The formation pathways of gamma-butyrolactone from the furan ring of tegafur during its conversion to 5-FU

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β-NAD⁺ (β-nicotinamide adenine dinucleotide), β-NADP⁺ (β-nicotinamide adenine dinucleotide phosphate), CDHP (5-chloro-2, 4-dihydroxypyridine), CYP (cytochrome P450), DNPH (2,4-dinitrophenylhydrazine), DPD (dihydropyrimidine dehydrogenase), Electrospray ionaization (ESI), *Escherichia coli* (*E.coli*), FT (tegafur), 5-FU (5-fluorouracil), GBL (γ-butyrolactone), GC/MS (gass mass spectrometry), GHB (γ-hydroxy butyrate), HPLC (High-performance liquid chromatography), LC/MS/MS (Liquid chromatography-tandem mass spectrometry), MS (mass spectrometry), NMR (nuclear magnetic resonance), 4-OH BTL (4-hydroxy butanal), QqTOF MS/MS (hybrid quadrupole time of flight mass spectrometer), SA (succinaldehyde), TPase (thymidine phosphorylase), UPLC (Ultra-performance liquid chromatography), VEGF (vascular endothelial growth factor)
Abstract

Tegafur (FT) is 5-fluorouracil (5-FU) prodrug that has been clinically used for various cancer chemotherapies. The following metabolites of FT were identified in patients: 5-FU, fluoro-β-alanine, and γ-butyrolactone (GBL) and its acidic form, γ-hydroxy butyrate (GHB). GBL/GHB, which is probably generated from the furan ring of FT, inhibits tumor cell angiogenesis, contributing to the antitumor effect of FT-based therapies. In the present study, we identified the metabolites formed from the furan ring of FT by the CYP2A6 and thymidine phosphorylase (TPase) using 2,4-dinitrophenylhydrazine (DNPH)-derivatization procedures and clarified the metabolic pathway of FT to GBL/GHB. Succinaldehyde (SA) and 4-hydroxy butanal (4-OH BTL) were produced as the metabolites because of the cleavage of the furan ring of FT during its conversion to 5-FU in cDNA-expressed CYP2A6 and purified TPase, respectively; however GBL/GHB was hardly detected in cDNA-expressed CYP2A6 and purified TPase. GBL/GHB was formed after human hepatic microsomes, or cDNA-expressed CYP2A6 mixed with cytosol were incubated with FT. Further, 4-OH BTL was converted to GBL/GHB in the microsomes and cytosol. These results suggest that GBL/GHB is generated from FT through the formation of SA and 4-OH BTL, but not directly from FT. Furthermore, the amount of 5-FU and GBL/GHB formed in the hepatic S9 was markedly decreased in the presence of a CYP2A6 inhibitor, suggesting that GBL/GHB may be mainly generated through the CYP2A6-mediated formation of SA.
Introduction

Tegafur [5-fluoro-1-(2-tetrahydrofuryl)-2, 4(1H, 3H)-pyrimidinedione] (FT) is a prodrug of 5-fluorouracil (5-FU) and has been clinically used for various cancer chemotherapies. FT is metabolized to its active form 5-FU in the liver. 5-FU inhibits the growth of cancer cell by being incorporated into RNA or by inhibiting the thymidylate synthase, which is the only de novo source of thymidylate required for DNA synthesis (Danenberg and Lockshin, 1982, Ullman and Kirsch, 1979). Previous studies have revealed that the conversion of FT to 5-FU is catalyzed by CYPs and thymidine phosphorylase (TPase) in the microsomes and cytosol, respectively (El Sayed and Sadée, 1982 and 1983; Kawata et al., 1984 and 1987; Kono et al., 1981; Sugata et al., 1986; Komatsu et al., 2001). Furthermore, it has been reported that CYP2A6 is mainly responsible for the bioactivation of FT to 5-FU in human hepatic microsomes (Ikeda et al., 2000; Komatsu et al., 2000). The following metabolites of FT have been identified in the plasma and urine of patients: 5-FU, fluoro-β-alanine, γ-butyrolactone (GBL), and its acidic form, γ-hydroxy butyrate (GHB) (Benvenuto et al., 1978; Au and Sadée, 1980; Peters et al., 2003; Emi et al., 2007). The metabolism of 5-FU to fluoro-β-alanine involves multi steps, including the action of various enzymes such as dihydropyrimidine dehydrogenase (DPD), and subsequently, it is excreted into urine. GBL/GHB, which is present endogenously (Tabakoff and von Wartburg, 1975; Bessman and Fishbein, 1963), is considered to be generated from the furan ring of FT. In addition, it has
been reported that GBL/GHB inhibits vascular endothelial growth factor (VEGF)-mediated angiogenesis in tumor cells, and thus contributes to the antitumor effect of FT-based therapies (Yonekura et al., 1999; Basaki et al., 2000; Nagai et al., 2008). During the conversion of FT to 5-FU, the furan ring of FT is thought to undergo hydroxylation at the 2’- or 5’-position, followed by the sequential decomposition to 5-FU; this is because both the hydroxy metabolites of FT are chemically unstable (Lin et al., 1979). During the decomposition of chemically unstable 2’ and 5’-hydroxy metabolites of FT to 5-FU, the furan rings of these metabolites are speculated to be converted to GBL and succinaldehyde (SA), respectively (Lin et al., 1979; EI Sayed and Sadée, 1983). Further, 4-hydroxy butanal (4-OH BTL) is generated by the spontaneous hydrolytic cleavage at the N-1-C-2’ bond of the furan moiety of FT (Au and Sadée, 1980). Although GBL/GHB was detected in vitro after the incubation of FT with mouse and rabbit hepatic homogenates (Au and Sadée, 1980), the metabolic pathways of the furan ring of FT to form GBL/GHB remain to be clarified.

