Predictive in vitro cardiotoxicity and hepatotoxicity screening system using neonatal rat heart cells and rat hepatocytes

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Abstract
To establish a predictive in vitro cardiovascular toxicity and hepatotoxicity screening system, whole cells from the neonatal rat heart and rat hepatocytes were cultured with known cardiotoxic and hepatotoxic compounds and cytotoxicities were assessed. The heart cells were isolated from neonatal SD rats by enzyme digestion and cultured on type I collagen gel. After a several days of preculture, the cells were exposed to test compounds for 24 or 48 hr and LDH activity in the cells was measured. The hepatocytes were isolated from adult SD rats by in situ perfusion with collagenase and cultured in type I collagen-coated plates. After several hours of preculture, the cells were exposed to test compounds for 24 hr and LDH activity in the cells was measured. The IC50 values for cardiotoxic anthracyclines in rat neonatal heart cells showed very good correlation with in vivo cardiotoxic doses in rats. IC50 values for cardiotoxic anthracyclines were much lower in the heart cells than in the hepatocytes, and IC50 values for known hepatotoxic compounds were lower in the hepatocytes than in the heart cells. The ratio of IC50 values (heart cells/hepatocytes) would be a good predictor of cardiotoxic and hepatotoxic compounds.

Keywords: rats, cardiomyocytes, hepatocytes, in vitro, cytotoxicity

Introduction
In early stages of drug discovery, in vitro toxicity evaluation is very important to obtain quick evaluation with small amount of test compounds and reduce animal use. Primary rat hepatocytes are widely used for in vitro hepatotoxicity evaluation. The cytotoxic concentrations in rat hepatocytes are different from that in cell lines such as HepG2, HeLa, and BALB/c 3T3 especially in compounds in which metabolites have hepatotoxicity (Wang, 2002). Human hepatocytes are also used for in vitro hepatotoxicity evaluation, but maintaining viability and liver-specific functions is difficult because of cryopreservation and shipping. Primary culture of rat cardiomyocytes has been used for in vitro cardiotoxicity evaluation (Toseland, 1996). Neonatal, rather than adult, rat cardiomyocytes have been used because they are easy to disperse and have higher viability after isolation and culturing (Fu, 2005). These in vitro evaluations are usually performed separately for each organ; parallel assay in different organs is not widely carried out. In this investigation, to establish a predictive in vitro cardiovascular toxicity and hepatotoxicity screening system, whole cells from the neonatal rat heart and rat hepatocytes were cultured with known cardiotoxic and hepatotoxic compounds and cytotoxicities (LDH contents) were compared.

Materials and methods
Chemicals: Epirubicin (EPI), daunorubicin (DNR), idarubicin (IDA), doxorubicin (DOX), acetaminophen (APAP), cyclophosphamide (CPA), diclofenac (DCF), disulfiram (DSF), lithocholic acid (LCA) were purchased from Sigma (Tokyo, Japan). Minimal essential medium (MEM), William’s E medium, penicillin (10000 U/mL)-streptomycin (10000 µg/mL) solution, L-glutamine solution (200 mM), HEPES buffer (1 M, pH 7.3), penicillin (10000 U/mL)-streptomycin (10000 µg/mL) solution, L-glutamine solution (200 mM), HEPES buffer (1 M, pH 7.3), heat-inactivated fetal bovine serum (FBS), Hank's balanced-salt solution (HBSS), Ca, Mg-free Hank's balanced-salt solution (HBSS(-)) were purchased from Invitrogen (Tokyo, Japan). Minimal essential medium (MEM), William's E medium, penicillin (10000 U/mL)-streptomycin (10000 µg/mL) solution, L-glutamine solution (200 mM), HEPES buffer (1 M, pH 7.3), heat-inactivated fetal bovine serum (FBS), Hank's balanced-salt solution (HBSS), Ca, Mg-free Hank's balanced-salt solution (HBSS(-)) were purchased from Invitrogen (Tokyo, Japan).

