Acute metabolic switch assay using glucose/galactose media in HepaRG cells to detect mitochondrial toxicity.

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Significance Statement This protocol provides a first tier screening model for detecting direct mitochondrial toxicity of an unknown compound using HepaRG cells. HepaRG cells are a differentiated cell line with hepatocyte-like characteristics such as CYP-enzymes and hepatic transporters required for the metabolism and disposition of drugs in the liver, which are lacking in other hepatoma cell lines such as HepG2 cells. They also provide opportunity to study chronic toxicity of compounds for up to 4 weeks after differentiation, compared to short incubation periods offered by other cell models, such as primary hepatocytes or HepG2 cells.

ABSTRACT

Using galactose instead of glucose in the culture media of hepatoma cells lines such as HepG2 cells has been utilised for a decade in order to unmask the mitochondrial liability of chemical compounds. We have previously reported a modified glucose-galactose assay on HepG2 cells, reducing the experimental period for screening of mitochondrial toxicity to 2 -4 hours. HepaRG cells are one of the few cell lines that retain some of the important characteristics of human hepatocytes, at the same time as offering advantages of working with a cell line, thus are considered as an alternative for HepG2 cells in drug toxicity screening. We have described a method here to use HepaRG cells in an acute metabolic switch assay using specific glucose/galactose media for this cell line, a combined ATP-Protein-LDH assay measuring three endpoints from one 96 well plate, and the criteria to label a compound as a mitochondrial toxin.

Keywords: mitochondria; drug-induced liver injury; HepaRG; glucose/galactose

INTRODUCTION

The Crabtree effect (Crabtree 1929), a situation where the energy requirements of a cell can be met by glycolysis alongside oxidative phosphorylation (OXPHOS) makes it difficult to detect early onset mitochondrial toxicity of drugs in hepatoma-originated cell lines because they are able to switch to glycolysis when OXPHOS is compromised (Diaz-Ruiz et al. 2011). In a glucose-rich environment mitochondrial damage is initially masked until ATP produced by glycolysis is no longer sufficient to support cell survival leading to a drop in ATP which coincides with other indications of cell death. Thus it becomes impossible to distinguish between mitochondrial damage as the primary cause or the subsequent effect of the cell toxicity (Kamalian et al. 2015).

Over the last decade the Crabtree effect has been exploited to enable the detection of mitotoxicants *in vitro* (Marroquin et al. 2007). Specifically, cells are deprived of glucose, the

main substrate of glycolysis, and are instead cultured in an alternative fuel source, galactose, that produces a net ATP production close to zero from glycolysis. In this altered environment, drug-induced mitochondrial dysfunction cannot be masked by glycolytic ATP production (Swiss et al. 2011).

In our previous work we introduced an acute metabolic modification assay in HepG2 cells, which is a modified short glu/gal assay (Kamalian et al. 2015). This highly specific method, however, suffered from limitations due to a lack, or very low amount, of metabolizing enzymes in HepG2 cells, that might give false negative results when investigating drug metabolite-driven mitochondrial toxicity. Furthermore, due to the proliferative nature of HepG2 cells, long term incubation (> 7 days) of compounds can be difficult to model. HepaRG cells, a hepato-carcinoma originated cell line (Gripon et al. 2002), offer an attractive alternative *in vitro* model due to some more hepatocytelike characteristics including more abundant cytochrome P450 (CYP450) enzymes, bile canaliculi structures and higher expression of bile acid transporters compared to HepG2 cells (Kanebratt et al. 2008, Kanebratt et al. 2008). Furthermore, once fully differentiated, these cells stay in non-proliferative state for up to 4 weeks which can be used for studying drugs that only show toxicity after long term incubation (Kamalian et al. 2018).

In this protocol we have adjusted the HepG2 acute metabolic modification assay to be used on HepaRG cells. After acute metabolic switch using glucose or galactose media specifically suitable for HepaRG cells for 2 hours, the compound of interest is added to the cells for a short period of 2 hours, before the ATP, LDH and protein level of the cells are measured, which then are represented as a percentage of the levels in the vehicle treated group. The compound is marked as a potential mitochondrial toxicant if the ATP, LDH and or protein IC50 concentrations fall within a specific criteria.

