Chapter 4

Human NAD(P)H:quinone oxidoreductase 1 (NQO1)-mediated inactivation of reactive quinoneimine metabolites of diclofenac and mefenamic acid

Reprinted with permission from:


Copyright 2014 American Chemical Society
doi:10.1021/tx400431k
ABSTRACT

NAD(P)H:quinone oxidoreductase 1 (NQO1) is an enzyme capable of reducing a broad range of chemically reactive quinones and quinoneimines (QIs) and can be strongly upregulated by Nrf2/Keap1-mediated stress responses. Several commonly used drugs implicated in adverse drug reactions (ADRs) are known to form reactive QI metabolites upon bioactivation by P450, such as acetaminophen (APAP), diclofenac (DF), and mefenamic acid (MFA). In the present study, the reductive activity of human NQO1 toward the QI metabolites derived from APAP and hydroxy-metabolites of DF and MFA was studied, using purified bacterial P450 BM3 (CYP102A1) mutant M11 as a bioactivation system. The NQO1-catalyzed reduction of the QI metabolites was quantified relative to spontaneous glutathione (GSH) conjugation. Addition of NQO1 to the incubations strongly reduced the formation of all corresponding GSH conjugates, and this activity could be prevented by dicoumarol, a selective NQO1 inhibitor. The GSH conjugation was strongly increased by adding human GSTP1–1 in a wide range of GSH concentrations. Still, NQO1 could effectively compete with the GST catalyzed GSH conjugation by reducing the QIs. In conclusion, we identified the QI metabolites of the 4′- and 5-hydroxy-metabolites of DF and MFA as novel substrates for human NQO1. NQO1-mediated reduction proves to be an effective pathway to detoxify these QI metabolites in addition to GSH conjugation. Genetically determined deficiency of NQO1 therefore might be a risk factor for ADRs induced by reactive QI drug metabolites.

INTRODUCTION

The human NAD(P)H:quinone oxidoreductase 1 (NQO1) is a flavoenzyme capable of reducing a broad range of substrates via two-electron reduction. Known substrates include quinones, quinoneimines (QI), glutathionyl-substituted naphthoquinones, azo-, and nitro-compounds (1). Depending on the chemical properties of the substrate and the product formed, reduction by NQO1 can be either considered a bioactivation or detoxification route. Well-studied bioactivation reactions catalyzed by human NQO1 are those of antitumor drugs containing a quinone pharmacophore, such as mitomycin C, β-lapachone, and 17-N-allylamino-17-demethoxygeldanamycin (17-AAG). Upon reduction by NQO1, these drugs form unstable hydroquinones that may either alkylate biomolecules like DNA or undergo redox-cycling reactions resulting in the generation of reactive oxygen species (ROS) (2). In contrast, formation of a stable hydroquinone product by NQO1 may prevent the toxicity of chemically reactive substrates or avoid one-electron reactions that may otherwise lead to reactive semiquinones and ROS (1). Besides this catalytic role, NQO1 is involved in multiple biological processes such as direct scavenging of superoxide anion radicals and the regulation of proteasomal degradation of specific proteins like the tumor suppressor p53 (3, 4).

Two single nucleotide polymorphisms of NQO1 have been identified within the human population, C609T (NQO1*2 allele) and C465T (NQO1*3 allele). The frequency of the NQO1*2 allele ranges from 16% in Caucasians to 49% in Chinese populations (5). Individuals carrying the NQO1*2/*2 genotype show very low or even undetectable levels of the mutant protein, resulting from an accelerated degradation of the protein by polyubiquitination and subsequent proteasomal
Chapter 4  
NQO1-mediated inactivation of reactive metabolites of diclofenac and mefenamic acid

degradation (2). Furthermore, heterozygous NQO1*1/*2 will likely have a lower NQO1 expression level compared to that of NQO1*1/*1 (6). The NQO1*2 polymorphism has been associated with an increased risk to benzene toxicity and increased susceptibility to cancer (7, 8). The effect of the NQO1*3 polymorphism on the phenotype is not extensively studied. Although comparable in stability to the wild type, changes in activity were shown, most notably a decreased activity for mitomycin C (9). The allele frequency of NQO1*3 is much lower, the highest frequency (0.05) being reported in Caucasians (10).

For several drugs causing adverse drug reactions (ADRs), including rare idiosyncratic drug reactions (IDRs), bioactivation to reactive QI metabolites is believed to play an important role (11, 12). NQO1-catalyzed reduction of these QI metabolites may therefore play a protective role, and consequently, the genetically determined deficiency of NQO1 may be a risk factor in these IDRs. Moreover, expression of NQO1 is regulated by its antioxidant response element (ARE) and can be strongly upregulated by Nrf2/Keap1-mediated stress responses (1, 4). NQO1 expression is thus likely to be induced by chemically reactive drug metabolites and has been shown to be strongly increased by, e.g., overdose with the hepatotoxic drug acetaminophen (paracetamol, APAP) (13).

