ATP Serves as an Endogenous Inhibitor of UDP-Glucuronosyltransferase (UGT): A New Insight into the ‘Latency’ of UGT

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Abbreviations: UGT, UDP-glucuronosyltransferase; ER, endoplasmic reticulum; RLM, rat liver microsomes; HLM, human liver microsomes; GRP, glucose-regulated protein; 4-MU, 4-methylumbellifone; 4-MUG, 4-Methylumbelliferyl-β-D-glucuronide; Brij-58, polyoxyethylene cetyl alcohol ether; PDI, protein disulfide isomerase; EGFR, epidermal growth factor receptor
Abstract:

We have suggested that adenine-related compounds are allosteric inhibitors of UGT in rat liver microsomes (RLM) treated with detergent. To clarify whether the same occurs with a pore-forming peptide, alamethicin, the effects of adenine-related compounds on 4-metylumbelliferone (4-MU) glucuronidation were examined using RLM and human liver microsomes (HLM). ATP inhibited 4-MU glucuronidation when Brij-58-treated RLM were used (IC\textsubscript{50} = around 70 µM). However, alamethicin-treated RLM exhibited a lower susceptibility (IC\textsubscript{50} = around 460 µM) than Brij-58-treated RLM. A similar phenomenon was observed when pooled HLM were used. Then, the endogenous ATP content of RLM was determined in the presence and absence of alamethicin or detergent. While no ATP remained in the microsomal pellets after Brij-58-treatment, more than half the microsomal ATP remained even after treatment with alamethicin. Further, the V\textsubscript{max} in the absence of an adenine-related compound was approximately three times higher in Brij-58-treated than in alamethicin-treated RLM. The difference in the inhibitory potency observed would be due to the difference in remaining endogenous ATP and the accessibility of exogenous ATP to the luminal side of the endoplasmic reticulum (ER) where the active site of UGT is located. Gefitinib,
a protein tyrosine kinase inhibitor, markedly inhibited human UGT1A9 activity. Interestingly, AMP antagonized Gefitinib-provoked inhibition of UGT1A9 and ATP exhibited an additive inhibitory effect at a lower concentration. Therefore, Gefitinib inhibits UGT1A9 at the common ATP binding site shared with ATP and AMP. Releasing adenine nucleotide from the ER is suggested to be one of the mechanisms to explain the 'latency' of UGT.
Introduction:

Glucuronidation is one of the most important steps in drug metabolism. Many endo- and xeno-biotics and/or their metabolites are converted to water-soluble glucuronides (Dutton, 1980; Bock et al, 1987; Ritter, 2000). This step is catalyzed by UDP-glucuronosyltransferase (UGT) which is expressed mainly in the liver and gastro-intestinal tract (Tukey and Strassburg, 2000). UGT is a family of enzymes which transfer the glucuronic acid moiety of the co-substrate, UDP-glucuronic acid (UDP-GlcUA), to a substrate (Mackenzie et al, 2005). This enzyme is localized in the endoplasmic reticulum (ER) and experimental evidence has suggested that the most important domain of UGT is located within the ER (Shepherd et al., 1989; Radomsinska-Pandya et al, 2005). A membrane-spanning domain of UGT is located near the carboxy-terminus which extrudes cytosolic tail consisting of about 20 amino acid residues (Meech and Mackenzie, 1997). In agreement with the topology of UGT in the ER membrane, it has been suggested that a UDP-GlcUA transporter is expressed on the ER membrane, and this protein translocates UDP-GlcUA from the cytosol to the lumen of the ER (Hauser et al., 1988). Muraoka et al. (2007) have cloned a UDP-GlcUA transporter, UDP-galactose transporter-related protein.
(UGTrel7; solute carrier family 35, SLC35D1), which is involved in the above process.

It has long been known that UGT activity is increased following treatment of microsomes with detergent (Winsnes, 1969), and this phenomenon is referred to as the 'latency' of UGT. The same is also observed when cells were treated with a pore-forming peptide, alamethicin (Bánhegyi et al., 1983). The reason why this latency is observed is thought to be due to the presence of a membrane (ER membrane) obstructing the free access of substrates to the active site of UGT. It is generally accepted that the latency of UGT is due to the limited transport of UDP-GlcUA from cytosol to the lumen of ER, the process of which is mediated by a UDP-GlcUA transporter(s) (Muraoka et al., 2007). This explanation sounds reasonable. If this is true, the kinetic Michaelis constant (Km) should be lower under detergent-treating conditions than under conditions not involving detergent treatment. However, the Km of the substrate (aglycone) is not often reduced even after detergent treatment (Puig and Tephly, 1986). It is, therefore, unlikely that the removal of the membrane obstruction is the sole reason for the latency of UGT.

