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Hepatic UDP-Glucuronosyltransferases Responsible for Glucuronidation of Thyroxine T_4 in Humans

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Running title: Human UGTs responsible for T_4-glucuronidation

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ABBREVIATIONS: ST232, 26,26,26,27,27,27-hexafluoro-1α,23(S),25-trihydroxyvitamin D_3; T_4, thyroxine; UGT, UDP-glucuronosyltransferase.

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Abstract

To clarify the UDP-glucuronosyltransferase (UGT) isoform(s) responsible for the glucuronidation of thyroid hormone thyroxine T_4 (T_4) in the human liver, the T_4-glucuronidation activities of recombinant human UGT isoforms and 7 individual human liver microsomes were comparatively examined. Among the 12 recombinant human UGT1A and UGT2B subfamily enzymes examined, UGT1A1, UGT1A3, UGT1A9 and UGT1A10 showed definite activities for T_4-glucuronidation. These UGT1A enzymes, with an exception of UGT1A10, were detected in all the human liver microsomes examined. Interindividual difference in T_4-glucuronidation activity was observed among the 7 individual human liver microsomes, and the T_4-glucuronidation activity was closely correlated with β-estradiol 3-glucuronidation activity. Furthermore, Spearman correlation analysis for a relationship between the T_4-glucuronidation activity and the level of UGT1A1, UGT1A3, or UGT1A9 in the microsomes revealed that levels of UGT1A1 and UGT1A3, but not UGT1A9, were closely correlated with T_4-glucuronidation activity. T_4-glucuronidation activity in human liver microsomes was strongly inhibited by 26,26,26,27,27,27-hexafluoro-1α,23(S),25-trihydroxyvitamin D_3 (an inhibitor of UGT1A3), moderately by either bilirubin (an inhibitor of UGT1A1) or β-estradiol (an inhibitor of UGT1A1 and UGT1A9), but not by propofol (an inhibitor of UGT1A9). These findings strongly indicated that glucuronidation of T_4 in the human liver was mediated by UGT1A subfamily enzymes, especially UGT1Al and UGT1A3, and further suggested that the interindividual difference would come from that in the expression levels of UGT1A1 and UGT1A3 in individual human livers.

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Introduction

Thyroid hormone, a thyroxine T_4 (T_4), is metabolized via deiodination, *O*-glucuronidation, *O*-sulfation, ether bond cleavage and/or oxidative deamination (Visser, 1996; Wu et al., 2005). Among these metabolisms, *O*-glucuronidation is considerably important, because it is responsible for the metabolism of many endogenous and exogenous chemicals (Iyanagi, T., 2007; Radominska-Pandya et al., 1999). Visser (1996) had firstly reported that T_4-glucuronidation was mediated by UDP-glucuronosyltransferase 1A (UGT1A) subfamily enzymes, especially UGT1A1 and UGT1A6, in the rat liver. On the other hand, it had been reported that T_4-glucuronidation in the human liver was mediated mainly by UGT1A1 and UGT1A9 (Visser et al., 1993; Findlay et al., 2000). Quite recently, Yamanaka et al. (2007) have reported that the T_4-glucuronidation activity in human liver is mainly catalyzed by UGT1A1.

However, these previous studies on contribution of the UGT subfamily enzymes responsible for T_4-glucuronidation in rats and humans have been performed using only limited samples and/or techniques. The UGT isoform(s) for the T_4-metabolism is not clearly determined. In addition, the UGT genes are divided into two families, *UGT1* and *UGT2*, based on a homology of the amino acid sequence (Mackenzie et al., 2005). In the present study, to further clarify the human UGT isoform(s) responsible for T_4-glucuronidation, we examined comparatively the activities of the 12 recombinant human UGTs, including UGT1 and UGT2 family enzymes, and further determined a relationship between T_4-glucuronidation activity and levels of UGT subfamily enzymes in 7 individual human liver microsomes.

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Materials and Methods

Materials. UDP-glucuronic acid, alamethicin, and propofol were purchased from Sigma-Aldrich (St. Louis, MO). 26,26,26,27,27,27-hexafluoro-1α,23(S),25-trihydroxyvitamin D_3 (ST232), a probe substrate for UGT1A3 (Kasai et al., 2005), was kindly donated from Sumitomo pharmaceuticals (Osaka, Japan) and used a selective inhibitor of UGT1A3. Its chemical structure is shown in Fig. 1. The $[^{125}I]T_4$ (116 Ci/mmol) radiolabeled ^{125}I at 5' position of the outer ring was obtained from PerkinElmer Life Sci., Inc. (Boston, MA).

Human liver microsomes from 7 individual human livers (HH2, HH13, HH47, HG3, HG32, HG64, and HG74) with the data on UGT activities toward β-estradiol, trifluoperazine, and propofol were purchased from BD Gentest (Woburn, MA). The recombinant human UGT isoforms, such as UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17, which are expressed in the insect cells (Supersomes) infected with the corresponding UGT gene-inserted baculovirus, were purchased from BD Genetest. Likewise, the microsomes from the insect cells infected with wild-type baculovirus (without a UGT gene) were purchased and used as a control (without UGTs).

