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Inhibition of bile canalicular network formation in rat sandwich cultured hepatocytes by drugs associated with risk of severe liver injury

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ABSTRACT

Idiosyncratic drug-induced liver injury is a clinical concern with serious consequences. Although many preclinical screening methods have been proposed, it remains difficult to identify compounds associated with this rare but potentially fatal liver condition. Here, we propose a novel assay system to assess the risk of liver injury. Rat primary hepatocytes were cultured in a sandwich configuration, which enables the formation of a typical bile canalicular network. From day 2 to 3, test drugs, mostly selected from a list of cholestatic drugs, were administered, and the length of the network was semi-quantitatively measured by immunofluorescence. Liver injury risk information was collected from drug labels and was compared with *in vitro* measurements. Of 23 test drugs examined, 15 exhibited potent inhibition of bile canalicular network formation (<60% of control). Effects on cell viability were negligible or minimal as confirmed by lactate dehydrogenase leakage and cellular ATP content assays. For the potent 15 drugs, IC_{50} values were determined. Finally, maximum daily dose divided by the inhibition constant gave good separation of the highest risk of severe liver toxicity drugs such as troglitazone, benzbromarone, flutamide, and amiodarone from lower risk drugs. In conclusion, inhibitory effect on the bile canalicular network formation observed in *in vitro* sandwich cultured hepatocytes evaluates a new aspect of drug toxicity, particularly associated with aggravation of liver injury.

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1. Introduction

Drug-induced liver injury (DILI) is a serious adverse reaction in clinical settings (Fontana, 2008; Navarro and Senior, 2006). Diagnosis of DILI is based on various clinical observations including serum enzyme markers such as alanine aminotransferase (ALT) and alkaline phosphatase (ALP) (Aithal et al., 2011). As a matter of convenience, DILI is classified into three types on the basis of these markers: hepatocellular type (elevated ALT), cholestatic type (elevated ALP), and mixed type (elevated ALT plus elevated ALP) (Navarro and Senior, 2006). It is desirable to identify drugs that are at risk of causing DILI as early as possible in the drug development process because DILI can be fatal in some patients, and unpredictable severe DILI will lead to withdrawal of the drug from further drug development or from the market. This may have a negative impact on the company, injured patients, and other non-DILI patients who would have benefited from the drug if it had remained on the market. Most DILI cases are idiosyncratic, which is quite unpredictable and considered a result of multiple overlapping risk factors including production of reactive oxygen species, lipid peroxidation, mitochondrial dysfunction, covalent binding of the drugs to cell proteins potentially leading to direct cell death or an immune reaction, imbalance of cellular death and survival pathways, bile salt transport inhibition, and so on (Luedde et al., 2014; Urban et al., 2014). Focusing on these various mechanisms, DILI risk screening assay systems have been extensively proposed (Bale et al., 2014). These assays not only monitor hepatocyte death but also collect data on functional markers including membrane permeability, cell proliferation, mitochondrial activity, endoplasmic reticulum stress, and so on (Donato et al., 2012; Persson et al., 2014; Tolosa et al., 2015). Such multifactorial analyses provide improved predictability and mechanistic information; however, methods to clearly identify drugs at risk of causing severe DILI have not yet been established.

DILI in most patients is asymptomatic, and patients may recover if the causative drug is discontinued or even when continuing treatment with the causative drug. Such variable responses might be attributable to the versatile nature of the liver, including high spare capacity, high regeneration ability, and immune tolerance (Jenne and Kubes, 2013; Michalopoulos and DeFrances, 1997). However, as described above, a fatal outcome can occur with particular drugs, and prevention of a

Abbreviations: DILI, drug-induced liver injury; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BBW, black box warning; WDN, withdrawn; BC, bile canalicular; BSEP, bile salt export pump; MRP2, multidrug resistance-associated protein 2; SCHs, sandwich cultured hepatocytes; WME, Williams' Medium E; DMEM, Dulbecco's Modified Eagle's Medium; TCA, taurocholic acid; LCA, lithocholic acid; LDH, lactate dehydrogenase; IC_{50}, half maximal inhibitory concentration; FDA, Food and Drug Administration; MDD, maximum daily dose; PMDA, Pharmaceuticals and Medical Devices Agency; $C_{max,u}$, maximum unbound concentration in plasma; NM, no mention; AR, adverse reaction; WNG, warning; PC, precaution; EC_{50}, half maximal effective concentration.

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fatal outcome (fulminant DILI) is the ultimate goal in clinical settings. According to a historical survey, hepatocellular-type DILI with jaundice is the most serious type of DILI and is associated with a high fatality rate (10–50%) (Navarro and Senior, 2006). Drugs that induce such severe DILI are considered the highest risk and are issued with a black box warning (BBW) and may eventually be withdrawn (WDN) from the market. Such drugs include amiodarone (BBW), flutamide (BBW), benzbromarone (WDN, but still used in Japan), and troglitazone (WDN). Although the detailed mechanisms underlying aggravation of DILI by these high-risk drugs are not yet completely understood, one can speculate that aggravation of hepatocyte cell death (manifested as injury) may lead to compromised liver function, including biliary excretion, that further promotes liver injury and prevents hepatocyte regeneration, ultimately leading to fatal liver failure.

