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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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ABSTRACT

Reactive metabolites have been suggested to play a role in the idiosyncratic hepatotoxicity observed with diclofenac (DF). By structural identification of the GSH conjugates formed after P450-catalyzed bioactivation of DF, it was shown that three types of reactive intermediates were formed: *p*-benzoquinone imines, *o*-imine methide and arene-oxide. 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 whose inactivation by GSH is completely dependent on presence of glutathione S-transferase. 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. 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. DDF-SG formation was also 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.

1. 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 appeared 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 several hydroxylated metabolites. The main hydroxylation product is 4′-hydroxydiclofenac (4′-OH-DF), whereas 5-hydroxydiclofenac (5-OH-DF) is a minor metabolite [3,7], Fig. 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 non-enzymatic oxidation of 4′-OH-DF and 5-OH-DF results in their corresponding *p*-benzoquinone imines, which have been detected as GSH conjugates M1 and M3 from diclofenac-2,5-quinone imine (5-OH-DF-QI) and GSH-conjugate M2 from diclofenac-1′,4′-quinone imine

Abbreviations: 4′-OH-DF, 4′-hydroxydiclofenac; 5-OH-DF, 5-hydroxydiclofenac; CYP, cytochrome P450; DDF-SG, 2′-(glutathion-S-yl)-deschloro-diclofenac; DF, diclofenac; FMO, flavin-containing monooxygenase; hGST P1-1, human glutathione-S-transferase P1-1; HRP, horseradish peroxidase; RM, reactive metabolite.

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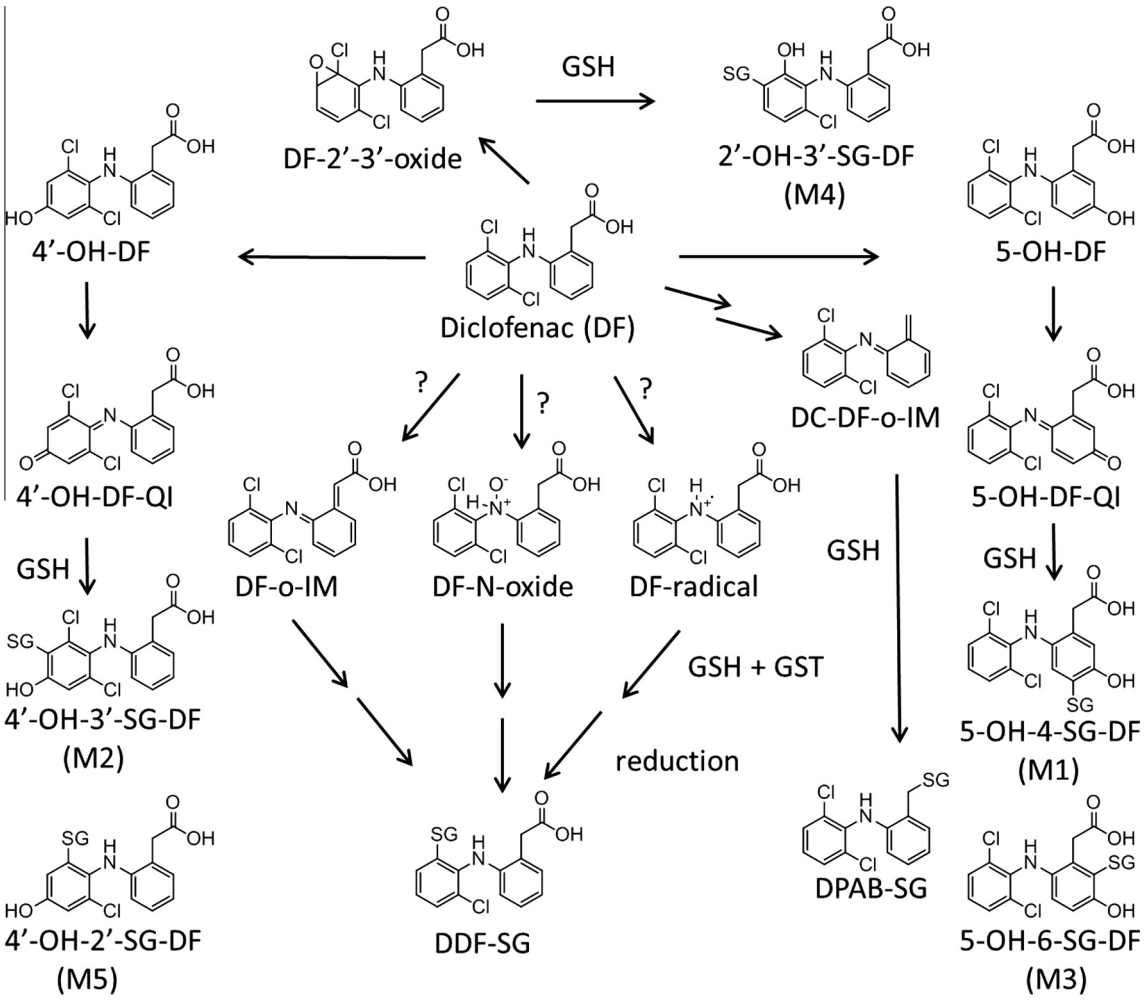

