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Characterization of Digoxin Uptake in Sandwich-Cultured Human Hepatocytes

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Abbreviations: DDI, drug-drug interaction; MDR, multidrug resistance; OATP, organic anion transporting polypeptide; SLCO, solute carrier organic anion transporter; SCHH, sandwich-cultured human hepatocytes; OAT, organic anion transporter; OCT, organic cation transporter; SLC, solute carrier; ABC, ATP binding cassette; GLUT, glucose transporter; MCT8, monocarboxylate transporter 8; HBSS, Hanks' balanced salt solution; E-17β-G,

estradiol-17β-D-glucuronide; ES, estrone-3-sulfate; CCK-8, cholecystokinin-8; TEA, tetraethylammonium; SDS, sodium dodecyl sulfate; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; PAH, *p*-amino hippuric acid; CCCP, carbonyl cyanide-m-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; CsA, cyclosporin A; BSP, bromosulfophthalein.

Abstract

Digoxin is a commonly used drug to treat congestive heart failure. With its narrow therapeutic index, patients are susceptible to drug-drug interaction-mediated cardiotoxicity. Digoxin is primarily renally cleared; however, a significant component of clearance is due to multidrug resistance 1 (MDR1)-mediated transport into bile. Digoxin is reported to be actively transported into human hepatocytes by the organic anion transporting polypeptide 1B3 (OATP1B3); however, further characterization has not been fully described. The purpose of the present study was to investigate the hepatic uptake mechanisms of [^{3}H]digoxin using sandwich-cultured human hepatocytes (SCHH) and transporter-expressing cells. Digoxin uptake in SCHH involves both a saturable (carrier-mediated) process and a passive (non-saturable) process. At low concentrations, the saturable component exhibited an apparent K_m of 2.39 μM and a V_{max} of 4.49 pmol/min/mg protein. The calculated passive diffusion clearance was 1.25 μL/min/mg protein. Uptake of [^{3}H]digoxin in SCHH was not inhibited by a variety of substrates or inhibitors for OATP1B1, OATP1B3, OATP2B1, organic anion transporter 2 (OAT2), organic cation transporter 1 (OCT1) and monocarboxylate transporter 8 (MCT8). Cytochalasin B, which inhibits glucose transporters (GLUT), did not significantly inhibit digoxin uptake, whereas the flavonoids quercetin and rutin inhibited uptake by ~50%. Nonlabeled digoxin inhibited [^{3}H]digoxin uptake by ~50%. Studies with OATP-transfected HEK cells or oocytes showed that digoxin is not a substrate of OATP1B1, 2B1 or 1B3. In conclusion, the data suggest that digoxin uptake in SCHH involves both saturable and passive processes. The saturable process is mediated by an as yet undetermined digoxin transporter(s).

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Introduction

Transporter-mediated uptake of drugs in the liver can be a major determinate of hepatic clearance. The majority of hepatic drug uptake in humans is mediated by members of the solute carrier superfamily of transporters. These transporters include organic anion transporting polypeptides OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), and OATP2B1 (SLCO2B1), the organic anion transporter OAT2 (SLC22A7), and the organic cation transporter OCT1 (SLC22A1). Of these, the OATP transporters are the most relevant for uptake of drugs into the liver. In addition to the role uptake transporters play in defining hepatic clearance, it is also recognized that inhibition of uptake transporters can reduce hepatic drug clearance and this represents a potential source of drug-drug interactions (DDIs) (Shitara et al., 2003; Poirier et al., 2007). For these reasons, it is important to identify the contribution of transporters to the overall uptake of drugs, and to identify the specific transporter(s) involved.

Digoxin is a cardiac glycoside which is commonly used in clinical practice to treat congestive heart failure (Gheorghiade and Pitt, 1997). Digoxin has a narrow therapeutic range and has been associated with numerous drug interactions with co-administered drugs (Koren, 1985; Rodin and Johnson, 1988); consequently, patients can be particularly susceptible to DDI-mediated cardiotoxicity. Digoxin is primarily eliminated in urine, however 30-50% is cleared via the liver (Goodman and Gilman, 2006). Digoxin is a substrate for the efflux transporter multidrug resistance 1 (MDR1/ABCB1) and is the recognized probe substrate to assess MDR1 DDI in the clinic (US DHHS FDA, 2006). Evidence suggests digoxin is primarily taken up actively into both rat and human hepatocytes. In rats, hepatic disposition of digoxin has been fairly well characterized. It has been shown to be a substrate

of rat Oatp1a4 (Noé at al., 1997; Kodawara et al., 2002) and mouse oatp1a4 (Ose et al., 2010), and hepatocyte uptake K_m values range from 0.18 – 36 μM (Hedman and Meijer, 1998; Lam and Benet, 2004; Liu et al., 2005). In human hepatocytes, digoxin uptake is decreased by 95% at 4°C (Olinga et al., 1998). It has also been reported that digoxin is a substrate for OATP1B3 using *Xenopus laevis* oocytes (Kullak-Ublick et al. 2001). However, further characterization of digoxin uptake into human hepatocytes has not been fully described.

Sandwich-cultured hepatocytes form intact bile canalicular networks, and they maintain functional expression levels of uptake and efflux transporter proteins for several days (Hoffmaster et al., 2004; Li et al., 2009). We previously reported the ability to use cryopreserved human hepatocytes in sandwich-culture (Bi et al. 2006) and have shown that uptake and efflux transporter activities are similar to those in fresh hepatocytes. The purpose of this study was to investigate the hepatic uptake of digoxin using cryopreserved human hepatocytes in sandwich culture.

Materials and Methods

Chemicals and Reagents. In VitroGro-HT, In VitroGro-CP and In VitroGro-HI hepatocyte media were purchased from Celsis In Vitro Technologies Inc. (IVT) (Baltimore, MD). Hanks' balanced salt solution (HBSS) and Williams' medium E were purchased from Invitrogen (Carlsbad, CA). BioCoat 24-well plates and Matrigel were purchased from BD Biosciences (Bedford, MA). Triton X-100 was purchased from Bio-Rad Laboratories (Hercules, CA). Dulbecco's phosphate-buffer saline, Krebs-Henseleit buffer and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). [^{3}H]Digoxin (35.4 Ci/mmol), [^{3}H]estradiol-17β-D-glucuronide (E-17β-G) (41.8 Ci/mmol), [^{3}H]estrone-3-sulfate (ES) (54.26 Ci/mmol) and [^{3}H]midazolam (5 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). [^{3}H]Cholecystokinin-8 (CCK-8) (89 Ci/mmol) was purchased from GE Healthcare UK Limited (Buckinghamshire, UK). [^{3}H]Rosuvastatin (10 Ci/mmol) and [^{14}C]tetraethyl ammonium (TEA) (55 mCi/mmol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Amiodarone was purchased from Sequoia Research Products (Pangbourne, UK). All other compounds were purchased from Sigma-Aldrich (St. Louis, MO).

