Novel In Vitro Dye Based High-Throughput Toxicity Assay on Human Primary Cells

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Abstract

Codex BioSolutions has developed a homogeneous cell based assay to measure the mitochondria membrane potential change using a novel fluorescent dye, m-MPI. It was achieved by mixing a masking dye with the m-MPI. The assay has been miniaturized to 384-well format and been tested on four different cell lines with 11 different compounds each. It is a robust assay with Z' value > 0.5 on both HepG2 and Hela cells and can be used for high throughput screening. The m-MPI homogeneous assay kit does not have any toxic effect on the cells, as was shown on Hela and HepG2 cells using Codex Enercount ATP assay kit. The m-MPI assay kit was further tested on 4 human primary cells with at least four compounds each. The assay worked excellently on these primary cells. Using this assay, we picked 90 compounds from LOPAC library and did a mini screening on human hepatocytes. The hits were confirmed with the follow up studies. Three potential toxic compounds were identified on human hepatocytes which have no effect on HepG2 cells.

Introduction

Chemically induced mitochondrial toxicity has become a critical part of the evaluation of chemical cytotoxicity in eukaryotic cells because of the central role they play in energy production and in the regulation of apoptosis and necrosis (1,2). Toxic effects on mitochondria are manifest by changes in mitochondrial membrane potential and thus toxicity assays have been developed that use membrane permeable, fluorescent lipophilic cations to determine the integrity of the membrane potential. Although these assays are important tools in a toxicological screen, the dyes used due have limitations. For instance, they can accumulate in membrane components other than mitochondria (3) and they can require a long time to achieve equilibrium across the mitochondrial membrane, especially when cellular accumulation is reduced by the multi-drug resistance pump (4). In addition, they can also have their own toxic effects on mitochondria (5,6).

Examples of fluorescent dyes used to measure MMP include nonylacridine orange (NAO), safranine O, rhodamine 123, chloromethyl-tetramethyl-rosamine, tetramethylrhodamine methyl and ethyl esters (TMRM and TMRE). Two popular dyes are 3,3'-Dihexiloxadicarbocyanine iodide [DiOC6(3)] and JC1, a cyanine dye (7). DiOC6(3) works effectively in isolated mitochondria but not in whole cells because it detects both plasma and mitochondrial membrane potential changes (7).

JC-1 (8), a cyanine dye, has advantages over rhodamines and other carbocyanines, because it is more selective to mitochondria. The time for JC-1 to reach equilibrium is relatively short and it has minimal toxic effect on the mitochondrial electron transport chain (9). JC-1 accumulates in healthy mitochondria as aggregates, but remains in the cytoplasm in the monomeric form in less healthy cells with lower MMP. JC-1 is relatively specific and sensitive with low background. The biggest limitation of JC-1 is its poor water solubility. JC-1 starts to precipitate in aqueous buffers at concentrations as low as 1 μM. Its poor water solubility makes it very difficult to load consistent amounts of JC-1 into cells, resulting in large experimental variation. JC-1 is also not suitable for all cell types. For instance, it can be used to measure MMP changes in HeLa cells but not in HepG2, CHO and primary rat hepatocytes due to its low signal to background window (6).

In order to overcome the limitations of the MMP assays described above, we have developed a cell-based assay using a novel dye, m-MPI (6,10). This dye rapidly partitions between the cytoplasm and mitochondria and the accumulation of dye reflects the status of the mitochondrial membrane potential. Codex BioSolutions has developed a homogeneous assay based on m-MPI. It was achieved by mixing a masking dye with the m-MPI. The assay has been miniaturized to 384-well format and been tested on four different cell lines with 11 different compounds each. It is a robust assay with Z' value > 0.5 on both HepG2 and Hela cells and can be used for high throughput screening. The m-MPI homogeneous assay kit does not have any toxic effect on the cells, as was shown on Hela and HepG2 cells using Codex Enercount ATP assay kit. The m-MPI assay kit was further tested on 4 human primary cells with at least four compounds each. The assay worked excellently on these primary cells. Using this assay, we picked 90 compounds from LOPAC library and did a mini screening on human hepatocytes. The hits were confirmed with the follow up assays. Three potential toxic compounds were identified on human hepatocytes which have no effect on HepG2 cells. From the data obtained in this contract work, we believe that the m-MPI assay can be further miniaturized to 1536-well format with human primary cells.