We have reported previously that adenine nucleotides, such as ATP,
NADP⁺ and NAD⁺, are endogenous inhibitors of UGT (Nishimura et al., 2007). These adenine nucleotides inhibit UGT in a non-competitive manner, and they exhibited the effect only when microsomes are disrupted by detergent. On the basis of a structure-inhibitory effect relationship, while NADPH and NADH exhibit no or only a minor inhibitory effect on UGT (IC₅₀>1 mM), their oxidized forms, NADP⁺ and NAD⁺, are potent UGT inhibitors. There are NAD(P)⁺-dependent dehydrogenases, such as 11β-hydroxysteroid dehydrogenase and hexose-6-phosphate dehydrogenase, in the lumen of the ER (Hewitt et al., 2005). Furthermore, molecular chaperones, such as glucose-regulated protein 78 (GRP78) and GRP94, are expressed within the ER, and they bind to ATP and hydrolyze it (Dierks et al., 1996; Rosser et al., 2004). Therefore, significant amounts of ATP, NAD⁺ and NADP⁺ should be present in the lumen of the ER, and they are needed for the oxidoreductase and chaperone functions. If adenine nucleotides work as endogenous inhibitors of UGT, their concentration in the ER is considered to be a determinant regulating the action of certain hormones which are UGT substrates. However, the actual concentration of adenine nucleotides within the ER has not been accurately determined.
Previously, we observed the nucleotide-dependent inhibition of UGT using detergent-treated microsomes. To clarify whether the same occurs with a pore-forming peptide, alamethicin, the effects of adenine-related compounds on 4-methylumbelliferone (4-MU) glucuronidation were examined using liver microsomes from rats (RLM) and humans (HLM). In addition, we measured the ATP content in the ER with and without alamethicin/detergent treatment. Through these experiments, we investigated the hypothesis that a change in the concentration of adenine nucleotides within the ER is the reason for the latency of UGT. On the other hand, agents such as Gefitinib inhibiting epidermal growth factor receptor (EGFR)-protein tyrosine kinase are UGT inhibitors (Liu et al., 2010; Fujita et al., 2011; Piu et al., 2011). However, the reason why Gefinitib and related compounds exhibit an inhibitory effect on UGT has not been elucidated. Since their pharmacological target site is the ATP binding domain of the protein tyrosine kinase, we also examined whether Gefitinib inhibits UGT through the ATP binding site on UGT.
Materials and Methods

Materials. ATP, NADH, NADPH, 4-MU, D-saccharic acid-1,4-lactone, and L-α-phosphatidylcholine (egg yolk, type XI-E) were purchased from Sigma-Aldrich (St. Louis, MO). NAD⁺, NADP⁺, and UDP-GlcUA trisodium salt were obtained from Wako Pure Chemicals, Co. Ltd. (Osaka, Japan). 4-Methylumbelliferyl-β-D-glucuronide (4-MUG) and polyoxyethylene cetyl alcohol ether (Brij-58) were purchased from Nakalai Tesque (Kyoto, Japan). ENLITEN® rLuciferase/Luciferin reagent and 5xPassive lysis buffer were purchased from Promega (Madison, WI). Gefitinib (Iressa®) was purchased from Tocris Bioscience (Bristol, UK). All other reagents were of the highest grade commercially available.

Animals and treatment. Animal experiments in this study were conducted following the approval of the Ethics Committee for Animal Experiments of Kyushu University. Male Sprague-Dawley rats (5 weeks-old) were purchased from Kyudo (Kumamoto, Japan) and they were maintained for one week with free access to water and a standard diet under a 7 am to 7 pm light/dark cycle. The liver was removed and perfused with ice-cold saline, and then the
microsomes were prepared by differential centrifugation as described previously (Oguri et al., 1996). The microsomes were then re-suspended in 0.25 M sucrose.

**Protein assay.** The protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

**Determination of UGT activity.** The activity of 4-MU glucuronidation was determined by the published methods (Hanioka et al., 2001) with slight modifications (Nishimura et al., 2007). Human UGT1A9 Supersome™ and human liver microsomes (pooled microsomes from 150 donors) were purchased from BDGentest (Woburn, MA).

**Determination of ATP by luciferin and luciferase.** Rat liver microsomes were diluted to give a protein concentration of 5 mg/mL and then treated with Brij-58 (0.25 mg/mg protein) or alamethicin (0.05 mg/mg protein). The mixture was kept on ice for 1 h, then centrifuged at 105,000 xg (4°C) for 60 min, and the supernatant and pellets were separated. Both fractions were mixed/diluted with 20-volume relative to the original microsome amount (protein base) of 1xPassive Lysis Buffer (Promega, Madison, WI). The mixture was kept at room temperature for 30 min then a 5 μL aliquot of the diluted sample was mixed with 50 μL Luciferase/Luciferin reagent. The emission of ATP-dependent
chemiluminescence was measured in a Turner Biosystems Luminometer (Model TD-20/20) (Promega, Madison, WI). The detection limit was 0.1 nmol ATP/5 μL sample.

**Data analyses.** Statistical significance, kinetic parameters and IC₅₀ were calculated using the computer software, GraphPadPrism5 (GraphPad, La Jolla, CA).

**Prediction of the three-dimensional structure and ligand binding sites of UGT1A9.** The amino acid sequence of UGT1A9 (GenBank: AAG30418.1) was subjected to a modeling system on the Web linking to a server installed with Phyre² (Protein Homology/analogy Recognition Engine V 2.0) (http://www.sbg.bio.ic.ac.uk/phyre2/html/; Kelly and Stemberg, 2009). The following six proteins were selected as the templates based on the Structural Classification of Proteins and ASTRAL release 1.75A (March 2012): flavonoid 3-O-glucosyltransferases (d2c1xa1, Offen et al, 2006; and c3hbjA, Modolo et al., 2009), hydroquinone glucosyltransferase (d2vcha1, Brazier-Hicks et al, 2007), (iso)flavonoid glycosyltransferase (d2pq6a1, Li et al, 2007), vancosaminyltransferase (Gtf glycosyltransferase) (d1rrva, Mulichak et al, 2004), and triterpene/flavonoid glycosyltransferase (d2acva1, Shao et al, 2005). This
selection was also based on a heuristics to maximize the confidence, percentage identity and alignment coverage. In the modeling, the three-dimensional structure of about 87% of whole UGT1A9 body could be constructed by overlapping with templates. The confidence of this modeling was expected to be over 90%. Because the similar way could not be applicable to the structural simulation of the other region involving the highly variable C-terminal domain, the structure of this region was tentatively predicted by ab initio calculation. The construct obtained was then submitted to a 3DLigandSite server (http://www.sbg.bio.ic.ac.uk/~3dligandsite/; Wass et al, 2010) for the estimation of ligand binding site.
Results

