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Real-time concurrent monitoring of apoptosis, cytosolic calcium, and mitochondria permeability transition for hypermulticolor high-content screening of drug-induced mitochondrial dysfunction-mediated hepatotoxicity

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HIGHLIGHTS

- High content screening was suggested for monitoring of drug-induced hepatotoxicity.
- Nefazodone, tolcapone, and troglitazone caused mitochondrial dysfunction.
- Piroxicam-treated cells showed apoptotic cell death without the MPT formation.

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ABSTRACT

A quantitative high-content screening (HCS) was suggested for the real-time monitoring of drug-induced mitochondrial dysfunction-mediated hepatotoxicity. This HCS is very advantageous in that it allows simultaneous observation of drug-induced activations of hepatotoxic pathways using hypermulticolor cellular imaging. The mitochondrial permeability transition (MPT), cytosolic calcium, and caspase-3 were selected as functional markers to verify drug-induced hepatotoxicity and were concurrently monitored in HepG2 cells in a real-time manner. Nefazodone, tolcapone, and troglitazone caused mitochondrial dysfunction and subsequent apoptotic HepG2 cell death in addition to marked cytosolic calcium increase. On the other hand, extrinsic pathway-mediated apoptotic cell death was monitored when HepG2 cells were treated with piroxicam. It was found that piroxicam-treated HepG2 cells showed apoptotic cell death without the MPT formation, while a cytosolic calcium increase was clearly observed. This finding was confirmed by the caspase-8 inhibition assay. These results demonstrated the unique potential of real-time hypermulticolor HCS to screen hepatotoxic drugs at the in vitro stage rather than the later in vivo stage based on an animal model and to ultimately reduce the probability of drug failure.

1. Introduction

Many new pharmaceuticals have been withdrawn from the pharmaceutical market due to their unexpected side effects. Drug-induced hepatotoxicity or cardiotoxicity are examples of representative side effects. Particularly, drug-induced mitochondrial dysfunction has received attention as a main contributor to hepatotoxic side effects (Masubuchi et al., 2006; Hynes et al., 2006; Haasio et al., 2001). Mitochondria are plentiful in cells which require active cellular respiration such as hepatocyte, myocyte, and cardiac cells. Recently many liquidated new drugs have had a tendency to cause mitochondrial dysfunction in hepatocyte or cardiac cells. Traditionally, large pharmaceutical companies have carried out drug safety tests at a fairly late stage in the drug discovery process (Dykens and Will, 2007). Drug safety evaluations are delayed until after a lead compound has been nominated based on its target and in vivo efficacy. Recently, some pharmaceutical companies have decided that it is preferable for drug safety evaluations to be executed during the process of lead compound selection, in order to diminish late stage attrition. When cell-based screening works properly as a tool for the evaluation of drug-induced mitochondrial toxicity, it is a practical solution to reduce the economic and time loss that may occur at the in vivo stage. Drugs that cause serious mitochondrial toxicity should be excluded at the in vitro level and this procedure, followed by in vivo efficacy monitoring, can contribute greatly to the reduction of the probability of drug failure.

Mitochondria, as factories of energy production, play a number of pivotal roles in cell survival such as ATP generation, cytosolic Ca^{2+} uptake and storage, and reactive oxygen species (ROS) detoxification (Song et al., 2009). Mitochondria maintain a particular

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mitochondrial membrane potential (MMP) formed through the transfer of protons from the mitochondrial matrix to the intermembrane space by mitochondrial complexes that are activated via the transfer of electrons from NADH and $FADH_2$ (Scheffler, 1999). The reduction of MMP is deeply related to disruption of cellular respiration and ATP production and leads to mitochondrial permeability transition (MPT). A remarkable characteristic of cellular Ca^{2+} is its versatility in binding and regulating various cellular proteins and subcellular structures. Although cellular Ca^{2+} homeostasis normally persists, the disruption of Ca^{2+} homeostasis activates multiple damaging processes under pathological conditions. Dramatic increase of intracellular Ca^{2+} level is one of the features that occur in damaged cells (Berridge et al., 2003). MPT and intracellular Ca^{2+} increase, in addition to cell death of apoptosis or necrosis, are effective indicators for the assessment of drug-induced hepatotoxicity. Particularly, the increase of the cytosolic Ca^{2+} level is closely related to MPT in the process of drug-induced hepatotoxicity.