Human Hepatocytes. Cryopreserved human hepatocyte lot 109 was purchased from BD Biosciences (Woburn, MA) and lot Hu4165 was purchased from CellzDirect (Pittsboro, NC).

Sandwich-Cultured Human Hepatocytes (SCHH). In VitroGro-HT (thawing), In VitroGro-CP (plating) and In VitroGro-HI (incubation) media were supplemented with

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Torpedo Antibiotic Mix (IVT) per the manufacturer's instructions. Cryopreserved hepatocytes were thawed and plated as described previously (Bi et al., 2006). Briefly, hepatocytes were thawed in a water bath at 37°C then placed in ice, after which the cells were poured into 37ºC In VitroGro-HT medium at a ratio of one vial/50 mL in a conical tube. The cells were then centrifuged at 50 × g for 3 minutes and resuspended to 0.7×10^6 cells/mL in In VitroGro-CP medium. Cell viability was determined by trypan blue exclusion. On day 1, hepatocyte suspensions were plated in BioCoat 24-well plates at a density of 0.35×10^6 cells/well in a volume of 0.5 mL/well. After 18 to 24 h of incubation at 37ºC, cells were overlaid with ice-cold 0.25 mg/mL Matrigel in In VitroGro-HI medium at 0.5 mL/well. Cultures were maintained in In VitroGro-HI medium, which was replaced every 24 hr.

Determination of uptake clearance in hepatocyte suspension. Cryopreserved hepatocytes were thawed at 37°C, then immediately suspended in Williams' medium E. The suspension was centrifuged (50 × g) for 3 minutes at room temperature, and the cells were re-suspended in Krebs-Henseleit buffer. Cell viability was determined by trypan blue exclusion and the suspension was diluted to 2×10^6 cells /mL. A 200 µL aliquot of cells was placed in test tubes and pre-warmed in a slow-motion 37°C water bath for 3 minutes with and without inhibitors. Uptake incubations were initiated by the addition of 200 µL of 2 µM [^{3}H]digoxin or 2 µM [^{3}H]CCK-8 pre-warmed (37°C) with and without inhibitors, which resulted a final substrate concentration of 1 µM, and a cell density of 1×10^6 cells/mL in 0.4 mL incubation volume. The incubations were terminated at 0.5, 1 and 2 minutes by collection of 100 µL of incubation mixture into a centrifuge tube that was previously prepared with two layers: a bottom layer of 50 µL of 2N NaOH and an upper layer of 100 µL oil (density =1.015,

a mixture of silicone oil and mineral oil). The tube was immediately centrifuged at 14,000 rpm for 10 seconds (Beckman Microfuge E) so that hepatocytes pass through the oil layer into the bottom layer. The centrifuge tubes were cut at the middle of the oil layer to separate the lower portion containing cells from the upper portion containing incubation buffer. Quantification of compounds in both portions was determined by scintillation spectroscopy.

Uptake Study in SCHH. The incubation medium used for the uptake study was Ca^{2+}-containing HBSS buffer. After rinsing the cells twice with Ca^{2+} HBSS buffer, the cells were pre-incubated for 10 minutes at 37°C or 4°C with Ca^{2+} HBSS buffer in the absence or presence of inhibitors. After aspirating the buffer, 0.5 mL of incubation buffer containing a substrate in the absence or presence of inhibitors was added at 37°C or 4°C. The uptake was terminated at a designated time by adding 0.5 mL of ice cold Ca^{2+} HBSS buffer after removal of the incubation buffer. Cells were then quickly washed three times with 0.5 mL of ice cold Ca^{2+} HBSS buffer, then solubilized with 0.5 mL of 0.5% Triton X-100 in Dulbecco's phosphate buffer saline. After shaking plates, aliquots (0.5 mL) were transferred to scintillation vials. The radioactivity was measured by a liquid scintillation counter (WALLAC 1409 DSA; Perkin Elmer, Waltham, MA) after adding 6 mL of scintillation fluid (Ready Safe; BECKMAN COULTER, Brea, CA) to the scintillation vials. For determination of digoxin uptake kinetics, various concentrations of [^{3}H]digoxin (0.05 – 300 μM) were incubated for 5 minutes at 37°C as described above. Data were then fit to equation 1 using GraphPad Prism (La Jolla, CA), where P_{dif} represents non-saturable or passive diffusion in units of μL/min/mg protein.

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$$v = \frac{V_{max} \bullet S}{K_m + S} + P_{dif} \bullet S \qquad (1)$$

Transport Study in Transfected HEK293 Cells. Studies with the three hepatic transporters OATP1B1, OATP1B3, OATP2B1 were conducted based on a previously described method (Kalgutkar et al., 2007). Human OATP1B1 and 1B3 expressed in human embryonic kidney 293 (HEK293) cells were obtained from Prof. Yuichi Sugiyama (University of Tokyo, Japan) and human OATP2B1 expressed in HEK293 cells were obtained from Prof. Dietrich Keppler (DKFZ, Heidelberg, Germany). Briefly, transporter-transfected and wild-type HEK293 cells were seeded onto 24-well poly-D-lysine–coated plates at a density of ~2.5×10^5 cells per well. OATP2B1-transfected cells were cultured with 10 mM sodium-*N*-butyrate 24h before conducting the transporter assay. OATP1B3-transfected cells were cultured with and without 10mM sodium-N-butyrate for 24h before conducting the transporter assays. After the cells were confluent, the cells were washed three times with 1 mL of uptake buffer. The transport study was initiated by incubation of the cells with 0.3 mL of incubation buffer containing each test compound in the absence or presence of inhibitors at 37°C. At the completion of the incubation, uptake was stopped by washing the cells three times with ice-cold buffer. The cells were then lysed in 1% sodium dodecyl sulfate (SDS) and 0.1 M NaOH in water, and the radioactivity was measured by liquid scintillation spectroscopy.

