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Characterization of Escherichia coli nitroreductase NfsB in the metabolism of nitrobenzodiazepines

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ABSTRACT

Nitrobenzodiazepine (NBDZ) is a sedative-hypnotic drug used in the treatment of anxiety and sleep problems. Overdose of NBDZ may cause severe neurological effects, especially for people in drug abuse or addiction. In the present study, we investigated NBDZ nitroreduction in rat enteric contents and characterized the role of enterobacterial nitroreductase in the reductive pathway. Nitroreduction of flunitrazepam (FZ) was studied in the microsomal membrane fractions of rat liver, jejunum and jejunal microflora using HPLC analysis. In the jejunal microflora, FZ was demonstrated to be significantly reduced to its amino derivative under anaerobic condition. Escherichia coli type I nitroreductase NfsB (EC 1.5.1.34) was found in rat jejunal microflora via PCR technique and Western blotting. The participation of NfsB in FZ nitroreduction was demonstrated from inhibition studies. Kinetic study of the purified recombinant NfsB indicated that nitroreduction of FZ, nitrazepam (NZ) and clonazepam (CZ) are mediated by NfsB, where CZ shows lower k_{cat}/K_{M} ratio than that of the other two. Finally, two other nitroreductases E. cloacae NR (EC 1.6.99.7) and S. typhimurium Cnr were also found to be responsible for FZ nitroreduction. These results provide that the reduction of NBDZ in normal flora is catalyzed by type I nitroreductase NfsB.

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1. Introduction

Nitrobenzodiazepines (NBDZs) are a group of nitroaromatic benzodiazepines (BDZs) that include flunitrazepam (FZ), nitrazepam (NZ) and clonazepam (CZ) (Fig. 1A) [1], and serve as important sedative-hypnotic drugs in clinic [2]. These compounds bind to GABA_A receptor in the central nervous system (CNS) to enhance GABA-mediated inhibitory effect, resulting in anxiety reduction and sleep encouragement [2,3]. However, misuse of NBDZ can have amnesic effect, memory impairment and behavioral disinhibition, especially for people with drug abuse and addiction problems [4]. An example of this is the abuse of FZ, which is reported with increased risky behavior such as violence and drug-facilitated

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sexual assault (DFSA) [5]. In addition, the combined use of FZ and other drugs such as opioid dependence therapeutic drug buprenorphine has been shown to have severe respiratory toxicity or even cause death [6,7], implying a drug-drug interaction in the body [8].

In humans, metabolism of NBDZ occurs mainly in liver, which involves oxidative and reductive pathways [9]. For example, FZ undergoes oxidative metabolism to N-desmethyl-flunitrazepam (DMFZ) and 3-hydroxy-flunitrazepam (3-OHFZ), or is reduced to 7amino-flunitrazepam (7AFZ) within liver microsomes (Fig. 1B) [10]. The oxidation of FZ to DMFZ and 3-OHFZ is catalyzed by the hepatic CYP2C9 and CYP3A4, respectively [11], where DMFZ is the main active metabolite correlating to the respiratory defect [8] and anesthetic effect [9]. Interestingly, 7AFZ appears to be the major metabolite detected in urine with less adverse effects [8,12], suggesting a possible route of FZ disposition for those intentional high-dosage users. We previously showed that human hepatic NADPH-cytochrome P450 reductase (P450R; EC 1.6.2.4) was involved in the reductive pathway of FZ, providing an evidence of nitroreductase activity for NBDZ clearance in the body [13]. Several studies have shown the distribution of nitroreductive activity toward nitroaromatic compounds in rat [14-16]. For

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Fig. 1. (A) Structures of FZ, CZ and NZ; (B) nitroreductive pathway of NBDZ.

example, prostate cancer therapeutic drug nilutamide is proved to be nitroreduced in rat organs particularly in small intestine and lung, and also in intestinal contents [15,16]. Furthermore, the antibiotic drug nitrofurazone has been reported being nitroreduced by liver microsomes and enteric bacteria of rat, where the bacteria show higher nitroreductase activity over the liver [17].

The role of intestinal bacteria in NBDZ metabolism has also been reported in mammals [1,18]. Robertson and Drummer [1] showed that NBDZs are readily metabolized to 7-amino-benzodiazepines (7ABDZs) in eight enteric bacteria isolated from postmortem specimens of a man. Takeno and Sakai [18] reported the reduction of NZ to 7-amino-nitrazepam (7ANZ) in rat by anaerobic microbial action of cecal contents at rates seven times higher than that of liver microsomes, implying the rapid conversion of NBDZ to 7ABDZ when exposed to the intestinal bacteria. However, bacterial enzymes governing the nitroreduction of NBDZ in gastrointestinal (GI) tract have not yet been identified.