High-content screening (HCS) has attracted attention due to its ability to profile potent toxic liability at an earlier stage than the traditional animal model histopathology in the process of drug development (Wlodkowic et al., 2011). HCS simultaneously measures multiple biomarkers in a single cell with multiplexing fluorescence measurement (Michelini et al., 2008). Monitoring of functionally significant spatial and temporal intracellular dynamics is one of features of HCS measurement (Schembri et al., 2009). Application of HCS to organ-specific cell models provides deeper biological information suitable for better decisions on progressing compounds. This is because early safety evaluation by HCS reveals the complex cellular responses triggered by potentially harmful molecules in the cells of target organs. Gaining a deep understanding of the mechanisms underlying these cellular toxicological responses is valuable before a series of lead compounds are progressed to time-consuming and expensive animal tests. In this work, a real-time hypermulticolor HCS is suggested to concurrently monitor multiple biomarkers related to drug-induced hepatotoxicity. Cytosolic Ca^{2+}, MPT, and caspase-3 were simultaneously observed in HepG2 cells. Nefazodone, troglitazone, tolcapone, and piroxicam were tested. Despite HCS's capability it is not common to simultaneously observe multiple biomarkers in an intact cell based on real-time monitoring. This is because HCS measurement is dependent on the use of probing materials. Concurrent monitoring of multiple biomarkers is practically limited due to the spectral overlap among probing materials having broad absorption and emission spectrums. In this work, hypermulticolor cellular imaging was suggested to solve this practical problem. Hypermulticolor HCS is capable of supplying cellular imaging at particular wavelengths and each wavelength can be scanned rapidly. This cellular imaging is very advantageous in that it can select particular wavelengths that do not overlap among the probing materials and concurrently monitor a larger number of drug targets or biomarkers. This approach contributed greatly to the prediction of drug safety risk with high specificity, as well as assisting in gaining understanding of the action mechanism of drug-induced hepatotoxicity.

2. Materials and methods

2.1. Chemicals

Nefazodone, piroxicam, dexamethasone, dimethyl sulfoxide (DMSO), and methanol were purchased from the Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Tolcapone and troglitazone were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Calcium indicator (Ex/Em: 549/576 nm) fluorescent probes and a MitoProbe™ transition pore assay kit (Ex/Em: 494/517 nm) were obtained from Molecular Probes Inc. (Eugene, OR, USA). A Magic Red™ [$(Z\text{-}DEVD)_2$-Magic Red] detection kit (Ex/Em: 550/610 nm) was purchased from Immunochemistry Technologies, LLC (Minneapolis, MN, USA). Caspase-8 substrate [$(Z\text{-}IETD)_2$-Rh110] was purchased from AnaSpec, Inc. (San Jose, CA, USA).

2.2. Cell culture

The HepG2 cell line was obtained from the Korean Cell Line Bank (KCLB®, Seoul, South Korea) for the screening of drug-induced hepatotoxicity. Cells were cultured in standard minimum essential medium (MEM) (Gibco, Grand Island, NY, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), penicillin (60 μg ml^{-1}), and streptomycin (100 μg ml^{-1}) in cell-culture flasks (75 cm^2). Cells were grown under the standard conditions, i.e. at 37 °C in a humidified incubator containing 5% CO_2 (US AutoFlowt, NuAire, Plymouth, MN, USA). After attaining confluence, the cells were subcultured following trypsinization with a 0.25% trypsin–EDTA solution (Gibco, Grand Island, NY, USA). HepG2 cells were plated onto a 35 mm petri-dish at a density of 1×10^6 overnight and then treated with the following probes in the MEM medium.

