ABSTRACT

Cyclophosphamide (CPA) is a chemotherapeutic agent that is primarily activated in the liver by cytochrome P4502B6 (CYP2B6) and then transported to the tumor via blood flow. To prevent deleterious secondary effects, P450-based gene-directed enzyme prodrug therapy (GDEPT) consists of expressing CYP2B6 in tumor cells before CPA treatment. The aim of our work was to modify CYP2B6 to increase its catalytic efficiency ($V_{\text{max}}$/$K_m$) to metabolize CPA into 4'-OH CPA. A molecular model of CYP2B6 was built, and four residues in close contact with the substrate were subjected to mutagenesis. Canine CYP2B11 exhibiting a particularly low $K_m$ to CPA, the amino acids exclusively present in the CYP2B11 substrate recognition sequences were substituted in human CYP2B6. All mutants ($n = 26$) were expressed in Saccharomyces cerevisiae and their enzymatic constants ($K_m$, $V_{\text{max}}$) evaluated using CPA as substrate. Five mutants exhibited a 2- to 3-fold higher catalytic efficiency than wild-type CYP2B6. A double mutant, comprising the two most effective mutations, showed a 4-fold increase in $K_m$/ $V_{\text{max}}$. Molecular dynamic simulations of several mutants were found to be consistent with the observed modifications in catalytic efficiency. Finally, expression of the CYP2B6 114V/477W double mutant, contrary to wt CYP2B6, allowed switching of a resistant human head and neck cancer cell line (A-253) into a sensitive cell line toward CPA. Thus, we were able to obtain a new efficient CYP2B6 mutant able to metabolize CPA, an important step in the GDEPT strategy for human cancer treatment.

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ABBRVIATIONS: CPA, cyclophosphamide; P450, cytochrome P450; SRS, substrate recognition sequences; CPI, 4-(4-chlorophenyl)imidazole; MD, molecular dynamics; RED, NADPH cytochrome P450 reductase; MOI, multiplicity of infection; RMSD, root-mean-square deviation; SMAS, solvent molecular accessible surface; wt, wild type.
tively low affinity of CYP2B6 for CPA, which shows a high $K_m$. The aim of this work was to modify the CYP2B6 enzyme to increase its catalytic efficiency ($V_{\text{max}}/K_m$) for 4-hydroxylation of CPA and, therefore, to improve its therapeutic effect. The concept of creating “improved” enzymes is not new and previous structure-function studies on mammalian P450s have shown that some mutations can enhance existing activities or confer new specificities (Domanski and Halpert, 2001; Melet et al., 2003; Spatzenegger et al., 2003; Chen et al., 2004). To engineer CYP2B6 with increased catalytic efficiency for 4-OH-CPA hydroxylation, two approaches were used.

First, as there is no crystal structure of CYP2B6 available in the Protein Data Bank (PDB, http://www.rcsb.org), a molecular model of CYP2B6 was built with the help of refined procedures to identify putative active site residues. This model was derived by sequence alignment with the crystalized CYP2B4, which shares the highest sequence homology with CYP2B6 (Scott et al., 2003). Moreover, the crystal structures of CYP2B4 with different ligands more or less bulky revealed its remarkable plasticity (Scott et al., 2003; Zhao et al., 2006) and demonstrated how the active site of the enzyme can be reshaped to accommodate ligands of different sizes and shapes while maintaining the overall P450 folding. The crystal structure of CYP2B4 (PDB code 1SUO) complexed with the specific inhibitor 4-(4-chlorophenyl) imidazole (CPI) was chosen as a unique template for the rebuilding process. This template is a reasonable choice, because CPA and CPI are substrates of comparable size and chemical structure. After docking CPA into the CYP2B6 homology model, four amino acid residues were identified to be in close contact with the substrate (Ile114, Val367, Val477 and Gly478) and were selected for mutagenesis. For each position, a large variety of amino acids was tested (hydrophobic, polar, charged, neutral side chains).

Second, similar to the human orthologous CYP2B6, several CYP2B isoforms catalyze 4-OH-CPA hydroxylation: CYP2B1 in rat, CYP2B4 and CYP2B5 in rabbit, and CYP2B11 in dog (Chen et al., 2004). The activity of rat CYP2B1 was 10- to 35-fold higher than that of rabbit CYP2B4 and CYP2B5, whereas canine CYP2B11, exhibiting a particularly low $K_m$ (80–160 \(\mu\)M), was 7- to 8-fold more active than CYP2B1. The primary structure alignment of these different isoforms (Fig. 1) suggested few additional positions for site-directed mutagenesis experiments in CYP2B6. We focused on mutations of amino acids that are present exclusively in the CYP2B11 substrate recognition sequences (SRS).

Following both strategies, we designed various single and double mutants obtained by site-directed mutagenesis. These mutants were cloned into the yeast expression vector pYeDP60 (V60) and expressed in Saccharomyces cerevisiae (Pompon et al., 1996). Site-directed mutant catalytic activities were evaluated using CPA as substrate. Molecular dynamic (MD) simulations of several model complexes (CPA/wild-type or mutated CYP2B6) were achieved to support interpretation of the observed modifications in catalytic efficiency at the molecular level, through the computed dynamic behavior of the substrate within the active site. At last, the potential possibility to enable a CPA-resistant head and neck cancer cell line (A-253) sensitive toward CPA by the GDEPT strategy was examined. Using the most efficient mutant to metabolize CPA, this strategy was found to be quite effective.
gradient termination of 0.005 kcal/(mol·Å). In this step, local minimization under SYBYL was operated with an aggregate defined by the whole protein, including the heme, deprived of a spherical area of 10 Å surrounding the substrate. Global minimization of 1000 steps without constraints was finally performed to generate the final structure of the CYP2B6 model/CPA complex in subsequent steps.

**Mutational Work in Silico.** Mutations were performed in silico using the Biopolymer module of the SYBYL 7.1 software (Tripos Inc., St Louis, MO). For each mutation, the side-chain conformation was fixed, using selection of the best rotamer for adjustment of side-chain positions and local minimization to remove close contacts and locally relax the system. Parameters used for minimization were the same as above. No aggregate was used at this stage, and no global minimization was performed because all models were submitted to MD simulation for subsequent docking calculations.

