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# Use of Cryopreserved Human Hepatocytes in Sandwich-Culture to Measure Hepatobiliary Transport

Yi-an Bi, Diana Kazolias and David B. Duignan

ADME Technology Group

Department of Pharmacokinetics, Dynamics & Metabolism

Groton/New London Laboratories, Pfizer, Inc.

Groton, CT 06340

**Running Title:**

**Sandwich-Culture of Cryopreserved Human Hepatocytes**

David B. Duignan

Pfizer Global Research & Development,

Groton/New London Laboratories, Pfizer, Inc.

Eastern Point Road, MS 8118W-116

Groton, CT 06340

Tel: 860-441-5608 Fax: 860-715-7377

E-mail: david.b.duignan@pfizer.com

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**Abstract**

Fresh hepatocytes cultured in a sandwich configuration allow for the development of intact bile canaliculi and the ability to measure hepatic uptake and biliary clearance. A disadvantage of this model is its dependence upon hepatocytes from fresh tissue. Therefore, the ability to use cryopreserved human hepatocytes in this model would be a great advantage. Multiple variables were tested and the recommended conditions for culturing cryopreserved human hepatocytes in a sandwich configuration in 24-well plates are: BioCoat™ plates, a cell density of $0.35 \times 10^{-6}$ cells/well in 500 µL, an overlay of Matrigel™ and InVitroGRO™ media. These conditions resulted in good hepatocyte morphology and the formation of distinct bile canaliculi. The function of multiple uptake and efflux transporters was tested in multiple lots of cryopreserved and fresh human hepatocytes. For taurocholate (NTCP/OATPs uptake/BSEP efflux) the average apparent uptake ($uptake_{app}$), apparent intrinsic biliary clearance ($CL_{bile,int,app}$) and biliary excretion index (BEI) among five cryopreserved hepatocyte lots was high, ranging from 11–17 pmol/min/mg protein, 5.8 – 10 µL/min/mg protein and 41 – 63%, respectively. The corresponding values for digoxin (OATP-8 uptake/MDR1 efflux) were 0.69 – 1.5 pmol/min/mg protein, 0.60 –1.5 µL/min/mg protein, and 37 – 63%. Both substrates exhibited similar results when fresh human hepatocytes were used. In addition, substrates of BCRP and MRP2 were also tested in this model, and all cryopreserved lots showed functional transport of these substrates. The use of cryopreserved human hepatocytes in 24-well sandwich culture to form intact bile canaliculi and to exhibit functional uptake and efflux transport has been successfully demonstrated.

## Introduction

Biliary excretion of therapeutic agents is often an important component of total drug clearance *in vivo* (Siegers and Bumann, 1991). Similarly, compounds that undergo little metabolism *in vivo* nevertheless can exhibit a significant degree of biliary elimination (Corsini et al., 1999; Martin et al., 2003). In the pharmaceutical industry within particular discovery therapeutic areas (i.e. cardiovascular, cancer, anti-infectives) one or more series of compounds may suffer from extensive biliary excretion. Therefore, being able to address this issue would help to understand mechanisms of clearance and help in the drug discovery process. Use of cannulated animals to assess biliary clearance is possible, yet the throughput is low and species differences in biliary clearance are known (Pahlman et al., 1998). Since the ultimate species of concern is human, it is necessary to use an *in vitro* human model to assess biliary elimination. Ideally this model should allow for at least a moderate number of compounds to be easily tested.

Use of fresh hepatocytes in culture to assess hepatic transport has gained much support in recent years. Initial work by LeCluyse and co-workers showed that culturing hepatocytes in a sandwich configuration resulted in the repolarization of hepatocytes over time and that this therefore was a good model to assess hepatic function, including that associated with the establishment of intact bile canaliculi (LeCluyse et al., 1994). Subsequent refinement of the culture conditions led to the development of a valuable *in vitro* model to assess hepatic transport using fresh rat hepatocytes cultured in a sandwich configuration (Liu et al., 1999a; Liu et al., 1999b) In contrast to a typical culture with no overlay, the sandwich culture model allows the hepatocytes to assume a more three dimensional orientation and to more easily form intact bile canaliculi and proper

localization of efflux transporters (Hoffmaster et al., 2004). Brouwer and co-workers have shown a good correlation exists in rats between *in vitro* biliary excretion and *in vivo* biliary excretion, as well as *in vitro* predicted biliary clearance and *in vivo* biliary clearance (Liu, et al., 1999c). The sandwich culture technique has been applied to human hepatocytes with some success as well, and to date the plate format has been in 60 mm dishes or 6-well plates. However, the dependency on commercially available fresh human tissue is a significant limitation of this model.

The commercial availability of good quality cryopreserved human hepatocytes has increased in recent years such that they are now readily available. Multiple vendors offer cryopreserved human hepatocytes, and plateable lots are also available. Cryopreserved hepatocytes have been successfully used for metabolism studies (Lau et al., 2002), for uptake studies in suspension (Houle et al., 2003; Shitara et al., 2003; Nakai et al., 2001) and for induction studies (Garcia et al., 2003). The ability to culture cryopreserved human hepatocytes in a sandwich configuration to assess hepatic uptake and biliary elimination would be a great advantage, since this would allow for studies on demand and this would also allow for the establishment of routine studies or screens. Therefore, the purpose of the present study was to determine if cryopreserved human hepatocytes could be cultured in a sandwich configuration in a small-well format to form intact bile canaliculi and to characterize the transport of well-studied compounds. The results indicate that human cryopreserved hepatocytes can be used in a 24-well plate sandwich culture format to assess uptake and efflux transport.

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## Materials & Methods

*Chemicals and Reagents*

InVitroGro™-HI, InVitroGro™-CP and InVitroGro™-HT hepatocyte media were purchased from In Vitro Technologies, Inc. (IVT) (Baltimore, MD). Hepatocyte maintenance media (HMM) was purchased from Cambrex Corporation (Baltimore, MD). Williams' media E (WME), Dulbecco's Modified Eagle Media (DMEM), glutamine, penicillin, streptomycin and Hank's balanced salt solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). BioCoat™ 24-well plates, Matrigel™ and ITS+ culture supplement (human recombinant insulin, human transferrin (12.5 mg each), selenous acid (12.5 µg), BSA (2.5 g), and linoleic acid (10.7 mg) was purchased from BD Biosciences (Bedford, MA). Mouse anti-MRP2 ($M_2$ III-6) monoclonal antibodies were obtained from IQ Products (Groningen, The Netherlands). Mouse anti-MDR1/3 (C219) and mouse anti-BCRP (BXP-21) monoclonal antibodies were obtained from ID Labs, Inc. (London, ON, Canada). 5-(and-6)-carboxy-2', 7'-dichiloro-fluorescein diacetate (CDFDA) was obtained from Molecular Probes (Eugene, OR). Triton X-100 was purchased from Bio-Rad Laboratories (Hercules, CA). Dulbecco's phosphate buffered saline (PBS), taurocholate (TC), digoxin, estradiol-17β-D-glucuronide (EG), sulfobromophthalein (BSP), and salicylate were purchased from Sigma-Aldrich (St. Louis, MO). [$^3$H]Taurocholate (1.19 Ci/mmol), [$^3$H]Digoxin (21.8 Ci/mmol), [$^3$H]Estradiol-17β-D-glucuronide (53.0 Ci/mmol) and [$^{14}$C]Salicylate (55.5 mCi/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). [$^3$H]BSP (17.5 Ci/mmol) was obtained from Hartmann Analytic (Köln, Germany).

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*Human Hepatocytes*

Cryopreserved human hepatocytes were purchased from IVT. Fresh hepatocytes in a BioCoat™/Matrigel™ sandwich configuration in 24-well plates were purchased from IVT and from CellzDirect (Pittsboro, NC). Characteristics of the hepatocyte lots are shown in Table 1.