Fig. 1. Overview of the metabolic pathways of diclofenac bioactivation by P450 enzymes. DF, diclofenac; QI, quinone-imine; DC-DF-*o*-IM, decarboxylated diclofenac *o*-imine methide; DPAB-SG, 2-(2,6-dichlorophenylamino)-benzyl-S-thioether glutathione; DF-*o*-IM, diclofenac *o*-imine methide; DDF-SG, 2′-(glutathion-S-yl)-deschloro-diclofenac.

(4'-OH-DF-QI) [13–15]. Another conjugate, assigned M5, was shown to result from GSH substitution of a chlorine atom of DF-1′,4′-QI followed by reduction to restore aromaticity [16]. Another GSH-conjugate, previously assigned M4, has exactly the same mass as M5 and was tentatively proposed to result from conjugation of GSH to diclofenac-2′,3′-oxide [17]. Another 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.

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]. More recently, we showed that also hGSTM1-1 and hGSTA1-1 were able to catalyze formation of DDF-SG, although at lower activity than hGSTP1-1 [22].

Three possible RMs of DF were previously proposed to explain the chlorine substitution reaction mechanistically, Fig. 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.

2. Materials and methods

2.1. 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.

2.2. Enzyme expression

For mechanistic studies, CYP2C9 and CYP3A4 were also co-expressed with human NADPH cytochrome P450 reductase in *Escherichia coli* DH5α. The expression and isolation of the P450s was conducted as previously described [23].

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 [24].

2.3. 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 were 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 over a period of 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% (v/v) 250 mM ascorbic acid in water to prevent further non-enzymatic oxidation after reactions were terminated. Protein fractions were pelleted by centrifugation (20 min, 20,000*g*) 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.

Previously, formation of an *o*-quinone methide was proposed as one of the alternative bioactivation mechanisms for DDF-SG. To investigate whether this mechanism is involved, incubations were also performed using buffers and cofactors prepared in deuterium oxide. Incorporation of a deuterium ion at the benzylic position would be expected if an *o*-quinone methide is involved.

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

Heat-inactivated P450s unexpectedly showed significant DDF-SG formation. To investigate whether DDF-SG formation might be the result from heme-iron, we also studied whether this GSH conjugate could also be formed in reactions with other hemeproteins, such as horseradish peroxidase (HRP), hemoglobin, myoglobin and hemin. As negative control corresponding incubations were carried out with human serum albumin. 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 H_2O_2 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 20,000*g* 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, 20,000*g*) and supernatants were stored at −20 °C until analysis by HPLC–UV or LC–MS/MS.

