Chapter 5

Activation of the anticancer drugs cyclophosphamide and ifosfamide by cytochrome P450 BM3 mutants

doi:10.1016/j.toxlet.2014.11.005
ABSTRACT

Cyclophosphamide (CPA) and ifosfamide (IFA) are widely used anticancer agents that require metabolic activation by cytochrome P450 (CYP) enzymes. While 4-hydroxylation yields DNA-alkylating and cytotoxic metabolites, N-dechloroethylation results in the generation of neuro- and nephrotoxic byproducts. Gene-directed enzyme prodrug therapies (GDEPT) have been suggested to facilitate local CPA and IFA bioactivation by expressing CYP enzymes within the tumor cells, thereby increasing efficacy.

We screened bacterial CYP BM3 mutants, previously engineered to metabolize drug-like compounds, for their ability to catalyze 4-hydroxylation of CPA and IFA. Two CYP BM3 mutants showed very rapid initial bioactivation of CPA and IFA, followed by a slower phase of product formation. N-dechloroethylation by these mutants was very low (IFA) to undetectable (CPA). Using purified CYP BM3 as an extracellular bioactivation tool, cytotoxicity of CPA and IFA metabolism was confirmed in U2OS cells. This novel application of CYP BM3 possibly provides a clean and catalytically efficient alternative to liver microsomes or S9 for the study of CYP-mediated drug toxicity. To our knowledge, the observed rate of CPA and IFA 4-hydroxylation by these CYP BM3 mutants is the fastest reported to date, and might be of potential interest for CPA and IFA GDEPT.

INTRODUCTION

Cyclophosphamide (CPA) and its structural isomer ifosfamide (IFA) are widely used anticancer agents of the oxazaphosphorine class. CPA and IFA are both administered as inactive prodrugs and require metabolic activation by cytochrome P450 (CYP) enzymes in order to exert their chemotherapeutic effect. As both drugs have been available for over 50 years, their metabolism has been studied in detail and reviewed extensively (1–3). Initial CYP-mediated activation results in the hydroxylation of CPA and IFA at the C-4 position of the oxazaphosphorine ring (Fig. 1). Several human CYP isoforms have been shown to catalyze the formation of 4-hydroxy-CPA (4-OH-CPA), CYP2B6 being the main contributor responsible for approximately 45% of the total CPA bioactivation observed, followed by CYP3A4 (25%) and CYP2C9 (12%) (4). In case of IFA, CYP3A4 plays the major role (65%) in bioactivation toward 4-hydroxy-IFA (4-OH-IFA) followed by CYP2A6 (13%), while CYP2B6 only contributes for about 7% of the total IFA 4-hydroxylation. The 4-hydroxylated metabolites of CPA and IFA equilibrate with their ring-opened tautomers aldophosphamide and aldoifosfamide, which can decompose through spontaneous beta-elimination yielding the therapeutically active phosphoramid mustard (PM) and ifosforamide mustard (IM) respectively, and the byproduct acrolein. Both nitrogen mustards are bifunctional alkylating agents that exert their cytotoxic effect by cross-linking DNA.

As CYP-mediated activation of CPA and IFA takes primary place in the liver, active metabolites can enter the blood circulation and are able to reach tumor- and healthy tissue alike, thereby increasing the risk for adverse drug reactions such as cardiotoxicity and bone marrow suppression (1). Moreover, while CPA and IFA and their ultimate cytotoxic metabolites PM and IM do not
readily diffuse across cell membranes, 4-OH-CPA and 4-OH-IFA do. However, half-life times of the two hydroxylated metabolites in plasma are short (5), leading to rapid decomposition to PM and IM that are subsequently unable to efficiently enter tumor cells. Alternative to 4-hydroxylation, CPA and IFA are also subject to CYP catalyzed N-dechloroethylation (Fig. 1). This side chain oxidation of CPA and IFA produces N-dechloroethylated CPA- and IFA-derivatives and chloroacetyladdehyde (CAA), a neuro- and nephrotoxic byproduct. For IFA, N-dechloroethylation by mainly CYP3A4 (71%) and to lesser extent by CYP2B6 (27%) may consume up to 60% of the therapeutic dose, while for CPA this secondary pathway is only significantly catalyzed by CYP3A4 (96%) and limited to approximately 10% of the dose (3, 4).

To overcome dose-limiting adverse drug reactions and to increase efficacy of CPA and IFA, CYP-based gene-directed enzyme prodrug therapy (GDEPT) has been proposed (6). The GDEPT strategy facilitates local intracellular bioactivation by introducing a vector that allows expression of a chosen CYP within the tumor cells. Besides increased levels of active metabolite in the CYP-expressing cells themselves, 4-OH-metabolites and their tautomers may readily cross cell membranes and elicit cytotoxicity in neighboring untransfected cells. This phenomenon, known as the bystander effect, is crucial for complete tumor eradication since transduction levels higher than 10% of tumor cells are difficult to establish (7, 8). A number of phase I clinical trials have been conducted utilizing the GDEPT approach combining CPA with human CYP2B6 and IFA with rat CYP2B1, proving these strategies to be safe and well tolerated (9, 10). However, affinities of human CYP2B6 and CYP2B1 for CPA and IFA are low with reported $K_M$ values between 1 and 2 mM, which is presumably related to the moderate results observed in the clinical trials so far (4, 11).