Preparation of anti-human UGT antibody. The peptide KWLPQNDLLGHPKA deduced from a UGT2B15 cDNA sequence (356–369) (Green et al., 1994) was used as a UGT-antigen and immunized to rabbits as described previously (Ikushiro et al., 1995). The established antibody, which is cross-reactive with all the UGT1A and UGT2B

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subfamily isoforms examined, was designated as an anti-human UGT.

Immunoblot analysis. Amount of each UGT isoform expressed in insect cells was determined by the Western blotting with an anti-human UGT. To obtain clear band on the immunoblot, a portion (20 μl) of microsomal fraction containing human UGT(s) was treated with endoglycosidase H (200 units) for 60 min at 37°C, and the reaction was terminated by heating at 98°C for 5 min. The endoglycosidase H-treated microsomal fraction was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed using a 4% stacking and 10% separating gel. After SDA-PAGE, the separated proteins on a gel were transferred to a nitrocellulose sheet by a semi-dry blotting method. The proteins bound to an anti-human UGT on a sheet were detected using chemical luminescence (ECL detection kit; Amersham Biosciences Inc., Piscataway, NJ). Amounts of UGT1A and UGT2B proteins in the microsomes were estimated using maltose binding protein (MBP)-UGT1AC and MBP-UGT2B7C fusion proteins (New England Biolabs), respectively, as standards. In addition, amounts of the fusion proteins were determined by a bicinchoninic acid protein assay.

Levels of UGT isoforms in human liver microsomes were measured by the Western blotting with either anti-h1AC (anti-human UGT1A antibody) or isoform-specific antibodies (anti-h1A1, anti-h1A3, anti-h1A9, and anti-2B7), as described previously (Ikushiro et al., 2006). In addition, a portion (20 μg or 40 μg protein/lane) of microsomal preparation was treated with endoglycosidase H and then used for the Western blot analysis. Levels of UGT isoforms in individual human microsomes were shown as a ratio to that of the respective isoforms expressed in a human sample HG3.

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Glucuronidation Assay. Microsomal T_4-glucuronidation activity was determined according to the method of Barter and Klaassen (1992). Briefly, the microsomal preparation (2.0 mg protein) with each human recombinant UGT or human liver microsomal preparation (2.0 mg protein) was added to the reaction medium (final volume 1ml) containing 0.1 mg alamethicin, 13.2 mM $MgCl_2$, 66 mM Tris-HCl (pH 7.4), 1.26 mM saccharic acid-1,4-lactone and 4 mM UDP-glucuronic acid. The reaction was initiated by the addition of 90 μM T_4 solution containing $[^{125}I]T_4$ (40-65 μCi/μmol), performed at 37℃ for 4 h, and terminated by the addition of ice-cold ethanol (500 μl). After centrifugation of the reaction mixture, the resultant supernatant was used for the assay. An aliquot (50 μl) of a supernatant fraction was applied to an LK6DF silica gel-coated TLC plate (Whatman) and then developed with the solution containing ethyl acetate, methyl ethyl ketone, formic acid, and water (50 : 30 : 10 : 10). After separation by the TLC, the entire plate was scraped in 5 mm fractions, and radioactivity on each fraction was measured by γ-scintillation spectrometry (PerkinElmer, Inc., Waltham, MA).

Effects of several UGT inhibitors on microsomal T_4-glucuronidation. Bilirubin, ST232, and propofol were used as typical inhibitors of UGT1A1 (Senafi et al., 1994; King et al., 1996), UGT1A3 (Kasai et al., 2005), and UGT1A1/UGT1A9 (Burchell et al., 1995), respectively. A human liver microsomal preparation (HH13), which contained a lot of UGT1A1, UGT1A3, and UGT1A9, was selected as an enzyme source, and inhibitory effects of bilirubin, ST232, and propofol on the microsomal activity for T_4-glucuronidation were examined by the method described in the "Glucuronidation

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assay".

Correlation analysis. Correlation analyses between the glucuronidation activity toward T_4 or other substrates and the level of UGT1A isoforms in 7 individual human liver microsomes were performed by linear regression.

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Results

Expression levels of the UGT 1A and 2B isoforms in the insect cells transfected human UGT genes. The quantitative immunoblot analyses with the anti-UGT cross-reactive with microsomal UGT1A and UGT2B isoforms were performed using the corresponding MBP-UGT fusion proteins as standards. As judged by Western blot analysis, amounts of microsomal UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15 and UGT2B17 in the insect cells expressing the corresponding human recombinant UGT isoforms were 1.6, 0.9, 1.2, 1.4, 1.3, 1.1, 1.1, 1.2, 0.8, 0.6, 0.6 or 0.8 nmol/mg protein, respectively.

T_4-glucuronidation by recombinant human UGT isoforms. T_4-glucuronidation activities of 12 human UGTs, including 8 UGT1A subfamily and 4 UGT2B subfamily isoforms, were examined. All the UGT isoforms examined showed definite activities for T_4-glucuronidation (Fig. 2). As judged by glucuronidation activity [v/E_0 (min^{-1})], UGT1A1, UGT1A3, UGT1A8, UGT1A9, UGT1A10 and UGT2B7 showed higher capacities than the other UGT isoforms examined. In addition, UGT1A8, an extrahepatic enzyme (Cheng et al., 1998; Gregory et al., 2004), showed the highest activity.