**Molecular Dynamics simulations.** The structural models of CYP2B6 and the mutants were equilibrated by extensive MD simu-

### Table: Amino Acid Sequence Alignment

| CYP2B11 | MELSVMILLA LIQTGVLLMA RGHKPKAYGRL PPGPRPFLPLG GNFLQMDKRG LLKSLFLQAE 60 |
| CYP2B6 | MEFSLILLIA LIQTGVLLLA RGHKPKANGL PPGPRPFLPLG GNKLQMDLRG LLKSLFLRKE 60 |
| CYP2B2 | MFSSILLLA LIQTGVLLLY RGHKPKSNGF PPGPRPFLLLG GNLQGDLQRG LNSGFMLRRE 60 |
| CYP2B1 | MEPTILLLLA LIQTGVLLLY RGHKPSNGFP PPGPRPFLLLG GNLQGDLQRG LNSGFMLQRE 60 |
| CYP2B6 | MELSVMILLA LIQTGVLLLY QHHRNTLEDL PPGPRPFLLLG GNLQGMDRGG LLKSLFRLRE 60 |
| CYP2B4 | MEFSSILLLA LIQTGVLLLY RGHKPKANGL PPGPRPFLPLG GNKLQMDLRG LLKSLFLRRE 60 |

### CYP2B4

- **CYP2B4**
  - DLIPFGVPHT
  - ETTSTTLRYG FLLMLK
  - SIVF
  - KY

### CYP2B1

- **CYP2B1**
  - DLVPIGVPHR VTKDTMFRGY LLPKNTEVYP ILSSALHDPQ YFDHPDSFNP EHFLDANGAL 420
  - ETSSTTLRYG FLLMLKYPHV AEKVQKEIDQ VIGSHRLPTL DDRSKMPYTD AVIHEIQRFS 360
  - MEPTILLLLA LIQTGVLLLY RGHKPKANGL PPGPRPFLPLG GNKLQMDLRG LLKSLFLRKE 60
  - KYGDVFTVHL GPRPVVMLCG TDTIKEALVG QAEDFSGRGT IAVIEPIFKE YGVIFANGER 120

### CYP2B2

- **CYP2B2**
  - DLAPIGLPHR VTKDTMFRGY LLPKNTEVYP ILSSALHDPQ YFDHPDTFNP EHFLDADGTL 420
  - ETGSTTLRYG FLLMLKYPHV TEKVQKEIDQ VIGSHRPPSL DDRTKMPYTD AVIHEIQRFA 360
  - MEPTILLLLA LIQTGVLLLY RGHKPKANGL PPGPRPFLPLG GNKLQMDLRG LLKSLFLRKE 60
  - KYGDVFTVHL GPRPVVMLCG TDTIKEALVG QAEDFSGRGT IAVIEPIFKE YGVIFANGER 120

### CYP2B5

- **CYP2B5**
  - DLVPFGAPHM VTKDTQFRGY VIPKNTEVFP VLSSALHDPR YFETPNTFNP GHFLDADGAL 420
  - ETTSTTLRYG FLLMLKYPHV TERVQKEIEQ VIGSHRPPAL DDRAKMPYTD AVIHEIQRFG 360
  - MEPTILLLLA LIQTGVLLLY RGHKPKANGL PPGPRPFLPLG GNKLQMDLRG LLKSLFLRKE 60
  - KYGDVFTVHL GPRPVVMLCG TDTIKEALVG QAEDFSGRGT IAVIEPIFKE YGVIFANGER 120

### CYP2B11

- **CYP2B11**
  - DLLPIGVPHM VTKDICFRGY IIPKGTEVFP ILHSALNDPH YFEKPDVFNP DHFLDANGAL 420
  - ETTSTTLRYG FLLMLKYPHI AERIYKEIDQ VIGPHRLPSL DDRAKMPYTD AVIHEIQRFG 360
  - MEPTILLLLA LIQTGVLLLY RGHKPKANGL PPGPRPFLPLG GNKLQMDLRG LLKSLFLRKE 60
  - KYGDVFTVHL GPRPVVMLCG TDTIKEALVG QAEDFSGRGT IAVIEPIFKE YGVIFANGER 120

### CYP2B6

- **CYP2B6**
  - INAYIGHSVE KHRETLDPSA PKDLIDTYLL HMEKEKSNAH SEFSHQNLNL NTLSLFFAGT 300
  - PPSYQIRFLA PPTYQIRFLP R--- 491
  - SIVFGERFDY TDRQFLRLLE LFYRTFSLLS SFSSQVFEFF SGFLKYFPGA HRQISKNLQE 240
  - KYGDVFTVHL GPRPVVMLCG TDTIKEALVG QAEDFSGRGT IAVIEPIFKE YGVIFANGER 120

### CYP2B1

- **CYP2B1**
  - ILDYIGHIVE KHRATLDPSA PRDFIDTYLL RMEKEKSNHH TEFHHENLMI SLLSLFFAGT 300
  - PPTYQICFSA R--- 491
  - SIVFGERFDY TDRQFLRLLE LFYRTFSLLS SFSSQVFEFF SGFLKYFPGA HRQISKNLQE 240
  - KYGDVFTVHL GPRPVVMLCG TDTIKEALVG QAEDFSGRGT IAVIEPIFKE YGVIFANGER 120

### CYP2B5

- **CYP2B5**
  - INTFIGQTVE KHRATLDPSN PRDFIDVYLL RMEKDKSDPS SEFHHRNLIL TVLTLFFAGT 300
  - PPTYQICFSA R--- 491
  - SIVFGERFDY TDRQFLRLLE LFYRTFSLLS SFSSQVFEFF SGFLKYFPGA HRQISKNLQE 240
  - KYGDVFTVHL GPRPVVMLCG TDTIKEALVG QAEDFSGRGT IAVIEPIFKE YGVIFANGER 120

### CYP2B4

- **CYP2B4**
  - KYGDVFTVHL GPRPVVMLCG TDTIKEALVG QAEDFSGRGT IAVIEPIFKE YGVIFANGER 120

### CYP2B11

- **CYP2B11**
  - WKTLRRFSLA TMDFPNGMRK SVERRIQEEA QCLVEELRKT EGVLDQRTFPF HFSNTIIC 180
  - PPVYQISFLS RGGC 494
  - WKALRRFSLA TMRDFGMGKR SVEERIQEELA QCLVEELRKS QGAPLDPTFL F QCITANIIC 180