*Hepatocyte Sandwich Culture*

InVitroGRO™-HT (thawing), InVitroGRO™-CP (plating) and InVitroGRO™-HI (incubation) media were supplemented with Torpedo Antibiotic Mix, as per the manufacturer's instructions. HMM was supplemented with $10^{-7}$ M dexamethasone, $10^{-7}$ M insulin, 100 units/mL penicillin G and 100 µg/mL streptomycin. WME and DMEM were supplemented with $10^{-7}$ M dexamethasone, 2 mM glutamine, 100 units/mL penicillin G, 100 µg/mL streptomycin and ITS+. For HMM, WME and DMEM, 10% and 2 % fetal bovine serum (FBS) were added for thawing and plating, respectively. There was no FBS in the incubation medium. Cryopreserved human hepatocytes were thawed based upon IVT's standard method – hepatocytes were thawed at 37°C then placed in ice, after which the cells were poured into 37°C InVitroGRO™-HT thawing medium at a ratio of one vial (~5 million cells):50 mL in a conical tube. The cells were then centrifuged at 50 xg for 3 minutes and resuspended to 0.7 x $10^6$ million cells/mL in InVitroGRO™-CP plating 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 x $10^6$ cells/well in a volume of 0.5 mL/well. After 18-24 h of incubation at 37°C, cells were overlaid with cold 0.25 mg/mL Matrigel™ in incubation medium at 0.5 mL/well. Cultures were maintained in FBS-free media, which was replaced every 24 hours.

*Observation of Bile Canaliculi Formation and Substrate Function Assay*

CDFDA was used for detecting the formation of bile canaliculi. On day 5, the cells were rinsed[1] with regular Hank's balanced salt solution (HBSS) and 0.5 mL of 5 μM CDFDA in HBSS was added. Following a 20-minute incubation, the cell cultures were rinsed three times with HBSS. Sandwich-cultured hepatocyte morphology and CDF accumulation in bile canaliculi were assessed by phase contrast microscopy and fluorescent microscopy, respectively, using a Nikon TE-300 or TE-2000U microscope with a 20x objective, and a Retiga 1300 or EXi digital CCD camera (QImaging, Burnaby, BC, Canada). Digital images were analyzed by MetaVue software (Molecular Devices Corporation, Downingtown, PA) or Clemex Vision PE software (Clemex Technologies, Inc., Longueuil, QC, Canada). For transporter function assays, on day 5 parallel cell cultures were rinsed twice with 0.5 mL of either regular HBSS or $Ca^{2+}$/$Mg^{2+}$-free HBSS containing 1 mM EGTA, and then incubated in the same buffers for 10 minutes. Substrates in regular HBSS were then added to both sets of cultures. At various time points, cells were rinsed three times with ice-cold regular HBSS. For radiolabeled compounds, cells were lysed with 0.5 mL of 0.5% Triton-100 in PBS. For unlabeled compounds, cells were lysed with 0.5 mL of methanol. The samples were analyzed by liquid scintillation spectroscopy or by LC/MS/MS.

*LC/MS/MS Conditions*

Samples were concentrated on an EVX-192 Apricot Evaporex® apparatus (Apricot Designs, Monrovia, CA). The residue was reconstituted to half the original volume in aqueous mobile phase prior to LC/MS/MS analysis. A Gilson 215 Multiprobe autosampler (Gilson Instruments, Middleton, WS) was used to deliver 25 μl injection volumes. Further sample concentration and

[1] Unless indicated, all rinse solutions were at 37°C.

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on-line sample clean up was achieved using a dual-column/column-switching system (Janiszewski *et al*., 2001) that consisted of a pair of columns (Showa Denko 1.5 x 5 mm cartridge style, custom packed with Showa Denko ODP polymeric material with a 13 μm particle size), plumbed using a 10 port 2-position switching valve (Valco Instruments, Houston, TX). Two HPLC pumps (model PU-1580, Jasco Inc., Easton, MA) delivered flow rates of 2 ml/min. The aqueous mobile phase (load step) was 98% 2mM ammonium acetate/2% methanol. The organic mobile phase (elute step) was 10% 2 mM ammonium acetate/90% 50/50 (acetonitrile/methanol). Mass spectrometry analysis was performed on a SCIEX API 4000 triple quadruple mass spectrometer (PE Sciex, Ontario, Canada) equipped with a turbo ion spray interface. Data was acquired in positive ion mode with an ESI probe voltage of 5.5 kV. Selected reaction monitoring was used to simultaneously monitor for analyte and internal standard. The following SRM transitions were used for the detection of analytes; 422 m/z → 377 m/z for topotecan, 482.2 m/z → 258 m/z for rosuvastatin, 393.2 m/z → 349.4 m/z for SN-38 and 687.3 m/z → 319.7 m/z for internal standard. A Q2 offset voltage of 5 V was used and collision energy was set to be 40 eV for rosuvastatin and 25 eV for topotecan and SN-38.

*Data Analysis*

The equations used to calculate apparent uptake rate ($Uptake_{app}$), apparent intrinsic biliary clearance ($CL_{bile,int,app}$) (Liu, et al., 1999c) and biliary excretion index (BEI)[2] (Liu, et al., 1999b) over a 10-minute interval are shown below. In the presence of $Ca^{++}/Mg^{++}$ the bile canaliculi remain intact (closed), while in the absence of $Ca^{++}/Mg^{++}$ the bile canaliculi tight junctions are disrupted (opened). Consequently, quantifying the accumulation of a test compound in the

[2] BEI was determined using B-CLEAR™ technology (Qualyst, Inc., Raleigh, NC).

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presence and absence of divalent cations allows one to determine the amount of test compound in the bile canaliculi.

$$\text{Uptake}_{app} = \frac{\Delta\text{Accumulation}_{(+Ca^{++}/Mg^{++},\,10-2\ min)}}{\Delta T_{(10-2\,min)}} \qquad (1)$$

$$CL_{bile,int,app} = \frac{\text{Accumulation}_{(+Ca^{++}/Mg^{++})} - \text{Accumulation}_{(Ca^{++}/Mg^{++}-free)}}{\text{Incubation Time} \bullet \text{Concentration}_{(media)}} \qquad (2)$$

$$BEI = \frac{\text{Accumulation}_{(+Ca^{++}/Mg^{++})} - \text{Accumulation}_{(Ca^{++}/Mg^{++}-free)}}{\text{Accumulation}_{(+Ca^{++}/Mg^{++})}} \bullet 100 \qquad (3)$$

## Results

### *Cell Culture Conditions*

To determine cell culture conditions, various plate coatings, cell densities, overlay matrices and media were examined using 24-well plates. Four separate plate coatings were tested and it was generally found that hepatocytes were more uniformly distributed on rigid collagen (RC) and BioCoat™ plates compared to gelled collagen (GC) or Matrigel™ plates (Figure 1). Cell densities of $0.2 \times 10^{-6}$, $0.35 \times 10^{-6}$ and/or $0.5 \times 10^{-6}$ cells/well (in 500 µL) were also tested on rigid collagen-coated plates and BioCoat™ plates. The lowest density of cells exhibited poor plate coverage and poor cell-cell contact resulting in little or no bile canaliculi formation. Use of a cell density of $0.35 \times 10^{-6}$ cells/well consistently resulted in near confluent distribution of cells, while use of $0.5 \times 10^{-6}$ cells/well exhibited a similar degree of confluence and a similar BEI for taurocholate relative to that observed with $0.35 \times 10^{-6}$ cells/well (data not shown). Regarding the overlay matrices, a gelled collagen overlay was tested on gelled collagen-coated plates; a Matrigel™ overlay was tested on Matrigel™-coated plates (Figure 1) and BioCoat™ plates; and a rigid collagen overlay was tested on rigid collagen-coated plates and BioCoat™ plates. Cell morphology was well maintained with GC/GC and similar to that observed with BC/Matrigel™ (Figure 1); however, using GC is difficult in a 24-well format and therefore not desirable for use in a routine assay. Both the RC/RC and BioCoat™ /RC configurations exhibited similar cell morphology and bile canaliculi formation. However, bile canaliculi formation appeared less extensive compared to the BioCoat™ /Matrigel™ configuration (Figure 2), which is consistent with a single study in which the BEI of 1 µM taurocholate in both the RC/RC and BC/RC configurations (34% and 39%, respectively) was significantly lower than that observed using the

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BioCoat™ /Matrigel™ configuration (63 ±15%, N=8). An additional disadvantage of using a collagen overlay is that extra care is needed when rinsing the cells, since the collagen overlay is easily dislodged, whereas this is not the case with a Matrigel™ overlay.

