Bioactivation of 3-n-Butylphthalide via Sulfation of Its Major Metabolite 3-Hydroxy-NBP: Mediated Mainly by Sulfotransferase 1A1

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ABSTRACT

3-n-Butylphthalide (NBP) [(±)-3-butyl-1(3H)-isobenzofuranone] is an anti-cerebral ischemia drug. Moderate hepatotoxicity has been observed in clinical applications. One of the major metabolites, 3-N-acetylcyesteine-NBP, has been detected in human urine, indicating the formation of a reactive metabolite. We elucidated the formation mechanism of the reactive metabolite and its association with the hepatotoxicity of NBP. In vitro incubations revealed that 3-glutathione-NBP (3-GSH-NBP) was observed only in fresh rat liver homogenate rather than in liver microsomes, liver cytosol, or liver, 9,000g supernatant supplemented with NADPH and GSH. We also detected 3-GSH-NBP when 3′-phosphoadenosine-5′-phosphosulfate was added in GSH-fortified human liver cytosol (HLC). The formation of 3-GSH-NBP was 39.3-fold higher using 3-hydroxy-NBP (3-OH-NBP) as the substrate than NBP. The sulfotransferase (SULT) inhibitors DCNP (2,6-dichloro-4-nitrophenol) and quercetin suppressed 3-GSH-NBP formation in HLC by 75 and 82%, respectively, suggesting that 3-OH-NBP sulfation was involved in 3-GSH-NBP formation. Further SULT phenotyping revealed that SULT1A1 is the major isozyme responsible for the sulfation. Dose-dependent toxicity was observed in primary rat hepatocytes exposed to 3-OH-NBP, with an IC50 of approximately 168 μM. Addition of DCNP and quercetin significantly increased cell viability, whereas L-buthionine-sulfoximine (a GSH depleter) decreased cell viability. Overall, our study revealed the underlying mechanism for the bioactivation of NBP as is follows. NBP is first oxidized to 3-OH-NBP and further undergoes sulfation to form 3-OH-NBP sulfate. The sulfate spontaneously cleaves off, generating highly reactive electrophilic cations, which can bind either to GSH to detoxify or to hepatocellular proteins to cause undesirable side effects.

Introduction

Sulfation catalyzed by the sulfotransferase (SULT, EC 2.8.2) superfamily is an important phase II conjugation in the metabolism of xenobiotics, the modulation of biologic activity, and the inactivation and elimination of potent endogenous chemicals, including thyroid hormones, steroids, and monoamine neurotransmitters (Weinshilboum, 1986; Coughtrie et al., 1998; Wang and James, 2006). The sulfation pathway is generally considered to be a detoxification pathway, making more polar and water-soluble products and thereby aiding in their excretion via kidneys or bile (Gamage et al., 2006). However, in certain cases, sulfation increases toxicity. It is increasingly recognized that sulfation mediates the bioactivation of many drugs such as tamoxifen and nevirapine via the formation of highly reactive electrophilic species attributed to the good leaving property of the sulfate group (Weinshilboum et al., 1997; Glatt, 2000; Kim et al., 2005; Sharma et al., 2013).

3-n-Butylphthalide (NBP) [(±)-3-butyl-1(3H)-isobenzofuranone] is an oral agent for the treatment of cerebral ischemia (Yan et al., 1998; Chong and Feng, 1999; Peng et al., 2008). The recommended oral dose of NBP is 200 mg taken three times a day, and the treatment course is 10 days (NBP Pharmaceutical, 2009). In 6-month toxicity studies in Sprague-Dawley rats (250 and 500 mg/kg per day NBP) and beagle dogs (500 mg/kg per day NBP), hepatotoxicity was observed, as indicated by elevation in the serum enzyme levels of alanine aminotransferase, alkaline phosphatase, and γ-glutamyl transferase (NBP Pharmaceutical, 2009). Histopathologically, the livers showed evidence of slight hyper trophy and vacuolar degeneration in focal hepatocytes (NBP Pharmaceutical, 2009). Moderate hepatotoxicity, indicated by alanine aminotransferase elevation, was also reported in humans after chronic administration of NBP (Cui et al., 2005, 2008; NBP Pharmaceutical, 2009; Xu and Zhao, 2011); the incidence ranged from 3.0% to 17.5% in phase II, III, and IV clinical trials (Cui et al., 2005, 2008, 2013; NBP Pharmaceutical, 2009). Our previous studies demonstrated that after single administration of 200 mg of NBP soft capsules, NBP underwent extensive metabolism in humans (Diao et al., 2013a,b). No parent drug was detected in the urine or feces, and urinary metabolites accounted for about 81.6% of the administrated dosage (Diao et al., 2013a). Out of the various urinary metabolites, one NAC (N-acetylcyesteine) conjugated metabolite, 3-NAC-NBP, which accounted for 4.7% ± 2.1% of the administered dose, drew our concern (Diao et al., 2013a).

