Screening for Drug Induced Phospholipidosis using Assay Ready HepG2 Cells

summary

To screen for drug induced accumulation of phospholipids (phospholipidosis) animal testing is still required for a predictive risk assessment. However, *in silico* models and cell-based assays can help to evaluate the phospholipidosis potential of a drug at an early stage of the preclinical development and may reduce animal testing. Cells used in cell-based assays are usually obtained from a continuous culture. When handled carefully and cryopreserved according to optimized protocols, the functionality of the cells can be maintained and is fully restored after thawing of the cells. Assay Ready Cells which are frozen at highly viable and functional state are ready to use directly after thawing and do not need to be cultivated or passaged before. We used Assay Ready HepG2 Cells in the phospholipidosis assay to demonstrate that they are equal to fresh cells from a growing culture. This approach provides a higher flexibility, greater reliability and increase in assay reproducibility.

introduction

Excessive accumulation of phospholipids in multi-lamellar bodies (phospholipids) can be induced by cationic amphiphilic drugs and is associated with clinical toxicities (e.g. hepatotoxicity, kidney, and lung injury) [1]. A working group, formed by the FDA in 2004 to study the phospholipidosis, provided a better understanding but no clear evidence of the molecular mechanisms [3]. Nevertheless, it is well known that phospholipidosis can predict drug accumulation that cause liver, kidney or pulmonary disfunctions. Detection of drug-induced phospholipidosis has been carried out using electron microscope and real-time PCR. More recently, fluorescent dyes have been used to assess phospholipidosis in cells *in vitro*. Here we utilized a fluorescently labeled phospholipid (Lyso-ID Red) which was added to Assay Ready HepG2 cells along with compounds exhibiting positive and negative phospholipidosis. We demonstrated that Assay Ready HepG2 cells are a useful and highly reproducible *in vitro* model for evaluating drug-induced phospholipidosis.

preparation of Assay Ready HepG2 Cells

To prepare high quality Assay Ready Cells, the cells must be expanded under optimal culture conditions and need to be kept in the logarithmic growth phase. HepG2 cells were cultivated in HAM's F12 (Sigma Aldrich), 20% FBS (PAN Biotech), and 2mM L-Glutamine (Sigma Aldrich). For further expansion sub-confluent cultures were detached with TrypLE Express (Thermo Fischer) and plated at a density of 2E4cells/cm^2 in CellStacks (Corning). Cells were cultivated at 37°C in a humidified atmosphere with 5% CO_2.

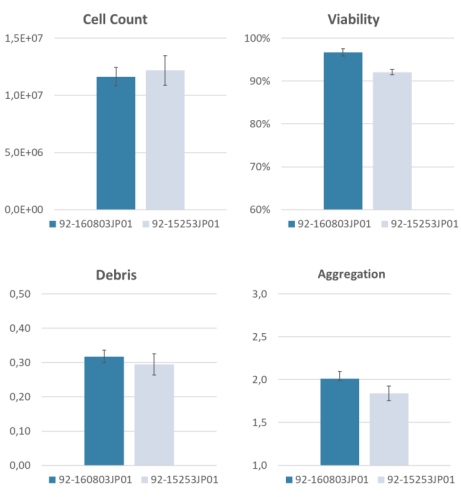

Fig.1.:Viability parameters of assay ready HepG2 cells.

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.

For quality control of the Assay Ready HepG2 Cells, a vial of frozen cells was 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 (Roche) (Fig. 1). An aliquot of the cells was seeded at 2E4 cells/cm^2 in a tissue culture flask. Morphology and confluence were determined 24h after plating by microscopic imaging (Fig. 2).

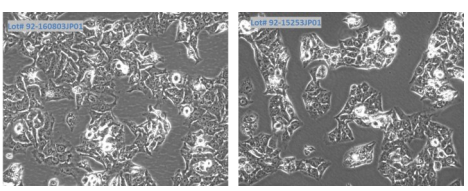

Fig.2.:HepG2 cells 24 hours after plating.

results

Imaging of phospholipidosis in Assay Ready HepG2 Cells

To measure drug-induced phospholipidosis, a vial of Assay Ready HepG2 Cells was quickly thawed in a water bath (37°C) and washed once in recovery buffer. After 24h of pre‑cultivation, Sertraline - a potent in-

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ductor of phospholipidosis (5 µM), Cyclosporine A - as a negative control (6 µM) and DMSO - as a vehicle control (0.5%) were added to the cells in assay medium. The cells were incubated at 37°C for 48h in a humidified atmosphere at 5% CO_2. The cells were stained with the LysoID Red detection kit (Enzo), which contains a specific dye for phospholipids and DAPI. Cells were fixed in 4% paraformaldehyde before imaging.