### CYP2B5

- **CYP2B5**
  - WRALRRFSLA TMRDFGMGKR SVEERIQEELA RCLVEELRKS KGALLDLTFL F HSVTSNIIC 180
  - WKALRRFSLA TMRDFGMGKR SVEERIQEELA QCLVEELRKS QGAPLDPTFL F QCITANIIC 180

### CYP2B1

- **CYP2B1**
  - WKALRRFSLA TMRDFGMGKR SVEERIQEELA QCLVEELRKS QGAPLDPTFL F QCITANIIC 180
  - WKTLRRFSLA TMDFPNGMRK SVERRIQEEA QCLVEELRKT EGVLDQRTFPF HFSNTIIC 180

### CYP2B4

- **CYP2B4**
  - WKTLRRFSLA TMDFPNGMRK SVERRIQEEA QCLVEELRKT EGVLDQRTFPF HFSNTIIC 180

### CYP2B6

- **CYP2B6**
  - WKTLRRFSLA TMDFPNGMRK SVERRIQEEA QCLVEELRKT EGVLDQRTFPF HFSNTIIC 180

### CYP2B1

- **CYP2B1**
  - WKTLRRFSLA TMDFPNGMRK SVERRIQEEA QCLVEELRKT EGVLDQRTFPF HFSNTIIC 180
lations of 2 ns in explicit solvent in the absence and in the presence of CPA docked in the active site. All MD simulations were carried out using the GROMACS v 3.1.4 molecular dynamics simulation package (Schulte-Ladbeck et al., 2001). For the generation of topology files under GROMACS, the structure template coordinates file was split into two structure files, one for the protein and one for the ligand, to keep the two molecules in the same frame of reference. Protein and CPA coordinates structure files were then separately converted into gro file (coordinates) and top file (topology file) as input files for GROMACS. The CPA file was converted by the PRODRG server, a utility that provides topology parameters of organic compounds while respecting the coordinates in the initial frame of reference (http://davacp1.bioch.dundee.ac.uk/programs/prodrg/). The protein file was converted by the pdb2gmx script using the parameter set ffgmx (GMX force field of Gromos87), which also provides topology parameters for the heme and various cofactors. The topology of the heme residue (HEME) was the default generated using pdb2gmx, with a total net charge of −2 for the protoporphyrin IX moiety associated to iron(II). Attachment of the heme to the proximal cysteine residue through Cys-thiolate bond was also performed by the pdb2gmx script, and all MD simulations were carried out with the heme macrocycle in the penta-coordinated state (5-c), corresponding to the high-spin state of the enzyme before ligand binding. The choice of GMX as a force field was driven by the fact that it is the only force field compatible with GROMACS’s ligand topologies obtained from the PRODRG server. In the prodrg script, chirality, full charges, and energy minimization flags were set, respectively, to “yes,” “yes,” and “no.” CPA gro and top files were then merged to the protein files according to the procedure described in Kerrigan’s Tutorial for the Drug-Enzyme complex (http://www2.umd.nj.edu/~kerri/gro). The presence of water molecules in the active site was taken into account by solvation of the complex CPA2B6/CPA in explicit solvent, using a truncated octahedral periodic box (editconf and top script dimensions of the box based upon setting the box edge 10 Å from the protein (i.e., 92 × 85 × 75 Å³)).

The whole system was then charge-neutralized by adding three counter-ions and energy-minimized without restraints for 5000 steps using the steepest minimization to remove residual steric clashes. After minimization, position-restrained MD simulation of 20 ps was performed (protein atom positions restrained; CPA, solvent molecules, and ions not restrained) for equilibration and free diffusion of water molecules into the protein structure. Conditions for MD simulation were: NPT ensemble, the PME (Particle Mesh Ewald) electrostatics method (Darden et al., 1993) for computing long-range electrostatics with a real space cutoff of 9 Å, temperature and pressure coupling using Berendsen’s algorithm (Berendsen et al., 1984) with temperature of the system fixed at 300 K with a coupling constant τp = 0.1 ps, and pressure set at 1 bar with a coupling constant τp = 0.5 ps and compressibility set to 4.5 × 10⁵ bar⁻¹ in all simulation box directions. Protein, heme, CPA, solvent, and ions were coupled separately. The time-step for integration was 2 fs, and coordinates and velocities were saved every 0.5 ps. For constrained MD simulations, the all-bond option was set, applying the Linear Constraint algorithm (Hess et al., 1997) to restrain all bond lengths in the system. After this equilibration, unconstrained extensive MD simulations of 2 ns using wild-type or mutants CYP2B6 complexed with CPA were carried out with the same parameters.

**Construction of CYP2B6 mutants.** Site-directed mutagenesis was based on the QuikChange kit (Stratagene, Amsterdam, The Netherlands). A single PCR reaction using mutagenic primers and wt V-60CPY2B6 (Gervot et al., 1999) as template was performed, followed by DpnI digestion and transformation into competent DH5α bacteria. The mutagenic PCR program was as follows: 10 min at 92°C, followed by 17 cycles at 92°C for 45 s, 59°C for 45 s, 68°C for 26 min, followed by final extension of 10 min at 68°C. Mutations were performed using Pfu turbo polymerase (Stratagene, La Jolla, CA). All mutants generated were sequenced by direct sequencing using an automatic sequencer (PerkinElmer Life and Analytical Sciences, Waltham, MA) to be sure that the desired mutant was obtained.

**Expression in Yeasts.** The yeast expression plasmid pYeDP60 and the W(R) S. cerevisiae strain, constructed by substitution of the natural W303-1B yeast reductase promoter by the galactose inducible GAL10-CYC1 hybrid promoter (Truan et al., 1993) were gifts from D. Pompon (Gif-sur-Yvette, France).

Mutated and wild-type CYP2B6 were expressed in the W(R) yeast strain, in which yeast NADPH cytochrome P450 reductase was over-expressed. The pYeDP60 plasmid was introduced into intact yeast cells based on a refined lithium acetate-mediated protocol (Truan et al., 1993). Yeast culture conditions have been described previously (Bellamine et al., 1994). Site-directed mutagenesis (Bellamine et al., 1994).

**Cell Line.** Human A-232 epidemoid carcinoma cells from the submaxillary gland (American Type Culture Collection number HTB 41), purchased from LGC Promocem (Molsheim, France), were grown as a monolayer in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, nonessential amino acids for Dulbecco’s modified Eagle’s medium, penicillin at 200 U/ml, and streptomycin at 50 µg/ml.

