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Supplemental Data

Chemical Inhibition of the Mitochondrial Division Dynamin Reveals Its Role in Bax/Bak-Dependent Mitochondrial Outer Membrane Permeabilization

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Supplemental Experimental Procedures

Assays in Yeast and Mammalian Cells

Yeast mitochondrial morphology: RDY84, transformed with pVT100 GFP (expresses mitochondrial-targeted GFP), was incubated for 5-30 minutes with the indicated concentrations of the compounds, and mitochondrial morphology was assessed by fluorescence microscopy as described (Nunnari et al., 2002).

Yeast plate growth assay: YPGlycerol plates were topped with 10 ml YPGlycerol containing 1% low melt agar and 75 μM mdivi-1, and cells were spotted 12 hours later using a 48 well pinning device. After pinning cells, plates were incubated at 24°C or 37°C and imaged using an Eagle Eye II imaging system (Stratagene).

Yeast actin morphology: Cells were treated with DMSO, 100 μM mdivi-1 or 100 μM mdivi-1 and 200 μM Latrunculin-A. F-actin was visualized with Alexa fluor 488 phalloidin as described (Adams and Pringle, 1991) and imaged using a DeltaVision deconvolution microscope.

Mammalian mitochondrial morphology and GFP-Drp1 behavior: COS cells were plated at a concentration of $5x10^4$ cells/ml onto 0.01% poly-L-lysine (P4832, Sigma) treated cover-slips in 6 well plates containing 2ml DMEM medium (DMEM, 10% fetal bovine

serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamate, 5 mM sodium pyruvate, Cellgro) per well, and were incubated in 37°C, 5% CO_2 for 24 hrs before the start of the experiment. Cells were treated with the specified concentration of the compounds or DMSO as a control, and, where indicated, 1 µM STS for 30 min-1 hr and were then stained with 0.02 µM MitoTracker Red CMXRos (Molecular Probes) 30 min prior to imaging. For examining the behavior of GFP-Drp1 behavior COS cells were transfected using Lipofectamine 2000 (Invitrogen) with GFP-Drp1/pcDNA3 (a gift from Alex van der Bliek, UCLA) and processed as described. To examine the effects of Drp1K38A expression on mitochondrial morphology and apoptosis, COS or HeLa cells, respectively, were transfected with Drp1/pcDNA3 or Drp1K38A/pcDNA3 (gifts from Alex van der Bliek, UCLA) using Lipofectamine 2000 (Invitrogen) and were analyzed as described. To create Drp1 RNAi knockdown cells, cells were transfected using Oligofectamine (Invitrogen) with siGENOME duplex DNM1L (Dharmacon, Cat. #D-012092-01) or non-targeting siRNA #1 (Dharmacon, Cat. #D-001210-01) and observed 48hrs after transfection. Image stacks were taken using a DeltaVision deconvolution fluorescent microscope fitted with a 100X objective, deconvolved and projected using SoftWorx 2.50 software (Applied Precision).

Annexin V staining: HeLa cells were plated in 6 well plates at a concentration of $5x10^5$ cells/ml in 2ml DMEM medium per well, and incubated in 37°C, 5% CO_2 for 24 hrs before the experiment. Cell were then treated with the drugs at the specified concentrations and 1 µM STS where indicated for 4hrs, trypsinized, washed once with phosphate-buffered saline and then with Annexin-Binding Buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4), and stained with Annexin V-Alexa Fluor 488(A-13201, Molecular Probes) at a concentration of 5 µl per 100 µl cell suspension ($1x10^6$

cells/ml Annexin Binding Buffer) at RT for 15 min in the dark. 400 µl of Annexin-Binding Buffer and propidium iodide (final concentration 0.5 µg/ml) were then added to the 100 µl cell suspension. Samples were kept on ice and analyzed by flow cytometry (FACS Calibur, Becton Dickinson), and the data were analyzed using WinMDI software.

