Chapter 7

Study Liver Cytochrome P450 3A4 Inhibition and Hepatotoxicity Using DMSO-Differentiated HuH-7 Cells

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Abstract

Metabolically competent, inexpensive, and robust in vitro cell models are needed for studying liver drug-metabolizing enzymes and hepatotoxicity. Human hepatoma HuH-7 cells develop into a differentiated in vitro model resembling primary human hepatocytes after a 2-week dimethyl sulfoxide (DMSO) treatment. DMSO-treated HuH-7 cells express elevated cytochrome P450 3A4 (CYP3A4) enzyme gene expression and activity compared to untreated HuH-7 cells. This cell model could be used to study CYP3A4 inhibition by reversible and time-dependent inhibitors, including drugs, food-related substances, and environmental chemicals. The DMSO-treated HuH-7 model is also a suitable tool for investigating hepatotoxicity. This chapter describes a detailed methodology for developing DMSO-treated HuH-7 cells, which are subsequently used for CYP3A4 inhibition and hepatotoxicity studies.

Key words HuH-7, DMSO, CYP3A4, Inhibition, Hepatotoxicity

1 Introduction

Metabolism is a key function of liver, which is carried out by drug-metabolizing enzymes, such as cytochromes P450. Over the years, several in vitro cell models have been developed to mimic liver function, but many lack drug-metabolizing enzyme activities, which is true, for example, with HepG2 cells [1]. Other models, such as primary human hepatocytes and HepaRG cells, express drug-metabolizing enzyme activities, but are expensive, scarce, or require higher levels of maintenance [2]. Therefore, a cost-effective, metabolically competent, and robust in vitro cell model is needed for medium to high-throughput screening purposes.

Human hepatoma HuH-7 cells were derived from a Japanese male with well differentiated hepatocellular carcinoma in 1982 [3]. Studies have shown that HuH-7 cells could be induced by DMSO and differentiate into primary human hepatocyte-like cells [4, 5]. The DMSO-treated HuH-7 cells resemble primary human hepatocyte characteristics, such as, polygonal shape and binucleated cells

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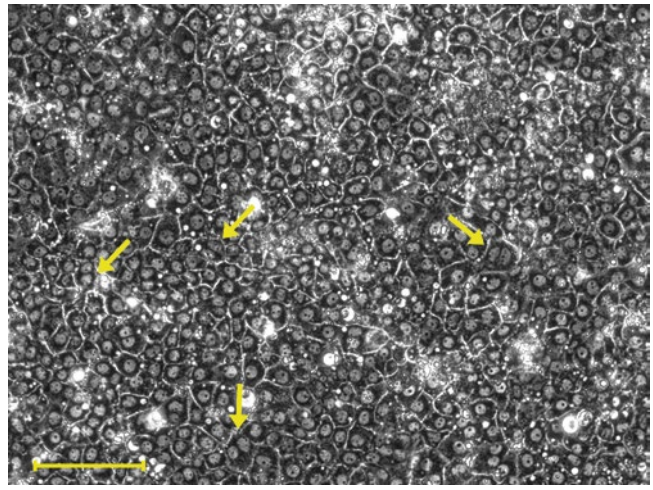

Fig. 1 Phase contrast photomicrographs of DMSO-treated HuH-7 cells with *arrows* pointing to binucleated cells. Bar represents 100 μm

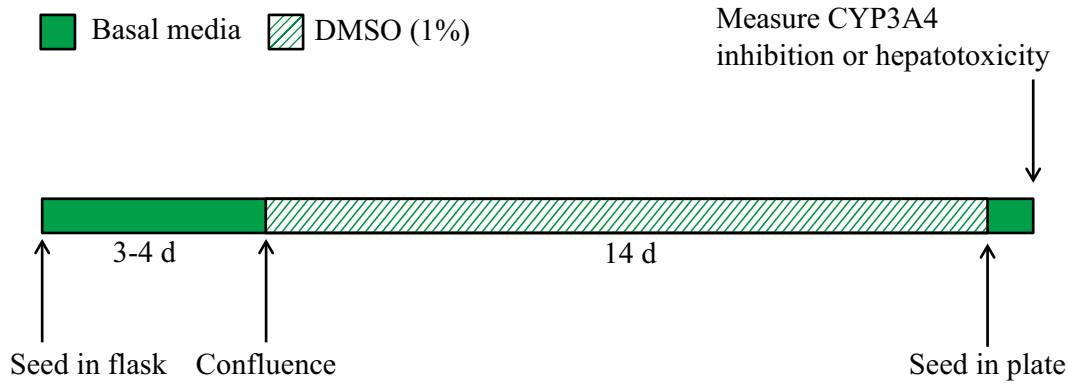

Fig. 2 Development of DMSO-treated HuH-7 cells. Timeline begins two to three passages after initial thawing of cells

(Fig. 1). Furthermore, DMSO-treated HuH-7 cells express the functional CYP 3A4 enzyme, the most abundant liver enzyme which mediates the metabolism of more than 50% of all marketed drugs [5]. Inhibition of CYP3A4 activity could cause drug–drug or food–drug interactions, which may lead to adverse effects [6].