Using a BioCoat™ / Matrigel™ sandwich configuration, various cell culture media – InVitroGRO™, HMM, WME and DMEM – were examined and evaluated based upon transporter substrate function assays. $Uptake_{app}$ of taurocholate was highest with InVitroGRO™ and WME, but use of InVitroGRO™ resulted in the highest $CL_{bile,int,app}$ and BEI for taurocholate, while use of DMEM resulted in the lowest values for $uptake_{app}$ and $CL_{bile,int,app}$ (Figure 3). Overall, the recommended conditions (based on experimental results and ease of use) for sandwich culture of cryopreserved human hepatocytes in a 24-well format are: BioCoat™ plates, a cell density of $0.35 \times 10^{-6}$ cells/well in 500 µL[3], an overlay of Matrigel™ and InVitroGRO™ media. As shown in Figure 2, using these conditions sandwich-cultured cryopreserved human hepatocytes maintained good morphology and formed distinct bile canaliculi. In addition, a time course study showed that bile canaliculi were well formed by day 5 and that the extent of formation began to decline after day 6 (Figure 4).

*Substrate Efflux*

Several compounds were chosen to assess the function of multiple uptake and efflux transporters in cryopreserved human hepatocytes cultured in a sandwich configuration. Representative plots of accumulation of compound over time are shown in Figure 5. In this example, taurocholate, a substrate of the uptake transporters NTCP and OATPs and the efflux transporter BSEP, was

[3] In Vitro Technologies, Inc. recommends this cell density and volume.

rapidly taken up into human hepatocytes and effluxed with a BEI of 75%. Digoxin, a substrate for the uptake transporter OATP-8 and the efflux transporter MDR1, showed a slower $uptake_{app}$ but a significant degree of efflux as evidenced by a BEI of 59%. Estradiol-17β-D-glucuronide, a substrate of the uptake transporters OATPs and the efflux transporter MRP2, exhibited a BEI of 43%. In contrast, salicylate exhibited essentially no efflux into bile canaliculi, consistent with this compound not being excreted into bile *in vivo* (Laznicek and Laznickova, 1994). In addition to transport activity, RT-PCR of day 5 samples from hepatocyte lots 130 and QKR showed expression of all tested transporters (OATP-B, OATP-C, BCRP, BSEP, MDR1 and MRP2) and Western blotting of day 5 samples from hepatocyte lots 130, QKR or FEP using antibodies against MDR1/3, MRP2 or BCRP, respectively, showed positive bands (data not shown).

The uptake and efflux of taurocholate and digoxin were assessed in multiple lots of cryopreserved human hepatocytes as well as two lots of fresh human hepatocytes. As shown in Figure 6, across five lots of cryopreserved hepatocytes, average taurocholate $uptake_{app}$ was high and ranged from 11-17 pmol/min/mg protein. Similarly, taurocholate $uptake_{app}$ in the two fresh hepatocytes lots was also high at 11 and 17 pmol/min/mg protein. The range of values for taurocholate $CL_{bile,int,app}$ among cryopreserved hepatocyte lots was 5.8 – 10 µL/min/mg protein, while the two fresh lots exhibited $CL_{bile,int,app}$ values of 6.9 and 9.9 µL/min/mg protein. Average BEI for taurocholate ranged from 41 – 63%, while the BEI for this substrate in each of two fresh human hepatocyte lots was 50% and 59%. Similar to the results presented in Figure 5, the time course of accumulation of taurocholate in fresh and cryopreserved human hepatocytes in sandwich culture was similar (Figure 7). As shown in Figure 8, average $uptake_{app}$ for digoxin ranged from 0.69 – 1.5 pmol/min/mg protein, while $uptake_{app}$ in the two fresh hepatocytes lots

were 0.75 and 1.8 pmol/min/mg protein. The range of values for digoxin $CL_{bile,int,app}$ among cryopreserved hepatocyte lots was 0.60 – 1.5 µL/min/mg protein, while the two fresh lots exhibited $CL_{bile,int,app}$ values of 0.5 and 2.4 µL/min/mg protein. The average BEI for digoxin in the six cryopreserved lots ranged from 37 – 63%, while the BEI in each of two fresh human hepatocyte lots was 33% and 56%.

Substrates of BCRP and MRP2 were also tested in this model. As seen in Table 2, all BCRP substrates tested showed uptake and efflux. Both rosuvastatin and mitoxantrone exhibited high $uptake_{app}$ in two separate lots of cryopreserved hepatocytes. In all lots tested, $CL_{bile,int,app}$ and BEI for rosuvastatin were relatively high. Cryopreserved hepatocytes also exhibited functional MRP2 activity as shown in Table 3. BSP exhibited very rapid $uptake_{app}$ in both lots of hepatocytes (35 and 37 pmol/min/mg protein) and high $CL_{bile,int,app}$, yet BEI values were low (11-23%). Estradiol-17-β-D-glucuronide was taken up readily into cryopreserved hepatocyte lot 130, while $uptake_{app}$ for lot QKR was ~6-fold lower. This compound exhibited an $CL_{bile,int,app}$ of 3.8 and 1.8 µL/min/mg protein, and a BEI 36% and 37%, in hepatocyte lots 130 and QKR, respectively.

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## Discussion

The use of sandwich-cultured fresh hepatocytes to study hepatic uptake, metabolism and efflux has previously been reported. However, to date, this model has only been used with fresh hepatocytes. This is a significant disadvantage, particularly with regard to human hepatocytes, since the availability of fresh human tissue of high quality is unpredictable and when it is available it is often at odd times. The ability to circumvent this limitation by using cryopreserved human hepatocytes is now possible due the increased commercial availability of cryopreserved human hepatocytes. Equally important, being able to conduct sandwich-culture with cryopreserved hepatocytes will allow investigators to conduct multiple studies on multiple days using hepatocytes from a single donor in order to assess intra-assay variability and to study multiple endpoints (i.e. transport and metabolism).

The results described in this study demonstrate that cryopreserved human hepatocytes can be used in a sandwich configuration to repolarize and form intact bile canaliculi, and that this model can successfully be used to measure hepatic uptake and efflux transport. The substrates tested show that there is functional NTCP- and OATP-mediated uptake and that there is functional efflux due to MDR1, MRP2, BSEP and BCRP. Importantly, the $uptake_{app}$ and efflux of digoxin and taurocholate in multiple lots of cryopreserved hepatocytes were similar to that observed with two lots of fresh human hepatocytes. The ability to conduct these studies in 24-well plates has also been demonstrated, which reduces the number of hepatocytes needed per test compound and which allows for more compounds to be tested in a given study. The results also show that LC/MS/MS can routinely be used to quantify the transport of unlabeled test compounds at low micromolar concentrations.

