

**Preincubation time-dependent, long-lasting inhibition of drug transporters and impact on
the prediction of drug-drug interactions**

Yoshitane Nozaki and Saki Izumi

Global Drug Metabolism and Pharmacokinetics, Tsukuba Research Laboratories, Eisai Co., Ltd.,

5-1-3, Tokodai, Tsukuba, Ibaraki, 300-2635, Japan (Y. N., S. I.)

Running Title Page

Running title: Preincubation effect on transporter inhibition potency

Corresponding author: Yoshitane Nozaki, PhD

Global Drug Metabolism and Pharmacokinetics, Tsukuba Research Laboratories, Eisai Co., Ltd.

5-1-3, Tokodai, Tsukuba, Ibaraki, 300-2635, Japan

Telephone: +81-70-2474-2916

Fax: +81-29-847-5672

E-mail: y2-nozaki@hhc.eisai.co.jp

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Abbreviation:

AUC, area under the plasma concentration-time curve; BSP, sulphobromophthalein; CsA, cyclosporine A; CYP, cytochrome P450; DDI, drug-drug interaction; E₁S, estrone-3-sulfate; E₂G, estradiol-17β-glucuronide; EMA, European Medicines Agency; FDA, U.S. Food and Drug Administration; HBV, hepatitis B virus; HDV, hepatitis D virus; HEK293, human embryonic kidney 293; IC₅₀, half maximal inhibitory concentration; K_i, inhibition constant; K_{p,uu}, intracellular-to-extracellular or tissue-to-plasma unbound drug concentration ratio; LAT, L-type amino acid transporter; MATE, multidrug and toxin extrusion; MDCK, Madin-Darby canine kidney; MHLW, Ministry of Health, Labour and Welfare; NTCP, Na⁺/taurocholate cotransporting polypeptide; OAT, organic anion transporter; OATP/Oatp, organic anion transporting polypeptide; OCT, organic cation transporter; SLC, solute carrier; statin, 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor; TCA, taurocholic acid; TDI, time-dependent inhibition; TLC, tauroolithocholic acid.

Abstract

Transporter-mediated drug-drug interaction (DDI) is of clinical concern, and the quantitative prediction of DDIs is an indispensable part of drug development. Cell-based inhibition assays, in which a representative probe substrate and a potential inhibitor are coincubated, are routinely performed to assess the inhibitory potential of new molecular entities on drug transporters. However, the inhibitory effect of cyclosporine A (CsA) on organic anion transporting polypeptide (OATP) 1B1 is substantially potentiated with CsA preincubation, and this effect is both long-lasting and dependent on the preincubation time. This phenomenon has also been reported with transporters other than OATP1Bs, but it is considered more prevalent among OATP1Bs and organic cation transporters. Regulatory agencies have also noted this preincubation effect and have recommend that pharmaceutical companies consider inhibitor preincubation when performing in vitro OATP1B1 and OATP1B3 inhibition studies. Although the underlying mechanisms responsible for the preincubation effect are not fully understood, a *trans*-inhibition mechanism was recently demonstrated for OATP1B1 inhibition by CsA, in which CsA inhibited OATP1B1 not only extracellularly (*cis*-inhibition), but also intracellularly (*trans*-inhibition). Furthermore, the *trans*-inhibition potency of CsA was much greater than that of *cis*-inhibition, suggesting that *trans*-inhibition might be a key driver of clinical DDIs of CsA with OATP1B substrate drugs. Although confidence in transporter-mediated DDI prediction is

generally considered to be low, the predictability might be further improved by incorporating the *trans*-inhibition mechanism into static and dynamic models for preincubation-dependent inhibitors of OATP1Bs and perhaps other transporters.

Significance Statement

Preincubation time-dependent, long-lasting inhibition has been observed for OATP1B1 and other solute carrier transporters *in vitro*. Recently, a *trans*-inhibition mechanism for the preincubation effect of CsA on OATP1B1 inhibition was identified, with the *trans*-inhibition potency being greater than that of *cis*-inhibition. The concept of *trans*-inhibition may allow us to further understand the mechanism of transporter-mediated DDIs not only for OATP1B1, but also for other transporters and to improve the accuracy and confidence of DDI predictions.

Introduction

In current pharmacotherapy, patients are commonly treated with multiple drugs to achieve therapeutic purposes, but the potential risk of drug-drug interactions (DDIs) demands caution. When the metabolism and/or excretion processes of a drug with a narrow therapeutic index are inhibited by concomitant medications, the increased systemic exposure of the affected drug may cause toxicities, including life-threatening adverse effects in a worst-case scenario. Indeed, severe toxicities caused by DDIs have been reported clinically (Honig et al., 1993; Diasio, 1998; Mullins et al., 1998; Bruno-Joyce et al., 2001); such toxicities can result in the withdrawal of approved drugs from the market, even if therapeutic benefits are available to some patient populations. To avoid such clinical safety issues, pharmaceutical companies characterize the DDI potentials of new molecular entities (NMEs) at various stages of drug development according to regulatory DDI guidance or guidelines (EMA, 2013; MHLW, 2018; FDA, 2020).

Recently, significant research progress has revealed that drug transporters as well as drug metabolizing enzymes, such as cytochrome P450 (CYP), are potential sites of DDIs, and an increasing number of transporter-mediated DDIs have been reported (Gessner et al., 2019). Of the transporters involved in drug pharmacokinetics (PK), P-glycoprotein (P-gp, *ABCB1*), breast cancer resistance protein (BCRP, *ABCG2*), organic anion transporting polypeptide (OATP) 1B1 (*SLCO1B1*), OATP1B3 (*SLCO1B3*), organic anion transporter (OAT) 1 (*SLC22A6*), OAT3

(*SLC22A8*), organic cation transporter (OCT) 1 (*SLC22A1*), OCT2 (*SLC22A2*), multidrug and toxin extrusion (MATE) 1 (*SLC47A1*), and MATE2-K (*SLC47A2*) are commonly acknowledged as being clinically relevant transporters (Giacomini et al., 2010; Yonezawa and Inui, 2011; Zamek-Gliszczyński et al., 2018). These transporters are involved in the active efflux of various drugs in the intestine and blood-brain barrier (P-gp, BCRP), hepatobiliary excretion (basolateral uptake by OATP1B1, OATP1B3, and/or OCT1 followed by active efflux by P-gp, BCRP, MATE1, and/or other efflux transporters via the canalicular membrane), or renal tubular secretion (basolateral uptake by OAT1, OAT3, and/or OCT2 followed by luminal efflux by P-gp, BCRP, MATE1, MATE2-K, and/or other efflux transporters), and the altered function of these transporters by DDIs could impact the clinical PK as well as the safety of the substrate drugs. One well-known example is the interaction of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) with cyclosporine A (CsA) (Neuvonen et al., 2006; Shitara and Sugiyama, 2006). The OATP1B-mediated hepatic uptake of statins is potently inhibited by CsA (Billington et al., 2019), resulting in increased systemic exposure to statins and a higher incidence rate of statin myotoxicity (Ballantyne et al., 2003; Link et al., 2008). Selecting drug candidates that have no or an acceptable risk of DDI before first-in-human clinical studies is the most straightforward approach to minimize such risk. To this end, drug candidates are routinely evaluated for their inhibition potency against drug transporters at nonclinical stages.

In conventional cell-based inhibition assays for uptake transporters, a representative probe substrate and an NME as a potential inhibitor are simultaneously added to transporter-transfected cells, and the uptake of the probe substrate is then examined for a short period of time in the presence of the inhibitor, based on the assumption that the inhibitor can inhibit transporter molecules from outside the cells (*cis*-inhibition) only. However, the examples from multiple inhibitors have demonstrated that employing a preincubation with cells prior to the conventional inhibition study can increase their inhibitory impact on certain transporters. OATP1B1 inhibition by CsA is a typical example, where the inhibitory effect of CsA on OATP1B1 was potentiated depending on the preincubation time, and the reduced OATP1B1 function slowly recovered even after the removal of CsA from the incubation buffer (Shitara and Sugiyama, 2017). This phenomenon has also been noted for other OATP1B inhibitors and other transporters (Tátrai et al., 2019).

The preincubation effect may result in an altered inhibition constant (K_i) or half maximal inhibitory concentration (IC_{50}) values compared to conventional method, and consequently can have an impact on clinical DDI risk assessments. The *in vitro* transporter inhibition study design should be carefully planned to reflect this. The preincubation time-dependency observed during the transporter inhibition is apparently very similar to the time-dependent inhibition (TDI) of drug metabolizing enzymes such as CYPs while a different mechanism (i.e., *trans*-inhibition)

was recently demonstrated for some transporter-inhibitor pairs (Lowjaga et al., 2021; Izumi et al., 2022). Although this research area is still actively evolving, in this minireview we summarized the current knowledge on the preincubation effects observed in transporter inhibition studies and discussed the potential impact on the quantitative prediction of transporter-mediated DDIs with a reference to the proposed *trans*-inhibition mechanism.

Preincubation time-dependent, long-lasting inhibition of OATP1Bs

The preincubation time-dependent, long-lasting inhibition of drug transporters was first reported by Shitara et al., who examined the inhibition of rat Oatp transporters by CsA in vitro and in vivo using sulfobromophthalein (BSP) as a probe substrate (Shitara et al., 2009). In the liver uptake index experiment, the in vivo hepatic uptake of BSP was impaired for 3 days after CsA treatment and returned to the baseline level on day 5. The unbound plasma concentration of CsA could not account for this long-lasting inhibition. They also found that the inhibitory effect of CsA on BSP uptake into primary cultured rat hepatocytes was potentiated after CsA preincubation, with an IC_{50} value of 0.126 $\mu\text{mol/L}$ under the no preincubation condition and decreasing to 0.0583 and 0.0422 $\mu\text{mol/L}$ after 20 and 60 minutes of preincubation with CsA, respectively. Subsequently, Amundsen and coworkers demonstrated that the inhibitory effect of CsA on human OATP1B1 was greatly potentiated by the addition of a CsA preincubation step in

vitro (Amundsen et al., 2010). Preincubating OATP1B1-transfected HEK293 cells with CsA for 60 minutes before the substrate (atorvastatin) and CsA coincubation potentiated the effect on OATP1B1 by a factor of 22, compared with a no-preincubation condition (IC_{50} of 0.021 and 0.47 $\mu\text{mol/L}$, respectively). These findings prompted multiple groups, including us, to investigate the preincubation effect on the OATP1B1 and OATP1B3 inhibition potency.