Inter-individual variation of T_4-glucuronidation activity in the human livers. In general, microsomal glucuronidation activity is increased by perturbation of microsomal membranes (Guéraud and Paris, 1998). Therefore, effect of alamethicine, a pore-forming oligopeptide, on the T_4-glucuronidation activity of human liver

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microsomes (HH13) was firstly examined. As shown in Table 1, addition of alamethicin (0.04-0.20 mg/ml reaction mixture) resulted in increase in the glucuronidation activity of the human micorosomes. Therefore, in the present experiment, the T_4-glucuronidation activity was measured in the assay system with alamethicin (0.1 mg/ml reaction mixture).

Microsomal fractions were independently prepared from 7 individual human livers, HH2, HH13, HH47, HG3, HG32, HG64 and HG74, and their T_4-glucuronidation activities were comparatively examined. Although all the human microsomes examined showed definite activities for T_4-glucuronidation, there is an inter-individual difference (Fig. 3). The microsomes from HH13, HH64, and HH74 had the highest activities among the samples examined.

UGT activities toward T_4, β-estradiol, trifluoperazine and propofol of the human liver microsomes. A correlation between T_4-UGT activity and the UGT activities toward other typical UGT substrates, such as β-estradiol, trifluoperazine and propofol, of human liver microsomes was examined. To this purpose, the data on the UGT activities toward β-estradiol, trifluoperazine, and propofol of each human given from BD Genetest were used. As shown in Fig. 4, T_4-UGT activity was closely correlated with the activity of β-estradiol 3-glucuronidation (r=0.729, P<0.05), while it showed no correlation with the UGT activities for trifluoperazine and propofol.

Western blot analysis of UGT1A subfamily enzymes in human liver microsomes. Western blot analyses with anti-h1AC and several anti-UGT1A isoform-specific antibodies were performed to determine the UGT isoforms expressed in human liver

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microsomes. The anti-h1AC could cross-react with the human UGT1A isoforms, while anti-h1A1, anti-h1A3, anti-h1A9, and anti-2B7 antibodies are specifically reactive with UGT1A1, UGT1A3, UGT1A9, and UGT2B7 respectively (Ikushiro et al., 2006).

Amount of total UGT1A isoforms (UGT1As) detected with an anti-h1AC was the greatest in the human liver sample HH47 among the 7 individual human samples (Fig. 5). On the other hand, amount of UGT1A1 determined with anti-UGT1A1 was in the order as follows: HH13>(HG74, HH2, HH47, HG64)>HH32, HG3. Concerning the UGT1A3, the order was HG64>(HG74>HH13, HG32, HH47)>HH2, HG3. In addition, amounts of UGT1A9 and UGT2B7 were not significantly different among the human samples examined.

UGT isoforms responsible for T_4-glucuronidation in human liver microsomes. The relative expression levels of UGT1A, UGT1A1, UGT1A3, and UGT1A9 in individual human liver microsomes were determined, and a relationship between the level of each UGT isoform and the T_4-UGT activity was examined. The T_4-UGT activity was closely correlated with the level of either UGT1A1 (r= 0.738, P<0.05) or UGT1A3 (r= 0.804, P<0.01) (Fig. 6). On the other hand, no correlation between the T_4-UGT activity and the level of UGT1A9 or total UGT1As was observed.

Inhibition of T_4-UGT activity by UGT inhibitors in human liver microsomes. Effects on microsomal T_4-UGT activity of the UGT inhibitors with different specificities were examined using a human liver sample HH13, which had definite levels of UGT1A1, UGT1A3, and UGT1A9. The inhibitors, with an exception of propofol, significantly decreased the T_4-UGT activity (Fig. 7). Especially, addition of

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ST232 (0.4 mM) to the reaction mixture resulted in 75%-decrease in T_4-UGT activity. In contrast, propofol (0.4 mM) showed little inhibitory effect on the microsomal T_4-UGT activity.

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Discussion

In the present experiments, T_4-UGT activities of human UGT isoforms, such as UGT1A1, UGT1A3, UGT1A4, UGT1A6, UIGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15 and UGT2B17, were comparatively examined. The results revealed that all the UGTs examined showed definite T_4-glucuronidation activities. Among the UGTs examined, UGT1A8 showed the strongest activity. However, UGT1A8 was not detected in human liver microsomes, as reported previously (Cheng et al., 1998; Gregory et al., 2004). Among the UGT isoforms detected in the human liver microsomes, recombinant UGT1A1, UGT1A3, and UGT1A9 enzymes had considerably high T_4-UGT activities. Furthermore, microsomal T_4-glucuronidation activity in the human liver was closely correlated with the activity of β-estradiol 3-glucuronidation activity, which is mediated by either UGT1A1 or UGT1A9 (Senafi et al., 1994), while it showed no correlation with the glucuronidation activities toward trifluoroperazine, a typical substrate of UGT1A4 (Court 2005; Dehal et al., 2001) and propofol, a typical substrate of UGT1A9 (Burchell et al., 1995). In addition to the results, T_4-glucuronidation activity in human liver microsomes was significantly inhibited by either bilirubin, an inhibitor of UGT1A1 (Senafi et al., 1994; King et al., 1996), or ST232, an inhibitor of UGT1A3 (Kasai et al., 2005), but not propofol, an inhibitor of UGT1A9 (Burchell et al., 1995), indicating strongly that UGT1A1 and UGT1A3 enzymes, but not UGT1A9, mainly mediated the T_4-glucuronidation in human microsomes. All these findings indicate that T_4-UGT activity in the human liver is mainly dependent on the levels of UGT1A1 and UGT1A3 and further suggest that the inter-individual difference among humans in T_4-glucuronidation activity would come