Transport Study in *Xenopus laevis* Oocytes. OATP1B3*1 (Konig et al., 2000)- and water-injected oocytes and a reagent pack were purchased from BD Biosciences (Woburn, MA). Oocytes were used 5 days after injecting and then the transport study was conducted at room temperature. All uptake studies were performed in Na^+ buffer from a reagent pack containing 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Hepes buffer adjusted to pH 7.4. Eight to ten oocytes were prewashed in 3 mL of uptake buffer three times and then incubated at room temperature in 0.1 mL of uptake buffer containing each test compound at the indicated concentrations. Water injected oocytes were used as controls for nonspecific uptake and binding of the test compound. Uptake was stopped by adding 3 mL of ice-cold uptake buffer, and oocytes were washed three times in 3 mL of ice-cold uptake buffer. Individual oocytes were transferred to scintillation vials and lysed in 0.15 mL of 10% SDS in uptake buffer. Radioactivity was measured by liquid scintillation spectroscopy.

Statistical Analysis. Two methods were used to assess statistical significance of differences, dependent upon the type of data being analyzed. One method was to use an unpaired two-tailed Student's *t* test for data generated in single studies with vehicle control and treatment groups. For data generated over multiple studies, each including vehicle control and treatment groups, an ANOVA was conducted within each study and then all treatments were compared versus control using Dunnett's procedure. A value of $p < 0.05$ was predetermined as the criterion for significance for both statistical methods.

Results

Time Course of Digoxin Uptake in SCHH. The time course of digoxin uptake into SCHH is shown in Fig. 1. The uptake of digoxin increased linearly up to 5 minutes. Digoxin uptake showed significant temperature dependence. The uptake rate over 5 minutes at 37°C was 153 fmol/min/mg protein, while the uptake rate at 4°C was only 1.33 fmol/min/mg protein.

Uptake Kinetics of Digoxin in SCHH. To characterize the uptake kinetics of digoxin, the concentration-dependence of uptake into SCHH was examined. The uptake of digoxin was characterized as having a saturable component at low concentrations, and a non-saturable or passive component at high concentrations (Figures 2A and 2B). In the concentration range 0.05 – 10 μM, uptake followed Michaelis-Menton kinetics with a K_m of 2.39 μM and a V_{max} of 4.49 pmol/min/mg protein, which results in an uptake clearance of 1.88 μL/min/mg protein. Using equation (1), the calculated passive diffusion clearance was significant at 1.25 μL/min/mg protein, and represents 40% of total uptake clearance.

Inhibitory Effect of Various Compounds on the Uptake of Digoxin in SCHH. To characterize the saturable uptake transport mechanism of digoxin, an extensive study was undertaken to assess the potential of a wide variety of known transporter substrates and inhibitors to affect the uptake of digoxin (Table 1). The uptake of digoxin in SCHH was not inhibited by classic OATP substrates and inhibitors, including BSP, which has recently been

shown to also be a MCT8 inhibitor (Kinne et al., 2010). Digoxin uptake was also not affected by a high concentration of the OATP1B3 substrate CCK-8. In addition, neither ES, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), *p*-amino hippuric acid (PAH), probenecid nor quinidine inhibited digoxin uptake. The mitochondrial uncouplers carbonyl cyanide-m-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNP) did not inhibit digoxin uptake, while carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) appeared to have a weak, yet variable, inhibitory effect. The antiarrhythmic agents amiodarone (known to elicit a non-renal DDI with digoxin in humans (Nademanee et al., 1984)) and quinidine (an OCT1 inhibitor), had no effect on the uptake of digoxin in SCHH. Although the glucose transporter (GLUT) inhibitor cytochalasin B did not inhibit digoxin uptake, the known GLUT2 inhibitor quercetin and its glycosylated analog rutin (quercetin-3-rutinoside) did show inhibition of digoxin uptake. The cardiac glycosides digoxin (unlabeled) and ouabain, also inhibited the uptake of [^{3}H]digoxin. The inhibitory effects of several compounds in a different cryopreserved hepatocyte lot are shown in Table 2. In lot Hu4165, the classic OATP inhibitors cyclosporin A (CsA), bromosulfophthalein (BSP) and rifampicin did not inhibit digoxin uptake. FCCP had a modest inhibitory effect and non-labeled digoxin inhibited the uptake of [^{3}H]digoxin by ~50%. All compounds (CsA, BSP, FCCP, rifampicin and digoxin) significantly inhibited the uptake of the known OATP1B3 substrate CCK-8.

Uptake Clearance of Digoxin and CCK-8 in Suspension and SCHH. The uptake clearance of digoxin in SCHH was compared to that observed in hepatocyte suspensions. As seen in Table 3, the uptake clearance of digoxin in day 5 SCHH was similar to that in

suspensions. In addition, day 5 SCHH retained appreciable OATP1B3-specific activity relative to suspensions, as evidenced by the uptake clearance of CCK-8. In hepatocyte suspensions, 100 µM Rifamycin SV did not affect digoxin uptake clearance, while CCK-8 uptake clearance was inhibited 86%.

Uptake of Substrates and Digoxin by OATP1B1, 1B3 and 2B1-Transfected Cells. Table 4 lists the transfected/wild type uptake ratios obtained for various substrates using OATP-expressing HEK293 cells. OATP1B1 mediated ES and rosuvastatin transport, whereas it did not transport digoxin or the negative control midazolam. OATP1B3 efficiently mediated E-17β-G, CCK-8 and rosuvastatin transport, but the uptake of digoxin by OATP1B3 was much lower and similar to that observed for the negative control midazolam. OATP2B1 efficiently mediated ES and rosuvastatin transport, while it did not show uptake of digoxin.

The Effect of Rifamycin SV on the Uptake of Digoxin by OATP1B3 -Expressing HEK293 Cells. The effect of rifamycin SV on OATP1B3-mediated uptake was determined (Fig. 3). The uptake of E-17β-G into OATP1B3-expressing HEK293 cells was 89-fold higher than that into wild type HEK293 cells, and this uptake was nearly completely inhibited by 100 µM rifamycin SV. The uptake of rosuvastatin into OATP1B3-expressing HEK293 cells was 9-fold higher than that into wild type HEK293 cells. The uptake of rosuvastatin was significantly inhibited by 100 µM rifamycin SV. The uptake of digoxin into OATP1B3-expressing HEK293 cells was very low (2.2 fold higher than wild type) and 100 µM rifamycin SV had no effect on the uptake of digoxin

Uptake of Substrates and Digoxin by *Xenopus laevis* Oocytes Expressing OATP1B3*1. To further determine the potential role of OATP1B3 in digoxin uptake, we investigated uptake by *Xenopus laevis* oocytes expressing OATP1B3*1 in the presence and absence of the OATP inhibitors CsA and rifamycin SV (Fig. 4). OATP1B3-mediated uptake of E-17β-G was 36-fold higher compared to water injected oocytes. Two inhibitors, 10 μM CsA and 100 μM rifamycin SV, significantly inhibited the uptake of E-17β-G. OATP1B3-mediated uptake of CCK-8 was 4.9-fold higher compared to the water injected oocytes, and both CsA and rifamycin SV significantly inhibited uptake. In studies 1 and 2, the uptake of digoxin was similar to that observed in water injected oocytes. Neither CsA nor rifamycin SV affected digoxin uptake.

