

DMD #63495

TITLE: Metabolite profiling and pharmacokinetic evaluation of hydrocortisone in a perfused 3D human liver bioreactor

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DMD #63495

RUNNING TITLE: Hydrocortisone metabolism in a 3D bioreactor

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Abbreviations:

HC, hydrocortisone; RP-UHPLC-QTOF-MS, reversed phase-ultra high performance liquid chromatography-quadrupole time of flight-mass spectrometry; $t_{1/2}$, half-life; k_{el} , elimination rate constant; CL, clearance; AUC, area under the curve; HSA, human serum albumin; CL_{int} , intrinsic clearance; CL_h , predicted hepatic clearance; GR, glucocorticoid receptor; PK/PD, pharmacokinetics/pharmacodynamics; CBG, corticosteroid binding globulin; 11 β -HSD, 11-beta-hydroxysteroid dehydrogenase; WEM, Williams E medium; LPS, lipopolysaccharide; SDS, sodium dodecyl sulphate; IL6, interleukin 6; TNF α , tumor necrosis factor alpha; CYP3A, cytochrome P450; EIC, extracted ion chromatogram; MFE, molecular feature extractor; KEGG, Kyoto encyclopedia of genes and genomes; MSC, molecular structure correlator; V_d , volume of distribution; LOD, limit of detection; LOQ, limit of quantification; CID, collision-induced dissociation; P450 UGT, uridine diphosphate (UDP) glucuronosyltransferase; DARPA; Defense Advanced Research Projects Agency; Q, hepatic blood flow; f_u , fraction of drug unbound to plasma protein.

DMD #63495

ABSTRACT

Endotoxin lipopolysaccharide (LPS) is known to cause liver injury primarily involving inflammatory cells such as Kupffer cells, but few in vitro culture models are applicable for investigation of inflammatory effects on drug metabolism. We have developed a 3D human microphysiological hepatocyte-Kupffer-cell coculture system and evaluated the anti-inflammatory effect of glucocorticoids on liver cultures. LPS was introduced to the cultures to elicit an inflammatory response and assessed by the release of pro-inflammatory cytokines, IL6 and TNF α . A sensitive and specific RP-UHPLC-QTOF-MS method was used to evaluate hydrocortisone disappearance and metabolism at near physiological levels. For this, the systems were dosed with 100 nM hydrocortisone and circulated for two days; hydrocortisone was depleted to approximately 30 nM, with first-order kinetics. Phase I metabolites, including tetrahydrocortisone and dihydrocortisol, accounted for 8-10 % of the loss, and 45-52 % was phase II metabolites, including glucuronides of tetrahydrocortisol and tetrahydrocortisone. Pharmacokinetic parameters, i.e., half-life ($t_{1/2}$), rate of elimination (k_{el}), clearance (CL), and area under the curve (AUC), were 23.03 h, 0.03 h⁻¹, 6.6x10⁻⁵ L. h⁻¹ and 1.03 $\frac{\text{mg}}{\text{L}} \cdot \text{h}$ respectively. The ability of the bioreactor to predict the in vivo clearance of hydrocortisone was characterized and the obtained intrinsic clearance values correlated with human data. This system offers a physiologically-relevant tool for investigating hepatic function in an inflamed liver.

DMD #63495

INTRODUCTION

Inflammation of the liver may result from infection, autoimmune disorders, alcohol abuse or fat accumulation (Adams et al., 2010). Severe inflammation can be life-threatening while mild inflammation can change drug metabolism profiles (Adams et al., 2010) and may play a role in liver injury (Park et al., 2004; Deng et al., 2009). Drug induced liver injury has resulted in late-stage clinical trial failures and post-market withdrawals (Guengerich, 2011).

A postulated mechanism for toxicity involves leakage of bacterial products from the gut to the liver, causing inflammation-related susceptibility to liver injury (Deng et al., 2008). This has been demonstrated in animal models (Roth et al., 2003; Deng et al., 2009; Roth and Ganey, 2010) but could be supported by a human-derived in vitro model that recapitulates the response of liver to inflammation. The simplest model is the 2D culture of primary hepatocytes, either as a monolayer or overlaid with extracellular matrix components in multiwell plates. Through exogenous addition of cytokines or other inflammatory stimuli to the cultures, 2D hepatocyte monocultures can probe inflammation-associated drug hepatotoxicity (Cosgrove et al., 2009). The absence of macrophages, however, makes it unsuitable for modelling anti-inflammatory effects.

Numerous advanced liver models, which improve the long term functional performance of hepatocytes compared to monocultures, have been described (Groothuis and Meijer, 1992; LeCluyse et al., 2012); a sub-set of these, incorporates liver non-parenchymal cells in coculture-(Maier and Milosevic, 1999; Milosevic et al., 1999; Zinchenko et al., 2006; Dash et al., 2009a; Domansky et al., 2009; Kostadinova et al., 2013). For inflammatory models, cultures containing Kupffer cells - either added as a separate fraction

DMD #63495

or as part of a whole non-parenchymal fraction - are most relevant. In these systems the non-parenchymal cell fraction or Kupffer cells may be mixed with hepatocytes to form heterogeneous aggregates (Drewitz et al., 2011; Messner et al., 2013; Thoma et al., 2014), supported on a scaffold (Dash et al., 2009b; Kostadinova et al., 2013) or separated through micropatterning (Zinchenko et al., 2006). Transwell devices have also been evaluated (Milosevic et al., 1999). Hoebe, et al., (Hoebe et al., 2000; Hoebe et al., 2001) showed that direct cell-to-cell contact between porcine hepatocytes and Kupffer cells increased the inhibitory effects of LPS stimulation on testosterone metabolism. Hepatocyte and Kupffer cell models have also been applied to the study of Kupffer cell suppression of cytochrome P450-mediated metabolism in humans (Sunman et al., 2004) and rat cells (Milosevic et al., 1999). Models based on hepatocytes and a mixed non-parenchymal cell fraction system have also been used to explore exaggerated hepatotoxicity in conjunction with an underlying inflammation (Dash et al., 2009b; Drewitz et al., 2011; Messner et al., 2013; Thoma et al., 2014),

Cortisol and, more typically the more potent synthetic glucocorticoid dexamethasone, are used at nanomolar to micromolar concentrations in cultures of primary hepatocytes to maintain a differentiated state (Hewitt et al., 2007; Scheving et al., 2007; Godoy et al., 2010). In a coculture of rat hepatocytes and epithelial cells, cortisol increased albumin secretion and extra-cellular matrix production (Baffet et al., 1982; Guillouzo et al., 1984).