Currently, the following compounds have exhibited a 3-fold or greater potentiation of OATP1B1 inhibition after preincubation in vitro in at least one examination: venetoclax (fold potentiation, >258), CsA (3.2–61), nilotinib (>42), regorafenib (>24), rifampin (0.5–9.3), everolimus (2.1–8.3), pazopanib (2.7–6.7), asunaprevir (4.9), AM1 (a metabolite of CsA, 4.4), and saquinavir (3.5) (Table 1 and Supplementary Table 1). CsA, AM1, saquinavir, and venetoclax also showed a 3-fold or greater preincubation effect on OATP1B3 inhibition potency (Table 1). Rifampin is a prototypical inhibitor for OATP1Bs in vitro and in vivo, but the preincubation effect was modest, compared with that of CsA. Of note, not all inhibitors exhibited a preincubation effect (Supplementary Table 1).

In addition to the preincubation effect, substrate-dependent inhibition is also a unique characteristic of the OATP1B1 molecule, in which the inhibition potency of an inhibitor on OATP1B1 can vary greatly depending on the probe substrates used in vitro (Noe et al., 2007; Izumi et al., 2013). Using five probe substrates (E_2G [estradiol-17 β -glucuronide], E_1S [estrone-

3-sulfate], BSP, atorvastatin, and pitavastatin), we investigated the preincubation effect of CsA on OATP1B1 (Izumi et al., 2015). A lower K_i value of CsA was given when E₂G was used as a probe substrate; however, the degree of the K_i shift was similar (3.2- to 5.1-fold) regardless of the probe substrates that were examined (Figure 1).

As reported earlier (Shitara et al., 2009), the preincubation effect was detected not only in transfected cells, but also in hepatocyte systems (Supplementary Table 2). Although the degree of potentiation was not examined, 30 minutes of preincubation with CsA inhibited the uptake of E₁S into plated human hepatocytes even after the removal of CsA from the incubation buffer (Shitara et al., 2012). In the sandwich-cultured human hepatocytes, 60 minutes of preincubation with everolimus potentiated the inhibitory effect by 3.6-fold or greater when pitavastatin and cholecystokinin octapeptide (CCK-8) were used as probe substrates for OATP1B1 and OATP1B3, respectively (Farasyn et al., 2021). A preincubation effect was also noted in monkey hepatocytes. By preincubating plated monkey hepatocytes with CsA for 60 minutes before the substrate and inhibitor coincubation, the inhibitory effect on the uptake of pitavastatin was potentiated by 3.7-fold (Ufuk et al., 2018), the degree of which was for unknown reasons weaker than that observed in cynomolgus monkey OATP1B1- and OATP1B3-transfected cells (23- and 7.7-fold, respectively) (Takahashi et al., 2016). Therefore, a preincubation effect has also been seen in in vitro hepatocyte systems, implying an in vivo relevance for nonhuman

primates and humans, in addition to rats (Shitara et al., 2009; Taguchi et al., 2016).

To predict the potential of NMEs to inhibit OATP1B1 and OATP1B3 in vivo, the R-value is calculated according to the static model using the in vitro K_i value as follows:

$$R = 1 + \frac{[I]}{K_i} \quad (1)$$

where [I] represents the inhibitor concentration. The latest regulatory DDI guidance and guidelines recommend the following $I_{u,in,max}$ (the maximum unbound inhibitor concentration at the inlet to the liver) for [I]:

$$I_{u,in,max} = f_p \cdot \left(I_{max} + \frac{F_a \cdot F_g \cdot k_a \cdot Dose}{Q_h \cdot R_B} \right) \quad (2)$$

where f_p is the unbound fraction in plasma, I_{max} is the maximum total plasma concentration, F_a is the fraction absorbed, F_g is the fraction of absorbed dose escaping from the intestinal metabolism, k_a is the absorption rate constant, Dose is the clinical dose of the inhibitor, Q_h is the hepatic blood flow rate (97 L/h), and R_B is the blood-to-plasma concentration ratio (Ito et al., 1998). When the calculated R-value is equal to or greater than the cut-off value (1.1, proposed by FDA and MHLW), the NME is considered a potential in vivo inhibitor of OATP1B1 or OATP1B3 that warrants further evaluation including a dynamic physiologically-based pharmacokinetic (PBPK) model analysis and/or clinical DDI study. However, the confidence in OATP1B-mediated DDI predictions based on the static and dynamic models is generally acknowledged to be relatively low, compared with that of CYP-mediated DDI predictions

(Yoshida et al., 2012; Vaidyanathan et al., 2016; Taskar et al., 2020). In addition to the uncertainties around the $I_{u,in,max}$ estimation, large variabilities have been observed in the reported in vitro K_i and IC_{50} values for OATP1B1 (Figure 2). Complex inhibition mechanisms such as the preincubation effect and substrate-dependency may make accurate determinations of in vitro IC_{50} and K_i values difficult, but the exact reason for the low confidence remains to be elucidated. Although the accurate prediction of OATP1B-mediated DDIs remains challenging, we would be able to reduce the risk of false-negative DDI predictions at least for preincubation effect-positive inhibitors by using lower K_i values obtained after inhibitor preincubation. Regulatory agencies have also acknowledged the preincubation effect (MHLW, 2018; FDA, 2020), and 30 minutes or longer of preincubation with NMEs is recommended for evaluating the in vitro inhibition potencies for OATP1B1 and OATP1B3 in the latest MHLW DDI guideline.

Preincubation effect observed for other transporters

In addition to OATP1B1 and OATP1B3, Tátrai and coauthors systematically examined the IC_{50} values of various compounds for OAT1, OAT3, OCT1, OCT2, MATE1, and MATE2-K in vitro with or without 3 hours of inhibitor preincubation (Tátrai et al., 2019). After giving careful consideration to the nonspecific binding of the inhibitors to labware as well as cell viability, they found that the potentiation of transporter inhibition by preincubation was

prevalent among OCTs and OATP1Bs, but not among OATs or MATEs. One of the most pronounced preincubation effects observed in their study was the inhibition of OCT1 by ledipasvir, with a >255-fold potentiation (Table 2).

CsA exhibited a preincubation effect not only for OATP1Bs, but also for OCT1 (Panfen et al., 2019). When metformin was used as a substrate, the inhibition potency of CsA on OCT1 was increased by 50.2-fold after a 30-minute preincubation with CsA (IC_{50} of 0.43 versus 21.6 $\mu\text{mol/L}$). However, this effect greatly depended on the substrate, with only a 3.2-fold or less potentiation observed for the OCT1-mediated uptake of sumatriptan and cycloguanil (Table 2 and Supplemental Table 1). We may need to select OCT1 probe substrates carefully when evaluating the preincubation effect. For OCT2, crizotinib showed a preincubation effect (Arakawa et al., 2017). With or without crizotinib preincubation, a lower IC_{50} value of crizotinib was obtained when creatinine, rather than 1-methyl-4-phenylpyridinium (MPP^+), was used as a substrate, but the degree of potentiation after crizotinib preincubation was similar between creatinine and MPP^+ (4.6- and 3.5-fold, respectively) (Table 2).

Although information is limited for OATs and MATEs, crizotinib and imatinib showed a preincubation effect on MATE1 with a 3.1- to 3.8-fold and a 7.2-fold potentiation, respectively (Omote et al., 2018) (Table 2). Some anthraquinone derivatives from a traditional Chinese medicine showed a preincubation-dependency for the OAT1 (chrysophanol, physcion) and

OAT3 (emodin, aloe-emodin, chrysophanol, physcion) inhibition potencies (Ma et al., 2015).

OATP2B1 (*SLCO2B1*) is thought to play a role in the intestinal and hepatic uptake of anionic drugs, but its clinical relevance remains debatable (Zamek-Gliszczyński et al., 2022).

Apple juice and (to a lesser extent) orange juice exerted a preincubation effect on OATP2B1 inhibition *in vitro*, which may account for the mechanism of the food-drug interaction between apple/orange juice and fexofenadine that has been observed clinically (Shirasaka et al., 2013).

L-type amino acid transporter 1 (LAT1, *SLC7A5*) is expressed in various types of tumor cells and is thought to contribute to tumor progression by supplying essential amino acids. JPH203 was developed as an LAT1 inhibitor and exhibits a preincubation effect on LAT1 in human colon adenocarcinoma HT-29 cells, yielding a 2.9-fold potentiation after a 2-hour preincubation (Okunushi et al., 2020).

Therefore, preincubation time-dependent, long-term inhibition is not unique to OATP1Bs, but is commonly observed for SLC transporters to varying degrees. When a borderline R-value is obtained for drug candidates from a static model-based DDI prediction (Eq. 1) following a conventional coincubation method, additional testing of the preincubation effect may help to avoid false-negative predictions for non-OATP1B transporters. By using the K_i values obtained after inhibitor preincubation, the outcomes of DDI risk assessments reportedly changed from “no risk” to “risk” for OCT2 inhibition by dolutegravir, irinotecan, isavuconazole, and

vandetanib according to the EMA criteria (Tátrai et al., 2019). For non-OATP1B transporters, however, currently available data on the preincubation effect is limited (Supplemental Table 1). More data sets are needed to determine the need for an inhibitor preincubation step even for non-OATP1B transporters.

***Trans*-inhibition as an underlying mechanism for the preincubation effect of CsA on OATP1B1 inhibition**

Even though this preincubation effect of transporters and metabolic enzyme-mediated TDI apparently share characteristics in common, such as (preincubation) time-dependency and long-lasting inhibition, the underlying mechanisms are considered to be different. Reactive intermediates are unlikely to be involved in the preincubation effect of transporters, due to the nature of lacking sufficient expression of metabolic enzymes with in vitro systems used in the transporter inhibition study (e.g., HEK293 cells). Very recently, a *trans*-inhibition mechanism for the preincubation effect of CsA on OATP1B1 inhibition potency was experimentally demonstrated (Izumi et al., 2022).

The intracellular CsA concentration was suggested to be a key driver of the preincubation effect on OATP1B1 observed in vitro. Shitara and coworkers spiked CsA onto either the apical or basal side of OATP1B1-transfected MDCKII cell monolayers that had been cultured on a

porous membrane filter. After a 1-hour preincubation, CsA was removed from the incubation buffer, and E₁S uptake was examined to evaluate the remaining transport function of OATP1B1. Although extracellular CsA was washed out to avoid *cis*-inhibition, similar degrees of OATP1B1 inhibition for almost the same intracellular CsA concentrations were detected regardless of the CsA-spiked side. No change in the protein expression levels or cellular localization of OATP1B1 was seen in the MDCKII cells after CsA preincubation (Shitara et al., 2012). These *in vitro* findings as well as the theoretical consideration given by the modeling and simulation analysis prompted Shitara and Sugiyama to propose *trans*-inhibition (i.e., CsA inhibits OATP1B1 molecules from the intracellular side) as a possible mechanism for the preincubation effect on OATP1B1 (Shitara and Sugiyama, 2017).