Microinjection: Cells were plated on glass bottom dishes (MatTek, Ashland, MA) the day before injection in DMEM (containing: 200 mM L-Glutamine, 10% heat inactivated FBS and 20 uM qVD) and kept at 37°C with 5% CO_2. At the time of injection, cells were approximately 90% confluent. Cells were coinjected with FL-BID or C8-BID and Texas Red dextran in HE buffer (10 mM HEPES pH 7.4, 1 mM EDTA); the needle concentrations were: 120 ng/uL BID (FL and C8) and Texas Red dextran 0.1 ug/uL. Cells were injected using an Eppendorf InjectMan NI2/FemtoJet microinjection system (Brinkmann Instruments, Westbury, NY), mounted on a Nikon TE2000-U inverted microscope (Nikon USA, Melville, NY), with an ELDW 40X Plan Fluor phase-contrast objective (NA 0.6). Cells were kept in the stage for less than 20 min at a time. Injection parameters: Pi: 30-120 hPa, Ti: 0.5 s, Pc: 20 hPa. Immediately after injection cells were allowed to recover at 37 °C, 7.5% CO_2. Confocal microscopy on live cells was performed with a Marianas spinning disk confocal imaging system (Intelligent Imaging Innovations/3i, Denver, CO) consisting of a CSU22 confocal head (Yokogowa Electric Corporation, Japan), DPSS lasers (CrystaLaser, Reno, NV) with wavelengths of 445nm, 473nm, 523nm, 561nm, and 658 nm, and a Carl Zeiss 200M motorized inverted microscope (Carl Zeiss MicroImaging, Thornwood, New York), equipped with spherical aberration correction optics (3i). Temperature was maintained at ~37°C and 5% CO_2 using an environmental control chamber (Solent Scientific, UK). Images were acquired

with a Zeiss Plan-Neofluar 40x 1.3 NA DIC objective on a CascadeII 512 EMCCD (Photometrics, Tucson, AZ), using SlideBook 4.2 software (3i).

In Vitro Assays

Recombinant protein purification: Dnm1, Dnm1 G385D, Dnm1 1-338 and dynamin-1 were purified from baculovirus-infected insect cells as described (Ingerman et al., 2005). The dynamin-1 baculovirus vector was a gift from Dr. Mark Lemmon, Department of Biochemistry and Biophysics, University of Pennsylvania, School of Medicine, Philadelphia.

GTP hydrolysis: A continuous regenerative assay was used to measure the GTPase activity of Dnm1 as described (Ingerman and Nunnari, 2005). For determination of the kinetic parameters, GTPase assays were carried out in 25 mM HEPES, 25 mM PIPES, pH 7, 150 mM NaCl, 30 mM imidazole, pH 7.4, 7.5 mM KCl, 5 mM $MgCl_2$, 1 mM phospho(enol)pyruvate (PEP), 20 U/mL pyruvate kinase/lactate dehydrogenase, 4% dimethyl sulfoxide (DMSO), and 600 μM NADH. Chemical inhibitor and GTP concentrations were varied, as specified. GTPase assay reactions were started by addition of approximately 10 μg of purified protein, in freezing buffer, to the GTPase assay reaction buffer containing the small molecule.

All GTPase assay reactions were started in a 200 μL volume, of which 150 μL was placed into the well of a 96-well plate. Depletion of NADH, as monitored by reading the A340 of the reaction, was measured every 20 s for a total of 40 min using a SpectraMAX 250 96-well plate reader (Molecular Devices). Spectrophotometric data were transferred to Excel and the measured steady state depletion of NADH over time was converted to protein activity. $K_{0.5}$, K_m, k_{cat}, K_i, Hill coefficient and other kinetic parameters were calculated numerically, using the Genfit function of Mathcad.

EM analysis of Dnm1 structures: Electron microscopy analysis of Dnm1 assembly was performed as described (Ingerman and Nunnari, 2005). Dnm1 in HCB 150 (Hepes column buffer 150: 20 mM Hepes, 1 mM $MgCl_2$, 2 mM EGTA, 150 mM NaCl, pH 7.2) was incubated with 50 μM mdivi-1 or DMSO as a control for 15 minutes at room temperature followed by incubation with 1 mM GMPPCP for an additional 30 minutes or in 1 mM GMPPCP only for 30 minutes. To prepare negative stain specimens, a carbon-coated grid was placed on a 10-μl sample drop of protein for 2 min, blotted with filter paper, stained with 2% uranyl acetate for 2 min, blotted again, and air dried. All images were obtained from a transmission electron microscope (Philips CM120; FEI Co.) operating at 100 kV and were taken at 35,000x with a focus range of 0.8–1.5 mm underfocus. Images were recorded digitally on CCD cameras (MultiScan 791 and 794; Gatan) with the Digital Micrograph software package (Gatan and (Danino et al., 2004)).