This chapter describes a protocol to establish DMSO-treated HuH-7 cells for measuring CYP3A4 inhibition and hepatotoxicity. A general timeline for developing DMSO-treated HuH-7 cell model is illustrated in Fig. 2. The cells are passaged for two or three times after thawing, then seeded in a flask for confluence and DMSO treatment. Finally, the cells are assayed for both reversible and time-dependent CYP3A4 inhibition, as well as tested for hepatotoxicity using control compounds.

2 Materials

2.1 Equipment

1. BMG FLUOstar Omega multimode microplate reader.
2. New Brunswick Galaxy 48R CO_2 incubator.
3. Inverted microscope.
4. Countess, automated cell counter; counting slides; Trypan blue stain 0.4%.
5. 96-well, black, clear flat bottom, tissue culture-treated polystyrene microplates, with lids, sterile.
6. 96-well, white, opaque flat bottom, untreated polystyrene microplates, nonsterile.
7. Vacuum filter/storage bottle system, 0.22 μm pore, cellulose acetate membrane filter, sterile.
8. 8-channel pipette and tips; multichannel reservoir.

2.2 Cells and Culture Medium

1. Human hepatoma HuH-7 cells, Health Science Research Resources Bank, Japan Health Sciences Foundation.
2. Dulbecco's Minimal Essential Medium (DMEM), low glucose, GlutaMAX Supplement, pyruvate.
3. 100× MEM nonessential amino acids and 1 M HEPES.
4. Fetal bovine serum, Atlanta Biologicals Premium Select.

2.3 Reagents and Solutions

1. Rat tail collagen type I.
2. Glacial acetic acid.
3. Dulbecco's phosphate-buffered saline (DPBS, no calcium, no magnesium) and Versene (0.02% EDTA).
4. Trypsin–EDTA (0.5%), without phenol red.
5. Promega P450-Glo CYP3A4 (luciferin-IPA substrate) and CellTiter-Glo assays; beetle luciferin, potassium salt.
6. DMSO, ketoconazole, troleandomycin, salicylamide, nitrofurantoin, and nefazodone hydrochloride.

3 Methods

3.1 Cell Culture

1. Coat tissue culture surface using rat tail collagen type I (Table 1). Dissolve collagen in 0.02 N acetic acid to make a 100 μg/mL solution. Coat tissue culture surfaces at 10 μg/cm^2. Incubate coated vessels at room temperature for at least 2 h and rinse with DPBS. Use fresh or air dry and store at 2–8 °C for up to 1 month.
2. Prepare basal medium (Table 2) and 1% DMSO supplemented basal medium. Measure and mix all components under sterile

Table 1
Collagen coating

Growth chamber	Surface area (cm^2)	Collagen concentration (μg/mL)	Volume (mL)	Collagen density ($\mu g/cm^2$)
96-well plate	0.32	100	0.032	10
T25 flask	25	100	2.5	10
T75 flask	75	100	7.5	10

Table 2
Basal medium

Basal medium component	Concentration	Amount per 500 mL
DMEM	88%	440 mL
100× MEM nonessential amino acids	1×	5 mL
1 M HEPES	10 mM	5 mL
Fetal bovine serum	10%	50 mL

conditions, followed by filter sterilization using a 0.22 μM filter. Medium is stable for 1 month if stored at 2–8 °C. 1% DMSO supplemented basal medium is prepared fresh for medium change after cells reach confluence.

3. Thaw and recover HuH-7 cells into T25 or T75 flasks using basal medium. Passage cells two or three times when reach 85–90% confluence.
4. When desired cell amount is reached, remove the basal medium from flask using aspiration and rinse twice with Versene solution.
5. Add 0.025% trypsin–EDTA reagent (prepared using 0.5% trypsin–EDTA and DPBS) and rinse the flask quickly. Remove extra trypsin reagent and leave 1 mL in the flask. Incubate the flask (37 °C) until cell detachment (3–5 min).
6. Use a microscope to observe. When cells are completely detached, add 3–4 mL prewarmed (37 °C) basal medium to stop trypsinization. Failure to add medium promptly can result in over-trypsinization and significant cell death.
7. Determine the viable cell concentration (cells/mL) using a cell counter. Adjust cell concentration using basal medium as needed.