Escherichia coli (E. coli) is a typical coliform bacteria of the family Enterobacteriaceae in normal flora of animals [19]. In rat, E. coli strains inhabit in small intestine for metabolism of xenobiotics from host, where nitroreductase activities occur toward many nitroaromatic compounds [20–22]. Nitroreductases are a family of NAD(P)Hdependent flavoenzymes (using flavin mononucleotide as cofactor) that catalyze the reduction of the nitro groups of a wide range of nitroaromatic compounds [23]. Two types of nitroreductases have been described based on their response to oxygen [17]. Type I or oxygen-insensitive nitroreductases catalyze the sequential reduction of the nitro groups by the addition of electron pairs from NAD(P)H to produce the nitroso, hydroxylamino and amino derivatives [23]. Type II or oxygen-sensitive nitroreductases catalyze the single-electron reduction of the nitro group to produce a nitro anion radical, which can be reoxidized aerobically to the superoxide anion in a futile cycle [23]. P450R belongs to type II nitroreductase, which has been reported to reduce several nitroaromatic compounds, including nitrofurazone [17,24], nitroimidazole [25] and flunitrazepam [13]. On the other hand, bacterial type I nitroreductases have attracted attention due to their potential environmental and clinical applications [26]. An example is E. coli nitroreductase NfsB (EC 1.5.1.34), which has been successfully used in the conversion of prodrug CB1954 for cancer therapy [26]

In this study, we investigated FZ nitroreduction in rat liver, jejunum and jejunal microflora. We found that *E. coli* nitror-

eductase NfsB catalyzes the nitroreduction of NBDZ under anaerobic condition, and such activity also occurs in other microfloral NfsB family nitroreductases. Our study thus showed that bacterial type I nitroreductase may be involved in the reductive pathway of NBDZ.

2. Materials and methods

2.1. Chemicals and reagents

Flunitrazepam (FZ), clonazepam (CZ), nitrazepam (NZ), NADPH and 2-iodosobenzoic acid (2-IBA) were obtained from Sigma– Aldrich (St. Louis, MO). Unless otherwise stated, other chemicals and reagents were also obtained from Sigma–Aldrich. 7-Aminoflunitrazepam (7AFZ), 7-amino-clonazepam (7ACZ), 7-aminonitrazepam (7ANZ) and bromazepam were obtained from Radian International (Austin, TX). Acetonitrile, chloroform, and methanol were obtained from RdH Laborchemikalien GmbH & Co. KG (Seelze, Germany).

2.2. Animals

Male Wistar rats were purchased from Animal Center, College of Medicine, National Taiwan University, and were housed in the airconditioned animal quarters on a 12-h light/dark cycle. The animals had free access to water and rodent laboratory chow (Purina Mills Inc., St. Louis, MO) until used. The animal care and experimental procedures were approved by the Institutional Care and Use Committee, College of Medicine, National Taiwan University.

2.3. Preparation of microsomal membrane fractions of rat liver, jejunum and jejunal microflora

Microsomal membrane fractions of rat liver and jejunum were prepared as described by Alvares and Mannering [27]. The liver and jejunum were removed and rinsed in the ice-cold homogenization buffer (100 mM potassium phosphate buffer, pH 7.4, containing 154 mM KCl). The jejunal tracts were flushed gently with 10 ml of sterile nutrient broth (BD, Becton, Dickinson & Co., Franklin Lakes, NJ) for several times and the collected jejunal contents were incubated in 100 ml nutrient broth at 37 °C under aerobic condition until stationary phase. The tissues were minced and homogenized using 4 ml of homogenization buffer per gram of tissue in a Teflon-glass homogenizer immersed in ice. The obtained homogenates were centrifuged at 9000 × g for 20 min at 4 °C. Supernatants were further centrifuged at 100,000 × g for 1 h at 4 °C to obtain the final fractions and stored at -70 °C.

To prepare microsomal membrane fractions of the jejunal microflora, culture media of the jejunal contents were centrifuged at 5000 × g for 15 min at 4 °C. The obtained microflora pellets were washed twice with PBS (100 mM phosphate-buffered saline, pH 7.4), and 1 ml of homogenization buffer was added to the pellet. The suspended floral cells were sonicated (Microson, XL-2000, Misonix Inc., Farmingdale, NY) at 4 °C. The disrupted cells were centrifuged at 9000 × g for 20 min at 4 °C. Supernatants were further centrifuged at 100,000 × g for 1 h at 4 °C to obtain the final fractions and stored at -70 °C.

Protein concentrations were determined using Pierce BCA protein assay calibrated against bovine serum albumin (Pierce, Rockford, IL).

2.4. FZ nitroreduction

The incubation was performed under aerobic or anaerobic condition. For anaerobic incubation, nitrogen was inlet into the anaerobic incubator (Concept 400, Biotrace Inc., Cincinnati, OH) as

described previously [13]. The reaction mixture consists of FZ (64 μ M), microsomal membrane fractions (2 mg), MgCl₂ (3.3 mM), and NADPH (0.2 mM for microsomal protein and 2 mM for purified protein) in 100 mM potassium phosphate buffer (pH 7.4). After incubation for 2 h at 37 °C, the reaction was terminated by the addition of 200 μ l of methanol. 7AFZ and FZ were extracted via sonication of the sample for 10 min, and then centrifuged at 12,000 \times g for 20 min at 4 °C. The obtained 100 μ l supernatant was taken for analysis.