In this study, we identified the metabolites formed from the furan ring of FT during its conversion to 5-FU mediated by CYP2A6 and TPase and clarified the metabolic pathway of FT leading to the formation of GBL/GHB in humans. We used the 2,4-dinitrophenylhydrazine (DNPH)-derivatization procedure, which is widely used in the analysis of aldehydes and ketones (Zwiener et al., 2002; Andreoli et al., 2003), to detect unstable metabolites formed from the furan ring of FT.
Material and Methods

Chemicals. FT, 5-chloro-2, 4-dihydroxypyridine (CDHP), 5-chloro-6-(2-iminopyrrolidin-1-yl) methyl-2, 4(1H,3H)-pyrimidinedione hydrochloride (TPI) and 4-OH BTL were synthesized at Taiho Pharmaceutical Co. (Saitama, Japan).

Succinaldehyde disodium bisulfite was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). 5-FU, β-NAD⁺, glucose-6-phosphate, GBL-d₆, DNPH, tranylcypromine hydrochloride (TCP), phenanthridine, menadione sodium bisulfite and 4-methyl pyrazole were purchased from Sigma-Aldrich Chemical Co. (St.Louis, MO, USA). Magnesium chloride hexahydrate, disulfiram, coumarin, thymidine, thymine, acetaldehyde, ethanol and GBL were purchased from Wako Pure Chemical Industries (Osaka, Japan). β-NADP⁺ and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast Co. (Tokyo, Japan).

Other chemicals used were of the highest grade commercially available.

Enzymes. Human liver samples were purchased from XenoTech, LLC. (Kansas City, KS, USA). Membranes prepared from E.coli expressing CYP2A6 (Bactosomes) was obtained from Cypex Ltd (Dundee, UK). Control membranes expressed only the vector.

Purified TPase from E.coli was purchased from Sigma-Aldrich Chemical Co. (St.Louis, MO, USA).

Preparation of standard of DNPH-derivatives. The standard DNPH-derivative of SA was prepared using the following procedures. Succinaldehyde disodium bisulfite was
dissolved in an HCl aqueous solution. The reaction was initiated by the addition of 3.1 mg/mL aliquot of DNPH/HCl solution to the SA/HCl aqueous solution, and the mixture was incubated at 37 °C for 15 min. Next, the DNPH-derivative was extracted with ethyl acetate. The organic layer was dried, and the resultant residue was purified by silica gel-column chromatography (Wakogel-C200; Wako Pure Chemical Industries, Osaka, Japan). The molecular structure of the derivative was confirmed by hybrid quadrupole time-of-flight mass spectrometer (QqTOF MS/MS) and 1H-nuclear magnetic resonance (1H-NMR) analysis.

The standard DNPH-derivative of 4-OH BTL was also prepared using the same procedure as that used for the preparation of SA-DNPH. 4-OH BTL was incubated in DNPH/HCl solution at 37 °C. The DNPH-derivative was extracted with ethyl acetate, and the collected organic layer was evaporated. The derivative of 4-OH BTL was characterized using its parent ion and the subsequent fragmentation pattern using LC/MS/MS.

**Assay to determine the formations of 5-FU and GBL/GHB from FT in human liver microsomes, cytosol or S9.** The decrease in the amount of 5-FU formed from FT, may be attributed to extensive metabolism of 5-FU by contaminated DPD. Therefore, a potent DPD inhibitor, CDHP, was added to all incubations to inhibit the degradation of 5-FU. The standard reaction mixture contained FT, 1 mM CDHP, an NADPH generating system consisting of 1.3 mM β-NADP+, 3.3 mM glucose-6-phosphate, 3.3 mM magnesium chloride and 0.4 U/ml glucose-6-phosphate dehydrogenase, in 100 mM phosphate buffer-0.1 mM
EDTA (pH 7.4). The reactions were initiated by the addition of human hepatic microsomes, cytosol or S9, following pre-incubation for 5 min at 37 °C. All incubations were performed for 30 min at 37 °C. Reactions were stopped by the addition of 3 vol. of ice-cold acetonitrile to the mixtures. After the centrifugation, the supernatant was collected and stored at -80 °C until the determinations of 5-FU and GBL. Both microsomes and cytosol were used at a concentration of 1 mg/ml, and the S9 was used at a concentration of 2 mg/mL. The spontaneous degradation of FT was evaluated using inactivated S9 which was prepared by boiling it at 100 °C for 5 min.

**Assay to determine the formations of 5-FU, GBL/GHB, SA and 4-OH BTL from FT in cDNA-expressed CYP2A6 or purified TPase.** The standard reaction mixture contained FT and 1 mM CDHP in 100 mM phosphate buffer-0.1 mM EDTA (pH 7.4). To examine CYP2A6-mediated metabolism of FT, cDNA-expressed CYP2A6 and an NADPH generating system were added to the reaction mixtures. The spontaneous degradation of FT was evaluated using control membranes. To investigate the effect of human liver cytosol on CYP2A6-mediated the formations of 5-FU and GBL/GHB, cytosol was added to bacterial membranes containing expressed CYP2A6 or control membranes, and the final concentration was 1 mg/ml. In the case of TPase-mediated metabolism, we used 10.5 U/mL of purified TPase. To determine the spontaneous degradation of FT, we performed the reaction without TPase. The reactions were initiated by the addition of cDNA-expressed CYP2A6 or purified
TPase, following pre-incubation for 5 min at 37 °C. The reactions were performed as described in the assay of FT metabolism in human hepatic microsomes, cytosol, and S9. To examine the formation of metabolites from the furan ring of FT, we added a 3.1 mg/mL aliquot of DNPH/HCl solution to the mixture after the reaction, followed by incubation for 15 min at 37 °C. Next, DNPH-derivatives were extracted with ethyl acetate, and the organic layer was dried under a nitrogen stream. The resultant residue was dissolved in mobile phase and injected into the LC/MS/MS system.

**Assay to determine the formation of GBL/GHB from 4-OH BTL in human liver microsomes or cytosol.** Standard mixture contained 62.5 μM 4-OH BTL and 1 mg/mL cytosol in 100 mM phosphate buffer-0.1 mM EDTA (pH 7.4). We used β-NAD⁺ or an NADPH generating system as co-factors in these assays. The spontaneous degradation of 4-OH BTL to GBL/GHB was evaluated using inactivated human liver cytosol which was prepared by boiling it at 100 °C for 5 min. After pre-incubation for 5 min at 37 °C, reactions were initiated by the addition of human liver cytosol, followed by the incubation for 3 min. Reactions were stopped by mixing the samples with 3-vol. of ice-cold acetonitrile. After the centrifugation, the supernatant was collected and stored at -80 °C until the determination of GBL.