In the last decade, this GDEPT approach has been further explored by engineering CYP enzymes with improved kinetics for CPA and IFA 4-hydroxylation. A number of mutants with improved affinity were created by site-directed mutagenesis of human CYP2B6 and CYP2B isoforms of rat, rabbit, and dog (11, 12). To date, CYP2B11 from dog is the most efficient CYP known to catalyze the 4-hydroxylation of CPA and IFA, with $K_M$ values of respectively 0.160 and 0.100 mM (13). Adenoviral expression of CYP2B11 in several human cancer cell lines confirmed that CPA-induced cytotoxicity was greatly increased compared to human CYP2B6 expressing cells (14). More recently Touati et al. engineered a CYP2B6 triple mutant (CYP2B6TM) with improved enzyme kinetics compared to the wildtype CYP2B6 (15). Moreover, they tackled the problem of low to absent expression of NADPH cytochrome P450 reductase (RED) in tumors by developing a fusion protein. The resulting CYP2B6-RED mutants showed a 8- to 10-fold higher efficiency ($V_{max}/K_M$) toward CPA 4-hydroxylation. In vivo models showed complete eradication of tumors containing 25% of CYP2B6TM-RED expressing cells following treatment.

In this study, we set out to screen a panel of CYP BM3 mutants for their potential future application within a GDEPT strategy, or more specifically their ability to efficiently catalyze the 4-hydroxylation of CPA and IFA while showing minimal N-dechloroethylation. The bacterial CYP BM3 (or CYP102A1) from Bacillus megaterium is a soluble single polypeptide in which the catalytic heme-domain is fused to a mammalian-like diflavin NADPH-CYP reductase (16). With the highest
Fig. 1. Metabolic scheme of CPA and IFA showing the cytochrome P450 mediated reactions.
activity ever reported for a cytochrome P450 monooxygenase, it has long been studied as a possible biocatalyst for many non-natural substrates (17). Our lab previously engineered several CYP BM3 mutants showing conversion of over 40 drugs-like compounds, some at very high catalytic rate and producing human-relevant metabolites (18–20).

In the present study, two highly active CYP BM3 mutants were identified, each showing a very rapid initial bioactivation of CPA and IFA, followed by a slower secondary phase of product formation. Determination of enzyme kinetics during this initial, dominant phase revealed a high affinity and catalytic rate for CPA and IFA 4-hydroxylation with minimal N-dechloroethylation. Cytotoxicity assays using extracellular bioactivation of CPA and IFA with these selected mutants showed an increased cytotoxicity in human osteoblastic osteosarcoma U2OS cells.

MATERIALS AND METHODS

Ifosfamide was purchased from Sigma (St. Louis, MO), cyclophosphamide was obtained from Alfa Aesar (Ward Hill, MA). All other chemicals were of analytical grade and obtained from standard commercial suppliers. Recombinant human CYP2B6 microsomes were purchased from Gentest (Woburn, MA). His-tagged CYP BM3 mutants were expressed and purified as described previously (18, 20, 21). Moreover, cell-free extracts of E. coli cultures expressing the different CYP BM3 mutants were prepared without subsequent purification; these lysates were used to screen the CYP BM3 mutants for CPA and IFA activation. CYP concentrations were determined according to the method of Omura and Sato (22).

Incubation of cyclophosphamide and ifosfamide by CYP BM3 mutants

Initial 4-hydroxylation of CPA and IFA was determined by analysis of the concentration acrolein formed after an incubation of 10 min at 37 °C, similar to previously described methods (11). Reactions contained 0.5 mM substrate and 1 mM NADPH in a 100 mM potassium phosphate buffer (KPi, pH 7.4), with a final volume of 100 μl. After 4 min preincubation, reactions were initiated by the addition of lysates of containing 100 nM of the different CYP BM3 mutants. After 10 min, the reactions were terminated by the addition of 40 μl ice-cold 5.5% ZnSO₄, 40 μl ice-cold saturated Ba(OH)₂, and 20 μl ice-cold 10 mM HCl, cooled on ice for 10 min and centrifuged at 14,000 rpm for 15 min at 4 °C. Acrolein formation was analyzed after derivatization with m-aminophenol as described below. For further characterization of selected CYP BM3 mutants, His-tag purified mutants were used. In addition to 4-hydroxylation of CPA and IFA (1 mM each), N-dechloroethylation was assessed by measuring formation of chloroacetylatedaldehyde (CAA). For determination of Michaelis–Menten parameters, incubations were prepared containing 250 nM CYP BM3 and twenty-three CPA or IFA concentrations, ranging from 4 to 1000 μM, and initiated by adding NADPH to a final concentration of 1 mM. After 10 s, reactions were stopped as described earlier in this section, samples were derivatized and analyzed as described below. Data analysis was performed using Graphpad Prism 4.0.
### Detection of acrolein and chloroacetylaldehyde formation

Acrolein formation was determined using a derivatization method previously described (23). Briefly, incubation mixtures were centrifuged for 10 min at 14,000 rpm. The supernatant was mixed with 0.5 volume of a reagent mix, freshly prepared by dissolving hydroxylamine (6 mg/ml) and 3-aminophenol (6 mg/ml) in 0.5 M HCl. Samples were heated to 90 °C for 30 min in the dark to form the fluorescent 7-hydroxyquinoline and subsequently cooled for at least 10 min followed by 5 min centrifugation at 14,000 rpm. The supernatant was either directly analyzed by HPLC or stored in the dark at −20 °C for analysis, the next day. The formed 7-hydroxyquinoline was separated on a Phenomenex Luna C18 column (5 μm, 150 mm × 4.6 mm) and eluted at 3.3 min using an isocratic mobile phase (18% methanol, 0.33% phosphoric acid, 1 ml/min) as detected by fluorescence (λ<sub>ex</sub> 350 nm, λ<sub>em</sub> 515 nm). Identity was confirmed by a reference standard of 7-hydroxyquinoline.