To validate the *trans*-inhibition mechanism experimentally, our group used four assay conditions to capture *cis*-, *trans*-, both *cis*- and *trans*-inhibition (*cis+trans*-inhibition), and long-lasting inhibition in OATP1B1-transfected HEK293 cells (Figure 3); we then tried to account for all the *in vitro* findings quantitatively using a cellular PK model that took both *trans*-inhibition and *cis*-inhibition into account (Figure 4) using CsA and rifampin as inhibitors (Izumi et al., 2022). As for CsA, we found that 1) incubation for 60 minutes or longer was necessary for CsA to reach a steady-state cellular uptake because of its high-affinity, high-capacity intracellular binding, possibly with cyclophilin A (the pharmacological target of CsA)

(Handschumacher et al., 1984); 2) CsA inhibited OATP1B1 competitively from the outside (*cis*-inhibition) and non-competitively from the inside (*trans*-inhibition) of cells; and 3) the inhibition of OATP1B1 by CsA was potentiated by a longer preincubation with CsA under the *trans*- and *cis+trans*-inhibition assay conditions. The preincubation time-dependent, long-lasting inhibition of OATP1B1 by CsA was well reproduced using the cellular kinetic model (Figures 4 and 5), which revealed that the *trans*-inhibition potency of CsA ($K_{i,trans}$, 0.00619 $\mu\text{mol/L}$) was 48-fold stronger than the *cis*-inhibition potency ($K_{i,cis}$, 0.297 $\mu\text{mol/L}$) for OATP1B1. In contrast, rifampin promptly reached a steady-state of uptake into HEK293 cells (within 10 minutes), showing a modest (only 2-fold) preincubation time-dependency for the potentiation of OATP1B1 inhibition. The modest effect of rifampin was also well accounted for by a cellular PK model that considered the OATP1B1-mediated active uptake of rifampin, in which the obtained $K_{i,trans}$ (1.56 $\mu\text{mol/L}$) was similar to the $K_{i,cis}$ (1.16 $\mu\text{mol/L}$). Based on these findings, we concluded that the CsA preincubation effect could be attributed to the potent *trans*-inhibition of OATP1B1 by CsA, the effect of which was potentiated as CsA accumulated intracellularly in a preincubation time-dependent manner.

In a long-lasting inhibition study, the slow and/or partial recovery of OATP1B1 transport function was observed for CsA (Figure 5E) and rifampin (Figure 5F); this recovery was also well simulated by our cellular PK model. A deeper investigation of the simulation results

showed that inhibitor persisting intracellularly after washing diffused into the extracellular buffer, and a new equilibrium was subsequently established between the cell and buffer compartments during incubation with an inhibitor-free buffer. Under the new equilibrium condition, the intracellular inhibitor concentration was lower than that in the first inhibitor preincubation step, but still high enough to exhibit *trans*-inhibition, resulting in the apparently slow and/or partial recovery of transport function. Thus, caution should be exercised when analyzing the recovery kinetics of transporter function *in vitro*.

Tátrai and coworkers also examined time profiles for cellular concentration and degree of transporter inhibition potency for some preincubation-dependent inhibitors of OATP1B1 (venetoclax and CsA) and OCT1 (ledipasvir) (Tátrai et al., 2019). The cellular concentrations and inhibition potencies of the inhibitors followed similar time-courses, suggesting that the intracellular inhibitor concentration is a determinant of the preincubation effects. Further in-depth analysis is warranted, but the *trans*-inhibition mechanism may account for at least some of the preincubation-dependent inhibitors that have been reported to date.

The *trans*-inhibition mechanism was also recently proposed as being responsible for the inhibition of Na⁺/taurocholate cotransporting polypeptide (NTCP, *SLC10A1*) by tauroolithocholic acid (TLC) (Lowjaga et al., 2021). NTCP is highly expressed at the basolateral membrane of human hepatocytes and is involved in the Na⁺-dependent hepatic uptake of bile acids and some

drugs (Dawson et al., 2009; Bi et al., 2019); it is also known as an entry receptor for the hepatitis B and D viruses (HBV and HDV) (Yan et al., 2012). The preincubation of NTCP-transfected HEK293 cells with TLC followed by intensive washing (to avoid *cis*-inhibition) showed the inhibition of not only NTCP transport function, but also the binding of the myristoylated preS1 domain peptide of the large HBV surface protein, without causing any internalization or degradation of NTCP protein. The maximum inhibitory effect of TLC on NTCP transport function was attained within 1 hour of preincubation, and the effect was long-lasting for at least 8 hours after extracellular TLC washout. TLC preincubation inhibited the NTCP-mediated uptake of taurocholic acid (TCA) and dehydroepiandrosterone sulfate only when the preincubation was performed in Na⁺-containing buffer, suggesting that the Na⁺-dependent cellular accumulation of TLC is a prerequisite for this effect. Interestingly, TLC preincubation did not influence the NTCP-mediated uptake of TLC itself. They also confirmed that the intracellular accumulation of TLC and the degree of inhibition of NTCP transport function followed a similar time-course. Furthermore, TLC preincubation suppressed HDV infection in NTCP-transfected HepG2 cells. These data strongly support the idea that TLC is intracellularly accumulated via Na⁺-dependent NTCP-mediated uptake and that the transport and receptor functions of NTCP are inhibited by TLC as a result of a *trans*-inhibition mechanism. The authors also mentioned that this *trans*-inhibition mechanism may help to

protect hepatocytes from the overload of toxic bile acids and could be a novel NTCP target site for potential long-acting HBV/HDV entry inhibitors.

Although the preincubation effect has been scarcely reported for ATP-binding cassette (ABC) transporters, the *trans*-inhibition mechanism was suggested for rat bile salt export pump (Bsep, ABCB11) using in vitro membrane vesicles (Stieger et al., 2000; Akita et al., 2001). Inhibitory effects of estrogen metabolite (E₂G) and sulfate-conjugated bile acids (TLC sulfate and taurochenodeoxycholic acid sulfate) on rat Bsep-mediated transport of TCA into inside-out vesicles were pronounced when rat Bsep and multidrug resistance-associated protein 2 (Mrp2) were co-expressed in the vesicles. Since the estrogen and bile acid metabolites were substrates for Mrp2, it was proposed that they exhibited a *trans*-inhibition of Bsep after accumulating into the vesicles via Mrp2. Thus, it is likely that the *trans*-inhibition mechanism is not specific to SLC transporters but can happen to ABC transporters. In the conventional inhibition studies for ABC transporters such as P-gp and BCRP, cell-based transcellular transport assays rather than vesicle assays have been commonly employed using Caco-2 or P-gp-transfected cell monolayers (LLC-PK1, MDCKII), in which relatively long substrate and inhibitor coincubation time (e.g., 45 – 180 minutes) is adopted (Bentz et al., 2013). During the coincubation period, the inhibitor can be distributed intracellularly and exhibit *trans*-inhibition in addition to *cis*-inhibition, leaving no room for preincubation effects. This may explain why the preincubation

effect has been infrequently reported for ABC transporters.

As shown in Table 1 and Supplementary Table 1, reported IC₅₀ fold change for OATP1B1 after preincubation are highly variable in CsA (3.2–61 fold) and rifampin (0.5–9.3 fold). Although the underlying reasons remain to be clarified, the *trans*-inhibition mechanism, in addition to possible substrate-dependency, may contribute to the inter-study or inter-laboratory variability. As discussed in the ABC transporters, substrate and inhibitor coincubation time period can affect the degree of preincubation effect even for uptake transporters (Tatrai et al. 2019). Depending on the coincubation time, *trans*-inhibition in addition to *cis*-inhibition may happen under the coincubation conditions, apparently attenuating the preincubation effect. Short coincubation time should be selected if we want to see the maximum IC₅₀ shift after preincubation. When a tested inhibitor is a substrate for OATP1B1 (e.g., rifampin), preincubation effect can be variable depending on the protein expression levels of OATP1B1 in the in vitro transfected cell systems used for each laboratory. Intracellular accumulation of such inhibitors is increased by higher OATP1B1 expressions, resulting in more potent *trans*-inhibition based on the buffer concentration. These assay conditions may be potential sources of the data variability.

The *trans*-inhibition mechanism has been demonstrated or suggested mainly for OATP1B1. Further studies are warranted to determine whether the *trans*-inhibition mechanism

is prevalent for other inhibitors and transporters. The cellular kinetic modeling that was used to study OATP1B1 inhibition by CsA and rifampin (Izumi et al., 2022) may be useful for clarifying the involvement of *trans*-inhibition. As for the mode of inhibition, noncompetitive inhibition has been demonstrated for the *trans*-inhibition of OATP1B1 by CsA, suggesting that CsA interacts with an allosteric site of the intracellular domain of OATP1B1 protein. Since *trans*-inhibition potency can be much greater (>10-fold) than that of *cis*-inhibition, as demonstrated using CsA (Figure 4), coincubation of the substrate and inhibitor might no longer be sufficient to evaluate the inhibitory potency, at least for OATP1B1, and an inhibitor preincubation step should be incorporated into inhibition assays to avoid underestimating the DDI risk.

Characteristics of preincubation-dependent inhibitors for OATP1B1

To extract key parameters that influence the OATP1B1 inhibitors preincubation effect, we compared the cellular kinetic parameters of CsA and rifampin that were obtained directly from *in vitro* experiments or estimated from kinetic modeling (Figure 4) (Izumi et al., 2022). Because of the high-affinity, high-capacity intracellular binding of CsA, the intracellular drug unbound fraction (f_T) of CsA (0.000254 under a linear condition) was much lower than that of rifampin (0.0311). Passive diffusion clearance (PS_{dif} in $\mu\text{L}/\text{min}/\text{mg}$ protein) was similar between

CsA (51.3) and rifampin (52.5). The cellular distribution was driven by passive diffusion for CsA, whereas rifampin was subjected to OATP1B1-mediated active uptake in addition to passive diffusion, resulting in an intracellular-to-buffer (extracellular) unbound concentration ratio ($K_{p,uu,in\ vitro}$) of 2.25 in OATP1B1-transfected HEK293 cells. As an index for the time to reach the half maximum intracellular concentration of inhibitors after the preincubation, we employed the following $T_{1/2,max}$:

$$T_{1/2,max} = \frac{0.693 \cdot V_{cell}}{PS_{dif} \cdot f_T} \quad (3)$$

where V_{cell} is the cellular volume (2 μ L/mg protein). According to Eq. 3, a longer preincubation time would be required to reach a steady-state cellular concentration for inhibitors that show low membrane permeability (PS_{dif}) and/or a low intracellular unbound fraction (f_T). Indeed, the calculated $T_{1/2,max}$ of CsA (106 minutes) was much longer than that of rifampin (0.849 minutes) because of the very low f_T value for CsA.