inhibitory effect of adenine-related compounds on glucuronidation by Brij-58- and alamethicin-treated RLM. The possibility whether adenine-related compounds inhibit UGT activity both in RLM treated with a pore-forming peptide, alamethicin, as well as a detergent, Brij-58, was examined using 4-MU as a substrate. To clarify the specificity of nucleotides and related substances, the inhibitory potential of 12 substances was assessed. Table 1 shows the IC$_{50}$ values of the compounds examined. Of the substances tested, ATP exhibited the strongest inhibitory effect in both Brij-58 and alamethicin-treated RLM. However, the IC$_{50}$ was 7-times higher in alamethicin-treated RLM than in a Brij-50-treated preparation: the IC$_{50}$ of ATP was approximately 70 and 460 µM in Brij-58- and alamethicin-treated RLM, respectively. Although ADP also exhibited an inhibitory effect in both preparations, its IC$_{50}$ was 11- and 3-times higher than ATP in Brij-58- and alamethicin-treated microsomes. However, the inhibitory effect of NADP$^+$ was stronger than ADP, and NAD$^+$ exhibited a stronger inhibitory effect than ADP only in Brij-58-treated RLM. CTP had an inhibitory effect close to that of ADP in Brij-58-treated RLM but not in alamethicin-treated RLM and no inhibitory effect
was observed for adenosine, NADH, NADPH, GTP and UTP in RLM treated with Brij-58 and alamethicin. Although the relative IC₅₀ (alamethicin/Brij-58) varied from 1.6 with ADP to 6.9 with ATP, alamethicin-treated microsomes were always less sensitive to nucleotides than Brij-58-treated preparations. Of the nucleotide triphosphates tested, only CTP had an inhibitory effect close to that of ADP in Brij-58-treated RLM. The inhibitory potential of adenine-nucleotides also depends on the number of phosphate groups [ATP >> ADP > AMP (= almost no effect)]. Thus, the number of phosphates at the 5'-position of ribose is thought to be one of the determinants of the inhibitory effect of the adenine nucleotides. Finally, neither NADH nor NADPH exhibited any inhibitory effect on UGT activity, while NAD⁺ and NADP⁺ did have an inhibitory effect. Therefore, the oxidized status of the nicotinamide moiety seems to be another factor affecting the inhibitory effect.

Previously, we have confirmed that the mode of inhibition of 4-MU glucuronidation catalyzed by Brij-58-treated RLM by adenine-related compounds was non-competitive. In this study, we examined whether the same is also true for the glucuronidation catalyzed by alamethicin-treated RLM. For this purpose, a kinetic experiment was performed by varying the 4-MU concentration (Fig. 1).
Even addition of ATP, or NADP⁺ to the reaction mixture did not change the Michaelis-Menten kinetics, and increased the concentrations of nucleotides producing more pronounced inhibition (Fig. 1A-D). These plots strongly suggest that 4-MU UGT in both Brij-58- and alamethicin-treated RLM is inhibited by ATP and NADP⁺ in a non-competitive manner. This assumption was supported by re-evaluation of the Eadie-Hofstee plots (Fig. 1E-H); that is, regression lines with different concentrations of inhibitor run parallel to each other. Taken together, these results suggest that ATP and NADP⁺ reduce 4-MU UGT function in both Brij-58- and alamethicin-treated RLM by the same mechanism and by interacting with the enzyme(s) at a site distinct from substrate-binding sites. In agreement with the data shown in Table 1, the inhibitory constants (Ki) of ATP and NADP⁺ were smaller in Brij-58-treated than alamethicin-treated RLM (Table 2).

**Effect of AMP on the inhibition caused by ATP and NADP⁺.** As described before, the 4-MU UGT activity in both Brij-58- and alamethicin-treated RLM is barely affected by AMP (Table 1). However, we have shown that AMP has an ability to modify the inhibitory effects of other nucleotides in Brij-58-treated RLM (Nishimura et al, 2007). To investigate whether the same is also true for
alamethicin-treated RLM, the effect of AMP on the 4-MU glucuronidation activity was examined. In both Brij-58- and alamethicin-treated RLM, the inhibitory effect of ATP on 4-MU UGT activity was antagonized by AMP (Fig. 2A-B). Such an antagonistic effect became stronger along with an increase in AMP concentration. AMP also antagonized the inhibitory effect of NADP⁺ (Fig. 2C-D).

These results suggest that AMP competes with adenine-related inhibitors at the binding site of 4-MU UGT in both Brij-58- and alamethicin-treated RLM, although AMP itself lacks any inhibitory potential.