NAC conjugates are generally considered to be derived from glutathione (GSH) conjugates through subsequent hydrolytic cleavage of glutamic acid.
and glycine residues to an S-substituted cysteine (Cys), which undergoes further N-acetylation—the classic mercapturic acid pathway (Clapp and Young, 1970; Renner and Nguyen, 1984; Poon et al., 2001). Abundant excretions of NAC or GSH conjugates in urine are convincing evidence for the presence of reactive metabolites or intermediates, which may be associated with hepatotoxicity (Gopal et al., 2000; Poon et al., 2001; Rinaldi et al., 2002). Here, we illuminate the formation mechanism of a reactive metabolite and evaluate its potential association with the hepatotoxicity of NBP.

Materials and Methods

NBP was kindly provided by the Shijiazhuang Pharmaceutical Company (Shijiazhuang, Hebei, People’s Republic of China). GSH, NAC, Cys, NADPH, DCNP (2,6-dichloro-4-nitrophenol), quercetin, dithiothreitol (DTT), PAPS (3’-phosphoadenosine-5’-phosphosulfate), and BSO (L-buthionine-sulfoximine) were purchased from Sigma-Aldrich (St. Louis, MO). The 3-OH-NBP (3-hydroxy-NBP) was synthesized as described in the literature with some modifications (Yang et al., 2007). Fresh rat liver and kidney homogenates were prepared from male Sprague-Dawley rats as previously described elsewhere (Bjorge and Baillie, 1991). The 3-GSH-NBP was isolated and purified from the biotransformation of 3-OH-NBP (50 μM) in fresh rat liver homogenate supplemented with GSH (1.5 mM). The 3-NAC-NBP was purified from human urine and subjected to NMR analysis. Human liver microsomes (HLM), human liver cytosol (HLC), human liver 9000g supernatant (S9), rat liver microsomes (RLM), rat liver cytosol (RCL), rat kidney microsomes (RKM), rat kidney cytosol (RKC), and rat kidney S9 were supplied by BD Gentest (Woburn, MA). Recombinant human SULT (SULT1A1*1, SULT1A1*2, SULT1A2, SULT1A3, SULT1B1, SULT1E1, and SULT2A1) were purchased from Cypex (Dundee, Scotland, UK). Cryopreserved primary human hepatocytes were obtained from Xeno-Tech (Lenexa, KS).

Trapping of Reactive Metabolite Using GSH, Cys, and NAC. The HLM, HLC, human liver S9, RLM, RLC, and rat liver S9 were thawed carefully on ice before the experiment. Fresh rat liver and kidney homogenates were kept on ice during preparation. The substrates NBP and 3-OH-NBP were dissolved in methanol and diluted with phosphate-buffered saline (PBS; pH 7.4, 100 mM) containing MgCl₂ (0.5 mM) and DTT (8.3 mM). The final concentration of methanol did not exceed 0.1%. NBP and 3-OH-NBP (50 μM) were separately incubated with 1.0 mg protein/ml of human (or rat) subcellular fractions, or 50 μl of freshly prepared rat liver (or kidney) homogenate supplemented with GSH (1.5 mM). The 3-OH-NBP in duplicate with recombinant human SULT (SULT1A1*1, SULT1A1*2, SULT1A2, SULT1A3, SULT1B1, SULT1E1, and SULT2A1) were prepared from male Sprague-Dawley rats as previously described elsewhere (Xie et al., 2013). Each incubation system, different trapping agents (i.e., GSH, NAC, or Cys), were set to a constant 17.5 and 15.0 ml/min, respectively; collagenase type II was used instead of type I (Seglen, 1976; Mohamed and Kaddoumi, 2013; Shukla et al., 2013). The fresh primary rat hepatocytes were also seeded in the 96-well collagen I-coated plates at a density of 3 × 10⁵ cells/ml. Primary rat hepatocytes were prepared with a previously published two-step collagenase perfusion technique with the following modifications: the perfusion buffer and digestion buffer flow rates were set to a constant 17.5 and 15.0 ml/min, respectively; collagenase type II was used instead of type I (Seglen, 1976; Mohamed and Kaddoumi, 2013; Shukla et al., 2013). The fresh primary rat hepatocytes were also seeded in the 96-well collagen I-coated plates at a density of 3 × 10⁵ cells/ml. The primary human or rat hepatocytes were then cultured for 24 hours before the addition of 3-OH-NBP. All cell incubations were performed in triplicate and were maintained at 37°C in a humidified incubator saturated with 95% O₂/5% CO₂.