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In addition to an absolute determination of uptake or efflux transport for a given substrate, this model may be used to rank compounds. For example, as seen in Figures 6 and 8, $uptake_{app}$ and $CL_{bile,int,app}$ for TC is much higher (~10-fold) compared to that observed for digoxin. This is consistent with a previous *in vivo* study showing high clearance of taurocholate (Cowen, et al., 1975) and previous in *vitro* and *in vivo* observations showing that digoxin has a relatively slow uptake into hepatocytes and it's *in vivo* biliary clearance is low (Olinga, et al., 1998; Angelin, et al., 1987). It is interesting to note with taurocholate that $uptake_{app}$ and $CL_{bile,int,app}$ across all lots of cryopreserved and fresh hepatocytes varied by <2-fold. In contrast, $uptake_{app}$ and $CL_{bile,int,app}$ of digoxin across all hepatocyte lots tested ranged from 2.6- to 5-fold, respectively. For digoxin, the larger variability is primarily due to the high $uptake_{app}$ and $CL_{bile,int,app}$ observed for the fresh hepatocyte lot F00927. Nevertheless, to better understand the source of this variability one would need to determine the expression of the uptake and efflux transporters in the various lots of hepatocytes in this model. Variability was also observed for estradiol glucuronide $uptake_{app}$ between hepatocyte lots 130 and QKR (Table 3). The 6-fold variability observed is similar to the 4- to 5-fold variability observed for uptake of this compound in suspension cultures of cryopreserved or fresh hepatocytes, respectively (Shitara, et al., 2003).

A common criticism of the sandwich culture model is that it is potentially cholestatic, since the model does not currently allow for the constant draining of the bile canalicular tree that occurs *in vivo*. Consequently, this may result in altered regulation/expression of transporters, especially NTCP. In fact, Brouwer and co-workers have shown that sandwich-culturing of fresh rat hepatocytes results in a significant down regulation of Ntcp expression (Liu, et al., 1998).

Although the temporal relationship of basolateral and apical transporter expression levels has not yet been completed with cryopreserved human hepatocytes, data from a recent publication suggest a trend toward better maintenance of NTCP expression in fresh human hepatocyte cultures compared to fresh rat hepatocyte cultures - after 3 days in culture NTCP mRNA declines <40% in human compared to >95% in rat. Likewise, NTCP-mediated activity in human hepatocyte cultures is somewhat maintained (although quite variable) after 5 days in culture (Jigorel, et al., 2005). This needs to be further investigated using our current conditions with cryopreserved hepatocytes, but it nevertheless highlights the potential for species differences in this model and therefore reemphasizes the advantage of being able to conduct studies, as needed, with cryopreserved human hepatocytes.

In this paper we have utilized two parameters to assess biliary elimination – $CL_{bile,int,app}$ and BEI. It is important to note that BEI is not merely the *in vitro* equivalent of the percent of an *i.v.* dose excreted in bile *in vivo*. BEI is the percent of drug *within the hepatocytes* that has been excreted into bile canaliculi - it is dependent solely on the transport of drug across the apical membrane. In contrast, *in vivo*, the amount of an i.v. administered drug recovered in the bile is dependent upon transport across the hepatic basolateral membrane and transport into the bile canaliculi, as well as any non-hepatic clearance mechanisms. Evidence has been given that, generally, BEI can be used to rank compounds as those that will exhibit a low or high percent of biliary excretion *in vivo*, and it may be expected that a compound that exhibits a high biliary clearance *in vivo*, should exhibit a high BEI (> ~50%) *in vitro* (Liu, et al., 1999c). However this is not always the case as evidenced by the data with BSP (Table 3). Historically, BSP was used extensively to monitor liver function since it is rapidly taken up into the liver and readily

excreted into the bile. In fact, early work with BSP showed that it accumulates in the liver to levels much higher than that in plasma, due to its very high uptake rate, and that biliary clearance, although high, quickly becomes saturated (Wheeler, et al., 1960). Our data indeed show a very rapid $uptake_{app}$ of BSP (Table 3). This $uptake_{app}$ is much higher than even that of the endogenous bile acid taurocholate (Figure 6). $CL_{bile,int,app}$ is also high and similar to that of taurocholate; however, the BEI for BSP is low and not consistent with *in vivo* observations. The likely reason for this discrepancy is that BSP is taken up very rapidly, accumulates in the hepatocyte and quickly saturates efflux, as observed *in vivo*. The level of drug is so high initially that during the short interval of the experiment (15 minutes), the amount excreted in the bile is small. Consequently, this highlights the need to carefully interpret BEI data when dealing with drugs that exhibit very high uptake rates. This example also highlights the utility, and preference, for determining $CL_{bile,int,app}$ instead of BEI.

In summary, the ability to use cryopreserved human hepatocytes in sandwich culture to form intact bile canaliculi and to exhibit functional uptake and efflux transport has been successfully demonstrated. The higher capacity afforded by the use of 24-well plates and the ability to quantify test compounds via LC/MS/MS make this an attractive model within the pharmaceutical industry. Use of cryopreserved hepatocytes allows studies to be conducted as needed. In addition, the use of cryopreserved hepatocytes could allow for pools of multiple donors to be tested in this model if desired. Studies are ongoing to further characterize hepatobiliary disposition using cryopreserved human hepatocytes in this model.

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# Figure Legends

**Figure 1. Effect of Various Plate Coatings on the Distribution of Sandwich-Cultured Cryopreserved Human Hepatocytes.** Sandwich cell cultures were prepared in 24-well plates using various plate coatings followed by an overlay of rigid collagen (RC), gelled collagen (GC) or Matrigel™ (M) and maintained using InVitroGRO™ media. The phase contrast photos were taken on day 5. A, hepatocyte lot QKR, RC coating / RC overlay; B, hepatocyte lot QKR, GC coating / GC overlay; C, hepatocyte lot 130, Matrigel™ coating / Matrigel™ overlay; D, hepatocyte lot 130, BioCoat™ plate / Matrigel™ overlay.

**Figure 2. Formation of Bile Canaliculi in Sandwich-Cultured Cryopreserved Human Hepatocytes.** Sandwich cell cultures were prepared using rigid collagen (RC) or Biocoat™/Matrigel™ in 24-well plates with InVitroGRO™ media. On day 5, cultures were incubated with 5 µM CDFDA for 20 minutes. CDFDA is hydrolyzed to fluorescent CDF (5-(and-6)-carboxy-2', 7'-dichiloro-fluorescein) inside the hepatocytes, which is then transported into bile canaliculi via MRP2. Panels on the left were obtained by phase contrast microscopy and panels on the right were obtained by fluorescent microscopy. Red arrows show examples of bile canaliculi.

**Figure 3. Effect of culture media on the uptake, biliary clearance and biliary excretion of 1 µM taurocholate in sandwich-cultured human hepatocyte lot QKR.** Hepatocytes were cultured in a sandwich configuration using various media and assayed for taurocholate efflux on

day 5. Data are expressed as the mean ± SD (N=3) for InVitroGRO™ media, or as the average of two separate studies for HMM, WME and DMEM.

**Figure 4. Formation of Bile Canaliculi in Sandwich-Cultured Cryopreserved Human Hepatocyte Lot FEP Over Multiple Days.** Sandwich cell cultures were prepared using Biocoat™/Matrigel™ in 24-well plates with InVitroGRO™ media. On each day from day 3 – 8, cultures were incubated with 5 µM CDFDA for 20 minutes. CDFDA is hydrolyzed to fluorescent CDF (5-(and-6)-carboxy-2', 7'-dichiloro-fluorescein) inside the hepatocytes, which is then transported into bile canaliculi via MRP2.

**Figure 5. Representative Accumulation Plots for Various Substrates Using Human Cryopreserved Hepatocytes in Sandwich-Culture.** Studies were conducted as described in Material and Methods. Each plot represents the mean ± SD of three replicates at each time point generated during a single experiment. BEI was determined at 10 minutes.

**Figure 6. Uptake, Biliary Clearance and Biliary Excretion of Taurocholate in Various Lots of Sandwich-Cultured Fresh or Cryopreserved Human Hepatocytes.** Data represent the average of separate studies (indicated in brackets). For lots 130, FEP and QKR, the data represent the mean ± SD. Fresh hepatocyte lots are indicated with an asterisk.