Probably one of the most-studied examples of drugs causing IDRs is diclofenac (DF). This nonsteroidal anti-inflammatory drug (NSAID) has been implicated in cases of rare but severe liver toxicity (14). Although the precise mechanism underlying this toxicity is unknown, bioactivation of DF into reactive metabolites is believed to play an important role. DF mainly undergoes glucuronidation by human UGT2B7 to reactive acyl glucuronides, which appear to be able to covalently modify proteins (15, 16). Oxidative metabolism of DF by CYP2C9 results in the formation of the major metabolite 4’-hydroxy-diclofenac (4’-OH-DF), which can be further oxidized enzymatically to the reactive diclofenac-1’,4’-quinoneimine (DF-1’,4’-QI) (17). A minor oxidative metabolite, 5-hydroxy-diclofenac (5-OH-DF), is formed by CYP3A4 and can autoxidize nonenzymatically to diclofenac-2,5-quinoneimine (DF-2,5-QI) (18). Both DF-1’,4’-QI and DF-2,5-QI react to GSH producing different GSH conjugates (19-22). Interestingly, only DF-2,5-QI was shown to bind covalently to proteins (18, 23).

Mefenamic acid (MFA) is an NSAID shown to be nephrotoxic in some patients (24). Moreover, several nonclinical studies indicate that MFA is also able to induce liver toxicity both in vivo and in vitro (25-27). In humans, MFA is directly and extensively metabolized to an acyl glucuronide and oxidized to 3’-hydroxymethyl and 3’-carboxyl metabolites, both of which are further converted into the corresponding acyl glucuronides (28). Additionally, GSH conjugates of MFA have been detected in vitro suggesting the formation of reactive metabolites (29). Recently, the formation of 4’-hydroxy- and 5-hydroxy-mefenamic acid (4’-OH-MFA and 5-OH-MFA) has been shown using recombinant human P450s and human liver microsomes (HLM), although these pathways are only minor compared to the formation of 3’-hydroxymethyl-MFA (30). The 4’-OH-MFA and 5-OH-MFA metabolites are further oxidized by P450 to their corresponding QIs, which are subsequently conjugated to GSH, analogous to the reactive DF metabolites (Fig. 1B).
Figure 1. Metabolic schemes describing the formation of QI metabolites and derived GSH conjugates of (A) DF, (B) MFA, and (C) APAP, and the proposed role of NQO1.

Although not idiosyncratic in nature, APAP overdose is a leading cause of liver failure (33). At low dose, APAP is primarily glucuronidated and sulfated. Because of cofactor limitation, oxidative metabolism by CYP2E1, 1A2, 2D6, and 3A4 becomes more important at a high dose of APAP, resulting in the formation of the cytotoxic NAPQI (34, 35). NAPQI can be reduced by NQO1 (31, 32) and GSH (36), and be conjugated to GSH resulting in the formation of 3-glutathionyl-
paracetamol (APAP-SG) (Fig. 1C). Hepatotoxicity results when these protective mechanisms have become insufficient to protect critical cellular target proteins against NAPQI.

In the present study, the NQO1-catalyzed inactivation of the QIs formed from the hydroxy-metabolites of DF and MFA was determined indirectly by measuring the NQO1-dependent reduction of GSH conjugates. Reduction of NAPQI was used as a positive control. The results of the present study show that human NQO1 can reduce these QIs, thereby strongly decreasing their GSH conjugation. Furthermore, the ability of NQO1 to compete with human GSTP1-1 catalyzed GSH conjugation was investigated. This GST isoform is reported to have a high catalytic activity toward the QIs of APAP and DF (36, 37). The results illustrate that NQO1 reduction of the QI metabolites is an efficient additional detoxification pathway in addition to GST catalyzed GSH conjugation.

MATERIALS AND METHODS

Chemicals and enzymes
4′-OH-MFA and 5-OH-MFA were produced enzymatically by a large-scale incubation of mefenamic acid with P450 BM3M11 and subsequent isolation of oxidative metabolites by preparative HPLC. The details of the biosynthesis of MFA metabolites will be described elsewhere (30). APAP-SG was synthesized based on a previously published method and purified using preparative TLC (38). 4′-OH-DF and 5-OH-DF were purchased from Toronto Research Chemicals (North York, Canada). The bacterial cytochrome P450 102A1 mutant M11 (P450 BM3M11) and human GSTP1-1 were expressed and purified as described previously (39, 40). Specific activity of P450 BM3M11, as determined by benzyloxyresorufin O-dealkylation (BROD) activity, was 5.4 nmol resorufin/min/nmol P450. The specific activity of GSTP1-1, measured by GSH conjugation of 1-chloro-2,4-dinitrobenzene, was 48 μmol CDNB/min/mg protein. All other reagents and chemicals were of analytical grade and purchased from standard commercial suppliers.

Cloning, Expression, and Purification of Human NQO1
Plasmid pCMV6-XL5, carrying the human NQO1 gene, was obtained from OriGene Technologies (SC119599; Rockville, MD). The NQO1 gene was excised from its original vector by performing a restriction digest using NcoI (blunted with a Klenow reaction) and NotI, and subcloned into the pET28(a)+ vector using BamHI (blunted with a Klenow reaction with a deoxynucleotide (dNTP) mix) and NotI restriction sites. The final construct encoding His-tagged human NQO1 was transformed into competent E.coli BL21 cells.

Overnight precultures of the BL21 cells containing the expression plasmid for human NQO1 were prepared in 15 mL of 2YT (1.6% (w/v) tryptone, 1.0% yeast extract (w/v), 0.8% NaCl (w/v)) medium supplemented with 30 μg/mL kanamycin, and grown at 37 °C and 175 rpm. For large scale enzyme expression, 600 mL of 2YT medium with 30 μg/mL kanamycin was inoculated with the overnight culture, and cells were grown at 37 °C until the OD600 value reached 0.6. Enzyme expression was induced by the addition of IPTG to a final concentration of 1 mM, and cells were incubated
overnight at 24 °C and 150 rpm. Cells were harvested by centrifugation (20 min, 4000g, 4 °C), resuspended in icecold KPi-glycerol buffer (100 mM potassium phosphate (KPi), pH 7.4, 10% (v/v) glycerol), and disrupted using Avestin Emulsiflex C3. The cytosolic fraction was separated from the membrane fraction by ultracentrifugation in a Beckmann 70Ti rotor (70 min, 120,000g, 4 °C).

