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**COMPARISON OF INHIBITION POTENTIALS OF DRUGS AGAINST
ZIDOVUDINE GLUCURONIDATION IN RAT HEPATOCYTES AND LIVER
MICROSOMES**

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Running title: GLUCURONIDATION INHIBITION IN TWO *IN VITRO* SYSTEMS

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List of abbreviations

AZT: 3'-Azido-3'-deoxythymidine, AZTG: AZT glucuronidation, CYP: Cytochrome P450, DMSO: Dimethyl sulfoxide, HLM: Human liver microsomes, LC-MS/MS: Liquid chromatography with tandem mass spectrometry, NSAIDs: Nonsteroidal anti-inflammatory drugs, RLM: Rat liver microsomes, UDPGA: UDP glucuronic acid, UGT: UDP glucuronosyltransferase, WME: Williams' Medium E.

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ABSTRACT

Hepatocytes and liver microsomes are considered to be useful for investigating drug metabolism catalyzed mainly via glucuronidation. However, there have been few reports comparing the glucuronidation inhibition potentials of drug in hepatocytes to those in liver microsomes. 3'-Azido-3'-deoxythymidine (AZT, Zidovudine) glucuronidation (AZTG) is the major metabolic pathway for AZT. In this study, the inhibition potentials of drugs against UDP-glucuronosyltransferase (UGT)-catalyzed AZTG in the hepatocytes and liver microsomes of rats are compared. The AZTG inhibition potentials of diclofenac, diflunisal, fluconazole, indomethacin, ketoprofen, mefenamic acid, naproxen, niflumic acid, and valproic acid in liver microsomes and hepatocytes were investigated using liquid chromatography with tandem mass spectrometry. Diflunisal (inhibition type: non-competitive) inhibited AZTG most potently in rat liver microsomes (RLM) with an IC_{50} value of 34 μ M. The IC_{50} values of diclofenac, fluconazole, indomethacin, ketoprofen, mefenamic acid, naproxen, niflumic acid, and valproic acid against AZTG in RLM ranged from 34 to 1791 μ M. Diclofenac, diflunisal, indomethacin, ketoprofen, naproxen, and valproic acid inhibited AZTG in hepatocytes with IC_{50}

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values of 58, 37, 88, 361, 486, and 281 μM , respectively. These values were similar to those obtained in RLM. In conclusion, the AZT glucuronidation inhibition potentials of drugs in the hepatocytes and liver microsomes of rats were found to be similar, and liver microsomes can be useful for evaluating UGT isozyme inhibition potentials.

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INTRODUCTION

Glucuronidation catalyzed by UDP-glucuronosyltransferase (UGT) is one of the major steps in the metabolism of endogenous substances and xenobiotics. It is generally recognized that UGTs are important isozymes in the glucuronidation of nonsteroidal anti-inflammatory drugs (NSAIDs). If UGTs are the primary isozymes involved in the clearance of drugs, their inhibition may cause enhanced drug exposure perhaps even that reaching toxic levels. It has been shown that 3'-azido-3'-deoxythymidine (AZT) glucuronidation (AZTG) is catalyzed primarily by UGT2B7 (Court et al., 2003; mano et al., 2007), and that concomitant dosages of fluconazole and valproic acid increase plasma AZT levels (Lertora et al., 1994, Sahai et al., 1994). This is attributed to the inhibition of UGT2B7 by these drugs. In order to predict *in vivo* drug-drug interactions, *in vitro* inhibition studies using matrix like liver microsomes and hepatocytes are very valuable.

The majority of inhibition studies on metabolic enzymes such as cytochrome P450 (CYP) and UGT has been performed using liver microsomes. Although the present popular opinion may be that hepatocytes yield the most

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reliable values for estimating the metabolizing enzyme inhibition potential for drugs, there has been no evidence presented to demonstrate that hepatocytes are the best predictors of inhibition potential (Jones et al., 2004). A recent report by Engtrakul et al. (2005) showed that the IC_{50} value of ibuprofen against AZT glucuronidation in human hepatocytes (170 μ M) was significantly lower than that in human liver microsomes (HLM) (975 μ M). In addition, the authors caution against relying on inhibition potentials extrapolated from data obtained using liver microsomes. However, their study was performed with only one substrate-inhibitor pair, which indicates that studies with more pairs are necessary to determine whether hepatocytes generally yield more potent inhibition values than liver microsomes. Although few studies have been reported in terms of comparing inhibitory potential of drug against glucuronidation, a number of reports comparing CYP isozyme-mediated metabolism inhibition in liver microsomes and hepatocytes are available. Di Marco et al. (2003) reported that pyrilamine, propafenone, verapamil, ketoconazole, and terfenadine inhibited dextromethorphan demethylation in rat liver microsomes and rat hepatocytes with similar potency. Jones et al. (2004) demonstrated that omeprazole inhibited diazepam metabolism to 4'-hydroxydiazepam, 3-hydroxydiazepam, and

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nordiazepam with similar IC_{50} values in RLM and hepatocytes.

In this study, the AZTG inhibition potencies of 9 drugs including diclofenac, diflunisal, fluconazole, indomethacin, ketoprofen, mefenamic acid, naproxen, niflumic acid, and valproic acid (Figure 1) in rat liver microsomes (RLM) and cryopreserved rat hepatocytes were investigated. The glucuronidation inhibition potential in both liver matrices was then compared. This study provides data on the issue whether *in vitro* testing of inhibitory potentials of drugs against drug metabolism in liver microsomes is representative of hepatic metabolism exemplified by the use of hepatocytes.

