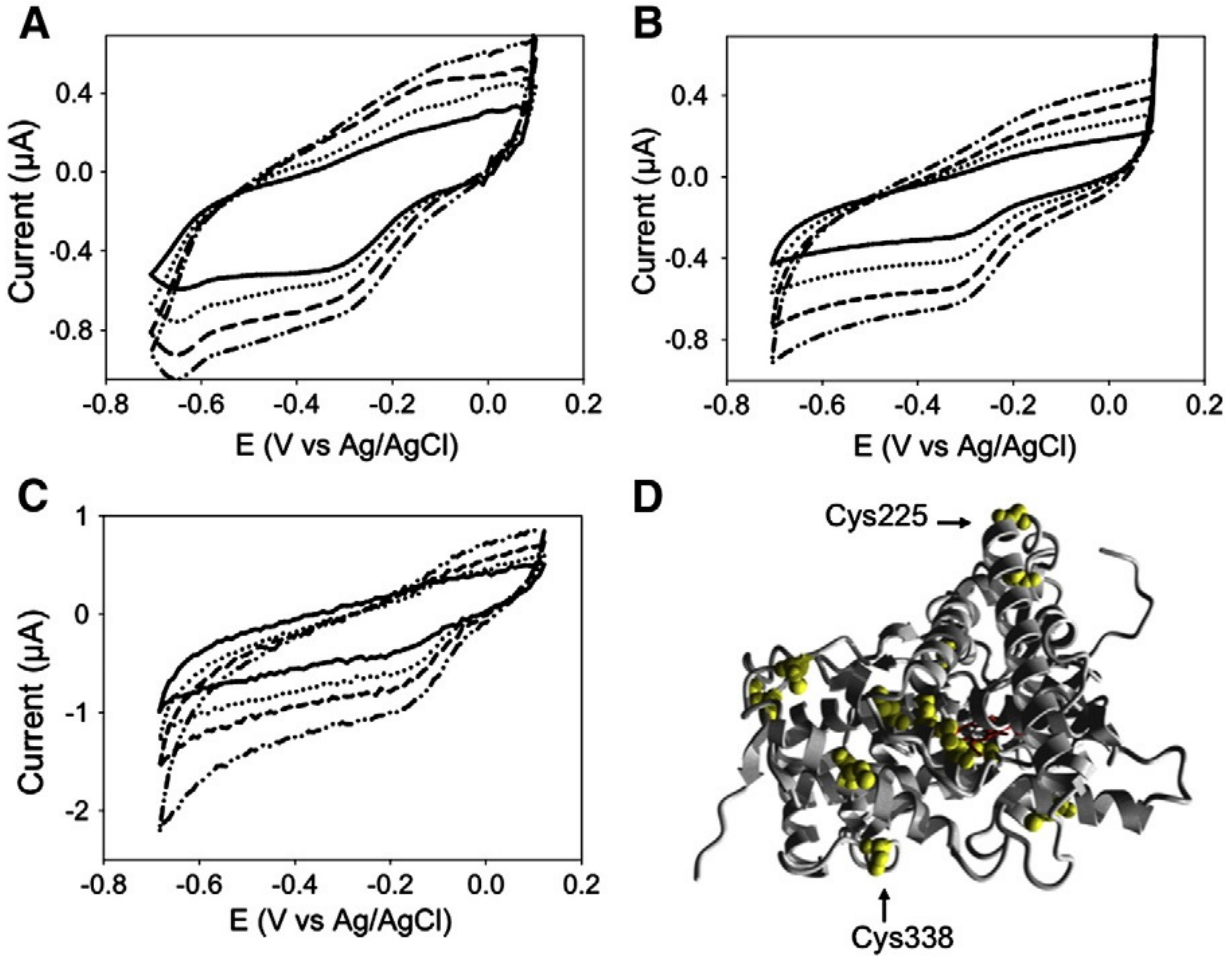

Fig. 3. Cyclic voltammograms of P450 2C20 on different electrode surfaces. (A) GC/2C20/BSA-GLUT, (B) GC/2C20/PDDA, and (C) GC/2C20/AuNPs. Scan rates: 30, 50, 70 and 110 mV/ sec. (D) 3D structure of the protein backbone of P450 2C20 (ribbon) showing the cysteine residues (yellow) in spacefill and the heme in sticks (red). The most exposed cysteines 225 and 338 are labeled for clarity.