His-tagged NQO1 protein was isolated using HIS-select nickel affinity gel (Sigma-Aldrich) according to the large-scale batch purification protocol provided by the supplier. Briefly, 2 mL of affinity gel was washed and equilibrated in 20 mL of equilibration buffer (KPi-glycerol, 0.3 M NaCl, pH 7.4). After centrifugation, the equilibrated affinity gel was added to the cytosolic fraction (supplemented with NaCl to a final concentration of 0.3 M) and incubated for 15 min on an orbital shaker. The affinity gel was washed with 10 gel volumes of equilibration buffer supplemented with 10 mM imidazole (pH 7.4). Washing was repeated until A$_{280}$ of the eluate no longer decreased. His-tagged protein was eluted twice by adding 2 gel volumes of equilibration buffer (supplemented with 250 mM imidazole, pH 7.4) and incubating for 10 min. Imidazole was removed from the pooled eluates by washing 4 times with 15 volumes of KPi-glycerol buffer supplemented with 0.1 mM EDTA using a Vivaspin 20 filtration tube (10,000 MWCO PES, Sartorius, 4000g). Protein concentration was determined using the bicinchoninic acid method (Pierce BCA protein assay kit, Thermo Scientific).

NQO1 activity was confirmed using 2,6-dichlorophenolindophenol (DCPIP). Briefly, incubations with 40 μM DCPIP, 100 nM NQO1, and 1 mM NADPH in 100 mM KPi buffer (pH 7.4) were performed at 24 °C. Reduction of DCPIP was determined by measuring the decrease in A$_{600}$ in time, resulting in a specific activity of 28 μmol DCPIP/min/mg protein. DCPIP reduction was completely inhibited by the addition of 20 μM dicoumarol.

**Reduction of QI Metabolites of APAP, DF, and MFA by Human NQO1**

In order to study the reducing activity of human NQO1 toward the different QI-metabolites, the substrates 4′-OH-DF, 5-OH-DF, 4′-OH-MFA, 5-OH-MFA, and APAP were bioactivated by purified P450 BM3M11, a CYP102A1 mutant engineered to mimic the drug-metabolizing potential of human CYPs for a range of drugs including APAP and DF (39). Using this bioactivation system, the contribution of confounding microsomal proteins present in HLM can be avoided. For each substrate, a concentration of P450 BM3 was used that resulted in rates of bioactivation similar to that observed in incubations with 2 mg/mL HLM, as determined by the total amount of GSH conjugates formed (data not shown). Substrates (50 μM 4′-OH-DF, 5-OH-DF, 4′-OH-MFA, and 5-OH-MFA, and 500 μM APAP) were incubated with P450 BM3M11 (25 nM for 4′-OH-DF, 5-OH-DF, 5-OH-MFA, and APAP, and 5 nM for 4′-OH-MFA to avoid complete substrate depletion).

Incubations were performed in 100 mM KPi buffer (pH 7.4) with 0.5 mM GSH and varying concentrations of NQO1 in a final volume of 100 μL and at 24 °C. At this temperature, we previously demonstrated the role of human GST’s in GSH conjugation of reactive metabolites of clozapine and diclofenac when using P450 BM3 as the bioactivating system (37, 40). To demonstrate that the reduction of GSH conjugates resulted from NQO1-activity, rather than direct scavenging of reactive QIs, incubations were also performed with 50 μM dicoumarol, a known inhibitor of NQO1.
Reactions were initiated by the addition of a NADPH regenerating system (250 μM NADP⁺, 20 mM glucose-6-phosphate, and 2 U/mL glucose-6-phosphate dehydrogenase). NADPH oxidation during preliminary incubations was determined continuously by monitoring the absorbance at 340 nm, which showed that NADPH concentrations remained well above the $K_m$ values of NADPH reported for P450 BM3 and human NQO1 \((41, 42)\). Incubations were terminated after 30 min by adding 100 μL of cold methanol or 10 μL of a 10% (v/v) perchloric acid solution for APAP incubations. Reactions were cooled on ice and subsequently centrifuged (10 min 20,800g). The supernatant was either stored at −20 °C or directly analyzed using HPLC or LC/MS as described below.

**Competition between NQO1 Reduction of QIs and GSTP1-Catalyzed GSH Conjugation**

To compare the rates of NQO1 reduction of reactive QI drug metabolites with GST-catalyzed GSH conjugation, incubations with purified human GSTP1-1 (5 μM) and NQO1 (1 μM) were performed as described in the section above. GSH concentration was, however, lowered to 100 μM to minimize nonenzymatic GSH conjugation relative to the enzymatic reaction, as was previously described for NAPQI \((36)\). Samples were analyzed as described below.

**Analytical Methods**

APAP incubations were analyzed using HPLC on a Shimadzu Prominence LC-20 HPLC system with a Shimadzu SPD-20A UV/vis detector set at 254 nm. APAP and its GSH conjugate (APAP-SG) were separated on a Varian ChromSpher 5 C18 column (G100 × 3.0 mm) using an isocratic method (18% methanol and 0.1% trifluoroacetic acid, 0.5 mL/min). Data analysis was performed using the Shimadzu LC solution software. APAP-SG was quantified using an analytical standard prepared for this study.

