Chapter 6

One-electron oxidation of diclofenac by human cytochrome P450s as a [potential] bioactivation mechanism for formation of 2’-(glutathion-S-yl)-deschloro-diclofenac

Adapted from:

One-electron oxidation of diclofenac by human cytochrome P450s as a [potential] bioactivation mechanism for formation of 2’-(glutathion-S-yl)-deschloro-diclofenac
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Chem Biol Interact, accepted
Chapter 6

ABSTRACT

Reactive metabolites have been suggested to play a role in the idiosyncratic hepatotoxicity observed with diclofenac (DF). P450-catalyzed bioactivation of DF previously led to identification of multiple GSH conjugates, which were considered to result from three types of reactive intermediates: \( p \)-benzoquinone imines, \( o \)-imine methide and arene oxides. Recently, detection of \( 2' \)-(glutathion-S-yl)-deschloro-diclofenac (DDF-SG), resulting from chlorine substitution, suggested the existence of a fourth type of P450-dependent reactive intermediate. In this study, fourteen recombinant cytochrome P450s and three flavin-containing monooxygenases were tested for their ability to produce oxidative DF metabolites and their corresponding GSH conjugates. Concerning the hydroxymetabolites and their GSH conjugates, results were consistent with previous studies. Unexpectedly, all tested recombinant P450s were able to form DDF-SG to almost similar extent. Hence, we further investigated the mechanism by which DF activation results in this conjugate. DDF-SG formation was found to be (partially) independent of NADPH and even occurred by heat-inactivated P450. However, product formation was fully dependent on both GSH and glutathione-S-transferase P1-1 (GST P1-1). DDF-SG formation could also be observed in reactions with horseradish peroxidase in absence of hydrogen peroxide. Because DDF-SG was not formed by free iron, it appears that DF can be bioactivated by iron in hemeproteins. This was confirmed by DDF-SG formation by other hemeproteins such as hemoglobin. As a mechanism, we propose that DF is subject to heme-dependent one-electron oxidation. The resulting nitrogen radical cation, which might activate the chlorines of DF, then undergoes a GST-catalyzed nucleophilic aromatic substitution reaction in which the chlorine atom of the DF moiety is replaced by GSH.

INTRODUCTION

The nonsteroidal anti-inflammatory drug diclofenac (DF) is commonly prescribed for the treatment of a number of inflammatory disorders, including rheumatoid arthritis. Therapy with DF is associated with severe hepatotoxicity in a very small percentage of patients (1). The exact mechanism of this idiosyncratic toxicity is still poorly understood. Nevertheless, metabolic activation of DF to reactive metabolites (RMs) is generally considered to play an important role in the development of hepatotoxicity (2).
DF metabolism in humans occurs predominantly by glucuronidation and oxidative biotransformation by P450s, both of which contribute to the formation of RMs (3). Glucuronidation of DF leads to reactive acyl-glucuronides, which have been found to be responsible for the majority of the protein modification observed in vitro and in test animals (4-6). Metabolism of DF by cytochrome P450s (CYPs) results in multiple hydroxylated metabolites. The main hydroxylation product is 4’-hydroxydiclofenac (4’-OH-DF), whereas 5-hydroxydiclofenac (5-OH-DF) is a minor metabolite (3, 7) (Figure 1). CYP2C9 was found to be mainly responsible for 4’-OH-DF formation (8-11). In contrast, 5-OH-DF may be formed by several P450s, including CYP2C8 and CYP3A4 (9-11). Furthermore, 4’-hydroxylation of DF-acylglucuronides by CYP2C8 is also considered to play an important role (12). Further enzymatic or spontaneous oxidation of 4’-OH-DF and 5-OH-DF results in their corresponding p-benzoquinone imines, which have been detected as GSH conjugates M1, M2 and M3 (13-15). A fourth GSH conjugate (M4) was tentatively proposed to result from conjugation of GSH to DF arene oxides (16). A further conjugate of identical mass (M5) was shown to result from GSH substitution of a chlorine atom of the p-benzoquinone imine of 4’-OH-DF (17). An alternative bioactivation pathway involves the oxidative decarboxylation of DF by CYP3A4 which results in a reactive o-imine methide that was identified after GSH conjugation (DPAB-SG) (18, 19).