− 162 ± 5 mV (vs Ag/AgCl) that was found to be more positive when compared to the protein entrapped on GC electrodes using PDDA. Dif- ferences in midpoint potential values can be attributed to the different electrode surfaces and their modifications which have been shown to affect the reduction potentials of P450 enzymes [31,36,37].

3.3.2. Immobilization on gold electrodes

Functionalization of the Au electrode surfaces with a self-assembled monolayer of sulfhydryl-reactive dithio-bismaleimidoethane (DTME) leads to the formation of maleimide-terminated groups that can cova- lently link to the protein via surface exposed cysteine residues. Among the different immobilization methods utilized, only this one results in an oriented and covalent attachment of the P450 2C20 to the electrode surface. In order to determine possible candidates for covalent linkage to Au electrodes i.e. surface exposed cysteine residue(s) and to visualize the surface of P450 2C20 a 3D structural model of this protein was gen- erated by homology modelling using as a template the published crystal structure of human P450 2C8 [24]. The solvent accessible areas of the 12 cysteine residues present in the primary amino acid sequence of P450 2C20 were then measured using the program YASARA [25]. The calcu- lated solvent exposure values showed that the majority of the cysteine residues are buried within the protein matrix with exposure values lower than 15 $Å^2$. Cysteine residues 225 and 338 resulted to be the most exposed with values of 58 $Å^2$ and 30 $Å^2$, respectively and their lo- cation is shown in Fig. 3D. Cysteine 338 is on the proximal side of the heme whereas cysteine 225 is located on the distal side. Therefore, it would be more advantageous if the DTME is covalently linked to cyste- ine 338, not interfering with the entry and binding of the substrates to the active site of P450 2C20.

Cyclic voltammetry experiments were carried out in order to char- acterize the electrochemical features of the protein covalently linked to the gold surface, but due to the high background current associated with the gold substrate used, no appreciable redox peak currents were detected.

Nevertheless, as we have previously observed for other P450 elec- trochemical systems, a weak protein current does not necessarily cor- relate to its catalytic activity on electrode surfaces [31] therefore the electrode-driven activity of the immobilized protein on the gold was tested in turnover studies as reported below.

3.4. Substrate turnover on electrochemical platforms

The catalytic properties of the enzyme immobilized using different strategies were measured by chronoamperometry or square-wave voltammetry in the presence of paclitaxel and amodiaquine. Bio- electrocatalysis experiments were carried out in triplicates for GC/ 2C20/BSA-GLUT, GC/2C20/PDDA and Au/2C20/DTME applying a po- tential bias of –650 mV (vs Ag/AgCl) for 30 min at 37 °C. After this time the products formed were analyzed by HPLC.

No product was detected for the protein immobilized on glassy carbon by entrapment in the co-cross-linking gel with BSA and glutaral- dehyde. On the contrary, when the protein was immobilized on GC/ PDDA or on Au/DTME surfaces, the specific hydroxylation of paclitaxel in position 6 (Fig. 4A–B) and the N-desethylation of amodiaquine (Fig. 4C–D) were detected by HPLC. In addition control experiments were also carried out with Au/DTME and GC/PDDA in the absence of the protein and as expected no product was detected. On glassy carbon and gold electrodes 0.04 ± 0.01 and 0.11 ± 0.02 nmol of 6α- hydroxy-paclitaxel and 0.018 ± 0.004 and 0.041 ± 0.006 nmol of N-desethylamodiaquine were produced, respectively (Table 2).