BASIC PROTOCOL 1

Acute metabolic switch assay with combined ATP-LDH-protein assay in HepaRG cells

The purpose of this protocol is to make use of Crabtree effect in hepatocyte-like hepatoma cell line, HepaRG, in order to screen for drugs with potential direct mitochondria liability. Briefly, cells already exposed to the tested drug for the required duration are incubated in glucose or galactose media specifically modified to be suitable for HepaRG cells for 2 hours. A combined ATP-LDH-Protein assay is then performed to measure the mentioned end-points.

Materials

1. Reagents and Solutions
    1.1. HepaRG growth media (Caltag Medsystems)
        1.1.1. HepaRG base medium (Cat. MIL700)
        1.1.2. HepaRG growth additive (Cat. ADD710)
    1.2. HepaRG differentiation medium (Caltag Medsystems)
        1.2.1. HepaRG base medium (Cat. MIL700)
        1.2.2. HepaRG differentiation additive (Cat. ADD720)
    1.3. HepaRG glucose or galactose media:
        1.3.1. Williams' medium E powder, w/L-Glutamine w/o Glucose (US Biological Life Sciences, Cat. W1105-05)
        1.3.2. D-(+)-Glucose Anhydrous (Fisher Scientific, Cat. G/0500/53)
        1.3.3. D-(+)-Galactose (Sigma, Cat. G5388)

1.3.4. Insulin (Sigma, Cat. I9278-5ml)

1.3.5. Hydrocortisone: (Sigma, Cat. H0888)

1.3.6. Sodium bicarbonate: (Sigma, Cat. S576)

1.4. Combined ATP-LDH-Protein assay:

1.4.1. Protein assay: Pierce™ BCA Protein Assay Kit (Thermo-Fisher Scientific, Cat. 23225)

1.4.2. ATP assay (Sigma)

1.4.2.1. Somatic cell ATP releasing reagent (Cat. FL-SAR)

1.4.2.2. ATP bioluminescent assay kit (Cat. FL-AA)

1.4.2.3. ATP standard (Cat. FL-AAS)

1.4.3. LDH assay: Cytotoxicity Detection Kit PLUS (LDH) (Sigma, Cat. 4744934001)

2. Equipment:

2.1. HepaRG growth and differentiation:

2.1.1. Nunc Flask vented (different sizes) (Thermo-Fisher Scientific)

2.1.2. 96-Well CytoOne® Plate, TC-Treated (STARLAB, Cat. CC7682-7596)

2.1.3. Standard tissue culture incubator (37^{o}C, 95% humidity, 5% CO_2)

2.2. Combined ATP-LDH-Protein assay:

2.2.1. Clear, flat bottom 96 well plates (any type)

2.2.2. White, flat bottom 96 well plates (clear or opaque bottom depending on the type of the plate reader available.

2.2.3. Plate reader (with luminescent, photometric and colorimetric reading capacity)

Protocol steps—*Step annotations*

HepaRG cells growth and differentiation:

1. Make HepaRG growth or differentiation media: Defrost HepaRG growth or differentiation additive (see material section) at room temperature or in a 37 ^{O}C water bath. Add the whole content of HepaRG growth or differentiation additive bottle into a 500 ml bottle of HepaRG base media and mix. The complete growth or differentiation media is stable for 1 month at 2-8 ^{O}C.

Culture and maintenance of HepaRG cells to full differentiation:

2. Plate undifferentiated HepaRG cells in wells of a 96 well plate at a cell density of 9000 cells per well, in HepaRG growth media, according to the manufacturers instruction.

   In our experience, HepaRG cells can be plated on collagen- or non-collagen coated plates with no effect on final growth and differentiated state.

   Plan your final assay configuration and plate the undifferentiated cell according your final experimental plate plan. Figure 1 shows an example of plating configuration for assessing 7 concentrations (conc. 1-7) of a test compound plus vehicle control (VC) in triplicates.

3. Change the media twice a week with growth media for two weeks to allow the cells to grow to 100 % confluence.
4. Change the media into a 50%:50% growth:differentiation media for first media change after the growth period is completed, then twice a week with differentiation media for two weeks.

   At this point (4 weeks after the initial plating) cells are fully differentiated and maintain their non-proliferative state and specific hepatocyte-like

characteristics for 4 weeks, during which the planned experiments can be performed.