**Preparation of the Yeast Microsomal Fraction.** Yeast microsomes were prepared based on the mechanical disruption method using glass beads as described elsewhere (Bellamine et al., 1994). The yeast microsomal protein concentration was determined by the bicinchoninic acid (BCA) procedure according to the manufacturer’s instructions (Pierce, Rockford, IL) using bovine serum albumin as a standard. Microsomal P450 content was spectrally determined by the method of Schoene et al. (1972).

**Recombinant Adenovirus.** CYP2B6, human cytochrome P450 reductase (RED), and the double mutant CYP2B6 114V/477W provided by our laboratory were sent to the University Hospital of Nantes supported by the Association Française contre les myopathies (AFM) and cloned into serotype five adenovirus (http://www.vectors.nantes.inserm.fr/). A replication defective E1 and E3 region-deleted adenovirus encoding CYP2B6, human RED, and the 2B6 double mutant were constructed and purified, and the titer was determined spectrophotometrically.

**CPA 4-Hydroxylase Activity.** The fluorometric determination of microsomal CPA 4-hydroxylase was adapted from a technique described previously (Roy et al., 1999) for a 96-well microplate with several modifications. Incubations were carried out for 1 h at 28°C in a total volume of 200 µl and included 100 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA, 10 pmol of CYP2B6, and 10 mM CPA. Reactions were initiated by adding the NADPH-generating system and stopped by the addition of 200 µl of 10% trichloroacetic acid. After centrifugation at 13,000 g and 4°C for 15 min to pellet the proteins, 300 µl of the supernatant was transferred to a clean test tube containing 160 µl of the fluorescence mixture (6 mg of 3-aminophenol and 6 mg of hydroxylamine hydrochloride freshly dissolved in 1 ml of 1N HCl). Samples were heated at 90°C for 20 min to form 7-hydroxquinoline by condensation of the 4-hydroxy-CPA with 3-aminophenol. After cooling to room temperature, fluorescence reading were performed on a Bio-tek FL600 microplate fluorescence reader (excitation at 350 nm and emission at 515 nm). Under these assay conditions, product formation was linear with time, and the enzyme concentration and amount of 4-hydroxy-CPA under these assay conditions was determined based on a standard curve of 4-hydroxy-CPA (0–20 μM) incubated with bovine serum albumin and treated in parallel under the same assay conditions.

The kinetic constants of cyclophosphamide hydroxylase were determined by a nonlinear regression with 15 substrate concentrations (0.05 to 25 mM). Data were analyzed using Prism software (GraphPad Software, Inc., San Diego, CA) to calculate kinetic parameters (Km, Vmax), with Vmax values expressed as moles of product formed per minute and Km values expressed in mM.

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Adenoviral Infections and In Vitro Cytotoxicity Assays. A-253 cells were seeded in six-well plates at 400,000 cells/well. After 24 h, they were infected for 4 h with adenovirus at a multiplicity of infection (MOI) of 300 (i.e., number of infectious particles/cell), in cell culture medium containing 2% fetal bovine serum. In the case of double infection (Ad-2B6 + Ad-Red), cells were infected with a total of 300 MOI (150 + 150), whereas Ad-LacZ at 150 MOI was used in combination with Ad-2B6 or Ad-Red (150 MOI) to get an even MOI concentration in each well. Cells infected with an adenoviral vector expressing LacZ at 300 MOI were used as a control. No toxicity of the control vector Ad-LacZ was observed at this dose in cells that were not treated with CPA. After 4 h, the adenovirus-containing medium was removed and replaced with the usual medium. Overexpression of the transgenes was checked 3 days after infection by Western blot using a polyclonal anti-CYP2B6 antibody (Gervot et al., 1999) and a polyclonal anti-RED antibody (Daichi Pure Chemicals Co. Ltd, Tokyo, Japan), which cross-reacts strongly with human RED (data not shown).

For the in vitro-cytotoxicity assays, cells were trypsinized 20 h after infection and plated in a 96-well plate at a concentration of 10,000 cells/well. After 24 h, they were treated with cyclophosphamide at 0 to 3 mM concentrations for six days. The cytotoxicity of CPA was measured with a MTS-colorimetric assay, using the Cell-Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer’s instructions. Cell viability was calculated as the ratio of the absorbance measured in infected cells to that measured in cells infected with the control (Ad-Lac Z), which were treated with identical CPA concentrations and then calculated as the percentage of the ratio obtained in untreated cells. Assays were repeated four times in triplicate.

NADPH Cytochrome P450 Reductase Activity Assay. RED activity was measured in the cellular microsomal fraction. The NADPH-dependent reduction of cytochrome c by RED was assayed as described previously (Yasukochi et al., 1980).

Results

CYP2B6 Modeling. The three-dimensional structure of CYP2B6 was generated by homology modeling based on the CYP2B4 structure as a unique template with 78% sequence identity (Fig. 1). With such homology, the resulting model of CYP2B6 produced by Modeler 7 according to the Modeler objective function can be considered a high-quality model as assessed by the structure-quality checking programs used (ProQ, Anolea, Prosa).

Moreover, the template used was an energetically optimized structure of CYP2B4 for improvement of side chain contacts and rotamer states in the initial structure (PDB code 1SUO). This relaxed structure of the template was obtained after equilibration in a water box of 1SUO and relaxation by 2 ns of MD simulation as described under Materials and Methods. As shown by the RMSD values of the backbone atom positions (Fig. 2), the minimized CYP2B6 model (red trace) proved to be very stable during dynamics production at 300 K after the first 250 ps of equilibration. For comparison, the MD trajectory is shown for the minimized crystal structure of CYP2B4 (1SUO, black trace), which was performed to generate the optimized template for CYP2B6 rebuilding. RMSD values of the backbone atom positions were calculated between trajectory structures and the starting structure (minimized 1SUO CYP2B4 and minimized CYP2B6 models, respectively). For both structures, the same tendency in the time evolution of RMSD values was observed: a plateau at around 1.8 Å was rapidly reached (within 250 ps for CYP2B6 and 500 ps for CYP2B4), and the structures were then stabilized in their respective conformation all the way to 2 ns. CYP2B6 reaches its equilibrium conformation in a shorter time compared with CYP2B4, because it was generated starting from a 2-ns-optimized MD template and not directly from a crystal structure. For CYP2B4, the observed stabilization at around 1.8 Å RMSD from the starting point (crystal structure) can be considered standard in this kind of simulation in explicit solvent at 300 K. For CYP2B6, considering the fact that the starting point was a MD-relaxed model, the RMSD profile indicates that the CYP2B6 model reached an equilibrium conformation that is not a strict copy of the template backbone from which it was designed. Indeed, the RMSD value of the backbone atom positions between the two final structures were found at 1.77 Å by ProFit (calculated on 464 aligned residues between CYPB4 and CYP2B6) revealing distinct configurations. These results are in favor of i) good stability and conservation of the global fold within the CYP2B subfamily (no detectable drift after 2 ns of MD simulation and no significant fold divergence, whereas CYP2B4 and CYP2B6 differ by more than 100 residues) and ii) accuracy and stability of the CYP2B6 structural model.