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MATERIALS & METHODS

Chemicals and reagents

AZT, diclofenac, fluconazole, indomethacin, ketoprofen, mefenamic acid, naproxen, and niflumic acid were purchased from Sigma (St. Louis, MO, USA).

Diflunisal and valproic acid were purchased from ICN Biomedicals (Aurora, Ohio, USA) and Wako Pure Chemicals (Osaka, Japan), respectively. AZT glucuronide was obtained from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada).

RLM and cryopreserved rat hepatocytes were purchased from Xenotech (Kansas City, KS, USA). All other chemicals were of analytical grade.

Glucuronidation in RLM

AZT (2 mM) was incubated with RLM (0.1 mg protein/ml) for 20 min in a final volume of 0.25 ml Tris-HCl buffer (50 mM, pH 7.5) containing 8 mM MgCl₂, 25 µg/ml alamethicin, and 5 mM UDPGA in both the presence and absence of drugs (diclofenac, diflunisal, fluconazole, indomethacin, ketoprofen, mefenamic acid, naproxen, niflumic acid, and valproic acid). The AZT concentration (2 mM) was

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below its K_m value (9.1 mM, Cretton et al., 1990). In a separate study, AZT (0.2 - 10 mM) was incubated with RLM under the same conditions described above in the presence and absence of diflunisal (40 μ M) to evaluate the type of inhibition diflunisal exerted against AZTG. Kinetic studies done in RLM using different buffers were performed as stated above. The buffers tested were 100 mM sodium carbonate and Williams' Medium E (containing 2 mM L-glutamine) (WME), both of which apparently altered AZTG in HLM, compared to the effects of Tris-HCl (Engtrakul et al., 2005). All buffers were prepared at pH 7.5. After the reaction mixture had been pre-incubated at 37 °C for 5 min, the reaction was started by adding UDPGA at a final concentration of 5 mM. After incubating for 20 min in RLM, the reaction was terminated by adding 0.05 ml acetonitrile to precipitate the protein. Formic acid (10%, 0.01 ml) and the internal standard [0.02 ml of the deuterium isotope of AZT glucuronide (5 μ g/ml)] were then added. Next, the samples were centrifuged at 1870 \times g for 10 min to obtain the supernatant, aliquots (20 μ l) of which were injected into a liquid chromatography with tandem mass spectrometry (LC-MS/MS) system. The substrate and inhibitors were dissolved in dimethyl sulfoxide (DMSO) to make an incubation mixture with a final DMSO concentration of 1% (v/v).

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Glucuronidation in hepatocytes

Cryopreserved rat hepatocytes were stored in liquid nitrogen until use. Thawing was done according to the instructions supplied by Xenotech, after which the hepatocytes were suspended in enough WME to make a concentration of 2×10^6 cells/ml. The viability, determined using the trypan blue exclusion method, was found to be >68.5%. The time profile for AZTG was assessed first. The hepatocyte suspension (2.5×10^5 cells in 125 μ l) was pre-incubated at 37 °C for 3 min, after which the reactions were initiated by adding 125 μ l of WME containing AZT (2 mM). After incubation at 37 °C for the designated time, the reaction was terminated by adding acetonitrile (50 μ l), followed by 10% formic acid (10 μ l) and the internal standard (100 ng). The reaction mixtures were centrifuged at 1870 \times g for 10 min, and aliquots of the supernatant (20 μ l) were injected into the LC-MS/MS to determine the levels of AZT glucuronide. The kinetic study for AZTG and the inhibition effects of drugs on AZTG in rat hepatocytes were then addressed. AZT (0.2 – 10 mM) was incubated with hepatocytes (2.5×10^5 cells) for 30 min using the same procedures described above. The inhibitory potentials of drugs (diclofenac, diflunisal, indomethacin, ketoprofen, naproxen, and valproic

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acid) were then evaluated. AZT (1 mM) was incubated for 30 min with hepatocytes (2.5×10^5 cells) spiked with varying concentrations of these drugs. As a separate study, the effect of UDPGA levels on AZTG in hepatocytes was investigated using externally added UDPGA with a final concentration of 5 mM. The kinetic study for AZTG was then performed as described above.

Assay

The peak areas of AZT glucuronide and its internal standard were analyzed using LC-MS/MS (Mano et al., 2007). A TSQ7000 triple quadrupole mass spectrometer with an atmosphere pressure ionization (API) source (Thermo Finnigan, San Jose, CA, USA) was used. The API source was fitted with an electrospray ionization inlet for ionizing the analytes. Nitrogen was used as both the sheath and auxiliary gas with pressures of 80 psi and 40 units, respectively. Electron spray voltage was set at 4.5 kV and the heated capillary temperature was maintained at 350 °C. AZT glucuronide and its internal standard were monitored in the negative ion mode using selected reaction monitoring by transmitting the molecular ions at m/z 442 and 445, respectively. These ions were subjected to collision-activated dissociation using argon (2.0 mtorr) at 20 eV, and then, the

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product ions were monitored at m/z 442 and 445 for AZT glucuronide and the internal standard, respectively. Chromatographic separation was achieved using a Capcellpak UG80 column (4.6 mm \times 150 mm, 5 μ m, Shiseido, Tokyo, Japan) with a flow rate of 0.5 ml/min. The mobile phase was comprised of 0.1% formic acid:acetonitrile (7:3, v/v). The standard curve for the AZT glucuronide was linear from 0.1 to 10 μ M, and the correlation coefficient was >0.99 . The relative errors of the back-calculated values at each concentration were less than 15%.