Acute metabolic switch assay:

1. Prepare HepaRG glucose and galactose media (please refer to REAGENTS AND SOLUTIONS section)
2. Pre-treat HepaRG cells with glucose or galactose media: Remove the media from the wells. Wash the wells with 100 µl of PBS once. Add 50 µl of either glucose or galactose media to the corresponding wells according to the planned assay configuration. Incubate in a cell culture incubator (37 ^{O}C, 95% humidity, 5% CO_2) for 2 h.
3. Prepare the drug solutions at twice the desired final concentration of the test compound in either glucose or galactose media.

   The volume needed for each test compound solution has to be calculated at 50 µl per well.

   If the compound stock diluent is DMSO make sure the final concentration of dimethyl sulfoxide (DMSO) in the wells would not exceed 0.5% of the final volume, because of the toxicity effect of DMSO on cells.
4. Add the 50 µl of media-compound concentration solution to the corresponding wells according to the planned assay configuration.
5. Incubate the plate in the cell culture incubator (37 ^{O}C, 95 % humidity, 5% CO_2) for the duration required (2-24 h).

Combined ATP-protein-LDH assay:

1. Prepare the cell lysate and supernatant plates: Transfer the media from all the wells into a fresh clear 96 well plate in the same plate format as the original cell plate. Label this plate as supernatant stock (SS) plate. Add 100 µl of Somatic cell ATP releasing reagent into each well containing cells. Agitate the plate on a plate shaker on medium speed for at least 5 minutes at room temperature. Label this plate as lysate stock (LS) plate. Label three clear flat bottom 96 well plates as; P (for protein), LDH-L (for lysate) and LDH-S (for supernatant). Label a white flat, opaque or clear bottom (according to your plate reader requirements) 96 well plate as ATP.

    For protein and ATP assays you will need to produce standard curves by running protein and ATP standards alongside the main experiments. Therefore you need to consider having enough wells for both your samples and standards on the same plate. If the samples cover the whole plates, standards can be loaded on separate plates, as long as both sample and standard plates are treated the same. In this case pay attention to label the sample and the standard plates carefully and clearly.

   Transfer 10 µl of the LS into the wells of the P, LDH-L and ATP plates. Transfer 50 µl of the SS into the wells of the LDH-S plate.

Protein assay:

1. Load 10 µl of protein standards on the P plate or a separate plate if there is not enough wells available on the P plate.
2. Measure the protein level using Pierce™ BCA Protein Assay Kit, according to the manufacturer's instruction.
3. Calculate the protein level of each well using the standard curve equation.

If BCA reagent is not compatible with the compound tested, it is possible to measure the protein using Bradford assay reagent. However, in this case the samples might need to be diluted further to make sure the measurements fall within the linear part of the standard curve.

ATP assay

1. Make up the ATP standards according to the manufacturer instruction. Load them on the ATP plate, or on a separate plate if there is not enough well on the ATP sample plate.
2. Make up the ATP assay mix according to the manufacturer instruction.
3. Add 50 µl of the ATP assay mix onto all the samples and the standards, shake the plate/s briefly on a plate shaker and read immediately using luminescence setting of the plate reader.
4. Calculate the ATP level of each well using the ATP standard curve.
5. Normalize the ATP level of each well to the protein level of the same well to estimate ATP level per each µg protein.
6. Calculate the average ATP level of the triplicate samples in each drug concentration.
7. Normalize the average ATP level for each drug concentration to the average calculated for the vehicle control using the formula (using the vehicle control in glucose and galactose media for each corresponding group).

$$ATP\ relative\ to\ the\ vehicle(\%) = \left(\frac{Average\ ATP\ level\ in\ each\ drug\ concentration}{Average\ ATP\ level\ in\ the\ vehicle}\right) x\ 100$$

8. Construct the ATP-dose response graph.

9. Calculate the IC_{50} concentration.

LDH assay

1. Add 40 µl of media into the wells of the LDH-L plate to make the samples 5 times diluted. This is so they remain on the standard curve due to signal saturation Make up the LDH reagent according to the manufacturer's instruction, allowing 50 µl volume of the reagent for each well containing sample on LDH-L and LDH-S plates.
2. Add 50 µl of the LDH reagent to all the lysate and supernatant wells. Cover the plates and incubate at room temperature and in a dark place for 30 minutes.