Features of the Binding Site. To characterize the geometry of the binding site, the Voidoo program was applied to calculate the solvent molecular accessible surface (SMAS) in both structures after minimization as well as after MD simulation of 2 ns. In each case, CYP2B6 displayed a larger active site compared with CYP2B4, accounting in part for the RMSD found between the backbones. According to Voidoo, the crystal structure (not minimized) of CYP2B4 displayed a volume defined by SMAS of 271 Å³, which corresponds to a quite narrow active site in P450 superfamily. When the structure was relaxed by MD in explicit solvent, the volume was found almost unchanged at 252 Å³. Conversely, CYP2B6 model displayed a SMAS volume of 363 Å³, a volume consistent with the binding of ligands bulkier than CPI, such as CPA. The three main subpockets described in a previously
published model (Bathelt et al., 2002) are also present in our optimized model and are formed by the same residues (Phe206, Ala298, Thr302, Leu363 for pocket A or heme pocket; Ile114, Phe115, Ser294, and Phe 297 for pocket B, Ile209, Val367, and Val477 for pocket C) with the exception of residue Leu216 (in C pocket), which we found was not oriented toward the active site.

Initial Positioning of CPA in the Active Site of CYP2B6 Model. The position of the CPI substrate in the CYP2B4 crystal structure 1SUO was used as an initial guess regarding the position of CPA in the active site of CYP2B6 rebuilt models. However, because of the difference in shape and volume between the two compounds and between the two active sites, it was necessary to adjust the position of the CPA for further MD calculations. In particular, the existence of a chiral center in the CPA molecule was taken into account. Because no experimental data on the effect of CPA stereoisomerism on biological activity were available, the two enantiomers were treated separately in the simulations. The position of the CPI ring close to the heme iron was used as a template to produce the initial positioning of CPA in the CYP2B6 active site. The metabolic (or inhibitory) atom sites of each molecule were superimposed (Fig. 3), and the CYP2B6 structure docked with CPA was relaxed by MD simulation (2 ns). Given that CPA displays a chiral center, initial positioning of both enantiomers was performed. The position of the ring of the two enantiomers relative to the heme was unchanged, and the chirality resulted mainly in the inversion of CPA C4 and C6 positions in front of the heme (by symmetry around the P2-C5 axis of the ring). After relaxation, in both cases, C4 and C5 atoms of CPA displayed the shortest distances to the heme iron. All MD trajectories were duplicated with \( R \)- and \( S \)-enantiomers. The three C atoms of the ring (C4, C5, and C6) exhibited variable distances with respect to heme iron during the 2-ns dynamics simulation, but the average tendency supported dominant metabolism leading to C4 hydroxylation for both enantiomers. The last structure (2 ns) of each simulation has been minimized under Gromacs force field (500 steps, converged).

In the wild type, the following simulation has been minimized under Gromacs force field for both enantiomers. The last structure (2 ns) of each ports dominant metabolism leading to C4 hydroxylation along helix I.

Fig. 3. Initial positioning of CPA in the active site of the CYP2B6 model: the position of the CPI ring close to the heme iron in CYP2B4 (1SUO) was used to produce an initial positioning for CPA (S-enantiomer) in the CYP2B6 model. The metabolic (or inhibitory) atom sites of each molecule were superimposed as starting point before MD simulation (C and N atoms, respectively, are indicated by black arrows). Each structure (1SUO and CYP2B6 model) was equilibrated and relaxed in the same conditions by MD (2 ns), resulting in slightly different positions of the ligands relative to the heme.

In the CYP2B6/CPA-computed model, CPA is surrounded by 16 residues located within 5 Å of one another: Val1104, Ile1110, Ile1114, Phe1115, Phe206, Phe208, Ile209, Ala298, Glu301, Thr302, Ser320, Leu363, Val367, Val477, and Gly478. These residues are at the edge of the active site and most of them have been shown to be important for substrate metabolism (Zhao and Halpert, 2007). Figure 4 shows some of these residues potentially involved in the recognition or positioning of CPA in the active site, as seen after 2-ns MD simulation of the complex.

Active Site Access. The access channels to the active site in the CYP2B6 model were explored using CAVER, a tool providing pathways leading from buried cavities to the outside solvent. CAVER was applied to various structures collected along the MD trajectory of 2 ns. As a result, three main entrance/exit pathways were detected in CYP2B6 (Fig. 5). A first group of channels (orange/red on Fig. 5) was found close to the B’ helix, on both sides of the B-C loop, a region known to be very flexible, adopting different conformations that follow the nature of the bound substrate (Honma et al., 2005). Depending on the frame of the simulation, several pathways of variable importance emerged in this region. The variability of the channel position around the B’ helix was clearly dependent upon the conformation of the B-C loop during the MD trajectory. A second pathway (blue, Fig. 5) was found between the E, F, and I helices and the \( \beta \)-turn of \( \beta_6 \) sheet. Similar to the first pathway, its relative importance was dependent on the frame of the MD going from a widely open state to an almost closed state. The third pathway (purple, Fig. 5) was found positioned among the A, I, and K helices, along helix I.

All these pathways have been described in the survey by Cojocaru et al. (2007), and our first, second, and third pathways correspond to channel (subclasses a, b, c, ac, e), channel S (or solvent channel), and channel five, respectively. In
CYP2B6, the main access is thus represented by three channels lined by the B-C loop/B'-helix that alternate dynamically. This adaptability is not surprising in a region of the protein structure known to be highly variable in sequence and structure between different P450s and is important for substrate recognition and specificity. Regarding the S channel, the egress between E, F, and I helices and the β₄ turn is frequently found in the P450 superfamily (Cojocaru et al., 2007). Conversely, channel five is a particular access of unknown role and found in only few structures of P450, including CYP2B4. The three same channels were found in the relaxed CYP2B4 structure indicating similar pathways for substrates in both enzymes, despite differences in their active sites.