2.5. Analytical methods

The metabolites of DF were separated by reversed phase chromatography using a C18 column (Symmetry Shield C18, 3.5 μm, 4.6 × 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 min 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 of metabolites, 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).

3. Results

3.1. 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 increased linearly for approximately 20 min in DF reactions of *E. coli* expressed CYP2C9 and CYP3A4, respectively, Fig. 2A and B. DDF-SG formation reached a plateau after 60 min of incubation, which might be explained by product-inhibition of GST P1-1. Sigmoidal curves were obtained when the time-course of formation of GSH conjugates M2 and M3 was plotted, Fig. 2C and D. These sigmoidal curves can be explained by the fact that the primary metabolites 4′-OH-DF and 5-OH-DF must undergo a second oxidation to produce the GSH-reactive quinone imines. Because at 60 min all GSH-conjugates of DF were formed at significant extent, all further incubations were also allowed to proceed for 60 min.

3.2. Bioactivation of DF by recombinant human P450s and FMOs

To evaluate which P450s contribute to the formation of 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, Figs. 3 and 4. The specific activities of the product formation can be found in Table A.1 of Appendix A. 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 (Fig. 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 (Fig. 3B).

As shown in Fig. 4A, the minor hydroxylation product 5-OH-DF was mainly catalyzed by CYP3A4 with 5- to 10-fold lower activity of CYP2B6, CYP2C18, CYP2C19, CYP2D6 and CYP3A5. The GSH conjugation products of the quinone imine of 5-OH-DF (M1 and M3) were also predominantly formed by CYP3A4, consistent with results of Tang and coworkers [14]. Evaluation of the CYPs which have not been tested previously (1A1, 1B1, 2B6, 2C18, 2J2 and 3A5) showed minor formation of M1 and M3 in incubations with CYP2B6 and CYP2C18, although activities were less than 15% of CYP3A4 (Fig. 4B and C).

DPAB-SG, which results from DF decarboxylation, was only formed in very 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.

3.3. 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 (Fig. A.1A in Appendix A). The mass spectrum of DDF-SG (m/z 567.1) exhibited a mono-chlorine isotope cluster (Fig. A.1B). 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].

Fig. 5 shows the formation of DDF-SG in the incubations with cDNA expressed P450s and FMOs. Unexpectedly, all tested P450s appeared to form DDF-SG in significant amounts, with CYP1A1 as most active, and CYP2A6 as least active isoform. In line with our previous findings [21], low levels of DDF-SG were also found in FMO incubations. However, the abundance of DDF-SG in reactions with FMOs appeared not to be different from the control micro-

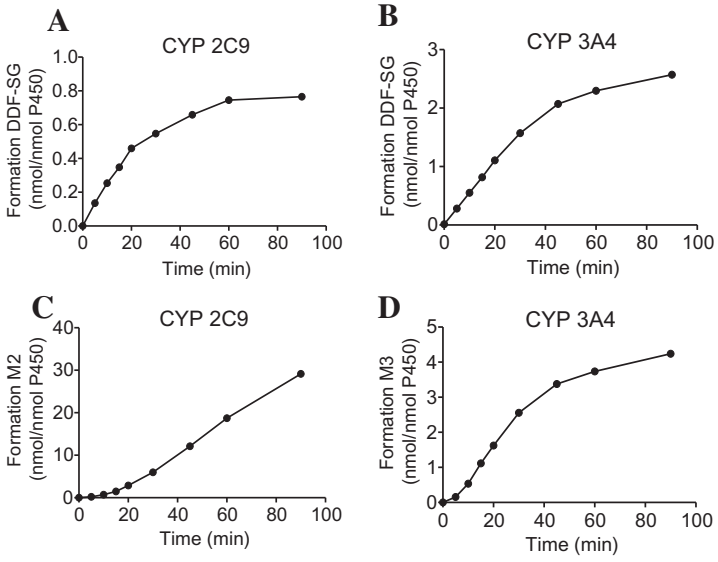

Fig. 2. Time-dependent formation of GSH conjugates of diclofenac by CYP2C9 and CYP3A4. (A) and (B): Formation of DDF-SG by CYP2C9 (A) and CYP3A4 (B). (C) and (D): Formation of GSH conjugate M2 by CYP2C9 (C) and conjugate M3 by CYP3A4 (D).