**Inhibition study.** We determined the effects of inhibitors of CYP2A6 and TPase on the formations of the FT metabolites, including 5-FU, GBL/GHB, SA and 4-OH BTL. TCP and
TPI were used as inhibitors of CYP2A6 and TPase, respectively (Zhang et al., 2001; Fukushima et al., 2000). Each inhibitor was added to the reaction mixture at a concentration of 10 μM.

We determined the effects of different oxidase inhibitors, disulfiram (an aldehyde dehydrogenase inhibitor), 4-methyl pyrazole (an alcohol dehydrogenase inhibitor) and menadione (an aldehyde oxidase inhibitor) on the formation of GBL/GHB from 4-OH BTL in human hepatic cytosol at a concentration of 100 μM (Lam et al., 1997; Lake et al., 2002; Obach et al., 2004; Pietruszko, 1975). The reactions were performed as described above.

**Assay to determine the enzyme activities of biological samples (positive control).**

CYP2A6 and TPase activities of biological samples were evaluated by measuring coumarin 7-hydroxylase activity and the formation rate of thymine (from thymidine), respectively. Coumarin 7-hydroxylase activity was determined by the fluorometric assay (Bogaards et al., 2000). The thymine formed from thymidine was measured with HPLC. The HPLC analysis was performed with the Prominence LC-20 systems (Shimadzu, Kyoto, Japan) equipped with a TSK-GEL ODS-100V column (4.6 mm i.d. × 150 mm, 3 μm; Tosoh, Tokyo, Japan). The flow rate was 1.0 mL/min and the column temperature was 25 °C. The mobile phases were A (4.5 % acetonitrile) and B (acetonitrile). The typical conditions for the elution were as follows: 100 % A (0-8 min), 20 % A (8.5 min), 20 % A (10.5 min) and 100 % A (11-25 min). The eluent was monitored at 256 nm to determine thymine and thymidine.
The activities of aldehyde dehydrogenase and alcohol dehydrogenase were assayed by monitoring the formation of β-NADH from β-NAD⁺ at a wavelength of 340 nm during the metabolism of acetaldehyde and ethanol, respectively. The aldehyde oxidase activity was measured using phenanthridine as the substrate, according to the published method (Lake et al., 2002).

**Measurement of DNPH-derivatives.** The LC/MS/MS system consisted of HP1100 series liquid chromatograph (Agilent Technologies, CA, USA) coupled with API4000 triple-quadrupole mass spectrometer (Applied Biosystems, CA, USA) equipped with Turbo V source and ESI interface. Chromatographic separation of DNPH derivatives was performed on a XBridge C-18 column (4.6 mm i.d. × 150 mm, 5 μm; Waters, MA, USA) using 10 mM ammonium acetate and acetonitrile under gradient elution conditions at a flow rate of 0.2 mL/min. We used the following gradient programs: 10 mM ammonium acetate/acetonitrile from 70:30 (v/v) to 10:90 (v/v) in 7 min, immediately back to 70:30 (v/v) and then hold for 8 min. Oven temperature was maintained at 40 °C. Turbo IonSpray was used for ionization with negative ion detection for the measurements of DNPH derivatives. The source temperature was set at 600°C, ionization voltage at -4 kV and orifice potential at -60 V. Multiple reaction monitoring (MRM) was performed in the negative ionization mode, following the reactions, m/z 247-m/z 181 (CE: 30 eV) for SA-DNPH and m/z 267-m/z 152 (CE: 30 eV) for 4-OH BTL-DNPH. Data acquisition was performed using Analyst 1.4.1.
QqTOF MS/MS and $^1$H-NMR analysis. The high mass resolution experiments were performed on a QSTAR Elite hybrid QqTOF mass spectrometer equipped with a Turbo IonSpray source (Applied Biosystems, CA, USA). The LC/MS/MS parameters were set as described above. NMR spectra of the synthetic product were obtained on either a JM-EX 270 spectrometer (JEOL Ltd., Tokyo, Japan) operating at 270.05 MHz, and chemical shifts were expressed relative to tetramethylsilane.

Measurement of 5-FU and GBL. Concentration of 5-FU was determined using an UPLC/MS/MS system. The UPLC/MS/MS system consisted of a Waters AQUITY UPLC® coupled with Quattro Premier™ XE triple-quadrupole mass spectrometer (both from Waters, Boston, MA, USA) equipped with ZSpray ion source and ESI interface. Sample separation was performed using a Unison UK-Amino column (2.0 mm i.d. × 100 mm, 3 μm; Imtakt, Kyoto, Japan) at a flow rate of 0.2 mL/min at 40 °C. The mobile phase consisted of 10 mM ammonium acetate/acetonitrile 10:90 (v/v). MS/MS analysis was performed in negative ionization mode under MRM, using mass transitions, m/z 128.6-m/z 41.8 (CE: 14 eV) for 5-FU and m/z 130.6-m/z 42.8 (CE: 14 eV) for Internal Standard (IS) ($^{15}$N$_2$-5-FU). Masslynx was used for instrument control and data acquisition. To measure the amount of 5-FU in the biological sample, we mixed 100 μL of the supernatant obtained in the metabolic assays with 50 μL of 500 ng/mL IS/75 % acetonitrile solution in an injection vial, and then injected 5 μL
of the aliquot into the UPLC/MS/MS system. Concentration of GBL was determined using a GC/MS system. GC/MS was performed using a Trace-GC gas chromatograph and AS2000 automatic sampler with a Trace-MS quadrupole mass spectrometer (all equipments from Thermo Fisher Scientific, San Jose, CA, USA). Negative ion chemical ionization (NICI) was performed using isobutane as the reagent gas. Ionization was initiated at 70 eV with an emission current of 150 mA. The source temperature was 210 °C, and the GC interface temperature was 250 °C. The GC column was interfaced directly to the ion source. Gas chromatographic separation was performed on a DB-WAX capillary column (30 m × 0.32 mm i.d.; film thickness 0.25 mm; J & W Scientific, Folsom, CA, USA). The column temperature was programmed by using the following a 2-ramp temperature program: the oven was heated at 50 °C/min to 280 °C and maintained at this temperature until analysis, then increased at the rate of 20 °C/min to 190 °C for the first ramp and at the rate of 40 °C/min to 250 °C for the second ramp, and then the oven temperature was maintained for 2 min. Xcalibur (version 1.2) was used for instrument control and data acquisition. GBL was partially converted to its acid form GHB in chemical equilibrium. Therefore, GBL was measured after the lactonization of GHB under acidic condition. We used the following procedure for sample preparation for the measurement of GBL/GHB. For in vitro assay, we added 0.5 mL aliquot of 6 M HCl and 50 μL of IS/methanol solution (2 μg/mL GBL-d6) to 0.2 mL of the supernatant. After 2 mL of CH2Cl2 was added to the above mixture, the
mixture was shaken for 10 min and centrifuged at 5 °C. The organic layer was pipetted out and transferred to another test-tube. CH₂Cl₂ extraction was repeated. The combined organic layer was evaporated under a gentle stream of nitrogen. The concentrated organic layer was transferred to an injection vial, and then 1 μL of aliquot was injected into the GC/MS system. GBL and IS were detected by a selected ion monitoring procedure at m/z 85 and m/z 90, respectively.