By application of a mass spectrometry-based method for the high-throughput screening of RMs, Wen and co-workers identified a new GSH conjugate of DF (2’-(glutathion-S-yl)-deschloro-diclofenac; DDF-SG) in incubations with human liver microsomes (20). The mass of the novel conjugate (m/z 567.1) can be explained by substitution of one of the chlorines on the dichlorophenyl ring of DF by GSH. Formation of this conjugate cannot be explained by GSH conjugation of the known P450-dependent reactive intermediates of DF (p-benzoquinone imine, arene oxide and o-imine methide resulting from decarboxylation). Nevertheless, the authors did not investigate nor discuss the possibility that an as yet uncharacterized intermediate of DF might be responsible for DDF-SG formation.
Figure 1: Overview of the metabolic pathways of diclofenac bioactivation by P450 enzymes. DF, diclofenac; QI, quinone imine; DC-DF-o-IM, decarboxylated diclofenac o-imine methide; DPAB-SG, 2-(2,6-dichlorophenylamino)-benzyl-S-thioether glutathione; DF-o-IM, diclofenac o-imine methide; DDF-SG, 2’-(glutathion-S-yl)-deschloro-diclofenac.
Recently, by using mass spectrometry to characterize the structure of DF-protein adducts, two peptide adducts with mass increment of 259 Da were found which result from adduction of DF by chlorine substitution. The corresponding GSH conjugate (DDF-SG) was only observed when DF incubations of CYP2C9 and CYP3A4 with GSH were supplemented with human glutathione-S-transferase P1-1 (hGST P1-1) (21).

Three possible RM5s of DF were previously proposed to explain the chlorine substitution reaction mechanistically (Figure 1). An electron-withdrawing nitrogen atom resulting from N-oxidation, o-imine methide formation or one-electron oxidation might allow for GST-catalyzed GSH substitution of the activated chlorine atoms (21).

The aim of the present paper is to characterize the bioactivation pathway resulting in the chlorine-substituted DF conjugate. It was investigated which of the commercially available human CYPs and flavin-containing monooxygenases (FMOs) is able to form DDF-SG and the other known metabolites of DF. The results from this study provide evidence for non-enzymatic heme-dependent bioactivation of DF into a radical cation, which is subject to GST P1-1-dependent chlorine substitution by GSH.

MATERIALS AND METHODS

Materials
DF sodium salt, bovine hemin, horseradish peroxidase (HRP) (type I), human hemoglobin, equine heart myoglobin and human serum albumin were obtained from Sigma (Steinheim, Germany). 4’-OH-DF and 5-OH-DF were from Toronto Research Chemicals (North York, Canada). Commercially available recombinant P450 enzymes and FMOs (Supersomes) were purchased from Gentest Corporation (Woburn, USA). CYP2A6, 2B6, 2C8, 2C9*1, 2C19, 2E1, 2J2, 3A4 and 3A5 were co-expressed with human P450 oxidoreductase and cytochrome b5. CYP1A1, 1A2, 1B1, 2C18 and 2D6*1 were co-expressed with human P450 oxidoreductase. Pooled human liver microsomes (20 mg/mL) were from Xenotech (lot no. 0710619). All other materials were from standard suppliers and of analytical grade.
Chapter 6

Enzyme expression
For mechanistic studies, CYP2C9 and CYP3A4 were co-expressed with human NADPH cytochrome P450 reductase in E. coli DH5α. The expression and isolation of the P450s was conducted as previously described (22).

An expression system composed of E. coli XL-1 Blue cells carrying a plasmid encoding GST P1-1 was a kind gift from Professor B. Mannervik (Department of Biochemistry and Organic chemistry, Uppsala University, Sweden). Expression and purification of GST P1-1 by GSH-affinity chromatography was performed as described previously (23).