MOMP assay: Outer membrane permeabilization in isolated mitochondrial was performed as described (Chipuk et al., 2005). Murine heavy membrane fractions (mitochondria) were purified from C57Bl/6 liver, female, less than 3 months, using dounce homogenization and differential centrifugation. For MOMP assays, mitochondria were incubated in mitochondrial isolation buffer (MIB: 200 mM mannitol, 68 mM sucrose, 10 mM HEPES-KOH pH 7.4, 100 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% BSA) plus or minus BH3-only proteins or peptides for 90 minutes at RT. For drug inhibition studies, mitochondria were pre-incubated with indicated compounds for 30 minutes prior to the addition of proteins. Reactions were then fractionated into supernatant and pellet by centrifugation at 5,500 x g for 5 minutes, and analyzed by SDS-PAGE and western blot with anti-cytochrome c (BD Pharmingen). For MOMP

reconstitution studies, heavy membrane fractions were isolated from the livers of polydIdC-treated MxCre $bak^{-/-}$ $bax^{f/-}$ animals.

LUV permeabilization assay: Large unilamellar vesicle (LUV) release assays were prepared as described (Kuwana et al., Cell 2002). Briefly, lipids were dried and resuspended in buffer containing fluorescein conjugated dextran (10kD) in a water bath sonicator. Unilamelar vesicles were formed by extrusion of the suspension through a 400 nm pore sized filter using an extruder (Avestin, Canada). Unincorporated dextrans were removed by float-up centrifugation in a sucrose gradient. Liposomes were resuspended in buffer and incubated with recombinant proteins (C8-BID, R&D Systems; full-length monomeric BAX, Suzuki et al., Cell 2000) and chemicals for 2.5 h at room temperature. The assay mix was filtered through .1μ pore sized membrane (Microcon, Amicon) and the released dextran was detected as fluorescence in the filtrate. The percentage of release was calculated between the baseline provided by the buffer control and 100 % release obtained by liposomes solubilized in 1% CHAPS.

Supplemental References

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A

$$\nu = \frac{V_{max} \cdot \frac{[S]}{K_s} \cdot (1+\frac{[S]}{K_s})^{n-1}}{\frac{[T_0]}{[R_0]} + (1+\frac{[I]}{K_i})^n + (1+\frac{[S]}{K_s})^n}$$

B

	0μM mdivi-1	2.5μM mdivi-1	5μM mdivi-1	10μM mdivi-1	20μM mdivi-1
$[T_0]/[R_0]$	5.01	5.01	5.01	5.01	5.01
k_{cat}	55.3	45.2	48.4	39.1	34.8
n_{app}	4.3	2	7.1	5.1	3.5
K_s	43.3	22.7	269	275	379
K_i	-	1.2	43	26	27