Data analysis

The IC_{50} value of each inhibitor was estimated from the inhibition against AZTG in the presence of inhibitors with varying concentrations by fitting the data to Equation (1), where I is the inhibitor concentration.

$$\% \text{ of control} = 100 \times \frac{IC_{50}}{I + IC_{50}} \quad \text{Equation (1)}$$

The K_i value of diflunisal was determined using Equation (2).

$$\frac{CL_{int}}{CL_{int}'} = 1 + \frac{I}{K_i} \quad \text{Equation (2)}$$

where CL_{int}' and CL_{int} represent the intrinsic clearance of AZTG in RLM in the presence and absence of diflunisal, respectively. Prism Ver. 3.02 software (Graph Pad Software, San Diego, CA, USA) was used for the calculations.

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Results

Glucuronidation in RLM

The effect on AZTG inhibition caused by diclofenac, diflunisal, fluconazole, indomethacin, ketoprofen, mefenamic acid, naproxen, niflumic acid, and valproic acid in RLM was investigated. Diflunisal had the most potent inhibitory effect with an IC_{50} value of $34 \pm 7.1 \mu\text{M}$ (mean \pm computer-calculated S.E.). The IC_{50} values of diclofenac, fluconazole, indomethacin, ketoprofen, naproxen, mefenamic acid, niflumic acid, and valproic acid were 40 ± 1.8 , 1791 ± 226 , 178 ± 37 , 103 ± 3.9 , 276 ± 7.7 , 332 ± 9.5 , 37 ± 0.9 , 102 ± 5.8 , and $538 \pm 35 \mu\text{M}$ (Table 1), respectively.

The type of inhibition diflunisal exerts against AZTG was investigated in RLM. In the absence of diflunisal, AZTG exhibited Michaelis-Menten kinetics with K_m and V_{max} values of $10 \pm 1.8 \text{ mM}$ and $1345 \pm 152 \text{ pmol/min/mg protein}$, respectively (Figure 2). In the presence of $40 \mu\text{M}$ diflunisal, the V_{max} value was reduced to $666 \pm 75 \text{ pmol/min/mg protein}$, while the K_m value ($8.2 \pm 1.6 \text{ mM}$) was similar to that without diflunisal. The inset in Figure 2, an Eadie-Hofstee plot,

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suggests that one or more isozymes with similar K_m values are involved in AZTG, and that diflunisal inhibits AZTG in a non-competitive manner with a K_i value of 61 μM .

The effects of different buffer conditions on AZTG with RLM were determined. The kinetics for AZTG in both carbonate buffer and WME obeyed Michaelis-Menten kinetics, and kinetic analysis revealed the K_m values to be 5.6 ± 0.5 and 6.6 ± 0.4 mM, respectively. The respective V_{max} values were 483 ± 20 and 528 ± 18 pmol/min/mg protein.

Glucuronidation in hepatocytes

The time profile for the formation of AZT glucurononide was investigated in cryopreserved rat hepatocytes. The data showed that the glucuronidation velocity was linear for at least 60-min during incubation (Figure 3A). The kinetics of AZTG was next evaluated using varying concentrations of AZT as a substrate for glucuronidation. The AZT concentration-velocity curve adheres to typical Michaelis-Menten kinetics, and the Eadie-Hofstee plot implies that more than one UGT isozyme is involved in AZTG. The K_m value for the high-affinity isozyme was estimated to be 1.0 ± 0.4 mM, and the V_{max} and CL_{ns} (clearance for low-affinity

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isozymes) values were 64 ± 15 pmol/min/ 10^6 cells and 4.4 ± 1.3 nl/min/ 10^6 cells, respectively (Figure 3B). The impact of UDPGA levels on AZTG was also assessed in cryopreserved hepatocytes. When hepatocytes were incubated with externally added UDPGA (5 mM), the resulting Eadie-Hofstee plot suggests that one or more isozymes with similar K_m values are involved in AZTG as they are in RLM, with K_m and V_{max} values of 3.2 ± 0.4 mM and 275 ± 12 pmol/min/ 10^6 cells, respectively.

The inhibitory effects of diclofenac, diflunisal, indomethacin, ketoprofen, naproxen, and valproic acid on AZTG were then assessed in rat hepatocytes. Diclofenac, diflunisal, indomethacin, ketoprofen, naproxen, and valproic acid inhibited AZTG with IC_{50} values of 58 ± 2.2 , 37 ± 2.2 , 88 ± 16 , 361 ± 34 , 486 ± 52 , and 281 ± 52 μ M, respectively (Table 1). The IC_{50} values for these drugs against AZTG in hepatocytes were compared to those in RLM. Representative AZTG inhibition profiles (indomethacin) in RLM and hepatocytes are presented in Figure 4. The AZTG inhibition potentials of the drugs investigated were found to be similar in both types of matrices (Figure 5).