**Statistical analysis.** All the results are represented as the mean ± standard deviation (S.D.). The statistical significance was analyzed by the Student’s t test, the Dunnet’s test, or Tukey’s test. Statistical Analysis System, version 6.12 software (SAS Institute, Inc., Cary, NC., USA) was used for all the statistical analyses.
Results

Enzyme activities of biological samples (positive control).

The results of the determinations of the enzyme activities of the biological samples and effects of enzyme inhibitors were presented in Table 1. These results served as positive control in the present investigations and demonstrated that cDNA-expressed CYP2A6, purified TPase, and human liver samples were active. Each typical inhibitor showed more than 80% inhibition against the marker enzyme activity.

The formations of 5-FU and GBL/GHB from FT in cDNA-expressed CYP2A6 and purified TPase.

Initial studies were performed to investigate the formations of 5-FU and GBL/GHB from FT catalyzed by CYP2A6 or TPase. The formation of 5-FU was observed after the incubation of FT (1 mM) with cDNA-expressed CYP2A6 in the presence of an NADPH generating system. Moreover, 5-FU was generated from FT (0.25 mM) in purified TPase. We observed a linear and time-dependent increase in the amount of 5-FU formed in both cDNA-expressed CYP2A6 and purified TPase. However, the formation of GBL/GHB was hardly observed in both enzymes.

The effects of CYP2A6 and TPase inhibitors on the conversion of FT to 5-FU are shown in Figure 1. The addition of a potent CYP2A6 inhibitor, TCP (10 μM), decreased the formation of 5-FU from FT to 42% of the control value (without the inhibitor), and the remaining
activity was similar to that in the control membranes. Similarly, the addition of a potent TPase inhibitor, TPI (10 μM), completely inhibited the conversion of FT to 5-FU.

**Characterization of DNPH-derivatives.** We applied the DNPH-derivatization approach to detect SA and 4-OH BTL in the reaction mixtures. We synthesized the derivatized standards of SA and 4-OH BTL and characterized their structures. Typical parent and product ion spectra of SA and 4-OH BTL derivatives were obtained by negative ESI/MS/MS analysis. The ion spectra of 4-OH BTL-DNPH in the negative ESI ion mode showed a predominant ion [M-H]⁻ at m/z 267, corresponding to the actual molecular weight of the derivative. We observed a predominant ion, [M-H]⁻ at m/z 247 for SA-DNPH in the negative ion mode, which was 18 atomic mass units (amu) lower than the molecular weight of the expected mono-DNPH attachment derivative at m/z 265. The time-of-flight mass analysis revealed that the [M-H]⁻ ion for SA-DNPH exhibited the molecular composition of C₁₀H₇N₄O₄, corresponding to the loss of H₂O molecule from the mono-DNPH attached adduct of C₁₀H₉N₄O₅ (Table 2). The formation of SA-DNPH possibly proceeds via the Paal-Knorr Pyrrole Synthesis reaction (Figure 2). First, the aldehyde group of SA reacted with DNPH to form the mono-DNPH derivative. Subsequently, a rapid reaction was triggered by a nucleophilic attack on the remaining carbonyl carbon in the amino group of the enamine intermediate, which results in the closed-ring structure, followed by dehydration to form the stable pyrrole compound. The results of QqTOF MS/MS analysis are shown in
Table 2; fragmentation of the ion at m/z 247 showed typical product ions at m/z 181 and m/z 66, which were identified as molecules with the molecular formulae, C₆H₃N₃O₄ and C₄H₄N, respectively. The radical anion [M]⁻ at m/z 181 was formed by the cleavage of SA-DNPH at the α-amino group of its hydrazine moiety and subjected to further fragmentation. As shown in Table 2, the series of the fragment ions at m/z 164, m/z 151, m/z 135, m/z 120 and m/z 105 were assigned to C₆H₂N₃O₃, C₆H₃N₂O₃, C₆H₂N₂O₂, C₆H₂NO₂, and C₆H₃NO, respectively, by QqTOF MS/MS analysis. These ions were probably formed through subsequent concomitant losses of NO, NO₂ or OH etc. from the product ion at m/z 181 by direct bond cleavage and the loss of HNO etc. The other molecular anion [M]⁻ at m/z 66 with the molecular formula, C₄H₄N, was attributed to the pyrrole ring of SA-DNPH. Furthermore, in its ¹H-NMR spectrum (270.05 MHz, CDCl₃), the characteristic signals at 6.34 (2H, t, J = 2.5 Hz) and 6.76 (2H, t, J = 2.5 Hz) ppm suggested symmetric heterocyclic structure of pyrrole ring, along with the proton signals at 6.34 (1H, d, J = 9.5 Hz), 8.25 (1H, dd, J = 2.5 and 9.5 Hz), 9.16 (1H, d, J = 2.5 Hz) and 10.27 (1H, s) ppm, those were assigned to DNPH moiety. The subsequent detection of DNPH derivatives in biological samples was obtained in MRM, following the reactions m/z 247-m/z 181 (CE: 30 eV) for SA-DNPH and m/z 267-m/z 152 (CE: 30 eV) for 4-OH BTL-DNPH.