Discussion

The liver plays an important role in drug metabolism and excretion. It is well known that hepatic drug-drug interactions can affect the therapeutic safety and efficacy of drugs. Although digoxin has been widely used in humans for many years, its therapeutic index is very narrow and drug-drug interactions with this agent have been reported (Leahey et al., 1978; Moysey et al., 1981; Dorian et al., 1988). Approximately 50-70% of digoxin is excreted in urine, while 30-50% is cleared by the liver, primarily via excretion of unchanged drug in bile (Goodman and Gilman, 2006). The mechanism of digoxin hepatic uptake in humans is not well understood, especially as it pertains to transporter-mediated uptake.

In this study, we further characterized the hepatic uptake of digoxin by utilizing cryopreserved human hepatocytes in sandwich-culture. Our data reveal two novel aspects of digoxin uptake – 1) there is a significant passive component of digoxin uptake in human hepatocytes, and 2) neither OATPs nor any other known drug uptake transporter is involved in digoxin uptake. These findings are significant and they have important implications on how one interprets in vitro data to predict clinical outcomes as described below.

Our data show that uptake of digoxin is mediated by both saturable (carrier-mediated) and passive (or non-saturable) processes. The passive contribution to uptake is significant, and represents 40–50% of total hepatic uptake, as evidenced by the high passive diffusion clearance relative to the total uptake clearance (Fig. 2A) and the fact that very high concentrations of unlabeled digoxin could only inhibit ~50% of [^{3}H]digoxin uptake (Tables 1 and 2). Even though uptake of [^{3}H]digoxin is nearly completely inhibited at 4°C (Fig. 1), that fact that very high concentrations of unlabeled digoxin could not inhibit more

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than ~50% of [^{3}H]digoxin uptake at 37°C strongly suggests the lack of uptake at 4°C is an artifact of a more rigid plasma membrane. This artifact has been noted previously for propranolol (Webborn et al., 2007). These examples highlight the caution one must use when interpreting uptake data at 37°C and 4°C.

Despite a significant passive component to digoxin uptake, a clinically relevant uptake transporter DDI with co-administered drugs is possible. Our data suggest active or saturable uptake represents 50-60% of total uptake. When one couples this with the fact that ~40% of digoxin is eliminated via hepatic mechanisms (primarily via efflux into bile), the theoretical maximum fold increase in systemic area under the plasma concentration-time curve (AUC) due to complete inhibition of digoxin carrier-mediated uptake into liver would be ~1.3. Because digoxin has a narrow therapeutic index, an AUC or C_{max} increase of ≥25% is considered significant (Zhang et al., 2008). The two flavonoids quercetin and rutin showed significant inhibition of [^{3}H]digoxin uptake similar to that seen with cold digoxin (~50%). Both flavonoids are present in the human diet, and both are available as dietary supplements. This data suggest that high concentrations of flavonoids could also impact digoxin AUC levels due to inhibition of digoxin hepatic uptake.

Our study has clearly demonstrated that digoxin is not a substrate of OATP1B1, 1B3 or 2B1. First, several classic pan-inhibitors of OATPs had no effect on digoxin uptake in SCHH. Second, using OATP transfected HEK293 cells, no significant uptake of digoxin was noted, while known substrates of these transporters performed as expected in these assays. Even though digoxin exhibited uptake ratios in the range of 1.6 – 2.3 in OATP1B3-transfected cells (Figure 3 and Table 4), these ratios were similar to those observed for the negative

control midazolam, and rifamycin SV had no effect on digoxin uptake. Lastly, two separate studies with OATP1B3 oocytes showed no difference in uptake compared to water-injected control oocytes. These two studies are in contrast to the previous study by Kullak-Ublick et al., which reported an OATP1B3/control oocyte ratio of 2.4 for digoxin. The reason for this difference is not clear. However, the several approaches taken in the current study to assess the potential for OATP1B3 mediated uptake of digoxin clearly show digoxin is not a substrate of OATP1B3 in transfected cells and in hepatocytes. Our data indicate that digoxin should not be used as a probe substrate for OATP1B3 in human hepatocyte systems. A previous clinical study suggested that an insert-variant allele of OATP1B3 was associated with an increase in the concentration/dose ratio of digoxin in hemodialysis (HD) patients measured 62-72 hours post dose (Tsujimoto et al., 2008). Although this appears to support a role for OATP1B3-mediated hepatic clearance in HD patients, additional studies comparing this allele to the measured digoxin systemic clearance are needed to confirm these findings.

This study has highlighted the utility of sandwich cultured hepatocytes as a model to measure OATP mediated uptake. We have previously shown that SCHH possess significant OATP activity (Bi et al., 2006) and a previous study showed that OATP1B1 and OATP1B3 protein levels are well maintained on day six in SCHH (Hoffmaster et al., 2004). Our data show that the uptake clearance of digoxin in sandwich culture is similar to that observed in non-cultured hepatocyte suspension, and that OATP1B3-specific activity (as measured by CCK-8 uptake clearance) in appreciable in sandwich culture. In addition, the effect of Rifamycin SV on the uptake of digoxin and the OATP1B3-specific substrate CCK-8 is identical in suspensions and sandwich culture.

The only two compounds that showed significant inhibition of [^{3}H]digoxin uptake are

the flavonoids quercetin and rutin. Each compound inhibited digoxin uptake by ~50% (Table 1). Both compounds have been shown to inhibit OATP2B1 (Satoh et al, 2005, Fuchikami et al., 2006). Quercetin inhibits the major hepatic glucose transporter GLUT2 (Song et al., 2002; Thorens et al., 1990), while rutin does not (Song et al., 2002). Rutin has been shown to increase the activity of OATP1B1, whereas quercetin had no effect on this transporter (Wang et al., 2005). Even though these two flavonoids interact with OATP1B1 and 2B1, our results show that digoxin uptake is not due to these transporters. Although the data *are* limited, it is interesting to speculate that physio-chemical properties of flavonoids may be important for an interaction with the putative digoxin transporter(s).

