Calcium retention capacity assay evaluates inhibition of mitochondrial permeability transition pore

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- **Calcium retention capacity is a measure of mitochondrial function**
- **Capacity and dose-dependence of MPTP inhibitors is measured in high throughput**
- **POLARstar® Omega enables multiple injections and kinetic assessment of fluorescent calcium sensor**

Introduction

Mitochondrial dysfunction is central to the pathogenesis of acute pancreatitis as well as other diseases including ischemia-reperfusion injury of the heart, brain and kidney, muscular dystrophies and neurodegeneration[1-3]. Mitochondrial dysfunction is the result of a sudden increase in permeability of the inner mitochondrial membrane (IMM), via persistent opening of a multi-protein channel known as the mitochondrial permeability transition pore (MPTP)[1]. This allows uncontrolled proton flow across the IMM and unregulated flux of water, ions and solutes up to 1.5 kDa into and out of the mitochondrial matrix. This results in loss of inner mitochondrial-membrane potential, which is essential for ATP production, disruption of calcium homeostasis, swelling of mitochondria and of the outer mitochondrial membrane (OMM). Eventually cells start dying through necrosis in an uncontrolled manner[4]. There-fore, MPTP could be an attractive target for maintenance of mitochondrial function and cell death prevention in a host of disease states.

Assay Principle

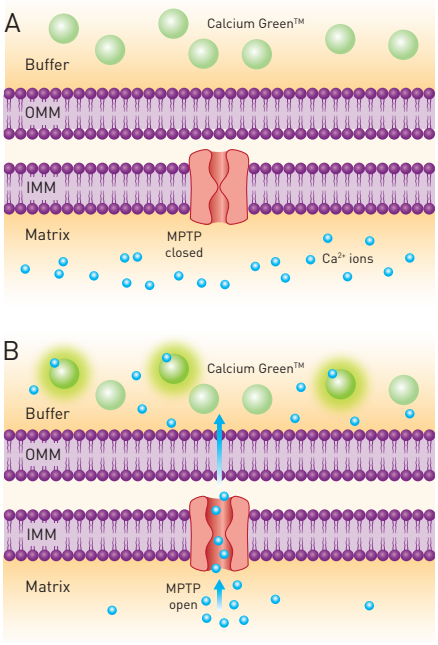

Fig. 1: Principle of calcium retention assay. The membrane-impermeable Calcium Green reports on extra-mitochondrial Ca^{2+}.

The MPTP opening and its inhibition can be evaluated by determining the calcium retention capacity (CRC) of mitochondria to Calcium Green™-5N, a low-affinity membrane imperme-able dye that exhibits an increase in fluorescence emission intensity upon binding to calcium (Fig 1).

A fixed volume of calcium chloride is added to the sample (mitochondrial suspension) at defined intervals, each eliciting a spike in signal that dissipates due to the uptake of calcium into the mitochondrial matrix. Opening of the MPTP collapses the mitochondrial membrane potential, which results in release of the accumulated calcium from the matrix, causing a sudden and sustained increase in fluorescence signal (Fig 2).

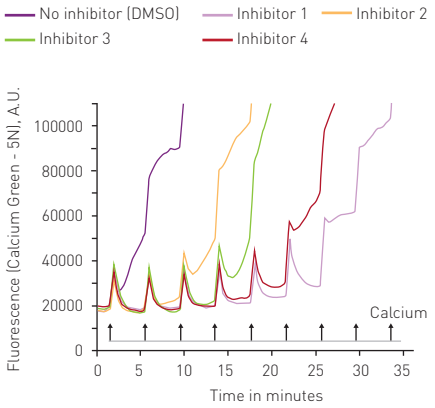

Fig. 2: Challenging isolated mitochondria with injections of $CaCl_2$ leads to fluorescent spikes until collapse of the mitochondrial permeability transition pore (MPTP) which is indicated by permanent high fluorescence value. Time to maximum fluorescence correlates with inhibition of MPTP.

Materials & Methods

- Freshly Isolated liver mitochondria
- Calcium Green-5N (Molecular Probes)
- Calcium chloride (Sigma)
- Inhibitors of interest
- Black 96-well microplates (Greiner)
- POLARstar Omega (BMG LABTECH)

Experimental procedure

Calcium was added every 4 minutes to mouse liver mitochondria in the presence of the fluorescent Ca^{2+} indicator, Calcium Green 5N. Calcium uptake was followed by measuring extra-mitochondrial calcium

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green fluorescence until MPTP opening was achieved. Each inhibitor was added to the mitochondria immediately prior to the start Ca^{2+} of the experiment.

Instrument settings

Optic settings	Fluorescence intensity, plate mode kinetic		
	Filter	Excitation 485 Emission 530	
	Gain	1800	
General settings	Number of flashes		10
	Settling time		0.1 s
Kinetic settings	Number of cycles		160
	Cycle time		30 s
Incubation	25 °C		
Injection settings	Smart dispensing used		
	Volume of pump		4 µl
	Pump speed		300 µl/s
	Injection cycle		4

Results & Discussion

Mostly, sustained mitochondrial matrix Ca^{2+} overload triggers prolonged high-conductance MPTP opening. Therefore, CRC assay using isolated mitochondria is an excellent tool for phenotypic screening of the MPTP inhibitors. As shown in Figure 2, different inhibitors resist opening of the MPTP to differing extents. The promising candidates can be tested in a concentration dependent manner. As demonstrated in Figure 3, one lead inhibitor showed MPTP inhibition in a concentration dependent manner in the CRC assay.

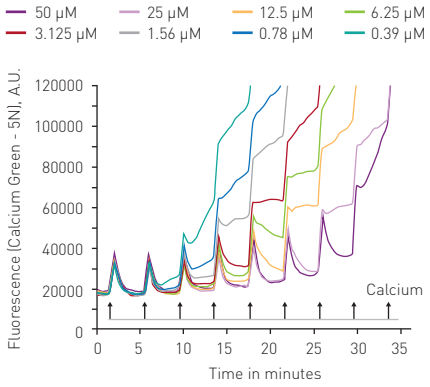

Fig 3: Concentration dependent response of the MPTP inhibitor in the CRC assay. Inhibitor was tested at 8 different concentrations 50 – 0.39 µM (with serial dilution).

Conclusion

CRC assay is a high throughput assay for a screening of the MPTP inhibitors with POLARstar Omega microplate reader. The assay can also be adopted for the live permeabilized cells. Owing to multiple injections and short fluorescent spikes, a robust microplate reader with injectors that detects fluorescence intensity fast and sensitive is required for reading the assay. The POLARstar Omega has proven to meet these requirements. Furthermore, the intuitive operation of the microplate reader by its control software assists in running such complex assays.

References

1. Bernardi P, Lisa FD (2015) The mitochondrial permeability transition pore: Molecular nature and role as a target in cardioprotection. J. *Mol. Cell. Cardiol.* **78**, 100-106.
2. Rao VK, Carlson EA, Yan SS (2014) Mitochondrial permeability transition pore is a potential drug target for neurodegeneration. *Biochim. Biophys. Acta*, **1842**, 1267-72.
3. Mukherjee R et al. (2016) Mechanism of mitochondrial permeability transition pore induction and damage in the pancreas: inhibition prevents acute pancreatitis by protecting production of ATP. *Gut* **65**, 1333-46.
4. Nakagawa T et al. (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* **434**, 652-8.