4′-OH-DF, 5-OH-DF, 4′-OH-MFA, 5-OH-MFA, and their metabolites were analyzed using LC/MS with parallel UV detection at 254 nm. Separation was performed using an Agilent 1200 Series Rapid resolution LC system with a Phenomenex Luna 5 μm C18(2) column (150 × 4.6 mm), protected by a Phenomenex security guard (5 μm) C18 guard column (4.0 mm × 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). For 4′-OH- and 5-OH-DF, the following gradient was programmed: first 1 min, isocratic 1% B; 1 to 23 min, a linear increase to 99% B; 23 to 23.5 min, a linear decrease to 1% B; and 23.5 to 30 min, maintained at 1% B for re-equilibration. For 4′-OH- and 5-OH-MFA, the gradient was set up as follows: first 1 min, isocratic 5% B; 1 to 30 min, a linear increase to 100% B; 30 to 30.5 min, a linear decrease to 5% B; and 30.5 to 40 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. 4′-OH-5′-SG-MFA was quantified using authentic standards prepared by complete
conversion of varying amounts of 4′-OH-MFA by 250 nM P450 BM3M11 and 5 mM GSH, yielding a single product corresponding to 4′-OH-5′-SG-MFA. The sum of the GSH conjugates derived from either 4′-OH-DF, 5-OH-DF, or 5-OH-MFA was quantified by calculating the corresponding substrate consumption.

RESULTS

Reduction of NAPQI by NQO1

NAPQI, a previously reported substrate for NQO1, was included as a positive control to validate the methods used in this study. Bioactivation of APAP by P450 BM3M11 in the presence of GSH resulted in the formation of a single GSH conjugate (APAP-SG) derived from NAPQI, the QI metabolite of APAP (Fig. 1C). Using GSH concentrations ranging from 100 μM up to 5 mM, APAP-SG formation increased with increasing concentrations of GSH, although approaching saturation above 0.5 mM GSH (Fig. 2A). At all GSH concentrations tested, addition of 1 μM NQO1 strongly reduced the formation of APAP-SG. Moreover, NADPH oxidation was determined spectrophotometrically at 340 nm, using 0.5 mM NADPH without regeneration. The rate of NADPH oxidation observed upon incubation of APAP with P450 BM3M11 was increased by the addition of NQO1 (data not shown).

The nonenzymatic GSH conjugation of NAPQI, assessed in more detail at 0.5 mM GSH, was decreased by NQO1 in a concentration-dependent manner (Fig. 2B). Also, the addition of dicoumarol, a well-known inhibitor of NQO1, significantly prevented the decrease in APAP-SG formation caused by NQO1. This confirms the catalytic role of NQO1 in the reduction of NAPQI rather than scavenging of NAPQI by the NQO1-protein.

Figure 2. Effect of NQO1 on APAP-SG formation after 30 min of incubation of APAP (500 μM) with P450 BM3M11 (25 nM). (A) Formation of APAP-SG without NQO1 (control, ●) and with 1 μM NQO1 (○) at various concentrations of GSH. Each replicate (n = 2) is shown; lines do not represent fits to the data and are only intended to guide the eye. (B) Formation of APAP-SG in incubations containing 0.5 mM GSH. Bars represent the means ± SD (n = 3).
Reduction of QI Metabolites of 4′-OH-DF and 5-OH-DF by NQO1

Bioactivation of 4′-OH-DF by P450 BM3M11 in the presence of 0.5 mM GSH yields 4′-OH-3′-SG-DF with a single protonated molecular ion \([M+H]^+\) of \(m/z\) 617.08 and the dechlorinated 4′-OH-2′-SG-DF with \(m/z\) 583.12 as reported previously (Fig. 1A) (43). Addition of NQO1 to the incubations with 4′-OH-DF showed a more than 90% decreased amount of both 4′-OH-3′-SG-DF and 4′-OH-2′-SG-DF (Fig. 3A) indicating efficient reduction of DF-1′,4′-QI. Addition of dicoumarol partially prevented the reduction of GSH conjugates, confirming the catalytic role of NQO1.

Bioactivation of 5-OH-DF resulted in two GSH conjugates with an \(m/z\) of 617.08, identified as 5-OH-4-SG-DF and 5-OH-6-SG-DF. As shown in Fig. 3B, addition of NQO1 to incubations with 5-OH-DF resulted in about 70% reduction of 5-OH-4-SG-DF and 5-OH-6-SG-DF, which also could be reversed to a significant extent by the addition of dicoumarol. As shown in Fig. 3, the reduction by NQO1 was significantly more efficient for DF-1′,4′-QI than for DF-2,5-QI.