In the case of GC/2C20/AuNPs titration experiments using paclitaxel and amodiaquine were carried out in square wave voltammetry. The latter method is a highly sensitive technique and gives better signal- to-noise ratio than cyclic voltammetry. The response of the immobilized protein to increasing concentrations of substrates resulted in an en- hancement of the reduction peak current as shown in Fig. 5A and C. Also in this case, control experiments were carried out

by titrating methanol alone (solvent used for dissolving the drugs) on GC/2C20/ AuNPs electrodes and also by titrating both drugs on GC/AuNPs

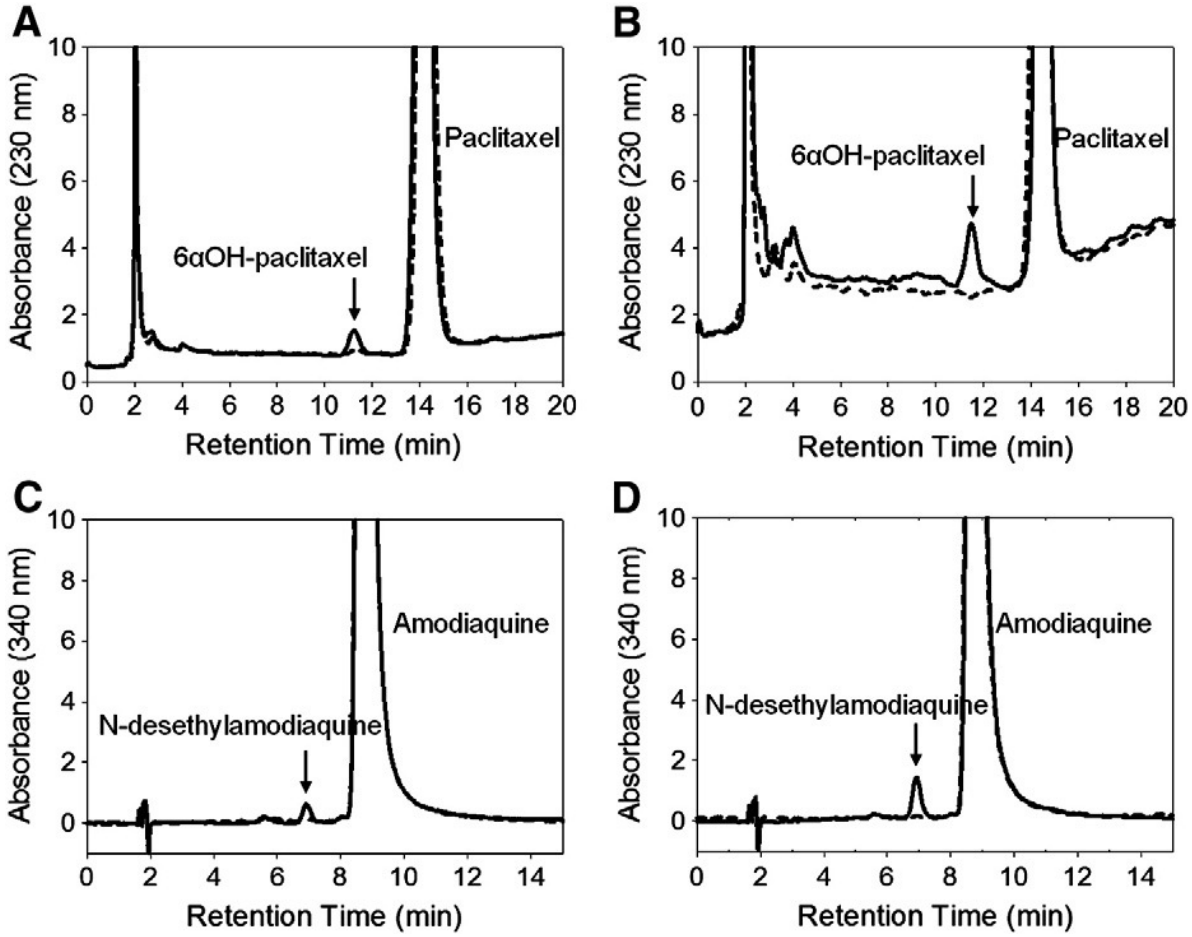

Fig. 4. HPLC chromatograms of the electrocatalysis product of paclitaxel turnover by P450 2C20 (solid line) immobilized on (A) GC/PDDA and (B) Au/DTME. Control experiments in the absence of P450 2C20 (dashed line). Retention times: 6α-hydroxy-paclitaxel = 11.5 min and paclitaxel = 14.5 min; HPLC chromatograms of the electrocatalysis product of amodiaquine turnover in the presence (solid line) and in absence of P450 2C20 (dashed line-control reaction) immobilized on (C) GC/PDDA and (D) Au/DTME, respectively. Re- tention times: N-desethylamodiaquine = 6.8 min and amodiaquine = 9.1 min

Table 2.

Electrochemical properties of P450 2C20 immobilized on different electrodes surfaces.

	GC/2C20/BSAa	GC/2C20/PDDAb	Au/2C20/DTMEc	GC/2C20/AuNPsd
Ea (mV)	– 287 ± 15	– 317 ± 5	n.d.	– 202 ± 8
Ec (mV)	– 148 ± 5	– 182 ± 7	n.d.	– 122 ± 5
Em (mV)	– 217 ± 8	– 249 ± 4	n.d.	– 162 ± 5
ΔE (mV)	139 ± 7	135 ± 5	n.d.	80 ± 4
Number of molecules/cm2	1.94 × 1012	1.26 × 1012	n.d.	n.d.
nmol 6αOH-paclitaxel	No product	0.04 ± 0.01	0.11 ± 0.02	0.51 ± 0.05
KM paclitaxel (μM)	n.d.	n.d.	n.d.	3.0 ± 0.5
nmol of N-des-ethylamodiaquine	No product	0.018 ± 0.004	0.041 ± 0.008	0.15 ± 0.02
KM amodiaquine (μM)	n.d.	n.d.		

a

GC/2C20/BSA: entrapment in a gel co-cross-linked with bovine serum albumin (BSA) and glutaraldehyde.

b

GC/2C20/PDDA: entrapment within the cationic polymer polydiallyldimethylammonium chloride (PDDA) on glassy carbon.

c

