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Supplementary Information for

A small molecule protects mitochondrial integrity by inhibiting mTOR activity

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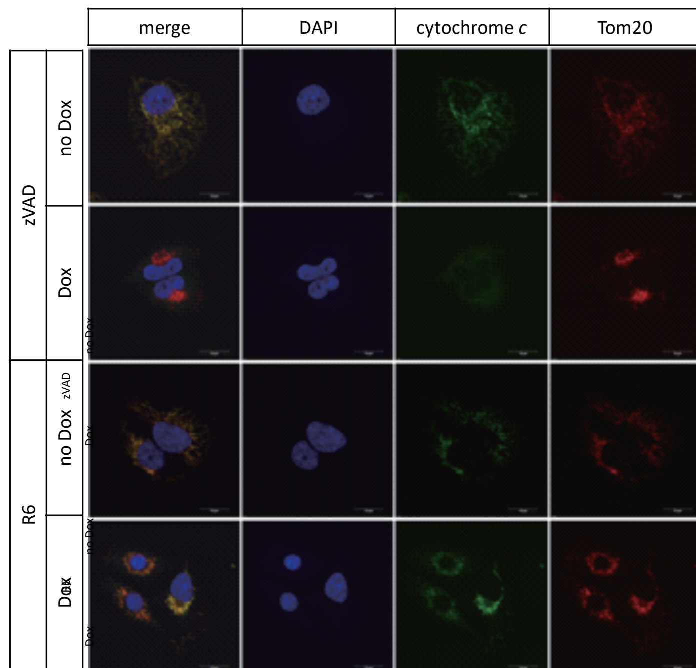

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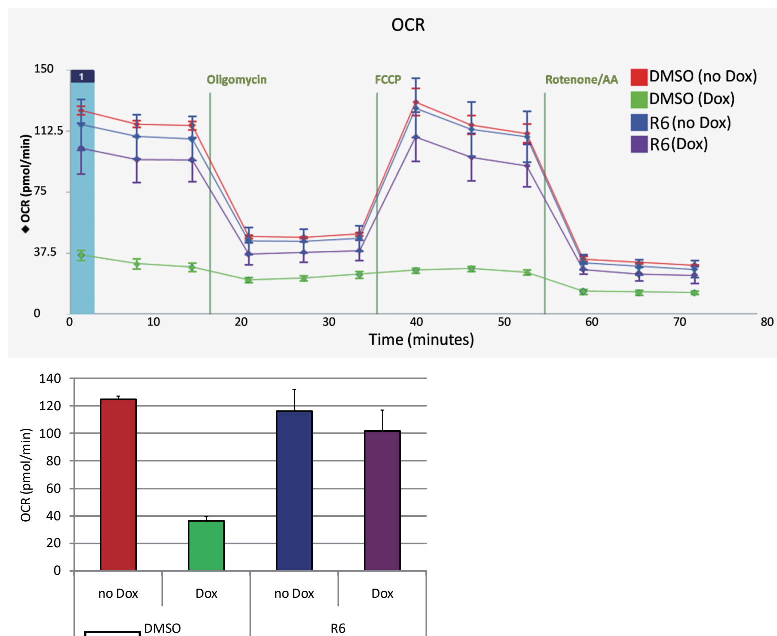
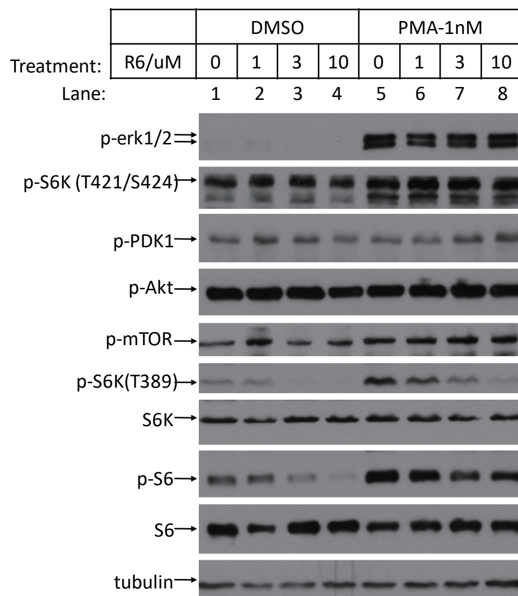