In an attempt to identify physicochemical descriptors that determine the preincubation effect, Tátrai and coworkers explored the correlation of physicochemical parameters with the degree of potentiation of transporter inhibition after preincubation using 30 compounds including preincubation effect-positive and -negative inhibitors for OATP1Bs and other SLC transporters (Tátrai et al., 2019). Of the seven physicochemical parameters that were examined, molecular weight, $\text{LogD}_{7.4}$, cellular K_p predicted from $\text{LogD}_{7.4}$ ($K_{p,pred}$), and the ratio of $K_{p,pred}$ to

apparent permeability via MDCK cell monolayers with a low efflux activity (MDCK-LE P_{app}) showed a significant correlation with the degree of potentiation while topological polar surface area, cLogP, and MDCK-LE P_{app} alone showed a weak or poor correlation. Since $K_{p,pred}/\text{MDCK-LE } P_{app}$ is a parameter corresponding to $1/(\text{PS}_{diff})$ in Eq. 3, the significant correlation that was reported seems reasonable.

In addition to the slow buildup of the cellular concentration, the intrinsic potency of *trans*-inhibition is essential for inhibitors to display a preincubation effect. Since the *cis*- and *trans*-inhibition of OATP1B1 by CsA follows competitive and noncompetitive inhibition, respectively (Izumi et al., 2022), OATP1B1-mediated uptake clearance in the presence of CsA ($CL_{OATP1B1+I}$) under the *cis+trans*-inhibition assay condition (Figure 3) can be described as follows:

$$CL_{OATP1B1+I} = \frac{V_{max} / (1 + f_T I_{cell} / K_{i,trans})}{K_m (1 + I_{buffer} / K_{i,cis}) + S} \quad (4)$$

where S , V_{max} , and K_m represent the substrate concentration, the maximum uptake velocity, and Michaelis constant, respectively. I_{cell} and I_{buffer} are the intracellular and buffer concentrations of CsA, respectively. Under the linear condition ($S \ll K_m$), the remaining fraction of OATP1B1 activity in the presence of CsA ($CL_{OATP1B1+I}/CL_{OATP1B1}$) can be described by the following equation:

$$\frac{CL_{OATP1B1+I}}{CL_{OATP1B1}} = \frac{1}{\left(1 + \frac{I_{buffer}}{K_{i,cis}}\right) \cdot \left(1 + \frac{f_T \cdot I_{cell}}{K_{i,trans}}\right)} \quad (5)$$

For an inhibitor that shows noncompetitive inhibition for both *cis*- and *trans*-inhibition,

$CL_{OATP1B1+I}$ can be described as follows:

$$CL_{OATP1B1+I} = \frac{V_{max}}{K_m + S} \cdot \frac{1}{\left\{ \left(1 + \frac{f_T \cdot I_{cell}}{K_{i,trans}}\right) \cdot \left(1 + \frac{I_{buffer}}{K_{i,cis}}\right) \right\}} \quad (6)$$

Under the linear condition ($S \ll K_m$), Eq. 6 can also provide Eq. 5. Thus, Eq. 5 holds true for noncompetitive inhibitors for both *cis*- and *trans*-inhibition. At a steady state after a sufficient preincubation time, the intracellular unbound concentration of the inhibitor ($f_T \cdot I_{cell}$) can be replaced by $K_{p,uu,in\ vitro} \cdot I_{buffer}$. By using the $K_{i,cis}$ -to- $K_{i,trans}$ ratio (α), the following equation can be drawn from Eq. 5:

$$\frac{CL_{OATP1B1+I}}{CL_{OATP1B1}} = \frac{1}{\left(1 + \frac{I_{buffer}}{K_{i,cis}}\right) \cdot \left(1 + \frac{\alpha \cdot K_{p,uu,in\ vitro} \cdot I_{buffer}}{K_{i,cis}}\right)} \quad (7)$$

where $(1 + I_{buffer}/K_{i,cis})$ and $(1 + \alpha \cdot K_{p,uu,in\ vitro} \cdot I_{buffer}/K_{i,cis})$ represent the *cis*- and *trans*-inhibition potencies, respectively, in OATP1B1-transfected cells. Therefore, $\alpha \cdot K_{p,uu,in\ vitro}$ is a determinant of the degree of potentiation after inhibitor preincubation. The $K_{p,uu,in\ vitro}$ of CsA can be assumed to be unity because of the passive diffusion-driven cellular distribution. Meanwhile, the *trans*-inhibition potency of CsA was much stronger than that of *cis*-inhibition (α of 48.0), resulting in an $\alpha \cdot K_{p,uu,in\ vitro}$ of 48. Because of OATP1B1-mediated active uptake, the $K_{p,uu,in\ vitro}$ of rifampin was 2.25, while symmetrical *cis*- and *trans*-inhibition potency yielded an α of 0.744, resulting in a modest preincubation effect with $\alpha \cdot K_{p,uu,in\ vitro}$ value of 1.7 (Figure 4). For NTCP inhibition

by TLC (Lowjaga et al., 2021), a higher $K_{p,uu,in\ vitro}$ of TLC driven by Na^+ -dependent NTCP uptake may contribute to the potent *trans*-inhibition.

Therefore, for preincubation-dependent inhibitors that have a *trans*-inhibition potency on OATP1B1, the time to reach a steady-state cellular concentration ($T_{1/2,max}$), the *trans*-inhibition potency relative to *cis*-inhibition (α), and the degree of the cellular accumulation of unbound inhibitor ($K_{p,uu,in\ vitro}$) are key factors of the time-dependency. Since these parameters are compound-specific, *in vitro* assay conditions such as the preincubation time period for reaching steady-state intracellular concentration may need to be optimized for each compound. When NMEs are tested for OATP1B1 substrate liability using the same cell systems, the time-course data on the cellular uptake may help to optimize the preincubation time for inhibition studies. According to Eq. 3, a low membrane permeability and/or strong intracellular binding contributes to a longer $T_{1/2,max}$. In addition to high-affinity, high-capacity binding to pharmacological target proteins (e.g., CsA), strong intracellular nonspecific binding of lipophilic compounds and lysosomal trapping of basic compounds could also result in long $T_{1/2,max}$ values in transfected cells. Further studies examining the cell retention mechanisms of preincubation time-dependent inhibitors are needed and may help us to understand why preincubation-dependent inhibition is most prevalent among OATP1B and OCT family transporters.

Clinical relevance of *trans*-inhibition mechanism to OATP1B1-mediated DDIs

To clarify the clinical relevance of the preincubation-time dependent, long-lasting inhibition of OATP1Bs, Mochizuki and coworkers performed clinical DDI studies, in which healthy volunteers orally received an OATP1B probe cocktail (pitavastatin, rosuvastatin, valsartan) 1 or 3 hours after an oral dose of CsA at 75 mg (Mochizuki et al., 2022b). Unlike in rats (Shitara et al., 2009), the inhibitory effect of CsA on OATP1Bs did not persist in humans; 1- and 3-hour dosing intervals yielded an area under the plasma concentration-time curve ratio (AUCR) of 3.46 and 2.38 for pitavastatin, and 2.16 and 1.81 for rosuvastatin, respectively. Although the exact reason for the inconsistency of long-lasting inhibition observations between human and rat results remains unknown, the different CsA blood exposure levels achieved in vivo (rats > human) and/or species differences in the metabolic clearance of CsA as well as the production of metabolite(s) that could inhibit hepatic Oatps/OATPs are potential factors. However, this clinical finding does not necessarily rule out the possibility of *trans*-inhibition mechanism in humans in vivo. Reportedly, the in vitro K_i values of CsA for OATP1B1 determined under the coincubation condition were much greater than the in vivo K_i values estimated using PBPK modeling of clinical DDIs with statins (pravastatin, pitavastatin, fluvastatin), but by applying the inhibitor preincubation method, the obtained in vitro K_i values

approached in vivo values (Varma et al., 2012; Yoshikado et al., 2016) (Figure 2). This analysis strongly suggested that the *trans*-inhibition of OATP1Bs by CsA, rather than *cis*-inhibition, is a major driver of the clinical DDIs with statins.

In an attempt to evaluate the impact of the *trans*-inhibition mechanism on OATP1B-mediated DDI prediction based on the static model, we have introduced the following equation from Eq. 7 to calculate the R-values of CsA and rifampin in a more mechanistic manner:

$$R = \left(1 + \frac{I}{K_{i,cis}}\right) \cdot \left(1 + \frac{K_{p,uu,in\ vivo} \cdot I}{K_{i,trans}}\right) \quad (8)$$

where $K_{p,uu,in\ vivo}$ represents the in vivo liver-to-plasma unbound concentration ratio in humans. The $K_{p,uu,in\ vivo}$ of CsA was assumed to be unity. For rifampin, the $K_{p,uu,in\ vivo}$ of 3.3 was reported previously (Chu et al., 2015). Although quantitative prediction of $K_{p,uu,in\ vivo}$ values is still a big challenge for the compounds that are subjected to active transport, several in vitro approaches using hepatocyte suspension have been proposed, including the initial uptake method (Yabe et al., 2011), the homogenization method (Riccardi et al., 2019), and the temperature method (Shitara et al., 2013; Izumi et al., 2017). The $K_{i,cis}$ value can be directly obtained from the coincubation (*cis*-inhibition) experiment in vitro. The apparent K_i value determined under the *cis+trans*-inhibition condition ($K_{i,app,cis+trans}$) corresponds to the inhibitor concentration in buffer (I_{buffer}) that yielded 50% inhibition of OATP1B1 in Eq. 7; thus, $K_{i,trans}$ can be estimated from the following equation:

$$K_{i,trans} = \frac{K_{i,cis} + K_{i,app,cis+trans}}{K_{i,cis} - K_{i,app,cis+trans}} \cdot K_{p,uu,in\ vitro} \cdot K_{i,app,cis+trans} \quad (9)$$

By using this equation, we can experimentally estimate $K_{i,trans}$ values, without cellular kinetic modeling. When there is no evidence of OATP1B1-mediated accumulation of NMEs into transfected cells, the $K_{p,uu,in\ vitro}$ can be assumed to be unity.