2.5. HPLC analysis

FZ and 7AFZ were analyzed as described previously [13]. All analyses were performed using a Gasukuro Kogyo Model 576 liquid chromatograph (Tokyo, Japan) equipped with a 4 μ m Cosmosil 5C-18-AR reverse phase column (4.6 × 250 mm, Nacalai USA Inc., San Diego, CA). The column was eluted for 25 min at a flow rate of 1.8 ml/min with a linear gradient from 90:10 (v/v) to 50:50 (v/v) of 50 mM sodium dihydrogen phosphate (pH 2.0)/ acetonitrile. All chromatographic separations were performed at 25 °C, and the column elute was monitored at 240 nm with a Gasukuro Kogyo 520 U detector (Tokyo, Japan).

The HPLC system with UV detection employed in the present study readily resolved 7AFZ (t_R 12.0 min), FZ (t_R 19.5 min) and internal standard bromazepam (t_R 14.0 min). Calibration plot (nine concentrations) prepared with standards was linear over the concentration range of 5–250 ng of 7AFZ or FZ with r value of 0.9997 and 0.9996 for 7AFZ and FZ. The detection limit for 7AFZ or FZ was less than 3–5 ng. Recovery of 7AFZ and FZ was 83–86% (three concentrations run in triplicate). Consequently, the concentrations of 7AFZ and FZ in the present study are likely to be within 10% of the reported values.

2.6. PCR amplification of bacterial genes

PCR amplification was performed by using DNA thermal cycler. Primer set for *E. coli* 16S *rRNA* (*E. coli*-f: 5'-GGGAGTAAAGTTAA-TACCTTTGCTC and *E. coli*-r: 5'-TTCCCGAAGGCACATTCT; a 584-bp region) was designed according to Tsen et al. [28], primer set for *E. coli* lacZ (ZL-1675: 5'-ATGAAAGCTGGCTACAGGAAGGCC and ZR-2548: 5'-CACCATGCCGTGGGTTTCAATATT; an 876-bp region) was designed according to Bej et al. [29], and *E. coli* nitroreductase *nfsB* (forward: 5'-ATGGATATCATTTCTGTCGC and reverse: 5'-TTA-CACTTCGGTTAAGGTG; a 651-bp region), *E. cloacae* nitroreductase *nfnB* (forward: 5'-ATGGATATCATTTCTGTCGC and reverse: 5'-TCAGCACTCGGTCACAATC; a 651-bp region) and *S. typhimurium* nitroreductase *nfsI* (forward: 5'-ATGGATATCGTTTCTGTCGC and reverse: 5'-TTAGACTTCCGTCAGTGTG; a 651-bp region) primer sets were designed.

Bacterial genomic DNA was isolated as described from Amit-Romach et al. [20]. The PCR solution used contained $1 \times$ PCR amplification buffer ($10 \times$ buffer contains 50 mM KCl, 15 mM MgCl₂ and 100 mM Tris–HCl, pH 8.13), dNTPs (200μ M each), the primers (1μ M each), the extracted DNA (0.5μ g), and *Tag* DNA polymerase (2.5 U, Stratagene, La Jolla, CA). The reactions were initially denatured at 94 °C for 5 min. Then a total of 30–40 PCR cycles were run under the following conditions: denaturation at 94 °C for 5 min, primer annealing at 52–55 °C for 1 min, and DNA extension at 72 °C for 1 min. The PCR-amplified DNA products were visualized by 1% agarose gel electrophoresis stained with ethidium bromide (Sigma–Aldrich, St. Louis, MO).

2.7. Preparation of anti-NfsB antibody

The pure *E. coli* NfsB was purchased from Sigma–Aldrich (St. Louis, MO). Young male New Zealand White rabbits (Animal

Center, College of Medicine, National Taiwan University) were used to generate anti-NfsB antibody according to a standard immunization protocol [30]. The obtained antiserum was purified using Immunopure IgG Purification kit (Pierce, Rockford, IL) including a Protein A affinity purification column according to manufacturer's instructions. The resulting antibody was used to recognize NfsB in the samples.

2.8. SDS-PAGE and Western blot analysis

For SDS-PAGE, rat microsomal membrane fractions (20 μ g) or the purified NfsB family proteins (0.1–1 μ g) were loaded onto a 12% SDS-PAGE gel (Invitrogen, Carlsbad, CA) and electrophoresed to separate proteins. In NfsB purification steps, the separated proteins were further stained with Coomassie brilliant blue solution (Invitrogen) to evaluate the purity.

