Fluorescent Parkin cell-based assay development for High Content Screening of compounds against Parkinson´s disease

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Abstract

Parkinson´s disease (PD) is a prevalent neurodegenerative disease characterized by selective degeneration of dopaminergic neurons in the substantia nigra, causing tremor and motor impairment. Parkin protein, whose mutants are the cause of Parkinson´s disease type 2 (PARK2), has been mechanistically linked to the regulation of apoptosis and the turnover of damaged mitochondria. Several studies have implicated aberrant mitochondria as a key contributor to the development of PD. In the attempt to discover new drugs, High Content Cell-based assays are becoming more important to mimic the nature of biological processes and their diversifications in diseases and will be essential for lead identification and the optimization of therapeutic candidates. We have developed a novel fluorescence cell-based assay for High Content Screening to find compounds that can promote the mitochondrial localization of Parkin without severe mitochondrial damage induction. In this work this model was used to screen a library of 1,280 compounds. After the screening campaign, the positive compounds were chosen for further testing, based on the strength of the initial response and lack of cytotoxicity. These results indicated that this Parkin cell-based assay is a robust (Z' > 0.5) and valid strategy to test potential candidates for pre-clinical studies.

Results

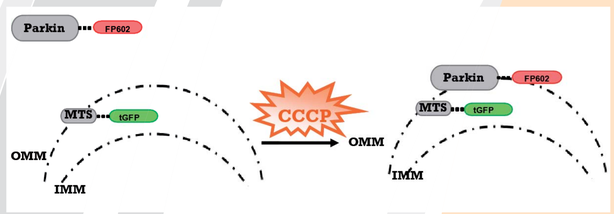

Fig.1. Schematic representation of Parkin recruitment model in living cells. Parkin model consist of a fluorescent fusion polypeptide capable of changing its localization within the cell from the cytosol to the outer mitochondrial membrane (OMM), after CCCP or positive compound addition. The second fluorescent fusion polypeptide (MTS-tGFP) allows visualizing integrity and location of mitochondria. MTS, mitochondrial-targeting signal.

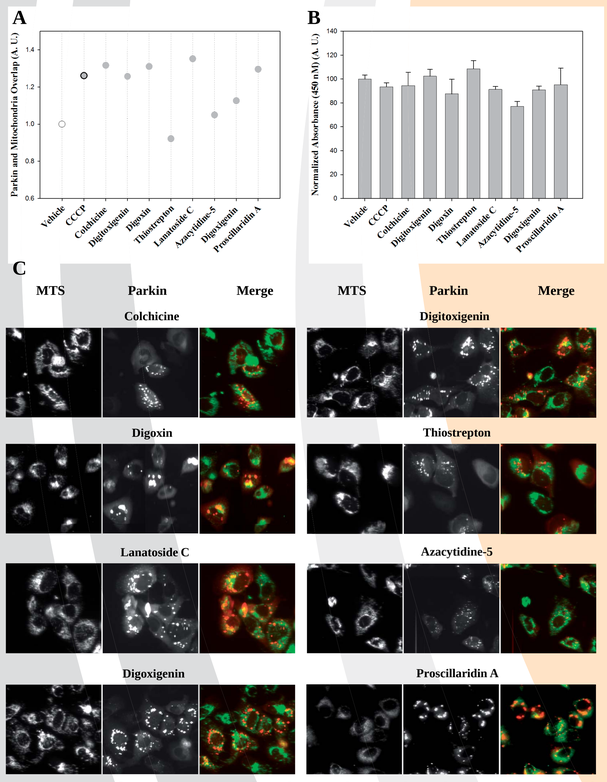

Fig.4. (A) Localization assay of Parkin and mitochondria in presence of redistribution-positive compounds. The recruitment of Parkin to mitochondria was measured by the overlap of Parkin-FP602 (red fluorescence) and MTS-tGFP (green fluorescence). Data were normalized to control (white). Positive control (CCCP, 5 µM) is represented in grey with solid edge and selected compounds are represented in grey without edge. **(B) Viability of the cells treated with the eight positive compounds was measured by WST-8 assay**. Absorbance at 450 nm was normalized to control. Data are shown as mean ± SD among 3 replicate wells.
(C) Representative images. U2OS double stably transfected cells after the addition of the eight positive compounds. Merged images are shown at right.