Au/2C20/DTME: covalent linkage on gold electrode via a self-assembled monolayer of sulfhydryl-reactive dithio-bismaleimidoethane (DTME) and surface-accessible cysteine residues of the enzyme.

d

GC/2C20/AuNPs: entrapment on glassy carbon electrodes modified with gold nanoparticles (AuNPs) and cationic surfactant.

electrodes in absence of enzyme. As expected, in all control experiments there was no appreciable increase in the cathodic current. The K_M values calculated from the plot of the increase in the cathodic current at each concentration of the drugs added were 3.0 ± 0.5 μM and 1.4 ± 0.2 μM for paclitaxel and amodiaquine, respectively (Fig. 5B and D).

A summary of the electrochemical data is reported in Table 2. 4. Discussion

During the drug development process numerous safety studies must be conducted in animals and M. fascicularis represents the most important non-human primate species used for this purpose. The development of alternative in vitro methods, while not yet a com- plete replacement for animal testing, can lead to improved and in some cases to complement existing techniques. Factors considered for the choice of an in vitro model include in vivo resemblance, ethical consideration, cost and also availability.

Here we have shown how it is possible to develop different in vitro methods using the M. fascicularis P450 2C20 as the model system corresponding to the human P450 2C8 homologue with 92% sequence

identity. The two approaches described include a self-sufficient chimera, P450 2C20/BMR, which functions without the need of any other redox protein and a direct electrochemical method whereby both the require- ment of redox partner and NADPH cofactor are eliminated.

In the first approach, the purified P450 2C20 and 2C20/BMR were both found to be active in vitro and able to turn over the specific human P450 2C8 substrates, paclitaxel and amodiaquine, with similar K_M values reported in literature for the human P450 2C8. These similar- ities for P450 2C20 and the self-sufficient P450 2C20/BMR chimera demonstrate that the chimera represents a good model that greatly simplifies the in vitro preclinical studies of cytochrome P450 2C20 me- diated drug metabolism.