For Western blotting [31], the proteins were transferred to a PVDF membrane (GE Healthcare, Amersham, UK) at 100 V for 1 h and the membrane was blocked overnight using blocking buffer (5% BSA in PBS). Tween 20 (final concentration in 0.5%) was added together with the primary antibodies. The membranes were incubated with rabbit anti-NfsB antibody (1:10,000) at 4 °C overnight, then the membrane was rinsed five times with PBS–0.5% Tween 20 (PBST) and incubated at 25 °C for 1 h with the antirabbit HRP secondary antibody (GE Healthcare) diluted 1:4000 in PBST. The membrane was rinsed again with PBS–0.5% Tween and stored in PBS at 4 °C until imaged. The proteins were visualized by chemiluminescence using ECL reagent (GE Healthcare).

2.9. Chemical and immunoinhibition studies

The reaction mixture and condition were the same as described in Section 2.4, except for the addition of the inhibitors 30 min prior to FZ. For chemical inhibition study, various concentrations of 2-iodosobenzoic acid (2-IBA; 2–200 μ M) were used to co-incubate with the reaction mixtures [32]. For immunoinhibition study, NfsB antibody was diluted with the reaction mixture (1:250 to 1:31; v/ v) [13]. After pre-incubation with the inhibitors, FZ was added and the mixtures were further reacted for 2 h.

2.10. Enterobacteriaceae screening

Cultures of rat jejunal microflora were applied to BBLTM EnterotubeTM II (BD Diagnostics, Heidelberg, Germany) and continue to inoculate for 24 h. The identification of the enterobacteriaceae is determined from the Interpretation Guide according to manufacturer's instructions.

2.11. Cloning, expression and purification of nitroreductase

The coding regions of nitroreductase nfsB, nfnB and nfsI were PCR amplified from the genomic DNA (as described in Section 2.6) of E. coli DH5a, E. cloacae and S. typhimurium TA1537, respectively. After confirmation of the correct nucleotide sequences, the individual gene products were cloned into the vector pET-46LIC (Novagen, Darmstadt, Germany). The recombinant proteins were expressed using E. coli BL21(DE3) competent cells (Novagen) and expression of the His-tagged recombinant proteins were carried out using LB medium (BD, Becton, Dickinson & Co., Franklin Lakes, NJ). A single colony was used to inoculate 800 ml of medium, and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma-Aldrich, St. Louis, MO) at 37 °C and 180 rpm shaking until stationary phase was reached. The resulting cell pellets, obtained via centrifugation at $5000 \times g$ for 15 min, were suspended in 50 mM Tris-HCl buffer (pH 7.5, containing 500 mM NaCl) and disrupted with a cell disrupter (Constant Systems, Daventry, UK) at 4 °C. Cellular debris was removed via centrifuged at 20,000 \times g for 30 min at 4 °C.

Expressed proteins were purified from the supernatant using HiTrap Ni-NTA packed column (GE Healthcare, Amersham, UK). The column was loaded with 100 mM NiSO₄ and equilibrated with 50 mM Tris–HCl buffer (pH 7.5, containing 500 mM NaCl and 10 mM imidazole). The supernatant was applied to the column and the bound protein was eluted with a gradient to 250 mM imidazole via FPLC (GE Healthcare). The collected fractions were pooled and further purified via DEAE ion-exchange column (GE Healthcare). The active fractions were dialyzed to 50 mM Tris–HCl buffer (pH 7.5, containing 50 mM NaCl) and stored at -70 °C.

2.12. Kinetic analysis

The rate of 7ABDZ formation as a function of NBDZ concentration was examined in purified recombinant NfsB (10 μ g). Incubations with NBDZ (7–128 μ M) were carried out under anaerobic condition with the addition of a fixed concentration of NADPH (2 mM).

2.13. Statistical analysis

Data were presented as arithmetic mean \pm S.D. Statistical analysis were established with Student's *t*-test for unpaired data (Fig. 2B), one-way ANOVA followed by Tukey's post hoc multiple comparison test for different organs under aerobic and anaerobic conditions (Fig. 2A), and one-way ANOVA followed by Dunnett's post hoc test for the groups compared with the same control (Figs. 2C and 7C). A value of P < 0.05 was considered significant.

3. Results

3.1. Nitroreduction of FZ in rat liver, jejunum and jujenal microflora

In order to elucidate the reductive metabolism of FZ in rat, microsomal membrane fractions of liver, jejunum and jejunal microflora were prepared. The reactions were performed in the presence of NADPH (0.2 mM) and FZ (64 μ M) for 2 h under aerobic and anaerobic conditions. By using HPLC analysis, the reductive activity of FZ in liver was a bit higher than that of jejunum in both conditions, and the anaerobic incubations had higher activities in both organs (Fig. 2A). In addition, the reductive activity increased

in the microfloral incubation (Fig. 2B), where the activity under anaerobic condition was six times higher than that of aerobic condition (52.58 ± 5.23 vs. 8.08 ± 1.43 pmol/mg protein/min), and at least 10 times higher than in liver under anaerobic condition (4.85 ± 0.23 pmol/mg protein/min).