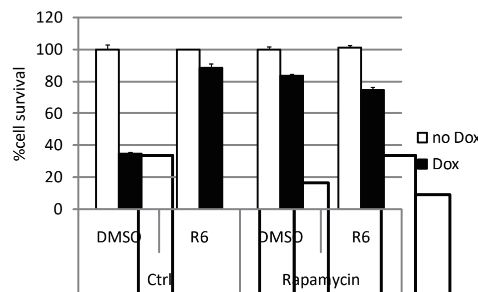
Fig. S1. Compound R6 protected mitochondrial integrity and function during Bim-induced intrinsic apoptosis. Related to Figure 1. (A) U2OS_Bim cells were treated with zVAD or R6 (1μM) for 1hr and then were treated with or without Dox for 4hr. Immunostaining was performed then. (B) U2OS_Bim cells were treated with DMSO or R6 (1μM) for 1hr and then were treated with or without Dox for 2hr. Oxygen consumption rates of the cells were examined by Seahorse XFe96 analyzer.

OCR (pmol/min)

no Dox Dox no Dox Dox

DMSO R6





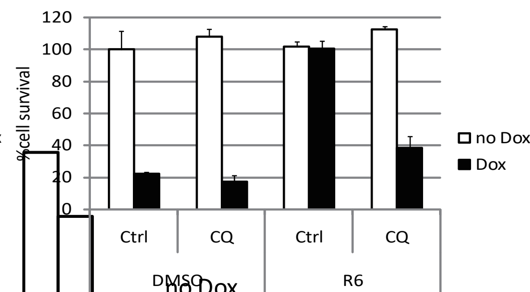
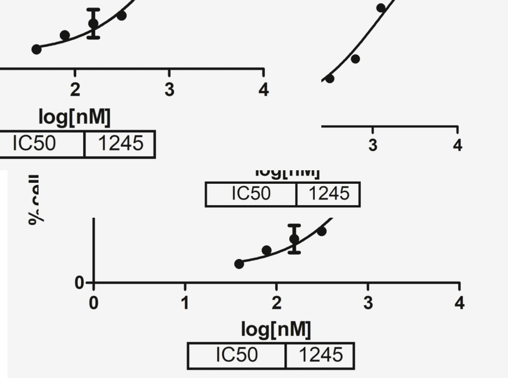


Fig. S2. Compound R6 worked through mTOR-mediated autophagy induction. Related to Figure 2. (A) Compound R6 blocked p-S6K-T389 phosphorylation by mTORC1 in a dose-dependent manner in Jurkat cells. Jurkat cells were treated with Compound R6 in different concentrations as indicated and then treated with DMSO or PMA for 10 min then collected by centrifuge. p-S6K-T421/S424 phosphorylation by p-erk is necessary to p-S6K-T389 phosphorylation by mTORC1 (ref (1)). PMA can activate p-mek/p-erk/p-S6K-T421/S424 pathway in Jurkat cells quickly so that there's enough p-S6K-T421/S424 for the next-step p-S6K-T389 phosphorylation by mTORC1. WCE were analyzed by western blotting using the indicated antibodies. (B) U2OS_Bim cells were treated with DMSO or R6 (1µM) with or without Rapamycin (20µM) for 1hr and then were treated with or without Dox for 24hr. Cell survival was determined and represented as the mean ± SD of duplicate wells. (C) U2OS_Bim cells were treated with DMSO or R6 (1µM) with or without CQ (50µM) for 1hr and then were treated with or without Dox for 24hr. Cell survival was determined and represented as the mean ± SD of duplicate wells.