Neonatal rat heart cell culture: The heart was isolated from neonatal (1 — 5 days old) Crl: CD (SD) rats (Charles Liver Japan, Tokyo, Japan) and dispersed on a rotation culture with an enzyme mixture of 2.5 mg/mL trypsin (1:250; Invitrogen), 1.5 mg/mL collagenase (Wako, Osaka, Japan), and 1.5 mg/mL crystallized...
bovine albumin (Serologicals Proteins Inc., IL, USA) in MEM at 37°C for approximately 60 min. The dispersed cells were collected every 15—20 min and the remaining undispersed tissues were re-suspended in the enzyme mixture solution and the rotation culture was continued. The dispersed cells were washed with HBSS(-) and suspended in MEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 10 mM HEPES (pH 7.3). The cell number was adjusted to 3×10^5/mL and cultured in 96-well plates coated with type I collagen gel (BD, Tokyo, Japan) with 10% Matrigel (BD). After several days of preculture, the supernatant was removed and the culture medium with a test compound was added to a well and incubated at 37°C for 24 or 48 hr. After incubation, LDH activities in the cells were measured. IC50 values were calculated from the concentration-response curve as the concentration of a test compound with a decrease in absorbance equivalent to 50% of the control value.

**Rat hepatocyte culture:** The hepatocytes were isolated by two-step in situ perfusion with collagenase from 6—10-week-old SD rats as previously described (Seglen, 1976, Wang, 2002). Briefly, the liver was perfused with 0.5% EGTA (Dojindo, Kumamoto, Japan) in HBSS(-) from the portal vein for approximately 5 min and then perfused with 0.05% collagenase + trypsin inhibitor (Sigma) in HBSS for 8—10 min. The dispersed cells were washed with ice-cold HBSS(-) and suspended in William's E medium supplemented with 10% FBS, 0.1 µM insulin (Sigma), 1 µM dexamethasone (Sigma), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 10 mM HEPES (pH 7.3). The cell number was adjusted to 3×10^4/mL and cultured in 96-well plates coated with type I collagen (IWAKI, Tokyo, Japan). After preculture for several hours, the supernatant was removed and the culture medium with a test compound was added and incubated at 37°C for 24 hr. After incubation, LDH activities in the cells were measured. IC50 values were calculated from the concentration-response curve as the concentration of a test compound with a decrease in absorbance equivalent to 50% of the control value.

**Results**

**Dose-response of neonatal rat heart cells to anthracyclines:** The dose-response curves of the cardiotoxic anthracyclines in neonatal rat heart cells are shown in Fig. 1 (24-hr exposure) and Fig. 2 (48-hr exposure).

**Table 1. In vivo and in vitro toxicities of anthracyclines**

<table>
<thead>
<tr>
<th>Anthracyclines</th>
<th>In vivo 24-hr IC50 (µg/mL)</th>
<th>In vitro 24-hr IC50 (µg/mL)</th>
<th>In vitro 48-hr IC50 (µg/mL)</th>
<th>In vitro 24-hr IC50 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNR</td>
<td>4.0</td>
<td>1.22</td>
<td>0.97</td>
<td>13.8</td>
</tr>
<tr>
<td>EPI</td>
<td>3.5</td>
<td>1.53</td>
<td>1.00</td>
<td>&gt;18.3</td>
</tr>
<tr>
<td>DOX</td>
<td>3.0</td>
<td>1.23</td>
<td>0.77</td>
<td>&gt;18.3</td>
</tr>
<tr>
<td>IDA</td>
<td>0.6</td>
<td>0.39</td>
<td>0.16</td>
<td>11.5</td>
</tr>
<tr>
<td>R^2 (with in vivo)</td>
<td>1.00</td>
<td>0.84</td>
<td>0.97</td>
<td>Poor correlation</td>
</tr>
</tbody>
</table>

*Doses giving major cardiac functional symptoms with an acceptable general toxicity with in SD rats from intraperitoneal administration for 12 days. (Plande, 2006)
The correlations of the in vitro data to published in vivo cardiotoxic doses in rats were shown in Fig. 3 (in vitro, 24-hr exposure) and Fig. 4 (in vitro, 48-hr exposure). The in vivo data are doses giving an acceptable toxicity with symptoms of major cardiac function (decreased left ventricular pressure developed under a constant perfusion pressure and the rate of variation of this parameter in contractility and relaxation) in SD rats intraperitoneally administered for 12 days (Plande, 2006). The R-square values between in vitro and in vivo were 0.8418 in in vitro with 24-hr exposure and 0.9740 in in vitro with 48-hr exposure.