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DISCUSSION

This paper describes the AZTG inhibition potentials of drugs in RLM and cryopreserved hepatocytes. AZTG is mainly catalyzed by UGT2B7 in humans, and clinical drug interactions via UGT2B7 have been reported when AZT is concomitantly administered with fluconazole and valproic acid (Lertora et al., 1994; Sahai et al., 1994). When trying to predict *in vivo* drug interaction via glucuronidation, animal models (e.g. rats) may be valuable tools for bridging *in vitro* and *in vivo* data. A recent paper by Engtrakul et al. showed that the K_m of AZTG and the IC_{50} value of ibuprofen against AZTG in human hepatocytes are lower than those in HLM, and suggests that liver microsomes appears to underestimate inhibitory potentials of drugs (Engtrakul et al., 2005). Thus, in this study, the AZTG inhibition potentials of drugs in rats in both RLM and hepatocytes, were investigated, and the results were compared in order to determine whether the IC_{50} values in the hepatocytes are lower than those in liver microsomes. Although AZTG is catalyzed mainly by UGT2B7 in humans, the UGT isozyme responsible for catalysis in rats remains to be investigated. Despite this deficit,

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the Eadie-Hofstee plot in Figure 2 suggests that one or more isozymes with similar K_m values are involved in AZTG. The K_m value for AZT in RLM (10 mM) is consistent with that in the previous report (9.1 mM, Cretton et al., 1990). It should be noted that the solubility of AZT is less than that necessary to obtain accurate kinetic parameters in RLM; however, the K_m value obtained is similar to the one reported. The K_m value for AZTG in rat hepatocytes (1.0 mM, Figure 3B) was lower than that in RLM (10 mM). This is a phenomenon similar to one that occurred in the previous study; the K_m value for AZTG in human hepatocytes was much lower than that in HLM (87 vs 760 μM , Engtrakul et al., 2005). The reason for this discrepancy remains to be determined, although the active transport systems involved in AZT uptake in the hepatocytes might be responsible. It has also been reported that AZT is a substrate for rat organic anion transporter 2, which is abundantly expressed in the liver (Morita et al., 2001). Buffer systems in HLM (Engtrakul et al., 2005) and differences in the environment surrounding the UGT isozymes might contribute to the differences in the K_m values. The levels of UDPGA, a glucuronidation co-factor, might be also be behind the differences between the two matrices. In RLM, enough UDPGA was added to the reaction mixtures to make a concentration of 5 mM, but no co-factor was added to the rat

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hepatocytes. Therefore, the UDPGA levels in the hepatocytes might have been insufficient, given the high concentration of AZT. In order to find potential factors which may contribute, at least partially, to the differences in the kinetic parameters of AZTG between hepatocytes and liver microsomes, the impact of UDPGA levels and buffer incubation conditions on AZTG in hepatocytes and RLM was determined. Externally added 5 mM UDPGA significantly increased the K_m value to 3.2 mM, which is closer to that in RLM, compared with that in hepatocytes that did not have UDPGA added (K_m : 1.0 mM). In addition, the Eadie-Hofstee plot suggests that one or more isozymes with similar K_m values are involved in AZTG in hepatocytes, as was observed in RLM (data not shown). Next, the effects of incubation buffers on AZTG were assessed in RLM. Incubation in sodium carbonate buffer and WME resulted in a K_m value that decreased to 5.6 and 6.6 mM in RLM, which is closer to that in hepatocytes incubated with 5 mM UDPGA (K_m : 3.2 mM). A decrease in the K_m value for AZTG in sodium carbonate buffer was also observed in HLM (Engtrakul et al., 2005). These findings suggest that buffer systems and UDPGA levels are at least partially involved in the differences in the K_m values for AZTG between RLM and hepatocytes.

In order to compare the AZTG inhibition potentials of drugs in liver

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microsomes with those in hepatocytes, the IC_{50} values were also determined in rat hepatocytes. Representative AZTG inhibition profiles (indomethacin) in RLM and hepatocytes are presented in Figure 4. The IC_{50} values of diclofenac, diflunisal, indomethacin, ketoprofen, naproxen, and valproic acid in hepatocytes were similar to those in RLM (Figure 5). This finding differs from the previous report in that the IC_{50} value of ibuprofen against AZTG in human hepatocytes was significantly lower than that in HLM (170 vs 975 μ M, Engtrakul et al., 2005). However, the study in human hepatocytes was carried out using only one substrate-inhibitor pair; thus studies with more pairs are necessary to determine whether hepatocytes generally yield more potent inhibition values than liver microsomes. In addition, the inhibition potentials of drugs against various UGT isozymes should also be investigated using probe substrates for each isozyme. This could yield insight into whether care should be taken when liver microsomes are used to assess metabolizing enzyme inhibition potentials for drugs.

When the inhibitory potentials of the drugs are estimated, the nonspecific binding of the inhibitors to the incubation matrix should be taken into account. McGinnity et al. reported that the unbound fraction is a major contributing factor in the differences in apparent IC_{50} values for recombinant CYP2C9 and hepatocytes

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in the incubation matrix (McGinnity et al., 2004). However, acidic drugs such as NSAIDs tend to exhibit low nonspecific binding to the matrix (Austin et al., 2002; McGinnity et al., 2004). Thus, the nonspecific binding of inhibitors is considered to have only a slight effect on the apparent IC_{50} value.

The previous report that similar IC_{50} values of drugs are obtained between liver microsomes and hepatocytes against CYP isozyme-mediated metabolism (Di marco et al., 2003; Jones et al., 2004) along with current study findings suggest that liver microsomes could be used as an *in vitro* system for assessing the metabolizing enzyme inhibition potentials of drugs.