The results from this study also highlight differences in digoxin hepatic uptake between rats and humans. In contrast to the human results reported here which show digoxin is not a substrate of hepatic OATPs, it has been shown that digoxin is a substrate of hepatic Oatp1a4 in rats (Noé et al., 1997), although there is no ortholog of Oatp1a4 in humans. In addition, amiodarone, quinidine, and rifampicin have been shown to inhibit uptake of digoxin in rat hepatocytes (Lau et al. 2004; Lambert et al., 1989; Kodawaera et al., 2002; Hedman and Meijer, 1998; Olinga et al.,2001; Lam and Benet, 2004). Neither of these compounds inhibited digoxin uptake in SCHH in the current study. A species difference for digoxin metabolism is well documented – digoxin is not significantly metabolized in humans, whereas CYP3A-mediated metabolism in rats is substantial (Salphati and Benet, 1999). Thus, the data suggest there are significant differences in the hepatic disposition of digoxin between rats and human. In rats, digoxin active uptake is mediated by Oatp1a4 and digoxin undergoes significant hepatic metabolism. In humans, digoxin uptake is not mediated by OATP1B1, OATP1B3 and OATP2B1 and very little hepatic metabolism occurs. Both species

excrete digoxin into bile via MDR1/Mdr1, however (Pauli-Magnus et al., 2001; Taipalensuu et al., 2004; Annaert et al., 2001).

In conclusion, the results of this study have highlighted important aspects of digoxin uptake in human hepatocytes. The results of this study are consistent with a significant passive component (40-50%) to digoxin uptake into human hepatocytes. The saturable (carrier-mediated) component of digoxin uptake does not involve typical hepatic drug transporters such as OATP1B1, OATP1B3, OATP2B1, OAT2, and OCT1. Consequently, drugs that only interact with one or more of these transporters would not be expected to affect digoxin blood concentrations. However, because of digoxin's narrow therapeutic index, drugs or dietary supplements that potently inhibit the putative digoxin transporter(s) responsible for its carrier-mediated uptake could lead to a clinically relevant DDI. More work is needed in order to identify the transporter(s) involved in the hepatic uptake of digoxin. Despite conflicting data on the role of GLUT2 in transporting digoxin (cytochalasin B did not affect uptake, but quercetin inhibited uptake by ~50%), future studies should focus on the putative role of GLUT2 by testing additional inhibitors in hepatocytes and in GLUT2-transfected cell models. In addition, due to the significant effect flavonoids appear to have on digoxin uptake, it would be of interest to test additional dietary flavonoids for their effect on digoxin uptake.

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Authorship Contributions

Participated in research design: Kimoto and Duignan

Conducted experiments: Kimoto, Chupka, Xiao and Bi.

Contributed new reagents or analytic tools: not applicable.

Performed data analysis: Kimoto, Chupka, Xiao, Bi and Duignan

Wrote or contributed to the writing of the manuscript: Kimoto, Bi and Duignan

Other: not applicable.

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Legends for Figures

Fig. 1. Time course of [^{3}H]digoxin uptake into SCHH. The uptake of [^{3}H]digoxin (0.05 μM) was measured at 37ºC (●) and 4ºC (○). Each point represents the mean ± S.D. of three separate measurements from a single study with hepatocyte lot 109.

Fig. 2. Kinetics of digoxin uptake in SCHH. The uptake rate of [^{3}H]digoxin was measured at 0.05, 0.1, 0.2, 1, 2, 10, 20, 40, 80, 100, 140, 180, 200, 250 and 300 μM at 37°C (2A), and kinetics were determined at 0.05, 0.1, 0.2, 1, 2 and 10 μM at 37°C (2B). Each experimental point represents the mean ± S.D. of 3-6 measurements from 1-2 studies in human hepatocyte lot 109. Data was modeled using the equation 1 as described in Methods.

Fig. 3. Inhibitory effect of rifamycin SV on OATP1B3-mediated uptake in HEK293 cells. The uptake of 1 μM [^{3}H]E-17 β -G or 0.05 μM [^{3}H]digoxin was measured at 37^{0}C for 3 minutes in the presence and absence of rifamycin SV. The uptake of rosuvastatin was measured with 1 μM [^{3}H]rosuvastatin at 37^{0}C for 1 minute in the presence and absence of rifamycin SV. Each value represents the mean + S.D. of three separate measurements from a single study.

Fig. 4. Substrate specificity of OATP1B3*1 in oocytes. The uptake of 2 μM [^{3}H]E-17 β -G, 1 μM [^{3}H]CCK-8 or 1 μM [^{3}H]digoxin was measured at room temperature for 60 minutes in the presence and absence of inhibitors. Each value represents the mean + S.D. of 3-9 oocytes. **; $P<0.01$, significantly different from control

TABLE 1

Inhibitory effect of various compounds on the uptake of [^{3}H]digoxin in SCHH lot 109

The uptake of 0.05 µM [^{3}H]digoxin was measured at 37ºC for 5 minutes in the presence and absence of inhibitors. The control values were 0.15^A, 0.17^B and 0.12^C pmol/mg protein/min, respectively. Data are expressed as a percent of the control values ± S.D. determined using all replicates (n=3-24) from 1-8 experiments. Statistical significance was determined using Student's t-test for amiodarone and 500 uM digoxin, while all other compounds utilized the ANOVA/Dunnett's approach as described in Methods. **; P<0.01 and *; P<0.05, significantly different from control.

Compounds	Inhibitor Concentration (µM)	[^{3}H] Digoxin Uptake (% of control)
Control^A (0.1% DMSO)		100 ± 4.6
Control^B (1% DMSO)		100 ± 3.1
Control^C (0.1% MeOH)		100 ± 7.8
OATP Substrates/Inhibitors		
Cyclosporin A	10	158 ± 29*
Rifamycin SV	100	100 ± 15
Rifampicin	100	163 ± 8.6*
Estrone Sulfate	100	90.5 ± 13
OATP & MCT8 Inhibitor		
BSP	100	96.1 ± 6.6
OATP1B3 Substrate		
CCK-8	100	100 ± 18
OAT Inhibitors		
PAH	500	115 ± 13
Probenecid	100	89.9 ± 7.3
OCT1 Inhibitor		
Quinidine	50	103 ± 8.3
Anion Transport Inhibitor		
DIDS	500	110 ± 27

TABLE 1 (continued)