Hepatocytes are polarized cells with a sinusoidal membrane facing the blood, and bile canalicular (BC) membrane facing the bile. BC spaces are formed between hepatocyte couplets, which are then connected to each other to form a continuous BC network. This network subsequently converges into bile ductules *via* canals of Herring near to the portal vein, which then converge into intrahepatic bile ducts and finally join the common bile duct (Hammad et al., 2014). Importantly, bile canaliculi formed by hepatocytes are the origin of the biliary excretion process, and primary active efflux transporters such as bile salt export pump (BSEP), multidrug resistance-associated protein 2 (MRP2), and so on are expressed on the BC membrane to excrete various endogenous and exogenous compounds into the bile (Cuperus et al., 2014). Genetic defects in some of these efflux transporters result in severe hepatic injury requiring liver transplantation in childhood (Srivastava, 2014). Indeed, liver-specific knockout of liver kinase B1, a kinase involved in BC structure formation (Fu et al., 2010; Fu et al., 2011), results in significant defects in the bile canaliculi and subsequent downstream bile duct structures (Woods et al., 2011). These knockout mice exhibit increased serum bilirubin glucuronide levels and extensive accumulation of bile acids in hepatocytes, and die at 4 weeks of age (Woods et al., 2011). Thus, biliary excretion structures and machinery are indispensable for maintenance of liver function and survival. Importance of the BSEP function is widely accepted and simple membrane vesicle transporter inhibition assay have been proposed (Dawson et al., 2012; Morgan et al., 2010; Pedersen et al., 2013; Warner et al., 2012). Recently, we have also reported unique *in vitro* bile acids-dependent hepatocyte toxicity assay (Susukida et al., 2015a). This method could assess the risk of serum enzyme marker increase, however it could not predict severity of DILI (Susukida et al., 2015a).

Considering the importance of biliary efflux systems in maintaining liver function, we hypothesized that compounds that inhibit the reorganization of BC structures during recovery from liver injury would exhibit a high risk of DILI aggravation, which is apparently recognized as hepatocellular jaundice in clinical situations (Navarro and Senior, 2006). Schematic diagram of our *in vitro* assay concept is depicted in Fig. 1 in relation to *in vivo* situation. Importantly, the drug inhibiting the BC network formation during the recovery process is not necessarily the same as the drug killing the hepatocytes during the initial injury process. On this basis, we attempted to establish an *in vitro* assay system where drug inhibition of BC network formation could be quantitatively evaluated. Sandwich cultured hepatocytes (SCHs) may be suitable for this purpose (LeCluyse et al., 1994). Primary hepatocytes cultured between extracellular matrices such as gelled collagen and Matrigel rapidly generate and re-organize an *in vivo*-like BC network lined by dense filamentous actin (F-actin) and sealed by tight junction proteins such as Zonula occluding-1 (ZO-1). Moreover, this structure is functionally active since some aforementioned biliary efflux transporters are specifically localized on the BC membrane (Fu et al., 2010; LeCluyse et al., 1994). SCHs culture system is prevalent in the fields of drug metabolism, transport, and toxicity research (De Bruyn et al., 2013; Shen and Meng, 2012; Swift et al., 2010). We selected test drugs known to induce cholestatic DILI and/or severe DILI, and then examined whether they inhibit BC network formation. Furthermore, their relationship with the clinical risk of severe DILI was discussed.

2. Materials and methods

2.1. Animals

Sprague-Dawley rats (SLC Japan Inc., Tokyo, Japan), 7–8 weeks old, were used throughout the experiments. Animals were treated humanely in accordance with guidelines issued by the National Institutes of Health. All procedures were approved by the Animal Care Committee of Chiba University.

2.2. Materials

Williams' Medium E (WME) and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Gibco® (Tokyo, Japan). Amiodarone,

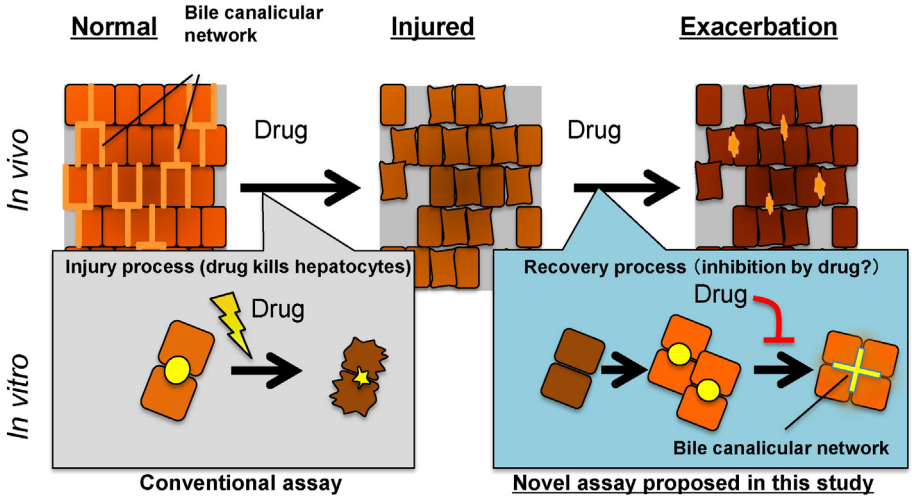

Fig. 1. Schematic diagram of our *in vitro* assay concept. Once liver is injured by drug, it is manifested as increase in hepatocellular marker increase in the serum (injury process). Subsequently, hepatocytes are replenished by proliferation and differentiation of the residual hepatocytes (recovery process). During the recovery process, liver specific structure such as BC network is formed. If the recovery process is inhibited, liver injury is prolonged or even exacerbated. Based on the concept, our present assay particularly focused on the recovery process by measuring the length of BC network formation in the presence of test drugs. Note that conventional assay mostly focused on the injury process but not on the recovery process.

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