In summary, the AZTG inhibition potentials of several drugs were investigated in RLM and cryopreserved rat hepatocytes. The IC_{50} values in liver microsomes and hepatocytes were found to be similar. These findings show that liver microsomes can be used to quantitatively determine the glucuronidation inhibition potentials of drugs.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Chemical structures of drugs investigated. (A) diclofenac, (B) diflunisal, (C) fluconazole, (D) indomethacin, (E) ketoprofen, (F) mefenamic acid, (G) naproxen, (H) niflumic acid, and (I) valproic acid.

Figure 2. Inhibitory effects of diflunisal on AZT glucuronidation in rat liver microsomes (RLM). AZT (0.2 – 10 mM) was incubated in the presence (●) and absence (○) of diflunisal (40 μM) in RLM (0.1 mg protein/ml) for 20 min. The *x* and *y* axes represent the substrate concentration and AZT glucuronidation velocity, respectively. The Eadie-Hofstee plot is presented as an inset. Each incubation was performed in duplicate, and the data represent the mean.

Figure 3. AZT glucuronidation in cryopreserved rat hepatocytes. Time profiles for the formation of AZT glucuronide (A) and kinetics for AZT glucuronidation (B) are presented. The Eadie-Hofstee plot is presented as an inset (B). AZT (2 mM) was incubated with rat hepatocytes (2.5×10^5 cells) for the times designated

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(A), and AZT (0.2 – 10 mM) was incubated with rat hepatocytes (2.5×10^5 cells) for 30 min (B). Each incubation was performed in triplicate, and the data represent the mean \pm S.D.

Figure 4. Inhibition of AZT glucuronidation by indomethacin in the liver microsomes (A) and cryopreserved hepatocytes (B) of rats. AZT (2 mM) was incubated with liver microsomes (0.1 mg protein/ml) for 20 min (A), and AZT (1 mM) was incubated with hepatocytes (2.5×10^5 cells) for 30 min (B), with varying concentrations of indomethacin. Each incubation was performed in duplicate, and data represent the mean.

Figure 5. Comparison of the AZT glucuronidation inhibition potentials of drugs in rat liver microsomes (RLM) and cryopreserved rat hepatocytes. AZT (2 or 1 mM) was incubated separately with RLM (0.1 mg protein/ml) and hepatocytes (2.5×10^5 cells) for 20 and 30 min, respectively, in the presence or absence of inhibitors. The x and y axes represent the IC_{50} values in RLM and hepatocytes, respectively, and the solid and dotted lines represent the line of unity and two-fold differences, respectively. Dic, Dif, In, K, Na, and V denote diclofenac, diflunisal, indomethacin,

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ketoprofen, naproxen, and valproic acid, respectively.

TABLES

Table 1 IC₅₀ values for drugs inhibiting AZT glucuronidation in the liver microsomes and hepatocytes of rats

Drugs	IC ₅₀ (μM)	
	Liver microsomes	Hepatocytes
Diclofenac	40 ± 1.8	58 ± 2.2
Diflunisal	34 ± 7.1	37 ± 2.2
Fluconazole	1791 ± 226	N.D.
Indomethacin	103 ± 3.9	88 ± 16
Ketoprofen	276 ± 7.7	361 ± 34
Mefenamic acid	37 ± 0.9	N.D.
Naproxen	332 ± 9.5	486 ± 52
Niflumic acid	102 ± 5.8	N.D.
Valproic acid	538 ± 35	281 ± 52

The data represent the mean ± computer-calculated S.E.