Cortisol, the major glucocorticoid in humans, is produced in the adrenal cortex; regulated both by diurnal rhythms and in response to stress (Morse and Davis, 1990). When used as a drug, it is referred to as hydrocortisone, and it acts *in vivo* as an anti-inflammatory

DMD #63495

and suppresses immune responses (Perogamvros et al., 2012). Most serum cortisol is bound to proteins, including corticosteroid binding globulin (CBG) and serum albumin (Perogamvros et al., 2011a; Perogamvros et al., 2011b), with both free and total concentrations varying diurnally. Free concentrations are between 10-300 nM (Lewis et al., 2005; Levine et al., 2007). Free cortisol passes easily through cellular membranes into the cytosol, where it can be converted to the inactive form - cortisone - or bind to intracellular cortisol receptors and reach the nucleus (Fig. 1). The anti-inflammatory effect and metabolism of cortisol on liver have not been studied extensively *in vitro*.

Here we introduce a cryopreserved primary human hepatocyte and Kupffer cell inflammatory liver model in which the cells are cultured in direct contact on an engineered scaffold recapitulating the liver capillary bed under perfusion (Fig. 2) (Powers et al., 2002a; Domansky et al., 2010). The model is used to examine the anti-inflammatory effect of glucocorticoids on liver cultures and, in conjunction with a sensitive and specific RP-UHPLC-QTOF-MS method, to study the metabolism of-hydrocortisone at near physiological levels. The distribution of metabolites is compared to that in human urine.

MATERIALS AND METHODS

Deuterated hydrocortisone (DLM-2218-0), used as internal standard, was from Cambridge Isotope Laboratories (Tewksbury, MA). HPLC grade ($\geq 99.9\%$) methanol (CAS: 67-56-1), chloroform (CAS: 67-66-3), acetonitrile (CAS: 75-05-8), formic acid, (CAS: 64-18-6), molecular biology grade DMSO (CAS: 67-68-5), dexamethasone (CAS: 50-02-2), hydrocortisone (CAS: 50-23-7) and 17- α -hydroxy-3,20-dioxopregn-4-en-21-yl- β -D-glucuronide were purchased from Sigma Aldrich (St. Louis, MO). Calibration/tuning

DMD #63495

standard for the QTOF was from Agilent Technologies (Santa Clara, CA). The Oasis HLB cartridges were from EMD Millipore Corporation (Billerica, MA). Distilled water and acetonitrile were prepared in-house with double distillation.

Coculture in the LiverChip

Cryopreserved human hepatocytes and human Kupffer cells were purchased from Life Technologies (Paisley, UK). Cells were thawed according to the instructions provided by the supplier. Viability was assessed using the trypan blue exclusion test (Strober, 2001) and was > 85 % for all lots.

Cells were seeded into LiverChip platforms (CNBio Innovations, UK) (Fig. 2A) housed in a humidified cell culture incubator at 37 °C with 5 % CO₂. The LiverChip platforms consist of 12 fluidically isolated bioreactors in which fluid is re-circulated by a pneumatically driven micro-pump controlled by LiverChip hardware (Fig. 2B) through a scaffold containing cells (Fig. 2C). The scaffold enables the formation of an array of 3D microtissues composed of either hepatocytes alone or hepatocytes in coculture with non-parenchymal cells (Kupffer cells) (Domansky et al 2010). The platform is covered with a single loose lid, as per a standard microtiter plate enabling access to each of the 12 bioreactors for cell seeding, media change and sampling. Each bioreactor can hold a maximum of 2.5mL and has a surface channel which allows for efficient re-oxygenation of the media by gaseous exchange with the atmosphere.

Cells were seeded with flow in the downward direction through the scaffold, for 8 h at a flow rate of 1.0 μ L/s (Fig. 2D). Downward flow encourages cells to seed within the

DMD #63495

scaffold and form microtissues. Following cell attachment within the scaffold, the flow was changed to the upward direction and maintained at 1.0 $\mu\text{L/s}$ for the remainder of the culture.

Hepatocytes monocultures were seeded at a density of 0.6×10^6 viable cells in 1.6 mL of medium per well. The cells were maintained in Williams' E Medium (WEM) containing primary hepatocyte thawing and plating supplements (Life Technologies, Paisley, UK) for the first 24 h of culture and in WEM containing primary hepatocyte maintenance supplements thereafter for the duration of the culture.

Hepatocyte and Kupffer cell cocultures were seeded simultaneously, maintaining the same number of hepatocytes while adjusting the number of Kupffer cells to achieve the desired ratios of hepatocytes: Kupffer cells (Supplemental Table 1). Cocultures were seeded in WEM containing primary human hepatocyte thawing and plating supplements for the first 24 h of culture, and then in WEM with primary human hepatocyte maintenance supplements. On day 3, for both mono and cocultures, where appropriate, hydrocortisone (100 nM) was substituted for 100 nM dexamethasone and the media was changed every 48 h. To induce an inflammatory state in the cultures lipopolysaccharide (LPS) (Sigma, Poole, UK) was added in the culture medium.

Total protein

Cells and scaffolds were washed once in phosphate buffered saline (PBS) and lysed using 0.5 mL of 0.1 M NaOH containing 2 % sodium dodecyl sulphate (SDS). Total cellular protein was then measured with a Pierce BCA Protein Assay kit (Thermo Fisher, Loughborough, UK).

DMD #63495

Hepatocyte and Kupffer cell phenotyping

Albumin secretion was measured with a human albumin ELISA (Assay Pro, St Charles, USA). Urea was quantified with a colorimetric assay kit (BioAssay Systems, Hayward, USA). Interleukin 6 (IL6) and tumor necrosis factor alpha (TNF α) production were quantified in culture medium using human DuoSet Kits (R&D Systems, Abingdon, UK).

CYP3A activity

CYP3A activity was measured with the P450-Glo™ CYP3A4 assay with Luciferin-IPA (Promega, Southampton, UK). A complete medium change to medium containing a 1000-fold dilution of Luciferin-IPA was performed and the plate returned to the incubator, with flow. After 1 hour, 50 μ L from each well was placed in an opaque 96 well assay plate and 50 μ L of the luciferin detection reagent was added. The plate was incubated at room temperature for 20 minutes, protected from the light, and luminescence measured relative to a standard curve of beetle luciferin potassium salt (Promega, Southampton, UK).

Metabolite extraction

Frozen samples for an analytical series were thawed at the same time and kept on ice. Unused samples were then immediately stored at -80 °C. A stock solution of 1.25 μ M of d₄-hydrocortisone (d₄-HC) was prepared and 2 μ L were spiked into a 50 μ L of sample for a final concentration of 50 nM. The samples were incubated in ice for 2 minutes after adding cold methanol and cold chloroform (HPLC grade) at a 1:1:4 ratio relative to sample volume. After vortexing for 20 seconds and shaking for another 5 minutes, the samples were

DMD #63495

incubated at -20 °C for 30 minutes. Subsequently, the samples were again vortexed for 20 seconds followed by centrifugation at 15,000 rpm for 10 minutes. The organic phase was collected carefully with a special narrow gel tip (Bioscience, Inc., Salt Lake City, Utah), and dried in a SpeedVac[®]. Samples were immediately prepared for LC-MS by resuspending in 50 µL of 2 % ACN containing 0.1 % formic acid.