**Figure 7. Comparison of [$^{14}$C]Taurocholate Accumulation Between Fresh and Cryopreserved Hepatocytes Cultured in a Sandwich Configuration on Day 5.** Results are expressed as the mean ± SD of four separate experiments run in triplicate (cryopreserved lot

QKR) or the mean of four replicates from a single experiment (fresh, IVT lot F00927). A BioCoat™/Matrigel™ sandwich configuration was used. The taurocholate concentration was 1 µM.

**Figure 8. Uptake, Biliary Clearance and Biliary Excretion of Digoxin in Various Lots of Sandwich-Cultured Fresh or Cryopreserved Human Hepatocytes.** Data represent the average of separate studies (indicated in brackets). For lot FEP, the data represent the mean ± SD. Fresh hepatocyte lots are indicated with an asterisk.

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**Table 1.**

**Characteristics of Fresh and Cryopreserved**

**Hepatocytes Obtained from Commercial Sources.**

The cryopreserved lots (130, FEP, LOF, NLR and QKR) and the fresh lot F00927 were purchased from In Vitro Technologies, Inc. Fresh lot Hu156 was purchased from CellzDirect™.

| Lot Number | Sex | Age | Race | Post Thaw Viability |
|---|---|---|---|---|
| 130 | F | 2 | C | 79% |
| FEP | F | 59 | AA | 92% |
| LOF | F | 54 | C | 86% |
| NLR | M | 13 | C | 84% |
| QKR | M | 35 | C | 97% |
| F00927 | F | 56 | C | – |
| Hu156 | M | 66 | C | – |

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**Table 2.**

**Uptake, Biliary Clearance and Biliary Excretion of BCRP Substrates in Sandwich-Cultured Cryopreserved Human Hepatocytes**

All compounds were tested as described in Materials and Methods. Compounds were tested at 2 μM, except for mitoxantrone (1 μM). Results are presented as the average of two separate experiments, except for lot 130 rosuvastatin (mean ± SD of three separate experiments).

| Hepatocyte Lot | Compound | $Uptake_{app}$ (pmol/min/mg protein) | $CL_{bile,int,app}$ (μL/min/mg protein) | BEI |
|---|---|---|---|---|
| 130 | Rosuvastatin | 26 ± 17% | 8.1 ± 3.5 | 58 ± 17% |
| | Mitoxantrone | 22 | 4.5 | 17% |
| QKR | Rosuvastatin | 31 | 12 | 45% |
| | Mitoxantrone | 23 | 2.9 | 11% |
| FEP | Rosuvastatin | 15 | 4.0 | 43% |
| | Topotecan | 2.8 | 1.1 | 33% |
| | SN-38 | 3.3 | 3.3 | 51% |

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**Table 3.**

**Uptake, Biliary Clearance and Biliary Excretion of MRP2**

**Substrates in Sandwich-Cultured Cryopreserved Human Hepatocytes**

All compounds were tested at 1 μM as described in Materials and Methods.

Results are presented as the average of two separate experiments.

| Hepatocyte Lot | Compound* | $Uptake_{app}$ (pmol/min/mg protein) | $CL_{bile,int,app}$ (μL/min/mg protein) | BEI |
|---|---|---|---|---|
| 130 | EG | 13 | 3.8 | 36% |
| | BSP | 35 | 9.9 | 23% |
| QKR | EG | 2.2 | 1.8 | 37% |
| | BSP | 37 | 4.9 | 11% |

* EG = estradiol-17β-D-glucuronide; BSP = sulfobromophthalein.

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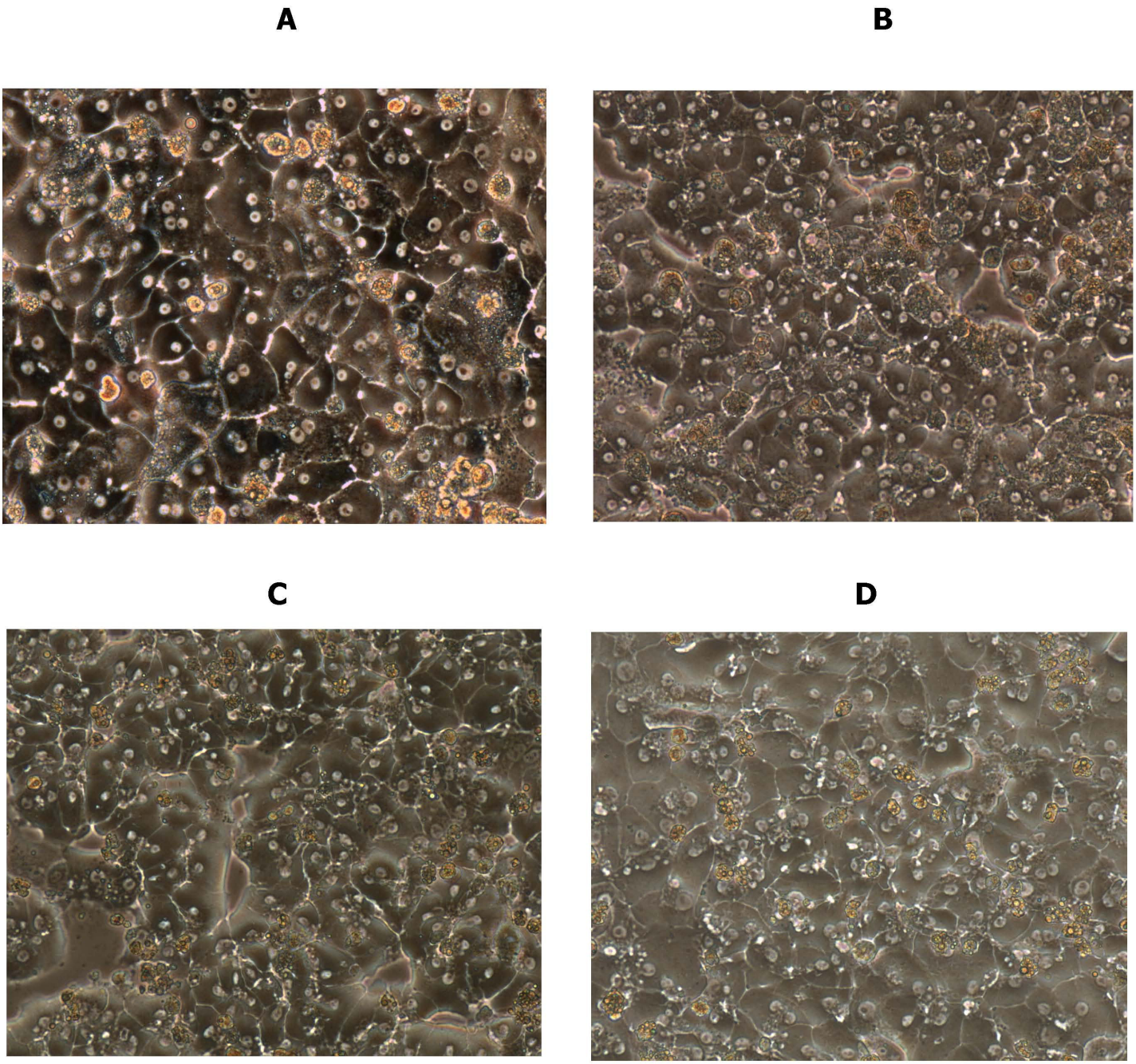
# FIGURE 1.