To further verify the reductive activity of the jejunal microflora in the hypoxia, FZ nitroreduction of rats at different ages (1-, 8-, 65and 103-week-old) were performed. The results showed that under anaerobic condition the reductive activities increased with age (in log scale), and the significance occurred markedly from 1 to 8 weeks of age and then was increased still further at 65 and 103 weeks (Fig. 2C).

3.2. Identification of E. coli nitroreductase NfsB in rat jejunal microflora

The most abundant microfloral bacteria *E. coli* and its known nitroreductase NfsB were investigated among rat microflora. Two *E. coli* multiple copy genes *16S rRNA* (partial sequence of 584 bp) and *lacZ* (partial sequence of 876 bp) were amplified from genomic DNA (0.5 μ g) of rat jejunal microflora (JM). The results showed that the amplified gene products from JM were all gel-shifted in the positions as well as control Ec (*E. coli* DH5 α) (Fig. 3A).

Furthermore, the identification of NfsB in rat jejunal microflora was determined in nucleotide and protein levels. *E. coli nfsB* (full-length of 651 bp) was amplified by PCR, which showed the same pattern in both JM and Ec (Fig. 3A). The existence of NfsB protein was then confirmed by using NfsB antibody. By Western blot analysis, we found that NfsB existed in rat jejunal microflora at all ages (Fig. 3B).

3.3. Inhibition of NfsB nitroreductase activity in rat jejunal microflora

In order to verify the role of *E. coli* nitroreductase NfsB in FZ nitroreduction, a known nitroreductase inhibitor 2-iodosobenzoic acid (2-IBA) [32] and NfsB antibody was used to co-incubate with the microsomal membrane fractions (2 mg) of rat jejunal microflora in the presence of NADPH (0.2 mM) and FZ (64 μ M). The effects of 2-IBA (2–200 μ M) and NfsB antibody (1:250 to 1:31; v/v) on nitroreductase activity are illustrated in Fig. 4, both showed the concentration-dependent inhibition. The percentage of inhibition with 100 μ M 2-IBA was 94%, and with 1:83 (v/v) NfsB antibody was 82%.



Fig. 2. FZ nitroreduction on microsomal membrane fractions of: (A) liver and jejunum and (B) jejunal microflora of 8-week-old rats. The reactions were performed under aerobic (\square) and anaerobic (\square) conditions in the presence of NADPH (0.2 mM) and FZ (64 μ M). The detail procedure is described in Section 2. (C) Age-dependent FZ nitroreduction on microsomal membrane fractions of rat jejunal microflora (1-, 8-, 65- and 103-week-old) under anaerobic condition. Log (week) is plotted in the *x* axis (0, 0.9, 1.8 and 2.0 accordingly) versus activity in the *y* axis. Each value represents mean \pm S.D. from six incubations. Asterisk (*) indicates significant difference (P < 0.05).



Fig. 3. (A) Amplification of *E. coli nfsB*, *16S rRNA* and *lacZ* genes in rat jejunal microflora. Genomic DNA from *E. coli* DH5 α and rat jejunal microflora (8-week-old) was extracted, and used as template (0.5 μ g each) for PCR reaction. The length of the amplified *nfsB*, *16S rRNA* and *lacZ* gene fragments were 651, 584 and 876 base pairs, respectively. The detail procedure is described in Section 2. Aliquot of PCR product (10 μ l) was loaded per lane. Lane Ec: *E. coli* DH5 α , Lane JM: rat jejunal microflora. (B) Western blotting of rat jejunal microflora (1-, 8-, 65- and 103-week-old) with NfsB antibody. Certain amounts of rat microsomal membrane fractions (20 μ g each) and the purchased pure NfsB (sNfsB; 0.1 μ g) were loaded on gel.

3.4. Kinetic study of NfsB in NBDZ

To investigate the relationships of NBDZ and NfsB in detail, *E. coli* recombinant NfsB was produced for the kinetic study. *NfsB* gene (651 bp) was amplified from the genomic DNA of *E. coli* DH5 α and, after confirmation of the nucleotide sequence, cloned into the



Fig. 4. (A) Chemical and (B) immunoinhibition of NfsB nitroreductase activity in the jejunal microflora of 8-week-old rats. Various amounts of 2-IBA (2–200 μ M) and NfsB antibody (1:250 to 1:31; v/v) were added to the reaction mixtures containing 0.2 mM NADPH, 60 μ M FZ and 2 mg microsomal protein under anaerobic condition. Each value represents the mean \pm S.D. of percentage relative to control group (no inhibitor; 51.25 \pm 4.18 pmol/mg protein/min) from six incubations.