**CYP2B6 Mutagenesis.** A total of 26 CYP2B6 site-directed mutants were constructed, as tabulated in Table 1. In all cases, the entire CYP2B6 coding sequence was checked to ensure that the expected base changes had been performed and that no undesired mutations were introduced during the process of site-directed mutagenesis. The 26 mutants are shared between 1) active site mutations at four positions as suggested by the modeling (114, 367, 477, 478, 19 single mutants, and 1 double mutant) and 1) canine-derived mutations at six positions as suggested by CYP2B2 SRS alignment of Fig. 1 (107, 199, 207, 236, 365, 475, all single mutants). The six “canine” mutants were constructed to replace related amino acids in human CYP2B6 with amino acids exclusively present in canine CYP2B11 (F107V, L199M, S207A, K236N, M365I, and C475I).

**Heterologous Expression of CYP2B6 Constructs.** All CYP2B6 site-directed mutants were successfully expressed in yeast microsomes. The levels of P450 expression spectrally determined show large variations from one construct to the other (ranging from 17 to 340 pmol/mg of microsomal protein) (Table 1). Note that spectral P450 expression was comparable in yeasts expressing wt CYP2B6 or the double mutant I114V/V477W. However, catalytic enzyme assays have been performed using the same amount of active P450 content for each construct and therefore were not affected by the various levels of expression.

**Enzyme Kinetic Analysis.** All mutants were investigated for CPA 4-hydroxylase activity to determine their kinetic parameters; V_{max}, K_m, and V_{max}/K_m ratio of CPA-4’hydroxylation by CYP2B6. The site-directed mutants are presented in Table 1 and a typical graphical analysis is displayed Fig. 6.

The first part of Table 1 displays the results obtained by mutations in the active site. I114V substitution increased the CPA-4OH efficiency (V_{max}/K_m) mainly by decreasing the K_m (~50%) of this activity. Amino acid Val367 was mutated into Leu, Phe, Ser, Thr, and His. An increase in V_{max} was only observed with the V367L mutant; however, because of the K_m increase, the catalytic efficiency of this mutant remained slightly lower than that of the wild-type CYP2B6. All other mutants at position 367 resulted in much lower CPA-4 hydroxylase activity (43–2% of wild-type CYP2B6). The substitution of Val477 with Ser, Thr, Tyr, Asn, Asp, and Glu also proved to be also detrimental for CPA-4OH hydroxylase activity. This activity was undetectable in V477N and V477S mutants. In contrast, replacement of Val with hydrophobic and aromatic amino acids resulted in a significant increase in CPA-4OH catalytic efficiency that was directly correlated with the size of the side chain [Ile (×1.89) < Phe (1.91) < Trp (×2.78)]. Replacement of Gly478 with Ala, Val, Ser, and Glu significantly reduced CPA-4OH hydroxylase activity from 36 to 5% of wild-type CYP2B6.

In the “canine” series (Table 1), only the L199M mutant showed a 2-fold increase in catalytic efficiency to metabolize CPA in 4-OH-CPA. However, the affinity of the six “canine” mutants remained comparable or lower than that of the wild-type CYP2B6.

From the results obtained with the different mutants, the construction of a new mutant, comprising the two mutations that proved to be most effective for metabolism of CPA (I114V and V477W), was undertaken. As shown in the last line of Table 1, this double mutant I114V/V477W showed a 4-fold increase in CPA-4OH catalytic efficiency, mainly as a result of an increase in enzyme affinity.

**Comparison of the Active Site Between wt CYP2B6 and CYP2B6 V477W/I114V.** Structural models were built to provide an interpretation of the variations observed in biological activities. One can notice that the replacement of residue Val477, which is positioned on the top edge of the active site in wild-type CYP2B6 with a tryptophanyl residue

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**Fig. 5.** CYP2B6 topology with the identified channels. The channels have been identified by CAVER based on various structures produced during the 2-ns MD simulations. The channels are shown using the CAVER-generated van der Waals representation within the PYMOL graphical environment. Three major pathways to the buried active site were identified: i, a class of channels (light orange, orange and red) close to the B-C loop on each side of helix B; ii, a channel among the E, F, and I helices (blue); and iii, a channel along the I helix (purple). The protein is shown in a shaded gray cartoon representation. α-Helices are annotated with their corresponding letters: A, top-view looking down on the heme cofactor from the distal side; B, side view looking down the I helix.
in the double mutant (Fig. 7) yielded a local lateral contraction of the cavity. Indeed, as a result of increased side-chain volume at position 477, the mutation led to a steric hindrance that provoked displacement of the ligand in the active pocket by stacking it against helix I. In addition, mutation of Ile114 to a valine, located on the lateral side near the bottom of the active site (BC loop), caused a widening near the heme. This double mutation forced a tilt of the CPA from its initial position. This observed tilt of the CPA provided a better positioning of the CPA over the three above-mentioned pockets. The volume of the mutated active site, as calculated by VOIDOO, increased to 636 Å³, a value that also reflects an extension toward the channels at the top. The same steric guidance effect was observed for both enantiomers.

**Dynamic Behavior of the CPA in the Active Site of wt CYP2B6 versus V477W/I114V CYP2B6 (Fig. 8).** The dynamic behavior of the substrate within the active site can be followed by analysis of the time evolution of all RMSD atoms during the MD simulation. Figure 8 shows RMSD fluctuations of CPA (S-enantiomer) during 2 ns of MD, as computed by GROMACS. At the plateau (reached at 100 ps for the mutant and 600 ps for the wild type), RMSD amplitudes reflect the deviation from the average position of the substrate. The R-enantiomer showed the same overall differential tendency (data not shown), although it was found to fluctuate more in the active site than the S-enantiomer, both in the wild type and in the double-mutant simulations. Figure 8 shows that the RMSD fluctuations of CPA (S-enantiomer) are significantly restricted in the mutant model compared with the wild type. Therefore, CPA showed restrained mobility in the mutant. It has to be mentioned that the levels of RMSD were dependent upon the initial positioning and therefore are not relevant as absolute values.