Preparation of standards for calibration curves

Hydrocortisone was dried overnight under vacuum to remove any residual water. Distilled water was used to prepare different concentrations of HC (e.g., 2, 5, 10, 20 µM) and verify the accurate concentration of a working stock solution by UV absorption. The concentration of each HC solution was determined by UV, at 242 nm with $\epsilon = 16000$ as the molar extinction coefficient.

LC-MS standards were prepared by diluting the working stock solution with Williams' E medium to give concentrations of 5, 10, 50, 75, 100 and 200 nM. All LC-MS standards and stock solutions were stored at 4 °C and allowed to equilibrate at room temperature for at least 15 min before use. For calibration, a 50 µL aliquot of each LC-MS standard was spiked with 2 µL of the 1.25 µM d₄-HC internal standard solution and the metabolites were extracted as previously described. Samples for LC-MS analyses were immediately resuspended in 50 µL of 2 % ACN containing 0.1 % formic acid. The calibration curve was constructed by plotting the peak area ratios of hydrocortisone relative to those of d₄-HC against the concentrations (nM) of the LC/MS standards.

LC-MS parameters and metabolite profiling

DMD #63495

The method validations are described in the Supporting material. LC-MS analyses were performed on an Agilent 6530 Accurate-Mass LC-Quadrupole-Time-of-Flight (QTOF) mass spectrometer with an Agilent Jet Stream electrospray ionization source and MassHunter workstation (v. B.06). The mass spectrometer was interfaced with an Agilent 1290 Ultra Performance Liquid Chromatography (UPLC) system. The column was an Agilent Extend-C18 (2.1 × 50 mm, 1.8 μm; Agilent Technologies, Santa Clara, CA). The column compartment temperature was set at 40 °C. The QTOF was calibrated daily using the standard tuning solution from Agilent Technologies. The mass spectra were acquired in positive ion mode for total and free hydrocortisone measurements and in negative ion mode for the analysis of glucuronides. Mass spectra were collected between m/z 70 and 1000 either at 2 scans/sec or 4 scans/sec. The ion spray voltage was set at 3800 V; the heated capillary temperature was 350 °C; drying gas was 8 L/min; nebulizer was 30 psi; sheath gas temp was set at 380 °C; sheath gas flow was 12 L/min. Two reference masses (m/z 121.0509: C₅H₄N₄; m/z 922.0098: C₁₈H₁₈O₆N₃P₃F₂₄) were continuously infused to allow mass correction during the run. MS/MS spectra were typically obtained at collision energy of 20 eV. Variations of retention times and m/z values were ≤ 0.2 min and 10 mDa, respectively, and the relative standard deviations (RSD) of peak areas were below 20 %, indicating good reproducibility and stability of the chromatographic separation and mass accuracy during a sequence (typically over 100 runs). The mobile phases were double-distilled water containing 0.1 % formic acid (A) and distilled acetonitrile containing 0.1 % formic acid (B). A linear gradient was run from 2 % to 95 % B over 12 minutes at 0.4 mL/min.

DMD #63495

Data processing

Hydrocortisone disappearance was followed on the QTOF in MS mode. Data were processed using Agilent MassHunter qualitative analysis software (v. B.06). Peak areas of HC (m/z 363.2171), d₄-HC (m/z 367.2423) and dexamethasone (m/z 393.2077) were obtained using the Extracted Ion Chromatogram (EIC) function. Hydrocortisone and metabolites data were analyzed both manually and using the Molecular Feature Extractor (MFE), i.e., all signals associated with a given analyte, with intensities > 1000, were used to profile metabolites, with a 10 ppm mass accuracy threshold. The data were further processed using Microsoft Excel 2011 and quantification was based on calibration curves built with known concentrations of hydrocortisone standards. GraphPad PRISM software was used to plot the data using the means of biological replicates with corresponding relative standard deviations.

Targeted MS/MS spectra, via matched exact masses and associated retention times, were generated on the Agilent QTOF 6530 mass spectrometer (Santa Clara, CA) to support the identities of the metabolites, followed by metabolic pathway or function analysis with the KEGG database (<http://www.genome.jp/kegg/>; <http://metlin.scripps.edu/index.php>). The MS/MS spectra were also analyzed with the Agilent Molecular Structure Correlator (MSC), which compares the accurate mass of the MS/MS fragment ions for a compound of interest with one or more proposed structures for that compound.

Analysis of glucuronides

LC-MS analyses of glucuronides were also performed on the Agilent 6530 mass spectrometer. Conditions for calibration and sample analyses were the same as those for

DMD #63495

metabolite profiling by LC-MS except that negative ion mode was used. To a 50 μL aliquot of the bioreactor culture medium was added 5 μL of the glucuronide-IS solution to make a final concentration of 50 nM 17- α -hydroxy-3,20-dioxopregn-4-en-21-yl- β -D-glucuronide and the mixture was then diluted with 100 μL of 5 mM ammonium acetate buffer (pH 5). The samples were thoroughly mixed and then applied to an Oasis HLB cartridge (10 mg, 1 mL) after washing the cartridges with methanol (1 mL) and water (1 mL). The cartridges were then washed with water (1 mL) to remove unbound material and the glucuronides eluted with methanol (1 mL). The samples were then dried in a SpeedVac[®] and immediately resuspended in 25 μL of water with 2 % ACN and 0.1 % formic acid.

Estimation of in vitro hydrocortisone pharmacokinetic parameters

Hydrocortisone pharmacokinetics parameters were calculated using the following equations (Eq. 1-4) where $t_{1/2}$ is the half-life, k_{el} is the rate of elimination, CL is the clearance, V_d is the volume of distribution, AUC is the area under the curve, t is time and C is concentration. The V_d of the system was 2.2 mL.

$$t_{1/2} = \frac{\text{Time Elapsed} * 0.30103}{\log \frac{\text{First Point}}{\text{Second Point}}} \quad (\text{Eq. 1})$$

$$k_{el} = \frac{\ln 2}{t_{1/2}} \quad (\text{Eq. 2})$$

$$CL = V_d * k_{el} \quad (\text{Eq. 3})$$

$$AUC = (t_1 - t_2) * \frac{C_1 + C_2}{2} \quad (\text{Eq. 4})$$

DMD #63495

Measurements of unbound (free) hydrocortisone

A fast UPLC-MS/MS method was developed to analyze free HC without sample preparation. The LC parameters were the same as previously described. The switching valve of the Agilent 6530 Accurate-Mass LC-Quadrupole-Time-of-Flight (QTOF) mass spectrometer was used to divert salt content to waste from 0-2.7 min and high protein content from 4-13 min (the HC elutes at 3.4 min). Two different samples were analyzed: 1) hepatocyte and Kupffer cell cocultures at time zero containing 100 nM HC and 1.25 mg/mL HSA; and 2) hepatocyte and Kupffer cell cocultures at time zero containing 100 nM HC and 25 mg/mL HSA. The injection volume was limited to 1 μ L to avoid overloading the column.