Incubations of diclofenac with human P450s and FMOs in presence of human glutathione-S-transferase P1-1
Incubations were conducted in a total volume of 125 µL 0.1 M potassium phosphate (KPi) buffer at pH 7.4. DF was added as a stock solution in DMSO (1% of incubation volume). A final concentration of 100 µM DF was used for incubations with commercial CYPs and FMOs and for incubations with isoform-specific inhibitors, whereas other incubations were conducted with 500 µM DF. All reactions were performed in presence of 100 µM GSH and 8 µM hGST P1-1. Commercial P450s and FMOs were added to a final concentration of 100 nM and 0.5 mg/mL, respectively. Incubations with in-house expressed P450s were conducted with 250 nM enzyme. Reactions were initiated by the addition of NADPH regenerating system (final concentrations: 50 µM NADPH, 2.5 mM glucose-6-phosphate and 0.5 U/mL glucose-6-phosphate dehydrogenase). First, the time course of product formation was investigated by analyzing aliquots (250 µL) taken from large-scale incubations (2.5 mL) at regular time points (0-90 min). Based on the results, subsequent reactions were performed for 1 h at 37 °C. All incubations were quenched by the addition of an equal volume of ice-cold methanol supplemented with 2% 250 mM ascorbic acid to prevent non-enzymatic oxidation after reactions were terminated. Protein fractions were pelleted by centrifugation (20 min, 20,000g) and supernatants were subsequently analyzed by HPLC-UV or LC-MS/MS. Incubations in absence of one of the reaction components were also conducted to investigate enzyme, GSH, and NADPH dependence of the formed DF metabolites.
To evaluate whether formation of DF metabolites was P450 dependent, incubations of CYP3A4 were performed in presence of 2 µM ketoconazole, whereas CYP2C9 reactions were
conducted with 20 μM sulfaphenazole. To study non-enzymatic formation of DF metabolites by CYPs, also heat-inactivated (5 min, 95 °C) enzymes were used.

**Incubations of diclofenac with horseradish peroxidase, heme-containing proteins and hemin in presence of human glutathione-S-transferase P1-1**

Because incubations with heat-inactivated P450s unexpectedly showed significant DDF-SG formation, it was investigated whether this GSH conjugate could also be formed in reactions with HRP and other hemeproteins. Incubations of DF with HRP were performed in a total volume of 250 μL 0.05 M KPi at pH 7.4 supplemented with 0.5 mM Detapac. Samples contained 100 μM DF, 20 U/mL HRP, 10 mM GSH and 8 μM GST P1-1. Reactions were started by addition of 50 μM H₂O₂ and allowed to proceed for 4 h at 25 °C. Incubations were terminated by addition of 250 μL ice-cold methanol containing 5 mM ascorbic acid and centrifuged for 20 min at 20,000g to remove precipitated protein.

To investigate whether observed metabolites were HRP dependent, the reversible inhibitor potassium cyanide was used at a final concentration of 100 μM. Non-enzymatic product formation by HRP was evaluated by use of heat-inactivated enzyme (5 min at 95 °C).

Reactions of DF with hemoglobin, myoglobin and hemin were conducted in 250 μL 0.05 M KPi at pH 7.4 supplemented with 0.5 mM Detapac. Incubations were performed with 500 μM DF, 5 mM GSH and 8 μM GST P1-1. The reactions were initiated by addition of a saturated solution of hemin (100 μM) or by hemeprotein (typically 0.2 mg/mL final concentration) and then incubated for 1 h at 37 °C. Incubations were stopped by the addition of an equal volume of ice-cold methanol containing 2% 250 mM ascorbic acid. Samples were centrifuged (20 min, 20,000g) and supernatants were stored at -20 °C until analysis by HPLC-UV or LC-MS/MS.

**Analytical methods**

The metabolites of DF were separated by reversed phase chromatography using a C18 column (Symmetry Shield C18, 3.5 μm, 4.6 x 100 mm i.d.; Waters) at a flow rate of 0.5 mL/min. The gradient was composed of solvent A (98.8% water/1% acetonitrile/0.2% formic acid) and solvent B (98.8% acetonitrile/1% water/0.2% formic acid). The first 5 min were isocratic at 0% B. The gradient was linear from 0% to 100% between 5 and 30 min, and the column was allowed to re-equilibrate from 30 to 40 minutes at 0% B. Samples were analyzed by HPLC-UV or by LC-MS/MS.
HPLC analysis was performed on a Shimadzu HPLC equipped with two LC-20AD pumps, a SIL20AC autosampler and SPD20A UV detector. Following injection of 50 µL, samples were chromatographed and detected by UV/Vis at 254 nm. A standard curve of DF (0.05-20 µM) was used to determine the concentrations of the formed GSH conjugates of DF, assuming the extinction coefficients of the conjugates are identical to that of DF. Peak areas of DF metabolites were analyzed using the Shimadzu LC solution software package (version 1.25).

For MS analysis, an injection volume of 25 µL was used. Samples were analyzed on an Agilent 1200 Series Rapid resolution LC equipped with a hybrid Quadrupole-Time-Of-Flight (Q-TOF) Agilent 6520 mass spectrometer (Agilent technologies, Waldbronn, Germany). Analytes were first detected by UV at 254 nm and subsequently ionized by electrospray ionization. The mass spectrometer was operated at a capillary voltage of 3500 V with nitrogen as drying gas (12 L/min) and nebulizer gas (pressure 60 psig). The gas temperature was 350 °C during operation. The Q-TOF was used in the positive mode and data was acquired using the Mass Hunter workstation software (version B.02.00).