    Always include at least 3 wells containing the media for background measurement. If there is not enough wells on the sample plates, this can be done on a separate plate.

3. Read the absorbance using colorimetric plate reader at 510 nm wavelength.

Subtract the average of the background measurements from all the lysate and supernatant measurements

4. Calculate the reserved LDH in the lysate using the formula:

$$Reserved\ LDH\ (\%) = \left(\frac{Lysate\ LDH}{Lysate\ LDH + supernatant\ LDH}\right) x\ 100$$

5. Calculate the average LDH level of the triplicate samples in each drug concentration.
6. Normalize the LDH level for each drug concentration to the average calculated for the vehicle control using the formula (using the vehicle control in glucose and galactose media for each corresponding group).

$$Reserved\ LDH\ relative\ to\ the\ vehicle(\%) = \left(\frac{Average\ LDH\ level\ in\ each\ drug\ concentration}{Average\ LDH\ level\ in\ the\ vehicle}\right) x\ 100$$

7. Draw the LDH-dose response graph.
8. Calculate the IC_{50} concentration.

Criteria for labelling a compound as a mitochondrial toxin

A compound is labelled positive mitochondrial toxin if:

1. $\frac{ATP\_IC50(Glucose)}{ATP\_IC50(Galactose)} \geq 2$
2. The difference between the ATP-IC_{50} (Glucose) and ATP-IC_{50} (Galactose) is statistically significant.

Mitochondria is the first or main target of a compound if:

1. $\frac{ATP\_IC50(Glucose)}{ATP\_IC50(Galactose)} \geq 2$
2. $\frac{LDH\_IC50(Galactose)}{ATP\_IC50(Galactose)} \geq 2$
3. The difference between the LDH-IC_{50} (Galactose) and ATP-IC_{50} (Galactose) is statistically significant.

REAGENTS AND SOLUTIONS

HepaRG glucose or galactose media:

1. Reconstitute glucose-free Williams E base media from powder by dissolving 0.88 g of the media powder in 80 ml of dH_2O. Adjust the pH to 7.4 (*the media changes colour from yellow to pink*), then adjust the volume to 100 ml by adding dH_2O. Add

3.7 mg/ml (w/v) bicarbonate sodium the media and mix well. Filter sterilise the media using a 0.22 µm syringe or bottle filter unit.

2. Make glucose or galactose stock 1 M solutions: Dissolve 1.8 g of glucose or galactose powder in 10 ml PBS, then filter sterilize. Aliquot and store at -20^{O}C.

   Galactose might not dissolve in PBS easily, in this case the solution can be placed in a sonic bath for 10-20 before filter sterilization.

3. Prepare hydrocortisone 5.5 mM stock solution: Dissolve 10 mg of the powder in 1 ml of 100 % ethanol. Make-up the volume to 5 ml by Williams' E glucose free base media (see step 1.1.). Aliquot and store at 2 - 8 ^{O}C.

4. To make glucose or galactose media add either glucose (11 mM) or galactose (10mM), insulin (5 µg/ml), L glutamine (4 or 6 mM) and hydrocortisone (50 mM) to the basal media (the values in the brackets indicate the final concentration of each compound). The amounts needed to prepare 10 ml of each media is summarized in Table 1.

   The media before addition of additives already contains 2 mM L-glutamine. Therefore, additional 2 or 4 mM is added to make the final desired 4 or 6 mM in glucose or galactose media respectively.

   The HepaRG glucose or galactose media are stable at least for one week after preparation at 2-8 ^{o}C, therefore could be prepared in advance.

COMMENTARY

Critical Parameters and Troubleshooting

Seeding density of HepaRG cells

HepaRG cells when differentiated consists of two different cell populations; hepatocyte-like and epithelial cell. The proportion of the two cell types need to stay in a specific ratio in order for the cells to retain their specific metabolic characteristics (Aninat et al. 2006, Cerec et al. 2007). In order to achieve this, the original seeding density of the cells in different vessels have been optimized by the manufacturers (Biopredics). It is important for this protocol to adhere to those seeding density, which for example is 9000 cells per well for a 96 well plate.