Fig. 5. (A) Construction of *nfsB* gene in the expression vector pET-46LIC. An extra fragment (1.7 kDa) containing a histidine tag and an Ek protease cleavage site is fused to the amino terminus of NfsB once protein expressed. (B) Purification and characterization of recombinant *E. coli* nitroreductase NfsB. The *nfsB* gene was overexpressed from BL21(DE3) and the recombinant NfsB was purified from Ni-NTA and DEAE columns sequentially. Proteins were run on SDS-PAGE (left panel) and compared to molecular markers (Lane Mk), which were stained with Coomassie brilliant blue. The purified NfsB was then recognized by NfsB antibody (right panel). Lane 1: non-induced crude cellular extract (20 μ g), Lane 2: IPTG induced crude cellular extract (20 μ g), Lane 2: IPTG induced crude cellular extract (20 μ g), Lane 5: the purified fraction (5 μ g), Lane 4: DEAE purified fraction (2 μ g), Lane 5: the purified recombinant NfsB (1 μ g; 25.6 kDa), and Lane 6: sNfsB (0.1 μ g; 23.9 kDa).

vector pET-46LIC (Fig. 5A). The deduced NfsB peptide sequence from the cloned gene was conserved to the declared in NCBI (accession no. NP_415110). Large amount of recombinant proteins were expressed using *E. coli* BL21(DE3) as competent cells, and then purified sequentially with Ni-NTA and DEAE columns (Fig. 5B). The purified recombinant protein showed yellow color as a flavin-containing enzyme. In addition, the recombinant NfsB had a molecular weight of 25.6 kDa as there was an extra tag of 14 residues (MAHHHHHHVDDDDK) in the amino terminus that gelshifted in higher molecular position than the purchased NfsB (23.9 kDa), and then was recognized by NfsB antibody, indicating the high purity (Fig. 5B).

In the presence of NADPH and NBDZs, *E. coli* NfsB followed Michaelis–Menten kinetics (Fig. 6), and the determined kinetic parameters for the three NBDZs were summarized in Table 1. The k_{cat}/K_{M} ratios for FZ and NZ under standard assay conditions were 3.09 ± 0.11 and 3.01 ± 0.11 [×10² μ M⁻¹ min⁻¹], respectively, and both were five times over CZ.

3.5. Nitroreductase of selected coliform enterobacteria

Using BBLTM EnterotubeTM II screening kit, three enterobacteria species, including *Escherichia spp.*, *Enterobacter spp.* and *Salmonella spp.* were successfully identified from rat jejunal microflora. The obtained isolates were further characterized with their individual nitroreductase genes (*i.e. nfsB*, *nfnB* and *nfsI*) via PCR amplification of the genomic DNA (0.5 μ g each). The amplified gene products were all gel-shifted in position as the representative bacteria



Fig. 6. Kinetics of the three NBDZs in recombinant nitroreductase NfsB. Anaerobic incubations of the recombinant NfsB were carried out in the presence of NBDZ compounds (FZ, NZ and CZ; 7–128 μ M) and NADPH (2 mM) for 2 h. Results were presented as 7ABDZ formation rate (μ M min⁻¹) in (A) from at least three incubations. Lineweaver–Burk plot is shown in (B).

strains *E. coli* DH5 α , *E. cloacae* and *S. typhimurium* TA1537, respectively (Fig. 7A).

The amplified *E. cloacae nfnB* and *S. typhimurium nfsI* gene products (both were 651 bp) from the representative strains were sequenced and the deduced peptides were all conserved to the declared protein sequences at NCBI website (accession nos. Q01234 and AAO69891). Both genes were also cloned into the vector pET-46LIC. After protein expression and purification, the obtained recombinant *E. cloacae* NR (rNR; EC 1.6.99.7) and *S. typhimurium* Cnr (rCnr) were significantly recognized by NfsB antibody (Fig. 7B). Nitroreductase activity assay of the purified rNfsB (recombinant NfsB), rNR and rCnr were performed under anaerobic condition in the presence of NADPH (2 mM) and FZ (64 μ M). The results showed that the three nitroreductases had



Fig. 7. Characterization and FZ nitroreduction of three enterobacterial nitroreductases. (A) Nitroreductase genes nfsB, nfnB and nfsI were PCR amplified from the genomic DNA of three screened species Escheriachia spp. (Es), Enterobacter spp. (En) and Salmonella spp. (Sa) as well as three representative strains E. coli DH5 α (Ec), E. cloacae (Ecl) and S. typhimurium TA1537 (St), respectively. Aliquot of PCR product (10 µl) was loaded per lane. (B) Recombinant nitroreductase proteins from nfsB-, nfnB- and nfsI-transformed BL21(DE3) cell lysates were purified and recognized by NfsB antibody. Certain amounts of the purified recombinant proteins (1 µg each) and the purchased sNfsB (0.1 µg) were loaded on gel separately. (C) FZ nitroreduction of the purified recombinant nitroreductases. An amount (10 µg) of the purified E. coli NfsB (rNfsB), E. cloacae (rNR) and S. typhimurium (rCnr) proteins were incubated with NADPH (2 mM) and FZ (64 µM) under anaerobic condition and compared to BL21(DE3) cell lysate (control, 2 mg). Each value represents mean \pm S.D. from three incubations. Mean control value 7AFZ formation was 25.84 ± 1.43 pmol/mg protein/min. Asterisk (*) indicates significant difference (P < 0.05).

similar activities in FZ nitroreduction, which were eight to eleven times higher than that of the control (Fig. 7C and Table 2).