To see the impact of the *trans*-inhibition mechanism on OATP1B1-mediated DDI prediction, the R-values of CsA and rifampin were calculated using static models and compared with the clinical AUCR values of OATP1B substrate drugs (Figure 6 and Supplemental Table 3). In this preliminary analysis, three different methods were used for the R-value calculations: Method 1, $R = (1 + I_{u,in,max}/K_{i,cis})$; Method 2, $R = 1 + (I_{u,in,max}/K_{i,app,cis+trans})$; and Method 3, $R = (1 + I_{u,in,max}/K_{i,cis}) \cdot (1 + K_{p,uu,in\ vivo} \cdot I_{u,in,max}/K_{i,trans})$. Method 2 is recommended for OATP1B1 and OATP1B3 in regulatory guidance and guidelines (MHLW, 2018; FDA, 2020). For applying Method 3, $K_{i,trans}$ values need to be estimated following Eq. 9, but $K_{p,uu,in\ vitro}$ values of CsA and rifampin were not determined in previous studies. Thus, the $K_{p,uu,in\ vitro}$ values of 1 for CsA and 2.2 for rifampin, which were estimated in our study (Figure 4), were used for calculating the $K_{i,trans}$ values in this preliminary analysis (Supplemental Table 3).

For CsA, the consideration of *cis*-inhibition only (Method 1) substantially underestimated the AUCR, in which 6 out of 11 cases were false negatives; however, the prediction ability was greatly improved using Methods 2 or 3 with minimal false-negative

predictions (only 1 false negative out of 11 cases). Since Methods 2 and 3 gave similar R-values for CsA, incorporating $K_{i,app,cis+trans}$ values into Eq. 1 (Method 2) seems to be a practical approach for assessing DDI risk (Figure 6A). Rifampin showed a modest preincubation effect in vitro, and it was judged to be a potential in vivo inhibitor of OATP1B1 (R-value ≥ 1.1) regardless of the prediction methods. The highest R-values were obtained using Method 3, which took the *trans*-inhibition mechanism as well as the active intracellular accumulation of rifampin ($K_{p,uu,in vivo}$) into consideration (Figure 6B). For inhibitors that are expected to be actively accumulated in human hepatocytes in vivo ($K_{p,uu,in vivo} > 1$), Method 3 may be a better approach than Method 2 for avoiding false-negative predictions.

This preliminary analysis suggested that for inhibitors that display a considerable preincubation effect on OATP1B1 in vitro (e.g., CsA), assessing *cis*-inhibition only might be insufficient for DDI prediction. The incorporation of $K_{i,app,cis+trans}$ values obtained by inhibitor preincubation followed by substrate and inhibitor cocubation into Eq. 1 is considered a reasonable approach for DDI risk assessment. A more mechanistic model (Method 3) was not necessary, at least for CsA or rifampin, from the perspective of avoiding false-negative predictions. However, this preliminary analysis was made based on a very limited data set. A comprehensive analysis using a more diverse compound set is needed to determine whether Method 2 consistently yields a good performance for OATP1B-mediated DDI predictions for

preincubation-dependent inhibitors and whether the current regulatory cut-off value (1.1) is appropriate for this approach. In addition to the static model, a dynamic PBPK model analysis is also an important element in determining the need for clinical DDI studies, but the *trans*-inhibition mechanism is typically not considered. PBPK models that incorporate *trans*-inhibition as well as *cis*-inhibition mechanisms should be developed, as this may further improve the predictability of OATP1B-mediated DDIs with preincubation-dependent inhibitors such as CsA. Most recently, it was suggested that in vivo K_i values of CsA and rifampin given by PBPK model analysis of clinical DDI data could be lower than in vitro K_i values experimentally obtained after preincubation (Mochizuki et al., 2022a; Yoshikado et al., 2022). There are still uncertainties in the in vitro-in vivo translatability of K_i values for OATP1Bs. Until the underlying mechanisms or reasons are fully clarified, endogenous biomarker-informed PBPK modeling approach would be one of the practical and effective approaches to quantitatively predict OATP1B-mediated clinical DDIs (Yoshikado et al., 2018; Kimoto et al., 2022).

Conclusions

Since the preincubation time-dependent, long-lasting inhibition of Oatp/OATP by CsA was first reported, this phenomenon has been observed for other OATP inhibitors as well as

other SLC transporters. The inhibitory effect on drug transporters can be substantially potentiated by adding an inhibitor preincubation step to the conventional cocubation method, potentially impacting the outcome of the DDI prediction. Regulatory agencies have also noted this effect, and the inhibitor preincubation method is mentioned or recommended for OATP1B1 and OATP1B3 inhibition studies in the latest DDI guidance and guidelines. Although the underlying mechanism for the preincubation effect has not been fully elucidated, the *trans*-inhibition mechanism was recently confirmed to account for OATP1B1 inhibition by CsA, and the same mechanism has been implicated for other inhibitors and transporters (e.g., NTCP inhibition by TLC). By incorporating the *trans*-inhibition mechanism into static and dynamic models, the accuracy and confidence of DDI prediction may be further improved for drug transporters like OATP1Bs and beyond, contributing to the delivery of safer drugs to patients.

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Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

Authorship Contribution

Wrote or contributed to the writing of the manuscript: Nozaki, Izumi

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Footnote

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Legends for Figures

Figure 1 Preincubation effect of CsA on OATP1B1 inhibition potency in vitro. CsA and a probe substrate, (A) [^3H]E₂G (0.1 $\mu\text{mol/L}$), (B) [^3H]E₁S (0.01 $\mu\text{mol/L}$), (C) [^3H]BSP (0.01 $\mu\text{mol/L}$), (D) pitavastatin (0.1 $\mu\text{mol/L}$), or (E) atorvastatin (0.1 $\mu\text{mol/L}$), were coincubated with OATP1B1-transfected HEK293 cells after 1-hour preincubation with (open squares) or without (open triangles) CsA. As a reference, cells were coincubated with CsA and a probe substrate without the preincubation step (closed circles). Thick, thin, and dashed lines represent fitted lines for coincubation of CsA and a probe substrate without preincubation, coincubation after 1-hour preincubation without CsA, and coincubation after 1-hour preincubation with CsA, respectively. Each point represents the mean \pm SD (n = 3). Reproduced from Izumi S, Nozaki Y, Maeda K, Komori T, Takenaka O, Kusuhara H, and Sugiyama Y (2015) Investigation of the impact of substrate selection on in vitro organic anion transporting polypeptide 1B1 inhibition profiles for the prediction of drug-drug interactions. *Drug Metab Dispos* **43**:235-247.

Figure 2 Reported in vitro and in vivo IC₅₀ or K_i values of CsA on OATP1B1. The IC₅₀ or K_i values of CsA on OATP1B1 determined in vitro and in vivo were collected from the literature and plotted. In vitro inhibitory effect of CsA on OATP1B1 was determined by substrate and inhibitor coincubation without (*cis*-inhibition) and with (*cis+trans*-inhibition) CsA

preincubation (for 30 minutes or longer) using atorvastatin, BSP, E₂G, E₁S, pemafibrate, pitavastatin, rosuvastatin, fluvastatin, or pravastatin as a probe substrate (Amundsen et al., 2010; Gertz et al., 2013; Izumi et al., 2015; Taguchi et al., 2019; Tátrai et al., 2019; Taguchi et al., 2020; Park et al., 2021). The in vivo K_i values of CsA, which were able to reproduce clinical DDIs with statins, were previously estimated using PBPK modeling (Varma et al., 2012; Yoshikado et al., 2016).

Figure 3 Inhibition assay conditions to demonstrate *cis*- and *trans*-inhibition mechanisms for OATP1B1. In the *cis*-inhibition assay, a substrate and an inhibitor were coincubated without inhibitor preincubation. Since the cellular distribution of the inhibitor is minimal, OATP1B1 can be inhibited only from the outside of cells (*cis*-inhibition). In the *trans*-inhibition assay, cells were preincubated with an inhibitor. After washing cells, the uptake of the probe substrate was examined in the absence of the inhibitor. The inhibitor can inhibit OATP1B1 only from the inside of cells (*trans*-inhibition). In the *cis*+*trans*-inhibition assay, the assay flow was consistent with that of *trans*-inhibition, except for coincubation of the probe substrate and inhibitor after the inhibitor preincubation step. The inhibitor can inhibit OATP1B1 from both outside (*cis*-inhibition) and inside (*trans*-inhibition) of cells. In the long-lasting inhibition assay, cells were preincubated with an inhibitor for designated time period, followed by washout and incubation

with fresh buffer at 37°C. At the designated times, cells were washed again and the uptake of a probe substrate was examined in the absence of the inhibitor to monitor the recovery of OATP1B1 activity. Reproduced from Izumi S, Nozaki Y, Lee W, and Sugiyama Y (2022) Experimental and Modeling Evidence Supporting the Trans-Inhibition Mechanism for Preincubation Time-Dependent, Long-Lasting Inhibition of Organic Anion Transporting Polypeptide 1B1 by Cyclosporine A. *Drug Metab Dispos* 50:541-551.

Figure 4 Diagram depicting *cis*- and *trans*-inhibition of OATP1B1 in vitro and cellular PK parameters of CsA and rifampin. In this model, an inhibitor in buffer (I_{buffer}) enters cells by passive diffusion (PS_{dif}) with or without OATP1B1-mediated active uptake (PS_{act}), and inhibits OATP1B1 from both outside (*cis*-inhibition) and inside (*trans*-inhibition) of cells. $K_{i,\text{cis}}$ represents the inhibition constant for the *cis*-inhibition of OATP1B1 based on the I_{buffer} . $K_{i,\text{trans}}$ represents the inhibition constants for the *trans*-inhibition of OATP1B1 based on the intracellular unbound concentration ($I_{\text{cell,u}}$). Intracellular unbound fraction (f_T) is set constant (rifampin) or nonlinear (CsA). For CsA showing nonlinear f_T , the intracellular bound concentration ($I_{\text{cell,b}}$) is saturable with K_d (dissociation constant), B_{max} (maximum number of binding site for intracellular binding), and A (non-saturable component). Parameters were obtained directly from in vitro experiments or estimated by data fitting (e.g., $K_{i,\text{cis}}$ was

experimentally determined by the substrate and inhibitor coincubation, and $K_{i,trans}$ was estimated by the cellular kinetic model analysis, which allowed the calculation of α [$K_{i,cis}$ -to- $K_{i,trans}$ ratio]). Reproduced with some modifications from Izumi S, Nozaki Y, Lee W, and Sugiyama Y (2022) Experimental and Modeling Evidence Supporting the Trans-Inhibition Mechanism for Preincubation Time-Dependent, Long-Lasting Inhibition of Organic Anion Transporting Polypeptide 1B1 by Cyclosporine A. *Drug Metab Dispos* **50**:541-551.