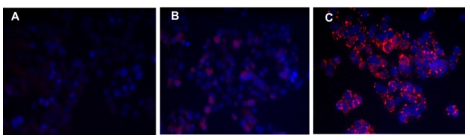

Fig. 3: Fluorescent imaging of phospholipidosis.

Sertraline induced a strong accumulation of phospholipids in the lysosomes of the cells as indicated by the red fluorescent Lyso-ID Red staining (Fig. 3B, C). In Cyclosporine A treated cells, which is supposed to induce steatosis but not phospholipidosis, no such staining could be detected (Fig. 3A).

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 10µM) and stained with Lyso-ID Red. Cyclosporine A (6mM) was used as a negative control again. After 48 hours, the cells were detached with TrypLE and washed in

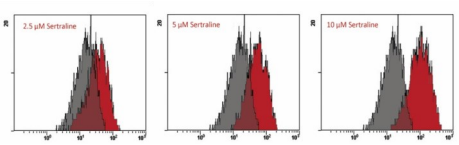

Fig. 4: Flow cytometry analysis of phospholipidosis.

PBS. Fluorescently labelled cells were analyzed in a Cytomics FC500 flow cytometer (Beckman Coulter). Cells treated with Sertraline displayed a strong signal for phospholipidosis in a dose-dependent manner (Fig. 4, red histograms). With 2.5µM Sertraline, 11.2% of the cells showed a significant increase in florescence when compared with Cyclosporine treated control cells (Fig. 4, grey histogram). When treated with higher concentrations of 5µM or 10µM Sertraline the proportion of fluorescent cells increased to 28% and 59% respectively.

Quantification of phospholipidosis in a plate -based assay

Assay Ready HepG2 Cells were thawed, washed once in recovery buffer, and dispensed into a 96□well plate at 3,4E4 cells/well. After 24 hours pre-cultivation, cells were treated with Sertraline (5µM), Amitriptyline (10µM) and Cyclosporin A (6µM) as a negative control and DMSO (0,5%) as a vehicle control for 48 hours. After the compound incubation, cells were stained with Lyso ID Red dye for 30 minutes. Thereafter the cells were washed once in assay medi-

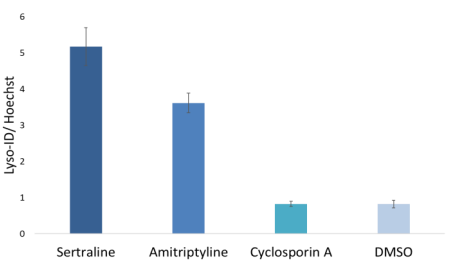

Fig. 5: Plate-based phospholipidosis assay

um and analyzed in fluorescence plate reader (Safire, Tecan) at 540Ex/680Em. Accumulation of phospholipids in acidic lysosomes was detected in a fluorescence plate reader. Treatment with Sertraline and Amitriptyline, the strong inducers of phospholipidosis, resulted in strong fluorescent phospholipid accumulation (Fig. 5). Assay Ready HepG2 Cells treated with steatosis inductor Cyclosporine A did not provide a fluorescent signal significantly above the vehicle control (0,5% DMSO). Dose-dependent increases in intracellular fluorescent phospholipid accumulation in Assay Ready HepG2 Cells were demonstrated. Assay Ready HepG2 Cells were treated for 48 hours with different

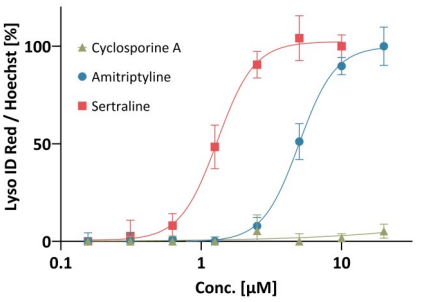

Fig. 6: Dose-dependent phospholipidosis induction

concentrations of test compounds and analyzed with Lyso-ID Red detection kit. A clear does-dependent increase of phospholipid accumulation could be demonstrated in Assay Ready Cells (Fig. 6)

discussion

Assay Ready HepG2 Cells, which are cryopreserved at a high viable and functional state, 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 inducers Sertraline and Amitriptyline to the same extend as expected but discriminate Cyclosporine A, which is known to induce steatosis but no phospholipidosis. The cells were applied with the phospholipidosis dye Lyso-ID Red and were successfully validated for different assay ready-outs (microscopic imaging, flow cytometry, plate-based read out).

related products

HepG2 Assay Ready Cells

prequalified cells for instant use, no propagation required. Including recovery buffer & assay medium. (Cat.N° RE561)

instaCELL® Phospholipidosis assay kit

validated kit with prequalified Assay Ready HepG2 Cells, media & buffer, control substances, staining dye, and 96-well assay plates. (Cat.N° SF130-05)