RESULTS

Coculture model characterization

Several ratios (Supplemental Table 1) of human cryopreserved hepatocytes and human cryopreserved Kupffer cells i.e., 15:1 (low inflammation), 10:1 (moderately inflamed) and 2.5:1 (highly inflamed) were cultured in the micro-bioreactor platform and assessed over an 8 day period. The cell health and differentiated state of the primary human hepatocytes maintained over 8 days in the bioreactor were monitored. Finally, LPS was introduced to the cultures to elicit an inflammatory response, as assessed by the release of pro-inflammatory cytokines, IL6 and TNF α via sandwich ELISAs.

Phase contrast imaging (Nikon, Ti-Eclipse) was used to visualize the morphology of the tissue formation within the scaffold after 8 days in culture. Figure 3 shows the tissue formation in mono- and 10:1 cocultures; no change in microtissue structure is observed as a result of the addition of Kupffer cells. CYP3A activity and total protein levels were

DMD #63495

measured at day 8 (Supplemental Figure 1). No significant differences were observed between the four conditions, demonstrating that the addition of Kupffer cells did not adversely affect cell health or inhibit the CYP3A activity in LPS naïve cultures. The albumin production rates after 8 days culture were greater than 30 $\mu\text{g}/\text{mg}$ total protein/day for all conditions, a level similar to human in vivo production (Supplemental Figure 1).

To assess the functionality of the Kupffer cells in cocultures with hepatocytes, cultures were exposed to LPS (1 $\mu\text{g}/\text{mL}$) for 48 h starting on day 6 (Fig. 4). $\text{TNF}\alpha$ was measured as a marker of Kupffer cell activation. In LPS naïve cultures, the $\text{TNF}\alpha$ concentration was ~ 19 pg/mL after 48 h period. The addition of LPS caused increased secretion of $\text{TNF}\alpha$ into the culture medium. The level of production of cytokines was dependent on the number of Kupffer cells seeded in the culture. The highest ratio of hepatocytes to Kupffer cells (2.5:1) resulted in a 42-fold increase in $\text{TNF}\alpha$ production, in comparison with 15: 1 which induced a 32-fold increase in production. Before stimulation the Kupffer cells were relatively quiescent but clearly remained viable and responsive to the addition of supra-physiological levels of LPS. IL6 production followed similar trends upon LPS stimulation. Stimulation of mono-cultures resulted in undetectable levels of cytokines in the media confirming Kupffer cells are responsible for cytokine production and the cryopreserved human hepatocyte lots used in this study are uncontaminated with residual amounts of Kupffer cells (data not shown).

Anti-inflammatory capacity of coculture in response to endogenous and synthesized glucocorticoids

DMD #63495

Hydrocortisone, a human endogenous glucocorticoid, has anti-inflammatory properties. Cocultures were exposed to various concentrations of hydrocortisone (0, 100 and 500 nM) up to day 7 in culture. To assess the effects of these concentrations, cytokine production was measured after the addition of 1 $\mu\text{g}/\text{mL}$ LPS for 24 h. As expected, in LPS naïve culture, low levels of TNF α and IL6 were detected (Supplemental Figure 2). Cultures without hydrocortisone produced the highest levels of cytokines with 1350 and 1043 pg for TNF α and IL6 respectively. The addition of 100 and 500 nM hydrocortisone caused a decrease in TNF α to 355 and 191 pg respectively. Similar trends were observed for IL6.

To determine if the model has utility as platform to screen anti-inflammatory compounds, the anti-inflammatory effects of hydrocortisone and dexamethasone were assessed at 100 nM (Supplemental Figure 3). After dosing with either hydrocortisone or dexamethasone from day 3 to 7, the cultures were stimulated with 1 $\mu\text{g}/\text{mL}$ LPS for 24 h. Hydrocortisone showed a modest anti-inflammatory effect whilst dexamethasone showed a greater reduction in the levels of both TNF α and IL6.

Experimental workflows

Figure 5 shows the experimental workflow for the quantification of hydrocortisone and metabolite profiling. The protocol is described in detail in the materials and methods sections; briefly, samples were spiked with internal standard, extracted with methanol and chloroform, dried and analyzed using RP-UHPLC-QTOF-MS. The aqueous layer was analyzed to confirm that there was no loss of hydrocortisone during the extraction process (Supplemental Figure 4). For the analysis of the glucuronides (Supplemental Figure 5), the

DMD #63495

samples were solid phase extracted in an Oasis HLB, then dried in a SpeedVac[®] and analyzed using RP-UHPLC-QTOF-MS.

Hydrocortisone clearance

Cocultures were seeded in LiverChip in duplicate to estimate biological variability within the bioreactor wells. Each well was treated with 100 nM of hydrocortisone and culture media samples collected at 0, 24, and 48 h were analyzed. Variation from technical sources, such as pipetting and liquid extraction was assessed. Each well was extracted two times and each extraction was run twice consecutively in positive ion mode in RP-UHPLC-QTOF-MS (Supplemental Figure 6). A concern in drug clearance studies in bioreactors is whether the drug is adsorbed onto any of the materials used in the apparatus. Two other wells without cells were therefore used as controls for non-specific binding experiments, using the same drug dose, sample collection and analysis. The concentration of hydrocortisone was virtually constant over a period of 4 h i.e., any loss of HC during coculture experiments can be attributed to cellular metabolism (Supplemental Figure 7).

Hydrocortisone disappearance in the bioreactor is plotted in Fig. 6. Under non-inflamed conditions, 100 nM hydrocortisone was depleted to approximately 30 nM in the culture medium after 48 h. A logarithmic plot ($\ln HC/HC_0$) vs time post dosing (Supplemental Figure 8); indicates that the clearance of HC follows first order kinetics.

Donor-to-donor variability

Hepatocytes and Kupffer cells from three different donor pairs (Supplemental Table 2) were plated as cocultures under non-inflamed conditions and treated with 100 nM

DMD #63495

hydrocortisone. The assessment was made by comparing the HC disappearance rates of each one of the donors (Supplemental Figure 9). The % RSD was $\leq 15\%$, confirming that the donor-dependent variability in HC clearance rates in the 3D microbio reactor platforms is acceptably low.

Effect of lipopolysaccharides (LPS) on HC clearance rate

Lipopolysaccharide is a component of Gram-negative bacterial cell walls and is associated with tissue injury and sepsis. To further unravel the mechanisms underlying inflammatory reactions in the liver, hepatocytes were plated in duplicate as cocultures with Kupffer cells at a 10:1 ratio and the media changed on day 4 to fresh media containing 100nM HC with LPS at concentrations of 0, 0.1 and 1 μ g/mL. Culture media samples were collected at 0, 24, and 48 h. The media was then replaced on day 6 with fresh media containing 100nM HC and LPS and samples taken to day 8. For technical variation assessment, each replicate was extracted twice and each of the two extracts was run in positive ion mode in the RP-UHPLC-QTOF-MS two times consecutively. HC concentrations in the bioreactor were plotted vs time (Supplemental Figure 10). The rates are equivalent in every case when comparing non-inflamed cocultures with LPS-induced, suggesting that inflammation neither inhibited nor induced the activity of reductases involved in the metabolism of hydrocortisone in the bioreactors. The HC clearance rates from day 5 to 7 were similar to those from day 3 to day 5, indicating that extended exposure to LPS did not affect clearance (data not shown).