Compounds	Inhibitor Concentration (μM)	[^{3}H] Digoxin Uptake (% of control)
Mitochondrial Uncouplers		
FCCP	50	79.2 ± 28
CCCP	50	87.4 ± 12
DNP	500	114 ± 25
Glucose Transporter Inhibitor		
Cytochalasin B	50	93.1 ± 4.8
Flavonoids		
Quercetin	50	55.4 ± 26*
Rutin	100	50.5 ± 4.1*
Cardiac Glycosides		
Digoxin	100	67.5 ± 14*
Digoxin (1% DMSO)	500	45.0 ± 13**
Ouabain	100	63.1 ± 2.2*
Antiarrhythmic		
Amiodarone (0.1% MeOH)	100	97.6 ± 4.6

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TABLE 2

Inhibitory effect of various compounds on the uptake of [^{3}H]digoxin and [^{3}H]CCK-8 in SCHH lot Hu4165

The uptake of 0.05 μM [^{3}H]digoxin or 1 μM [^{3}H]CCK-8 was measured at 37ºC for 5 minutes in the presence and absence of inhibitors. The digoxin control values were 0.18^A and 0.14^B pmol/mg protein/min, respectively. The CCK-8 control value was 1.06 pmol/mg protein/min. Data are expressed as a percent of the control values ± S.D. (n=3). Statistical significance was determined using Student's t-test as described in Methods. **; P<0.01 significantly different from control.

Inhibitors	Concentration (μM)	[^{3}H] digoxin (% of control)	[^{3}H] CCK-8 (% of control)
Control^A (0.1% DMSO)		100 ± 3.1	100 ±10.4
Control^B (1% DMSO)		100 ± 7.0	–
Cyclosporin A	10	93.2 ± 9.2	16.0 ± 0.6**
BSP	100	113 ± 4.4	11.8 ± 2.0**
FCCP	50	63.8 ± 1.1**	14.4 ± 0.3**
Rifampicin	100	120 ± 1.3**	26.7 ± 3.3**
Digoxin	100	48.9 ± 1.3**	29.0 ± 4.0**
Digoxin (1% DMSO)	500	55.5 ± 3.6**	-

TABLE 3.

Uptake Clearance of Digoxin and CCK-8 in Human Hepatocyte Suspensions and Day 5 Sandwich Culture.

[^{3}H]CCK-8 was tested at 1 µM. [^{3}H]Digoxin was tested at 1 µM and 0.05 µM in suspension and sandwich-culture, respectively. Uptake clearance was determined over 0.5, 1 and 2 minutes in suspension, while uptake clearance in sandwich culture was determined at a single 5 minute time point. Suspension data are presented as the mean of triplicate determinations from a single experiment (digoxin) or as the mean of two experiments each run in triplicate (CCK-8). Unless indicated, sandwich culture data represent the mean of triplicate determinations from a single experiment.

		Uptake Clearance (µL/min/mg protein)		
		Suspension		Day 5
Compound	Hepatocyte Lot	Vehicle[A]	+100 µM Rif	SCHH
Digoxin	109	3.70	4.06	1.88[B] - 3.08[C]
	Hu4165	4.15	4.53	3.60
CCK-8	Hu4165	2.44	0.35	1.06

[A] Vehicle = 0.11% DMSO

[B] Determined from Vmax/Km (Figure 2).

[C] Mean of eight separate studies (SE = 0.26, Table 1).

TABLE 4

Substrate specificity of OATP1B1, 1B3 and 2B1 in transfected cells

Uptake was measured at 1 and 10 µM at 37ºC for 3 minutes in HEK293 transfected cells and wid-type cells. The values are presented as uptake ratios (transfected / wild-type). The values represent the mean of 2-3 replicates from a single experiment (designated with *) or the mean of 2-3 experiments each run in duplicate or triplicate. Values in parentheses for OATP1B3 represent the uptake ratios in cells pretreated with sodium butyrate.

Compounds	Concentration (µM)	----------Uptake ratio---------- OATP1B1	OATP1B3	OATP2B1
E-17β-G	1		87 (74)	
	10		116 (78)	
ES	1	6.7		44
	10	2.2		50
CCK-8	1		173 (226*)	
	10		126 (198*)	
Rosuvastatin	1	3.4	35* (16*)	6.0
	10	1.9	39* (25*)	6.3
Midazolam	1	0.92	1.7 (2.1*)	
	10	0.92	1.7 (1.9*)	
Digoxin	1	0.66*	1.6 (1.8)	1.3
	10	0.63*	1.9 (2.3)	1.3