**Dose-response of rat hepatocytes to anthracyclines:** The dose-response curves of cardiotoxic anthracyclines in rat hepatocytes were shown in Fig. 5 (24-hr exposure). The IC50 values for each compound at 24 hr were EPI: >31.6 µM (>18.3 µg/mL), DNR: 24.5 µM (13.8 µg/mL), IDA: 21.5 µM (11.5 µg/mL), DOX: >31.6 µM (>18.3 µg/mL). The IC50 values for each assay of anthracyclines compared with in vivo cardiotoxic doses are summarized in Table 1. IC50 values for in vitro rat heart cells showed good correlation with the in vivo cardiotoxic doses, but the IC50 values for in vitro rat hepatocytes showed poor correlation with the in vivo cardiotoxic doses. The ratio of IC50 values for in vitro rat heart cells to in vitro rat hepatocytes are shown in Table 2. The ratios showed very low values, indicating that the cytotoxic concentrations of cardiotoxic anthracyclines in rat heart cells was very lower than in rat hepatocytes.

**IC50 values of hepatotoxic compounds in neonatal rat heart cells and in rat hepatocytes:** IC50 values of known hepatotoxic compounds APAP (McConnachie, 2007), CPA (DeLeve, 1996), DCF (Aithal, 2004), DSF (Jatoe, 1988), and LCA (Kitada, 2003) for in vitro neonatal rat heart cells and for in vitro rat hepatocytes and ratios of IC50 values (heart/hepatocytes) are shown in Table 3. Hepatotoxic compounds showed higher ratios than cardiotoxic anthracyclines, but some hepatotoxic compounds showed the ratios of less than 1, indicating that cytotoxicity in rat heart cells was higher than in rat hepatocytes.
The results suggest that discrimination of cardiotoxic compounds from hepatotoxic compounds might be partially possible using the IC50 ratio of heart cells to hepatocytes from in vitro assays using neonatal rat heart cells and rat hepatocytes.

**Discussion**

To clarify the ability of our in vitro cardiotoxicity assay using neonatal rat heart cells cultured on collagen gel to predict in vivo cardiotoxicity, cardiotoxicity of anthracyclines between in vitro and in vivo were compared. The IC50 values for LDH content in the cells exposed to anthracyclines for 24 or 48 hr were compared with in vivo data published by Plande et al. (Plande, 2006). The IC50 values of the known cardiotoxic anthracyclines (EPI, DNR, IDA, DOX) used in our experiment showed very good correlation with the in vivo results. The in vivo cardiotoxic doses from Plande et al. were determined as doses that cause major cardiac functional symptoms with acceptable general toxicity. In general, a comparison of in vitro with in vivo results is difficult because of the complex mechanisms of toxicities in vivo, different parameters measured, relatively longer treatment periods with in vivo repeated-dose studies, and different pharmacokinetic profiles of the test compounds. In this experiment, the correlation between in vitro results in rat heart cells and in vivo results in 12-day rat studies was very good probably because of similar mechanisms of toxicity and similar pharmacokinetic profiles of anthracyclines. DOX and EPI are reported to have similar disappearance from serum after intravenous injection in mice (Broggini, 1980). IDA and DNR are reported to have similar pharmacokinetic profiles in leukemic patients (Robert, 1992). In rat experiments, the disappearance of EPI was comparable with DOX (Broggini, 1980). The cardiotoxic dose, shown by reduction of cardiac output at 12 weeks after single administration in rats for DOX was approximately two times more cardiotoxic than EPI (Yeung, 1989); however the cytotoxicity of DOX was comparable with EPI in our in vitro cytotoxicity assay in neonatal rat heart cells and after 12 days administration in rats (Plande, 2006). The later cardiotoxicity of anthracyclines is considered to involve different mechanisms than the direct injury to cardiomyocytes from anthracyclines. It would take time to cause cardiac functional alterations after the injury to cardiomyocytes. On the other hand, acute (shorter exposure than our experiment) cytotoxicities of anthracyclines in vitro in adult rat cardiomyocytes have been reported (Andersson, 1999). Andersson assessed the cytotoxicity using a Trypan blue exclusion test until 2 hr of in vitro exposure. The IC50 values were 489 µM for EPI, 185 µM for DNR, 159 µM for IDA, and 887 µM for DOX, values are much higher than our results: and the relative order of the cytotoxicity of DNR was higher (a lower IC50 value) than in our experiment. Those high values may have been a result of the very short time exposure to high concentrations of the test compounds. Acute (within 2 hr) cytotoxicities would have a different mechanism than exposure longer than 24 hr. Higher correlation of our data to in vivo results (12-day administration in rats) suggest that an exposure period of more than a day is necessary for better prediction of in vivo cardiotoxicities at least for anthracyclines. The relative order of acute in vitro cytotoxicity reported by Andersson correlates well with our results in rat hepatocytes, suggesting that the cytotoxicity from a very short exposure with very high concentrations in rat cardiomyocytes and a 24-hr exposure time in rat hepatocytes (non-target organ of in vivo toxicity) would be from cell type-nonspecific mechanisms of injury for all types of cells.