Hydrocortisone metabolism: phase I/II metabolites

DMD #63495

High accuracy exact mass, retention time, and database searching, followed by manual interpretation of collision induced dissociation (CID) MS/MS spectra, were used to confirm the structures of the hydrocortisone metabolites. The MS/MS spectra revealed a characteristic fragmentation pattern with ions at m/z 267.1747, 121.0650 and 163.1119. For example, the MS/MS spectrum of cortisone, which is the oxidized product of hydrocortisone, showed the m/z 163.1119 fragment as the most intense and characteristic ion, which is not found in the MS/MS spectrum of hydrocortisone (Supplemental Figure 11). As noted above (Fig. 6), approximately 70% of the hydrocortisone disappeared over 48 h, and this was largely accounted for as 8% phase I metabolites, and 52 % as phase II metabolites. In the absence of authentic standards, tetrahydrocortisone, and dihydrocortisol (phase I; Fig. 7) and tetrahydrocortisol, and tetrahydrocortisone glucuronides (phase II; Fig. 8), were confirmed by comparing their retention times, exact monoisotopic masses, and MS/MS spectra with those of known hydrocortisone metabolites found in human urine. (Supplemental Figures 12 and 13).

Hydrocortisone pharmacokinetics

The pharmacokinetics of hydrocortisone was investigated after administration of 100 nM HC to a coculture of human hepatocytes and Kupffer cells in the bioreactor. The HC concentrations in culture media were measured by LC–MS/MS and the pharmacokinetics were determined using standard pharmacokinetic equations (Eq. 1-4).

The mean culture media concentration-time curve after administration of 100 nM hydrocortisone is shown in Fig. 6. The half-life ($t_{1/2}$), rate of elimination (k_{el}),

DMD #63495

hydrocortisone clearance (CL) and the area under the curve (AUC) were estimated to be 23.03 h, 0.03 h¹, 6.6x10⁻⁵ L. h⁻¹ and 1.03 $\frac{\text{mg}}{\text{L}} * \text{h}$ respectively (See also Table 1).

Albumin is the most abundant plasma protein, with a half-life of three weeks (Peters, 1970). Some drug molecules are rapidly excreted from the circulation, resulting in lower therapeutic efficacies. In this regard, albumin plays a role in increasing plasma half-life through non-covalent interaction with the small molecules (Sleep et al., 2013). Protein-drug binding may affect pharmacokinetic behaviors, including the half-life of a drug (Tayman et al., 2011). It is interesting to see whether protein concentration may show the similar effect in the 3D microbio reactors. To test this hypothesis, we used higher concentration of human serum albumin (25 mg/mL) and analyzed the HC clearance. The half-life calculated for high protein concentration was higher in comparison to 1.25 mg/mL HSA ($t_{1/2} = 34.36 \text{ h}$ vs $t_{1/2} = 23.03 \text{ h}$), indicating that the number of available interactions with the HC extended the half-life. Hydrocortisone was 50% bound with 25 mg/L albumin, and almost all free with 1.25 mg/mL albumin. The fraction unbound (f_u) was expressed using Eq. 5, where [D] is the free drug concentration and [DP] is the concentration of drug protein complex.

$$f_u = \frac{[D]}{[D]+[DP]} \quad (\text{Eq. 5})$$

Extrapolation of in vivo intrinsic clearance ($CL_{\text{int in vivo}}$) of hydrocortisone from in vitro data

The prediction of human clearance is a key objective in drug discovery. It is therefore important to establish an in vitro/in vivo correlation (IVIVC) for the clearance of hydrocortisone based on in vitro metabolism data. For this, in vitro data from the 3D

DMD #63495

microbioreactor was used to estimate expected in vivo data that could be compared with the in vivo clearance in humans. The in vitro half-life was used to calculate the in vitro intrinsic clearance using the scaling factors in Eq. 6:

$$CL_{int} = \frac{\ln 2}{t_{1/2}} * \frac{\text{liver weight}}{\text{standard body weight}} * \frac{\text{incubation volume}}{\text{hepatocytes/well}} * \text{hepatocytes/g liver} \quad (\text{Eq.6})$$

Scaling parameters were used for the calculations (Supplemental Table 3) (Davies and Morris, 1993; Obach et al., 1997). The hepatic clearance (CL_h) was calculated from CL_{int} using the well-stirred model (Eq. 7) (Pang and Rowland, 1977a; Pang and Rowland, 1977b). CL_h was calculated for hydrocortisone, correcting for the plasma protein binding (f_{ub}) from the literature (Robin et al., 1978), and using Eq. 7 where Q is the hepatic blood flow rate and $f_{u inc}$ is the unbound fraction in hepatocytes from the bioreactor. To compare in vivo intrinsic clearance values with in vitro in vivo clearance values, Eq. 7 was rearranged into Eq. 8 (Davies and Morris, 1993; Bayliss et al., 1999; Chiba et al., 2009; Chan et al., 2013).

$$CL_h = \frac{Q * f_{ub} * \left(\frac{CL_{int}}{f_{u inc}}\right)}{Q + \left[f_{ub} * \left(\frac{CL_{int}}{f_{u inc}}\right)\right]} \quad (\text{Eq. 7})$$

$$CL_{int-in vivo} = \frac{Q * CL_h}{(Q - CL_h) * f_{ub}} \quad (\text{Eq.8})$$

The intrinsic clearance (CL_{int}) calculated by equation 6 was 5.7 mL/min/kg, and the hepatic clearance was 1.1 mL/min/kg (assuming $f_u = 1$). The in vivo clearance data from LiverChip calculated in mL/min/kg was converted to L/h using the human body weight (average body weight = 70 kg). The $CL_{int-in vivo}$ was calculated to be 23.8 L/h, which is 1.3

DMD #63495

fold higher than human in vivo clearance, i.e., approximately 18 L/h (Derendorf et al., 1991).

DISCUSSION

Multiple approaches aimed at halting the relatively rapid de-differentiation of primary hepatocytes in monolayer in vitro cultures have been studied. Co-culturing of hepatocytes with feeder or liver non-parenchymal cells (NPCs) has proved effective, as has 3D culture in various formats, including bioreactors. Work in this area typically focuses on maintenance of hepatic viability and function with relatively less emphasis placed on deploying the models to explore disease states such as inflammation or in concert with high quality analytics to produce detailed pharmacokinetic information. A notable exception is the micropattern cocultures first published by Khetani and Bhatia (Khetani and Bhatia, 2006; Khetani and Bhatia, 2008), which have been used for extensive clearance and metabolite identification studies (Wang et al., 2010; Dash et al., 2012; Chan et al., 2013). Here we demonstrate an inflammatory liver model supported in a 3D bioreactor culture system to explore the metabolism and anti-inflammatory effects of hydrocortisone.