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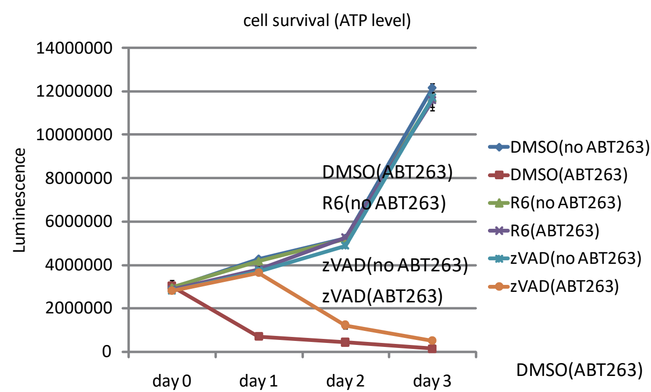

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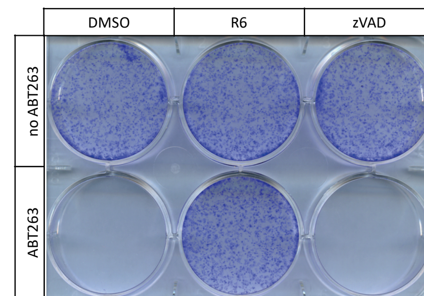

Fig. S3. Compound R6 is a general inhibitor of the intrinsic apoptosis pathway

(A) In serum withdrawal condition, U2OS cells were treated with Compound R6 start from 50uM, 3X dilute for 1hr and then were treated with or without ABT263 (5μM) each duplicate wells for 8hr. EC50 was calculated by software GraphPad Prism. (B) U2OS cells were seeded in 96-well plates at a density of 500 cells/well. On the next day, in serum withdrawal condition, cells were treated with the indicated compounds for 1hr and were treated with or without ABT263 for 8hr and then serum was added back to the medium. Cell survival was determined immediately after Dox treatment (day 0) or 1, 2, or 3 days later. (C). U2OS cells were seeded in a 6-well plate at a density of 3000 cells/well. On the next day, in serum withdrawal condition, cells were treated with the indicated compounds for 1hr and were treated with or without ABT263 for 8hr and then serum was added back to the medium. After a week, colony formation was stained by crystal violet.

A

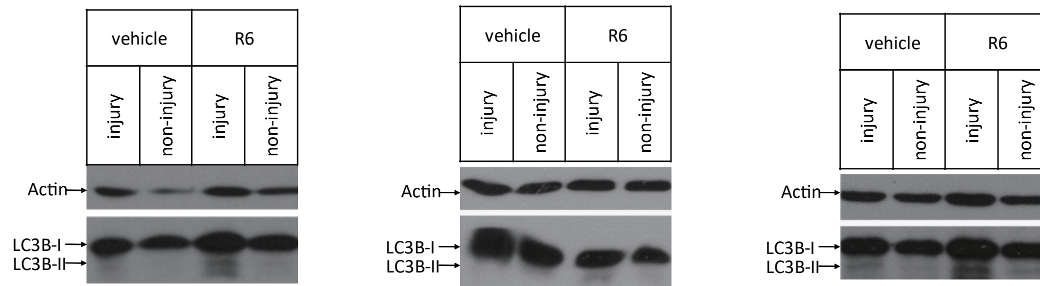

Fig. S4. Related to Figure 5. (A) Before TTC-staining, an injury or non-injury slice from each brain was kept for western blotting analysis. An injury or non-injury slice from vehicle or R6 treated brain was analyzed by western blotting using anti-LC3A/B and anti-β-Actin-HRP antibodies.

A

DMPK, NIBS

Pharmacokinetics Summary - Result and Chart

Compound R6's PK Parameters:

IV administration (7.50mg/kg)

No pts used for $t_{1/2}$	$t_{1/2}$ (hr)	C_0 (ng/mL)	AUC_{last} (hr*ng/mL)	AUC_{Inf} (hr*ng/mL)	AUC Extra (%)	V_z (L/kg)	V_{ss} (L/kg)	CL (mL/min/kg)	MRT (hr)	Last time point for AUClast (hr)	Time points for T1/2 (hr)
3	4.04	795	981	1229	20.1	35.6	28.1	102	4.61	8	2,4,8

IP administration (10.00mg/kg)