![Figure 3. Effect of NQO1 on GSH conjugate formation after 30 min of incubation of 4′-OH-DF and 5-OH-DF with P450 BM3M11 (25 nM) in the presence of 0.5 mM GSH. For each substrate, the sum of the GSH conjugates is quantified to illustrate total QI formation. (A) Sum of 4′-OH-3′-SG-DF and 4′-OH-2′-SG-DF formation upon bioactivation of 4′-OH-DF (50 μM). (B) Sum of 5-OH-4-SG-DF and 5-OH-6-SG-DF formation upon bioactivation of 5-OH-DF (50 μM). Bars represent the means ± SD (n = 3). Results of the individual GSH conjugates showed the same effects of NQO1 and dicoumarol as observed for the total of GSH conjugates.](image)

Reduction of QI Metabolites of 4′-OH-MFA and 5-OH-MFA by NQO1

Bioactivation of 4′-OH-MFA by P450 BM3M11 in incubations containing 0.5 mM GSH yielded a single GSH conjugate with a protonated molecular ion of \(m/z\) 563.18, which corresponds to the recently elucidated 4′-OH-5′-SG-MFA (MG1) formed in HLM incubations (Fig. 1B) (30). Coincubation with NQO1 resulted in a 80% decrease of 4′-OH-5′-SG-MFA, which could be significantly reversed by inhibiting NQO1 with dicoumarol (Fig. 4A). P450 BM3M11 metabolism of 5-OH-MFA in the presence of 0.5 mM GSH produced two GSH conjugates with a protonated molecular ion \([M+H]^+\) of \(m/z\) 563.18, previously identified as MG2 and MG3 in HLM incubations (Fig. 1B) (30). The sum of these GSH conjugates was decreased by more than 80% by NQO1, indicating the reduction of MFA-2,5-QI (Fig. 4B). Reduction of MFA-2,5-QI was clearly inhibited by dicoumarol confirming the catalytic role of NQO1.
NQO1-mediated inactivation of reactive metabolites of diclofenac and mefenamic acid

**Figure 4.** Effect of NQO1 on GSH conjugate formation after 30 min of incubation of 4′-OH-MFA and 5-OH-MFA in the presence of 0.5 mM GSH. For each substrate, the sum of the GSH conjugates is quantified to illustrate total QI formation. (A) 4′-OH-5′-SG-MFA (MG1) formation upon bioactivation of 4′-OH-MFA (50 μM) by P450 BM3M11 (5 nM, to avoid complete substrate depletion during incubation). (B) Sum of MG2 and MG3 formation upon bioactivation of 5-OH-MFA (50 μM) by P450 BM3M11 (25 nM). Bars represent the means ± SD (n = 3). Results of the individual GSH conjugates showed the same effects of NQO1 and dicoumarol as observed for the total of GSH conjugates.

**NQO1 Competes with GSTP1-1-Catalyzed GSH Conjugation of Reactive QI Metabolites**

To evaluate the intrinsic activity of NQO1 in the detoxification of reactive QIs, NQO1 reduction was compared with GST-catalyzed GSH conjugation. Incubations with NQO1 were performed in the presence of human GSTP1-1, which was previously shown to be the most active GST involved in inactivation of NAPQI and the QIs of DF (36, 37). Addition of 5 μM human GSTP1-1 enhanced the GSH conjugation of NAPQI significantly at all GSH concentrations with the highest effect at 100 μM (Fig. 5A). Although the concentration of NQO1 was 5-fold lower than that of GSTP1-1, it strongly decreased the effect of GSTP1-1 at all GSH concentrations, illustrating an effective competition between NQO1 reduction and GST-catalyzed GSH conjugation (Fig. 5A).

The effect of GSTP1-1 on GSH conjugation of the DF-1′,4′-QI and DF-2,5-QI was very large and showed regioselective behavior consistent with that observed by Dragovic et al. (37). Upon incubation of 4′-OH-DF, formation of 4′-OH-3′-SG-DF was increased 62-fold by GSTP1-1 compared to that of the control, whereas the second GSH conjugate 4′-OH-2′-SG-DF was not detectable (Fig. 5B). Addition of NQO1 lowered the GSTP1-1-catalyzed formation of 4′-OH-3′-SG-DF from 62- to 16-fold, confirming the very efficient inactivation of DF-1′,4′-QI by NQO1.

Formation of 5-OH-4-SG-DF and 5-OH-6-SG-DF from DF-2,5-QI was increased, respectively, 22- and 253-fold by the addition of 5 μM GSTP1-1 (Fig. 5C). Addition of 1 μM NQO1 could efficiently compete with the increased GSH conjugation of DF-2,5-QI by GSTP1-1, reverting GSH conjugation close to control levels for both GSH conjugates. A diglutathionyl conjugate, previously identified as 5-OH-4,6-diSG-DF (37), was formed in 5-OH-DF incubations with GSTP1-1 that was absent in the control incubation without GSTP1-1 (Fig. 6). This metabolite is believed to be the result of secondary oxidation of 5-OH-4-SG-DF and/or 5-OH-6-SG-DF to their corresponding QI and
subsequent GSH-addition (Fig. 7). Formation of 5-OH-4,6-diSG-DF was completely abolished by the addition of 1 μM NQO1. This might be caused by an NQO1-dependent decrease in 5-OH-4-SG-DF and/or 5-OH-6-SG-DF available for subsequent secondary oxidation. Alternatively, a secondary QI formed by (auto)oxidation of 5-OH-4-SG-DF and/or 5-OH-6-SG-DF might be a substrate for NQO1 reduction as well. It was shown previously that also GSH-substituted quinones are substrates for rat NQO1 (44, 45).