N.D.: Not determined

Fig. 1

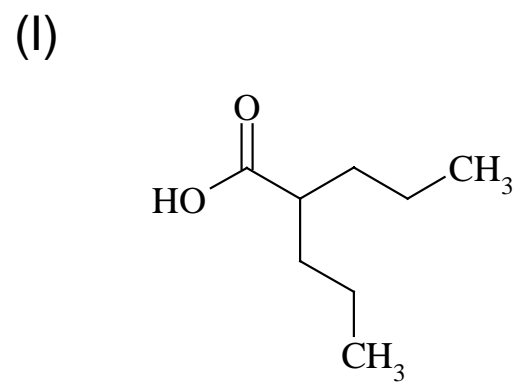
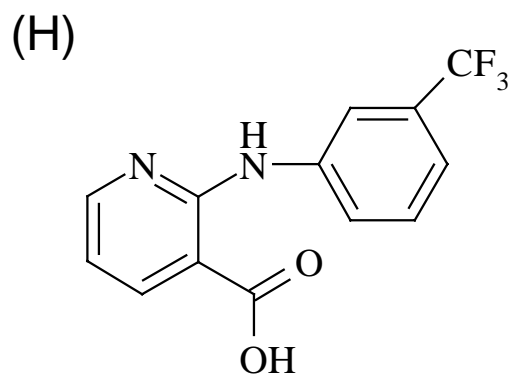
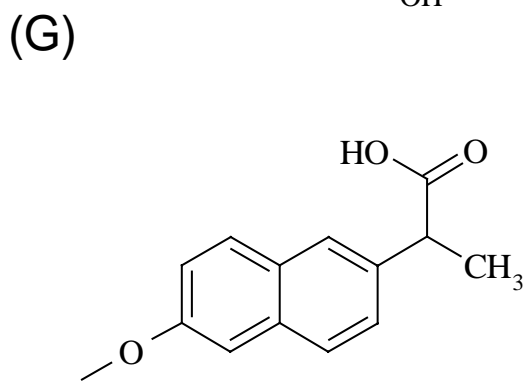
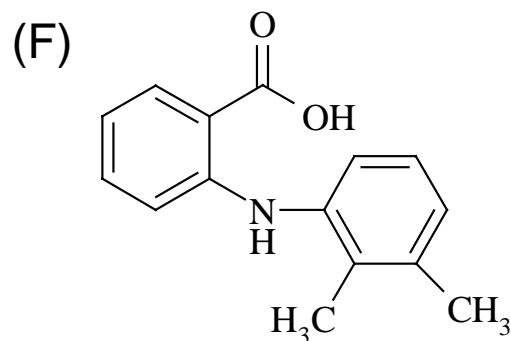
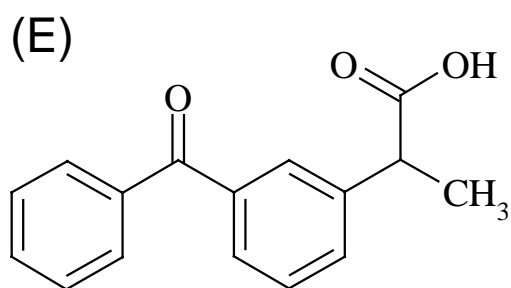
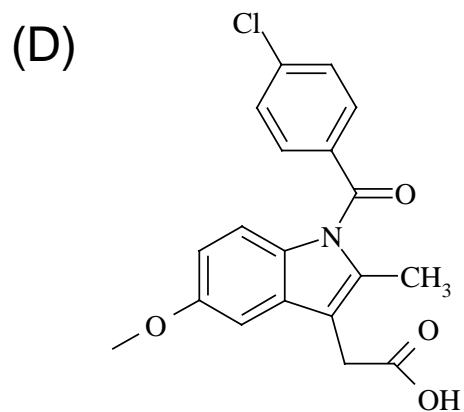
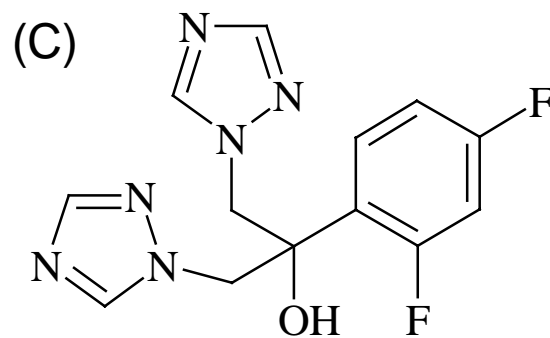
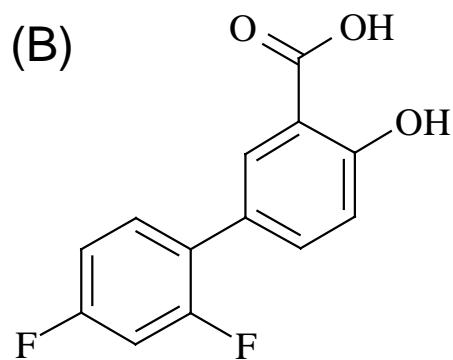
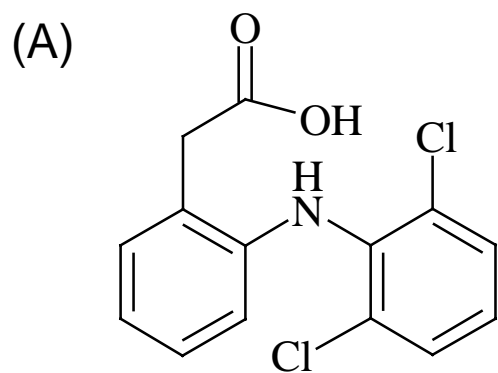