Fig. 1

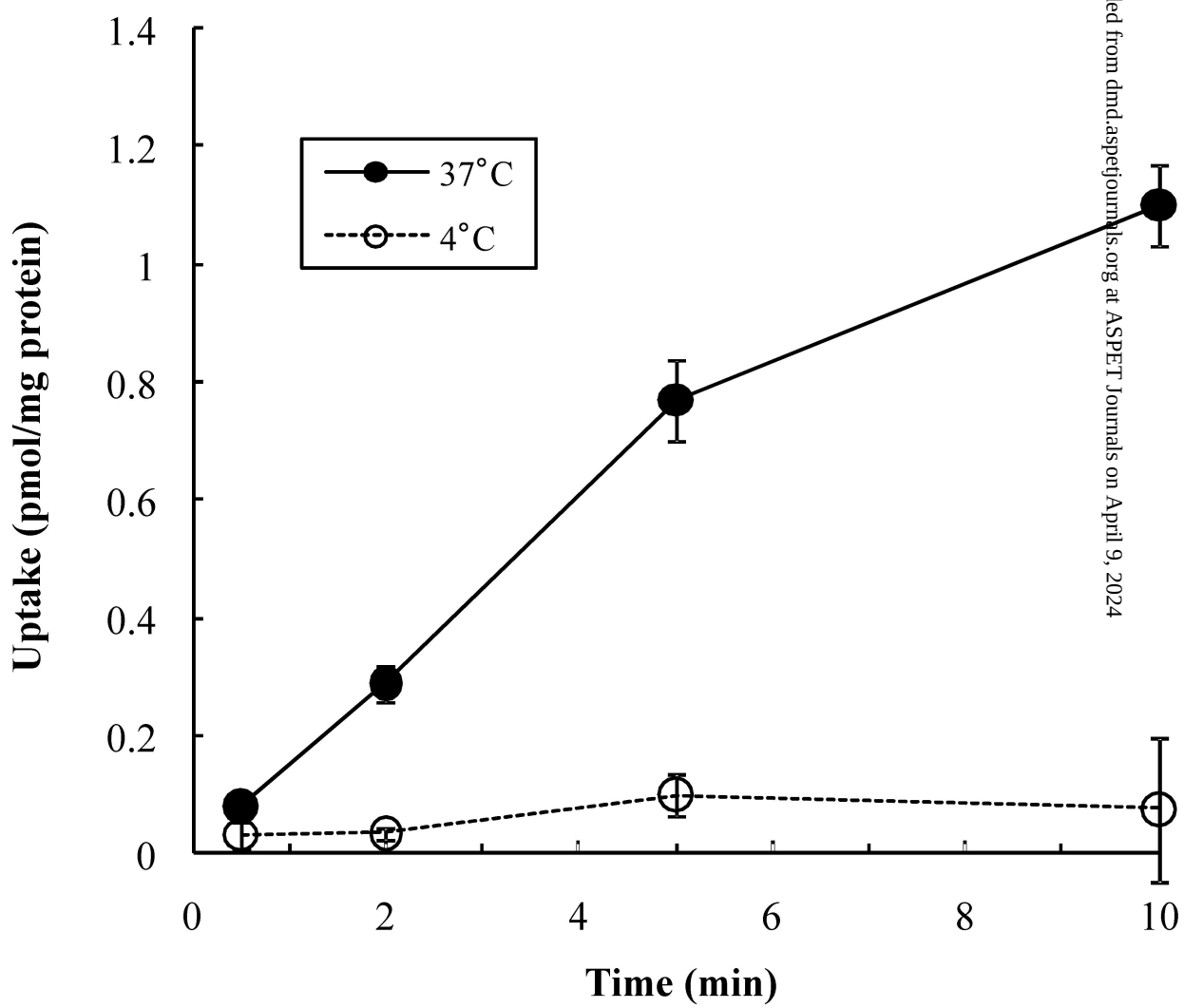

Fig. 2A

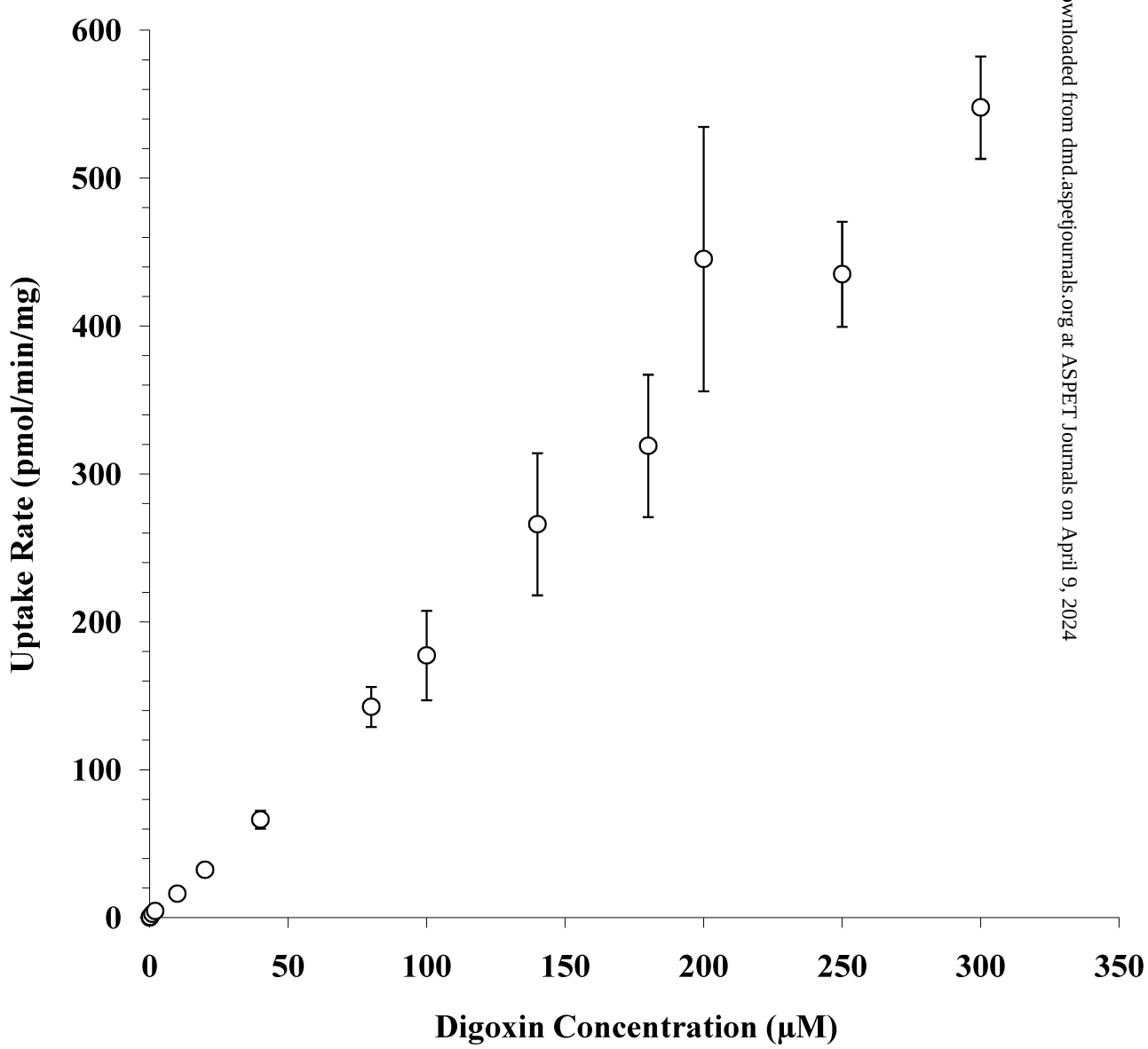