**Hepatocyte cultures are widely used for toxicological and pharmacological experiments** (Guillouzo, 1997, Paillard, 1999). In our own laboratories, we can easily obtain sufficient number of primary rat hepatocytes by collagenase digestion. Human hepatocytes can be obtained commercially, but viability and liver specific activities are decreased from long shipping period and cryopreservation. We examined primary rat hepatocyte monolayers cultured in type I collagen-coated plates. Recently, many types of culture systems have been developed to maintain liver-specific functions (Xu, 2006). In our experiment, we selected a basic hepatocyte monolayer culture system that has been widely used for a long time. The cytotoxicity of the anthracyclines in the rat hepatocytes was much less (higher IC50 values for LDH contents) than in the rat heart cells. It suggests that cardiotoxic compounds show higher cytotoxicity in the heart cells than in other cells, and screening of cardiotoxic compounds may be possible. In addition, the order of cytotoxicity of anthracyclines...
in the hepatocytes was different from that in the heart cells (low correlation), suggesting that cytotoxicity of cardiotoxic compounds in the hepatocytes may be detecting cell-type nonspecific cytotoxicities in high concentration of compounds. To confirm the criteria, we also examined known hepatotoxic compounds (APAP, CPA, DCF, DSF, LCA) in rat hepatocytes and rat heart cells. Specific cytotoxicities of these compounds in the rat hepatocytes were confirmed in comparison with mouse 3T3 fibroblasts, human cervical cancer HeLa cells, and human hepatoma HepG2 cells (Wang, 2002). Higher cytotoxicity in rat hepatocytes than in rat heart cells was not evident in all of these compounds. CPA and DCF showed an IC50 ratio (heart/hepatocyte) more than 2, possibly indicating a relative higher cytotoxicity in hepatocytes. CPA is known to be metabolized to toxic metabolites by liver P450 enzymes (Huang, 2000) that cause higher cytotoxicity in the hepatocytes. APAP (IC50 ratio of 0.277) is reported to be cardiotoxic in significant overdose (Armour, 1993). DSF (IC50 ratio of 0.781) is reported to cause arrhythmia (Fossa, 1983). LCA (IC50 ratio of 1.06) is reported to be toxic for cardiac mitochondria (Ferreira, 2005).

These results indicate that APAP, DSF, and LCA are not only hepatotoxic but also cardiotoxic. From these information, the IC50 ratio (heart/hepatocyte) around 1 suggest that the test compounds are both hepatotoxic and cardiotoxic. Organ-specific toxicity in vivo is thought to be caused by higher sensitivity of the specific cells or accumulation of the compounds in specific tissues. The former mechanism could be evaluated using an in vitro system with organ-specific cell types. Organ-specific toxicities might be screened from parallel experiments of in vitro culture systems using cells from different organs. An in vitro cytotoxicity assay using liver and kidney cell lines was reported to have good compatibility with target-organ toxicity in vivo (Li, 2004, Zhang, 2007).

Our experiment demonstrated the possible usage of separate multi-organ in vitro systems of the liver and the heart for screening hepatotoxic and cardiotoxic compounds. Further experiment using more reference compounds would be needed to further validate this in vitro organ-toxicity evaluation system.

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References