CAA formation was measured by derivatization with adenosine to yield the fluorescent 1-N<sup>6</sup>-ethenoadenosine (24). Incubations were mixed with 0.25 volume 100 mM adenosine and 0.25 volume 2 M sodium acetate (pH 4.5). Samples were heated at 80 °C for 2 h in the dark, cooled on ice for at least 10 min and centrifuged for 5 min at 14,000 rpm. The supernatant was stored in the dark at −20 °C and measured by HPLC. 1-N<sup>6</sup>-ethenoadenosine was separated on the Phenomenex Luna column described above and eluted at approximately 14 min using an isocratic mobile phase (5% acetonitril, 50 mM ammonium acetate, adjusted to pH 5.1 with HCl, at a flow rate of 1 ml/min) as detected by fluorescence (λ<sub>ex</sub> 275 nm, λ<sub>em</sub> 410 nm). Acrolein and CAA in the incubations were quantified by preparing derivatized acrolein and CAA standards in parallel.

### Identification of CPA and IFA metabolites using LC/MS

CPA, IFA and their metabolites were analyzed using LC/MS. Separation was performed using an Agilent 1200 Series Rapid resolution LC system with a Phenomenex Luna 5 μm C18(2) column (150 mm × 4.6 mm), protected by a Phenomenex security guard (5 μm) C18 guard column (4.0 mm x 3.0 mm). A binary gradient was used mixing solvent A (1% acetonitrile, 99% water, and 0.2% formic acid) and solvent B (99% acetonitrile, 1% water, and 0.2% formic acid), at a flow rate of 0.4 ml/min. The following gradient was programed: first 5 min isocratic 5% B; 5–25 min a linear increase to 95% B, followed by an immediate decrease to 5% B; 25–30 min maintained at 5% B for re-equilibration. MS analysis was performed using a time-of-flight (TOF) Agilent 6230 mass spectrometer, equipped with an electrospray ionization (ESI) source operating in positive mode (2 GHz, extended dynamic range). The MS ion source parameters were set with a capillary voltage at 3500 V; nitrogen was used as the desolvation and nebulizing gas at a constant gas temperature of 350 °C; drying gas, 10 l/min; and nebulizer, 50 psig. Data analysis was performed using the Agilent MassHunter Qualitative Analysis software.

### BROD activity of CYP BM3

CYP BM3 activity after preincubation with CPA and NADPH was assessed using the highly sensitive 7-benzoxylxyresorufin O-dealkylation (BROD) assay previously described (25). CYP BM3 M11 (50 nM) was preincubated with CPA (1 mM) and NADPH (0.6 mM) for 2 min, past the burst
phase of product formation. Subsequently, an aliquot of the preincubation mixture was taken and
diluted 5 times for measuring BROD activity. Incubations contained a final concentration of 10 μM
BROD, 0.25 mM NADPH and 10 nM BM3. Resorufin formation was monitored by fluorescence
\( \lambda_{\text{ex}} 530 \text{ nm}, \lambda_{\text{em}} 586 \text{ nm} \) at room temperature.

**CPA- and IFA-induced cytotoxicity in U2OS cells after extracellular bioactivation by CYP
BM3 mutants**

To assess CPA- and IFA- induced cytotoxicity in U2OS cells, two different cytotoxicity parameters
were used. To this end, an U2OS cell line constitutively expressing luciferase (Cytotox CALUX,
described in (26)) was used. U2OS cells were chosen because these cells have little or no endogenous
expression of metabolic enzymes, so the observed toxicity of CPA and IFA is known to originate
from BM3-generated metabolites rather than U2OS-generated metabolites. Cytotoxicity was
assayed on this cell line using either a standard MTT test, or by monitoring decreasing luciferase
levels associated with decreasing numbers of viable cells. U2OS cells were cultured essentially as
described before (27). Cells were routinely subcultured every 3–4 days in growth medium consisting
of DMEM (Gibco) supplemented with 7.5% fetal calf serum, 1× non-essential amino acids (Gibco)
and 10 U/ml penicillin and 10 μg/ml streptomycin. Cells were maintained at 37 °C and 5% CO₂ at
time.