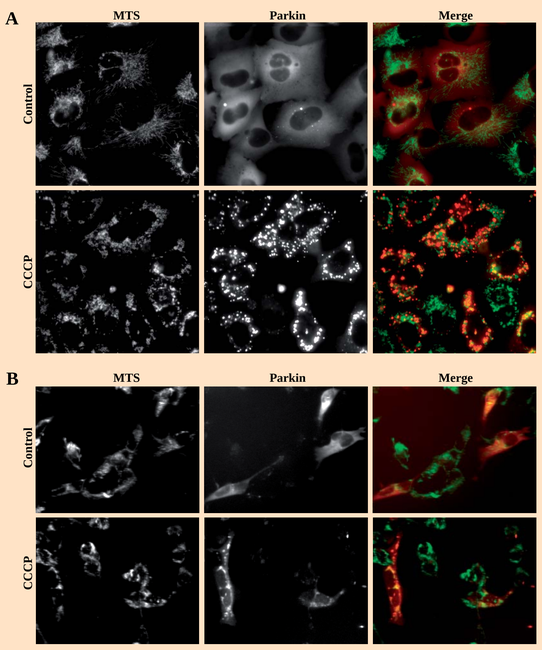

Figure 2. Cellular fluorescence redistribution after compound treatment.

(A) Representative images of negative control and positive compound CCCP in U2OS stably transfected cells. Negative control shows a cytoplasmic distribution of Parkin-FP602. By contrast, positive compound turns it into a vesicular pattern corresponding to mitochondrial distribution. Merged images are shown at right.

(B) Representative images of negative control and positive compound CCCP (5 mM) in MTS-tGFP and PARK2-FP602 expressing SH-SY5Y cells. Merged images are shown at right.

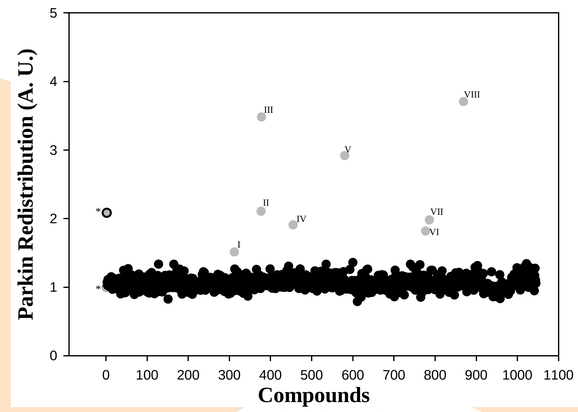

Figure 3. Representative data of Parkin redistribution screening. An initial screening of 1,280-compound library was performed. Negative control (DMSO) is represented in white and positive control (CCCP, 5 µM) is represented in grey with solid edge. Positive compounds (with an activity over 1.5 with respect to control) are represented in grey without edge. Those compounds with cell viability under 60% with respect to control were considered toxic and eliminated from the study. Parkin redistribution was quantified by measuring fluorescence granularity with Attovision software.

