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Identification of enzymes responsible for nitrazepam metabolism and toxicity in human

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ABSTRACT

Nitrazepam (NZP) is a hypnotic agent that rarely causes liver injuries in humans and teratogenicity in rodents. In humans, NZP is primarily metabolized to 7-aminonitrazepam (ANZP) by reduction and subsequently to 7-acetylamino nitrazepam (AANZP) by acetylation. ANZP can be regenerated from AANZP by hydrolysis in rodents, but it is still unclear whether this reaction occurs in humans. In rodents, AANZP may be associated with teratogenicity, while in humans, it is known that drug-induced liver injuries may be caused by NZP reactive metabolite(s). In this study, we attempted to identify the enzymes responsible for NZP metabolism to obtain a basic understanding of this process and the associated metabolite toxicities. We found that the NZP reductase activity in human liver cytosol (HLC) was higher than that in human liver microsomes (HLM). We purified the responsible enzyme(s) from HLC and found that the NZP reductase was aldehyde oxidase 1 (AOX1). The role of AOX1 was confirmed by an observed increase in the NZP reductase activity upon addition of N^1-methylnicotinamide, an electron donor of AOX1, as well as inhibition of this activity in HLC in the presence of AOX1 inhibitors. ANZP was acetylated to form AANZP by *N*-acetyltransferase (NAT) 2. An experiment using recombinant esterases in an inhibition study using HLM revealed that AANZP is hydrolyzed by arylacetamide deacetylase (AADAC) in the human liver. *N*-Hydroxylamino NZP, which is suspected to be a reactive metabolite, was detected as a conjugate with *N*-acetyl-L-cysteine through NZP reduction and ANZP hydroxylation reactions. In the latter reaction, the conjugate was readily formed by recombinant CYP3A4 among the various P450 isoforms tested. In sum, we found that AOX1, NAT2, AADAC, and CYP3A4 are the determinants for the pharmacokinetics of NZP and that they confer interindividual variability in sensitivity to NZP side effects.

1. Introduction

Nitrazepam (NZP) is a long-acting benzodiazepine hypnotic agent that is used as an effective treatment for insomnia [1]. Insomnia is referred to as a disorder of initiating or maintaining sleep, and its prevalence in older people is estimated to be 23–34% [2,3]. NZP works as a hypnotic to prolong sleeping time and decrease the time needed to fall asleep [3], but several side effects, such as liver injury and teratogenicity, can occur. Cholestatic liver failure has been reported as a result of NZP usage [4,5]. These adverse reactions are thought to be induced through the metabolism of NZP [6,7].

In humans, NZP is primarily metabolized to 7-amino nitrazepam (ANZP) by nitro-reductase(s) and subsequently to 7-acetylamino nitrazepam (AANZP) by *N*-acetyltransferase(s) (Fig. 1) [8]. In rodents, AANZP is hydrolyzed to ANZP in the liver, but it has not been determined whether this reaction occurs in humans [7]. Within 24 hours of an oral administration of 10 mg NZP in humans, urinary excretion of ANZP and AANZP represented 4.1% and 12.5% of the initial dose, respectively, [9]. Other possible NZP metabolites

Abbreviations: AADAC, arylacetamide deacetylase; AANZP, 7-acetylamino nitrazepam; ABT, 1-aminobenzotriazole; ANZP, 7-amino nitrazepam; AOX1, aldehyde oxidase 1; BNPP, bis-(*p*-nitrophenyl) phosphate; CES, carboxylesterase; CM, carboxymethyl; CYP, cytochrome P450; DEAE, diethylaminoethyl; DFP, diisopropyl phosphorofluoride; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; Eserine, sulfate physostigmine; HLC, human liver cytosol; HLM, human liver microsomes; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MNA, N^1-methylnicotinamide; MRM, multiple-reaction monitoring; NAC, *N*-acetyl-L-cysteine; $NADP^+$, nicotinamide adenine dinucleotide phosphate oxidized form; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; NAT, *N*-acetyltransferase; NZP, nitrazepam; PMSF, phenylmethylsulfonyl fluoride; S9, supernatant 9000*g*; SDS-PAGE, sodium dodecyl sulfate-poly acrylamide gel electrophoresis; XOR, xanthine oxidoreductase.

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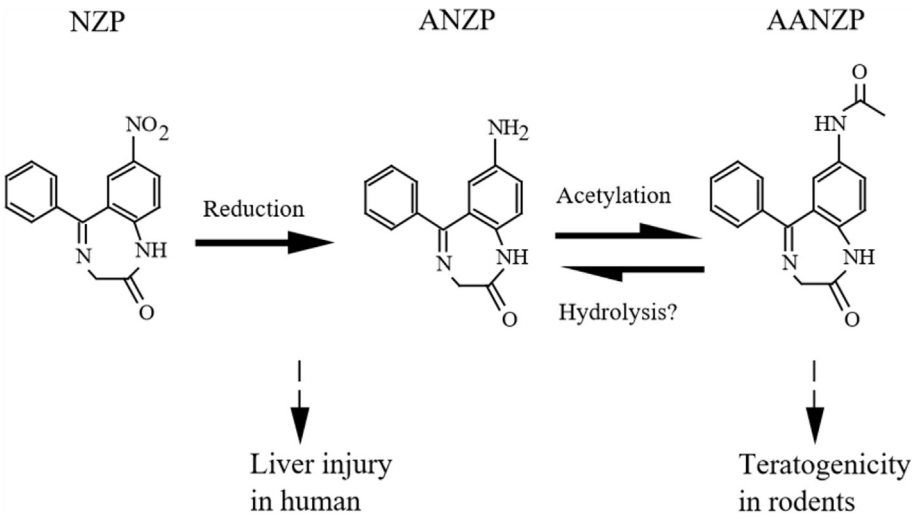

Fig. 1. Proposed metabolic pathways and related adverse reactions of NZP.

were detected to a much lesser extent in human urine compared to ANZP and AANZP.