After 24 hours of incubation with various concentrations of 3-OH-NBP, we determined the cell viability using the Cell Counting Kit-8 reagent (Dojindo Molecular Technologies, Gaithersburg, MD) following the manufacturer’s protocol (Hosomi et al., 2011; Iwamura et al., 2011) and measured at 450 nm. The absorbance of the treated cells was compared with that of the control, in which the cells were exposed only to the vehicle and considered as 100% viable.

We also evaluated the effects of several SULT inhibitors or the GSH depletor on their modulation of the cytotoxicity caused by 3-OH-NBP. These inhibitors included the nonspecific SULT inhibitor DCNP (10 μM) and the SULT1A1&SULT1E1-sensitive inhibitor quercetin (10 μM). The final concentration of BSO, a GSH depletor, was 20 μM (Xie et al., 2013). Each inhibitor or GSH depletor was preincubated with the cells for 30 minutes before the addition of 3-OH-NBP. Various concentrations of 3-OH-NBP (100, 200, and 400 μM) were then incubated with the pretreated cells at 37°C for 24 hours. Cell viability was then measured as mentioned earlier. The groups with the pretreatment of SULT inhibitors or GSH depletor were compared with their controls, which only contained 3-OH-NBP. The media from the incubations were collected and subjected to analysis of 3-GSH-NBP and 3-Cys-NBP by UPLC-Q-TOF MS.

Statistical Analysis. The formation rate of 3-GSH-NBP in subcellular fractions and recombinant human SULT was calculated as

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V \text{ (pmol/min/mg protein) } = \frac{C1 \mu M \times V (\mu l)}{C2 \text{ (mg/ml)} \times \bar{V} (\mu l) \times T (min)}
\]

where C1 (μM) and C2 (mg/ml) are the concentrations of 3-GSH-NBP and protein in the incubation sample, respectively; V (μl) is the incubation volume; and T (min) is the incubation time.

Data from culture of hepatocytes are presented as mean ± S.D. values. Differences between the means were tested by one-way analysis of variance with unpaired two-tailed Student’s t test using the Statistical Package for the Social Sciences version 20.0 software (SPSS, Inc., Chicago, IL). *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant.

Results

Trapping and Detection of Reactive Metabolite of NBP. First, we examined the MS fragmentation patterns of 3-NAC-NBP and 3-GSH-NBP.
3-NAC-NBP and 3-GSH-NBP exhibited \([M + H]^+\)/[M + Na]^+ ions at \(m/z\) 352.123/374.102 and 496.173/518.154 in electrospray ionization (+), respectively. 3-NAC-NBP and 3-GSH-NBP shared fragmentation properties similar to that of 3-OH-NBP (Diao et al., 2013a): cleavage occurred mainly on the C–S bond and the \(n\)-butyl side chain, generating major product ions at \(m/z\) 189.089 and 133.027 (Fig. 1, A and C). Detailed tentative fragmentation is demonstrated in the insets of Fig. 1, A and C. In electrospray ionization (–), 3-NAC-NBP and 3-GSH-NBP displayed a \([M – H]^-\) ion at \(m/z\) 350.107 and 494.158, respectively. The primary fragment ions of 3-NAC-NBP were 221.065 and 177.075, which were attributed to the cleavage of the C–S bond and the GSH moiety (Fig. 1D).