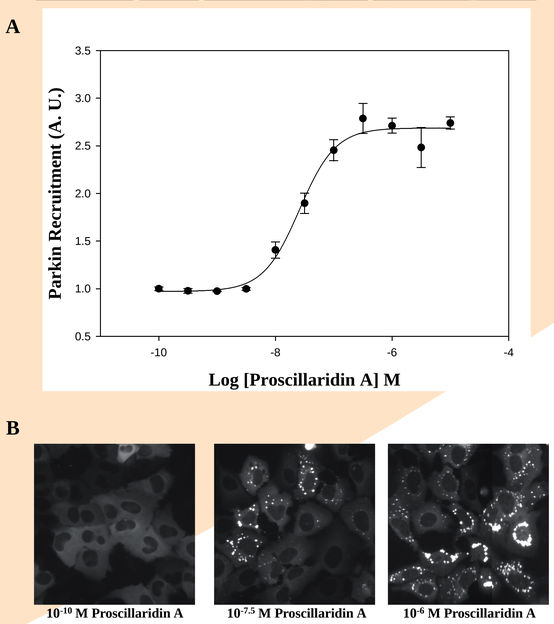

Figure 5. (A) Dose-response curve of the positive compound Proscillaridin A. Cells were treated with a range of concentrations from 100 pM to 10 µM during 24h. Data from measurements were fitted to a sigmoid 4-parameter logistic model (sigmoidal) with Sigma Plot 9 (Systat Software Inc., San Jose, CA) software. Error bars represent the standard deviation among 4 replicate wells. EC50 for Proscillaridin A was 2.59x10-8 M and Z' factor for this experiment was 0.85 +/-0.01.
(B) Representative images. Parkin-FP602 recruitment in U2OS double stably transfected cells after the addition of three representative concentrations (10-10 M, 10-7.5 M and 10-6 M) of Proscillaridin A.

Material & Methods

Cell cultures: U2OS human bone osteosarcoma cell line (DSMZ, Braunschweig Germany), derived from ATCC (Catalog No. HTB-96), was grown in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich, St. Louis, MO), MEM non-essential amino acids (Sigma-Aldrich, St. Louis, MO) and gentamicin (Sigma-Aldrich, St. Louis, MO) at 37°C in a humidified atmosphere supplemented with 5% CO2. For the screening image analysis, cell lines were cultured into 96 wells Imaging Plates (BD, Franklin Lakes, NJ) at a density of 10,000 cells/well.
Generation of stable cell line: PARK2-FP602 fusion protein was created by subsequent cloning of the FP602 red fluorescent protein (Evrogen, Moscow, Russia) and the human Parkin (PARK2) (Sino Biological, Beijing, China) cDNAs into the puromycin resistant pPURO vector designed in our lab, under the control of the CMV (cytomegalovirus) promoter. On the other hand, MTS-tGFP fusion protein was also created by the cloning of the turboGFP (tGFP) green fluorescent protein cDNA (Evrogen, Moscow, Russia) into the geneticin resistant MTS (mitochondrial targeting sequence, derived from the subunit VIII of human cytochrome C oxidase) expressing vector (Evrogen, Moscow, Russia), under the CMV promoter as well. After generating green and red fluorescent resistant clones with limit dilution, a 100% certified truly monoclonal cell line was obtained with the technology developed within FP7 PASCA EU project where cells are handled and analyzed one by one. These cells constitutively express the fusion polypeptides Parkin-FP602 and MTS-tGFP.
Liquid Handling and phenotype-based screening assay: For dispensing of the liquid media containing cells and compounds, the Hamilton's (Reno, NV) Microlab Star automated liquid handling workstation was used. Human tagged PARK2-FP602+MTS-tGFP stably expressing cells were treated with CCCP (positive control) at 5 µM for 3 hours and library compounds at 10 µM for 24 hours in Optimem medium (Thermo Fisher Scientific, Waltham, MA) before image acquisition. Redistribution of Parkin and its localization to mitochondria was quantified after treatment. Cellular viability was determined by counting nuclei.
Image acquisition and analysis: Plates were fixed with phosphate buffered saline (PBS; Sima-Aldrich, St. Louis, MO) containing 3.7% formaldehyde (FA; Sigma-Aldrich, St. Louis, MO) for 10 min at room temperature (RT), permeabilized with 0.3% Triton-X100 (Sigma-Aldrich, St. Louis, MO) in PBS for 3 min at RT and cell's nuclei were stained with DAPI (Sigma-Aldrich, St. Louis, MO) for 5 min at RT. Fluorescent images were acquired in the BD (Franklin Lakes, NJ) Pathway 855 High-Content automated image platform with a x20 dry objective.

Conclusions

PARK2+MTS U2OS cell-based model permits evaluating mitochondrial Parkin recruitment in living cells through the study of its location pattern in the space and time.
PARK2+MTS U2OS cell-based model can be applied for the screening of compound libraries in multiple High Content Bioimaging Platforms.
PARK2+MTS U2OS cell-based model has been adapted to HCS analysis based on image algorithms.
PARK2+MTS U2OS cell-based model provides a robust assay to evaluate compounds that promote mithocondrial Parkin localization in living cells and therefore, to test drugs against Parkinson's disease.
From 1280 compounds tested, 8 were selected to study their potential Parkin recruitment effect in further experiments