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from difference in the levels of the UGT isoforms.

Although UGT1A1 had been reported to be an important enzyme for hepatic T_4-glucuronidation in humans (Visser et al., 1993; Findlay et al., 2000; Yamanaka et al., 2007), the present findings further confirm this. On the other hand, importance of UGT1A3 for hepatic T_4-glucuronidation in humans was firstly demonstrated in the present experiments, while Yamanaka et al. (2007) had reported that UGT1A3 would little contribute to the T_4-glucuronidation. Thus difference concerning significance of UGT1A3 for T_4-glucuronidation would come from the difference in the experimental methods rather than that in human samples. Yamanaka et al. (2007) lead to the conclusion on the basis of the results that microsomal activity for chenodeoxycholic acid 24-*O*-glucuronidation, which is thought to be catalyzed by UGT1A3 (Trottier et al., 2006), was not correlated with the T_4-glucuronidation activity and not inhibited by imipramine, an inhibitor of UGT1A3 and UGT1A4 (Nakajima et al., 2002). However, the results obtained only by the enzyme assays would not be enough for establishment of the conclusion that UGT1A3 would not contribute to T_4-glucuronidation. In the present experiments, importance of UGT1A3 for T_4-glucuronidation was more directly demonstrated not only by the inhibition assays with a selective inhibitor (ST232) of UGT1A3 but also by the correlation analysis between microsomal T_4-glucuronidation activity and level of UGT1A3 in microsomes. In addition, the band detected by the Western blotting with anti-h1A3 antibody, which might show a cross-reactivity with UGT1A5, was judged as a UGT1A3 protein, because UGT1A3 mRNA, but not UGT1A5 mRNA, is detected in human liver (Tukey and Strassburg, 2000).

UGT1A8 and UGT1A10 are also reported as T_4-glucuronidation enzymes (Visser et al., 1993; Findlay et al., 2000). However, these UGT isoforms would hardly

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contribute to hepatic T_4-glucuronidation, because they are expressed in the intestine but little in the liver (Cheng et al., 1998; Gregory et al., 2004). In addition, UGT1A8 and UGT1A10 are considered to contribute to intestinal T_4-glucuronidation (Yamanaka et al. (2007).

In conclusion, we demonstrate herein that UGT1Al and UGT1A3 are important enzymes for hepatic T_4-glucuronidation in humans and further suggest that the inter-individual difference in hepatic T_4-glucuronidation activity would come from difference in the level of UGT1A1 and UGT1A3.

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Footnotes

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Legends for figures

FIG. 1. Chemical structure of 26,26,26,27,27,27-hexafluoro-1α,23(S),25-trihydroxyvitamin D_3 (ST232).

FIG. 2. T_4-glucuronidation by recombinant human UGT isoforms.

Glucuronidation activity of the microsomes containing a recombinant human UGT isoform was examined in the reaction mixture containing 90 μM T_4 with [^{125}I]T_4 (40-65 μCi/μmol), as described in "Materials and Methods". V/E_0 (min^{-1}) value of a UGT isoform was calculated on the basis of the content of UGT in microsomes.

FIG. 3. Comparison of microsomal T_4-glucuronidation activity in individual human livers.

Microsomal glucuronidation activity was examined in the reaction mixture containing 90 μM T_4 with [^{125}I]T_4 (40-65 μCi/μmol), as described in "Materials and Methods". Each column represents the mean $\pm$ SE (vertical bars) of triplicate determinations

FIG. 4. Relationship between hepatic microsomal activities for T_4-glucronidation and other chemicals (β-estradiol, trifluoperazine, and propofol)-glucuronidation in humans.

Human samples: HG3, ①; HH13, ②; HG32, ③; HH47, ④; HG64, ⑤; HG74, ⑥; HH2, ⑦. Lines represent the best-fit estimate of least-squares linear regression analysis. A correlation coefficient of regression (*r*) is shown for each plot.

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FIG. 5. Immunoblot analysis of hepatic microsomal UGT isoforms in 7 individual humans.

The immunoblot analyses with human-UGT1A antibody and isofom-specific antibody (anti-h1AC, anti-h1A1, anti-h1A3, anti-h1A9, or anti-2B7) were performed using 20 μg and 40 μg protein/lane of hepatic microsomes, respectively.