4. Discussion

While the prescription of NBDZ in clinic has long history, the reductive metabolism of NBDZ in mammals is seldom discussed. Our previous study proposed that human hepatic P450R is responsible for FZ reduction [13], suggesting a role of nitror-

Table 1

Kinetic parameters for the recombinant E. coli nitroreductase NfsB.

NBDZ	Activity				
	<i>K</i> _M (μM)	$V_{\rm max}$ (×10 ³ µM min ⁻¹)	$k_{\rm cat}~(imes 10^3~{ m min}^{-1}$)	$k_{\rm cat}/K_{\rm M}~(imes 10^2~\mu{ m M}^{-1}~{ m min}^{-1})$	
Flunitrazepam (FZ)	23.17 ± 1.60	5.96 ± 0.19	7.15 ± 0.03	$\textbf{3.09} \pm \textbf{0.11}$	
Nitrazepam (NZ)	$\textbf{6.57} \pm \textbf{1.04}$	1.65 ± 0.27	1.98 ± 0.40	3.01 ± 0.12	
Clonazepam (CZ)	39.78 ± 3.06	2.11 ± 0.20	2.53 ± 0.68	$\textbf{0.64} \pm \textbf{0.08}$	

Data were analyzed and calculated via linear plot as shown in Fig. 6B [26]. Values for NBDZ were obtained using the standard nitroreductase assay of *E. coli* NfsB with a fixed concentration of cofactor NADPH (2 mM).

Table 2

Summary of the screened enterobacteria and the corresponding nitroreductases in the present study.

	Screened species ^a			
	Escherichia spp.	Enterobacter spp.	Salmonella spp.	
Representative strain ^b Nitroreductase ^b NCBI accession no. Amino acids Protein identity (%) ^c Activity ^d	$\begin{array}{l} \textit{E. coli DH5} \\ \textit{NfsB} \\ \textit{NP_415110} \\ \textit{217} \\ \textit{100} \\ \textit{235.57} \pm \textit{15.01} \end{array}$	E. cloacae NR Q01234 217 88 295.48 ± 3.44	S.TA1537 Cnr AAO69891 217 88 205.45 ± 25.45	

 $^{\rm a}$ Three enterobacterial species were screened via BBL^{TM} Enterotube TM II screening kit.

^b The common enterobacteria strains described with nitroreductase activity and the NfsB family nitroreductase in each strain [23].

^c Protein sequences of NR and Cnr were compared to NfsB and presented as percentage of identity.

^d Activity was determined using 64 μ M FZ as substrate (a concentration reaches over 2.5× $K_{\rm M}$ for NfsB and tends to be steady in the kinetic study) and represented as mean \pm S.D. of pmol/mg protein/min.

eductase in NBDZ disposition. In the present study, we investigated FZ nitroreduction in rat liver and jejunum, and both showed nitroreductase activities under aerobic and anaerobic conditions, consisting with the previous findings of nitroreductive potential of rat organs toward nitroaromatic compounds [1,13,15,18]. However, whether P450R or other enzymes from rat liver and jejunum are responsible for FZ nitroreduction will need to be further confirmed.

Intestinal content has already been associated with nitroreductase activity toward some nitroaromatic compounds, where the anaerobic incubation is demonstrated to have higher activity than that of the aerobic incubation [15,33]. Here we found that FZ nitroreduction in the jejunal microflora of adult rat was significantly elevated under anaerobic condition, which agrees with the previous results of NZ nitroreduction in rat cecal content [18]. Furthermore, nitroreduction of FZ under anaerobic condition showed a developmental increase beginning at 8 weeks of age. This may support the idea that nitroreductase activity in rat intestinal bacteria is significantly elevated after 28 days of age [21] since the growth of microbial population in GI tract increases extensively after neonate [34].

In this study, the introduction of E. coli type I (oxygeninsensitive) nitroreductase NfsB for NBDZ nitroreduction in rat jejunal microflora is suggested by the following considerations. First, few of bacteria type II (oxygen-sensitive) nitroreductase is clearly identified, although the anaerobic incubation is proved to be necessary for this nitroreductase in the reduction of nitroaromatic compounds [17,24]. Second, anaerobic circumstance is required for some oxygen-insensitive nitroreductases to maintain the yield of amino metabolite [33]. It is believed that the shortage of amino metabolite in air results from the oxido-reductive instability of reductive intermediates during the reaction [35]. Third, P450R, an NADPH-dependent flavoprotein, was proved to be a nitroreductase for several nitroaromatic compounds in liver [13,24]. Similarly, type I nitroreductase such as E. coli NfsB is a flavoprotein that uses FMN and NADPH as electron donors for the reduction of many nitroaromatic compounds [26,36].

In mammals, there are over 400 bacterial strains in the intestinal microflora, and *E. coli* is the most abundant (\sim 1%) enterobacterium in GI tract [15]. Clearly, our study showed an existence of *E. coli* in rat jejunal microflora from PCR amplification of 16S rRNA and lacZ, where the presence of lacZ further implied the existence of other coliform enterobacteriaceae [29]. This is further confirmed in the latter study of screening of two other coliform bacteria species *Enterobacter spp.* and *Salmonella spp.* via Enterotube.