We developed 3D cultures of primary hepatocytes and a mixed fraction of liver NPCs as unsupported spheroids and in scaffold-containing bioreactors have been shown to maintain hepatic viability and produce pro-inflammatory cytokines on stimulation with high concentrations of LPS (Dash et al., 2009a; Messner et al., 2013). The NPCs in these models likely play a dual role, both supporting hepatic function and through the incorporated Kupffer cells recapitulating inflammatory response. In the model described here, a modular approach starting from separate stocks of cryopreserved human hepatocytes and Kupffer

DMD #63495

cells, has been taken. The bioreactor and 3D scaffold are sufficient to maintain hepatocyte viability for extended periods in the presence or absence of NPC (Powers et al., 2002b; Sivaraman et al., 2005; Vivares et al., 2014). Kupffer cells can therefore be added to the cultures in well-defined ratios, allowing the evaluation of models of non-inflamed, mild and highly inflamed livers with hepatocyte to Kupffer cells ratios ranging from 15:1 and 2.5:1.

Glucocorticoids are necessary in the media of primary hepatocyte cultures, as they assist in the preservation of the differentiated state as reflected, e.g., by the maintenance of CYP450 levels (Schuetz and Guzelian, 1984; Schuetz et al., 1984). In the media-for Kupffer cell monocultures, glucocorticoids are removed or kept at low nanomolar concentrations as their presence blunts cytokine production. Hydrocortisone, or cortisol, is the major human glucocorticoid; here we confirm that hepatocyte and Kupffer cell cocultures can be maintained in hydrocortisone-containing media and that hydrocortisone shows a dose dependent anti-inflammatory effect. When compared to hydrocortisone at 100 nM, dexamethasone has a more potent anti-inflammatory effect as judged by suppression of cytokine production, reflecting the situation *in vivo*.

The metabolism of hydrocortisone has not been extensively investigated *in vitro* in 3D perfusion models, particularly at concentrations near physiological free levels. To exemplify the utility of the model not only for anti-inflammatory drug testing but also for complex drug metabolism studies, a detailed characterization of hydrocortisone disappearance and metabolite profile has been undertaken. For metabolism studies of hydrocortisone, an initial concentration of 100 nM was used. The hydrocortisone was depleted to 30 nM after 48 h, with first order kinetics. LC-MS approaches were used to identify the metabolites produced in the bioreactor; tetrahydrocortisone and

DMD #63495

dihydrocortisone were identified as major and minor phase I metabolites, respectively. The majority of the metabolism, however, was glucuronidation; phase II metabolites were identified as tetrahydrocortisol glucuronide and tetrahydrocortisone glucuronides. Towards the end of the application period the formation rates of tetrahydrocortisol glucuronide and tetrahydrocortisone glucuronide were higher than at earlier time points in the bioreactor cultures, suggesting that UDP-glucuronosyltransferase (Kaji and Kume, 2005) and other oxidoreductases (reductive enzymes), were present in the bioreactor cultures.

Donor-to-donor variability was assessed by comparing the HC disappearance rates of several donors. The donor-dependent variability is low, supporting the promise of this microphysiological system as a tool for investigating drug metabolism and anti-inflammatory effects (Supplemental Figure 9).

To address whether the hydrocortisone nominal disappearance included a contribution from prior or metabolically-formed cortisone, we used [9,11,12,12-²H₄] hydrocortisone (d4-hydrocortisone) as a tracer (Supplemental Figure 14). The deuterated isotopomer can be distinguished from unlabeled hydrocortisone by the mass difference of 4 Da. Metabolism by the 11 β -HSD type 2 enzyme would yield cortisone, with the deuterium at the 11 β position being lost. The resulting [9,12,12-²H₃] cortisone (d3-cortisone) would have a mass 1 Da less than that of the d4-hydrocortisone. Subsequent conversion of cortisone via 11 β -HSD type 1 back to hydrocortisone would yield d3-hydrocortisone, which is 1 Da less than the starting isotopomers. Regeneration of d4-hydrocortisone is unlikely since this would require re-introduction of highly-diluted deuterium at the 11 β position. The 1-dalton mass difference, and the contribution of the ¹³C abundance of the d4 compound to the signal from the presumed d3-hydrocortisone, preclude a detailed

DMD #63495

interpretation. There appears, however, to be some cortisone-hydrocortisone inter-conversion, although the net effect on hydrocortisone disappearance is modest at best.

To further understand the functionality of the Kupffer cells in cocultures with hepatocytes, the system was exposed to LPS for 48 h to stimulate inflammation. The disappearance rate of hydrocortisone was not affected by stimulation of the cultures with LPS. We have demonstrated that hydrocortisone is primarily metabolized by phase II enzymes and hypothesize that this is the reason that LPS stimulation has little effect on clearance rate. LPS stimulation causes the release of pro-inflammatory cytokines which are known to down regulate CYP450 (Milosevic et al., 1999).

Pharmacokinetic parameters were estimated for the *in vitro* metabolism and to establish IVIVC of hydrocortisone behavior. The intrinsic clearance (CL_{int}) was ~5.7 mL/min/kg (calculated by using Eq. 6). Hepatic clearance was ~1.1 mL/min/kg (assuming $f_u = 1$), and the calculated value for $CL_{int - in vivo}$ was 23.8 L/h, which is 1.3 times that of human *in vivo* clearance, (approximately 18 L/h (Derendorf et al., 1991)). The intrinsic clearance values and the metabolites generated in the perfused human 3D microbio reactor correlated generally with human data.

In summary, CYP activities, total protein, albumin and urea levels were maintained for extended periods of coculture in the microphysiological system, a useful tool for pharmacological or toxicological investigations requiring a highly stable and reproducible physiological *in vitro* representation of the human liver.

We demonstrated the applicability of a recently developed bioreactor for long-term pharmacological investigations on human hepatocytes coculture system, using hydrocortisone as a steroidal anti-inflammatory drug model. Correlations between *in vitro*

DMD #63495

and in vivo data (IVIVC) were generated, suggesting that 3D microphysiological systems with mixed human cell populations could be used as tools for investigating drug metabolism and toxicity for drug development.

Overall, the biology in the coculture system and the in vitro and in vivo hepatic clearance correlation data suggest that the roles of various uptake and efflux transporters in drug efficacy and toxicity studies in these systems may also correlate with the human liver in vivo.

Thus, especially if interactions with non-parenchymal cells are involved, the miniaturized bioreactor may be a useful model system to increase predictivity in pharmacological and toxicological studies on a sub-chronic timescale. In addition, the culture system facilitates histological analyses of pharmacology-related structural characteristics of human liver tissue.

DMD #63495

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DMD #63495

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Sarkar, Rivera-Burgos, Large, Hughes, Ravindra,

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Performed data analysis: Sarkar, Rivera-Burgos, Large, Ravindra, Wishnok, Tannenbaum

Wrote or contributed to the writing of the manuscript: Sarkar, Rivera-Burgos, Large,

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DMD #63495

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FOOTNOTES

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DMD #63495

LEGENDS FOR FIGURES

Fig. 1: Diffusion of cortisol to the nucleus. Cortisol is the most common glucocorticoid and is essential for life. It supports and regulates cardiovascular, metabolic, immunologic, and homeostatic functions.