Figure 5. Competition between GSTP1-1-catalyzed GSH conjugation and NQO1 reduction of QI metabolites formed after 30 min of incubation of APAP (500 μM), 4′-OH-DF (50 μM), 5-OH-DF (50 μM), and 5-OH-MFA (50 μM) with P450 BM3M11 (25 nM). (A) Formation of APAP-SG without NQO1 or GSTP1-1 (●, dashed line), with GSTP1-1 (■), and with GSTP1-1 and NQO1 (□). Each replicate (n = 2) is shown; lines do not represent fits to the data and are only intended to guide the eye. (B) Formation of 4′-OH-2′-SG-DF (black bars) and 4′-OH-3′-SG-DF (white bars) in the presence of 0.1 mM GSH as the fold over control without NQO1 and without GSTP1-1. (C) Formation of 5-OH-4-SG-DF (black bars) and 5-OH-6-SG-DF (white bars) as the fold over control (0.1 mM GSH). (D) Formation of MG2 as the fold over control (0.1 mM GSH). Bars represent the means ± SD (n = 3).
Formation of MG2 from 5-OH-MFA-QI was increased 6.6-fold by GSTP1-1, which could be lowered to 2.3-fold by the addition of NQO1 (Fig. 5D). NQO1 is thus able to compete with GSTP1-1 for MFA-2,5-QI. In the case of MFA-1′,4′-QI, GSTP1-1 was not able to catalyze the formation of MG1 (data not shown). Therefore, competition experiments between GSTP1-1 and NQO1 were not performed for this substrate.

Figure 6. LC/MS analysis of P450 BM3M11 (25 nM) incubations with 5-OH-DF (50 μM), with GSTP1-1 and/or NQO1. Shown are extracted ion chromatograms (EIC) of control incubation without GSTP1-1 and NQO1 (gray lines), 5 μM GSTP1-1 without NQO1 (black lines), and 5 μM GSTP1-1 combined with 1 μM NQO1 (dashed lines). The upper three traces are EIC of m/z 617.08 (5-OH-4-SG-DF and 5-OH-6-SG-DF, [M+H]⁺); the bottom three traces are EIC of m/z 461.58 (5-OH-4,6-diSG-DF, [M+2H]²⁺).
**Chapter 4  
NQO1-mediated inactivation of reactive metabolites of diclofenac and mefenamic acid**

**DISCUSSION**

The formation of chemically reactive QIs has been implicated in several drug-induced ADRs and IDRs. Reduction of these QIs by NQO1 is therefore likely to play an important role in the protection against these reactive metabolites, especially considering the fact that NQO1 expression can be strongly upregulated by Keap1/Nrf2-mediated stress responses. As genetically determined deficiencies in NQO1 and GSTs may be risk factors in these ADRs and IDRs, we aimed to study the role of NQO1-mediated reduction of reactive QI drug metabolites relative to GSH conjugation catalyzed by human GSTs.

In the present study, efficient reduction of the different QIs of APAP, DF, and MFA by human NQO1 was observed, as reflected by the strongly decreased formation of the corresponding spontaneous GSH conjugates. Inhibition of the NQO1-mediated reduction by dicoumarol confirmed the catalytic role of NQO1 but was only partial. It is known that the concentration of dicoumarol required for effective NQO1 inhibition depends on the affinity of the electron-acceptor substrate (46). For the QIs in the current study, a higher concentration of dicoumarol may be needed for complete NQO1 inhibition than that for DCPIP as the substrate. However, increasing the dicoumarol concentration in control incubations without NQO1 resulted in significantly reduced formation of GSH conjugates, possibly due to the inhibition of P450 BM3M11 or due to the inhibition of glucose-6-phosphate dehydrogenase (data not shown).

Even when the GSH conjugation of the different QIs was strongly increased by the addition of human GSTP1-1, a 5-times lower concentration of NQO1 could efficiently compete with GSTP1-1 in the inactivation of the different QIs (Fig. 5). NQO1 should therefore be considered an important...
pathway in the detoxification of these QIs, in addition to GSH conjugation. Boots et al. previously studied the interplay between NQO1 reduction (at a level relevant to human liver) and chemical GSH conjugation (at 100 μM GSH) using the flavonoid quercetin (47). They showed that the spontaneous GSH conjugation of the oxidized ortho-quinone/quinone methide metabolite was much faster than the reduction by human NQO1. Therefore, the efficiency of NQO1 reduction relative to GSH conjugation is likely substrate-dependent. Reduction of a QI metabolite, formed by bioactivation of a parent compound, will regenerate the respective parent compound, which can be oxidized again or metabolized by phase 2-reactions thereby avoiding QI formation and futile cycling. These alternative phase 2-reactions include, e.g., glucuronidation and sulfation, well-known metabolic pathways for APAP. Glucuronide conjugates of 4’-OH-DF and 5-OH-DF have also been identified in urine of DF-treated patients and mice bile (48, 49). The oxidative 3’-hydroxymethyl- and 3’-carboxyl metabolites of MFA undergo glucuronidation as well, but this has not been reported for 4’-OH-MFA and 5-OH-MFA (28). However, the latter two metabolites have only recently been identified in vitro and have not been studied extensively (30).

NQO1 is ubiquitously expressed in human tissues, although large variations have been observed between individuals and tissues types (50). NQO1 expression is generally considered to be low in normal human liver, as has been shown using, e.g., immunodetection (51). Immunohistochemistry of human liver tissue showed mainly NQO1-staining of interlobular biliary epithelial cells (52). However, absolute quantification of NQO1 has only been performed for lung and colon tissue. The NQO1 activities in these tissues, as reported in several studies, appeared to be in a similar range compared to those measured in human liver samples (Table 1). On the basis of the reported NQO1 activities, the basal expression of NQO1 in human liver is estimated to be around 2 pmol/mg cytosolic protein, corresponding to an estimated intracellular concentration of approximately 0.3 μM (calculated using a hepatic cytosolic protein concentration of 80.7 mg/g liver and an intracellular hepatocyte volume of 0.55 mL/g liver (58, 59)). Because NQO1 is known to be strongly inducible by Nrf2/keap1-mediated stress