Fig. 2B

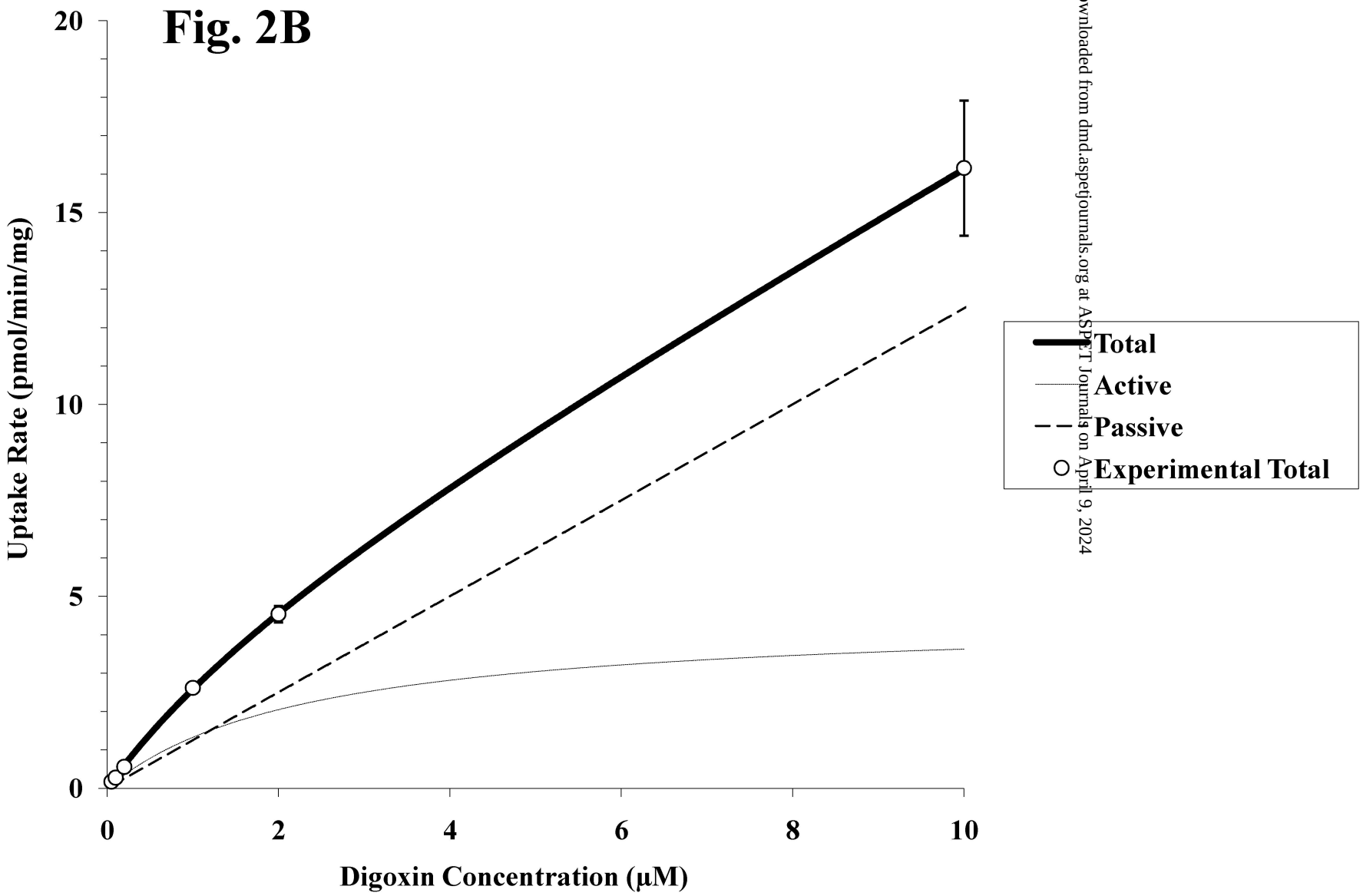

Fig. 3

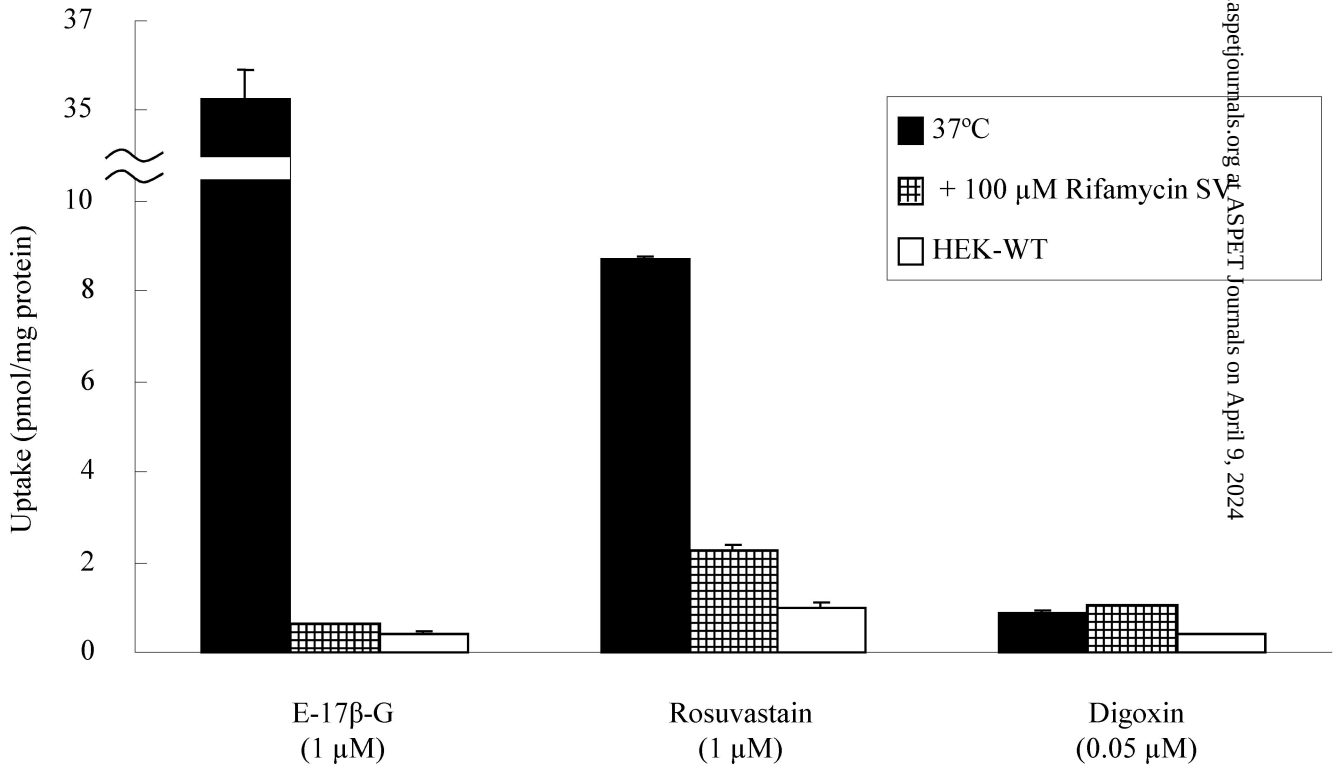

Fig. 4