Fig. 2: Microfluidic 3D tissue culture system. A) LiverChip: This device is a multiwell plate platform that enables maintenance of 3D liver tissue cultures under perfusion, B) LiverChip hardware, C) Scaffold containing 301 channels in which cells are seeded. Typically 0.6×10^6 human hepatocytes can be seeded per scaffold, D) Schematic of flow through a single LiverChip culture well.

Fig. 3: Tissue formation in cocultures plated at different cell type ratios. Phase contrast imaging (Nikon, Ti-Eclipse) was used to assess the morphology of the tissue formation after 7 days in culture. A) Monoculture, B) Coculture 10:1.

Fig. 4: Inflammation stimulated by the addition of 1 $\mu\text{g}/\text{mL}$ LPS to the mono-culture and coculture systems seeded at 2.5:1, 10:1 and 15:1 ratios. $\text{TNF}\alpha$ was measured as a marker of Kupffer cell activation. $n=3$ replicates, single donor. Results are reported as mean \pm SD.

DMD #63495

Fig. 5: Experimental workflow used for the quantification of hydrocortisone as metabolite profiling. The method consists of spiking with internal standard, liquid extraction and mass spectrometry analysis.

Fig. 6: Hydrocortisone disappearance over time. n=2 for biological and technical replicates. Lines represent mean \pm SD.

Fig. 7: Phase I metabolites. 8-10% of hydrocortisone was found as phase I metabolites including tetrahydrocortisone and dihydrocortisol.

Fig. 8: Phase II metabolites. 45-52 % of hydrocortisone was found as phase II metabolites including tetrahydrocortisol glucuronide and tetrahydrocortisone glucuronide.

DMD #63495

TABLE

Symbol	Characteristic	Value	Unit
$t_{1/2}$	Elimination half-life	23	h
k_{el}	Elimination rate constant	0.03	h^{-1}
V_d	Volume of distribution	2.2	mL
CL	Clearance	6.6×10^{-5}	$L \cdot h^{-1}$
AUC	Area under the curve	1.03	$\frac{mg}{L} * h$

Figure 1

protein, e.g., albumin or cortisol binding globulin (CBG)

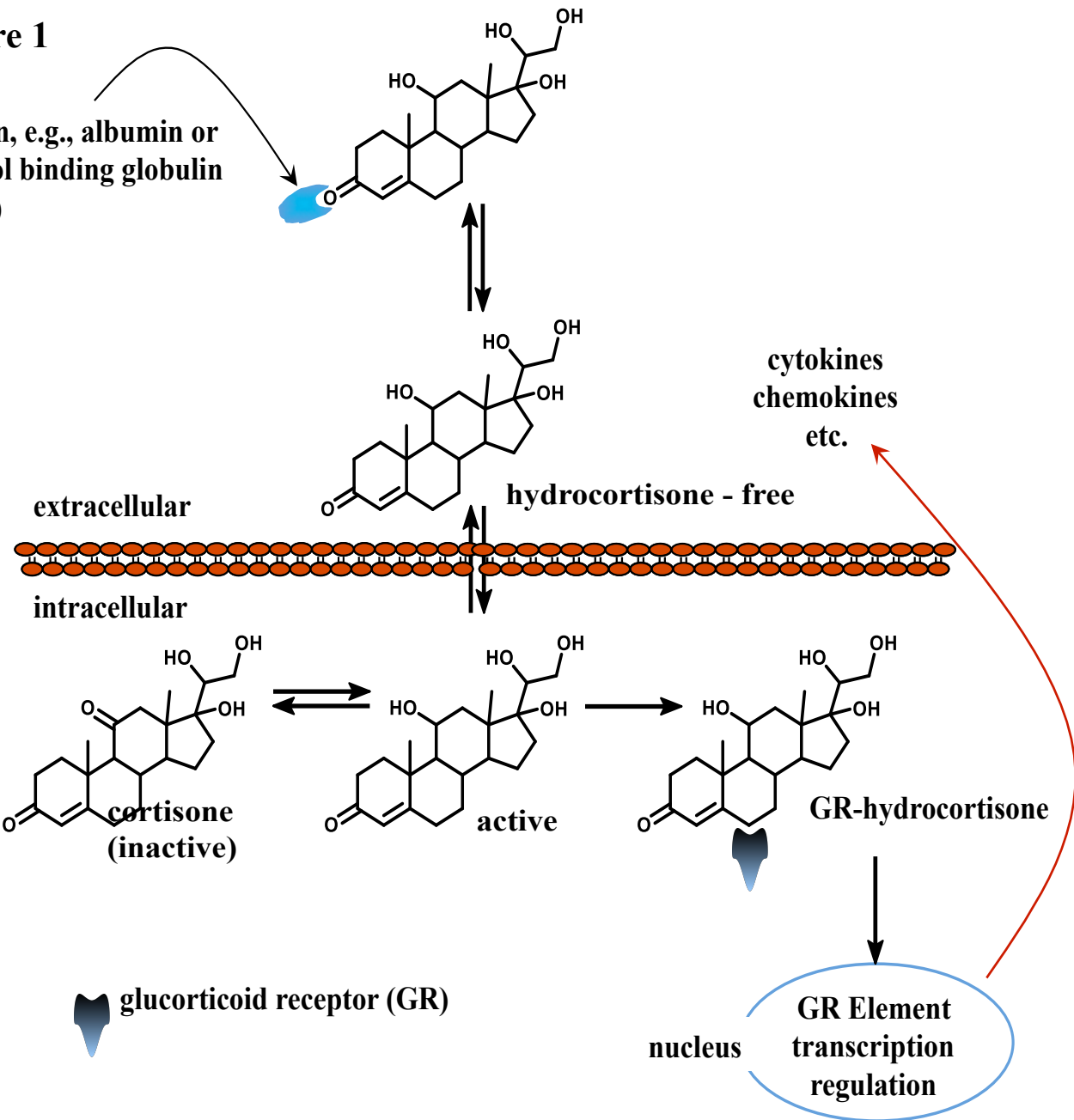


Figure 2

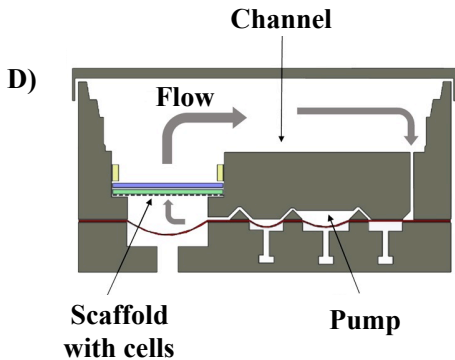
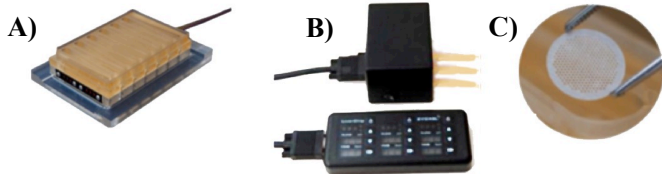
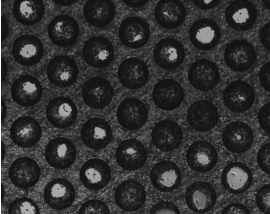