**Mechanism underlying the UGT latency caused by detergent- and alamethicin-treatment.** Our preliminary study showed that Brij-58-treatment causes a 17-fold increase in the Vmax of 4-MU glucuronidation in RLM (Supplemental Table S1). The Michaelis constant, Km, for 4-MU was slightly increased by Brij-58, while the Km for co-substrate, UDP-GlcUA, tended to be reduced by the treatment. Therefore, it is likely that an increase in the affinity for UDP-GlcUA may contribute to the enhancement of Vmax following detergent treatment. However, an increase in the Km for 4-MU does not agree with the hypothesis that removal of membrane obstruction is the sole reason for the latency of UGT. Table 3 shows the kinetic parameters for 4-MU glucuronidation.
which were compared for Brij-58- and alamethicin-treated RLM. The Km values for 4-MU and UDP-GlcUA were slightly higher in alamethicin-treated than Brij-58-treated RLM. When the kinetics were studied with different 4-MU concentrations, the Vmax in alamethicin-treated RLM was approximately one third that of Brij-58-treated RLM. As far as the activating conditions with Brij-58 (0.25 mg/mg protein) and alamethicin (0.05 mg/mg protein) were concerned, the Vmax obtained after varying the 4-MU/UDP-GlcUA concentrations was higher in Brij-58-treated than in alamethicin-treated RLM.

Although the ER appears to contain a substantial concentration of adenine nucleotides for molecular chaperons and dehydrogenases, it is not known whether the luminal adenine nucleotides are released by treating microsomes with detergent or alamethicin. If this is true, the inhibitory effect should be abolished or weakened following detergent- or alamethicin-treatment of microsomes. To examine this hypothesis, we determined the ATP content in the ER with and without Brij/alamethicin treatment. Table 4 shows the ATP content in microsomal pellets and supernatant after treatment with Brij-58 or alamethicin, the concentrations of which were the same as those used for determining UGT activity. ATP was released by Brij-58 treatment and no detectable ATP remained
in the detergent-treated microsomes. However, ATP remained in the microsomes in the control sample. Part of the microsomal ATP was also released by alamethicin treatment, but almost 60% of ATP remained unreleased even after alamethicin treatment. The lower Vmax in alamethicin-treated than in Brij-58-treated RLM (Table 3) seems to correlate with the extent of ATP remaining in the microsomes. For example, it is suggested that an ATP concentration sufficient for UGT inhibition still remains in microsomes even after alamethicin treatment. Conversely, it would be reasonable to believe that the release of ATP from microsomes after detergent treatment results in the abolition of its inhibitory effect on UGT. Therefore, it is suggested that the presence of inhibitory adenine nucleotides is one possible mechanism for the latency of UGT.

**The combined effect of adenine-related compounds on UGT activity.** Under normal conditions, the cellular ATP concentration is several mM (Schwiebert, 2000) and this is 5- to 10-fold higher than the ADP level (Taylor et al., 1975). The proportion of ATP, ADP and AMP concentrations has been assumed to satisfy the following equation (Hadie et al., 1998; 2001).

\[
\frac{[AMP]}{[ATP]} = \left(\frac{[ADP]}{[ATP]}\right)^2
\]

The concentration of adenine nucleotides in the ER lumen of living cells has
not been accurately determined. However, on the basis of the above equation, three ratios (ATP:ADP:AMP=1:1:1, 25:5:1 and 100:10:1) at four total concentrations (ATP+ADP+AMP) from 250 to 1500 µM were examined under UGT inhibition. In Brij-58-treated RLM, the inhibitory effect on UGT was increased depending on an increase in the ATP ratio (Fig. 3). Therefore, the inhibitory effect of ATP can be negatively modulated by its metabolites ADP and AMP. Although a similar trend was observed in alamethicin-treated RLM, the effect of combined nucleotides was more marked in Brij-58-treated RLM. Then, we examined the effect of more complicated mixtures on UGT activity. For this, all inhibitory substances listed in Table 1 were mixed at their respective IC50 and half IC50 concentrations and the effects of these cocktails on UGT activity were examined. In Brij-58-treated RLM, the ‘IC50/2’ cocktail reduced 4-MU glucuronidation to approximately 40% of the control, while the effect was almost abolished when alamethicin-treated RLM were used (Fig. 4). It is, therefore, likely that the access of inhibitory adenine-related compounds to the target site of UGT is limited in alamethicin-treated RLM.

**Inhibition of glucuronidation in HLM and human UGT1A9 by ATP.** The effect of AMP and ADP as well as ATP on UGT activity was examined in Brij-58
and alamethicin-treated HLM. ATP exhibited the strongest inhibitory effect in both Brij-58 and alamethicin-treated HLM (Table 5). However, the IC₅₀ was 27-times higher in alamethicin-treated HLM. No inhibitory effect was observed for ADP and AMP in HLM treated with Brij-58 or alamethicin. We then examined the effect of adenine nucleotides on human UGT1A9 which is one of the major UGT isoforms involved in 4-MU glucuronidation. In this case, we used alamethicin-treated UGT1A9 Supersome™ which is used widely for research purposes. As shown in Table 5, ATP inhibited UGT1A9-catalyzed 4-MU glucuronidation with an IC₅₀ lower than that obtained in alamethicin-treated HLM. The IC₅₀ of ADP and AMP was greater than 1000 µM (Table 5).