### Table 1. NQO1 expression in human tissues reported in literature

<table>
<thead>
<tr>
<th>Tissue origin</th>
<th>DCPIP activity b</th>
<th>Expression c</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>breast</td>
<td>50 ± 11 (17)</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17 ± 4 (24)</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 ± 20 (4)</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32 ± 1</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>colon</td>
<td>1.98 ± 0.23 (6)</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>kidney</td>
<td>117 ± 15 (5)</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>110 ± 15</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17 ± 4 (10)</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>liver</td>
<td>3.7 ± 0.7 (6)</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22 ± 4</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 ± 2 (31)</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.4 ± 4 (3)</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.40 ± 0.19 (7)</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>lung</td>
<td>40 ± 3</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>stomach</td>
<td>387 ± 75 (9)</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>head and neck</td>
<td>19.5 ± 7.2 (8)</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

a Data reported here was obtained from normal, nontumor tissue. Activities reported by ref 56 were obtained from pooled tissue samples and measured in at least triplicate. Data from refs 55 and 57 are expressed as the mean ± SD (n) and data from ref 54 as the mean ± SE (n).
b nmol 2,6-dichlorophenol indophenol (DCPIP)/min/mg cytosolic protein.
c pmol NQO1/mg cytosolic protein.
responses, a much higher hepatic concentration is expected in conditions of chemically induced stress. Indeed, hepatic NQO1 expression has been reported to be 16-fold induced in the case of liver injury after APAP overdose (13). Our results indicate that at concentrations estimated to reflect chemically induced stress, NQO1 efficiently reduces reactive QI drug metabolites and likely complements GSH conjugation in the detoxification of these QIs in vivo to a significant extent.

In addition to hepatotoxicity, DF and MFA have also been associated with ADRs occurring in other tissues. MFA is associated with cases of nephrotoxicity (24). DF, like many other NSAIDs, is commonly implicated in gastrointestinal toxicity, in which P450 metabolism was recently shown to play an important role (53). These tissues express considerably more NQO1 compared to that of the human liver (Table 1) (51). Our results thus demonstrate the potential of NQO1 to also protect these organs from toxicity induced by the QIs derived from DF and MFA. Nevertheless, the metabolic interplay among CYP-, NQO1-, and GST-catalyzed biotransformation observed in the present study remains to be confirmed in a cellular context, where, e.g., GSH is more abundant. Cellular models should reveal if GSH conjugate formation, and ultimately the cytotoxicity, of the reactive QI metabolites of APAP, DF, and MFA is indeed affected by NQO1 expression, the latter manipulated by overexpression or knockdown of the NQO1 gene.

The highest frequency polymorphism of NQO1 (NQO1*2) results in virtually a complete lack of activity because the mutant protein is rapidly degraded, and this polymorphism is correlated to increased risks in cancer and benzene toxicity (7, 8). Therefore, the NQO1*2 polymorphism may also affect the protection against the reactive QI drug metabolites identified as NQO1-substrates in this study. A third known polymorphism (NQO1*3) shows stability similar to that of the wild type, but changes in kinetic parameters have been shown to vary between substrates (9). Thus, the effect of the NQO1*3 polymorphism on the detoxification of the current, newly identified substrates cannot yet be predicted accurately and requires further investigation.

CONCLUSION

To summarize, we identified the reactive QI drug metabolites of the 4′- and 5-hydroxylated metabolites of DF and MFA as novel substrates for human NQO1 and confirmed the previously published reduction of NAPQI by NQO1. Moreover, we showed that NQO1-mediated reduction of these substrates is very efficient when compared with GSH conjugation of the respective QIs, even when the GSH conjugation was catalyzed by human GSTP1-1. Like human GSTs, detoxification of reactive QIs by NQO1 likely contributes to the balance between bioactivation and inactivation of drugs in the cell, and as a consequence modulates the toxicity of the QIs generated by P450 metabolism of drugs. Genetic polymorphisms of NQO1, especially NQO1*2/*2 that can be considered a null phenotype, may thus be a risk factor for ADRs and IDRs linked to these chemically reactive QI metabolites of DF and MFA. The clinical relevance of these findings remains to be investigated.
ACKNOWLEDGEMENTS
This work was partially funded by a grant of Assuring Safety without Animal Testing (ASAT) project 165600001.

APPENDIX
Introduction
Kinetic parameters of APAP metabolism by human CYPs and GSTs were determined to facilitate in silico PBTK modelling of cellular exposure to the reactive NAPQI metabolite within the Assuring Safety without Animal Testing (ASAT) program (60). Synthesis of N-acetyl-p-benzoquinoneimine and 3-(glutathione-S-yl) paracetamol-N-acetyl-p-benzoquinoneimine (NAPQI) was synthesized according to a method described by (61). The resulting NAPQI solution was subsequently used to synthesize 3-(glutathione-S-yl)paracetamol by the method previously described (62) and separated using preparative TLC. The product was positively identified using 1H-NMR and UV-Vis spectrophotometry; results were identical to those described in literature (63, 64). No residual paracetamol or glutathione was detected on TLC and purity was over 95% based on HPLC analysis.