In the second approach the protein was immobilized on different electrode surfaces using several approaches. This is the first time that a macaque P450 enzyme has been immobilized in an active form on both glassy carbon and gold electrodes. Although several other animal P450s [38–40] have been studied electrochemically (on carbon or gold electrodes in the presence of DDAB) none have been for the pur- pose of reducing animal testing but rather for determination of redox potentials or the catalytic behavior.

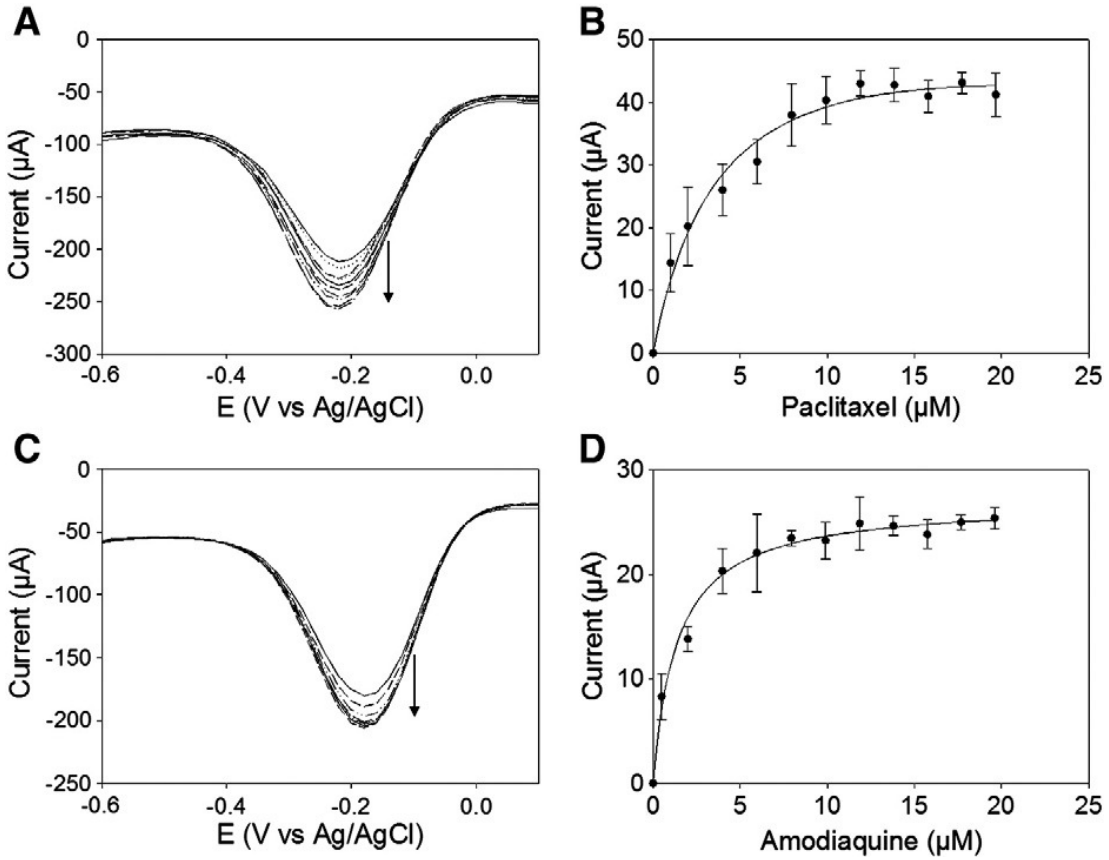

Fig. 5. Square wave voltammetry of Au/2C20/AuNPs titrated with increasing amounts of (A) paclitaxel (0–20 μM) and (C) amodiaquine (0–20 μM) and their respective Michaelis–Menten curves (B and D) calculated from the plot of the increase in the cathodic current at each concentration of the added substrate.