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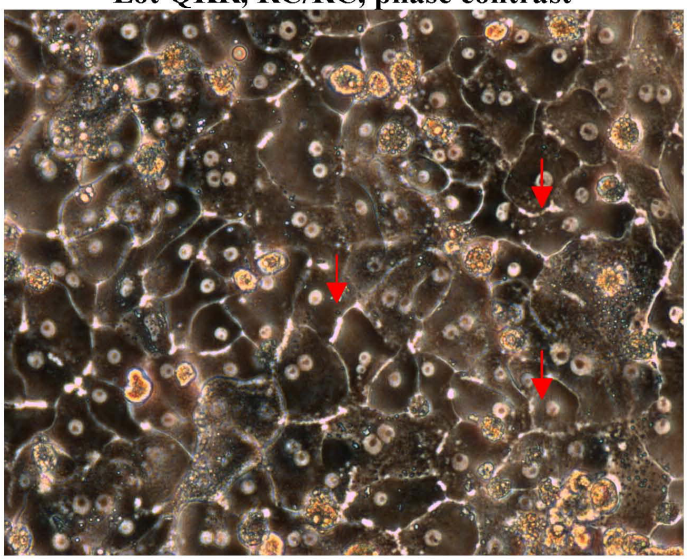
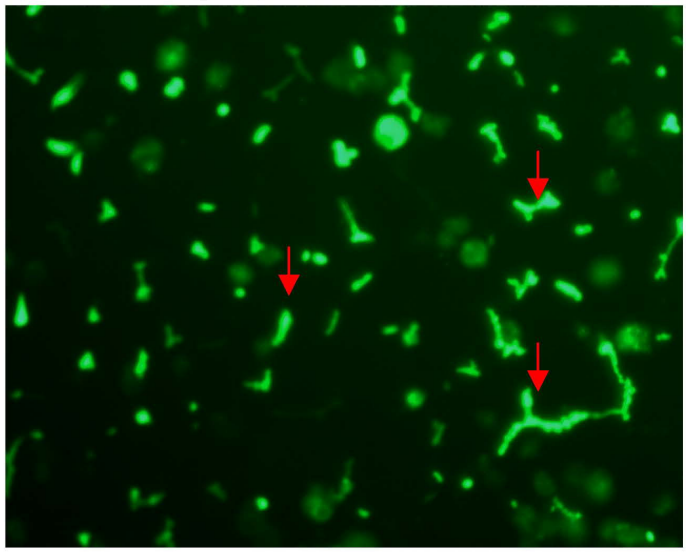
# Figure 2.

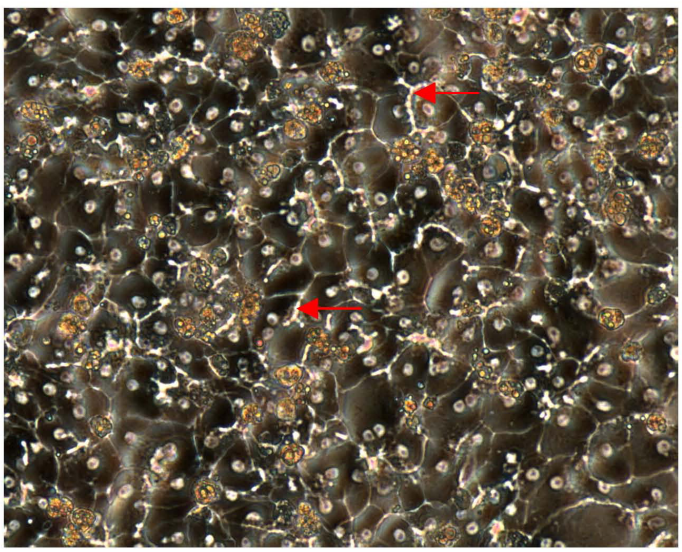
Lot QKR, RC/RC, phase contrast



Lot QKR, RC/RC, fluorescence



Lot 130, BioCoat™ /Matrigel™, phase contrast



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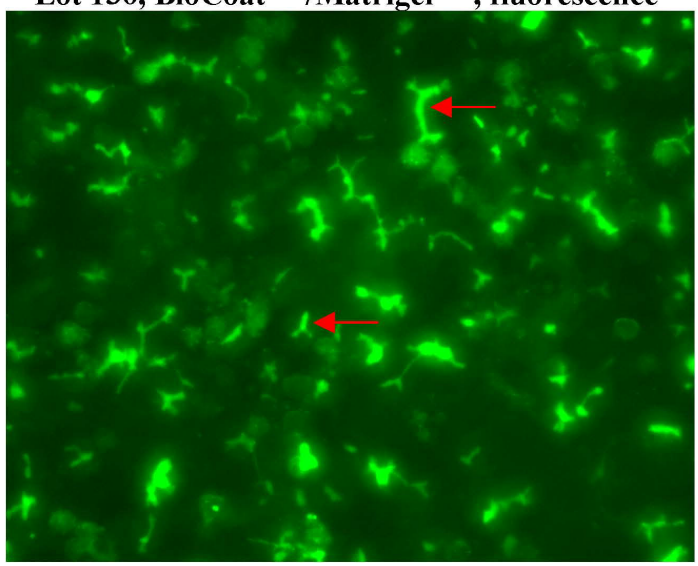

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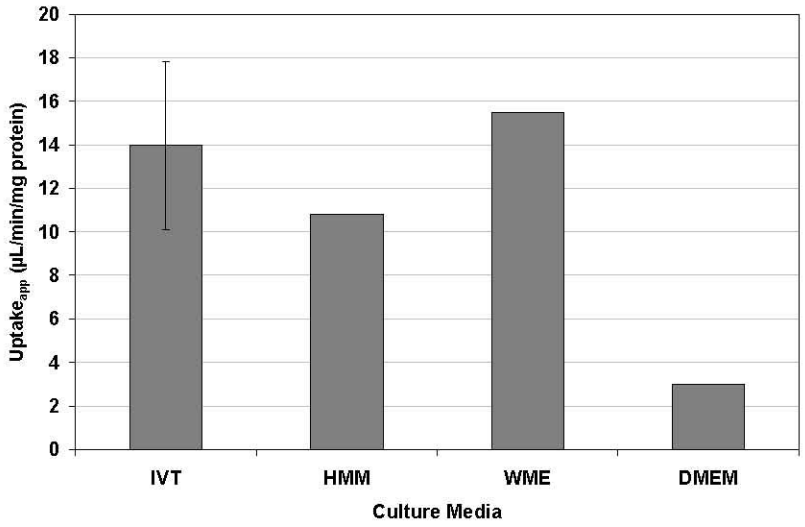