To elucidate the formation pathway of 3-NAC-NBP and its precursor, 3-GSH-NBP, NBP and 3-OH-NBP were separately incubated in liver microsomes, liver cytosol, or liver S9 from humans and rats in the presence of NADPH. GSH, NAC, and Cys were also added in the medium as trapping agents.

No formation of 3-GSH-NBP, 3-NAC-NBP, or 3-Cys-NBP ([M + Na]^+, \(m/z\) 332.093) was detected in the incubation of HLM, HLC, and human liver S9. The \([M + H]^+\) ion intensities of 3-NAC-NBP and 3-Cys-NBP were much lower than those of the \([M + Na]^+\) ions under current liquid chromatography–mass spectrometry conditions. Thus, the Na adducted ions were used to represent these two conjugates. Similar to the results from human liver subcellular incubations, 3-GSH-NBP, 3-NAC-NBP, and 3-Cys-NBP were also not detected in rat liver subcellular incubations.

However, the scenario became quite different in the freshly prepared rat liver homogenate incubation system (fresh human liver homogenate was not assayed because of its commercial unavailability and ethical issues). As shown in Fig. 2, left, when NBP was used as the substrate, 3-GSH-NBP was detected at trace amount (mass intensity 60.6) with the addition of NADPH and GSH. No 3-NAC-NBP or 3-Cys-NBP was observed in the presence of NADPH and NAC or Cys, respectively. However, when 3-OH-NBP was used as the substrate, 3-GSH-NBP, 3-NAC-NBP, and 3-Cys-NBP were formed (Fig. 2, right). The yield of 3-GSH-NBP was 39.3-fold higher using 3-OH-NBP as the substrate than using NBP. Notably, the yield of 3-GSH-NBP (mass intensity 2380) was much higher than those of 3-NAC-NBP (mass intensity 83.8) and 3-Cys-NBP (mass intensity 509). In terms of UPLC-UV analysis, the peak area of 3-GSH-NBP was 2.1-fold higher than the summed areas of 3-NAC-NBP and 3-Cys-NBP.

**Formation of 3-GSH-NBP in Incubation with PAPS.** To facilitate the interpretation of the underlying formation mechanism of the NBP reactive metabolite, GSH was employed as the trapping agent because of its much stronger nucleophilicity compared with NAC and Cys (Fig. 2). In the presence of PAPS, 3-GSH-NBP was detected in HLC and RLC with formation rates of 0.74 and 5.97 pmol/min/mg protein, respectively (Fig. 3, A and B). A trace amount of 3-GSH-NBP was also generated in HLM and RLM fortified with PAPS. In the absence of PAPS, almost no 3-GSH-NBP was observed in HLC or RLC. PAPS-dependence suggested the involvement of SULT (Gong et al., 2012; Yalcin et al., 2013).

Selective inhibitors of SULT were estimated to further confirm the role of SULT in mediating the formation of 3-GSH-NBP. As shown in Fig. 3A, DCNP, a general SULT inhibitor, suppressed the formation of 3-GSH-NBP in HCL by 75% at 1 \(\mu\)M, and almost completely blocked the yield of 3-GSH-NBP at 10 \(\mu\)M. Quercetin, a SULT1A1&–SULT1E1-sensitive inhibitor, vigorously inhibited the generation of 3-GSH-NBP in HLC by up to 82% at 10 \(\mu\)M. In addition, as displayed in Fig. 3B, formation of 3-GSH-NBP was also reduced in RLC by the addition of SULT inhibitors. DCNP suppressed the formation of 3-GSH-NBP by 27 and 46% at 1 and 10 \(\mu\)M, respectively; 10 \(\mu\)M of quercetin displayed a higher inhibition effect by up to 54%.

**Fig. 1.** Product ion scans of 3-NAC-NBP and 3-GSH-NBP. (A) 3-NAC-NBP in electrospray ionization (ESI) (+). (B) 3-NAC-NBP in ESI (–). (C) 3-GSH-NBP in ESI (+). (D) 3-GSH-NBP in ESI (–). Insets are their tentative fragmentation patterns.
Generation profiles of 3-GSH-NBP in rat kidney subcellular fractions were also evaluated in the presence of PAPS and GSH. No formation of 3-GSH-NBP was detected in the incubation of 3-OH-NBP with RKM, RKC, rat kidney S9, or rat kidney homogenate.