Assays were performed in assay medium, consisting of DMEM without phenol red indicator (Gibco)
supplemented with 5% DCC-stripped fetal calf serum, 1× non-essential amino acids (Gibco) and
10 U/ml penicillin and 10 μg/ml streptomycin. Cells were seeded in 96-wells plates at 10,000 cells/
well in assay medium. 24 h after seeding, the cells were exposed to different concentrations of CPA
or IFA. The final solvent (DMSO) concentration was kept constant at 1%. Subsequently, various
concentrations of His-purified CYP BM3 mutants M11 and M11 L437S and an NADPH-regenerating
system (final in well: 200 μM NADPH, 3 mM glucose-6-phosphate, 0.3 U/ml G6P-dehydrogenase,
and 5 mM MgCl₂) were added to each well. After 24 h incubation, cell viability was assessed either
by performing an MTT assay or by detecting the total amount of luciferase expressed (Cytotox
CALUX). The MTT assay was performed as follows: exposure medium was aspirated and replaced
by 30 μl/well assay medium containing 1 mg/ml MTT. After 4 h, the formation of formazan crystals
was confirmed microscopically. The formazan crystals were dissolved by adding 100 μl DMSO to
each well. Subsequently, cell viability was quantified by measuring the OD₅₉₅ in a microplate reader. The
Cytotox CALUX read-out was performed as described earlier (27); basically, the exposure
medium was removed from the cells and replaced by 30 μl/well lysis buffer. Subsequently, the
luciferase signal was measured in a luminometer.
RESULTS

Screening of the CYP BM3 mutant library for CPA and IFA 4-hydroxylation

The 39 tested lysates containing 100 nM of CYP BM3 mutants showed a large variability in 4-hydroxylation activity toward CPA and IFA, as was determined by measuring acrolein formation (Fig. 2). Two mutants were identified to be very active for 4-hydroxylation of both CPA and IFA: M11 and M11 L437S (18, 20).

The 4-hydroxylation activity (expressed as % of the most active mutant) of most mutants for CPA seemed to correlate well with the activity toward IFA, with clear exception of mutants M11 V87I, M11 V87A, and M11 V87G. Apparently substitution of residue 87 within the active site of CYP BM3 changes selectivity of 4-hydroxylation of either CPA or IFA. M11 is the third most active mutant for IFA 4-hydroxylation, but substitution of valine 87 to the bulkier isoleucine (M11 V87I) abolishes the activity toward this substrate while maintaining good activity toward CPA (approximately 70% of M11 activity). In contrast, substitution of valine 87 to a smaller alanine residue (M11 V87A) results
in a big loss in activity toward CPA (<25% of M11 activity) but retains decent IFA hydroxylation activity (about 60% of M11 L437S activity). Mutation of the 87 residue to the smallest possible amino acid glycine (M11 V87G) shows a similar preference for IFA conversion, although with much lower activity in general. These changes in activity might be due the interaction of the 87 residues with the 2-chloroethyl group that is either linked to the endocyclic nitrogen in IFA (close to the 4-position of the ring) or to the extracyclic nitrogen in case of CPA. Replacing the valine residue at position 87 with residues with a larger hydrophobic side chain or residues with different physicochemical properties (e.g., with a charged or a polar side chain) all resulted in a clear loss of activity. A crucial role of this 87 residue has been shown before with testosterone, where the same amino acid changes shifted stereoselectivity from a mixture of both 15- and 16-beta oxidation (M11) to primarily 15-beta oxidation (M11 V87A) or mainly 16-beta oxidation (M11 V87I) (21).

Table 1. Overview of screened CYP BM3 mutants in this study.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>CPA 4-hydroxylation*</th>
<th>IFA 4-hydroxylation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>R47L, F87V, L188Q (&quot;Triple&quot;)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>R47L, F87V, L188Q, E267V, G415S (&quot;M01&quot;)</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>R47L, L86I, F87V, L188Q, N319T, A964V (&quot;M02&quot;)</td>
<td>19</td>
<td>30</td>
</tr>
<tr>
<td>R47L, F87I, L188Q, E267V, G415S, G1049E (&quot;M05&quot;)</td>
<td>52</td>
<td>30</td>
</tr>
<tr>
<td>Triple L75W</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>M01 K440S</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>M01 L437E</td>
<td>16</td>
<td>24</td>
</tr>
<tr>
<td>M11 L437E</td>
<td>28</td>
<td>22</td>
</tr>
<tr>
<td>M11 L437N</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>M11 A74E</td>
<td>45</td>
<td>37</td>
</tr>
<tr>
<td>M11 L437S</td>
<td>86</td>
<td>100</td>
</tr>
<tr>
<td>M11 L437T</td>
<td>45</td>
<td>31</td>
</tr>
<tr>
<td>M11 A74D</td>
<td>38</td>
<td>28</td>
</tr>
<tr>
<td>M11 S72D</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>M01 A74D</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>M01 S72D</td>
<td>43</td>
<td>55</td>
</tr>
<tr>
<td>M01 S72E</td>
<td>23</td>
<td>34</td>
</tr>
<tr>
<td>L188Q</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>F87V, E267V, F87I</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>Triple L437E</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>M11 V87A</td>
<td>23</td>
<td>59</td>
</tr>
<tr>
<td>M11 V87I</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>M11 V87N</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>M11 V87C</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>M11 V87Q</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>M11 V87E</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>M11 V87G</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>M11 V87H</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>M11 V87L</td>
<td>72</td>
<td>10</td>
</tr>
<tr>
<td>M11 V87L</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>M11 V87K</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>M11 V87M</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>M11 V87F</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>M11 V87W</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>M11 V87Y</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>M11 A82W</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>M11 A82C</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>M01 A82W</td>
<td>16</td>
<td>9</td>
</tr>
</tbody>
</table>

