Cultured Hepatocyte Toxicity Assay

Introduction

Freshly isolated human hepatocytes represent a living biological system in which toxicity can be evaluated.[1]

Purpose

This assay is designed to study whether a new compound is toxic, and if so, the degree of toxicity. It is not designed to determine the mechanisms of the toxicity.

Principle of the Procedure

Cytotoxicity is evaluated by determining the mitochondrial function of the hepatocytes using the tetrazolium dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) following exposure to (or treatment with) the compound. MTT is a yellow vital dye that is actively converted by mitochondrial oxidation-reduction reactions into blue formazan crystals. The formation of the blue MTT crystals within the cell decreases in direct proportion to the viability of cells.[2]

Materials*

Item	Manufacturer	Name/Catalog/Model #
Biosafety cabinet	NuAire, Inc.	NU425FM600
CO_2 water-jacketed incubator	Forma Scientific	3110
Deionized water		
Orbital shaker	Bellco	7744-08096
Plastic multi-well plates with cover	Costar	
Plated hepatocytes	BioreclamationIVT	
Pump	GAST	DPA104AA
4 ^{o}C Refrigerator	Fisher Scientific	R134A
UV/Kinetic microplate reader	Molecular Devices	UV Max
Solvents/Solubilizers		
Acetonitrile (ACN)	Aldrich	27,071-7
Dimethyl Sulphoxide (DMSO)	Sigma Chemical Co.	D-2650
Ethanol	Aldrich	36,280-8
Methanol	Sigma Chemical Co.	270474
Buffers/Reagents		
*InVitro*GRO™ HI Medium	BioreclamationIVT	Z90009
Hydrochloric Acid (HCl)	JT Baker	9535-01
Isopropanol	EM Science	PX-183P-1
MTT	Sigma Chemical Co.	M-2128
Sodium Chloride (NaCl)	Sigma Chemical Co.	S-9625
Chlorpromazine	Sigma Chemical Co.	C-8138

*Items listed in this Materials section are for convenience; suitable materials and equipment from other manufacturers may be substituted as appropriate. Contact information for vendors used by BioreclamationIVT are listed in the Notes section at the end of this document.

Procedure

Reagent preparation

(To be completed in advance of assay.)

1. Prepare a 10X MTT stock solution. Dissolve 5 mg/mL MTT in 0.9% NaCl. The MTT stock solution may be stored in a 4 °C refrigerator for up to 3 months. **Note:** Since MTT is light sensitive, wrap the bottle in aluminum foil, or use an opaque bottle.

2. Prepare acidified isopropanol. Add 0.04 N HCl to isopropanol. This reagent is stable at 4 °C for up to 2 months.

Assay

3. Prepare the test article using BioreclamationIVT *InVitro*GRO HI Medium. To solubilize lipophilic test articles, use an organic solvent as necessary. Appropriate solvents include ACN, DMSO, ethanol, or methanol. Final concentration of the solvent should be kept at or below 1%.

4. Use BioreclamationIVT *InVitro*GRO HI Medium containing any solvent used to dissolve test materials as a vehicle control. BioreclamationIVT *InVitro*GRO HI Medium containing 100 mM chlorpromazine (1 % methanol) as a positive control.

5. Once test materials are prepared, transfer plate from incubator to biosafety cabinet, aspirate incubation media, and replace with an equal volume of each test material dissolved in incubation media. Appropriate volumes are:
   - 100 uL per well for 96-well plates.
   - 500 uL per well for 24-well plates.
   - 1.0 mL per well for 12-well plates.
   - 2.5 mL per well for 6-well plates.

6. Replace the lid on the plate. Return the plate to a 5% CO_2, 37 °C incubator for an appropriate length of time (16–24 hours is typical).

7. Prepare an MTT working solution. Dilute the MTT stock solution 1:10 in Celsis IVT Hepatocyte Incubation Media. Pre-warm the MTT reagent at 37 °C for at least 30 minutes.

8. Remove the plate from incubator, aspirate the media (containing test material), and replace with an equal volume of MTT working solution. Do this step quickly to prevent the cells from drying. Replace the lid on the plate.

9. Return the plate containing MTT reagent to a 5% CO_2, 37 °C incubator and incubate for 3 hours.

10. Remove the plate from the incubator, aspirate the MTT reagent from all wells, and replace with equal volumes of acidified isopropanol. Replace the lid on the plate.

11. Leave plate in refrigerator, covered with foil, for 12–24 hours to extract MTT colored reaction product from the cells.

12. Shake the plate on an orbital shaker at room temperature for several minutes to dissolve any formazan precipitate. Measure absorbance of solution in each well at 570 nm and 690 nm. This is particularly easy to do in 96-well plates by using a 96-well plate spectrophotometer.

13. Subtract the 690 nm OD in each well from the 570 nm OD in each well. This step corrects for non-specific background. Use the difference as the "OD" in the calculations in step 9.

14. Calculate "% vehicle control" for each well, using the following equation:

$$\% \text{ vehicle control} = \frac{\text{OD of treated well}}{\text{OD of control well}} \text{ x100}$$

(OD = optical density)

15. Once this "% vehicle control" measure is calculated for each of the treatments, a simple dose-response curve can be plotted and an "Effective Concentration 50%" (or "EC_{50}") can be calculated (EC_{50} = concentration of test material calculated to result in 50% reduced viability as compared with the vehicle control).

References

1. Li, A. P. Primary hepatocyte cultures as an in vitro experimental model for the evaluation of pharmacokinetic drug-drug interactions. *Adv. Pharmacol. Series* **1997**, *43*, 103–130.

2. Li, A. P.; Lu, C.; Brent, J. A.; Pham, C.; Fackett, A.; Ruegg, C. E.; Silber, P. M. Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. *Chem Biol Interact* **1999**, *121(1)*, 17–35.