The formations of SA and 4-OH BTL from FT in cDNA-expressed CYP2A6 and purified TPase. To investigate the formations of SA and 4-OH BTL from FT catalyzed by
CYP2A6 or TPase, metabolic studies were conducted using the DNPH-derivatization. The formation of DNPH-derivatives in the reaction mixtures was confirmed by comparing the retention times of SA-DNPH (8.1 min) and 4-OH BTL-DNPH (6.8 min) with those of their corresponding standards. We compared the peak area of each DNPH derivative obtained from the MRM analysis under the different conditions. TCP and TPI exerted inhibitory effects on the formations of SA-DNPH and 4-OH BTL-DNPH in cDNA-expressed CYP2A6 or purified TPase as shown in Figure 3. After FT (5 mM) was incubated in cDNA-expressed CYP2A6, SA-DNPH was observed as a much larger peak than that observed in other assays, and the formation of 4-OH-BTL-DNPH in cDNA-expressed CYP2A6 was similar to that in control membranes. As shown in Figure 3, a small amount of SA-DNPH observed in the absence of FT was derived from DNPH. The addition of TCP (10 μM) completely decreased the formation of SA-DNPH in cDNA-expressed CYP2A6, whereas the formation of 4-OH BTL-DNPH was unaffected (Figure 3A). Furthermore, the formation of 4-OH BTL in control membranes was similar to that in cDNA-expressed CYP2A6, which suggests that 4-OH BTL was generated by the spontaneous degradation of FT.

After FT was incubated in purified TPase, the peak of 4-OH BTL was much higher than that observed in other reaction mixtures. Further, the amount of SA-DNPH formed was similar to that formed in the mixture incubated without purified TPase or FT. The addition of TPI (10 μM) resulted in complete inhibition of the formation of 4-OH BTL from FT in
The formation of 5-FU and GBL/GHB in cDNA-expressed CYP2A6 or human liver cytosol. The effect of addition of human liver cytosol on the formation of GBL/GHB was assessed to investigate the conversion of SA and 4-OH BTL to GBL/GHB during the metabolism of FT to 5-FU. The formations of 5-FU and GBL/GHB from FT (1 mM) in cDNA-expressed CYP2A6 or human liver cytosol are shown in Figure 4. The formation of 5-FU was observed in all reaction mixtures, whereas GBL/GHB was not generated in the absence of human liver cytosol. The addition of cytosol increased the amount of 5-FU formed in cDNA-expressed CYP2A6 (from 9.9 ± 0.8 pmol/min to 13.2 ± 0.7 pmol/min) and control membranes (from 2.6 ± 0.1 pmol/min to 4.8 ± 0.2 pmol/min). The amount of 5-FU generated from FT in human liver cytosol, was 4.6 ± 0.1 pmol/min. After FT was incubated in cDNA-expressed CYP2A6 with human liver cytosol, the amount of GBL/GHB generated was higher (4.1 ± 0.2 pmol/min) than that in control membranes with human liver cytosol (2.4 ± 0.2 pmol/min), which indicates that metabolites such as SA and 4-OH BTL that are generated from the furan ring of FT in cDNA-expressed CYP2A6, are converted to GBL/GHB by cytosolic enzymes.

The formation of GBL/GHB from 4-OH BTL in human liver cytosol and microsomes. We investigated the involvement of human liver microsomes and cytosol in the conversion of 4-OH BTL to GBL/GHB. Formation of GBL/GHB from 4-OH BTL (67 μM) in human liver
cytosol and microsomes is shown in Figure 5. In human liver cytosol, 4-OH BTL was converted to GBL/GHB, and the additions of $\beta$-NAD$^+$ and NADPH enhanced the formation of GBL/GHB. We used the inhibitors at much higher concentrations than their Ki and IC50 values reported (Lam et al, 1997; Obach et al, 2004; Pietruszko, 1975). The typical inhibitors, disulfiram, 4-methyl pyrazole and menadione showed more than 80% inhibitory effect on marker metabolic enzymes in cytosol at the concentration of 100 $\mu$M (Table 1).

The formation of GBL/GHB was inhibited by the addition of disulfiram (100 $\mu$M), and menadione (100 $\mu$M), approximately by 72% and 45%, respectively, whereas the addition of 4-methyl pyrazole (100 $\mu$M) had a little effect on the formation of GBL/GHB (Figure 5A). The formation of GBL/GHB from 4-OH BTL in hepatic microsomes was similar to its spontaneous formation in the inactivated cytosol. The addition of $\beta$-NAD$^+$ resulted in an increase in the formation of GBL/GHB in human liver microsomes; the amount of GBL/GHB formed was similar to that in hepatic cytosol.

The formations of 5-FU and GBL/GHB from FT in human liver microsomes, cytosol or S9. To clarify the metabolism of FT in human liver, the formations of 5-FU and GBL/GHB from FT were investigated in human liver microsomes, cytosol and S9. The formations of 5-FU and GBL/GHB from FT (1 mM) in hepatic microsomes, cytosol or S9 after incubation with CYP2A6 and TPase inhibitors are shown in Figure 6 (A) and (B). In human liver microsomes, the amounts of 5-FU and GBL/GHB generated from FT, were 38.1
± 3.2 pmol/min and 5.3 ± 0.7 pmol/min, respectively; further, TCP (10 μM) showed 74 % and 87 % inhibition against the formation of 5-FU and GBL/GHB in hepatic microsomes, respectively. The amounts of 5-FU and GBL/GHB generated from FT in hepatic cytosol were 6.0 ± 0.1 pmol/min and 2.7 ± 0.3 pmol/min, respectively. TPI (10 μM) slightly decreased the formation of 5-FU in hepatic cytosol to a similar level as the inactivated S9.

