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Short communication

Novel multiple assessment of hepatocellular drug disposition in a single packaged procedure

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ABSTRACT

Better prediction of drug disposition prior to the clinical trial is critical for the efficient development of new drugs. The purpose of this study is to develop a novel multiple assessment methodology of hepatocellular drug disposition from drug uptake to efflux including biliary and basolateral excretion, in a single packaged procedure. We started a sandwich culture using rat primary hepatocytes. After five days culture, the hepatocytes were incubated with a dosing solution including CDF or Rhodamine 123. Three distinct sequences were then performed in parallel: disrupting and maintaining the tight junctions comprising a bile canalicular network at 37 °C, and maintaining the network at 4 °C. Supernatant fractions were collected from each sequence, and followed by the cell lysate collection. The disposition rates of basolateral efflux by diffusion, by transporter-mediation, biliary excretion, and residual cellular fraction of CDF and Rhodamine 123 were 38.2% and 11.0%, 26.6% and 12.1%, 18.6% and 4.9%, and, 16.7% and 72.0%, respectively. CDF was likely to excrete extracellularly whereas Rhodamine 123 tended to remain intracellularly. CDF showed a relatively higher biliary excretion rate than Rhodamine 123. This novel protocol may contribute to improve the predictability of pharmacokinetics eventually in human, and streamline new drug development.

Chemical compounds: 5(6)-Carboxy-2′,7′-dichlorofluoroscein (PubChem CID: 132525); Rhodamine 123 (PubChem CID: 65217).

1. Introduction

Clinically relevant prediction of drug disposition during the early stages of drug development is critical for the efficient development of new drugs. In recent years, two of the primary factors behind the current low clinical success rate of drug candidates (~11%) [13] are lack of efficacy (56%) and safety issues (28%) [2], which have been first detected in the late clinical study stages of the drug development process. In order to reduce the attrition rate, there is a growing need for an in vitro methodology that can clarify the entire pharmacokinetics of a drug eventually applied for better prediction in humans.

Primary hepatocytes have been widely used as effective cell sources for DMPK-related or toxicological study. The sandwich culture system was proposed in the late 1980s as a way to mimic the hepatocytes of an in vivo environment [3,4]. The sandwich-cultured hepatocytes exhibited the structural polarity with bile canalicular network and the localization of proper hepatobiliary transporters on the network, maintaining liver-specific functions for several days [9,18]. This culture method has been utilized for investigating the hepatic disposition of drugs. In vitro biliary clearance determined in sandwich-cultured hepatocytes correlates well with in vivo biliary clearance [7], and biliary clearance calculation based on the intracellular concentration of a drug has the potential to reflect more the in vivo process than that based on incubation medium [11]. In another report, basolateral and biliary clearance was revealed to be an alternative elimination route in sandwich-cultured hepatocytes by combining biliary excretion and basolateral efflux experiment individually [14].

The objective of this study is to consider these previous reports as a basis and develop an in vitro multiple assessment methodology using hepatocytes to determine the entire picture of drug dispositions including uptake, biliary excretion, and basolateral efflux disposition in a single packaged procedure. The evaluation of these endpoints at the same time in a single procedure has not been

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reported. Furthermore, diffusion and transporter-mediated efflux can be distinguished by controlling the specific experimental conditions.

2. Materials and methods

2.1. Chemicals

5(6)-Carboxy-2,7′-dichlorofluoroscein (CDF) and Aprotinin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Rhodamine 123 (Rh 123), Insulin, and Epidermal Growth Factor (EGF) were purchased from Sigma–Aldrich (St. Louis, MO). Penicillin–streptomycin solution and Dexamethasone was purchased from Nakalai Tesque, Inc. (Kyoto, Japan). Matrigel was purchased from BD Bioscience (San Jose, CA). All other chemicals and reagents were of analytical grade and readily available from commercial sources.

2.2. Hepatocyte culture

Hepatocytes were isolated from six- to eight-week-old specific viral-pathogen-free male SD rats weighing about 150–250 g (Charles River Japan Inc., Japan) by modified two-step *in situ* collagenase perfusion and purified by Percoll gradient separation [8,15]. Hepatocytes with over 90% viability were used for the following culture.

The hepatocytes were resuspended in William's E medium (Sigma–Aldrich) containing 10% fetal bovine serum, supplemented with 8.6 nM insulin, 255 nM dexamethasone, 50 ng/ml EGF, and 5 KIU/ml aprotinin, and seeded at a density of 1×10^5 cells/cm^2 into a type I collagen-coated 24-well culture dish (AGC Techno Glass Co., Ltd., Chiba, Japan). The seeded hepatocytes were incubated in a humidified chamber with 5% CO_2 at 37 °C. After 24 h of post-seeding, the culture medium was replaced with serum-free William's E medium containing Matrigel with the same supplements described above. Subsequently, the culture medium without Matrigel was changed daily. Hepatocytes were cultured for a total of four days.