FIG. 6. Correlation analysis between the T_4-glucuronidation activity and the content of UGT1A isoforms in the human liver microsomes.

Semiquantitative immunoblot analyses for UGT1A, UGT1A1, UGT1A3, and UGT1A9 in the human liver microsomes were performed according to the method as described in the legend of Fig. 4. Human samples: HG3, ①; HH13, ②; HG32, ③; HH47, ④; HG64, ⑤; HG74, ⑥; HH2, ⑦. Line represents the best-fit estimate of least-squares linear regression analysis. A correlation coefficient of regression (r) is shown for each plot. Net intensity values were expressed relative to the content of respective UGT isoforms in HG3.

FIG. 7. Effects of several UGT inhibitors on microsomal T_4-glucuronidation in the human liver.

Bilirubin, ST232, propofol, and β-estradiol were used as inhibitors for UGT1A1, UGT1A3, UGT1A9 and UGT1A1/UGT1A9, respectively. Basal activity (without an inhibitor) in the human liver (HH13) was 17.6 ± 0.3 pmol/mg protein/min. ○, Bilirubin; □, ST232; ▲, propofol; ◆, β-estradiol. Each point represents the mean ± SE (vertical bars) for 4 preparations.

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*P<0.01, significantly different from the control.

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Table 1. Effect of alamethicin on microsomal T_4-glucuronidation activity in the human liver

Alamethicin (mg/ml reaction mixture)	T_4-glucuronidation (pmol/mg protein/min)
0	5.9 ± 0.2
0.04	15.0 ± 1.0
0.1	15.6 ± 0.4
0.2	17.0 ± 0.3

Data represent the mean ± SE for 4 preparations.

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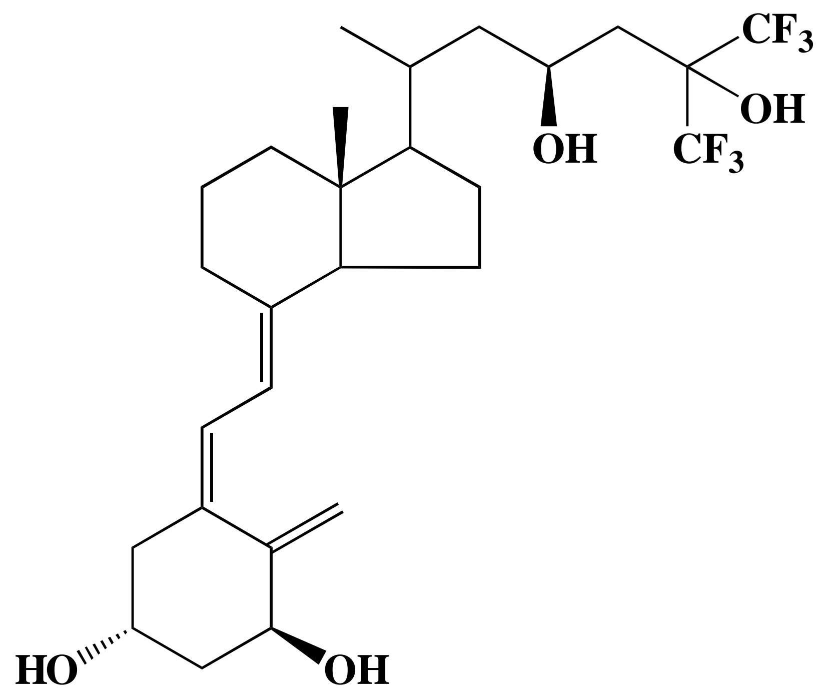
Fig. 1