Figure 5 Preincubation time-dependent, long-lasting inhibition of OATP1B1 by CsA and rifampin. The inhibitory effects of CsA (A) and rifampin (B) on OATP1B1-mediated uptake of [3 H]E₂G were examined in the *cis*-, *trans*-, and *cis+trans*-inhibition conditions using OATP1B1-transfected and control HEK293 cells. Detailed assay flow is given in Figure 3. In the *trans*- and *cis+trans*-inhibition conditions, CsA and rifampin were preincubated for 10 and 60 (rifampin) or 120 (CsA) minutes. Each symbol represents mean \pm SEM (n=3), and solid (*cis*-inhibition), dashed (*trans*-inhibition), and dash-dotted lines (*cis+trans*-inhibition) are fitted lines. *Trans*-inhibition of OATP1B1 by CsA (C) and rifampin (D). After preincubation with CsA (for up to 120 minutes) or rifampin (for up to 60 minutes), OATP1B1-mediated uptake of [3 H]E₂G was determined in the buffer lacking inhibitors. Observed % of control values (relative to OATP1B1-mediated uptake of [3 H]E₂G without inhibitor preincubation) were presented as

closed circles (mean \pm SEM, n=3). Solid lines are fitted lines. Long-lasting inhibition of OATP1B1 by CsA (E) and rifampin (F). After preincubation of CsA (for 120 minutes) or rifampin (for 60 minutes) with OATP1B1-transfected and control HEK293 cells, cells were washed and incubated with an inhibitor-free buffer at 37°C. At designated times, buffer was removed, followed by cell washing. Then, OATP1B1-mediated uptake of [³H]E₂G was examined in the buffer lacking inhibitors to see the recovery of OATP1B1 transport function. Each symbol represents observed % of control values (mean \pm SEM, n=3). Solid, dashed, and dash-dotted lines are simulated values given by constructed cellular PK model. Reproduced with some modifications from Izumi S, Nozaki Y, Lee W, and Sugiyama Y (2022) Experimental and Modeling Evidence Supporting the Trans-Inhibition Mechanism for Preincubation Time-Dependent, Long-Lasting Inhibition of Organic Anion Transporting Polypeptide 1B1 by Cyclosporine A. *Drug Metab Dispos* **50**:541-551.

Figure 6 Static model-based prediction of DDIs mediated by OATP1B1 inhibition by CsA and rifampin. The R-values of CsA (A) and rifampin (B) for OATP1B1 were calculated according to Method 1 ($R = 1 + I_{u,in,max}/K_{i,cis}$, closed circles), Method 2 ($R = 1 + I_{u,in,max}/K_{i,app,cis+trans}$, open squares), and Method 3 ($R = (1 + I_{u,in,max}/K_{i,cis}) \cdot (1 + K_{p,uu,in vivo} \cdot I_{u,in,max}/K_{i,trans})$, open triangles) and were compared with clinically observed AUCR values of OATP1B substrate drugs (atorvastatin,

pemafibrate, pitavastatin, rosuvastatin, and valsartan for CsA; pemafibrate, pitavastatin, and rosuvastatin for rifampin). Vertical and horizontal dashed lines represent AUCR of 1.25 and the cut-off for R-values (1.1). R-value calculations and data source for AUCR values are given in Supplemental Table 3.

Table 1 Preincubation-dependent inhibitors for OATP1B1 and OATP1B3

IC₅₀ or K_i values determined by substrate and inhibitor coinubation (*cis*-inhibition condition) were compared with those obtained by adding an inhibitor preincubation step (*cis+trans*-inhibition condition). Inhibitors that showed 3-fold or greater potentiation after inhibitor preincubation in at least one of the examinations are considered as positive preincubation-dependent inhibitors and listed in this table. All data were obtained from transfected cell systems with HEK293 or HEK293T as host cells. Individual data are given in Supplementary Table 1.

Transporters	Inhibitors ^a	Probe substrate	Preincubation time (<i>min</i>)	Mean IC ₅₀ or K _i ^b		Mean fold potentiation ^b (<i>fold</i>)
				w/o preincubation (<i>μmol/L</i>)	w/ preincubation (<i>μmol/L</i>)	
OATP1B1	CsA	E ₂ G	30 – 180	0.316 (0.0458 – 0.667)	0.0412 (0.0109 – 0.0983)	10 (3.3 – 27)
		E ₁ S	30 – 60	0.229 (0.134 – 0.383)	0.0437 (0.0264 – 0.0692)	5.1 (4.8 – 5.5)
		BSP	60	0.252	0.0799	3.2
		Atorvastatin	30 – 60	0.311 (0.112 – 0.47)	0.0263 (0.021 – 0.0319)	13 (4.3 – 22)
		Pitavastatin	30 – 60	0.401 (0.074 – 1.31)	0.0278 (0.013 – 0.048)	10 (3.9 – 27)
		Pemafibrate	30	2.5	0.041	61
		Rosuvastatin	30	0.397 (0.193, 0.60)	0.0631 (0.0312, 0.095)	6.3 (6.2, 6.3)
		Valsartan	60	0.064	0.008	8.0
		CP-I	60	0.163	0.019	8.6
		GCDCA-S	60	0.057	0.009	6.3
	GCDCA-G	60	0.048	0.011	4.4	
	AMI	E ₂ G	30	0.41	0.09	4.4
	Rifampin	E ₂ G	60 – 180	1.03 (0.74 – 1.20)	0.521 (0.22 – 0.818)	2.3 (1.5 – 3.4)

		Pemafibrate	30	3.62	0.39	9.3
		Pitavastatin	30	4.32	1.25	3.5
		Rosuvastatin	30	3.96	1.05	3.8
	Asunaprevir	E ₁ S	30	0.17	0.0349	4.9
	Everolimus	E ₁ S	60	0.61	0.16	3.8
		Rosuvastatin	60	1.58	0.19	8.3
	Nilotinib	E ₁ S	60	>10	0.235	>42
	Pazopanib	E ₂ G	60	13.5	2.03	6.7
	Regorafenib	E ₁ S	60	>10	0.403	>24
	Saquinavir	E ₂ G	180	3.65	1.03	3.5
	Venetoclax	E ₂ G	180	>171	0.663	>258
OATP1B3	CsA	E ₂ G	30 – 180	0.269 (0.14 – 0.505)	0.0797 (0.032 – 0.167)	3.9 (3.0 – 5.1)
		Valsartan	60	0.071	0.022	3.2
		CP-I	60	0.059	0.020	3.0
		GCDCA-S	60	0.094	0.031	3.0
	AM1	E ₂ G	30	0.191	0.059	3.2
	Saquinavir	E ₂ G	180	11.0	2.91	3.8
	Venetoclax	E ₂ G	180	>300	34.5	>8.7

BSP = sulphobromophthalein, CP-I = coproporphyrin I, CsA = cyclosporine A, E₁S = estrone-3-sulfate, E₂G = estradiol-17 β -glucuronide,

GCDCA-S = glycochenodeoxycholic acid sulfate, GCDCA-G = glycochenodeoxycholic acid glucuronide.

a: Simeprevir showed the preincubation effect on OATP1B1 and OATP1B3 inhibition, but the fold potentiation was not determined (Furihata et al., 2014).

b: When multiple data source is available, arithmetic mean was calculated for the substrate-inhibitor pair. Range of reported values (or individual values when n=2) are given in the parenthesis.

Table 2 Preincubation-dependent inhibitors for transporters other than OATP1Bs

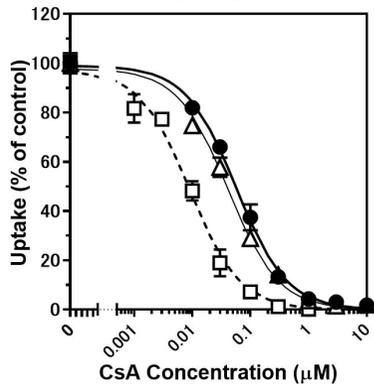
IC₅₀ or K_i values determined by substrate and inhibitor coincubation (*cis*-inhibition condition) were compared with those obtained by adding an inhibitor preincubation step (*cis+trans*-inhibition condition). Inhibitors that showed 3-fold or greater potentiation after inhibitor preincubation are considered as positive preincubation-dependent inhibitors and are summarized in this table. The data were obtained from transfected cell systems with HEK293 or MDCKII (for MATE2-K only) as host cells. Individual data are given in Supplementary Table 1.

Transporters	Inhibitors	Probe substrate	Preincubation time (<i>min</i>)	Mean IC ₅₀ or K _i		Mean fold potentiation (<i>fold</i>)
				w/o preincubation (<i>μmol/L</i>)	w/ preincubation (<i>μmol/L</i>)	
OCT1	CsA	Metformin	30	21.6	0.43	50.2
		Fenoterol	30	>20	2.4	>8.3
		Ranitidine	30	>20	4.6	>4.3
		Sumatriptan	30	2.5	0.77	3.2
	Daclatasvir	Metformin	180	0.533	0.0943	5.7
	Irinotecan	Metformin	180	0.121	0.0233	5.2
	Isavuconazole	Metformin	180	5.21	1.75	3.0
	Ledipasvir	Metformin	180	>50	0.196	>255
	Saquinavir	Metformin	180	18.2	4.45	4.1
	Verapamil	Metformin	180	0.459	0.150	3.1
OCT2	Crizotinib	Creatinine	15	1.58	0.347	4.6
		MPP ⁺	15	16.2	4.58	3.5
	Daclatasvir	Metformin	180	67.8	1.98	34

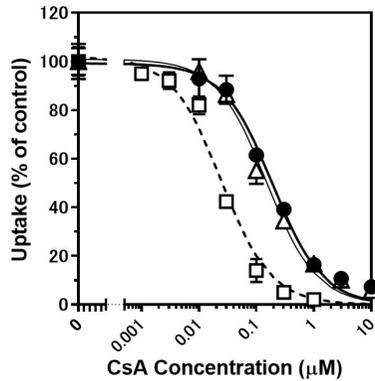
	Dolutegravir	Metformin	180	23.0	3.71	6.2
	Isavuconazole	Metformin	180	16.8	1.27	13.2
	Ledipasvir	Metformin	180	>200	22.9	>8.7
	Vandetanib	Metformin	180	19.8	4.72	4.2
MATE1	Crizotinib	Creatinine	60	2.16	0.573	3.8
		MPP ⁺	60	2.66	0.868	3.1
	Imatinib	Creatinine	60	0.466	0.0651	7.2
MATE2-K	Vandetanib	Metformin	180	0.880	0.293	3.0

CsA = cyclosporine A, MPP⁺ = 1-methyl-4-phenylpyridinium.