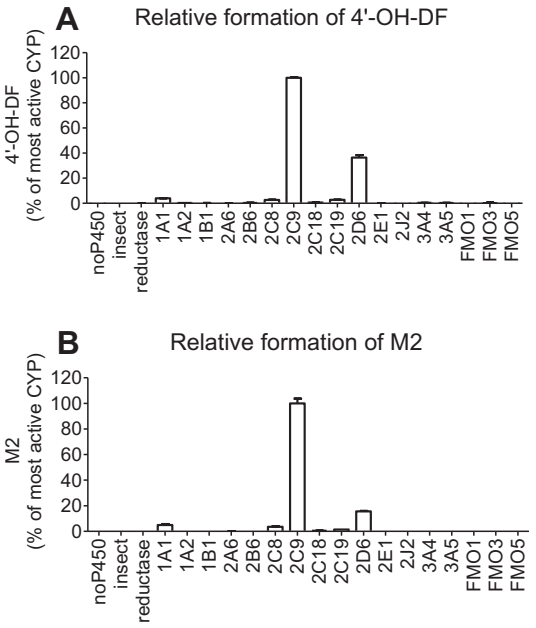

Fig. 3. Evaluation of recombinant human P450s and FMOs involved in the formation of 4′-OH-DF (A) and GSH conjugate M2 (B). Control microsomes (insect) and microsomes expressing reductase and cytochrome b5 (reductase) are indicated.

somes without expressed P450 or FMO. Not any DDF-SG was detected when microsomes were omitted from incubations.

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, addition of 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 inhibited by 54% when compared to the non-inhibited incubations. 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 also performed with heat-inactivated enzymes (Table 1). The formation of monohydroxylated DF metabolites and their corresponding GSH conjugates was 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 the non-heated

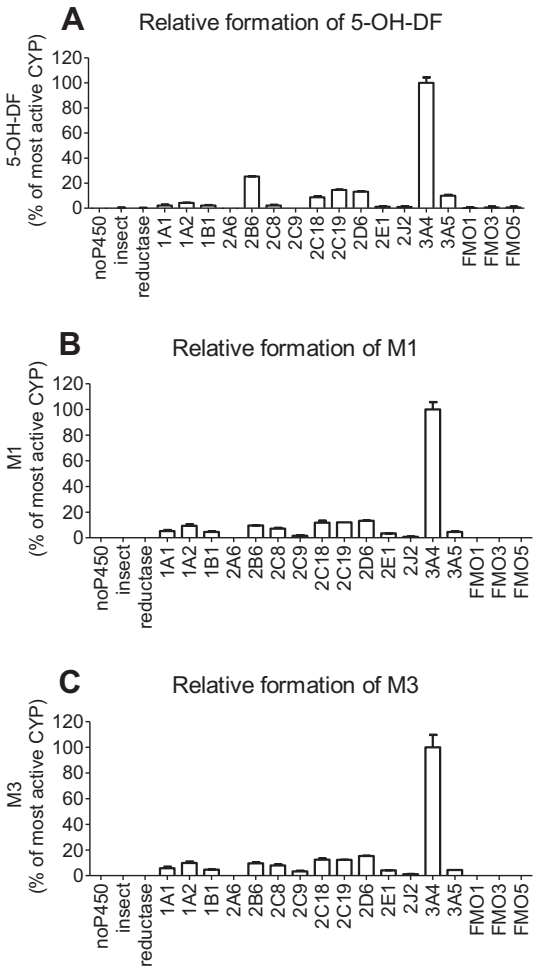

Fig. 4. Formation of 5-OH-DF (A) and its GSH conjugates M1 (B) and M3 (C) by cDNA expressed P450s and FMOs. Controls consisting of insect cell microsomes (insect) and microsomes expressing P450 reductase and cytochrome b5 (reductase) are shown.