For identification, samples were analyzed by automated MS/MS analysis. In each round, one MS$^1$ spectra (m/z 200-1000) was acquired, followed by fragmentation of the three most abundant ions. MS/MS analysis was conducted at a collision energy voltage of 25 V using nitrogen as the collision gas.

For quantitative analyses, only MS$^1$ spectra (m/z 300-700) were acquired in order to obtain sufficient data points for each ion. Clozapine (4 µM) was added to each of the samples as internal standard. All LC-MS/MS data was analyzed using the Mass Hunter software package (version B.01.03).
RESULTS

Time-dependent formation of GSH conjugates of diclofenac by cytochrome P450s

The time course of product formation was evaluated with CYP2C9 and CYP3A4, which were previously found to be responsible for formation of GSH conjugates resulting from 4’-OH-DF and 5-OH-DF, respectively (14). In addition, these P450s formed the GSH conjugate resulting from chlorine substitution (DDF-SG) in reactions performed with 100 µM GSH and 8 µM GST P1-1 (21). When time-dependent product formation was investigated under these conditions, DDF-SG formation was linear for about 20 and 30 min in DF reactions of E. coli expressed CYP2C9 and CYP3A4, respectively (Figure 2A and B). DDF-SG formation reached a plateau after 60 min of incubation.

![Graphs showing time-dependent formation of GSH conjugates](image)

**Figure 2:** Time-dependent formation of GSH conjugates of diclofenac by CYP2C9 and CYP3A4. A and B: Formation of DDF-SG by CYP2C9 (A) and CYP3A4 (B). C and D: Formation of GSH conjugate M2 by CYP2C9 (C) and conjugate M3 by CYP3A4 (D).
Interestingly, sigmoidal curves were obtained when time-dependence was plotted for GSH conjugates M2 and M3 (Figure 2C and D). In case of CYP2C9-catalyzed M2 formation, no plateau was reached within the time interval investigated. However, the rate of CYP3A4-mediated M3 formation significantly decreased after 60 min of incubation. The obtained sigmoidal curves most likely reflect the intermediacy of 4’-OH-DF and 5-OH-DF in the formation of GSH conjugates M2 and M3, respectively. Subsequent DF incubations were therefore allowed to proceed for 60 min.

**Bioactivation of DF by recombinant human P450s and FMOs**

To evaluate which P450s contribute to the different stable and reactive metabolites of DF, a panel of 14 commercially available cDNA expressed P450s was incubated with 100 µM DF in presence of 100 µM GSH and 8 µM GST P1-1 (Figure 3, 4 and Table S1 of the Supporting information). Consistent with previous data (8-10), CYP2C9 appeared the major isoform involved in 4’-OH-DF formation, whereas CYP2D6 was found to have 2.7-fold lower activity (Figure 3A). Compared to these enzymes, the contribution of the other CYPs to 4’-OH-DF formation was negligible. Conjugate M2 and M5 result from GSH conjugation to the p-benzoquinone imine of 4’-OH-DF. Similar to DF 4’-hydroxylation, M2 formation was mainly mediated by CYP2C9 (Figure 3B). Conjugate M5 was not detected under the current conditions, presumably because it is not catalyzed by GST P1-1.

The minor hydroxylation product 5-OH-DF was mainly catalyzed by CYP3A4 and at 4-fold and 7-fold lower activity by CYP2B6 and CYP2C19, respectively (Figure 4A). As described previously (9, 10), CYP2C18 also contributed to 5-hydroxylation. Only a low activity of CYP2C8 was detected. In line with results of Tang and co-workers (14), the GSH conjugation products of the quinone imine of 5-OH-DF (M1 and M3) were predominantly formed by CYP3A4. Evaluation of CYPs which have not been tested (1A1, 1B1, 2B6, 2C18, 2J2 and 3A5) showed some M1 and M3 formation in incubations with CYP2B6 and CYP2C18, although activities were less than 15% of CYP3A4 (Figure 4B and C).