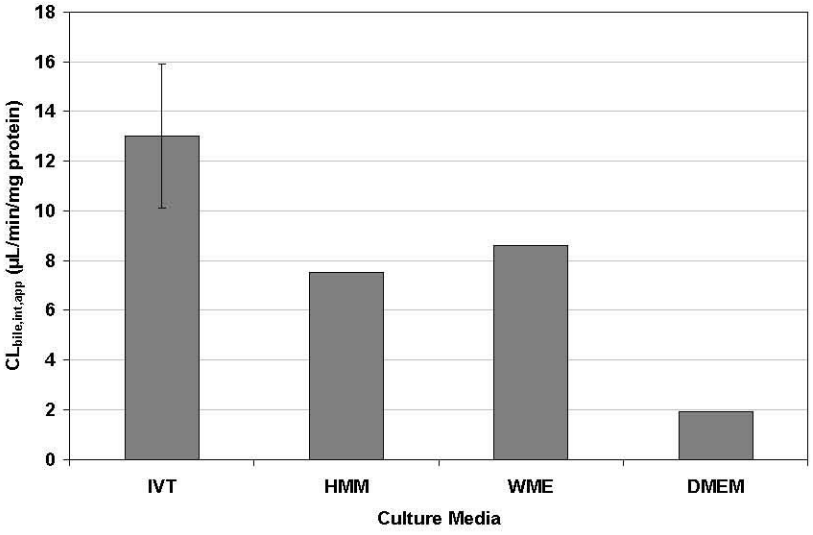


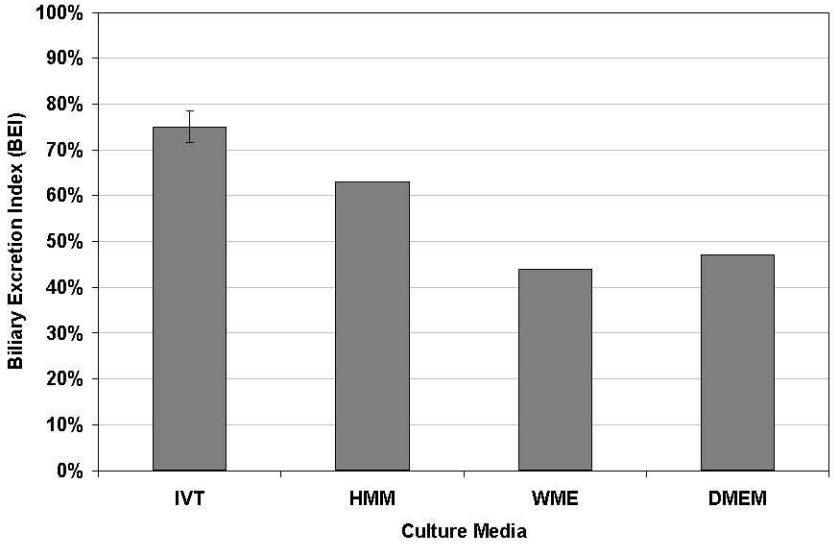

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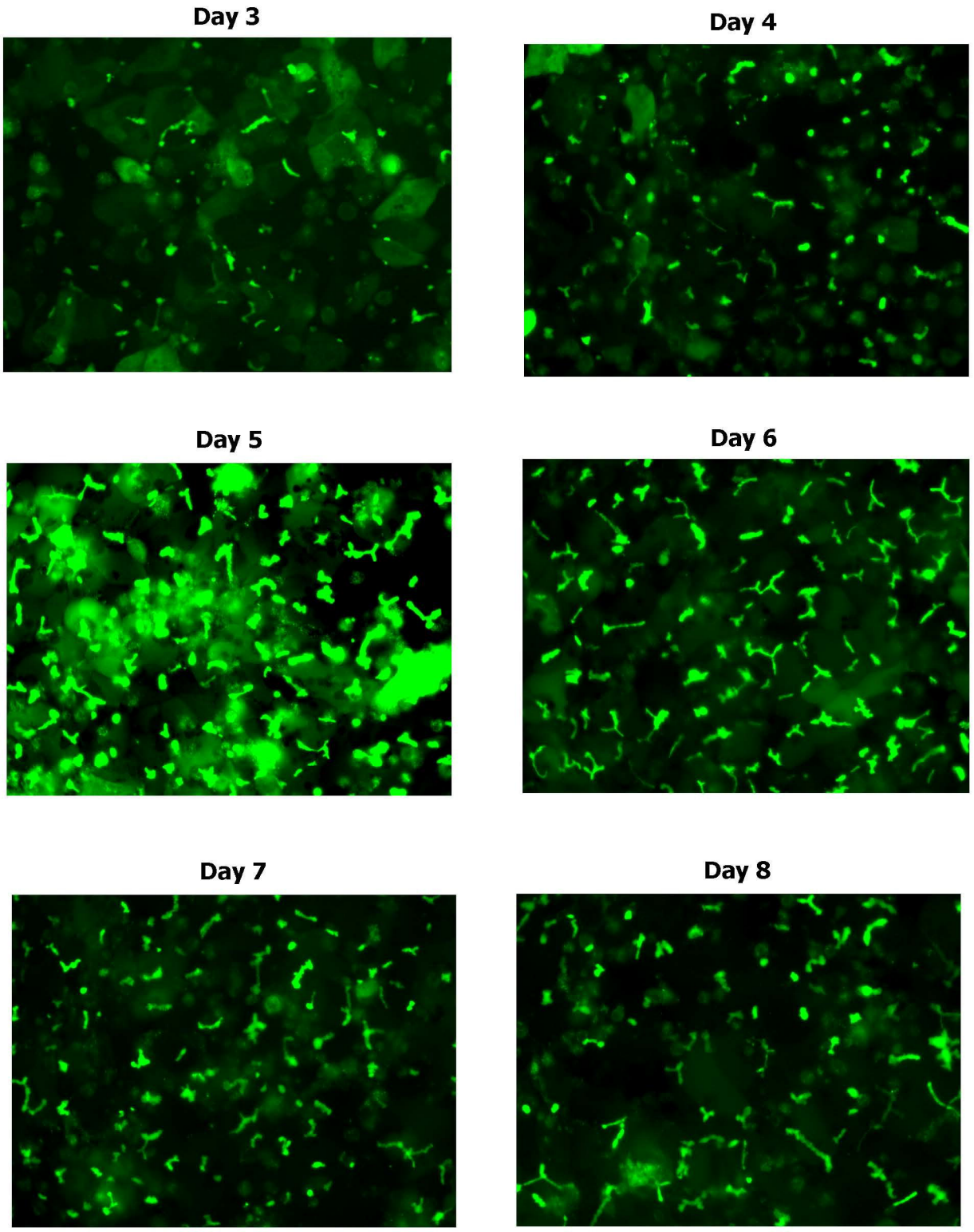
# Figure 4



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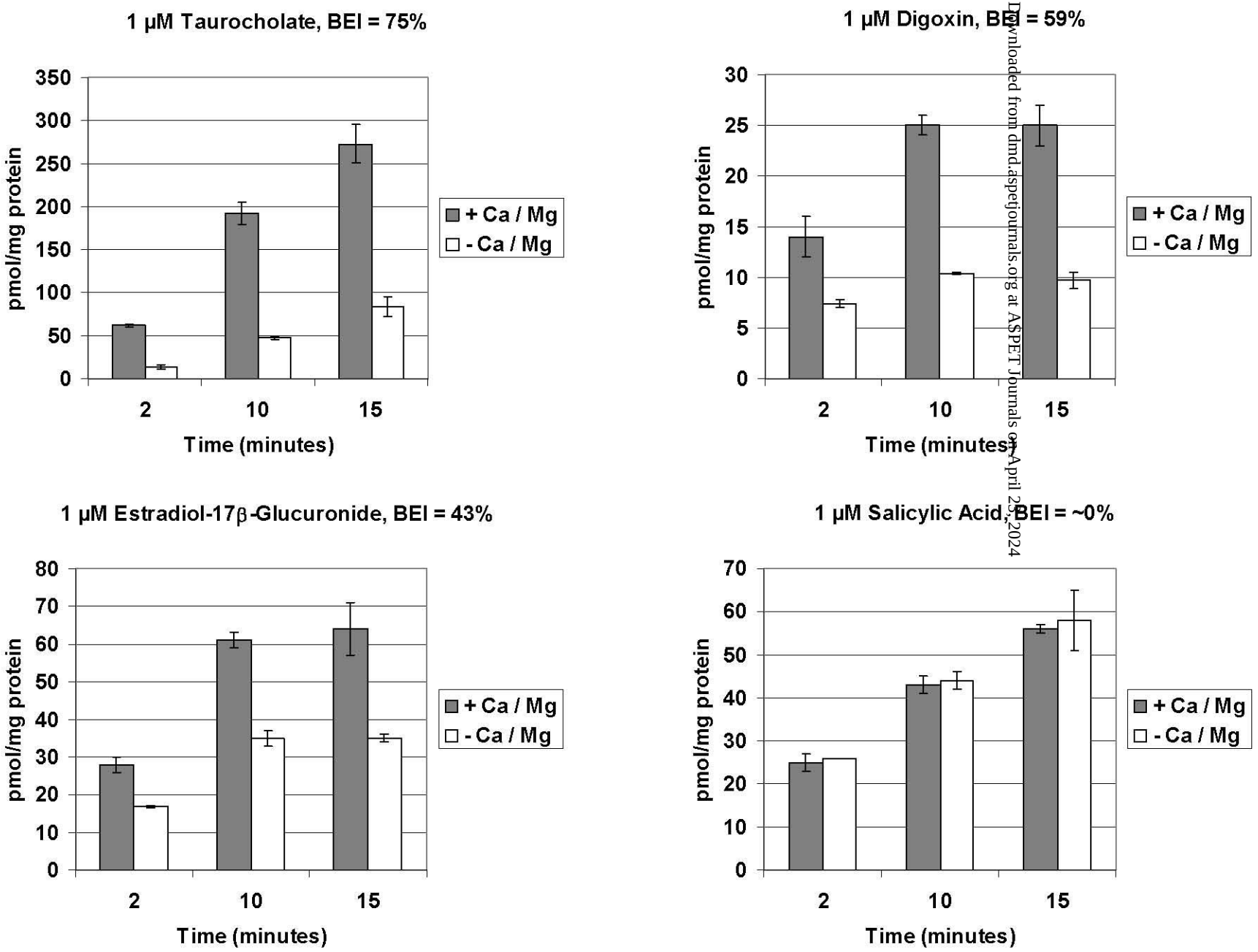