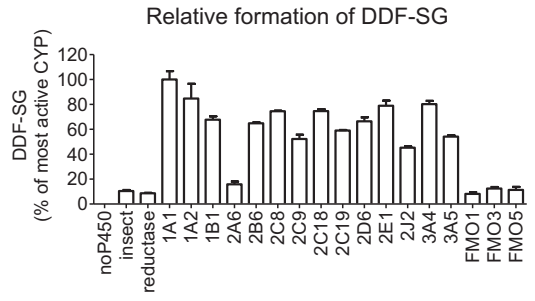

Fig. 5. Formation of DDF-SG in incubations of recombinant P450s and FMOs. Control microsomes without reductase and CYPs (insect) or expressing reductase and cytochrome b5 (reductase) were also evaluated.

controls. Heat denaturation of CYP2C9 even increased DDF-SG formation by more than 2-fold, clearly indicating that regular oxidative biotransformation is not applicable in formation of this GSH conjugate. No DDF-SG was detected when P450 reactions were performed with heat-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 amounts of formation of diclofenac metabolites under different experimental conditions.

Controls	CYP2C9			CYP3A4		
	4′-OH-DF	M2 + M5	DDF-SG	5-OH-DF	M1 + M3	DDF-SG
Complete	100(±4)[a]	100(±10)	100(±3)	100(±3)	100(±2)	100(±3)
−P450	ND[b]	ND	ND	ND	ND	ND
−GSH	94(±9)	ND	ND	376(±109)	ND	ND
−GST P1-1	92(±5)	25(±2)	ND	434(±127)	6(±1)	ND
−NADPH	ND	ND	108(±12)	ND	ND	63(±15)
P450 inhibition						
−Inhibitor	100(±3)	100(±7)	100(±4)	ND	100(±3)	100(±1)
+Inhibitor[c]	51(±13)	30(±5)	105(±2)	ND	1(±2)	46(±1)
Heat treatment						
−Boiling	100(±4)	100(±2)	100(±3)	100(±21)	100(±1)	100(±2)
Boiled P450	ND	ND	242(±35)	ND	6(±1)	54(±6)
Boiled GST	99(±6)	23(±2)	ND	814(±146)	7(±1)	ND

[a] 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).
[b] ND, not detected.
[c] Ketoconazole (2 μM) was used as inhibitor for CYP3A4, whereas sulfaphenazole (20 μM) was used to inhibit CYP2C9.

3.4. Formation of DDF-SG in incubations of horseradish peroxidase

Previously, hemeproteins have been found to perform oxidative reactions by mechanisms of peroxidases [25,26]. Therefore, incubations of DF were also conducted with HRP, which is known to catalyze one-electron oxidations [27]. 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_2O_2 (Fig. 6). Although incubations were performed with 10 mM GSH, no DDF-SG was detected in absence of HRP or GST P1-1, ruling out direct substitution of one of the chlorines of DF by GSH. However, product formation in reactions without H_2O_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 completely 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.

3.5. 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 may be required for DDF-SG formation. To investigate this, several hemeproteins were incubated with DF in presence of GSH and GST P1-1 (Fig. 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 (Fig. 7).