C

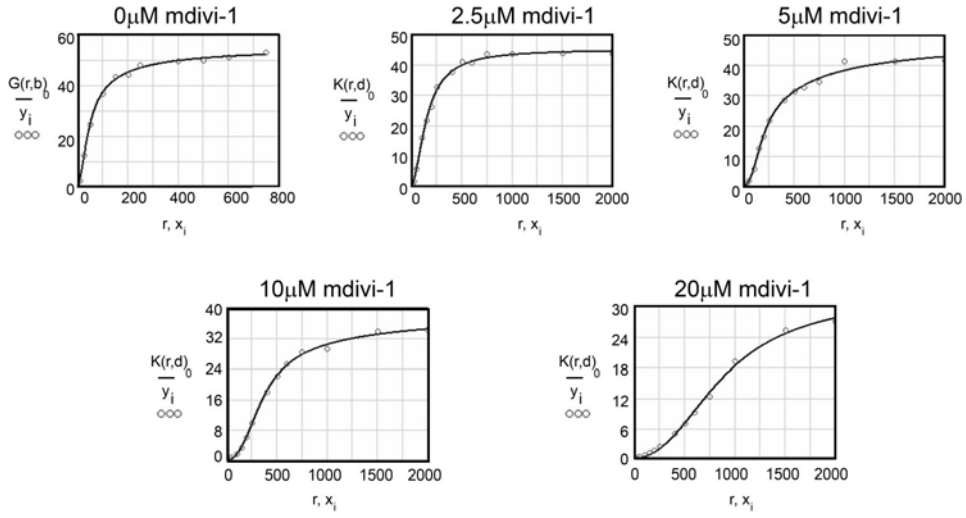
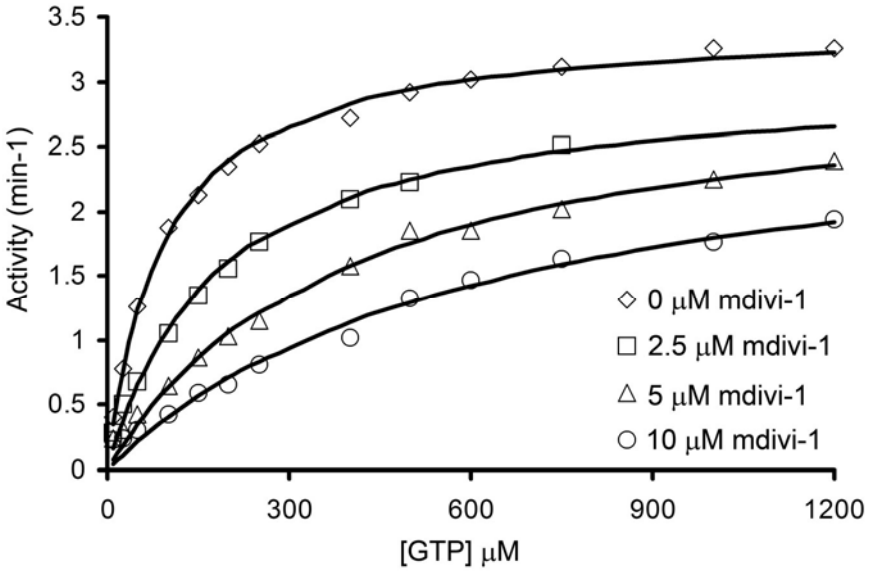


Figure S1. mdivi-1 Is an Allosteric Inhibitor of Dnm1

A. Inhibition of Dnm1 GTPase activity by mdivi-1 fit to the concerted transition model of Monod, Wyman, and Changeux using the mathematical equation (A). B. The kinetic parameters, and C. Curve fit of kinetic data.

A



B

Parameters	0 μM mdivi-1	2.5 μM mdivi-1	5 μM mdivi-1	10 μM mdivi-1
k_{cat} (min^{-1})	3.6	3.9	4.1	5.4
$K_{0.5}$ (μM)	100	340	770	2600
Apparent Hill Coefficient	.9	.8	.8	.7

Figure S2. mdivi-1 Is a Mixed Type Inhibitor of Dnm1 G385D GTPase Activity

Kinetic analysis (A and B) of the effect of mdivi-1 on Dnm1G385D GTPase activity.