**The mechanism of Gefitinib-induced inhibition of UGT1A9.** As shown in Fig. 5, Gefitinib significantly inhibited 4-MU UGT activity mediated by UGT1A9 as well as Brij-58- and alamethicin-treated HLM. The IC₅₀ of Gefitinib for UGT1A9-catalyzed 4-MU glucuronidation was about 20 µM (Table 6). To investigate whether Gefitinib inhibits UGT through the putative ATP binding site on UGT, the inhibitory effect of Gefitinib on UGT1A9 was examined in the presence or absence of ATP at its IC₅₀ concentration. Although ATP exhibited an additive inhibitory effect when the Gefitinib concentration was less than 50 µM,
no such effect was observed in the presence of Gefitinib at a concentration of 75 µM or more (Fig. 6). Furthermore, the Gefitinib-induced inhibition of UGT1A9-catalyzed 4-MU glucuronidation was antagonized in the presence of AMP which is a non inhibitory adenine nucleotide but a negative modulator of ATP-dependent inhibition (Fig. 6). From these observations, Gefitinib and adenine nucleotides are thought to share the binding site on UGT. Therefore, it is suggested that Gefitinib inhibits UGT1A9 through the putative ATP binding site on UGT.
Discussion

The inhibitory effect of adenine nucleotides and related compounds on 4-MU UGT was studied in RLM and HLM. ATP exhibited a strong inhibitory effect in HLM as well as RLM. On comparing the inhibitory effect of ATP on UGT between microsomes pre-treated with Brij-58 and alamethicin, the inhibitory effect was more marked in Brij-58-treated RLM than in alamethicin-treated RLM. As the mode of inhibition of 4-MU UGT was non-competitive in both Brij-58- and alamethicin-treated RLM, ATP is considered to be an allosteric inhibitor of UGT. The data suggest the poor ability of adenine-related compounds to gain access to the target site on UGT in alamethicin-treated microsomes (Fig. 4). Therefore, the IC50 observed in the detergent-treated microsomes is considered as a relevant index for the inhibitory potency of the adenine-related compounds.

It is suggested that there is an ATP transporter on the microsomal membrane which draws ATP into the lumen (Clairmont et al, 1992; Kim et al., 1996). ATP uptake into microsomes is saturable with a Km of 3 to 4 µM and a Vmax of 3 to 7 pmol/min/mg protein (Clairmont et al, 1992). When firefly-luciferase, which contains a signal sequence and a sequence for ER
targeting, was expressed in Chinese hamster ovary (CHO) cells, ATP-dependent chemiluminescence was observed by microscopy after addition of luciferin (Dorner and Kaufman, 1994). Thus, it is likely that the luminal side of the ER contains a substantial level of ATP, although its concentration remains unknown.

In addition, the ER lumen expresses proteins, such as protein disulfide isomerase (PDI), UGT1A1 and UGT1A7, which are sensitive to phosphorylation (Quéméneur et al, 1994; Basu et al., 2005). Furthermore, molecular chaperones, such as PDI, calreticulin, GRP78 and GRP94, capable of binding to ATP are present within the ER (Guthapfel et al., 1996; Dierks et al., 1996; Rosser et al., 2004). The dissociation constant of ATP and the Km for ATPase activity of GRP78 are reported to be 5.4 and 28.6 µM, respectively (Wearsch and Nicchitta, 1997). For PDI, the corresponding values are 9.7 and 7.1 µM, respectively (Guthapfel et al., 1996). Accordingly, the concentration of ATP needed for the functions of the above series of proteins is assumed to be around 30 µM, which is close to the IC50 determined in this study. Since these ATP-binding proteins are ATPases, it would be possible that they modulate UGT activity by hydrolyzing inhibitory ATP to less-inhibitory ADP and non-inhibitory AMP in the ER.
As shown in Fig. 3, the inhibitory effect of adenine nucleotides on UGT activity varied depending on the ratio of ATP:ADP:AMP. If the concentration of ATP in the ER is kept much higher than its IC$_{50}$, the UGT activity should be completely suppressed under such conditions. However, it is reasonable to assume that the concentration of ATP and antagonistic AMP varies dynamically in the lumen of the ER to switch the glucuronidation reaction on/off. Our preliminary study indicated that the ATP level within the hepatic ER is higher in the evening than in the morning (Supplemental Figure S1). The concentration of nocturnal ATP was close to 30 µM. Also, the extracellular production of adenosine from ATP in the retina exhibits a circadian variation (Ribelayga and Mangel, 2005), and we also found an intra-day variation in the ATP level in the lumen of the ER.

Although it is assumed that the limited transport of UDP-GlcUA from cytosol to the ER lumen renders UGT latent, the Vmax of UGT was markedly increased by Brij-58 treatment, and the Km values for 4-MU and UDP-GlcUA showed only a minor change (Supplemental Table S1). Thus, although it cannot be excluded that reducing the Km for UDP-GlcUA may make some contribution to an apparent increase in UGT function produced by detergent, increasing the
Km for substrates (glucuronic acid acceptors) seems to disagree with the idea that the removal of interference by the membrane is the absolute mechanism explaining the latency of UGT.