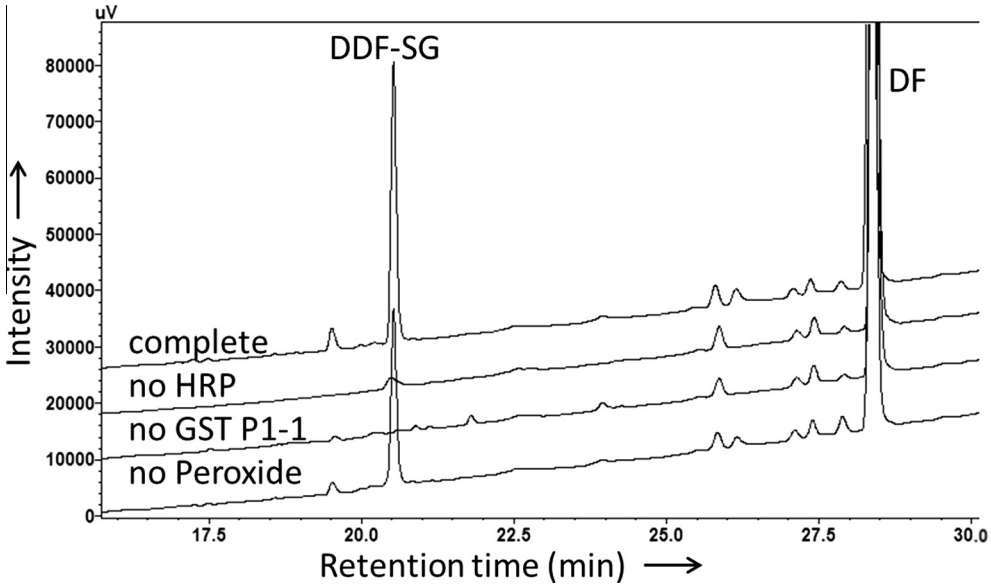

Fig. 6. HPLC–UV analysis of diclofenac conjugate DDF-SG in incubations of HRP. Shown UV traces (254 nm) are from incubations in which HRP, GST P1-1 or peroxide was omitted. A reaction containing all components required for catalytically active HRP (complete) is also shown.

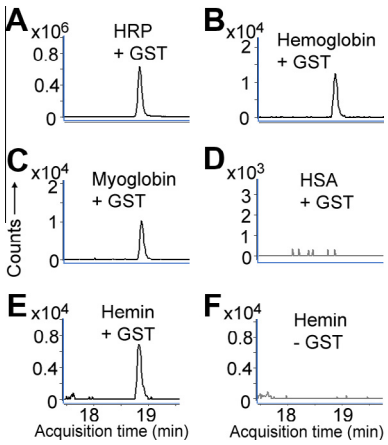

Fig. 7. Ion traces of DDF-SG (m/z 567.1) from diclofenac incubations performed with different (heme)proteins and hemin. Shown traces are from horseradish peroxidase (A), hemoglobin (B), myoglobin (C), human serum albumin (D) and hemin (E) incubated in presence of GSH and GST P1-1. (F) DF incubations of hemin in which GST P1-1 was omitted.

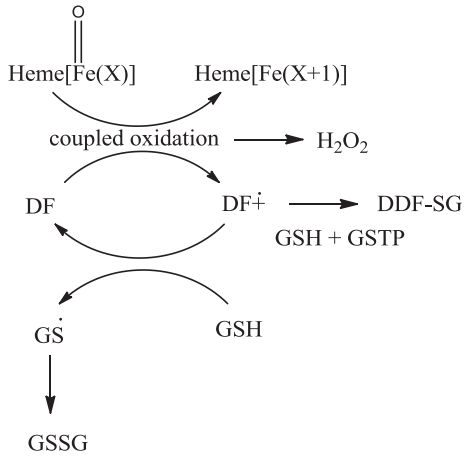

Fig. 8. Proposed mechanism for the bioactivation route of diclofenac that results in formation of DDF-SG.

3.6. 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].

4. 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 (Fig. 1). The aim of the present paper was to elucidate the mechanism by which DF bioactivation might result in 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 (Fig. 5). P450-expressing microsomes showed significantly higher levels of DDF-SG than FMOs.