*Activity expressed as % of most active mutant (M11 for CPA, M11 L437S for IFA); activity determined using cell-free extracts as described in materials and methods.
LC–MS identification of human relevant metabolites of CPA and IFA

The two most active mutants for both CPA and IFA 4-hydroxylation, M11 and M11 L437S, were selected for further characterization using the purified proteins instead of the crude cell-free extract. The metabolites formed during incubation of CPA and IFA with the two selected CYP BM3 mutants (without derivatization of acrolein or CAA) were identified using LC–MS. For each compound, incubation with either M11 or M11 L437S yielded the same human-relevant metabolites. Incubation of CPA (Fig. 3A) mainly resulted in the formation of 4-OH-CPA with a protonated molecular ion [M + H]+ of m/z 277.03, which readily loses water to form [M + H–H2O]+ with m/z 259.02. Both the parent drug (CPA, [M + H]+ of m/z 261.03) and 4-OH-CPA were also clearly abundant as their sodium adducts [M + Na]+ with an m/z of respectively 283.01 and 299.01. The extracted ion chromatogram (EIC) of m/z 221.00, corresponding with the [M + H]+ of the phosphoramide mustard metabolite, yielded two clear peaks in the chromatogram. Both these peaks matched the expected chlorine isotope pattern. However the second peak (annotated with an asterisk in Fig. 3B) clearly co-eluted with 4-OH-CPA, possibly the result of fragmentation of its tautomeric aldophosphamide. Addition of GSH in the incubation resulted in the GSH-conjugate of acrolein with an [M + H]+ of m/z 364.12. Moreover, spontaneous conjugation of GSH to CPA (CPA-SG, m/z 566.10) was observed. N-dechloroethyl-CPA with an expected [M + H]+ of m/z 199.04 could not be detected.

Similar to the CPA incubations, metabolism of IFA (Fig. 3B) yielded primarily the 4-OH-IFA metabolite detected as [M + H]+ with m/z 277.03, [M + H–H2O]+ with m/z 259.02, and [M + Na]+ with m/z 299.01. When compared to CPA and 4-OH-CPA, IFA and 4-OH-IFA eluted slightly earlier. Also the formed ifosforamide mustard metabolite of IFA ([M + H]+ of m/z 221.00) eluted before phosphoramide mustard. Addition of GSH to the IFA incubations resulted in the formation of the acrolein-SG conjugate (m/z 364.12), but did not lead to spontaneous conjugation of GSH to IFA. IFA incubation did however yield two metabolites with a protonated molecular ion of m/z 199.04, corresponding to the N-dechloroethylated metabolites (2- and 3-dechloroethyl-IFA, Fig. 1B).

Time dependence of enzyme activity

To further characterize the highly active M11 and M11 L437S mutant, 4-hydroxylation and N-dechloroethylation of CPA and IFA was measured in time. Analysis of the time dependence of 4-hydroxylation of CPA (Fig. 4A) and IFA (Fig. 4B) revealed a very high initial activity during approximately the first minute of the reaction, followed by a decrease to a much slower linear rate of product formation for both mutants. Moreover, M11 and M11 L437S catalyzed the N-dechloroethylation of IFA (but not of CPA, in agreement with the LC–MS results) as was detected by the formation of CAA. This N-dechloroethylation was approximately 20-fold lower compared to the 4-hydroxylation reaction. N-dechloroethylation activity also showed clear biphasic behavior (Fig. 4C). The rates of product formation for the initial fast phase, followed by a subsequent exponential decay to a secondary slow phase were estimated by fitting the data in Fig. 4 to an empirical function previously used to describe biphasic behavior of CYP BM3 mutants (18). Results of this nonlinear regression analysis are given in Table 2.
Chapter 5  

Activation of cyclophosphamide and ifosfamide by CYP BM3 mutants

Fig. 3. LC–MS extracted ion chromatograms (EICs) of CPA (A) and IFA (B) incubations with CYP BM3 mutant M11 L437S. CPA or IFA (1 mM) were incubated with 100 nM M11 L437S in presence of NADPH (3 mM). GSH (1 mM) was added to trap possible reactive metabolites as GSH-conjugates. CPA/IFA, m/z 261 + 283 ([M + H]+ + [M + Na]+); 4-OH-CPA/4-OH-IFA, m/z 277 + 258 + 299 ([M + H]+ + [M + H–H2O]+ + [M + Na]+); acrolein-SG, m/z 364 ([M + H]+); phosphoramide mustard/ifosfamide mustard, m/z 221 ([M + H]+); N-dechloroethyl-IFA, m/z 199 ([M + H]+); CPA-SG, m/z 566 ([M + H]+). Peaks in the m/z 221 trace annotated with an asterisk (*) co-eluted with 4-OH-CPA/4-OH-IFA.