On the basis of the inhibitory effect of ATP and its level in the lumen of the hepatic ER determined in this study, we propose a new hypothesis for the latency of UGT. As mentioned before, in untreated microsomes, the luminal concentration of ATP is around 30 µM (Supplemental Figure S1), which is comparable with its IC₅₀ value for UGT inhibition. This observation suggests that UGT function is partially suppressed by ATP under normal physiological conditions. However, when microsomes are treated with detergent or alamethicin, the enclosure of adenine nucleotides in the ER is disrupted, and the concentration of the nucleotides is markedly reduced. Therefore, the concentration of adenine nucleotides, especially in Brij-58-treated microsomes, becomes too low to inhibit UGT. This possibility seems to be consistent with the fact that the inhibition mode of adenine nucleotides is non-competitive and the V_max of UGT is increased by detergent treatment (Supplemental Table S1, Table 2, Fig. 1). Furthermore, when RLM were treated with Brij-58, ATP did not remain in the pellets obtained after ultra-centrifugation, while this nucleotide
remained in the microsomal pellets in untreated microsomes. Taking all above findings into consideration, it is suggested that a detergent/alamethicin-caused decrease in adenine nucleotides in the ER is one possible mechanism explaining the latency of UGT.

The inhibitors of EGFR-protein tyrosine kinase have an undesired inhibitory effect on UGT (Liu et al., 2010; Fujita et al., 2011; Piu et al., 2011). This may cause a drug-drug interaction in UGT-dependent metabolism (Fujita et al, 2011). However, it has not been discovered why these EGFR-protein tyrosine kinase inhibitors suppress UGT activity. In this study, we suggest that Gefitinib inhibits UGT1A9 by binding to the allosteric site shared with ATP and AMP. ATP at its IC₅₀ concentration additively enhanced the inhibition of UGT catalysis with Gefinitib at low concentrations, and AMP antagonized the inhibitory effect of Gefitinib. The inhibitory potency of Gefitinib was far stronger than that of ATP. Although ATP at IC₅₀ showed an additive effect on the inhibition of UGT1A9 by Gefitinib at a low concentration, the effect was diminished by increasing the concentration of Gefitinib. Therefore, Gefitinib and ATP seem to inhibit UGT1A9 through binding to a common site. No additive effect of ATP at the high concentration of Gefitinib would be owing to the occupation of the binding site
with Gefitinib. On the other hand, the Gefitinib-induced inhibitory effect on UGT1A9 was partially antagonized by 1 mM AMP, and the suppressive effect remained even in the presence of high concentration Gefinitib. Although AMP at a concentration more than 1 mM may completely eject Gefitinib from the competitive binding site an alternative possibility that AMP exerts its antagonistic effect against Gefinitib through an allosteric mechanism cannot be excluded. It is considered that the ATP and AMP contents of cells largely depend on the mitochondrial function which varies in response to a change in physiological conditions. However, many more studies will be needed to fully understand how disease conditions affect the ATP concentration within the ER, hormone metabolism and drug-drug interaction with EGFR-protein tyrosine kinase inhibitors. The three-dimensional structure of UGT1A9 has already been simulated using the crystal structure of TDP-epi-vancosaminyltransferase (PDB ID: 1PN3) as a template (Fujiwara et al, 2009). In their study, the prediction was carried out using procedures reported by Ogata and Ueyama (2000). On the contrary, in Phyre² system used in this study, multiple templates described in the Materials and Methods were used to predict the UGT1A9 structure. The templates used were the enzymes utilizing UDP-sugar as the co-substrate
belonging to UDP-glycosyltransferase/glycogen phosphorylase superfamily and a flavonoid 3-\(O\)-glucosyltransferase (crystal structure of UGT78G1 with binding UDP as a ligand). Interestingly, we have predicted the binding site of UGT for ADP in a 3D structural model, using an algorithm “3DLigandSite using similar structures” and multiple (six) templates substituted for UGT (Kelly and Sternberg, 2009; Wass et al., 2010)(Supplemental Figure S2).

Further studies are needed to confirm which residues are relevant for the modulation of UGT activity by adenine nucleotides.
Authorship Contributions

Participated in research design: Ishii, An, Nishimura, Yamada

Conducted experiments: Ishii, An, Nishimura

Performed data analysis: Ishii, An, Nishimura

Wrote or contributed to the writing of the manuscript: Ishii, An, Yamada
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Footnotes

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Legends for figures:

Fig. 1 Michaelis-Menten and Eadie-Hofstee plots for the inhibition by ATP and NADP⁺ of 4-MU glucuronidation catalyzed by RLM pretreated with Brij-58 or alamethicin: kinetics by varying 4-MU concentration.

Effect of ATP (A, E) and NADP⁺ (B, F) on 4-MU glucuronide formation in Brij-58-treated microsomes was estimated at seven concentrations of 4-MU (10 to 1000 µM). The effect of ATP (C, G) and NADP⁺ (D, H) in alamethicin-treated microsomes was also determined. The UDP-GlcUA concentration was fixed at 2 mM.

Fig. 2. Antagonist effect of AMP on an ATP- and NADP⁺-evoked reduction in 4-MU glucuronidation catalyzed by Brij-58- or alamethicin-treated RLM. Effects of ATP (A, B) and NADP⁺ (C, D) (10 to 2500 µM) on the 4-MU glucuronide formation were assayed in the presence or absence of AMP (closed circle, 0 µM; open square, 200 µM; closed triangle, 1000 µM). Microsomes were treated with Brij-58 (0.25 mg/mg protein) (A, C) or alamethicin (0.05 mg/mg protein) (B, D). 4-MU and UDP-GlcUA concentrations were fixed at 100 µM and 2 mM, respectively. Each plot
Fig. 3. **Effect of the ratio of exogeneously added adenine nucleotides on the UGT activity in detergent- or alamethicin-treated RLM.** Effects of the ratio of adenine nucleotides (ATP:ADP:AMP=100:10:1, 25:5:1, and 1:1:1) on 4-MU glucuronide formation were examined by varying the total concentration of ATP, ADP and AMP (250, 500, 1000 and 1500 μM). Microsomes were treated with Brij-58 (0.25 mg/mg protein) or alamethicin (0.05 mg/mg protein). 4-MU and UDP-GlcUA concentrations were fixed at 100 μM and 2 mM, respectively. Each bar represents the mean ± S.E. of a triplicate assay. Significantly different from the activity in the absence of adenine nucleotides (*, p<0.05; **, p<0.01; ***, p<0.001).