8. Seed HuH-7 cells at 6.3×10^4 cells/cm^2 onto collagen coated T25 or T75 flasks using basal medium. Wait 10 min at room temperature before returning to the incubator. Replenish medium twice per week until 100% confluence, which takes about 3–4 days after seeding.
9. Upon cell confluence, switch basal medium to 1% DMSO supplemented basal medium (fresh prepared each time). Replenish medium twice per week for 2 weeks using 1% DMSO supplemented basal medium.
10. After the 2-week DMSO treatment, seed DMSO-treated HuH-7 cells at 3.1×10^5 cells/cm^2 onto a collagen coated clear bottom 96-well plate using basal medium. Use plated cells for assays within 48 h after seeding (*see* **Note 1**).

3.2 CYP3A4 Reversible Inhibition Assay

1. Reagent preparation.

   Serum-free medium, prepare according to Table 2, leave out fetal bovine serum, and compensate volume with DMEM.

   Luciferin-IPA substrate, thaw 3 mM stock solution, protect from light.

   Salicylamide, Phase II conjugation inhibitor, prepare 3 M in DMSO fresh each time.

   Beetle luciferin, prepare 2 mM stock in H2O, serial dilute using serum-free medium (0.2–80 nM, *see* **Note 2**).

   Luciferin detection reagent, equilibrate to room temperature.

   Ketoconazole, prepare 2 mM stock in DMSO and conduct serial dilution using DMSO (0.01–2 mM).

   Dissolve luciferin-IPA substrate (3 μM), salicylamine (3 mM) and various concentrations of ketoconazole (0.01–2 μM) using serum-free medium (*see* **Note 3**) and prewarm to 37 °C. Preparation without ketoconazole serves as the control.
2. CYP3A4 reversible inhibition.

   Wash DMSO-treated HuH-7 cells on 96-well plates with serum-free medium and incubate with ketoconazole (0.01–2 μM) simultaneously with CYP3A4 substrate luciferin-IPA (3 μM, Km value) at 37 °C (100 μL/well) for 30 min. Include beetle luciferin standards (100 μL) in blank wells.
3. Assay detection.

   Terminate reaction by transferring 50 μL of incubation medium from each well to a separate white flat bottom plate with wells containing 50 μL of luciferin detection reagent at room temperature. Mix well and incubate the plate for 20 min, then measure luminescence using a plate reader (Fig. 3a).
4. Calculate IC_{50}, the half-maximal inhibitory concentration of ketoconazole determined using a nonlinear regression Hill model.

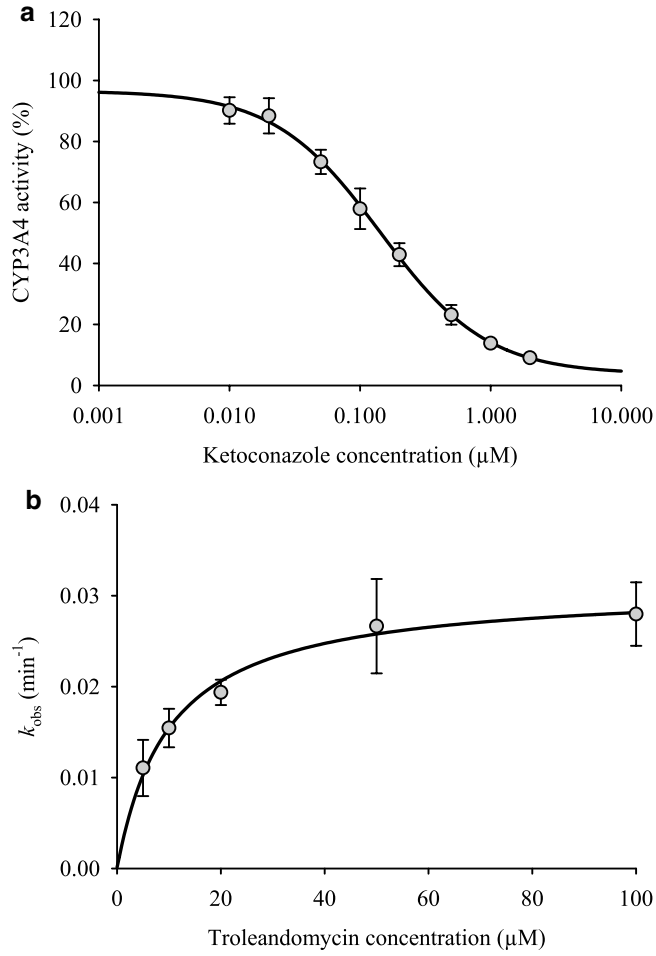

Fig. 3 (**a**) CYP3A4 reversible inhibition by ketoconazole and (**b**) time-dependent inhibition by troleandomycin in DMSO-treated HuH-7 cells. Figure reproduced from reference [5]

3.3 CYP3A4 Time-Dependent Inhibition Assay

1. Reagent preparation.

   Serum-free medium, prepare according to Table 2, leave out fetal bovine serum, and compensate volume with DMEM.