Table 2. Initial fast and secondary slow enzyme rates for CPA and IFA metabolism by CYP BM3 mutants. Enzyme rates were obtained by fitting the time dependent product formation curves in Fig. 3 to the equation product = \([P_1(1 - e^{-P_2t})]/P_2 + P_3t\).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Mutant</th>
<th>(P_1^{a})</th>
<th>(P_2^{b})</th>
<th>(P_3^{c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPA 4-hydroxylation</td>
<td>M11</td>
<td>59.1</td>
<td>0.08</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>M11 L437S</td>
<td>11.5</td>
<td>0.02</td>
<td>0.5</td>
</tr>
<tr>
<td>IFA 4-hydroxylation</td>
<td>M11</td>
<td>34.3</td>
<td>0.05</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>M11 L437S</td>
<td>9.6</td>
<td>0.03</td>
<td>0.2</td>
</tr>
<tr>
<td>IFA N-dechloroethylation</td>
<td>M11</td>
<td>2.4</td>
<td>0.07</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>M11 L437S</td>
<td>1.1</td>
<td>0.1</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\(^{a}\) Initial ‘fast’ rate in nmol product/s/nmol enzyme.  
\(^{b}\) Deactivation process in s\(^{-1}\).  
\(^{c}\) Secondary slow rate in nmol product/s/nmol enzyme.
Enzymatic kinetic parameters during initial rapid metabolic phase

Enzymatic kinetic parameters were determined during the initial phase showing high enzyme activity. Short incubation times of 10 s were chosen to ensure accurate measurements of the initial enzyme activity. The activity of the CPA 4-hydroxylation reaction at different substrate concentrations could be fitted to Michaelis–Menten kinetics using nonlinear regression analysis (Supplemental Fig. S1). From these analyses $K_M$ and $V_{max}$ were determined for mutants M11 and M11 L437S (Table 3). Compared to both CYP BM3 mutants the catalytic efficiency ($V_{max}/K_M$) of this reaction by human CYP2B6 was much lower, up to 280-fold in case of M11.

The activities of 4-hydroxylation and $N$-dechloro-ethylation of IFA did not reach saturation over the substrate concentration range studied (up to 1 mM), both with the M11 and M11 L437S mutant (Supplemental Fig. S2). Therefore, only the intrinsic clearances of these enzymatic reactions ($V_{max}/K_M$, Table 3) were determined by calculating the slopes of the regression lines fitted using linear regression.

NADPH consumption and BROD activity

To ensure the biphasic behavior of CPA and IFA metabolism by CYP BM3 mutants was not caused by NADPH depletion, NADPH consumption was monitored by measuring absorbance at 340 nm ($A_{340}$) during CPA incubations (Fig. 5A). When using an NADPH concentration of 1 mM without regeneration system, a fast NADPH consumption during the initial burst of product formation was observed, followed by a much smaller rate of consumption over time during the second, linear phase of metabolism. A similar initial rapid NADPH consumption was observed using an NADPH regenerating system. However, during the slower metabolic phase the $A_{340}$ again reached a steady-state level of initial absorbance. Product formation was very similar in both systems (Fig. 5B). The biphasic time-course was therefore not caused by NADPH depletion.

To investigate whether the observed time-course results from inactivation or inhibition of the CYP BM3 mutants by CPA- or IFA-metabolites, the residual enzyme activity of CYP BM3 mutant M11 was determined by the BROD assay after preincubation with CPA. Preincubation of M11 with CPA for 2 min, well after completion of the initial fast metabolic phase of CPA metabolism by M11, did not influence subsequent BROD activity (Supplemental Fig. S3).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>BM3 M11</th>
<th>BM3 M11 L437S</th>
<th>Human 2B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPA 4-OH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_M$ a</td>
<td>0.087 ± 0.012</td>
<td>0.160 ± 0.019</td>
<td>0.088 ± 0.013</td>
</tr>
<tr>
<td>$V_{max}$ b</td>
<td>896 ± 33</td>
<td>1614 ± 65</td>
<td>757 ± 32</td>
</tr>
<tr>
<td>$V_{max}/K_M$ c</td>
<td>10315 ± 1104</td>
<td>10078 ± 872</td>
<td>8620 ± 1036</td>
</tr>
<tr>
<td>IFA 4-OH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}/K_M$</td>
<td>1306 ± 43</td>
<td>1430 ± 37</td>
<td>577 ± 9</td>
</tr>
<tr>
<td>IFA N-deCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}/K_M$</td>
<td>74.1 ± 5.2</td>
<td>75.0 ± 4.1</td>
<td>27.0 ± 1.0</td>
</tr>
</tbody>
</table>

a In mM. b In mol product/min/mol CYP. c In mol product/min/mol CYP/mM. d Not determined.
Chapter 5 Activation of cyclophosphamide and ifosfamide by CYP BM3 mutants

Fig. 4 (left column). Time dependence of enzyme activity of CPA and IFA bioactivation by CYP BM3 mutants M11 (closed circles) and M11 L437S (open circles). CYP BM3 mutants (250 nM) were incubated with CPA or IFA (1 mM) and NADPH (1 mM). Shown is the biphasic behavior of 4-hydroxylation of CPA (A) 4-hydroxylation of IFA (B) and N-dechloroethylation of IFA (C). N-dechloroethylation was much lower compared to 4-hydroxylation, hence a different scale was used for (C). Data expressed as mean of two independent experiments.