Summary

We have developed a homogeneous assay to measure the membrane potential change of mitochondria

The homogeneous assay have been adapted to 384-well format with good data quality

The homogeneous assay can be used to measure the mitochondria membrane potential change on human primary cells

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Fig. 1 Principle of the m-MPI assay

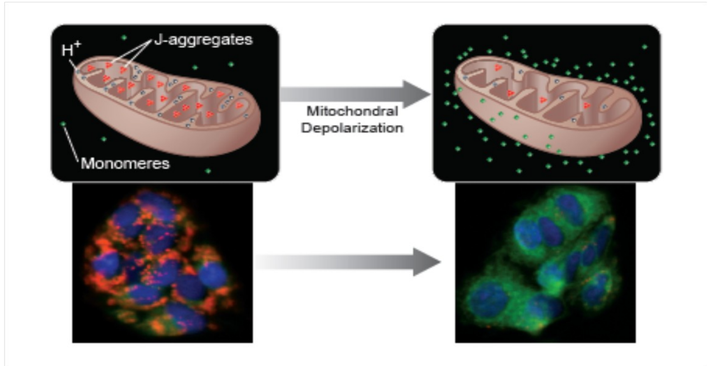

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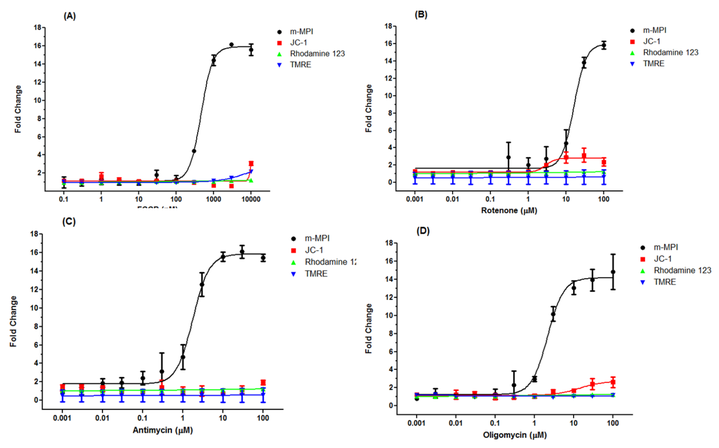
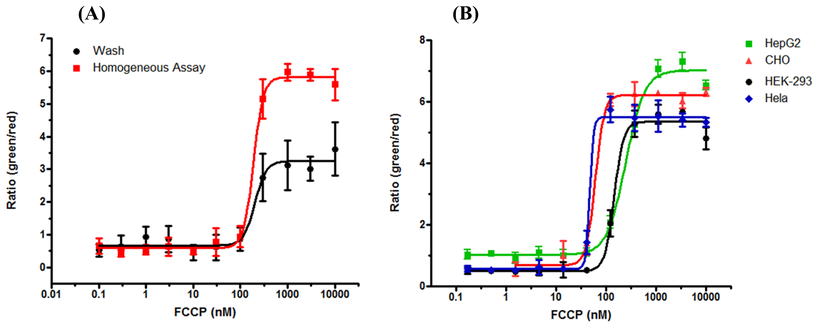
Fig. 2 Comparison of Different Dyes with Different Compounds on HepG2 Cells



Assay performance using 4 MMP dyes, m-MPI, JC-1, rhodamine 123, or tetramethylrhodamine ethyl ester (TMRE). MMP was measured after the cells were treated with various concentrations of FCCP (A), rotenone (B), antimycin (C) or oligomycin (D) for 1 h in 384-well plate format. The fluorescent intensities of both the green and the red channels were recorded on FlexStation. The fold change was defined as the value of DMSO control divided by the value of compound treatment. Values are means, SD from 3 experiments performed in quadruplicate.

Fig. 3 Homogeneous m-MPI assay on 384-well plate



A) 12K of HEK-293 cells were plated into each well of a 384-well plate. Different concentrations of FCCP (2X final concentrations) were prepared in 1X HBSS. Equal volume of such solutions was added into each well and incubated with the cells for 30 min. 3X m-MPI dye solution and 3X homogeneous m-MPI solution were prepared. Equal volume of above solutions (3X) was then applied to the cells treated with FCCP. The plate was incubated at 37°C for another 30 min. The wells incubated with 3X m-MPI dye solution were washed once with Codex's proprietary m-MPI enhancer solution and recorded on FlexStation, The wells incubated with 3X homogeneous m-MPI solution were directly recorded on FlexStation after 30 min incubation. B) 6K of HepG2, 8K of Hela, 10K of CHO-k1 and 12K of HEK-293 cells were plated into each well of a 384-well plate. On the 2nd day, the medium was removed. 1X of FCCP final concentrations were prepared in 1X HBSS. 20 μl of such solutions was added into each well and incubated with the cells for 30 min. 2X homogeneous m-MPI solution were prepared. 20 μl of above solutions (2X) was then applied to the cells treated with FCCP. The plate was incubated at 37oC for another 30 min and recorded on FlexStation