The toxicity of NZP has been evaluated by *in vitro* studies. When HepG2 cells (a hepatocarcinoma cell line) were treated with NZP and recombinant human CYP3A4, significant cytotoxicity was observed compared to the absence of CYP3A4 [6], suggesting that the metabolism NZP is associated with liver injury. However, it remained unclear whether CYP3A4 is the sole enzyme that forms a reactive NZP metabolite. Teratogenicity caused by NZP was reported in rats, but not in mice [7]. ANZP acetylase activity is 8.5-fold higher in rats than in mice, whereas AANZP hydrolase activity is 10-fold lower in rats than in mice. Therefore, AANZP was suggested to be the cause of the observed teratogenicity.

As mentioned above, metabolic reactions are thought to be linked with the side effects of NZP, but the enzymes responsible for NZP metabolism in humans are unknown. In rats, NZP has been reported to be reduced by intestinal bacteria [10–12], but it is also conceivable that NZP metabolism efficiently occurs within the liver after being absorbed from the intestinal tract. The identification of the enzymes responsible for NZP metabolism would be helpful to prove the association of NZP metabolism with the pathogenesis of adverse reactions. In this study, we attempted to identify the enzymes responsible for the metabolism of NZP, including reduction, acetylation, and hydrolysis, and to identify the reactive metabolite(s) that may cause liver injuries in humans.

2. Materials and methods

2.1. Materials

Pooled human liver cytosol (HLC) (prepared from 150 individuals), pooled human liver microsomes (HLM) (prepared from 50 individuals), pooled human 9000*g* supernatant (S9) (prepared from 22 individuals), recombinant human CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 Supersomes, and recombinant *N*-acetyltransferase (NAT) 1 and NAT2 cytosols were purchased from Corning (Corning, NY). IRDye 680 goat anti-mouse IgG was obtained from LI-COR Biosciences (Lincoln, NE). Acetyl CoA, diisopropyl fluorophosphate (DFP), *N*-acetyl-L-cysteine (NAC), NZP, phenylmethylsulfonyl fluoride (PMSF), and sulfate physostigmine (eserine) were purchased from Wako Pure Chemicals (Osaka, Japan). Ammonium sulfate, ANZP, and bis(*p*-nitrophenyl) phosphate (BNPP) were purchased from Sigma-Aldrich (St. Louis, MO). Carboxymethyl (CM) Sepharose and diethylaminoethyl (DEAE) Sephacel were purchased from GE Healthcare (Buckinghamshine, UK). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) were purchased from Oriental Yeast (Tokyo, Japan). 2D-Silver Stain Reagent II and N^1-methylnicotinamide (MNA) were purchased from Cosmo Bio (Tokyo, Japan). A mouse anti-human aldehyde oxidase 1 (AOX1) monoclonal antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). All other chemicals and solvents were of the highest grade commercially available.

2.2. Measurement of NZP reductase activity

NZP reductase activities were determined as follows: a typical reaction (final volume of 200 μL) contained 100 mM potassium phosphate buffer (pH 7.4), enzyme sources (0.4 mg/mL HLM or HLC; 20 μL of a 40–60% ammonium sulfate precipitated fraction and CM Sepharose fraction; or 50 μL of a DEAE Sephacel fraction) and a NADPH-generating system (0.5 mM $NADP^+$, 5 mM glucose-6-phosphate, 5 mM $MgCl_2$, and 1 U/mL glucose-6-phosphate dehydrogenase). For the evaluation of the involvement of AOX1, 1 mM MNA was added to the reaction mixture. We confirmed that the ANZP formation rates were linear up to a 1 mg/mL protein concentration and a 90 min (in the absence of MNA) or 30 min (in the presence of MNA) incubation time. NZP was dissolved in dimethyl sulfoxide (DMSO). The reactions were initiated by the addition of NZP at a final concentration of 20 μM after a 2-min preincubation at 37 °C. After a 45- or 10-min incubation (in the absence or presence of MNA, respectively), the reactions were terminated by the addition of 200 μL of ice-cold acetonitrile. After removal of the protein by centrifugation at 20,400*g* for 5 min, a portion of the supernatant was subjected to liquid chromatography–tandem mass spectrometry (LC–MS/MS). The LC equipment was comprised of a CBM-20A controller (Shimadzu, Kyoto, Japan), LC-20AD pumps (Shimadzu), an SIL-20AC HT autosampler (Shimadzu), a CTO-20AC column oven (Shimadzu), and a SPD-20A UV detector (Shimadzu) equipped with a Develosil ODS-UG-3 column (3 μm particle size, 4.6 mm i.d. × 150 mm; Nomura Chemical, Seto, Japan). The column temperature was set at 40 °C, and the flow rate was 0.2 mL/min. The mobile phase was 0.1% formic acid/5 mM ammonium formate (A) and acetonitrile containing 0.1% formic acid (B). The conditions for elution were as follows: 15% B (0–2 min), 70% B (2–4 min), 95% B (4–7 min), and 15% B (7–9 min). The LC was connected to a LC-MS 8040 (Shimadzu) and was used in the positive