Fig. 5 (right column). NADPH consumption and 4-OH-CPA product formation in incubations of CPA (1 mM) with CYP BM3 mutant M11 (250 nM). (A) NADPH consumption was measured by determining the absorbance at 340 nm during incubations with either NADPH (1 mM; dashed line) or an NADPH regenerating system (0.3 mM NADPH, 2.5 mM glucose-6-phosphate, 0.5 U/ml glucose-6-phosphate dehydrogenase; solid line). (B) 4-OH-CPA product formation was determined by measuring acrolein production with either NADPH (dashed line, black squares) or an NADPH regenerating system (solid line, open squares).

Curves were fitted using non-linear regression to the equation product = \[ P_t \left( \frac{1 - e^{-P_2t}}{P_2 + P_3t} \right) \].
Chapter 5 Activation of cyclophosphamide and ifosfamide by CYP BM3 mutants

CPA- and IFA-induced cytotoxicity upon extracellular bioactivation

Human osteoblastic osteosarcoma U2OS cells were used to study the cytotoxicity of the metabolic products of CPA and IFA formed extracellularly by CYP BM3 mutants M11 and M11 L437S. Cells were exposed to the parent drug without and with the addition of purified M11 or M11 L437S mutant to the culture medium, supplemented with an NADPH regenerating system. CPA-induced cytotoxicity, as determined by using the MTT assay, confirmed successful extracellular bioactivation by both mutants (Fig. 6). In absence of CYP BM3, no significant cytotoxicity was observed up to 1 mM CPA. Notably, the CYP BM3 bioactivation system itself does not induce loss of cell viability. Addition of CYP BM3 M11 and M11 L437S to the medium resulted in a clear CPA dose-dependent loss of cell viability, which was also dependent on the amount of CYP enzyme used. Mutant M11 was more potent in mediating this CPA-induced cytotoxicity compared to mutant M11 L437S, which is in agreement with the enzyme kinetics determined in this study. Based on these results, additional experiments were performed with mutant M11 and both CPA and IFA using the recently described Cytotox CALUX assay in which luciferase is expressed from a constitutive promoter (16). Compared to the MTT assay the Cytotox CALUX does not rely on the specific metabolic competency of the cell to reduce MTT, but rather reflects a general loss of viable cells by monitoring constitutive gene expression. For CPA, results (Fig. 7A) confirmed those observed in the MTT assay (Fig. 6). Likewise for IFA, cytotoxicity was only induced when mutant M11 was added to the culture medium (Fig. 7B). The Cytotox CALUX results are in good agreement with the MTT results. This confirms that the Cytotox CALUX assay is a faster, less laborious alternative to the frequently used MTT test. Moreover, these results show the potential of CYP BM3 to extracellularly bioactivate (pro-) drugs for toxicity studies.

DISCUSSION

CYP-based GDEPT approaches have been proposed to overcome dose-limiting adverse drug reactions of CPA and IFA and to increase their efficacy. In the present study we set out to identify CYP BM3 mutants showing high catalytic activity toward CPA and IFA. Two CYP BM3 mutants showed very high 4-hydroxylation activity: M11 (CYP BM3 M11) and M11 L437S (CYP BM3 M11 + L437S). Both these mutants showed a non-linear time-dependence of product formation: a very high initial activity during approximately the first minute was followed by a much slower, linear rate of product formation. A very similar phenomenon has been observed before for M11 mutant with MDMA and dextromethorphan as substrates (18), and the oxidation of lauric acid by CYP BM3 mutants F87G and F87Y, which was hypothesized to result from an irreversible conformational change (28). Our results however indicate that the observed biphasic behavior for CPA and IFA metabolism by CYP BM3 is not irreversible, since preincubation of mutant M11 with CPA did not influence subsequent benzyloxyresorufin O-dealkylation. Possible inhibition of CYP BM3 by the incubation products was explored briefly. After reaching the secondary slow phase of product formation during incubations of CPA with BM3 M11, fresh BM3 enzyme was added. This resulted in a second biphasic formation of 4-OH-CPA, suggesting that stable CPA-metabolites were not inhibiting the enzyme (data not shown). The underlying mechanism of the observed biphasic behavior thus remains to be elucidated.
Chapter 5

Activation of cyclophosphamide and ifosfamide by CYP BM3 mutants

Fig. 6. CPA-induced cytotoxicity in U2OS cells upon extracellular bioactivation by addition of CYP BM3 mutants M11 and M11 L437S to the culture medium, supplemented by an NADPH regenerating system. Cell viability was determined after 24 h using the MTT assay. Viability is expressed as percentage of the corresponding DMSO control ± SD (n = 3). Statistical analysis performed by two-way ANOVA with Bonferroni post-test, each bar compared to the corresponding DMSO control. **p < 0.01; ***p < 0.001.