The electrochemical platforms were tested using paclitaxel and amodiaquine as substrates and the electrocatalytic properties of the P450 2C20 immobilized on different surfaces was compared to the P450 reconstituted system in presence of the human P450 reductase and NADPH in solution. The best electrochemical platform was found to be the one where P450 2C20 was immobilized on glassy carbon elec- trodes using AuNPs.

When the AuNPs were used to increase the sensitivity of the system it was possible to obtain a platform where the protein functioned as well as in the in vitro assay with K_M values of 3.0 ± 0.5 μM and 1.4 ± 0.4 μM for paclitaxel and amodiaquine, respectively, in perfect agreement with the P450 2C20 values.

In conclusion, starting from the cloning of an intriguing animal P450 we have developed two alternative methods to study the M. fascicularis P450 2C20 enzyme in vitro. Both the chimeric P450 2C20/BMR and the electrochemical platforms are representative of the catalytic properties of the physiological monooxygenase system. Through the P450 2C20/BMR chimera and the immobilization on electrode surfaces we have bypassed the requirement for the redox partner and NADPH as electron source, the two main obstacles for de- veloping P450 enzymes for biotechnological applications. The data presented are a step forward toward alternative in vitro tests which will become more predictive of the in vivo situation and should be used wherever possible to replace animal models for rapid assess- ment of drug biotransformation and toxicity [41].

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References

[1] J.E. May, J. Xu, H.R. Morse, N.D. Avent, C. Donaldson, Br. J. Biomed. Sci. 66 (2009) 160–165.

[2] O. Varga, A.K. Hansen, P. Sandøe, I.A. Olsson, EMBO Rep. 11 (2010) 500–503.

[3] F.P. Guenguerich, Chem. Res. Toxicol. 21 (2008) 70–83.

[4] M. Ebeling, E. Küng, A. See, C. Broger, G. Steiner, M. Berrera, T. Heckel, L. Iniguez, T. Albert, R. Schmucki, H. Biller, T. Singer, U. Certa, Genome Res. 21 (2011) 1746–1756.

[5] X.S. Lai, L.P. Yang, X.T. Li, J.P. Liu, Z.W. Zhou, S.F. Zhou, Curr. Drug Metab. 10 (2009) 1009–1047.

[6] Y. Uno, H. Fujino, G. Kito, T. Kamataki, R. Nagata, Mol. Pharmacol. 70 (2006) 477–486.

[7] F. Roussel, D.B. Duignan, M.P. Lawton, R.S. Obach, C.A. Strick, D.J. Tweedie, Arch. Biochem. Biophys. 357 (1998) 27–36.

[8] M. Fairhead, S. Giannini, E.M. Gillam, G. Gilardi, J. Biol. Inorg. Chem. 10 (2005) 842–853.

[9] V.R. Dodhia, A. Fantuzzi, G. Gilardi, J. Biol. Inorg. Chem. 11 (2006) 903–916.

[10] A. Fantuzzi, M. Fairhead, G. Gilardi, J. Am. Chem. Soc. 28 (2004) 5040–5041.

[11] D.L. Johnson, B.C. Lewis, D.J. Elliot, J.O. Miners, L.L. Martin, Biochem. Pharmacol. 15 (2005) 1533–1541.

[12] V.R. Dodhia, C. Sassone, A. Fantuzzi, G. Di Nardo, S.J. Sadeghi, G. Gilardi, Electrochem. Commun. 10 (2008) 1744–1747.

[13] S.J. Sadeghi, S. Fererro, G. Di Nardo, G. Gilardi, Bioelectrochemistry 86 (2012) 87–91

[14] C. Helvig, J.H. Capdevila, Biochemistry 39 (2000) 5196–5205.

[15] J. Spratlin, M.B. Sawyer, Crit. Rev. Oncol. Hematol. 61 (2007) 222–229.

[16] X.Q. Li, A. Björkman, T.B. Andersson, M. Ridderström, C.M. Masimirembwa,

J. Pharmacol. Exp. Ther. 300 (2002) 399–407.

[17] T. Omura, R. Sato, J. Biol. Chem. 239 (1964) 2370–2378.