Fig. 2

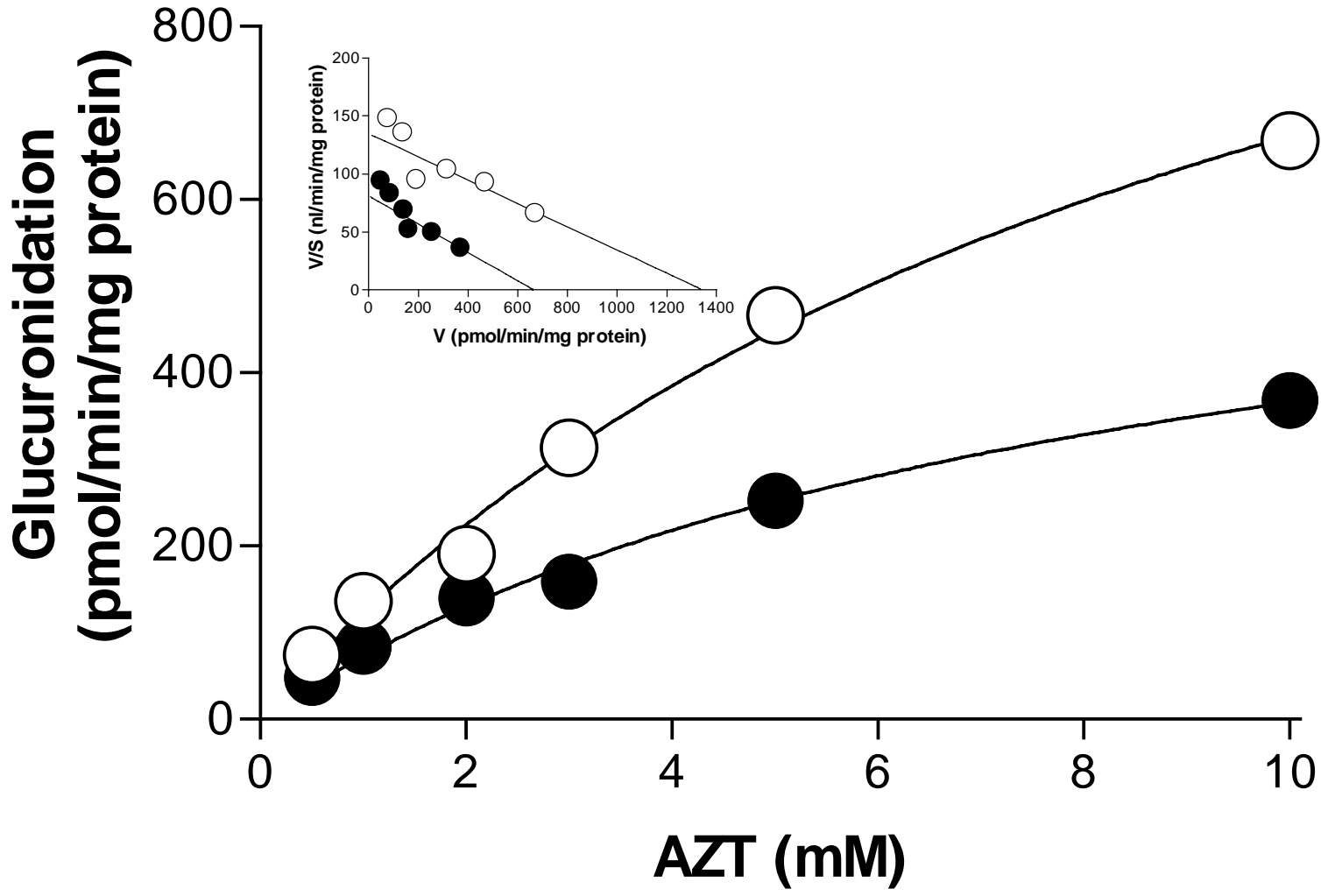


Fig. 3

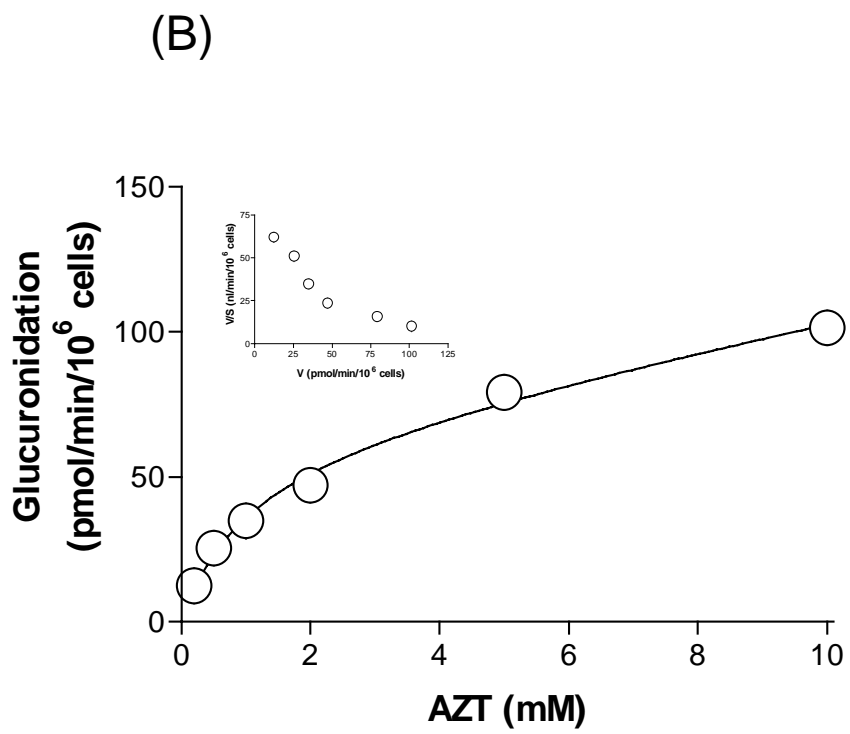
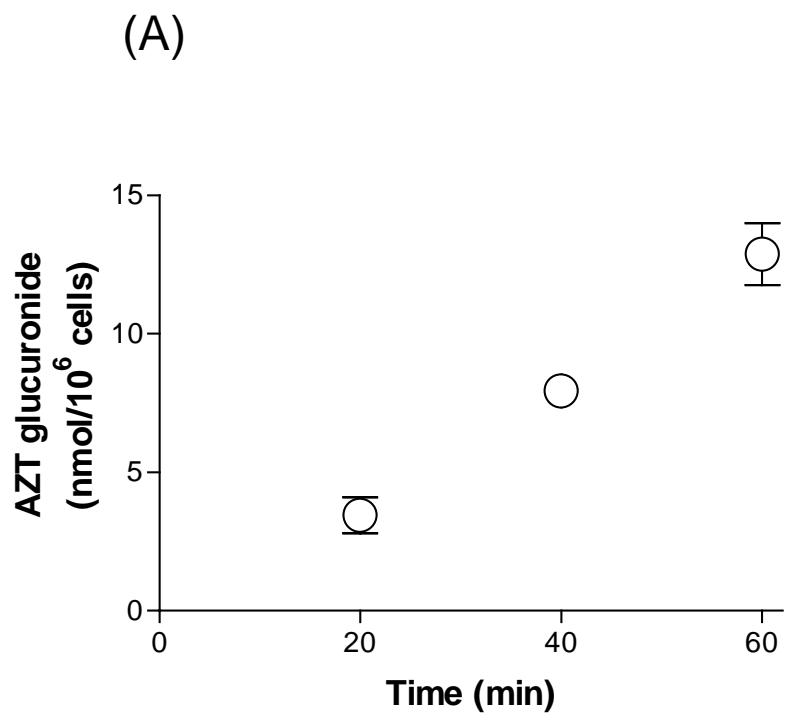