   Luciferin-IPA substrate, thaw 3 mM stock solution, protect from light.

   Salicylamide, Phase II conjugation inhibitor, prepare 3 M in DMSO fresh each time.

   Beetle luciferin, prepare 2 mM stock in H_2O, serial dilute using serum-free medium (0.2–80 nM, *see* **Note 2**).

   Luciferin detection reagent, equilibrate to room temperature.

   Troleandomycin, prepare 10 mM in DMSO and conduct serial dilution using DMSO (0.2–10 mM). Dissolve each concentration using serum-free medium (5–100 μM) and prewarm

to 37 °C. Incubation without troleandomycin serves as the control.

Dissolve luciferin-IPA substrate (3 μM) and salicylamide (3 mM) using serum-free medium and prewarm to 37 °C.

2. CYP3A4 time-dependent inhibition: preincubation.
   Wash DMSO-treated HuH-7 cells on 96-well plates with serum-free medium and incubate with troleandomycin (5–100 μM, 100 μL/well) for 0, 15, 30, and 60 min at 37 °C.
3. CYP3A4 time-dependent inhibition: substrate incubation.
   At different time points, wash cells with serum-free medium and incubate with CYP3A4 substrate luciferin-IPA (3 μM, Km value) at 37 °C (100 μL/well) for an additional 30 min. Include beetle luciferin standards (100 μL) in blank wells.
4. Assay detection.
   Terminate reaction by transferring 50 μL of incubation medium from each well to a separate white flat bottom plate with wells containing 50 μL of luciferin detection reagent at room temperature. Mix well and incubate the plate for 20 min, then measure luminescence using a plate reader (Fig. 3b).
5. Calculate Parameters.
   Obtain inactivation rate constant k_{obs} by plotting the natural logarithm of the remaining CYP3A4 activity (%) against preincubation time with troleandomycin [I].

   Calculate kinetic parameters k_{inact} and K_I by fitting data to the following equation using a nonlinear regression,

$$k_{obs} = \frac{k_{inact}[I]}{K_I + [I]}$$

3.4 Hepatotoxicity Assay

1. Reagent preparation.
   Nitrofurantoin and nefazodone, prepare 100 mM in DMSO, protect from light. Conduct serial dilution using basal medium (1–100 μM) on treatment day.
   CellTiter-Glo assay, reconstitute and equilibrate at room temperature on assay day (24 h after treatment day).
2. Treatment.
   Incubate DMSO-treated HuH-7 cells with nitrofurantoin or nefazodone (100 μL/well) for 24 h at 37 °C. Incubation without chemicals serves as the control.
3. Toxicity assay.
   After treatment, equilibrate HuH-7 cells at room temperature for 30 min. Add 100 μL of CellTiter-Glo reagent to each well and mix well. Incubate the plate for additional 10 min, then measure luminescence using a plate reader (Fig. 4).
4. Calculate EC_{50}, the half-maximal cytotoxic concentration of hepatotoxicants determined using a nonlinear regression Hill model.

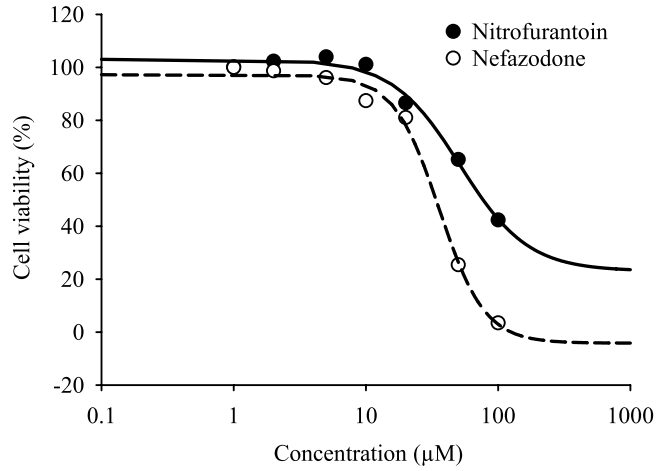

Fig. 4 Cytotoxicity of nitrofurantoin and nefazodone (24 h) in DMSO-treated HuH-7 cells. Figure reproduced from reference [5]

4 Notes

1. DMSO-treated HuH-7 cells maintain CYP3A4 activity in basal medium for at least 48 h after plating.
2. Add 100 μL/well of beetle luciferin standards (0.2–80 nM) to blank wells on the 96-well plate, proceed together with samples for inhibition studies and luminescence detection. When calculate enzyme activity, use beetle luciferin standard curve range 0.1–40 nM.
3. Prepare luciferin-IPA and salicylamide in serum-free medium, then dissolve various concentrations of ketoconazole using a multichannel reservoir.

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