Fig. 4. **Effect of the mixture of inhibitory adenine-related compounds on the UGT activity in Brij-58- or alamethicin-treated RLM.** Effects of a mixture of inhibitory compounds [ATP, ADP, adenine, NAD⁺, NADP⁺ and CTP (Brij-58); and ATP, ADP, adenine, NAD⁺ and NADP⁺ (alamethicin)]
on 4-MU glucuronide formation were examined. The 'IC$_{50}$' and 'IC$_{50}$/2'
cocks contain the inhibitory compounds described above at a IC$_{50}$
concentration and half the IC$_{50}$ concentration (see Table 1), respectively.
Microsomes were treated with Brij-58 (0.25 mg/mg protein) or
alamethicin (0.05 mg/mg protein). 4-MU and UDP-GlcUA concentrations
were fixed at 100 µM and 2 mM, respectively. Significantly different from
the activity in the absence of inhibitory compounds (**, p<0.01; ***,
p<0.001).

**Fig. 5. Effects of Gefitinib on pooled human liver microsome- and human
UGT1A9 microsome-catalyzed 4-MU glucuronidation.** Effects of
Gefitinib on 4-MU glucuronidation were assayed. Microsomal protein
(HLMs, 20 µg; UGT1A9 supersomes, 10 µg) was used in each assay.
The 4-MU concentration was fixed at 100 and 30 µM for HLM and
UGT1A9 supersomes, respectively. Each bar represents the mean ± S.E.
of triplicate assays. Abbreviation: HLMs, human liver microsomes.
Significantly different from the activity in the absence of Gefitinib (*,
p<0.05; **, p<0.01; ***, p<0.001).
Fig. 6. Effects of AMP and ATP on a Gefitinib-evoked reduction in 4-MU glucuronidation catalyzed by humen UGT1A9. Effect of Gefitinib on the 4-MU glucuronide formation was assayed in the presence of 500 μM ATP (A) and 1 mM AMP (B). UGT1A9 supersomes were treated with alamethicin (0.05 mg/mg protein). The 4-MU concentration was fixed at 30 μM. Each plot represents the average of triplicate assays.
Table 1. IC\textsubscript{50} value of nucleotides and adenine-related substances toward 4-MU UGT in RLM pretreated with either Brij-58 or alamethicin.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Brij-58 (μM)</th>
<th>Alamethicin (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>66.8±2.2</td>
<td>463±36 [6.9]</td>
</tr>
<tr>
<td>ADP</td>
<td>769±251</td>
<td>1258±54 [1.6]</td>
</tr>
<tr>
<td>Adenine</td>
<td>&lt;1000</td>
<td>1890±89 [-]</td>
</tr>
<tr>
<td>AMP</td>
<td>&gt;1000</td>
<td>&gt;2000 [-]</td>
</tr>
<tr>
<td>Adenosine</td>
<td>&gt;1000</td>
<td>&gt;2000 [-]</td>
</tr>
<tr>
<td>NADP\textsuperscript{+}</td>
<td>385±65</td>
<td>1079±65 [2.8]</td>
</tr>
<tr>
<td>NAD\textsuperscript{+}</td>
<td>334±116</td>
<td>1594±167 [4.8]</td>
</tr>
<tr>
<td>NADPH</td>
<td>&gt;1000</td>
<td>&gt;2000 [-]</td>
</tr>
<tr>
<td>NADH</td>
<td>&gt;1000</td>
<td>&gt;2000 [-]</td>
</tr>
<tr>
<td>CTP</td>
<td>853±47</td>
<td>&gt;2000 [-]</td>
</tr>
<tr>
<td>GTP</td>
<td>&gt;1000</td>
<td>&gt;2000 [-]</td>
</tr>
<tr>
<td>UTP</td>
<td>&gt;1000</td>
<td>&gt;2000 [-]</td>
</tr>
</tbody>
</table>

RLM pretreated with Brij-58 (0.25 mg/mg protein) or alamethicin (0.05 mg/mg protein) were used as the enzyme sources. 4-MU and UDP-GlcUA concentrations were fixed at 100 µM and 2 mM, respectively. Each value represents the estimated IC\textsubscript{50} ± S.E. Value in bracket is the IC\textsubscript{50} relative to that
determined using Brij-58-treated microsomes (=1.0). Control activities in the absence of inhibitor was 11.2 ± 0.3 and 4.63 ± 0.16 for Brij-58- and alamethicin-treated RLM, respectively.
Table 2. Ki values of ATP and NADP⁺ in terms of 4-MU glucuronidation activity in RLM.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Ki (μM)</th>
<th>Brij-58</th>
<th>Alamethicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>204 ± 17</td>
<td>635 ± 42</td>
<td></td>
</tr>
<tr>
<td>NADP⁺</td>
<td>298 ± 30</td>
<td>607 ± 23</td>
<td></td>
</tr>
</tbody>
</table>