ST232

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Fig. 2

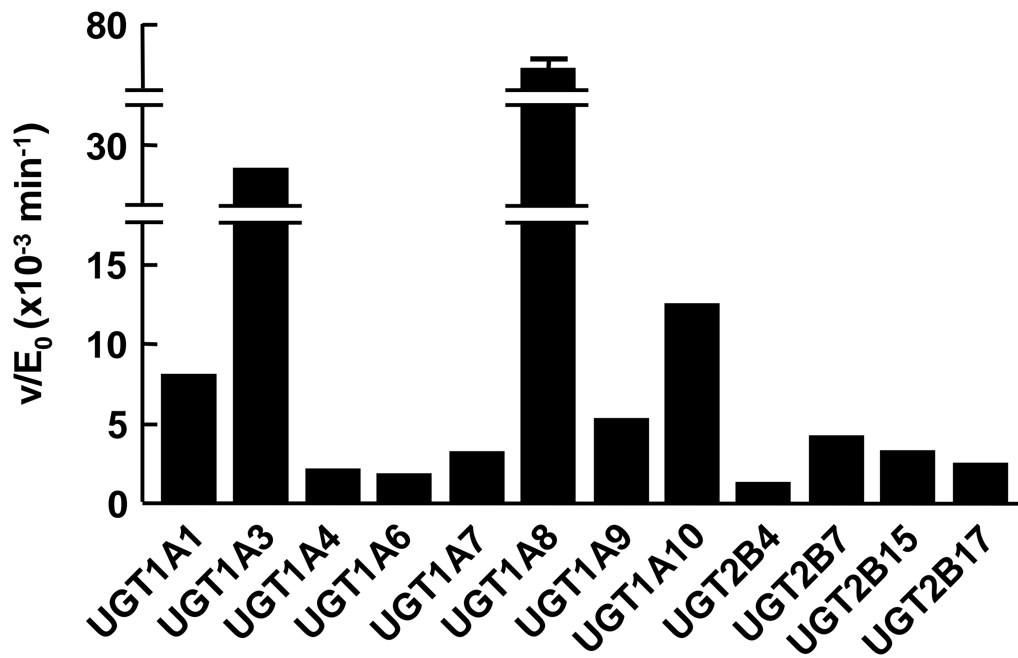

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Fig. 3

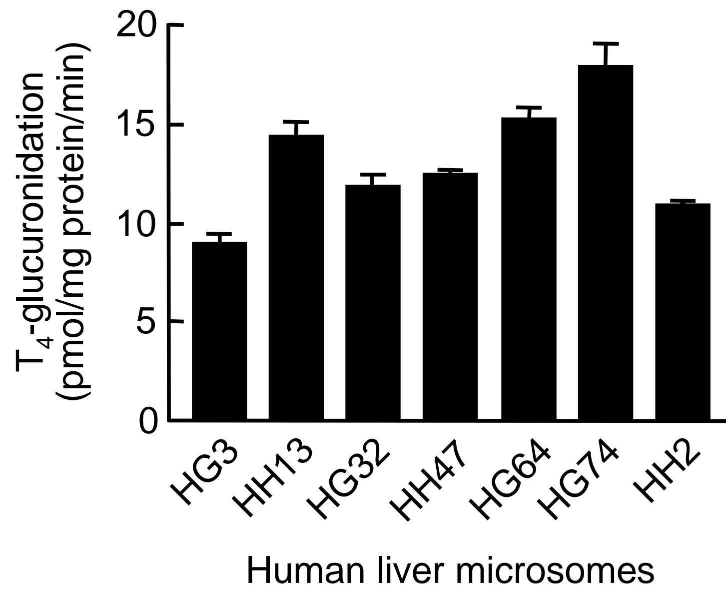