In hepatic S9, the amounts of 5-FU and GBL/GHB generated from FT were 46.0 ± 3.0 pmol/min and 7.4 ± 0.9 pmol/min, respectively. TCP showed approximately 70 % inhibition of the formations of 5-FU and GBL/GHB, whereas TPI had little effect. In inactivated S9, we did not observe the formation of GBL/GHB. Figure 6 (C) and (D) show the effects of various oxidase inhibitors on the formations of 5-FU and GBL/GHB in human liver microsomes, cytosol, and S9. Disulfiram and menadione inhibited the formation of 5-FU in microsomes by 68 % and 87 %, respectively. The amount of 5-FU formed in S9 was also significantly decreased to 44 % and 14 % of the control values by the additions of disulfiram and menadione, respectively. Further, we observed similar results on the formation of GBL/GHB in microsomes and S9. On the other hand, disulfiram showed complete inhibition against the formation of GBL/GHB in the cytosol without affecting the amount of 5-FU formed.
Discussion

GBL/GHB, the metabolite possibly formed from the furan ring of FT, suppresses the VEGF-induced chemotactic migration and tube formation in human umbilical vein endothelial cells stimulated at the 50% inhibitory concentration (IC50) of 0.31 μM (26.7 ng/mL) (Basaki et al., 2001). It has been reported that uracil-tegafur (UFT), which is an oral fluoropyrimidine formed by the combination of uracil and FT at a molar ratio of 4:1, has an antiangiogenic effect. Emi et al. measured the plasma concentration of GBL/GHB in gastric cancer patients, before and after the administration of UFT. The endogenous plasma concentration of GBL/GHB in patients was 16.8 ± 4.0 ng/mL. The mean maximum plasma concentration of GBL/GHB after the administration of UFT was 147.5 ng/mL, which was 5 times higher than its IC50 described above. Furthermore, at 4 hr after the administration of UFT, the plasma concentration of GBL/GHB (78.0 ng/mL) was 3 times higher than its IC50 of 26.7 ng/mL (Emi et al., 2007). Other reports reveal that there is an inverse correlation between the serum levels of GBL/GHB and VEGFs before and after UFT therapy (Nagai et al., 2008). Thus, the anti-angiogenesis effects of UFT may be attributed to the action of GBL/GHB, which specifically acts against VEGF-induced angiogenesis. Therefore, GBL/GHB has been expected to play an important role in FT-based chemotherapies; however, the mechanism of metabolic cleavage of the furan ring of FT to form GBL/GHB remains to be clarified. Results from previous studies indicate that 2’-and 5’-hydroxy metabolites of FT
are immediately decomposed to 5-FU, because of their chemical instability, and that their
furan rings would concomitantly be converted to GBL or SA (Lin et al., 1979; El Sayed and
Sadée, 1983). However, the metabolic pathways of the furan ring of FT have remained
unclear under physiological conditions.

In the present study, we identified the metabolites from the furan ring of FT during its
CYP2A6 or TPase-mediated conversion to 5-FU. 5-FU was generated from FT, whereas the
formation of GBL/GHB was not observed after the incubation of FT with both
cDNA-expressed CYP2A6 and purified TPase. This result indicates that the hydroxylation
of FT at the 2'-position does not occur, which results in the formation of GBL/GHB in both
enzymes, because the 2'-hydroxy metabolite of FT can be directly converted to GBL/GHB.
The chemical derivatization using hydrazine reagents has been reported to improve the
sensitivity of detection of poorly ionic carbonyl compounds with MS and overcome the
reactive and volatile nature of aliphatic-chain aldehydes and ketones (Vogel et al., 2000). To
identify the metabolites from the furan ring of FT, such as SA and 4-OH BTL, which are
short-chain dialdehyde and semialdehyde compounds, we employed the DNPH-derivatization
method. The structure of DNPH-derivative of SA was identified as an anilino pyrrole
structure by QqTOF MS/MS and $^1$H-NMR analysis. This finding is in agreement with the
previous report, which shows that 1,4-diketones reacted with DNPH to form
(N-2,4-dinitroanilino) pyrroles under the acidic condition (Binns and Brettle, 1966). The
4-OH BTL-DNPH yielded the parent ion with the expected molecular weight of the mono-DNPH-attached derivative previously reported (Chen et al, 1978). In cDNA-expressed CYP2A6, SA and 4-OH BTL were generated from FT during its conversion to 5-FU. TCP, a potent CYP2A6 inhibitor, completely inhibited the formation of SA, but it had no effect on the formation of 4-OH BTL. Furthermore, the formation of 4-OH BTL in control membranes was similar to that in cDNA-expressed CYP2A6, which indicates that 4-OH BTL was generated by the spontaneous degradation of FT. These results suggest that FT is hydroxylated at 5'-position by CYP2A6, resulting in the formation of 5-FU. On the other hand, the furan ring of FT was converted to 4-OH BTL, but not SA by TPase. Thus, the hydrolytic cleavage of the furan ring of FT at the N-1-C-2’ bond may be catalyzed by TPase, which results the formation of 4-OH BTL.

