Testing of Compounds for Drug Induced Phospholipidosis Using Assay Ready Cryopreserved HepG2 Cells

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introduction

Excessive accumulation of phospholipids in multi-lamellar bodies (phospholipidosis) can be induced by cationic amphiphilic drugs and is associated with clinical toxicities (e.g. hepatotoxicity, QT prolongation, kidney and lung injury, and myopathy). For a predictive risk assessment of drug candidates animal testing is still required. However, in silico models and cell based assays can help to evaluate the phospholipidosis potential of a drug at an early stage of preclinical development and may reduce animal testing.

Here we demonstrate the use of assay ready cryopreserved HepG2 cells to detect phospholipidosis in vitro by fluorescent probes which specifically stain phospholipids accumulated in the lysosomes. The cells which are frozen at a functional state are ready to use instantly after thawing and don't need to be cultivated or passaged before. Alternative protocols were developed to use assay ready frozen HepG2 cells to quantify the grade of phospholipidosis either by fluorescent microscopy, flow cytometry, or in a plate based assay.

preparation of assay ready frozen cells

To prepare high quality assay ready cryopreserved cells, the cells have to be expanded under optimal culture conditions and need to be kept in the logarithmic growth phase. HepG2 cells were grown in CellStacks with HAM's F12 (Sigma Aldrich), 20% FBS (PAN Biotech), and 2mM L-Gln (Sigma Aldrich). Sub-confluent cultures were harvested enzymatically using TrypLE Express (Thermo Fischer). For cryopreservation the cells were suspended in a serum free medium formulation containing 5% DMSO for cryoprotection. The cells were automatically dispensed at 10 million cells per vial (Nunc) using an XSD-Biofill decapping and filling device (FuidX) and frozen in a Cryomed 7452 controlled rate freezer (Thermo Fisher) at a cooling rate of 1°C per minute. Cryopreserved cells were stored in vapor phase of liquid nitrogen (Fig.1).

Fig. 1: Production of assay ready frozen cells

quality control of assay ready HepG2 cells

For quality control of the assay ready HepG2 a vial of frozen cells were thawed and washed once in recovery buffer. The cell pellet was resuspended in culture medium and the cell count, the viability, the amount of debris and the grade of aggregation was determined in a CASY TT cell counter (Fig. 2). An aliquot of the cells was seeded at 2E4 cells/cm² in a tissue culture flask. Morphology and confluences was determined 24 hours after plating by microscopic imaging (Fig. 3).

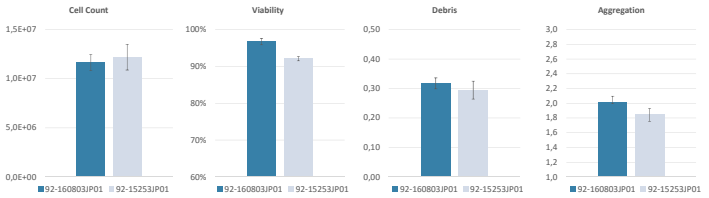

Fig. 2.: viability parameters
assay ready HepG2 cells of two different lots were analyzed in a CASY TT to determine cell count, viability, debris and aggregation.

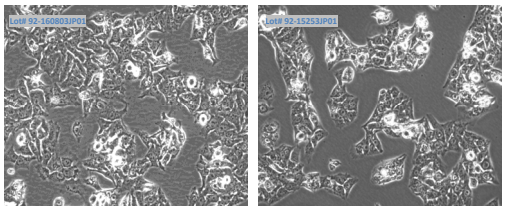

Fig. 3.: microscopic imaging
Morphology and confluence of assay ready HepG2 cells 24 hours after plating.

imaging of phospholipidosis in assay ready HepG2

To measure drug induced phospholipidosis a vial of assay ready HepG2 cells were thawed quickly in a water bath (37°C) and washed once in recovery buffer. The cell pellet was resuspended in assay medium and 5.000 cells were dispensed into each well of a 96-well plate. Sparing any pre-cultivation of the cells Sertraline a potent inductor of phospholipidosis (final conc. 10 µM), or Cyclosporine A as a negative control (final conc. 6 µM) were added directly to the cells in assay medium. LipidTox Red (Thermo Fisher), a specific dye of phospholipids, was added to the cells at the same time. The cells were incubated at 37°C for 72 hours in a humidified atmosphere at 5% CO_2. Thereafter the medium was removed and the cells were fixed with 4% paraformaldehyde and analyzed by florescence microscopy.

Assay ready HepG2 cell treated with the drugs directly after plating attached normally and displayed an typical morphology 72 hours after plating (not shown). Sertraline induced a strong accumulation of phospholipids in the lysosomes of the cells as indicated by the red fluorescent LipidTox staining (Fig. 4B). In Cyclosporine A treated cells which is supposed to induce steatosis but not phospholipidosis, no such staining could be detected (Fig. 4A).