**In Vitro Cytotoxicity Assays.** Preliminary experiments showed that A-253 cells, originating in the head and neck epidermoid carcinoma, remained insensitive to CPA treatment up to 3 mM (data not shown). CYP2B6 was not expressed in this cell line and RED activity remained relatively weak (12.1 ± 1.5 nmol/min/mg). Results of the in vitro cytotoxicity assays are summarized in Fig. 9. No cytotoxicity was observed after treatment of Ad-LacZ infected cells with up to 3 mM CPA. Infection of cells with Ad-RED alone or with Ad-2B6 alone resulted in no cytotoxic effects in the A-253 cell line at any CPA concentration used. At 3 mM CPA, infection with the adenovirus expressing the double mutant V477W/I114V alone or in association with Ad-RED led to a cytotoxic effect of CPA. This cytotoxic effect increased with the CPA concentration, reaching 70% at 3 mM.

**Discussion**

Local metabolism of a prodrug within tumor cells provides a potential mechanism for delivering high local concentrations of the active metabolites while minimizing systemic drug concentrations and thus toxicity. The concept of over-expressing individual forms of prodrug-metabolizing P450 enzymes in tumor cells is now becoming well recognized (McFadyen et al., 2004), in particular the CYP2B6/CPA strategy (Waxman et al., 1999; Jounaidi, 2002; Tychopoulos et al., 2005). However, one of the major drawbacks of this approach has been its inherent toxicity, which is likely due to the over-expressing of P450 enzymes with a high turnover number, leading to increased production of reactive metabolites.

**Table 1**

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<th>mM</th>
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N.Q., not quantifiable.
strategy is the relatively low affinity of CYP2B6 for CPA. The present work was then undertaken to improve the catalytic activity of CYP2B6 for activation of CPA by site-directed mutagenesis using a rebuilt structural model of CYP2B6.

MD simulations demonstrated that the stability of CYP2B4 and the constructed model of CYP2B6 were comparable, as evidenced by time evolution of RMSD (Fig. 2), which strengthens the choice of CYP2B4 as a unique template for homology modeling. CYP2B4 was cocrystallized with CPI (Scott et al., 2004), which can be considered structurally close to CPA (Fig. 3). In all MD simulations that were carried out, the final position of CPA in the active site was always the same and was not dependent upon its initial manually created position. Although CPA displays a chiral center, no data were available in the literature about the respective biological activities and metabolism of R- and S-enantiomers thus preventing comparison with results obtained in silico. Nevertheless all simulations were duplicated for (R)- and (S)-CPA and showed no marked difference between the two enantiomers in the average position in the active site and in the potential metabolism resulting from the position metabolism. Given that a racemic mixture is always used in clinical therapy, we chose not to emphasize this point of discussion in this work.

Some CYP2B6 active site residues have already been subjected to site-directed mutagenesis (Domanski et al., 1999; Spatzenegger et al., 2003). For example, the V477F CYP2B6 mutant has been reported to have increased catalytic activity for 7-ethoxycoumarin (2.1-fold increase) and 7-ethoxy-4-trifluoromethylcoumarin-O-deethylase (1.8-fold increase). From our CYP2B6 model, we selected amino acids close to the substrate (Ile114, Val367, Val477, and Gly478) to build a range of mutants. Our initial goal was to stabilize the substrate in the active site pocket by hydrogen bonding and thus

![Fig. 6. CPA hydroxylase activity of double mutant V477W/I114V CYP2B6. Enzymatic kinetic analysis of cyclophosphamide 4-hydroxylation was performed using microsomes from yeasts expressing the double mutant V477W/I114V CYP2B6 according to Materials and Methods. Kinetic constants of CPA hydroxylase were determined by a nonlinear regression with 15 substrate concentrations (0.05 to 25 mM) $K_m$ and $V_{max}$ were calculated using GraphPad Prism. Data shown were based on duplicate determinations for each data point.](image)

![Fig. 7. Geometry of the active site of wt CYP2B6 (A) and CYP2B6 V477W/I114V (B) (side and top views). A, some residues shown to be important for substrate metabolism and selected for mutagenesis studies (114, 367, 477, 478) are labeled and displayed as purple sticks. Heme is shown as orange sticks and CPA (S-enantiomer) as blue sticks. The contour of the active site computed by Voidoo is shown in grey mesh. B, changes in the orientation of the active site are shown in the mutant model. Mutated residues 114 and 477 are labeled and displayed as magenta sticks. Heme is shown as orange sticks and CPA as blue sticks. The contour of the active site computed by Voidoo is shown in grey mesh. After double mutation, the active site is flattened by the presence of a tryptophan side chain, which causes steric displacement at the top, and widened at the basis of the site in the vicinity of the heme.](image)
hydrophobic amino acids Val367, Val477, and Gly478 were mutated into polar amino acids that could establish hydrogen bonds with the substrate (V367S, V367T, V367H, V477S, V477T, V477Y, V477N, V477D, V477E, G478S, G478E). The results showed that the introduction of polar amino acids into the active site was often detrimental for catalytic activity; this result highlighted that it was necessary to preserve the global hydrophobic nature of the active site.

In addition, replacement of these residues with hydrophobic amino acids gave contrasting results for CPA-4' hydroxylase activity depending on the amino acid considered. Substitution of Val367 with Phe or Gly478 with Ala decreased this activity, whereas substitution of Val367 with Leu or Gly478 with Val showed no significant effect on this activity. In contrast, mutants more efficient than wt CYP2B6 were obtained by replacement of Val477 with Ile, Phe, and Trp, leading to a decrease in the $K_m$ and an increase in the $V_{max}$ of CPA-4' hydroxylase activity (Table 1). The CYP2B6 active site was found to be relatively large (compared with CYP2B4), allowing for amplified CPA motions inside. For instance, the two aliphatic chains (CH$_2$CH$_2$Cl) can rotate around a virtual axis defined by the CPA acyclic P-N bond and can also freely tilt up and down. Replacement of Val477 with bulky residues restricted these motions, and this restraint was correlated with the size of the residue (V477I, V477F, V477W, Table 1), the most efficient being the V477W mutant. In this latter mutant, the steric control exerted by the mutated side chain could allow for more effective presentation of the hydroxylation site to the heme iron by reducing the number of statistically nonproductive orientations. This is supported by the observed increase in affinity, and higher metabolism efficiency.