Fig. 7. (A) CPA- and (B) IFA-induced cytotoxicity in U2OS cells upon extracellular bioactivation by addition of CYP BM3 mutant M11 to the culture medium, supplemented by an NADPH regenerating system. Cell viability was determined after 24 h using the Cytotox CALUX previously described (26) and is expressed as relative light units (RLU) ± SD (n = 3). Statistical analysis performed by two-way ANOVA with Bonferroni post-test compared to the corresponding DMSO control. **P < 0.01; ***P < 0.001.
Assessment of the enzyme kinetic parameters during the fast initial phase of CPA 4-hydroxylation by M11 and M11 L437S revealed a low $K_M$ ranging between 0.09 and 0.16 mM, comparable to those reported for canine CYP2B11 (0.16 mM), the most efficient CYP isoform reported to date for CPA and IFA 4-hydroxylation (11). These values are well within the plasma concentration range of CPA and IFA (0.1–0.5 mM) typically observed in patients (4, 11). In comparison, recombinant human CYP2B6 showed a much lower affinity for CPA ($K_M$ of 1.9–2.0 mM), similar to that reported previously for CYP2B6 (1.89 mM) (4). Compared to CYP2B11, $V_{\text{max}}$ values of both CYP BM3 mutants were much higher, resulting in an almost 60-fold increased catalytic efficiency ($V_{\text{max}}/K_M$). Compared to the recently developed CYP2B6TM-RED mutants, which are similarly to the CYP BM3 mutants fused to a reductase domain, our CYP BM3 mutants showed a 70- to 80-fold higher efficiency in CPA 4-hydroxylation (15). In contrast to human CYPs, $N$-dechloroethylation of CPA was not catalyzed by the two selected CYP BM3 mutants.

Although $V_{\text{max}}$ was not achieved for IFA 4-hydroxylation and $N$-dechloroethylation within the substrate concentration range studied, the catalytic efficiency $V_{\text{max}}/K_M$ of the hydroxylation reaction was about 40-fold higher compared to CYP2B11 while $N$-dechloroethylation was only increased 1.3-fold (11). For M11 and M11 L437S alike, the $N$-dechloroethylation reaction constituted approximately 5% of the total metabolism. This contribution of $N$-dechloroethylation is much lower than that for the dog CYP2B11 (~50%). As the $N$-dechloroethylation pathway yields neuro- and nephrotoxic byproducts, such an increased ratio of 4-hydroxylation to $N$-dechloroethylation is advantageous in avoiding adverse drug reactions that are often dose-limiting for CPA and IFA in clinical practice.

Mammalian expression of a CYP BM3 F87V mutant has successfully been performed by infection with recombinant adeno-associated viruses, both in vitro in several different cell types and in vivo in rats (29–31). Application of the currently identified CYP BM3 mutants in a similar adeno-viral expression system could be explored. If feasible, this would allow the investigation of the effects of the rapid biphasic kinetics in a cellular context: it remains to be seen if the biphasic behavior is also observed in a more complex intracellular matrix. High initial intracellular bioactivation of CPA and IFA is likely to result in rapid killing of the transfected cell itself, hampering prolonged bioactivation. However, at the same time this would lead to a burst formation of cytotoxic metabolites that are known to pass cell membranes and elicit a bystander effect. Sudden formation of large amounts 4-OH-CPA and 4-OH-IFA by CYP BM3 mutants may lead to a more pronounced and faster killing of local neighboring cells.

We confirmed that the CPA and IFA metabolites formed by our CYP BM3 mutants are indeed able to induce a bystander effect by using extracellular bioactivation of CPA and IFA in mammalian cell cultures, using the recently published Cytotox CALUX system (26). Extracellular bioactivation of CPA has also been performed using rat liver S9 fraction, showing cytotoxicity over a similar dose range (32). Whereas the use of S9 is associated with cytotoxicity, the CYP BM3 system for extracellular bioactivation is non-toxic. As our lab has shown that CYP BM3 mutants can metabolize a broad range of drugs (18–20), their application for extracellular bioactivation may provide a clean and catalytically efficient alternative to liver S9 fraction for the study of CYP-mediated drug toxicity.
In conclusion, we identified two highly active mutants showing very rapid initial 4-hydroxylation of CPA and IFA, followed by a slower secondary phase of product formation. While their very high catalytic efficiency of 4-hydroxylation combined with minimal N-dechloroethylation is promising, the true potential of these mutants in GDEPT requires additional investigation.

SUPPLEMENTARY DATA

Fig. S1 (left column). Substrate-concentration dependence of CPA 4-hydroxylation by M11 (A) and M11 L437S (B). Purified CYP BM3 mutant (250 nM) was incubated with various concentrations of CPA and NADPH (1 mM) for 10 seconds. Data of two independent experiments are shown. Curves are fitted using nonlinear regression according to the Michaelis-Menten equation.

Fig. S2 (middle column). Substrate-concentration dependence of IFA 4-hydroxylation and N-dechloroethylation by M11 (closed circles) and M11 L437S (open circles). Purified CYP BM3 mutant (250 nM) was incubated with various concentrations of IFA and NADPH (1 mM) for 10 seconds. (A) 4-hydroxylation activity was determined by measuring formation of acrolein. (B) N-dechloroethylation was determined by measuring CAA formation. Replicates of two independent experiments are shown. Lines are fitted using linear regression. $r^2$ describes goodness of fit.

Fig. S3 (right column). Benzyloxyresorufin O-dealkylation (BROD) activity of M11 after preincubation with CPA. Preincubation of M11 with CPA (+ CPA) did not influence subsequent BROD activity compared to incubations without preincubation (- CPA). Bars represent mean ± SD (n=2).
REFERENCES


Chapter 5 Activation of cyclophosphamide and ifosfamide by CYP BM3 mutants