Each value represents the estimated Ki ± S.D. Details are described in Fig. 1.
Table 3. Kinetic parameters of 4-MU glucuronidation catalyzed by RLM pretreated either with Brij-58 or alamethicin: kinetics by varying the UDP-GlcUA concentration

<table>
<thead>
<tr>
<th>Activator</th>
<th>Km (µM)</th>
<th>Vmax (nmol/min/mg protein)</th>
<th>Vmax/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinetics by varying 4-MU&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brij-58</td>
<td>93.6 ± 0.3</td>
<td>26.3 ± 0.3</td>
<td>0.28</td>
</tr>
<tr>
<td>Alamethicin</td>
<td>163 ± 9</td>
<td>8.32 ± 0.15</td>
<td>0.05</td>
</tr>
<tr>
<td>Kinetics by varying UDP-GlcUA&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brij-58</td>
<td>348 ± 22</td>
<td>19.0 ± 0.3</td>
<td>0.05</td>
</tr>
<tr>
<td>Alamethicin</td>
<td>586 ± 49</td>
<td>4.73 ± 0.11</td>
<td>0.01</td>
</tr>
</tbody>
</table>

RLM pretreated with Brij-58 (0.25 mg/mg protein) and alamethicin (0.05 mg/mg protein) were used as the enzyme source. <sup>a</sup>4-MU glucuronide formation was estimated at ten concentrations of 4-MU (10 to 5000 µM). The concentration of UDP-GlcUA was set at 2 mM in this assay. <sup>b</sup>4-MU glucuronide formation was estimated at ten concentrations of UDP-GlcUA (50 to 7500 µM). The concentration of 4-MU was set at 100 µM in this assay. Each value represents the optimized value ± S.E.

<table>
<thead>
<tr>
<th></th>
<th>ATP (pmol)</th>
<th></th>
<th>Total [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pellet</td>
<td>Sup.</td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>36.8 ± 1.0</td>
<td>4.83 ± 0.73</td>
<td>41.6 [100]</td>
</tr>
<tr>
<td>Brij-58</td>
<td>N. D.</td>
<td>13.4 ± 0.1</td>
<td>13.4 [32.2]</td>
</tr>
<tr>
<td>Alamethicin</td>
<td>21.1 ± 0.5</td>
<td>11.5 ± 2.0</td>
<td>32.6 [78.4]</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of three determinations. Value in bracket is the percentage of the sum of the ATP recovered in the pellet and sup. relative to that determined using intact microsomes (=100). Abbreviation: sup., supernatant; N.D., not detected.
Table 5.  IC₅₀ of adenine nucleotides for 4-MU glucuronidation catalyzed by pooled HLM and UGT1A9.

<table>
<thead>
<tr>
<th>compounds</th>
<th>HLM</th>
<th>UGT1A9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (µM)</td>
<td>Ratio (Brij-58=1.0)</td>
</tr>
<tr>
<td>Brij-58 alamethicin</td>
<td>33.8±5.2</td>
<td>27.7</td>
</tr>
<tr>
<td>ATP</td>
<td>&gt;1000</td>
<td>-</td>
</tr>
<tr>
<td>ADP</td>
<td>&gt;1000</td>
<td>-</td>
</tr>
<tr>
<td>AMP</td>
<td>&gt;1000</td>
<td>-</td>
</tr>
</tbody>
</table>

Microsomes (20 µg) were pretreated with Brij-58 (0.25 mg/mg protein) or alamethicin (0.05 mg/mg protein). Values represent the mean ± S.E. of triplicate assays. ‘Ratio’ is the IC₅₀ relative to that determined using Brij-58-treated microsomes (=1.0). For UGT1A9 supersomes, microsomal protein (10 µg) was pretreated with alamethicin (0.05 mg/mg protein). Values represent the mean ± S.E. of triplicate assays.
Table 6.  *IC*<sub>50</sub> of Gefitinib for 4-MU glucuronidation catalyzed by pooled HLM and human UGT1A9 microsomes.

<table>
<thead>
<tr>
<th></th>
<th><em>IC</em>&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLMs (Brij-58-treated)</td>
<td>&gt;250</td>
</tr>
<tr>
<td>HLMs (alamethicin-treated)</td>
<td>&gt;250</td>
</tr>
<tr>
<td>UGT1A9 (alamethicin-treated)</td>
<td>19.4±6.6</td>
</tr>
</tbody>
</table>

Human microsomal protein (20 μg) was pretreated with either Brij-58 (0.25 mg/protein) or alamethicin (0.05 mg/mg protein). UGT1A9 microsomes (10 μg protein) were pretreated with alamethicin (0.05 mg/mg protein). Values represent the mean ±S.E. of triplicate assays.
Figure 1
Figure 2
Figure 3

Brij-58-treated microsomes

4-MUG formed (nmol/min/mg protein)

ATP: ADP: AMP (ratio)
- control (0 µM)
- 1:1:1
- 25:5:1
- 100:10:1

Total concentration of ATP, ADP and AMP (µM)

Alamethicin-treated microsomes

4-MUG formed (nmol/min/mg protein)

ATP: ADP: AMP (ratio)
- control (0 µM)
- 1:1:1
- 25:5:1
- 100:10:1

Total concentration of ATP, ADP and AMP (µM)
Figure 4
Figure 5
Figure 6