DPAB-SG results from DF decarboxylation and was only formed in low levels by recombinant CYP3A4 (data not shown), which is in line with previous observations (19). As expected, human FMOs were unable to form monohydroxylated DF metabolites or GSH conjugates thereof.
Figure 3: Evaluation of recombinant human P450s and FMOs involved in the formation of 4’-OH-DF (A) and GSH conjugate M2 (B). Control microsomes (insect) and microsomes expressing reductase and cytochrome b5 (reductase) are indicated.
Figure 4: Formation of 5-OH-DF (A) and its GSH conjugates M1 (B) and M3 (C) by cDNA expressed P450s and FMOs. Controls consisting of insect cell microsomes (insect) and microsomes expressing P450 reductase and cytochrome b5 (reductase) are shown.
Chapter 6

Formation of DDF-SG by human liver microsomes, recombinant human P450s and FMOs

Formation of DDF-SG was only observed when DF incubations with human liver microsomes were supplemented with GST P1-1 (Supporting information, Figure S1A). The mass spectrum of DDF-SG (m/z 567.1) exhibited a clear mono-chlorine isotope cluster (Figure S1B). Presence of a GSH-moiety in DDF-SG was confirmed by MS/MS fragments of m/z 492 and 438, which correspond to loss to loss of glycine and pyroglutamate, respectively (20, 21).

DDF-SG formation was also evaluated in the incubations with cDNA expressed P450s and FMOs (Figure 5). Interestingly, all tested P450s formed DDF-SG in significant amounts, with CYP1A1 as most active, and CYP2A6 as least active isoform. In line with our preliminary findings (21), low amounts of DDF-SG were also found in FMO incubations. However, the abundance of DDF-SG in reactions with FMOs appeared not different from control microsomes. No DDF-SG was detected when microsomes were omitted from incubations.

Figure 5: Formation of DDF-SG in incubations of recombinant P450s and FMOs. Control microsomes without reductase and CYPs (insect) or expressing reductase and cytochrome b5 (reductase) were also evaluated.
Chapter 6

Since it appeared highly unlikely that all P450 isoforms contribute to DDF-SG formation to significant extent, additional mechanistic studies were conducted with CYP2C9 and CYP3A4 to further investigate how different components of the reaction affected DDF-SG formation.

First, it was investigated whether DDF-SG and other DF metabolites were formed under conditions in which one of the incubation constituents was omitted from the reaction (Table 1). CYP2C9 and CYP3A4 formed DDF-SG in DF reactions containing P450, GSH, GST P1-1 and NADPH. DDF-SG and other DF metabolites were not detected in absence of P450, whereas 4'-OH-DF and 5-OH-DF were the only products formed in absence of GSH. The GST-dependent GSH conjugate DDF-SG was not found in P450 incubations without GST P1-1. Other GSH conjugates could be detected under these conditions but conjugate formation was at least 4-fold lower in absence of GST P1-1. In case of CYP2C9, NADPH had no effect on DDF-SG formation. In contrast, DDF-SG was about 1.5-fold less abundant when NADPH was omitted in reactions of CYP3A4. In absence of NADPH, 4'-OH-DF, 5-OH-DF and their corresponding GSH conjugates were not observed.

To evaluate whether DDF-SG formation was dependent on binding to the P450-active site, reactions were also conducted in presence of isoform-specific inhibitors (Table 1). When CYP3A4 was incubated with ketoconazole (2 µM), DDF-SG formation was only 46% of non-inhibited controls. Other DF metabolites of CYP3A4 were fully inhibited by ketoconazole. In contrast, DDF-SG formation in CYP2C9 incubations was not inhibited by sulfaphenazole (20 µM), whereas the formation of 4'-OH-DF and its GSH conjugates was decreased by at least 50%.

To investigate the possibility of non-enzymatic DDF-SG formation by P450s and GST P1-1, incubations of DF were performed with heat-inactivated enzymes (Table 1). The monohydroxylated DF metabolites and their corresponding GSH conjugates were completely abolished upon heat treatment of the P450s at 95 °C. Unexpectedly, in incubations with heat-inactivated CYP3A4, DDF-SG formation was still 54% of non-heated controls. Heat denaturation of CYP2C9 even increased DDF-SG formation by more than 2-fold, clearly indicating that regular oxidative biotransformation is not involved in formation of this GSH conjugate. No DDF-SG was detected when P450 reactions were performed with denatured
GST P1-1, suggesting that the native conformation of this enzyme is required for DDF-SG formation. Similar to incubations without GST P1-1, heat treatment of GST P1-1 resulted in low levels of GSH conjugates derived from the monohydroxylated metabolites of DF.