To evaluate whether an imine-methide intermediate of DF is responsible for DDF-SG formation (Fig. 1), P450 incubations were also performed in phosphate buffer prepared with D_2O. 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 the product was observed in absence of NADPH and in reactions of heat-inactivated P450s (Table 1). The finding that other hemeproteins and protoporphyrin IX could also catalyze DDF-SG formation (Fig. 7) strongly suggests that this conjugate results from a DF radical cation formed by one-electron oxidation followed by GST P1-1 catalyzed GSH conjugation. Unambiguous evidence for the involvement of a radical cation of DF may be obtained from electron spin resonance studies.

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 [28,29]. 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 [26,30]. In the aforementioned studies, peroxide was used to form higher oxidation states of the hemeproteins, which is necessary for one-electron oxidation to occur [25,29]. In the present study, however, DDF-SG was formed when reactions of HRP and other hemeproteins were incubated in absence of hydrogen peroxide (Figs. 6 and 7). To evaluate a potential role for H_2O_2 in DDF-SG formation, CYP2C9 incubations were also supplemented with catalase (200 U/mL) or with 500 μM H_2O_2. 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 H_2O_2 (Fig. 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 [31–34]. Alternatively, the radical intermediate of DF might result from autoxidation. However, DDF-SG could not be detected in absence of hemeproteins (Figs. 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 [35]. 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 H_2O_2 resulted in co-oxidation of NADH, which they explained by formation of prooxidant radicals from these drugs [36]. 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 H_2O_2 to DF incubations with rat hepatocytes significantly increased lipid peroxidation and GSSG formation [37]. Reduction of DF radical cations by concomitant oxidation of

GSH regenerates DF, Fig. 8. The resulting redox cycling may deplete cellular GSH pools, impairing cellular defenses and disrupting the normal redox balance [38]. Besides reactions involving GSH, we have previously shown that the putative radical cation of DF also adducts to cysteine residues of a model protein [21]. Because of its apparent preference for cysteine thiolates, the radical cation of DF may react with highly nucleophilic cysteines in catalytic triads of proteins such as the molecular chaperone protein disulfide isomerase. The essential dithiol pairs in this protein each contain a reactive cysteine, modification of which was shown to result in complete enzyme inactivation [39]. In the present study and in our previous work, we have used an *in vitro* experimental approach to investigate GSH conjugation and protein modification of the radical cation of DF [21]. Therefore, it remains to be established whether thiol modification by the radical cation of DF is relevant *in vivo* and whether this mechanism contributes 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, Fig. A1). This may provide a likely explanation for the fact that DDF-SG was previously not observed in previous DF studies using recombinant P450s or human liver microsomes [14,16,17,19,35]. Nevertheless, by analysis of concentrated DF incubations using a sensitive LC–MS method, DDF-SG was previously also detected in human liver microsomes [20]. This finding suggests that microsomal GSTs might also catalyze formation of this conjugate.

In conclusion, as the mechanism of DDF-SG formation we propose a nucleophilic aromatic substitution reaction of the chlorine atom of the DF radical cation formed by heme-iron containing proteins. 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 [40]. However, unlike GSH conjugation to 1-chloro-2,4-dinitrobenzene, which also occurs non-enzymatically to significant extent at pH 7.4, the chlorine substitution of the proposed DF radical cation requires activation of GSH (to GS^-) by GSTs. Interestingly, a fully GST P1-1-dependent chlorine substitution reaction was previously observed for the clozapine nitrenium ion [24]. Hence, this type of GST-catalyzed substitution reactions could also apply to other chlorinated reactive drug metabolites. The high selectivity of the radical cation of DF for activated cysteine residues in proteins might be of toxicological relevance, since activated cysteines generally have high functional importance [41,42]. The cytotoxicity of DF might be the result of the combination of the different P450-dependent reactive intermediates. However, the occurrence of the novel bioactivation mechanism *in vivo* remains to be established by analyzing DDF-SG and/or corresponding thioethers in bile or urine of DF-exposed individuals, or by analyzing corresponding protein adducts.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbi.2013.11.001.

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