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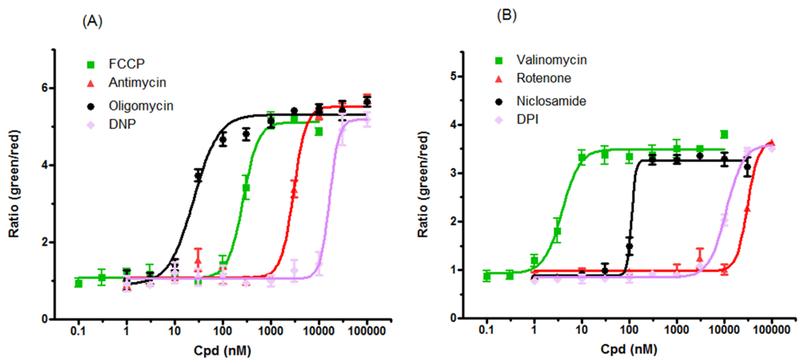
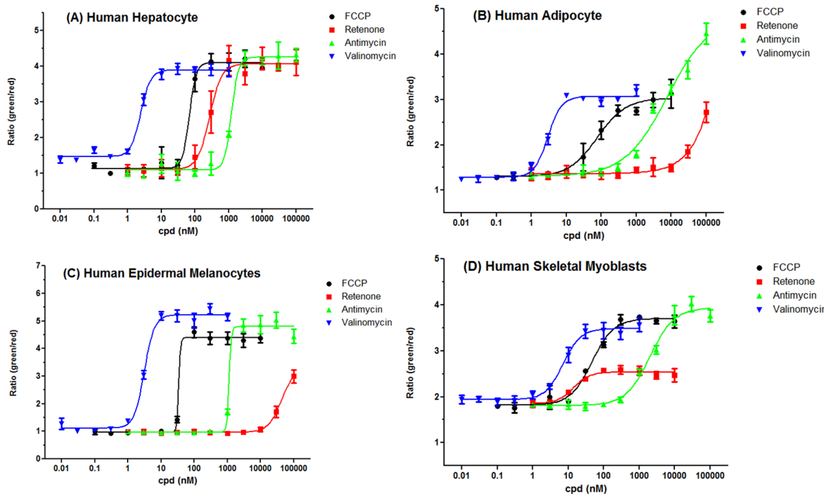
Fig. 4 Homogeneous Assay with Different Compounds on Hela Cells



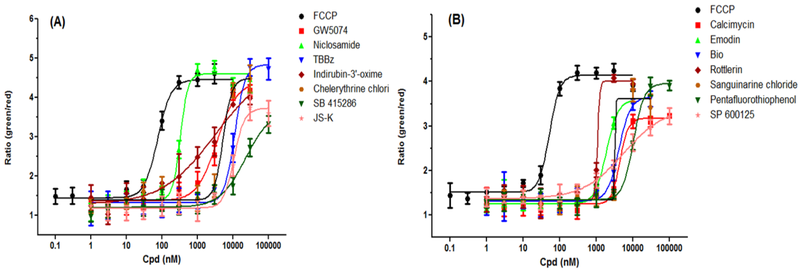
8K of Hela cells in 20 μl culture medium were plated into each well of a 384-well plate. Different concentrations (2X of final concentrations) of each compound were prepared in 1X HBSS. 20 μl of such solutions was added into each well and incubated with the cells for 30 min. 3X homogeneous m-MPI solution was prepared. 20 μl of above solutions (3X) was then applied to the cells treated with FCCP. The plate was incubated at 37°C for another 30 min. The plate was placed on a FlexStation and the fluorescent intensities of both the green and the red channels were recorded using the endpoint mode.

Fig. 5 m-MPI Assay with Different Human Primary Cells



(a) Human hepatocytes, (b) Human adipocytes derived from the adult stem cells, (c) Human epidermal melanocytes cells and (d) Human skeletal myoblasts cells were plated on 384-well plates. Different concentrations of the compounds (2X) were prepared in 1X HBSS. Equal volume of the compound solution was added and incubated with the cells for 60 min at 37°C. Afterwards, equal volume of 3X dye m-MPI homogeneous dye solution was added into each well and incubated with the cells at 37°C for another 30 min. The plates were placed on a FlexStation and the fluorescent intensities of both the green and the red channels were recorded using the endpoint mode.

Fig. 6 Confirm the Hits from LOPAC Library with Human Hepatocytes



The positive hits from LOPAC Library were identified and the individual compounds were ordered again from Sigma. Human hepatocytes were plated on 384-well collagen coated plates at the density of 9K/well (in 25 μl Plating Medium) and incubated at a 37°C incubator. On the 2nd day, the plates were taken out of the CO2 incubator. The medium was removed and 10 μl of the fresh Plating Medium was added back into each well. Different concentrations of the compounds (2X) were prepared in 1X HBSS (all start from 100 μM except FCCP). 10 μl of the compound solution was added into each well and incubated with the cells for 60 min at 37°C. 10 μl of the 3X homogeneous m-MPI dye solution was added into each well and incubated with the cells at 37°C for another 30 min. The plates were placed on a FlexStation and the fluorescent intensities of both the green (Excitation: 485 nm; Emission: 530 nm; Cutoff 515 nm) and the red (Excitation: 485 nm; Emission: 590 nm; Cutoff 570 nm) channels were recorded using the endpoint mode. The ratio of Green/Red signal was used to plot the graph.

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