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Fig. 4

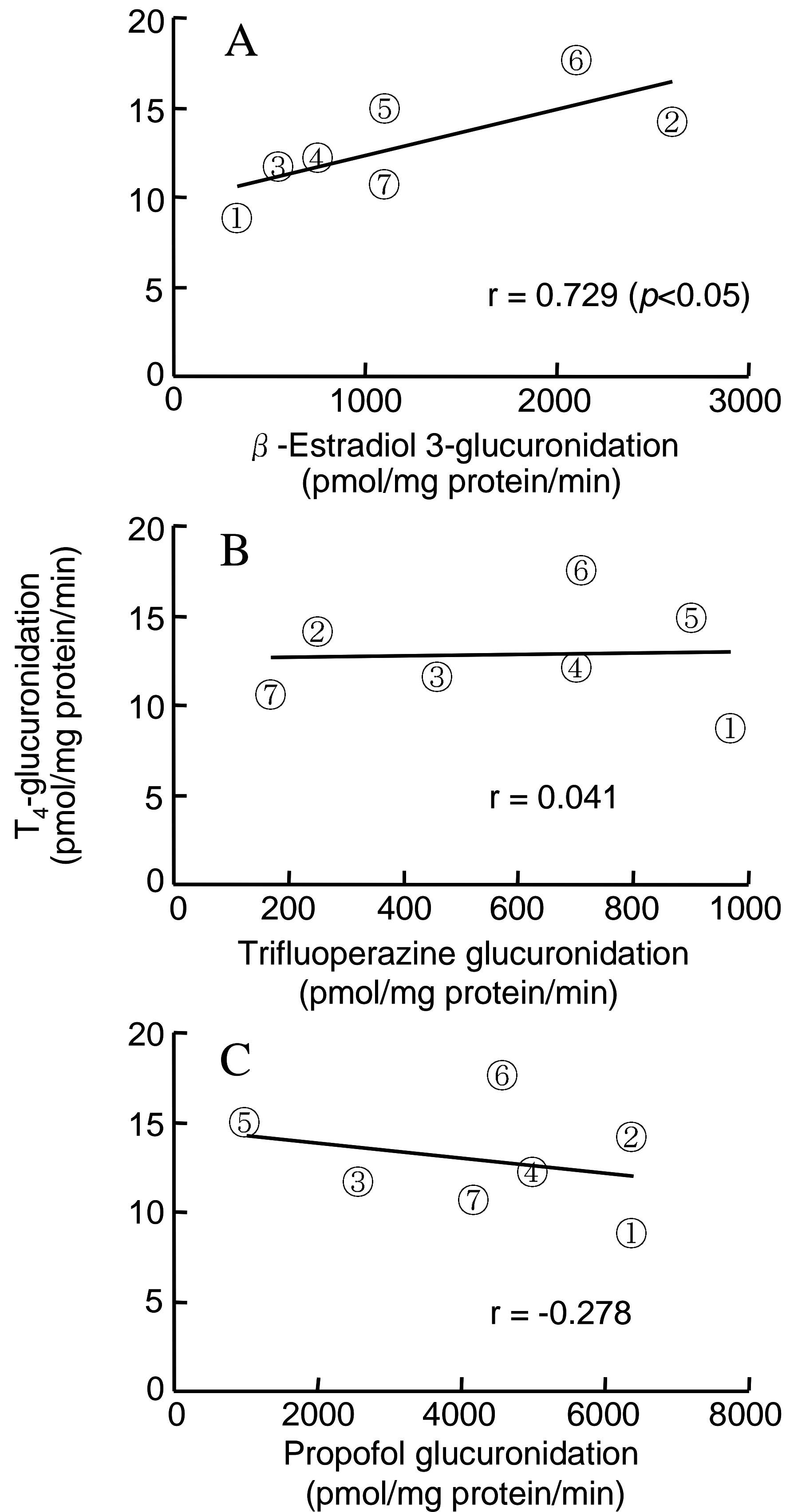

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Fig. 5

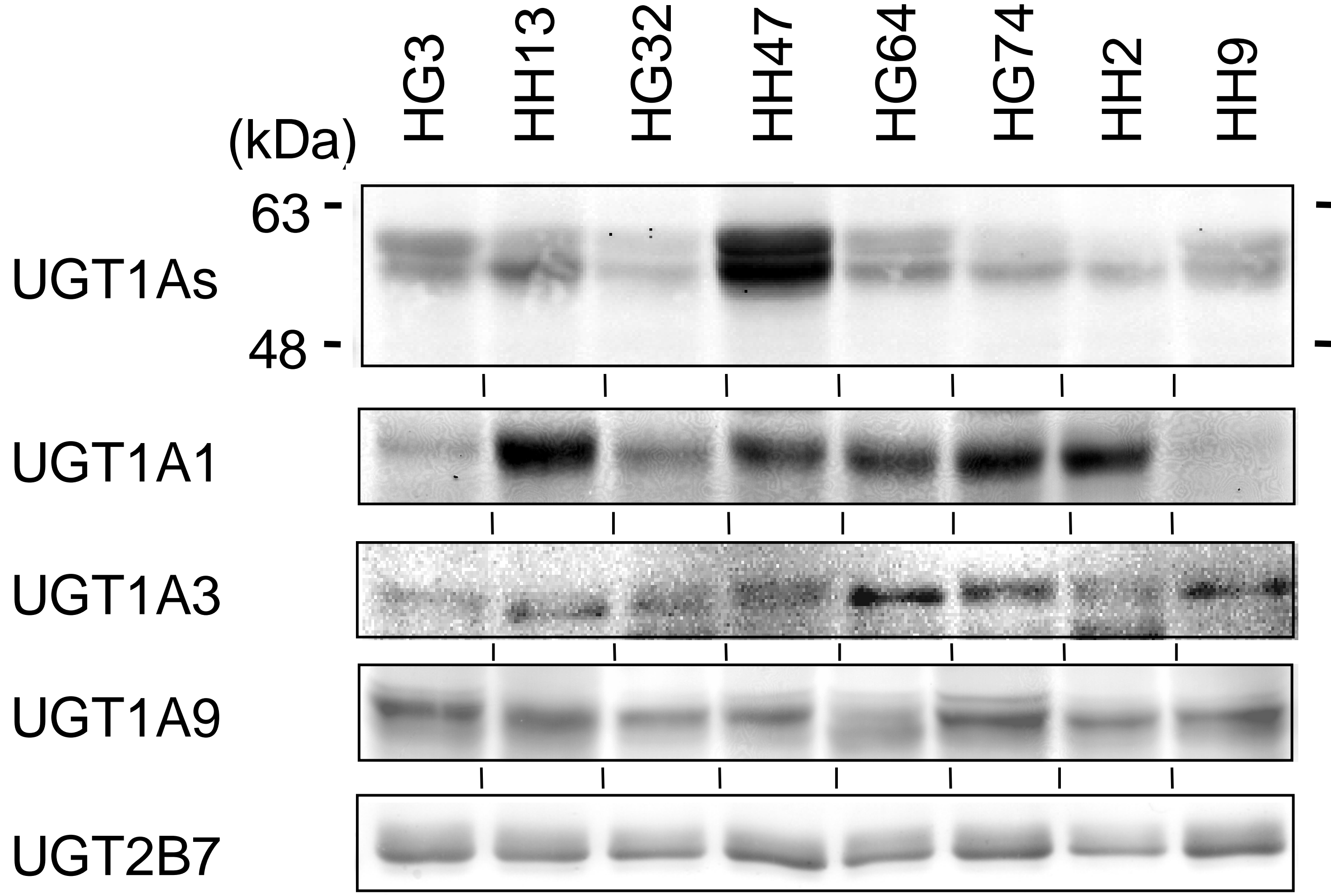

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Fig. 6