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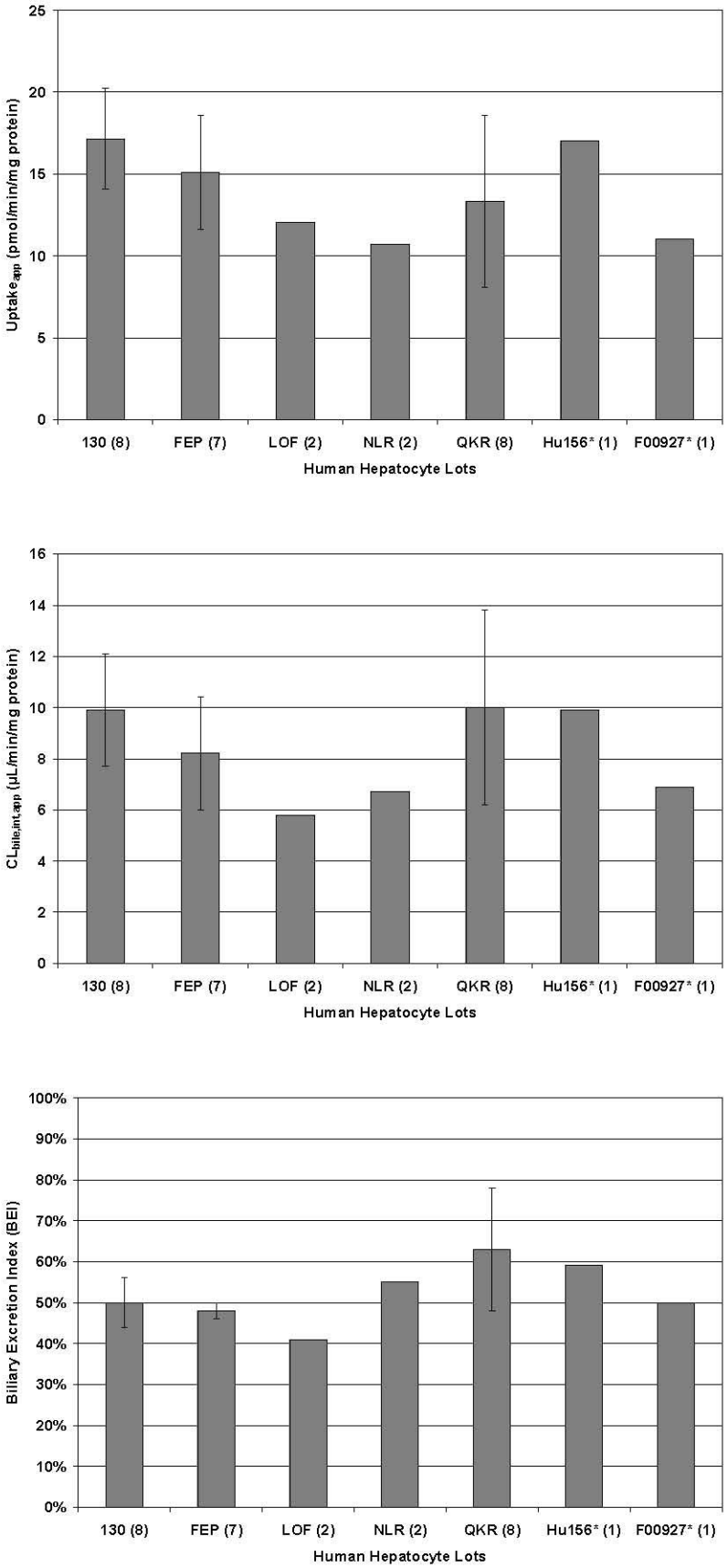

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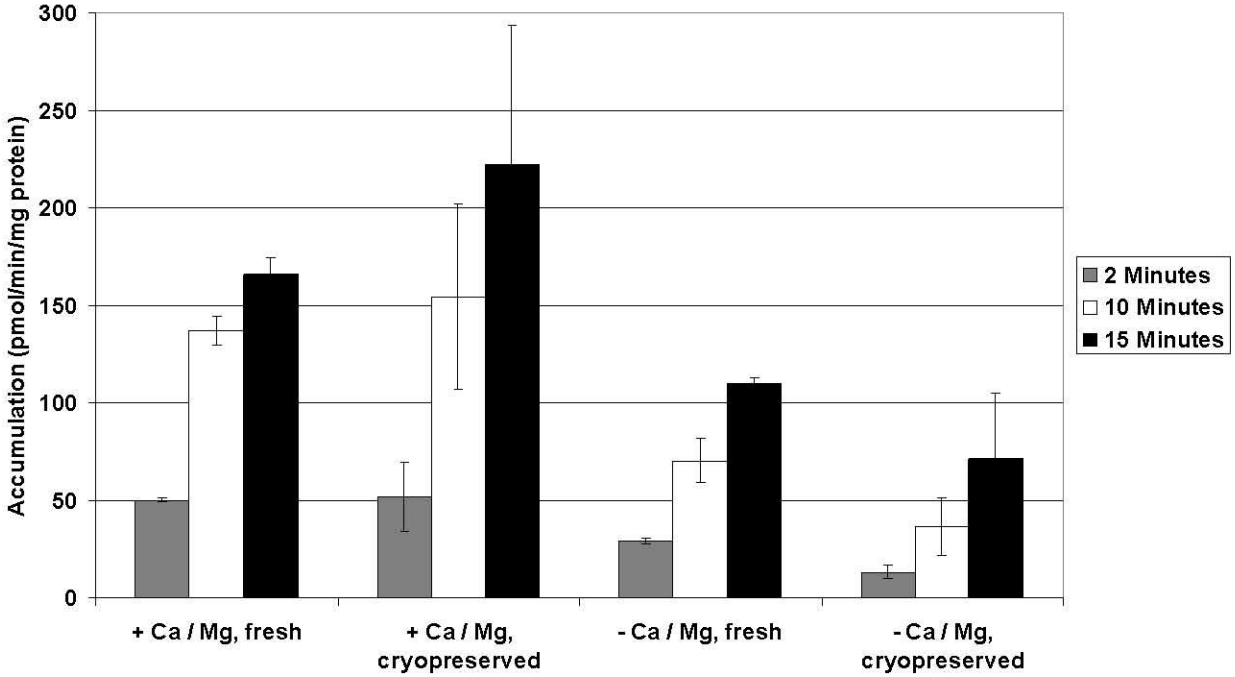

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# Figure 8.