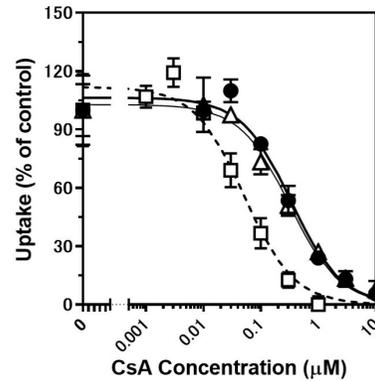
A. [³H]E₂G



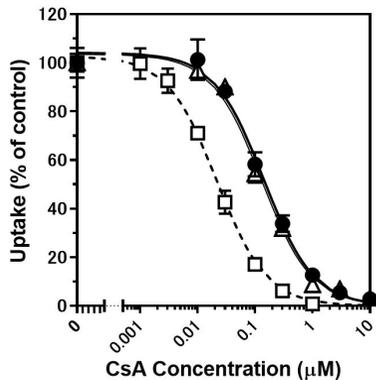
B. [³H]E₁S



C. [³H]BSP



D. Pitavastatin



E. Atorvastatin

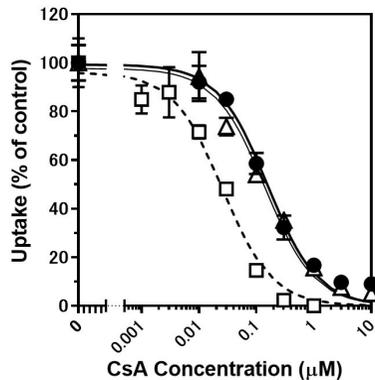


Figure 1

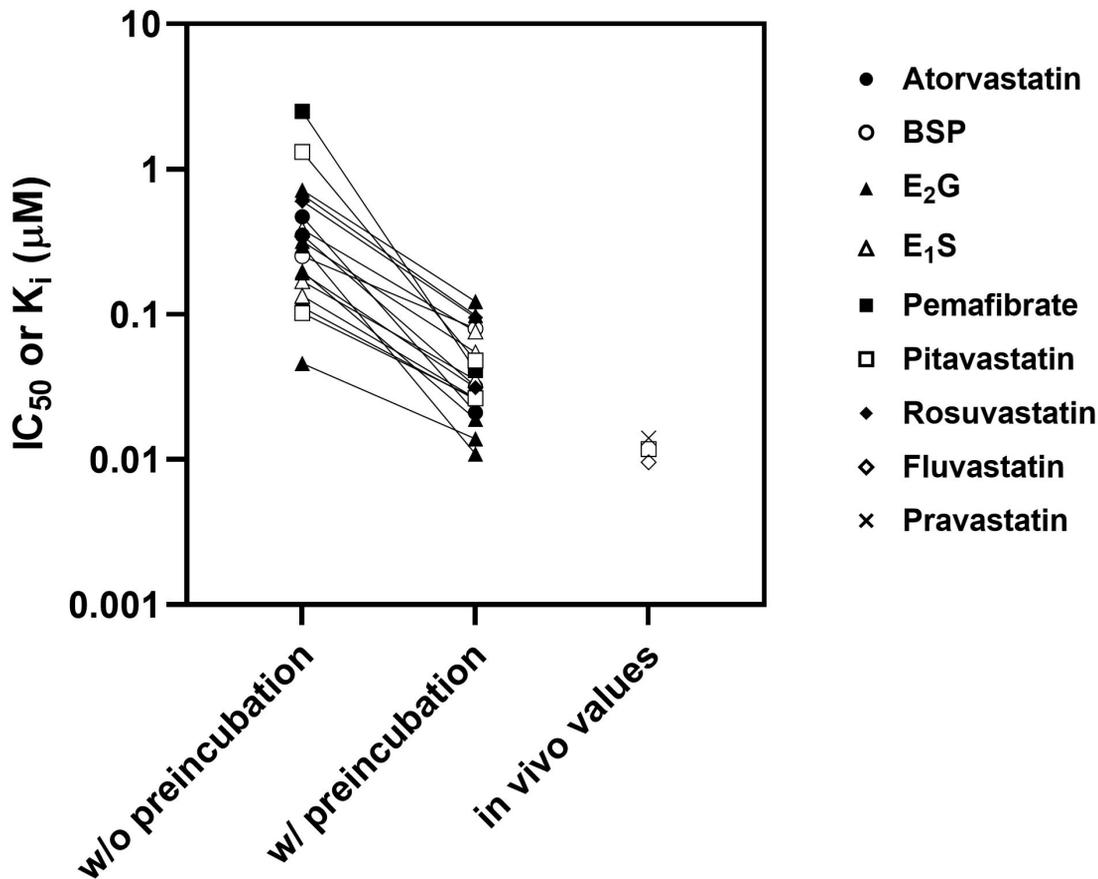


Figure 2

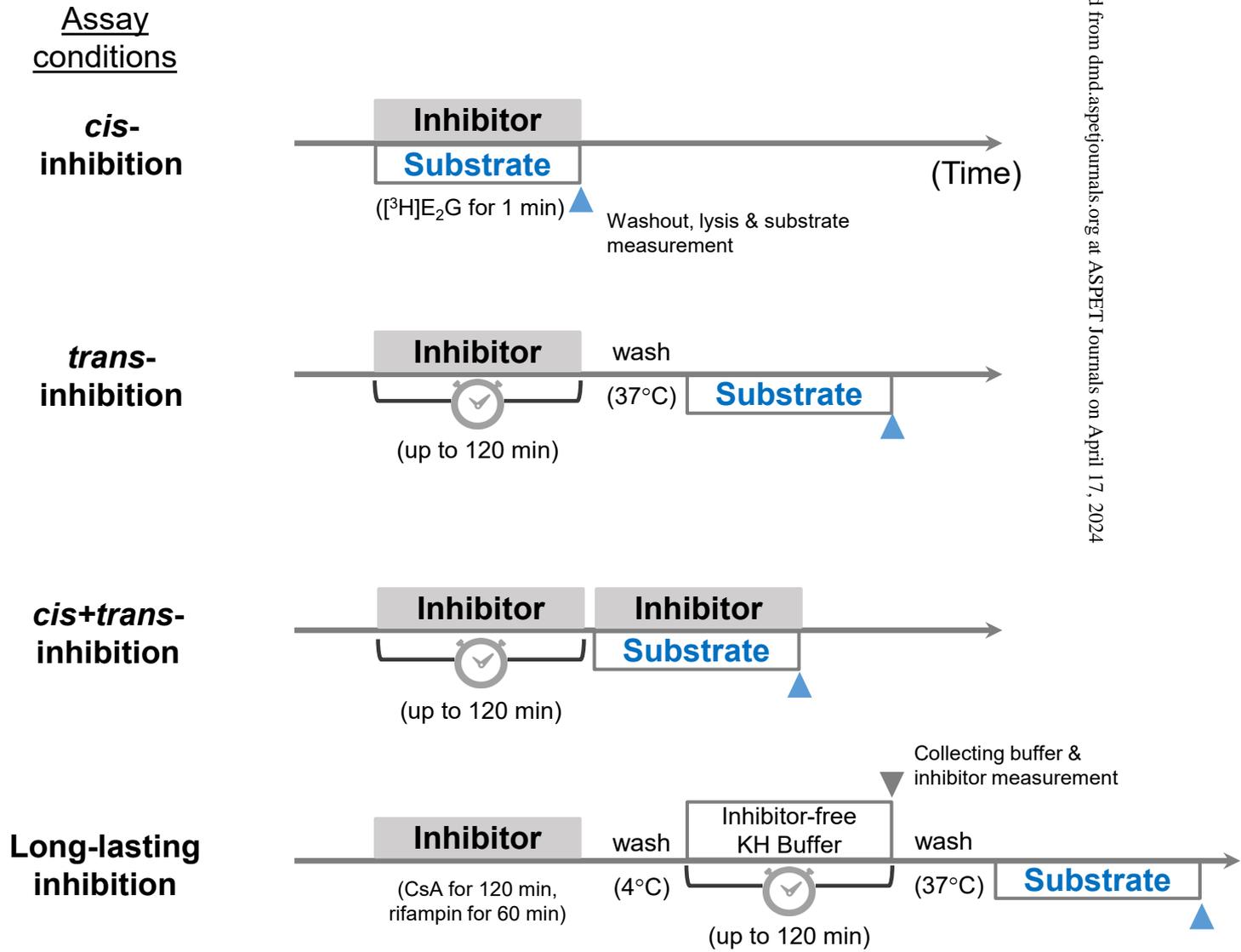
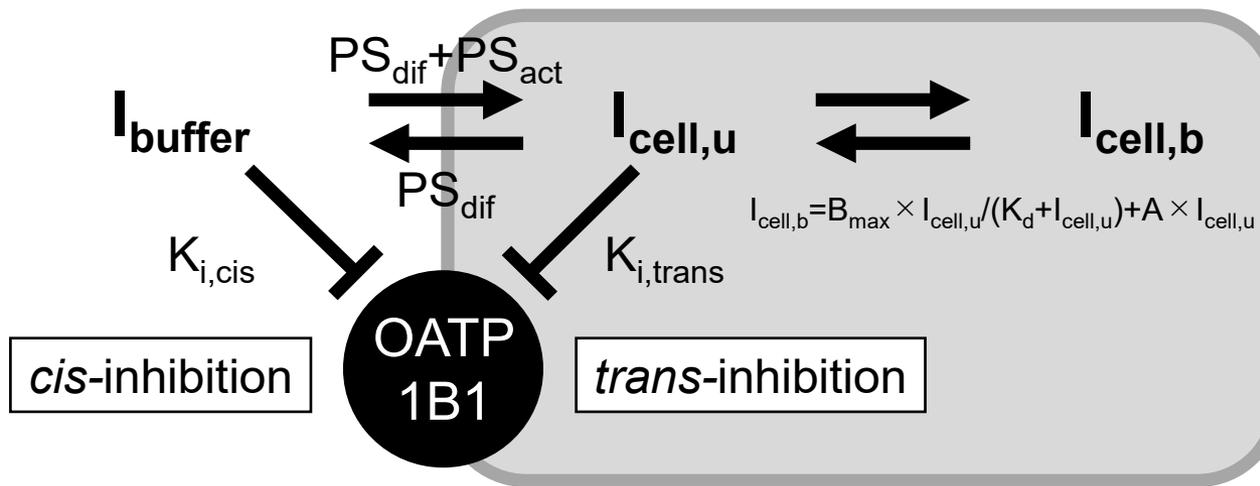