2.3. HepG2 cell staining with calcium binding probes

HepG2 cells in a 35 mm petri-dish were treated with calcium binding probes. 5 mM stock solution of the calcium binding probe (Invitrogen, Carlsbad, CA, USA) was prepared by dissolving 50 μg of calcium indicator powder in a vial with DMSO. The stock solution was then diluted with 1 mL MEM medium to produce a 3 μM working solution. After the incubation of HepG2 cells with the 3 μM working solution for 45 min at 25 °C, the cells were washed with a 1× PBS buffer. The entire staining process was executed in the dark condition. The non-fluorescent calcium indicators become fluorescents after their binding to intracellular calcium. As the amount of intracellular calcium increases, the cellular fluorescence intensity increases as a result of the production of fluorescents.

2.4. HepG2 cell staining with calcein-acetoxymethyl ester (AM) for the monitoring of MPT pore opening

Calcein-AM in the MitoProbe™ transition Pore Assay kit (Invitrogen, Carlsbad, CA, USA) was used as a probe to observe the intracellular MPT depending on drug toxicity. All of the calcein-AM powder was dissolved in a vial with DMSO to make a 1 mM stock solution. The calcein-AM stock solution was diluted 500 times in Hank's buffered salt solution (HBSS)/Ca^{2+} to make a 2 μM working solution. The calcein-AM working solution of 5 μL was mixed with 5 μL of $CoCl_2$ (supplied with the kit) and further diluted in MEM media so that total volume of the solution was 1 mL. HepG2 cells having the calcium indicator were consecutively treated with this solution for 15 min at 37 °C. The whole staining process was performed in the dark condition. The calcein-AM uptaken in HepG2 cells are accumulated in cytosolic compartments including mitochondria and cleaved by intracellular esterase to liberate the very polar fluorescent calcein. The produced calcein has a tendency to not penetrate mitochondria membrane or plasma membrane in appreciable amounts over relatively short periods of time. The fluorescence of cytosolic calcein is quenched by $CoCl_2$ while that of mitochondrial calcein is retained. The HepG2 cells were then washed with 1× PBS buffer.

2.5. Treatment of HepG2 cells with caspase-3 substrate

A vial containing caspase-3 substrate in the Magic Red™ detection kit was dissolved with 50 μL of DMSO. This stock solution was diluted 5 times in deionized water. This solution of 10 μL was further diluted with 500 μL MEM. The HepG2 cells having calcium indicators and calceins were consecutively incubated with this caspase-3 substrate solution for 30 min at 37 °C. The HepG2 cells were then washed with 1× PBS buffer. The staining process was executed in the dark condition. The caspase-3 substrate is a conjugate of fluorescent dye and two tetra peptides and exists as a non-fluorescent. The caspase-3 substrates uptaken in the HepG2 cells reacted with the caspase-3 formed under the apoptotic condition. As a result, the conjugates were cleaved and produced fluorescent dyes.

2.6. Drug treatment

For the monitoring of drug-induced hepatotoxicity in a real time manner, HepG2 cells which contained calcium indicators, calceins, and caspase-3 substrates were treated with the individual drug to be tested at different concentrations (nefazodone, tolcapone, troglitazone, piroxicam, and dexamethasone). The stock solutions of nefazodone (40 mM), troglitazone (100 mM), tolcapone (10 mM), and dexamethasone (100 mM) were prepared by dissolving their powders in DMSO. The stock solution of piroxicam (0.5 M) was prepared in methanol. The HepG2 cells were cultured overnight to achieve log phase growth prior to the drug treatment. When the cell confluency reached around 80% the drug treatment was executed. The stock solutions were diluted with MEM containing 10% FBS and subsequently the cells were treated with the following drugs: nefazodone (1 μM, 38.4 μM, 200 μM), troglitazone (400 μM), tolcapone (10 μM), dexamethasone (100 μM), and piroxicam (4 mM). During the cellular measurements, the cells were enclosed in a live cell incubator system (Chamlide TC, Live Cell Instrument, Seoul, South Korea) mounted