In this study, we showed that GBL/GHB is not directly generated from FT by CYP2A6 and TPase. However, the formation of GBL/GHB has been reported after the incubation of FT with animal hepatic homogenate. In addition, previous studies revealed that GBL/GHB was detected in the plasma of patients in the form of a metabolite from the furan ring of FT. Consequently, we investigated the metabolic pathways of SA and 4-OH BTL in human hepatic samples. 4-OH BTL occurs as an intermediate during the conversion of 1,4-butanediol to GBL/GHB (Roth and Giarman, 1969). SA is an oxidized form of succinic semialdehyde, which is an endogenous compound and can be reduced to GBL/GHB by GHB.
synthase (Lyon et al., 2007). Therefore, SA and 4-OH BTL generated from the furan ring of FT are thought to be converted to GBL/GHB under physiological conditions. In the present study, the addition of cytosol increased the amounts of 5-FU and GBL/GHB formed in cDNA-expressed CYP2A6. The increase in the formation of 5-FU may be attributed to TPase in the cytosol, considering that TPI decreased the formation of 5-FU in the cytosol to a similar level as its spontaneous formation in the inactivated S9, which suggests that TPase is responsible for the metabolism of FT in the cytosol. After the addition of cytosol, the amount of GBL/GHB formed in cDNA-expressed CYP2A6 was much greater than that in control membranes with cytosol. Furthermore, the formation of GBL/GHB in human hepatic microsomes was inhibited by TCP. These findings suggest that SA from the furan ring of FT is converted to GBL/GHB in the cytosol and microsomes. Similarly, 4-OH BTL was converted to GBL/GHB in the cytosol and microsomes. Our study with different inhibitors revealed that aldehyde dehydrogenase and aldehyde oxidase were mainly involved in the conversion of 4-OH BTL to GBL/GHB in the cytosol. In addition, the amount of GBL/GHB formed from 4-OH BTL in the cytosol with β-NAD⁺, which is a co-factor necessary for aldehyde dehydrogenase in catalyzing the oxidation of aldehydes, was considerably higher than that in microsomes, which suggests that cytosolic dehydrogenases would mainly contribute to the oxidation of 4-OH BTL to GBL/GHB. These inhibitors strongly inhibited the formation of 5-FU in microsomes and S9, whereas its formation in the

26
cytosol was not affected. The metabolite of disulfiram, diethyldithiocarbamate, and menadione efficiently inhibit CYP2A6 and NADPH-dependent cytochrome P450 reductase, respectively, in microsomes (Ono et al., 1996; Sadowski et al., 1986), which would result in the decrease of the formation of 5-FU in the present study. The amount of GBL/GHB formed in the cytosol was decreased by addition of disulfiram without inhibiting the formation of 5-FU, which indicates that 4-OH BTL formed during the conversion of FT to 5-FU can be converted to GBL/GHB by aldehyde dehydrogenase. In human hepatic S9, the addition of TCP exerted a strong inhibitory effect on the formation of 5-FU, whereas TPI had little effect, which suggests that in the human liver, CYP2A6, but not TPase, is mainly responsible for the conversion of FT to 5-FU. We observed similar results during the formation of GBL/GHB in the hepatic S9, which indicates that the CYP2A6-mediated metabolic pathway of the furan ring of FT, followed by the sequential conversion of SA to GBL/GHB may mainly contribute to the formation of GBL/GHB under physiological conditions. The studies on endogenous GBL/GHB catabolism have shown the oxidation of GBL/GHB to succinic semialdehyde by GHB dehydrogenase, followed by its oxidation to succinate by succinic semialdehyde dehydrogenase in mitochondria (Kaufman et al., 1979). Therefore, GBL/GHB generated after the administration of FT, may be finally metabolized to succinate in mitochondria in \textit{in vivo}.

The proposed metabolic pathway of the furan ring of FT is shown in Figure 7. In this
study, we clarified that SA and 4-OH BTL were generated from the furan ring of FT by CYP2A6 and TPase, respectively, during its conversion to 5-FU. Further, we showed that these metabolites were converted to GBL/GHB in human hepatic samples. In human liver, the CYP2A6-mediated conversion of the furan ring of FT to SA, followed by the oxidation to GBL/GHB, may be the main pathway responsible for the formation of GBL/GHB.


References


Bessman SP, and Fishbein WN (1963) gamma-Hydroxybutyrate, a normal brain metabolite.


*Xenobiotica.* 30: 1131-1152.


[R,S-1-(tetrahydro-2-furanyl)-5-fluorouracil]: the microsomal oxidative pathway.


El Sayed YM, and Sadée W (1983) Metabolic activation of

R,S-1-(tetrahydro-2-furanyl)-5-fluorouracil (ftorafur) to 5-fluorouracil by soluble enzymes.

*Cancer Res.* 43: 4039-4044.


Legends for Figures

Figure 1. The effects of inhibitors of (A) CYP2A6 and (B) TPase on the formation of 5-FU in cDNA-expressed CYP2A6 and purified TPase. FT (1 mM for CYP2A6 and 0.25 mM for TPase) was incubated in cDNA-expressed CYP2A6 (25 pmol/mL) or purified TPase (10.5 U/mL) in the absence or presence of inhibitors at 37 °C for 30 min. Data are expressed as percentages of the formation of 5-FU in the incubation mixture without inhibitors. Each bar represents the mean ± S.D. (n = 3). The formations of 5-FU in cDNA-expressed CYP2A6 and purified TPase were 2.3 ± 0.4 pmol/min/pmol P450 and 18.9 ± 4.0 pmol/min/U, respectively. **Significantly different (p < 0.01) from CYP2A6 or TPase.

Figure 2. The proposed derivatization pathways for SA (A) and 4-OH BTL (B) with DNPH.

Figure 3. The formations of SA and 4-OH BTL from FT in cDNA-expressed CYP2A6 (A) or purified TPase (B). FT was incubated in cDNA-expressed CYP2A6 (25 pmol/mL) or purified TPase (10.5 U/mL) in the absence or presence of the inhibitor at 37 °C. After the reaction, the derivatives of metabolites were prepared using DNPH. Data was expressed as percentages of the formations of SA-DNPH and 4-OH BTL-DNPH in the incubation mixture of FT without inhibitors (control). Each bar represents the mean ± S.D. (n = 3). **Significantly different (p < 0.01) from control in each reaction.

The retention times of peaks of DNPH-derivatives in incubations were preliminary.
determined by co-elution of biological samples with the derivatized standards.

**Figure 4. The formations of 5-FU (A) and GBL/GHB (B) from FT in cDNA-expressed CYP2A6 (2A6) or human liver cytosol (Cyt).** FT (1 mM) was incubated in cDNA-expressed CYP2A6 (2A6), CYP2A6 with human liver cytosol (2A6 + Cyt), control membranes (CMS), control membranes with cytosol (CMS + Cyt) or cytosol (Cyt) at 37 °C for 30 min. Each bar represents the mean ± S.D. (n = 3). **Significantly different (p < 0.01) from the incubation without cytosol in each reaction.