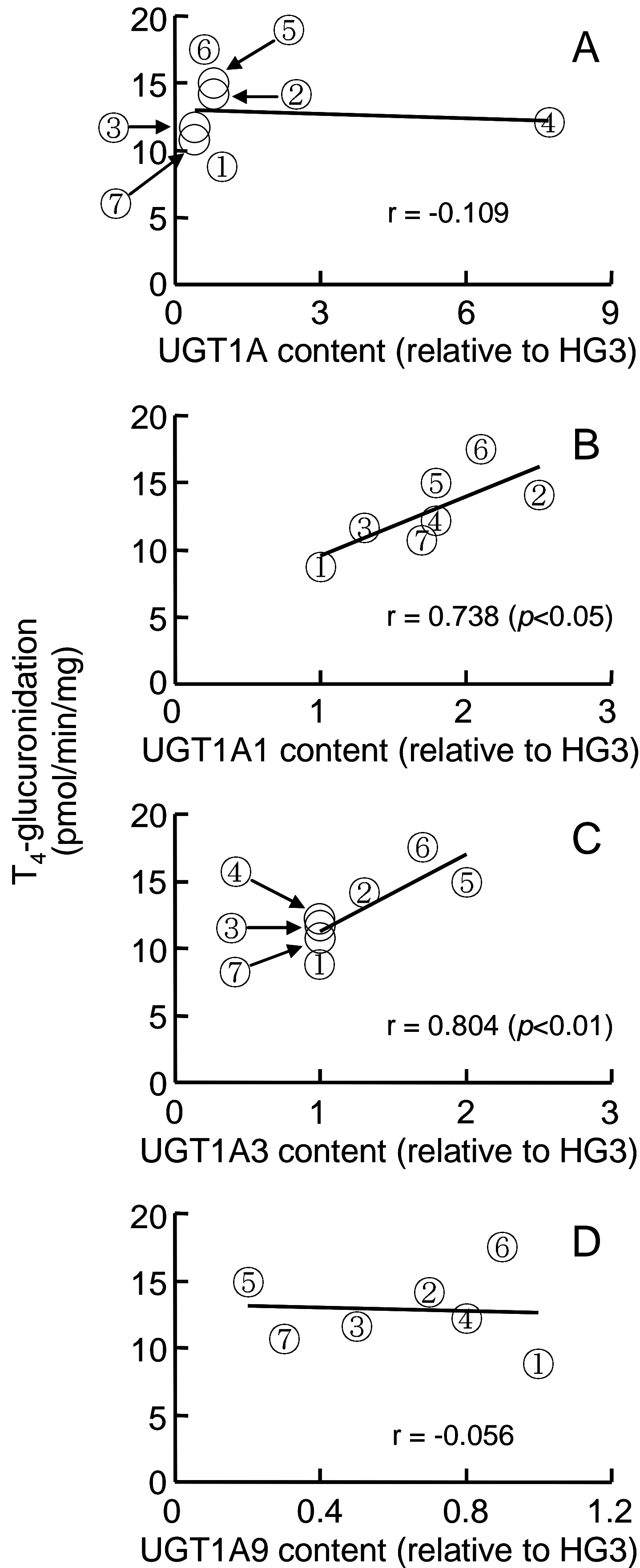

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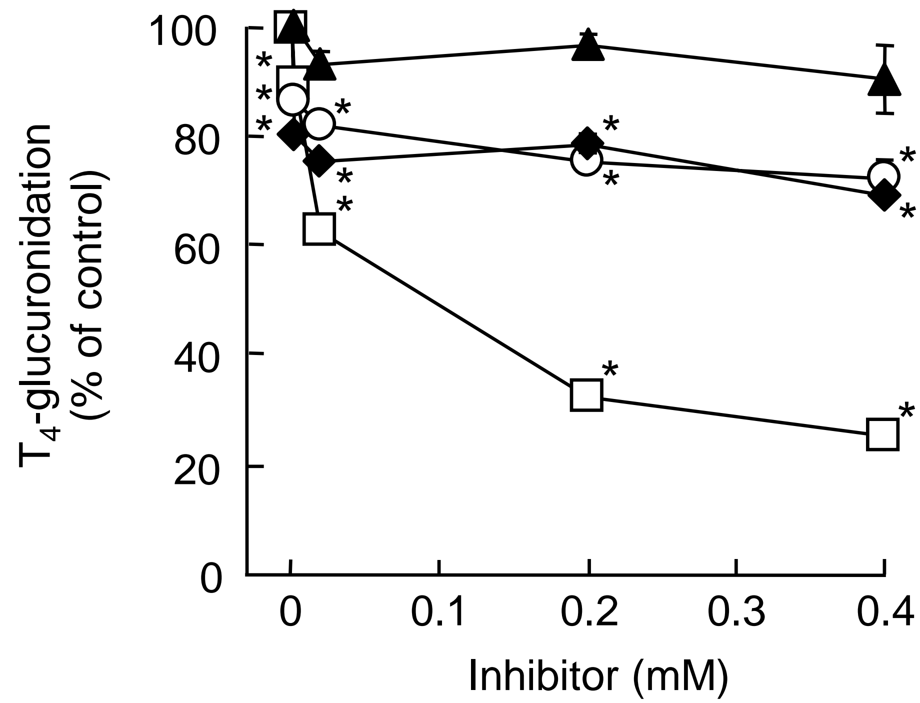
Fig. 7



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