Kinetic study of NAPQI-GSH conjugation by human glutathione S-transferases
A stopped-flow kinetic study of the enzymatic and spontaneous conjugation of NAPQI to glutathione was performed as described previously (65). Small portions of the NAPQI chloroform solution were mixed with acetonitrile and the chloroform was evaporated to obtain NAPQI in acetonitrile. Concentration was determined prior to use by measuring the absorbance at $\lambda_{max}$ 260-264nm, using the previously reported extinction coefficient of $3.33 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ at 260nm. hGSTs A1-1, M1-1, P1-1 and T1-1 were expressed in and purified from E.coli as described previously (66). Stopped-flow kinetics was performed using a two-syringe, pneumatic driven mixer (KinTek Corporation); absorbance was measured on a Pharmacia LKB Ultrospec Plus spectrophotometer equipped with a Shimadzu CBM-20A data acquisition system with the LCsolution software. A solution of reduced glutathione (GSH, 100μM final) either without or with purified hGSTs was mixed with various concentrations of NAPQI (1-20μM final). All measurements were performed at pH6.5 (50mM potassium phosphate buffer) and room temperature. Decay of absorbance at 264nm was recorded over time; measurements were performed in at least triplicate.

Observed enzymatic rate constants were corrected for spontaneous conjugation and used to calculate the initial velocities. Data was analyzed using Graphpad Prism 5. From these data the Michaelis-Menten parameters were derived using non-linear regression (Table A-1). No significant effect of human GST T1-1 on the glutathione conjugation was observed. Intrinsic clearances ($V_{max}/K_M$) were determined from the slopes of these curves, as the curves of these two isoforms remained close to linear over the concentration range studied.

Table A-1. Kinetic parameters of GST-catalyzed NAPQI-GSH conjugation

<table>
<thead>
<tr>
<th></th>
<th>hGSTA1-1</th>
<th>hGSTM1-1</th>
<th>hGSTP1-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (μmol/mg/s)</td>
<td>3.45 ± 0.62</td>
<td>4.00 ± 0.82</td>
<td>1.95 ± 0.16</td>
</tr>
<tr>
<td>$K_M$ (μM)</td>
<td>48.5 ± 11.5</td>
<td>47.3 ± 12.9</td>
<td>11.2 ± 1.79</td>
</tr>
<tr>
<td>$CL_{int}$ (L/s/mg enzyme)</td>
<td>0.066</td>
<td>0.080</td>
<td>0.14</td>
</tr>
</tbody>
</table>
Kinetic study of APAP oxidation by human CYP2E1 and CYP3A4

Using similar incubation methods described under methods and materials earlier in this chapter, Michaelis-Menten parameters of APAP metabolism by recombinant human CYP2E1 and CYP3A4 (Gentest Supersomes, Woburn, MA) were determined by measuring the velocity of APAP-SG formation using HPLC at varying concentrations of APAP. Measurement were performed in duplicate in two independent experiments. Michaelis-Menten curves were constructed by non-linear regression in Graphpad Prism 5. Results are presented in Table A-2.

Table A-2. Kinetic parameters of human CYP-catalyzed APAP oxidation

<table>
<thead>
<tr>
<th></th>
<th>CYP2E1</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (nmol/min/nmol enzyme)</td>
<td>14.6 ± 0.94</td>
<td>11.1 ± 0.40</td>
</tr>
<tr>
<td>$K_M$ (mM)</td>
<td>6.27 ± 0.93</td>
<td>0.23 ± 0.029</td>
</tr>
</tbody>
</table>

Discussion and conclusion

When initiated, the ASAT program identified kinetic parameters of human enzymes involved in the metabolism of APAP from literature, including major pathways such as glucuronidation and sulfation. However, data on GSTs and CYPs was incomplete and/or lacking. The current study described in this appendix aimed to experimentally determine these missing values needed for PBTK modelling (60).

In this study four different hGSTs (A1-1, M1-1, P1-1 and T1-1) have been investigated. However, several other hGST isoenzymes are expressed in the human liver and should ideally be included. Especially with the addition of hGST A2-2, responsible for 24% of total hepatic GST expression, our set of hGSTs would more closely represent the total GST expression in the human liver. By including known mutants of hGSTs the influence of polymorphisms may be studied as well. To obtain enzymatic conjugation rates clearly distinguishable from the spontaneous conjugation, pH, glutathione concentration and temperature were chosen below physiological conditions. As a result kinetic parameters determined are likely to be underestimated. Coles et al. (65) showed a clear pH dependence of the spontaneous glutathione conjugation rate. However, the effects of pH and temperature on the enzymatic rate of NAPQI conjugation have not been studied. Scaling factors towards physiological conditions can possibly be derived from studying the enzymatic conjugation of less reactive GST substrates under multiple conditions.

Although for several human CYPs kinetic parameters of APAP metabolism have been published, we were unable to find clear literature data for human CYP2E1 and CYP3A4. Together with the results described in the first part of this chapter, namely the NQO1-catalyzed reduction of NAPQI and its competition with hGST-catalyzed NAPQI-GSH conjugation, data acquired in these additional studies may aid the construction of an in silico PBTK model used to describe the formation and fate of the reactive NAPQI metabolite. Such a model may aid the understanding of hepatotoxicity induced by APAP, a frequently studied model drug with a known chemically reactive metabolite, NAPQI.
Chapter 4

NQO1-mediated inactivation of reactive metabolites of diclofenac and mefenamic acid

REFERENCES


Chapter 4  NQO1-mediated inactivation of reactive metabolites of diclofenac and mfenamic acid


Chapter 4

NQO1-mediated inactivation of reactive metabolites of diclofenac and mefenamic acid


APPENDIX REFERENCES