HepaRG glucose and galactose media

All the ingredients specified in the material section and within the annotated protocol for HepaRG glucose and galactose media are required in order to achieve the results expected from this protocol. For example simple glucose free DMEM media supplemented by galactose or glucose which is normally used for HepG2 cells will not be suitable for use for HepaRG cells, even though the length of incubations on the assay day are short. Once the glucose or galactose media are prepared they are stable for at least one week at 2-8^{o}C. Therefore these media can be prepared in advance, in the interest of speeding the process on the day of the assay.

Statistical Analyses

To test the significance of the difference between ATP-IC50 values in glucose and galactose media, or ATP-IC50 and LDH IC50 values in galactose medium the authors the test that was used by was un-paired student t-test, assuming non-equal variances, comparing the results of at least three independent experiments.

Understanding Results

Comparing results for a compound labelled as a positive mitochondrial toxin, rotenone versus a negative compound, digitonin.

Rotenone (a mitochondrial complex I inhibitor) reduced the ATP level to 50% of the vehicle control in galactose media at 0.17 ±0.04 µM. The ATP in glucose media, and LDH level in glucose or galactose media never declined to 50% of the vehicle control. This resulted in a IC_{50}ATPglu/IC_{50}ATPgal and IC_{50}LDHgal/IC_{50}ATPgal ratios of >650 (Table 2). Both the differences were statistically significant. Conversely, using digitonin (a detergent) the ATP and LDH levels in both glucose and galactose media reached 50% of the vehicle at similar concentrations. According to the criteria in section 5 of the protocol, rotenone and digitonin are labelled as positive and negative for mitochondrial toxicity. Figure 1 provides typical dose response graphs created for rotenone and digitonin.

Time Considerations

HepaRG growth and differentiation take four weeks to complete after which cells will stay in non-proliferative fully differentiated status for four weeks, during which the protocol can be performed. The acute metabolic switch assay including pre-treatment in glu/gal media, incubation with the compounds, and combined protein-ATP-LDH assay is completed in less than a working day (5-8 hours depending on the speed of the operator and the preparations that may be made beforehand).

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FIGURE LEGENDS

Figure 1: Changes in ATP and LDH levels of HepaRG cells when exposed to rotenone or digitonin for 2 hours in either HepaRG glucose or galactose media after being pretreated with the

same media for 2 hours. Values are reported as percentages of the vehicle control. Figure taken from the original manuscript by Kamalian et al https://doi.org/10.1016/j.tiv.2018.08.001, available under the creative commons licence http://creativecommons.org/licenses/by/4.0/.

TABLES

Table 1: Summary of the reagents needed for making HepaRG glucose or galactose media.

Ingredient	Glucose media (10 ml)			Galactose media (10 ml)		
	Stock	Final	Volume	Stock	Final	Volume
Glucose	1 M	11 mM	110 µM	---	---	---
Galactose	---	---	---	1 M	10 mM	100 µM
Insulin	V*	5 µg/ml	V*	V*	5 µg/ml	V*
L-glutamine	200 mM	4 mM**	100 µM	200 mM	6 mM**	200 µM
Hydrocortisone	5.5 mM	50 µM	90.8 µl	5.5 mM	50 µM	90.8 µl
Williams-E	N/A	N/A	Up to 10 ml	N/A	N/A	Up to 10 ml

* V is for variable: the insulin stock concentration varies from batch to batch, therefore the volume needed has to be calculated for each batch separately.

** Please refer to the notes under "Reagents and Solution" section.

Table 2: The ATP and LDH IC_{50} values and statistical analysis calculated from dose response curves after incubation of HepaRG cells with rotenone or digitonin for 2 hours in glucose or galactose media. Data taken from the original manuscript by Kamalian et al

	IC_{50}-ATP (µM) ± S.D.		IC_{150}-LDH (µM) ± S.D.		IC_{50}ATPglu/IC_{50}ATPgal (p value)*	IC_{150}LDHgal/IC_{50}ATPgal (p value)*
	Glucose	**Galactose**	**Glucose**	**Galactose**		
Rotenone	>100	0.17 ± 0.04	>100	>100	>605 (< 0.001)	>605 (< 0.001)
Digitonin	7.65 ± 1.64	8.82 ± 2.01	6.80 ± 0.07	8.00 ± 2.07	0.87 (0.478)	0.90 (0.646)

***** p value is estimated using unpaired Student t-test, assuming non-equal variances.