Identification of SULT Isoenzymes Responsible for 3-OH-NBP Bioactivation. To identify the SULT isoform(s) involved in 3-GSH-NBP formation, a panel of seven recombinant human SULTs (SULT1A1*1, SULT1A1*2, SULT1A2, SULT1A3, SULT1B1, SULT1E1, and SULT2A1) was screened for their activities. The results indicated that all tested SULT isoforms could mediate the yield of 3-GSH-NBP (Fig. 4A). The formation rate of 3-GSH-NBP ranged from 1.94 to 10.58 pmol/min/mg protein, following a decreasing order of SULT1A1*2/C25 SULT1A1*1/C25 SULT1B1/SULT1E1/SULT1A2/SULT1A3/SULT2A1 (Fig. 4A). After the normalization of the 3-GSH-NBP formation rate to the native content of each SULT isoform in human liver cytosolic fractions (Riches et al., 2009), the sulfation of 3-OH-NBP was mainly catalyzed by SULT1A1 (Fig. 4B) and to a lesser extent by SULT1B1. Two other isoforms, SULT1E1 and SULT2A1, also made minor contributions to the sulfation.

Cytotoxicity and Bioactivation of 3-OH-NBP in Primary Human and Rat Hepatocytes. Bioactivation of xenobiotics via sulfation involves the leaving of the sulfate group and formation of electrophilic cations, which may attack endogenous proteins or DNA and cause undesirable toxic effects. Cytotoxicity assays were performed in primary human or rat hepatocytes to evaluate the safety of the sulfation pathway of 3-OH-NBP. The primary human and rat hepatocytes were exposed to 50–400 μM of 3-OH-NBP for 24 hours. No significant toxic effects were observed in primary human hepatocytes (Fig. 5A). However, dose-dependent toxicity was observed after treatment with 3-OH-NBP in primary rat hepatocytes (Fig. 5B). Notably, cell viability in the group treated with 400 μM 3-OH-NBP was as low as 27% that of the vehicle group (Fig. 5B). The calculated IC50 of 3-OH-NBP on the rat hepatocytes’ viability was approximately 168 μM (calculated by SPSS, Inc.).

To determine the mechanisms of cytotoxicity, we added the SULT inhibitors DCNP and quercetin, or GSH depleter BSO in the primary rat hepatocytes’ culture medium to examine their effects on the modulation of the toxic response. As displayed in Fig. 6, the addition of the general SULT inhibitor DCNP at 10 μM increased cell viability by 22.8, 7.6, and 35.3% with an 3-OH-NBP concentration of 100, 200, and 400 μM, respectively. Quercetin, another SULT inhibitor, also attenuated the cytotoxicity by increasing cell viability up to 50.4, 24.6, and 34.2%. By contrast, the presence of the GSH depleter BSO dramatically decreased cell viability by 52.1, 31.1, and 30.1% with an 3-OH-NBP concentration of 100, 200, and 400 μM, respectively. Additionally, in the cell culture of 3-OH-NBP at 400 μM, 3-GSH-NBP and 3-Cys-NBP formation in rat hepatocytes pretreated with BSO decreased significantly compared with those in rat hepatocytes without BSO (Supplemental Fig. 1).

Discussion

NBP undergoes extensive metabolism before its excretion as metabolites in human or rat urine. A high proportion of 3-NAC-NBP, a mercapturic acid metabolite, has been observed, which might...
be associated with formation of reactive metabolites and sequential toxicity. Herein, we proposed a metabolic bioactivation pathway of NBP. We initially incubated NBP and 3-OH-NBP in liver microsomes, liver cytosol, or liver S9 from humans and rats with NADPH and the trapping agents GSH, NAC, and Cys. No 3-GSH-NBP, 3-NAC-NBP, or 3-Cys-NBP was formed in these subcellular fraction incubations. When NBP was incubated in freshly prepared rat liver homogenate, except for a trace amount of 3-GSH-NBP, no 3-NAC-NBP or 3-Cys-NBP was formed. When 3-OH-NBP was incubated in fresh rat liver homogenate, the formation of 3-GSH-NBP was 39.3-fold higher than using NBP as the substrate. Moderate formation of 3-NAC-NBP and 3-Cys-NBP was also observed. The most likely reason is that the freshly prepared rat liver homogenate contains all drug metabolizing enzymes and prerequisite cofactors intact. Hence, we concluded that 3-GSH-NBP was generated from 3-OH-NBP instead of directly from NBP. Therefore, we used 3-OH-NBP as the substrate in subsequent tests to elucidate the bioactivation pathway. Furthermore, it can also be concluded that HLM, HLC, or human liver S9 may lack some cofactors that are obligatory for certain enzyme(s) responsible for mediating 3-GSH-NBP generation.