**Figure 5. The formation of GBL/GHB from 4-OH BTL in cytosol (A) or microsomes (B).** 4-OH BTL (67 μM) was incubated in the cytosol (0.3 mg/mL) or microsomes (0.5 mg/mL) in the absence or presence of co-factors at 37 °C for 3 min. Each inhibitor was added to the incubation mixture at a final concentration of 100 μM. Data are expressed as percentages of the formation of 5-FU in the incubation mixture without inhibitors. Each bar represents the mean ± S.D. (n = 3). **Significantly different (p < 0.01) from the incubation with β-NAD⁺ in cytosol.

**Figure 6. The formations of 5-FU and GBL/GHB from FT in human hepatic microsomes, cytosol or S9. Effects of TCP and TPI on the formations of 5-FU (A) and GBL/GHB (B), and disulfiram (DF), 4-methyl pyrazole (4MP) and menadione (MD) on the formations of 5-FU (C) and GBL/GHB (D).** FT (1 mM) was incubated in microsomes, cytosol or S9 with an NADPH generating system and CDHP (1 mM) at 37 °C for 30 min in
the absence or presence of inhibitors. Each point represents the mean ± S.D. (n = 3).

**Significantly different (p < 0.01) from control in each material.

**Figure 7. Proposed metabolic pathway of FT.**
### Table 1. Enzyme activities of biological samples and effects of typical inhibitors.

Each value represents the mean ± S.D. (n = 3).

<table>
<thead>
<tr>
<th>Marker enzyme</th>
<th>Enzyme source</th>
<th>Probe substrate</th>
<th>Inhibitor</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2A6</td>
<td>cDNA-expressed</td>
<td>Coumarin</td>
<td>+ TCP (10 μM)</td>
<td>7.1 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>0.7 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Microsomes</td>
<td>Coumarin</td>
<td>+ TCP (10 μM)</td>
<td>424.0 ± 30.3&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
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<td></td>
<td>12.0 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>S9</td>
<td>Coumarin</td>
<td>+ TCP (10 μM)</td>
<td>262.7 ± 9.7&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
<td>11.4 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>TPase</td>
<td>Purified TPase</td>
<td>Thymidine</td>
<td>+ TPI (10 μM)</td>
<td>6.6 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>N.D.&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>TPase</td>
<td>Cytosol</td>
<td>Thymidine</td>
<td>+ TPI (10 μM)</td>
<td>9.0 ± 0.3&lt;sup&gt;e&lt;/sup&gt;</td>
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</tr>
<tr>
<td>TPase</td>
<td>S9</td>
<td>Thymidine</td>
<td>+ TPI (10 μM)</td>
<td>5.5 ± 0.7&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>ALDH</td>
<td>Cytosol</td>
<td>Acetaldehyde</td>
<td>+ Disulfiram (100 μM)</td>
<td>5.8 ± 0.8&lt;sup&gt;e&lt;/sup&gt;</td>
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<td></td>
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<td></td>
<td>0.4 ± 0.4&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>ALH</td>
<td>Cytosol</td>
<td>Ethanol</td>
<td>+ 4-Methyl pyrazole (100 μM)</td>
<td>18.0 ± 1.5&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>3.1 ± 0.7&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>ALO</td>
<td>Cytosol</td>
<td>Phenanthrine</td>
<td>+ Menadione (100 μM)</td>
<td>2.8 ± 0.2&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>0.2 ± 0.1&lt;sup&gt;e&lt;/sup&gt;</td>
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</table>

ALDH, Aldehyde dehydrogenase; ALH, Alcohol dehydrogenase; ALO, Aldehyde oxidase; cDNA-expressed, cDNA-expressed CYP2A6; Microsomes, Human liver microsomes; S9, Human liver S9; Cytosol, Human liver cytosol.

<sup>a</sup>pmol/min/pmol P450; <sup>b</sup>pmol/min/mg; <sup>c</sup>nmol/min; <sup>d</sup>ND, not detected; <sup>e</sup>nmol/min/mg.
Table 2. Product ion mass spectra analysis of SA-DNPH using QqTOF MS/MS under the negative ESI condition. An accuracy error threshold of ± 5 mDa was set as a limit to the calculation of possible elemental compositions.

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<th>Measured mass</th>
<th>Calculated mass</th>
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<th>Electron state</th>
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<td>MS² [M-H]</td>
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<td>MS²</td>
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<td>C₅H₄N</td>
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Figure 1.
Figure 2.

A)  
\[
\begin{align*}
\text{SA} & \quad + \quad \text{DNPH} \\
& \quad \xrightarrow{37^\circ C \text{ for } 15 \text{ min}} \quad \xrightarrow{\text{[H}^+\text{]}} \\
& \quad \xrightarrow{-[\text{H}_2\text{O}]} \\
& \quad \xrightarrow{\text{[H}^+\text{]}} \\
& \quad \xrightarrow{\text{[H}^+\text{]}} \\
& \quad \text{SA-DNPH}
\end{align*}
\]

B)  
\[
\begin{align*}
\text{4-OH BTL} & \quad \xleftrightarrow{37^\circ C \text{ for } 15 \text{ min}} \quad \xrightarrow{-[\text{H}_2\text{O}]} \\
& \quad \xrightarrow{\text{[H}^+\text{]}} \\
& \quad \text{4-OH BTL-DNPH}
\end{align*}
\]
Figure 3.

A) SA-DNPH

4-OH BTL-DNPH

B) SA-DNPH

4-OH BTL-DNPH
Figure 4.

A) 

5-FU formation (pmol/min)

- 2A6
- 2A6 + Cyt
- CMS
- CMS + Cyt
- Cyt

B) 

GBL/GHB formation (pmol/min)

- 2A6
- 2A6 + Cyt
- CMS
- CMS + Cyt
- Cyt
Figure 5.
Figure 6.

A) 5-FU formation (pmol/min)

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B) GBL/GBH formation (pmol/min)

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<td>S9(-)</td>
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C) 5-FU formation (pmol/min)

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D) GBL/GBH formation (pmol/min)

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Figure 7.