Table 1: Relative formation of diclofenac metabolites under different experimental conditions

Formation of DF metabolites as percentage of the amount formed in control incubations (AUC_{metabolite} / AUC_{metabolite, control} * 100%). Control reactions consisted of all incubation components (complete), were conducted without inhibitor (-inhibitor) or were not subjected to heat treatment (-boiling). ND, Not detected. Ketoconazole (2 µM) was used as inhibitor for CYP3A4, whereas sulfaphenazole (20 µM) was used to inhibit CYP2C9.

<table>
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<th>Controls</th>
<th>CYP2C9</th>
<th>CYP3A4</th>
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<tr>
<td></td>
<td>4'-OH-DF</td>
<td>M2+M5</td>
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<tr>
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<td>-NADPH</td>
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P450 inhibition

| -inhibitor | 100(±3) | 100(±7) | 100(±4) | ND      | 100(±3) | 100(±1) |
| +inhibitor | 51(±13) | 30(±5)  | 105(±2) | ND      | 1(±2)   | 46(±1)  |

Heat treatment

|          | 100(±4) | 100(±2) | 100(±3) | 100(±21) | 100(±1) | 100(±2) |
| -boiling | ND      | ND      | 242(±35) | ND      | 6(±1)   | 54(±6)  |
| boiled P450 | ND      | ND      | 242(±35) | ND      | 6(±1)   | 54(±6)  |
| boiled GST  | 99(±6)  | 23(±2)  | ND      | 814(±146) | 7(±1)   | ND      |
Formation of DDF-SG in incubations of horseradish peroxidase

Previously, hemeproteins have been found to perform oxidative reactions by mechanisms of peroxidases (24, 25). Therefore, incubations of DF were also conducted with HRP, which is known to catalyze one-electron oxidations (26). HRP did not form 4’-OH-DF and 5-OH-DF or GSH conjugates resulting from these metabolites. Interestingly, HRP was able to produce DDF-SG in DF reactions performed in presence of GSH, GST P1-1 and H$_2$O$_2$ (Figure 6). DDF-SG was not detected in absence of HRP or GST P1-1. However, product formation in reactions without H$_2$O$_2$ was still 77% of complete incubations, suggesting DDF-SG formation by HRP is non-enzymatic. In line with this, neither boiling of HRP prior to the reaction nor addition of the reversible HRP inhibitor potassium cyanide abolished product formation. Under these conditions, DDF-SG formation was still 41% and 44% of controls, respectively. As observed in the P450 reactions, no DDF-SG was detected upon heat inactivation of GST P1-1.

**Figure 6:** HPLC-UV analysis of diclofenac conjugate DDF-SG in incubations of HRP. Shown UV traces (254 nm) are from incubations in which HRP, GST P1-1 or peroxide was omitted. A reaction containing all components required for catalytically active HRP (complete) is also shown.
Chapter 6

Effect of iron and hemeproteins on the formation of DDF-SG

The observation that both P450s and HRP can form DDF-SG in absence of cofactor raises the possibility that this conjugate results from iron-mediated activation of DF. DDF-SG was not observed when DF was incubated with 100 µM Fe$^{3+}$. Alternatively, coordinated iron from the prosthetic heme groups in P450 and HRP is responsible for DDF-SG formation. To investigate this, several hemeproteins were incubated with DF in presence of GSH and GST P1-1 (Figure 7). LC-MS analysis showed formation of DDF-SG when reactions were conducted with hemoglobin, myoglobin or HRP. No conjugate was found with human serum albumin or when hemeproteins were incubated in absence of GST P1-1. To further corroborate these findings, reactions were also performed with iron-containing protoporphyrin IX (hemin), which is highly similar to the heme-moiety of the tested hemeproteins. DDF-SG was detected in DF reactions of hemin containing GSH and GST P1-1, but not in similar reactions in which hemin or GST P1-1 was omitted (Figure 7).

**Figure 7:** Ion traces of DDF-SG (m/z 567.1) from diclofenac incubations performed with different (heme)proteins and hemin. Shown traces are from horseradish peroxidase (A), hemoglobin (B), myoglobin (C), human serum albumin (D) and hemin (E) incubated in presence of GSH and GST P1-1. (F) DF incubations of hemin in which GST P1-1 was omitted.
Effect of ascorbic acid on the formation of DDF-SG by P450 and HRP

Because DF bioactivation by hemeproteins presumably involves non-enzymatic one-electron oxidation resulting in formation of a DF radical cation, it was investigated whether ascorbic acid could inhibit product formation. DDF-SG was not observed when DF incubations of P450 or HRP were supplemented with ascorbic acid. GSH conjugates M1 and M3 were also strongly decreased when ascorbic acid was added to P450 incubations (data not shown). This is in line with previous observations that ascorbic acid reduces \( p \)-benzoquinone imine back to 5-OH-DF (15).