In the present study, the lacking cofactor was PAPS because the 3-GSH-NBP formation in HLC was PAPS dependent (Fig. 3A), indicating the involvement of SULT. A trace amount of 3-GSH-NBP was also detected in PAPS-fortified HLM, probably because of the contamination of microsomes with minor cytosolic enzymes. The addition of the SULT general inhibitor DCNP or quercetin potently suppressed the yield of 3-GSH-NBP in HLC (Fig. 3A), further confirming the

Fig. 3. Generation of 3-GSH-NBP following incubation of 3-OH-NBP (50 μM) with GSH (1.5 mM) and PAPS (250 μM) in HLM and HLC (A), and in RLM and RLC (B) with or without SULT inhibitors (DCNP, quercetin). Each column represents the mean of two independent samples.

Fig. 4. (A) Formation rate of 3-GSH-NBP in the incubation of 3-OH-NBP (50 μM) with seven recombinant human SULTs (250 μg protein/ml) supplemented with PAPS (250 μM) and GSH (1.5 mM). (B) Contribution of each SULT isoform to the sulfation of 3-OH-NBP after normalization with regard to their native abundance in human liver cytosolic fractions. Data are displayed as the mean of two separate samples.
contribution of SULT to the formation of 3-GSH-NBP. A similar formation profile of 3-GSH-NBP was observed in RLM and RLC (Fig. 3B). Notably, the formation rate of 3-GSH-NBP in RLC (5.97 pmol/min/mg protein) was approximately 8.1 times higher than that in HLC (0.74 pmol/min/mg protein), indicating higher SULT activity in RLC than in HLC. This result is consistent with the higher urinary excretion rate of 3-NAC-NBP in rats than in humans (8.7% versus 4.7%). The species difference in SULT activity was also observed with the formation of tamoxifen-DNA adducts, which were catalyzed via \( O^-\)-sulfation of hydroxytamoxifen (Kim et al., 2005).

Reaction phenotyping analysis with seven recombinant human SULTs demonstrated that SULT1A1 made the largest contribution to the formation of 3-GSH-NBP, and, to a lesser extent, there were contributions by SULT1B1, SULT1E1, and SULT2A1. The bioactivation pathway of NBP was proposed to be through the sulfation of its major metabolite 3-OH-NBP, as shown in Fig. 7. Genetic polymorphism of SULT1A1 was reported with three allelic variants: SULT1A1*1, SULT1A1*2, and SULT1A1*3 (Carlini et al., 2001). The allele frequency ratio of SULT1A1*1:*2:*3 was 0.656:0.332:0.012 in Caucasians, 0.477:0.294:0.229 in African Americans, and 0.914:0.080:0.006 in Chinese, respectively (Raftogianis et al., 1997; Carlini et al., 2001). These three alleles are associated with altered enzymatic activities; the general trend of \( V_{\text{max}} \) values as determined in previous studies is *1 > *3 > *2 (Raftogianis et al., 1997; Nagar et al., 2006). Although SULT1A1*2 showed 40% lower activity toward the sulfation of troglitazone than SULT1A1*1 (Honma et al., 2002), nearly equivalent enzymatic activities were observed between SULT1A1*1 and SULT1A1*2 toward the formation of 3-GSH-NBP (Fig. 4A). We did not assay SULT1A1*3 because of its commercial unavailability.

In addition to SULT, we also evaluated the role of acetyl transferase in the generation of 3-GSH-NBP, because both sulfate and acetoxy are good leaving groups (Dasaradhi and Shibutani, 1997; Kim et al., 2005). 3-GSH-NBP was not detected in the incubation of 3-OH-NBP with HLC or RLC in the presence of acetyl coenzyme A and GSH, excluding the potential participation of acetyl transferase.