[18] C.L. Crespi, T.K. Chang, D.J. Waxman, Methods Mol. Biol. 320 (2006) 103–107. [19] M. Ntale, M. Mahindi, J.W. Ogwal-Okeng, L.L. Gustafsson, O. Beck, J. Chromatogr. B

Analyt. Technol. Biomed. Life Sci. 859 (2007) 137–140.

[20] S. Castrignanò, S.J. Sadeghi, G. Gilardi, Anal. Bioanal. Chem. 398 (2010)

1403–1409.

[21] V.E. Ferrero, L. Andolfi, G. Di Nardo, S.J. Sadeghi, A. Fantuzzi, S. Cannistraro, G.

Gilardi, Anal. Chem. 80 (2008) 8438–8446.

[22] S.J. Sadeghi, R. Meirinhos, G. Catucci, V.R. Dodhia, G. Di Nardo, G. Gilardi, J. Am.

Chem. Soc. 132 (2009) 458–459.

[23] L.H. Mak, S.J. Sadeghi, A. Fantuzzi, G. Gilardi, Anal. Chem. 82 (2010) 5357–5362. [24] G.A. Schoch, J.K. Yano, S. Sansen, P.M. Dansette, C.D. Stout, E.F. Johnson, J. Biol.

Chem. 20 (2008) 17227–17237.

[25] E. Krieger, G. Vriend, Bioinformatics 18 (2002) 315–318.

[26] R.A. Laskowski, J.A. Rullmannn, M.W. MacArthur, R. Kaptein, J.M. Thornton, J. Biomol.

NMR 8 (1996) 477–486.

[27] T. Cresteil, B. Monsarrat, J. Dubois, M. Sonnier, P. Alvinerie, F. Gueritte, Drug

Metab. Dispos. 30 (2002) 438–445.

[28] A. Rahman, K.R. Korzekwa, J. Grogan, F.J. Gonzalez, J.W. Harris, Cancer Res. 54

(1994) 5543–5546.

[29] R.L. Walsky, E.A. Gaman, R.S. Obach, J. Clin. Pharmacol. 45 (2005) 68–78.

[30] N. Hanioka, K. Matsumoto, Y. Saito, S. Narimatsu, Basic Clin. Pharmacol. Toxicol.

107 (2010) 565–569.

[31] S.J. Sadeghi, A. Fantuzzi, G. Gilardi, Biochim. Biophys. Acta 1814 (2011) 237–248. [32] A. Fantuzzi, L.H. Mak, E. Capria, V.R. Dodhia, P. Panicco, S. Collins, G. Gilardi, Anal.

Chem. 15 (2011) 3831–3839.

[33] P. Panicco, V.R. Dodhia, A. Fantuzzi, G. Gilardi, Anal. Chem. 15 (2011) 2179–2186. [34] E. Laviron, J. Electroanal. Chem. 101 (1979) 19–23.

[35] X. Cao, Y. Ye, S. Liu, Anal. Biochem. 417 (2011) 1–16.

[36] B.D. Fleming, Y. Tian, S.G. Bell, L.L. Wong, V. Urlacher, H.A.O. Hill, Eur. J. Biochem.

270 (2003) 4082.

[37] A.K. Udit, N. Hindoyan, M.G. Hill, F.H. Arnold, H.B. Gray, Inorg. Chem. 44 (2005) 4109–4111.

[38] Y.O. Rudakov, V.V. Shumyantseva, T.V. Bulko, E.V. Suprun, G.P. Kuznetsova, N.F. Samenkova, A.I. Archakov, J. Inorg. Biochem. 102 (2008) 2020–2025.

[39] S.Y. Rhieu, D.R. Ludwig, V.S. Siu, T.R. Palmore, Electrochem. Commun. 11 (2009) 1857–1860.

[40] D.L. Johnson, A.J. Conley, L.L. Martin, J. Mol. Endocrinol. 36 (2006) 349–359.

[41] M. Martignoni, G.M. Groothuis, R. de Kanter, Expert Opin. Drug Metab. Toxicol. 2 (2006) 875–894.