Moreover, when comparing CPA hydroxylase activities between V477Y and V477F, our results confirmed that in addition to the size of the residue, which is comparable between tyrosine and phenylalanine, its hydrophobicity was essential to maintain enzymatic activity. Indeed, in the V477Y mutant, it was observed by MD simulations, both with and without CPA, that the hydroxyl group of the tyrosine can occasionally establish a stable H-bond with the glutamic acid (Glu301) (Fig. 10). In the V477Y mutant, the Glu301 side chain was reoriented toward Tyr477 on the top of the active site, and this locking mechanism could hinder the entrance/egress of the CPA. According to CAVER analysis, the two channels located next to the Phe helix and along the Ile helix (blue and purple, Fig. 5) can be closed by this 477/301 H-bond in the mutant.

The last “active site” mutation investigated (Ile114) also involved a possible gating of the other group of access channels (red/orange in Fig. 5). Replacement of Ile114 with a less bulky hydrophobic residue (valine) yielded enhanced catalytic activity (Table 1). In fact, this effect can be related to the key position of this residue on the B'-C loop by promoting CPA accessibility to the active site through the major pathway (channel 2 class in R. Wade nomenclature [Cojocaru et al., 2007]).

**Fig. 8.** Dynamic behavior of CPA in the active site of wt CYP2B6 vs CYP2B6 V477W/I114V: RMSD fluctuations of CPA (S-enantiomer) during 2 ns of MD simulation calculated by the g_rms function of GROMACS and, showing the deviation from the average position of the substrate as a function of time. Amplitudes of fluctuations are attenuated in the mutant model (maximum amplitude observed is represented in the margin by red and black arrows for the wt and double mutant, respectively). All structures are fitted pairwise by g_rms with starting structure taken as reference. Note that the RMSD levels reached at equilibrium (1 and 1.2 Å for wt and double mutant, respectively) are different and cannot be compared because they are dependent upon the initial positioning.

**Fig. 9.** MTS cytotoxicity assays for A-253 cells. CPA-induced cytotoxicity assays were performed in cells infected with different adenoviral vectors expressing LacZ, CYP2B6 (2B6), double mutant V477W/I114V CYP2B6 (2B6 double mutant) or the human reductase (Red). Cytotoxicity was assayed using a MTS colorimetric test, which measures the dehydrogenase activity in the metabolically active mitochondria of viable cells as described under Materials and Methods. Cell viability is expressed as the percentage of viable cells compared with those infected with control vector (Ad-LacZ) treated at identical CPA concentrations and compared with untreated cells for each infection. The assays were repeated four times in triplicate and the bars represent standard deviation.

**Fig. 10.** Active site in the V477Y mutant, side and top views. The black arrow indicates potential hydrogen bonding between the OH group of the Tyr477 and the carboxylate group of Glu 301. Side chains and heme are displayed as green sticks.
Given that canine CYP2B11 presented a higher affinity for CPA (Chen et al., 2004), a set of six mutants were built up by introduction in CYP2B6 SRS sequences of residues exclusi-
vously present in CYP2B11. Unfortunately, none of them exhibited improved affinity over CPA, and the $K_m$ of K236N was even 10-fold higher than that of wt CYP2B6 (Table 1). This latter effect could be related to the position of the Lys236 side chain in the structure of CYP2B6. Indeed, this residue was found located in the G helix, outside of the active site. This structural region (F-G loop and F and G helices) is, according to hydrophobicity surface potential (data not shown) and other literature arguments, thought to be located at the membrane interface. Because the lysyl side chain was found oriented toward the outside (i.e., potentially toward the membrane), the substitution with asparagine might perturb the interaction with lipids. Because this interaction is likely to influence movements of the F-G crown and access to substrates from the lipid phase, such a mutation can have consequences in P450 activity modulation by membrane, and affect substrate metabolism.

Nevertheless, among the six “canine” mutants, one mu-
tant, L199M, proved to be more efficient than wt CYP2B6 in CPA metabolism. In this case, the mutation is located in a region of the F helix involved in helix-helix contacts with the E helix. Leucine is replaced with a polar residue that can establish new interactions with vicinal side chains on the E helix, either Gln172 or Ser173, which are close enough to establish H-bonds with 199M, thus leading to rigidifying of the E-F scaffold. Such a motional restriction can once again generate improved channeling for the substrate and thus improved catalytic efficiency.

The double mutant, which contains two of the most efficient mutations, I114V and V477W, showed an additive 4-fold increase in 4′-hydroxylation of CPA, mainly because of an increase in enzyme affinity. In Fig. 7, the active site in the double mutant compared with wt CYP2B6 is deviated and narrowed at the top, leading to stacking of CPA against the I helix, and becomes wider at the base of the active site in the vicinity of the heme moiety; as a result, the double mutation forced a tilt of the CPA from its initial position. This consequence of this steric displacement is a restricted motion of the substrate in the active site pocket, thus allowing for longer presentation of its metabolic site to the heme iron. Moreover, as indicated by RMSD amplitudes (Fig. 8), CPA seemed to be less mobile in the mutant structural model. This restrained motion could favor higher metabolism efficiency (see Supplemental Data).

To complete our work, ex vivo cytotoxicity assays were performed on the A-253 cell line currently used as a proto-
type of squamous cells from human head and neck cancer (Kawakami et al., 2001). WT CYP2B6 or the double mutant CYP2B6 Val114IleTrp477 alone or in combination with RED were coexpressed into A-253 cells using adenoviral vectors. Coexpression of CYP2B6 and RED led to a maximum cytotoxicity that could be observed at lower CPA concentrations in these cells compared with cells trans-

tected with wt CYP2B6. This cytotoxicity was dose-depend-
ent and reached 75% at 3 mM CPA. Coexpression of RED with the CYP2B6 mutant did not increase CPA cytotoxicity compared with cells infected with the CYP2B6 mutant alone. This can be explained by a higher efficiency of electron transfer from the weakly expressed endogenous RED to the CYP2B6 mutant than to wt CYP2B6. This hypothesis, how-
ever, remains to be demonstrated.

In conclusion, this work demonstrated that it was possible to improve the activity of CYP2B6 toward CPA by site-di-
rected mutagenesis. Moreover, from our constructed model of CYP2B6, MD simulations were found to be consistent with the observed modifications in catalytic efficiency of different mutants and showed their ability to guide the design of new mutants. Given the encouraging results obtained, this study will be pursued by evaluating our strategy in vivo against subcutaneous A-253 tumor xenografts in athymic mice of advanced tumor stage. According to the results obtained in this in vivo model, clinical protocols could be undertaken.

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