Amplification of *nfsB* gene from rat jejunal microflora has indicated that there may exist of other bacterial NfsB-like proteins except for *E. coli* NfsB. It is convincing that two NfsB family nitroreductases *E. cloacae* NR and *S. typhimurium* Cnr were characterized genetically from the identified species (Fig. 7A), and the produced recombinant proteins were immunologically recognized by NfsB antibody (Fig. 7B). The specificity of NfsB antibody is acceptable in our experiment since it recognizes NR and Cnr (Fig. 7B) but fails to interact with two other *E. coli* type I nitroreductases NfsA [37] and YdjA [38] (data not shown).

Several studies have described the inhibition of bacterial nitroreductase activity by 2-iodosobenzoic acid (2-IBA) [32,39]. Rafil et al. reported the nitroreductase activities of five bacterial isolates from human GI tract are inhibited by 70% with 2-IBA (0.32 mg/ml) [32], and latter they demonstrated the activity of flavin-containing nitroreductase from *C. perfringens* is also significantly inhibited by 90% [39]. Here we showed that FZ nitroreduction in rat jejunal microflora was inhibited by 94% with 2-IBA (100 μ M), indicating the role of bacterial nitroreductase in the reduction of FZ. The inhibition further implies the participation of NfsB in the reduction of FZ since nicotinic acid, an analog of 2-IBA, is shown to stack on FMN for the repression of NfsB activity [40]. This hypothesis was demonstrated by NfsB antibody inhibition in our experiment, which showed an extensive repression of 82% in maximum.

To our knowledge, the major metabolic activity of NfsB is the nitroreduction for a broad range of nitroaromatic compounds [23]. In our experiment, 7ABDZ appears to be the predominant reductive metabolite of NfsB seen in the anaerobic incubation. Kinetic parameters were obtained from *E. coli* NfsB with the three NBDZ compounds. Comparison of k_{cat}/K_{M} ratios indicates that FZ and NZ are the preferred substrates for NfsB. The poor catalytic efficiency on CZ may be attributed to the higher K_{M} value (lower substrate affinity) over FZ and NZ. This can be further speculated from the chemical structures of NBDZ (Fig. 1), where R_2 group of CZ (chlorine) is obviously larger than that of FZ (fluorine) and NZ (hydrogen) in atom size that may influence CZ interaction with NfsB. Thus, in contrast to the previous studies [1,18], our experiment clarifies the relationships between bacterial nitror-eductase and NBDZ.

In spite of this, we do not exclude the involvement of other microfloral NfsB family enzymes or type I nitroreductases in NBDZ reduction. We identified three coliform enterobacteriaceae species Escherichia spp., Enterobacter spp. and Salmonella spp. from rat jejunal microflora, which all showed type I nitroreductases with high sequence homology [26]. Protein structures of NfsB, NR and Cnr are highly conserved in the whole molecules as well as the active sites [41-43], implying their similar reductive activities toward nitroaromatic compounds. This can be seen in our finding that the three recombinant NfsB family proteins have similar FZ reductive activities (Fig. 7C and Table 2). Moreover, NfsB proteins in the jejunal microflora from different ages of rats were against by the antibody and showed similar levels (Fig. 3B), yet reductive activity increased markedly with age (Fig. 2B), indicating that except for NfsB, there may exist other bacterial reductive enzymes contributing to FZ nitroreduction (Fig. 2C). For instance, E. coli nitroreductases NfsA and YdjA are identified to have nitroreductive activities as does NfsB [37,38]. To test this, E. coli NfsA and YdjA were produced in recombinant for their reductive activities, but only NfsA proceeded FZ nitroreduction (data not shown). Interestingly, NfsA shares poor sequence and structure identities with NfsB but has reductive activity toward some nitroaromatic compounds [37]. These studies demonstrate that most type I nitroreductases can reduce NBDZ, however, the detailed NfsB-NBDZ interaction and the metabolic divergence of NBDZ among different nitroreductases need to be further identified.

In conclusion, we performed nitroreductase assay in rat jejunal microflora, which showed an extensive reduction on FZ. The identified three jejunal bacteria NfsB nitroreductases had similar activities toward FZ, indicating the feasibility of bacterial type I nitroreductase in NBDZ disposition. NBDZ is one of the most potent hypnotic benzodiazepines in clinic but has drawbacks such as acute toxicity, primary addiction potential and drug–drug interactions whenever misuse [4,8]. In humans, the major place for NBDZ conversion is in liver except long half-life [9]. Instead, intestinal flora is for the short-term run of NBDZ, and it appears to be more active in metabolizing NBDZ as demonstrated in our experiment and others [1,18]. Altogether, the present study suggests that bacterial NfsB family nitroreductases are the active enzymes in NBDZ detoxification in the future.

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