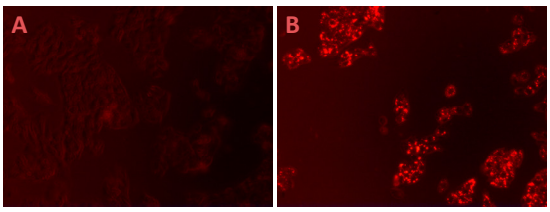

Fig. 4: Imaging of phospholipidosis. Assay ready HepG2 cells were treated with 6µM Cyclosporine A (A) or 10 µM Sertraline (B) and were stained with LipidTox Red (Thermo Fisher). Fluorescently stained phospholipids were analyzed by florescence microscopy (100 x)

analysis of phospholipidosis by flow cytometry

To quantify the grade of phospholipidosis assay ready HepG2 cells, prepared as described before, were treated directly with increasing concentrations of Sertraline (2.5, 5, and 10 µM) and stained with LipidTox Red. Cyclosporine A (6mM) was used as a negative control again. After 72 hours the cells were detached with TrypLE, washed in PBS and fixed with ice cold 2% paraformaldehyde in PBS. Fluorescently labelled cells were analyzed in a Cytomics FC500 flow cytometer (Beckman Coulter).

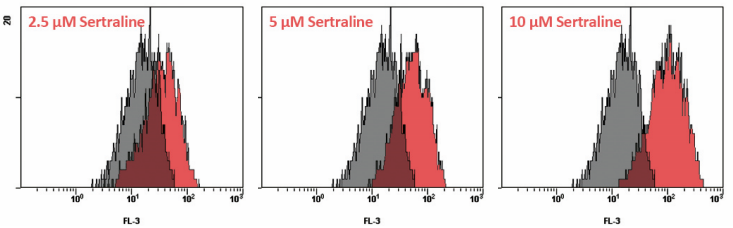

Fig. 5: FACS analysis of phospholipidosis. assay ready HepG2 cells were treated with increasing concentration of Sertraline, stained with LipoTox Red and analyzed by flow cytometry. The fluorescence intensity of cells was plotted as a histogram (red) and compared with the fluorescence intensity of Cyclosporine A treated control cells (grey).

Cell treated with Sertraline displayed a strong signal for phospholipidosis in a dose dependent manner (Fig. 5, red histograms). With 2.5 µM Sertraline 11.2 % of the cells showed a significant increase in florescence when compared with Cyclosporine treated control cells (Fig.5, grey histogram). When treated with higher concentrations of 5 µM or 10 µM Sertraline the proportion of fluorescent cells increased to 28% and 59% respectively.

quantification of phospholipidosis in a plate based assay

An alternative detection reagent for phospholipidosis was used for a plate based read-out. Red fluorescent Lyso-ID® (ENZO Life Sciences) is a cationic amphiphilic tracer, which specifically accumulates in acidic lysosomes. Drug induced phospholipidosis results in an strong increase in fluorescence of stained cells. 1.5E4 assay ready HepG2 cells were seeded into each well of a 96-well plate. The cells were directly treated with Sertraline (7.5 µM) and Verapamil (100 µM) for 72 hours. Cyclosporine A was again used as control. After the incubation the Lyso-ID staining solution was added to the cells and incubated for 30 minutes. Thereafter the cells were washed twice in assay medium and analyzed in fluorescence multiwell plate reader (Safire, Tecan) at $540_{Ex}/680_{Em}$.

Assay ready HepG2 cells treated with steatosis inductor Cyclosporine A did not provide a fluorescent signal significantly above the vehicle control. However, treatment with Verapamil or Sertraline, two strong inducers of phospholipidosis, results in a strong and reproducible fluorescence of stained phospholipids 30x above control (Fig. 6).

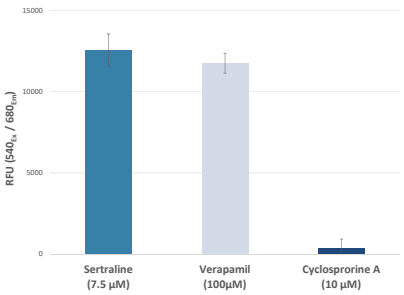

Fig. 6: Plate based phospholipidosis assay.
assay ready HepG2 cells were treated with Sertraline, Verapamil, and Cyclosporine A and were stained with Lyso-ID. Accumulation of phospholipids in acidic lysosomes was detected in a fluorescence plate reader.

discussion

assay ready HepG2 cells which are cryopreserved at a high viability and function provide a very robust and reproducible tool when used for the testing of new drug candidates for their potential to induce phospholipidosis. Accumulation of phospholipids is not altered by the cryopreservation process. The cells respond to phospholipidosis inducing drugs (e.g. Sertraline and Verapamil) to the same extend as expected but discriminate drugs which are known to induce steatosis but no phospholipidosis (e.g. Cyclosporine A). The cells could be applied with different phospholipidosis dyes (LipidTox, Lyso-ID) and were successfully validated for different assay ready-out (microscopic imaging, flow cytometry, plate based ready out).

Assay ready cells release the user from preparatory cell culture work and enables any operator to perform a phospholipidosis assay without being an expert in cell culture techniques. Because the assay ready cells are prepared and validated externally in homogenous batches, a big factor which generates inter laboratory variability is eliminated.

SAFETY

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