Sulfation and glucuronidation are competitors for the conjugation of 3-OH-NBP. In human urine, 3-OH-NBP glucuronide conjugate was detected, accounting for 3.4% of the administered dose, which was comparable to 3-NAC-NBP (derived from 3-OH-NBP sulfate) (Diao et al., 2013a). However, 3-OH-NBP glucuronide conjugate was not detected in rat urine, and a higher excretion ratio of 3-NAC-NBP was observed, which can probably be attributed to the higher SULT activity in rats.

3-NAC-NBP could be formed via two pathways: 1) derived from 3-GSH-NBP through the classic mercapturic acid pathway, as mentioned in the Introduction (Poon et al., 2001); 2) direct binding of Cys to the cation to yield 3-Cys-NBP, which undergoes further N-acetylation to 3-NAC-NBP. As shown in Supplemental Fig. 1, abundant formation of 3-GSH-NBP and a trace amount of 3-Cys-NBP were observed in the culture of 400 \( \mu \)M 3-OH-NBP with fresh primary rat hepatocytes. The presence of the GSH depleter BSO led to a sharp decrease in 3-GSH-NBP and 3-Cys-NBP formation, excluding the latter tentative formation pathway of 3-NAC-NBP, in which case the formation of 3-Cys-NBP would increase due to more exposure to the cations.
After oral administration of 200 mg of NBP soft capsules, 3-OH-NBP and 3-NAC-NBP were detected mainly in human urine. Given that SULTs are also distributed in the kidney in addition to the liver (Wang and James, 2006), we further assessed the role of kidney SULT in the formation of 3-GSH-NBP. No formation of 3-GSH-NBP was observed in the incubation of 3-OH-NBP with RKM, RKC, rat kidney S9, or fresh rat kidney homogenate in the presence of PAPS and GSH. Therefore, we proposed that 3-GSH-NBP was generated in the liver and further hydrolyzed to 3-Cys-NBP. The 3-Cys-NBP was later acetylated to 3-NAC-NBP in the kidney and excreted in urine.

In addition, we evaluated the cytotoxicity of 3-OH-NBP in primary human and rat hepatocytes. 3-OH-NBP was used in this study because it is the start compound of the metabolic bioactivation pathway and also a major metabolite in humans and rats. In primary human hepatocytes, no significant toxic effects were observed with 3-OH-NBP up to 400 μM. In primary rat hepatocytes, dose-dependent cytotoxicity was observed with 3-OH-NBP, with an estimated IC50 of approximately 168 μM. The absence of cytotoxicity in primary human hepatocytes is probably due to the much lower SULT enzyme activity in human hepatocytes than in rat hepatocytes. This finding is also consistent with higher formation rate of 3-GSH-NBP in RLC relative to HLC. The addition of the SULT inhibitors DCNP or S9, or fresh rat kidney homogenate in the presence of PAPS and GSH.

In summary, we have characterized the metabolic bioactivation pathway of NBP. NBP is first oxidized to its major metabolite 3-OH-NBP by cytochromes P450; then 3-OH-NBP undergoes sulfation to 3-OH-NBP sulfate, mainly catalyzed by SULT1A1. The 3-OH-NBP sulfate is chemically unstable, with the sulfate group spontaneously cleaving off to form highly reactive electrophilic cations. The cations bind either to endogenous GSH to detoxify or to the thiol groups of hepatocellular proteins, causing hepatocyte dysfunction. Although the lower SULT activity in human hepatocytes renders NBP safer in humans than in rats, the 3-OH-NBP concentration may accumulate to high amounts after chronic administration of NBP and lead to liver damage, especially in cases of GSH depletion. Therefore, follow-up observation of liver function is recommended, as described in the package insert of NBP soft capsules (NBP Pharmaceutical, 2009).

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Authorship Contributions

Participated in research design: Diao, Chen, Pang, Xie.
Conducted experiments: Diao, Pang, Guo.
Contributed new reagents or analytic tools: Diao.
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Bioactivation of NBP via Sulfation of 3-Hydroxy-NBP


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