Figure 3

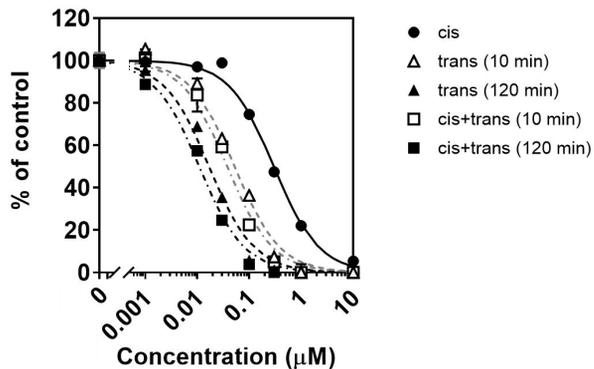


Parameters	Units	Inhibitors	
		CsA	Rifampin
K_d	μM	0.0914	-
B_{max}	μM	326	-
A		362	-
f_T		0.000254	0.0311
PS_{dif}	$\mu\text{l}/\text{min}/\text{mg protein}$	51.3	52.5
V_{max}	$\text{pmol}/\text{min}/\text{mg protein}$	-	25.1
K_m	μM	-	0.382
$K_{p,uu,\text{in vitro}}$		1	2.25
$K_{i,cis}$	μM	0.297	1.16
$K_{i,trans}$	μM	0.00619	1.56
$K_{i,cis}$ -to- $K_{i,trans}$ ratio (α)		48.0	0.744

Figure 4

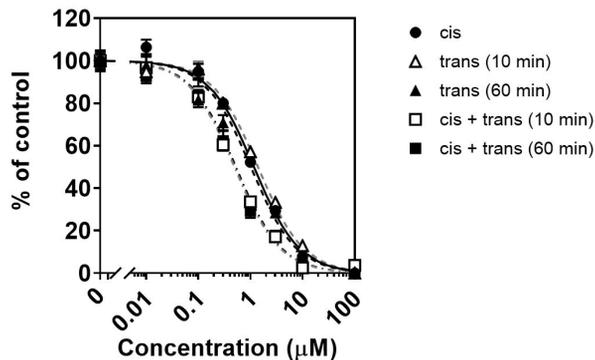
CsA

(A)

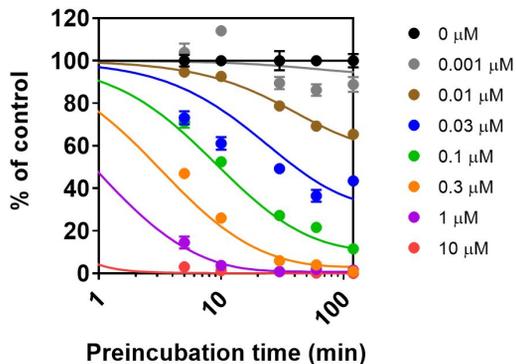


Rifampin

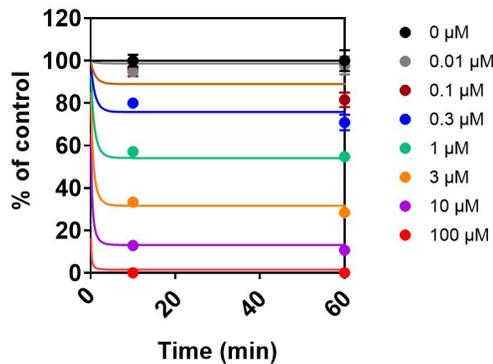
(B)



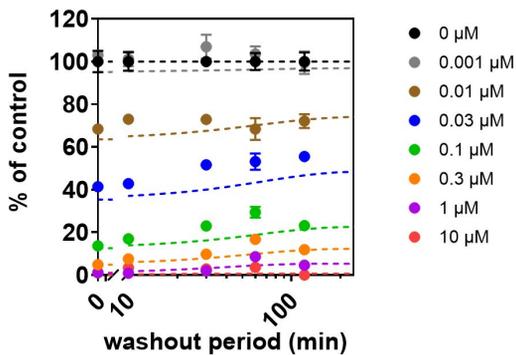
(C)



(D)



(E)



(F)

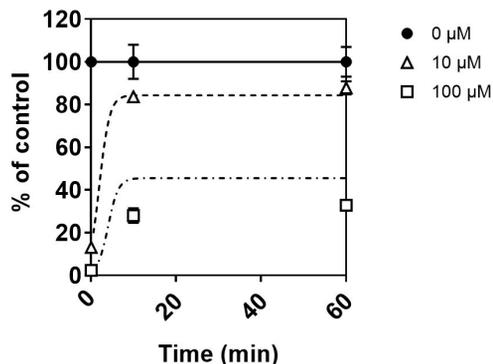
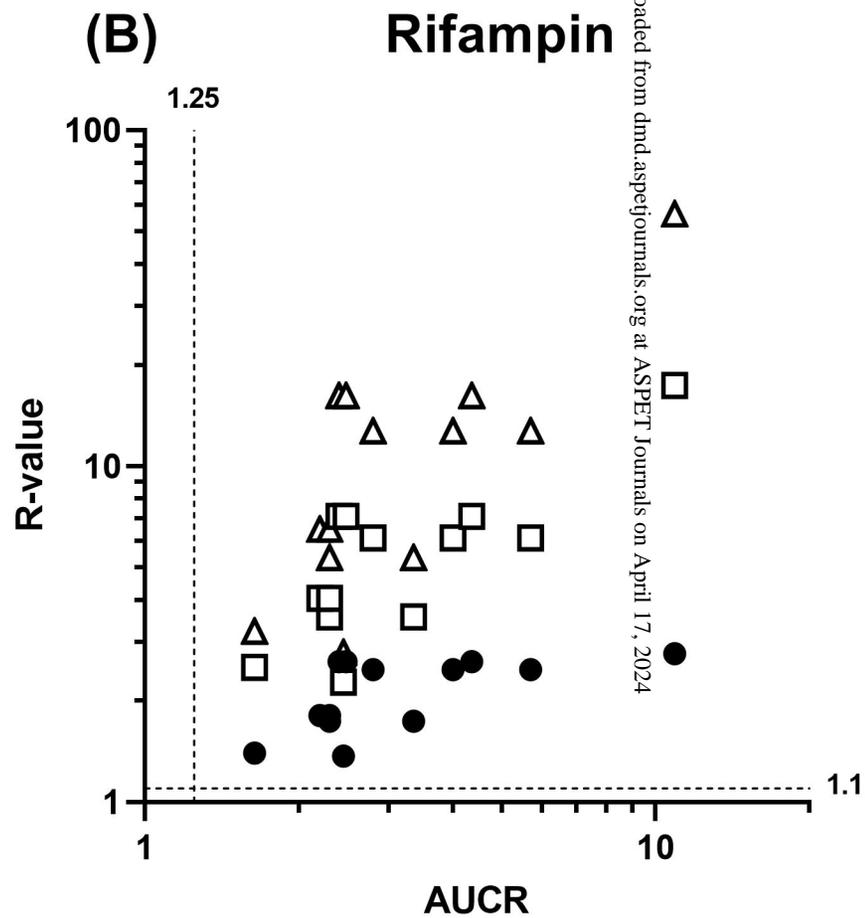
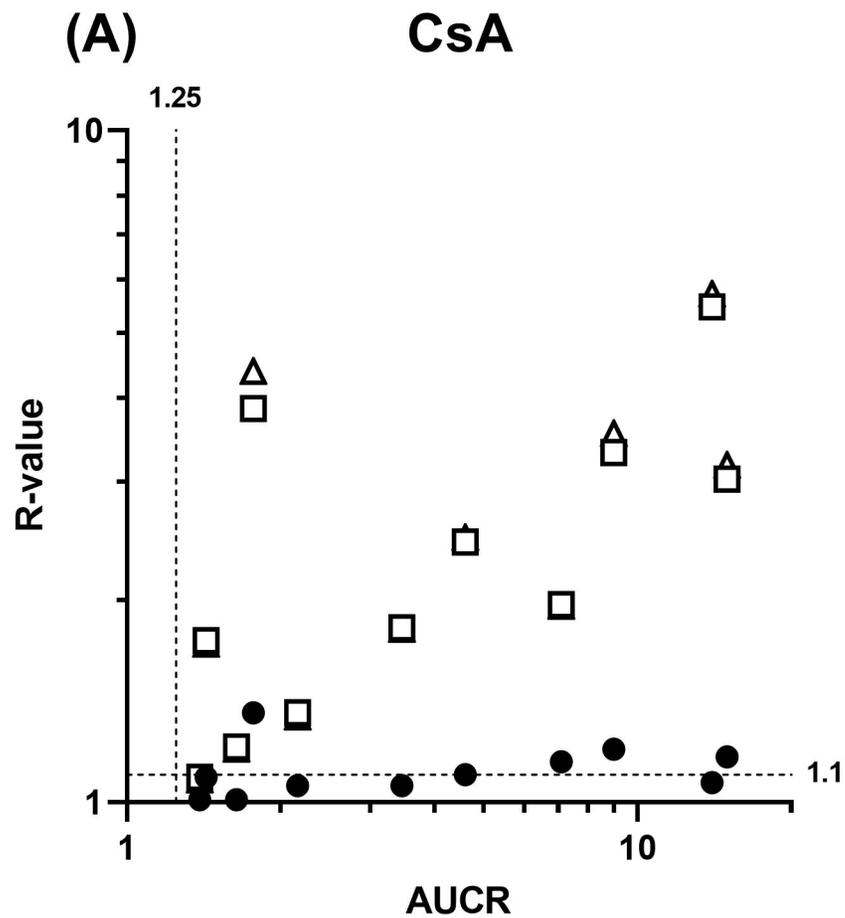


Figure 5



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Figure 6