Fig. 4

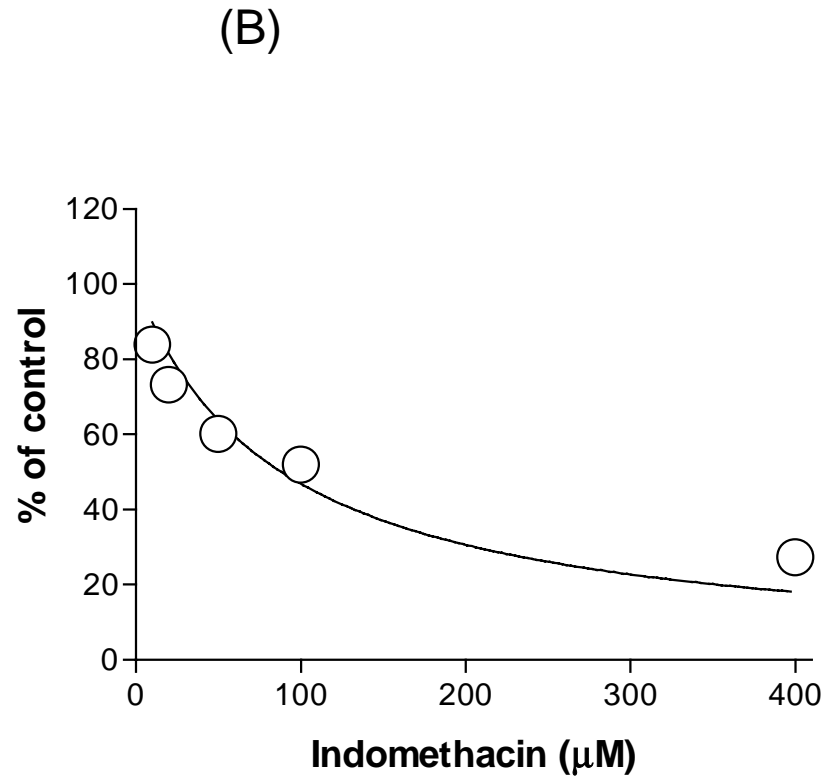
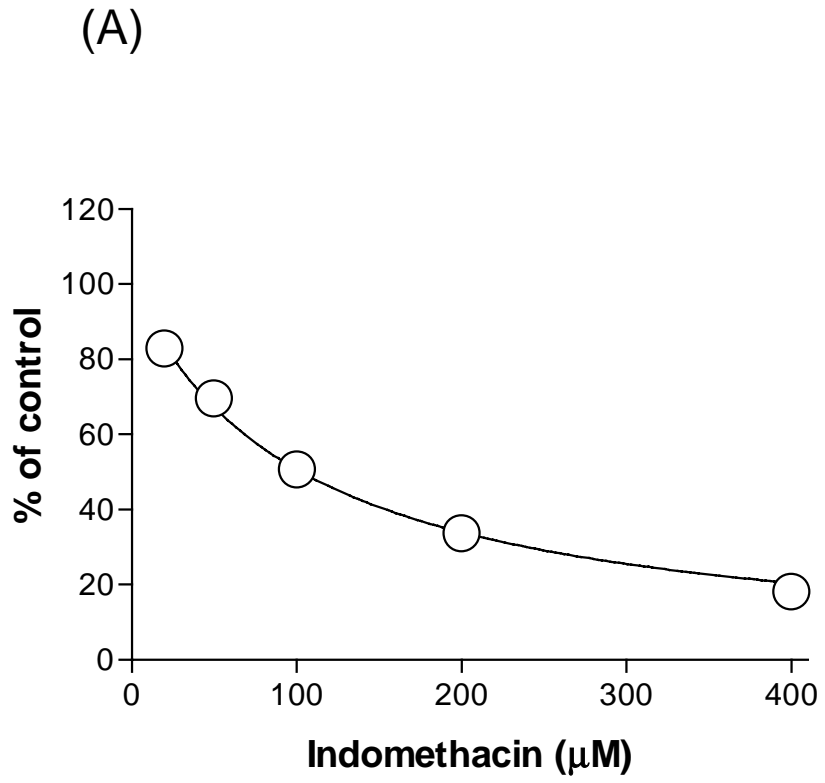


Fig. 5