DISCUSSION

Recently, protein adducts of DF were identified in which the chlorine atom of DF was substituted by protein thiol. The corresponding GSH conjugate (DDF-SG) was subsequently only identified in incubations in presence of GST P1-1, suggesting that the reactive entity involved is only reactive to activated thiol groups (21). As possible explanation for DDF-SG formation, three reactive intermediates have been proposed in which an electron withdrawing nitrogen atom activates the chlorines (Figure 1). The aim of the present paper was to elucidate the mechanism by which DF bioactivation results in the previously uninvestigated GSH conjugate DDF-SG. Preliminary findings indicated that recombinant human FMOs formed low amounts of DDF-SG, which was interpreted as support for \( N \)-oxygenation as a possible bioactivation pathway (21). However, in the present study, no difference in product formation was observed between recombinant FMOs and their corresponding control microsomes (Figure 5). P450-expressing microsomes showed higher levels of DDF-SG than FMOs.

To evaluate whether an imine methide intermediate of DF is responsible for DDF-SG formation (Figure 1), P450 incubations were also performed in phosphate buffer prepared with \( \text{D}_2\text{O} \). However, no deuterium incorporation was observed at the benzylic position in DDF-SG (data not shown), suggesting the postulated DF imine methide is not formed. Remarkably, formation of DDF-SG appeared non-enzymatic in P450 incubations, because product was observed in absence of cofactor and in reactions of heat-inactivated P450s (Table 1). The finding that other hemeproteins and protoporphyrin IX could also catalyze DDF-SG formation
(Figure 7) strongly suggests that this conjugate is formed by GST P1-1 catalyzed GSH conjugation to a DF radical cation resulting from one-electron oxidation.

Peroxidase-like activity of hemeproteins may be responsible for one-electron oxidation of substrates. The higher oxidation states (compound I and II) of HRP were found to accept electrons from nitrogen compounds, resulting in formation of nitrogen-centered radical cations (27, 28). Peroxidase-type mechanisms have also been observed in reactions of the antipsychotic chlorpromazine with methemoglobin, and were implicated in the bioactivation of the antidepressant nomifensine by hemoglobin and myeloperoxidase (25, 29). In the aforementioned studies, peroxide was used to form higher oxidation states of the hemeproteins, which is necessary for one-electron oxidation to occur (24, 28). In the present study, however, DDF-SG was formed when reactions of HRP and other hemeproteins were incubated in absence of hydrogen peroxide (Figure 6 and 7). To evaluate a potential role for H₂O₂ in DDF-SG formation, CYP2C9 incubations were also supplemented with catalase (200 U/mL) or with 500 µM H₂O₂. Nevertheless, these conditions did not affect DDF-SG formation by CYP2C9 (data not shown).

A possible mechanism to explain DDF-SG formation is coupled one-electron oxidation of DF and hemeprotein, resulting in electron transfer to molecular oxygen and formation of H₂O₂ (Figure 8). The DF radical cations formed may either be reduced back to DF by GSH or may undergo GST-catalyzed chlorine substitution. Previously, similar mechanisms were proposed to rationalize one-electron oxidation of various substrates in incubations with hemoglobin (30-33). Alternatively, the radical intermediate of DF might result from autoxidation. However, DDF-SG could not be detected in absence of hemeproteins (Figure 6 and 7), even after incubation at room temperature for 4 h under air.
The formation of a nitrogen centered radical cation has been proposed as the initial step in the electrochemical oxidation of DF (34). It was previously suggested that this radical might contribute to DF toxicity (2). Galati and co-workers showed that incubation of DF and other nonsteroidal anti-inflammatory drugs with HRP and \( \text{H}_2\text{O}_2 \) resulted in co-oxidation of NADH, which they explained by formation of prooxidant radicals from these drugs (35). The activation of molecular oxygen by NAD radicals might result in formation of reactive oxygen species which can induce mitochondrial injury. In support of a role for prooxidant DF radicals in cell toxicity is the finding that addition of noncytotoxic concentrations of HRP and \( \text{H}_2\text{O}_2 \) to DF incubations with rat hepatocytes significantly increased lipid peroxidation and GSSG formation (36). Reduction of DF radical cations by concomitant oxidation of GSH leads to reformation of the DF parent compound (Figure 8). The resulting redox cycling may deplete cellular GSH pools, impairing cellular defenses and disrupting the normal redox balance (37). Besides reactions involving GSH, we have previously shown that the putative radical cation of DF also adds to cysteine residues of a model protein (21). It remains to be established whether this mechanism of protein modification is relevant to DF toxicity.
Contrary to other GSH conjugates of DF, DDF-SG was only detected when DF incubations of recombinant P450s or human liver microsomes were supplemented with GST P1-1 (Table 1, Figure S1). This may provide a likely explanation for the fact that DDF-SG was not observed in previous DF studies using recombinant P450s or human liver microsomes (14, 16, 17, 19, 34). Nevertheless, by analysis of concentrated DF incubations using a sensitive LC-MS method, DDF-SG was previously also detected in human liver microsomes in one study (20). This finding suggests that microsomal GSTs might also catalyze formation of this conjugate.