2.3. Fluorescent and phasecontrast microscopy

Sandwich-cultured hepatocytes were rinsed with standard (Std) Hanks' balanced salt solution (HBSS) containing Ca^{2+} and Mg^{2+} on day 5 and then incubated with a dosing solution including 10 μM CDF or 10 μM Rh 123 in the 5% CO_2 incubator. After 30-min incubation, HBSS was removed and the hepatocytes were washed with Std HBSS. Fluorescent and phasecontrast micrographs of the hepatocytes were acquired with a Zeiss Axiovert 200 inverted optical microscope (Carl Zeiss Microscopy GmbH, Germany).

2.4. Disposition study

Sandwich-cultured hepatocytes were rinsed and preincubated in the 37 °C, 5% CO_2 incubator with Std HBSS containing Ca^{2+} and Mg^{2+} (Std HBSS) on day 5, which the MRP2 expression still maintained in a high rate [16]. Hepatocytes were then incubated with a dosing solution including 10 μM CDF or 10 μM Rh 123 (Fig. 1) in the CO_2 incubator for 30 min. After incubation, the dosing solution was aspirated from the cells and the uptake process was stopped by washing the cells with ice-cold Std HBSS. Subsequently, three different sequences were performed in parallel: one, disrupting the tight junctions comprising a bile canalicular network at 37 °C by Ca^{2+}, Mg^{2+}-free HBSS with EGTA (seq. 1), two, maintaining the network at 37 °C by Std HBSS (seq. 2), and three, maintaining the network at 4 °C by Std HBSS (seq. 3). The supernatant fractions were collected from each sequence after 30 min incubation. Finally, cells were lysed with 1% Triton X-100 in H_2O and cell lysate was collected. The samples were analyzed for relative luminescence unit (RLU) of CDF and Rh 123 by microplate reader.

2.5. Data analysis

The disposition rates of each fraction were calculated using the obtained fluorescent data. B2 corresponds to the amount of total basolateral efflux, B3 corresponds to that of basolateral efflux by diffusion, B2 − B3 corresponds to that of basolateral efflux mediated by transporters, B1 − B2 corresponds to that of biliary efflux, and C1 corresponds to that of intracellular residue. In this analysis, B1 + C1 which represents putative intracellular drug amount in the beginning of the efflux fractionation process was counted as 100%. The disposition rate of total basolateral efflux (BEfx-Total), basolateral efflux by diffusion (BEfx-Dif), basolateral efflux by transporter (BEfx-TP), biliary efflux (BCEfx), and intracellular residue (Cell) were calculated by Eqs. (1)–(5), respectively.

$$\text{BEfx-Total} = B2/(B1 + C1) \quad (1)$$

$$\text{BEfx-Dif} = B3/(B1 + C1) \quad (2)$$

$$\text{BEfx-TP} = (B2 - B3)/(B1 + C1) \quad (3)$$

$$\text{BCEfx} = (B1 - B2)/(B1 + C1) \quad (4)$$

$$\text{Cell} = C1/(B1 + C1) \quad (5)$$

3. Results and discussion

CDF and Rh 123 were independently exposed to sandwich-cultured rat hepatocytes to examine the localization of these two compounds (Fig. 1), which are not subject to hepatic metabolism and primarily excreted in an unchanged form [1,21]. CDF has been used as a model fluorescent compound to evaluate the excretion into bile canaliculi by Multidrug Resistant-associated Protein (MRP) 2 [10,21]. Rh 123, also a fluorescent substance, has been used to determine mitochondrial membrane potential [20] and is currently used to label mitochondria in living cells [5]. CDF was accumulated in the bile canalicular network of sandwich-cultured hepatocytes for 30-min incubation (Fig. 1). In contrast, Rh 123 was localized in the cytoplasm (Fig. 1). These results indicate the characteristics of the two tested compounds.

Multiple drug disposition assay was performed on day 5 according to the protocol described in Materials and methods and Fig. 2. We incubated with CDF or Rh 123 for 30 min at the uptake process. Due to the existing reports, uptake time is generally applied 10–20 min [6,12,19]. Whereas their uptake condition requires two different SCRH buffers with Ca^{2+}/Mg^{2+} and without Ca^{2+}/Mg^{2+}, our protocol is performed only with Ca^{2+}/Mg^{2+}, which is favorable to maintain hepato-cellularly stable condition with intact intercellular adhesion. Accordingly, our uptake condition enables prolonged absorption of the drugs in SCRH. We applied 30 min for efflux experiment because of the results based on the preliminary experiment (data not shown). The time-course of supernatant fluorescence of efflux fractions saturated at about 30 min. We concluded 30 min was suitable for efflux study.

In order to calculate the disposition rate of biliary excretion, the supernatant fraction of B2 was subtracted from the B1 fraction (Fig. 2). The calculation of transporter-mediated basolateral efflux was performed by subtracting B3, a supernatant of the 4 °C