Figure 3

A)



B)

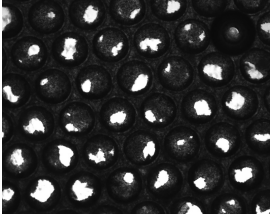


Figure 4

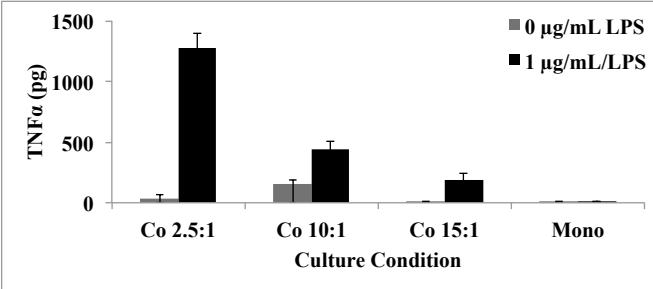


Figure 5

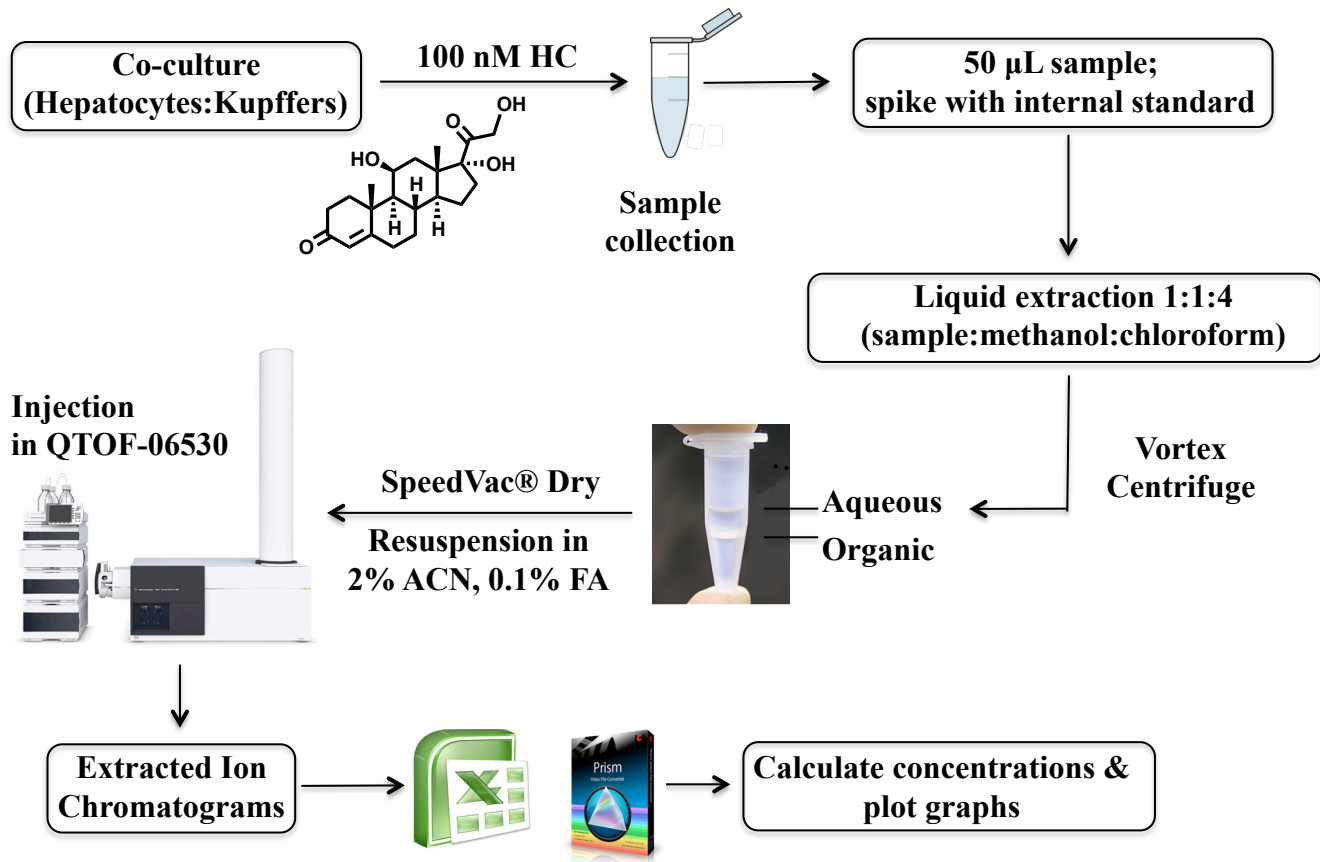


Figure 6

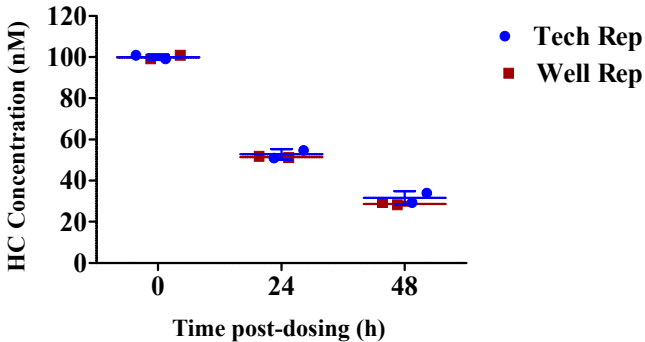
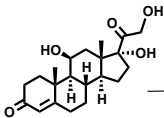
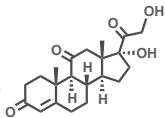


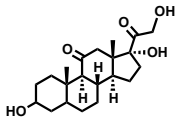
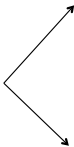
Figure 7



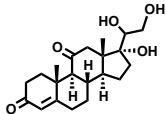
Hydrocortisone



Cortisone

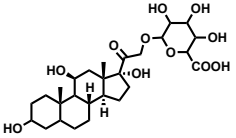


Tetrahydrocortisone

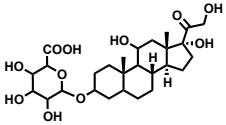


Dihydrocortisol

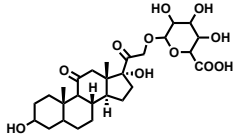
Figure 8



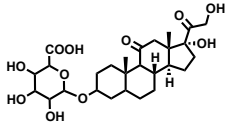
or



Tetrahydrocortisol glucuronide



or



Tetrahydrocortisone glucuronide