In conclusion, the proposed mechanism of DDF-SG formation involves a nucleophilic aromatic substitution reaction of the chlorine atom of the DF radical cation. GSTs can catalyze the substitution of chlorine atoms on electrophilic compounds as is most well-known by the example of 1-chloro-2,4-dinitrobenzene (38). However, unlike GSH conjugation to 1-chloro-2,4-dinitrobenzene, which occurs chemically to some extent, chlorine substitution of the DF radical cation requires activation of GSH (to GS\(^{-}\)) by GSTs. Interestingly, a similar GST P1-1-dependent chlorine substitution reaction was previously observed for the clozapine nitrenium ion (23). Hence, this type of GST-catalyzed substitution reactions could also apply to other chlorinated reactive drug metabolites.

REFERENCES


Chapter 6


Figure S1: Formation of the chlorine-substituted GSH conjugate of diclofenac (DDF-SG) in incubations of human liver microsomes. (A) Ion traces (m/z 567.1) from incubations of human liver microsomes with GSH in presence (i) and absence (ii) of hGST P1-1. (B) Isotope pattern of the parent ion (m/z 567.1) of DDF-SG in LC-MS.
Table S1: Quantitative determination of diclofenac metabolites formed by recombinant P450s and FMOs

Quantification of 4'-OH-DF and 5-OH-DF was based on comparison of peak areas in LC-UV chromatograms at 254 nm with standard curves of the commercially obtained metabolites. For GSH conjugates, clozapine-spiked standards of known concentration were analyzed by LC-MS to calculate $AUC_{\text{conjugate}} / AUC_{\text{clozapine}}$. The obtained ratio was used to calculate the amount of conjugate formed in spiked samples of recombinant P450s and FMOs. Control incubations were performed in absence of microsomes (no P450) or with microsomal preparations from vehicle-infected insect cells (insect) or cells expressing only human P450 reductase and cytochrome b5 (reductase).

<table>
<thead>
<tr>
<th>Bioactivation enzyme</th>
<th>4'-OH-DF</th>
<th>5-OH-DF</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>DDF-SG</th>
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<tr>
<td>No P450</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt; ND ND ND ND ND</td>
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<tr>
<td>Insect&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND ND ND ND ND 0.07 (±0.01)</td>
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<tr>
<td>Reductase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND ND ND ND ND 0.06 (±0.00)</td>
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<tr>
<td>1A1</td>
<td>30.92 (±0.29) 1.78 (±0.91) 0.63 (±0.11) 2.34 (±0.5) 0.91 (±0.22) 0.65 (±0.06)</td>
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<tr>
<td>1A2</td>
<td>2.08 (±0.11) 3.60 (±0.15) 1.09 (±0.17) ND 1.55 (±0.18) 0.55 (±0.11)</td>
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<tr>
<td>1B1</td>
<td>ND 1.82 (±0.41) 0.55 (±0.05) ND 0.73 (±0.01) 0.44 (±0.02)</td>
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<tr>
<td>2A6</td>
<td>0.34 (±1.14) ND ND 0.04 (±0.05) ND 0.10 (±0.02)</td>
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<td>2B6</td>
<td>1.74 (±2.93) 20.89 (±0.26) 1.13 (±0.01) ND 1.50 (±0.15) 0.42 (±0.01)</td>
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<td>2C8</td>
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<td>3A5</td>
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<td>FMO1</td>
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<td>FMO3</td>
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<td>FMO5</td>
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