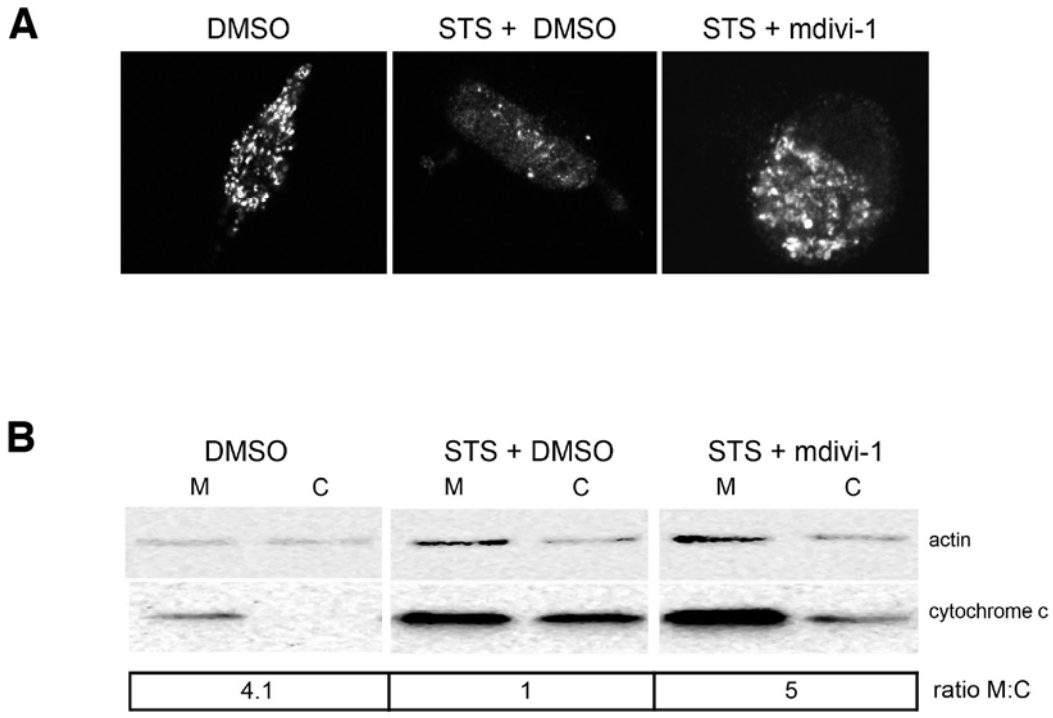


Figure S3. mdivi-1 Partially Blocks Cytochrome *c* Release after Stimulation of Apoptosis with Staurosporine

A. HeLa cells were grown in DMEM on cover slips at 37°C with 5% CO_2 overnight, then incubated with DMSO, 1μM staurosporine (STS) plus DMSO or 1μm STS plus 50 μm mdivi-1 for four hours. Cytochrome c was visualized by indirect immunofluorescence with anti-cytochrome c (Pharmingen) and secondary goat anti-mouse Alexa fluor 488 (Molecular Probes) on a Leica confocal microscope. **B.** Mitochondria and cytosol from HeLa cells grown as in A were isolated by differential centrifugation, then separated using PAGE, blotted to nitrocellulose, probed with anti-cytochrome c and anti-actin antibodies and quantified. Each cytochrome c band was normalized to the actin band and then the ratio of cytochrome c in the mitochondrial fraction (M) to cytosolic fraction (C) was determined for each condition.

Table S1. Effects of Compounds on Mitochondrial Morphology in COS Cells

Compound[a]	Morphological categories, percentage[b]		
	Reticular	Loose Nets	Degenerate/ perinuclear
A	41	40	19
B	34	43	22
C	81	19	0
D	72	18	10
E	53	45	3
F	87	13	0
G	81	15	4
H	80	20	0
DMSO	89	11	0

[a] As indicated in Figure 2. All compounds at 50 μM.
[b] Represents n ≥ 90 cells.

Table S2. Mitochondrial Morphology in STS-Treated COS Cells

[mdivi-1] μM	Morphological Categories, percentage[a]			
	Fragmented	Reticular	Loose Nets	Degenerate/ perinuclear
0	76	24	0	0
1	70	27	3	0
10	65	30	5	0
50	52	37	10	0
100	46	40	13	0

[a] Represents n ≥ 90 cells.

Table S3. Quantification of Bid Induced Cytochrome *c* Release in HeLa Cells

Treatment	% cytochrome c release[a]
DMSO	9.1+/-1.3%[b]
C8-BID	95.6+/-6.3%
B	10.7+/-1.0%
B plus C8-BID	20+/-4.1%
F	7.6+/-3.5%
F plus C8-BID	87+/-18.4%

[a] ≥50 cells were scored for punctate (mitochondrial) or diffuse (cytosolic) localization of stably-expressed cytochrome c-GFP by confocal microscopy of the microinjected fields. Data represent at least three independent experiments.

[b] SD