No pts used for $t_{1/2}$	$t_{1/2}$ (hr)	t_{max} (hr)	C_{max} (ng/mL)	AUC_{last} (hr*ng/mL)	AUC_{Inf} (hr*ng/mL)	AUC Extra (%)	MRT (h)	AUC/D (hr*kg*ng/mL/mg)	F (%)	Last time point for AUClast (hr)	Time points for T1/2 (hr)
4	6.36	0.500	257	1311	2246	41.63	9.10	224.6	100.2	8	1,2,4,8

Note: 1. F was based on the calculation of AUClast

B

DMPK, NIBS

Pharmacokinetics Summary - Result and Chart

Compound R6's Rat iv 7.5 mg/kg 8h Brain conc. Result

FileName	Calculated Conc (ng/ml)	*5 (5 times dilution) (ng/g)	AVE ng/g
Rat_Brain_IV_8h_1	25.7	128	195
Rat_Brain_IV_8h_2	42.0	210	
Rat_Brain_IV_8h_3	49.2	246	

Standard Range 2-500 ng/mL

Compound R6's Rat IP 10 mg/kg 8h Brain conc. Result

FileName	Calculated Conc (ng/ml)	*5 (5 times dilution) (ng/g)	AVE ng/g
Rat_Brain_IP_8h_1	108	540	310
Rat_Brain_IP_8h_2	48.0	240	
Rat_Brain_IP_8h_3	30.2	151	

Standard Range 2-2000 ng/mL

Table S1. Pharmacokinetics study of Compound R6 by IV and IP injection.

Materials and Methods:

Reagents:

Compound R6 was synthesized by Zichen Cao in Dr. Zhiyuan Zhang's lab at the National Institute of Biological Sciences, Beijing.

The Cell Titer-Glo kit was purchased from Promega.

The following antibodies were used: anti-Bim (CST, 2819), anti-cleaved caspase 9 (CST, 9501), anti-PARP (CST, 9532), anti-VADC (CST, 4866), anti-Bax (CST, 5203), anti-Bak (CST, 12105), anti-phospho-S6K-T389 (CST, 9234), anti-phospho-S6K-T421/S424 (CST, 9204), anti-S6K (CST, 9202), anti-LC3B (CST, 3868), anti-LC3A/B (CST, 12741), anti-phospho-mTOR (CST, 5536), anti-mTOR (CST, 2972), anti-Akt (CST, 4685), anti-phospho-Akt(CST, 4060), anti-phospho-erk1/2 (CST, 4370), anti-phospho-PDK1 (CST, 3438), anti-phospho-S6 (CST, 4858), anti-S6 (CST, 2317), anti-cytochrome *c* (BD, 556433 for western blotting, 556432 for immunostaining), anti-Tom20 (Santa Cruz,sc-11415), anti-β-Actin-HRP (MBL,D291-7),and anti-β-tubulin (EASYBIO, BE3312-10).The following small molecules were used: 3-MA (MCE, HY-19312), Rapamycin (MCE,HY-10219), and PMA (MCE, HY-18739).

Cell Culture and Stable Cell Lines:

U2OS, HeLa, and MEF cells were cultured in DMEM containing 10% (V/V) FBS. Jurkat cell was cultured in RPMI 1640 containing 10% (V/V) FBS. The U2OS_Bim cell line was generated as described in ref (2).

Cell Survival Assay:

Cell survival was analyzed by measuring ATP levels with a Cell Titer-Glo kit from Promega, according to the manufacturer's instructions.

Crystal Violet Staining:

We modified the Crystal Violet Staining described in ref (3). Briefly, 3,000 Cells/well were seeded in 6-well plates and treated on the following day, as indicated in the figure legends. Cells were washed twice with PBS, followed by incubation with 0.05% crystal violet in H_2O for 10 min at room temperature. Subsequently, the crystal violet solution was removed and the samples were again washed twice with PBS, followed by removal of PBS and drying of the plate at room temperature.

Immunostaining:

We modified the immunostaining method described in ref (2). Briefly, Cells were seeded in Lab-Tek eight-chambered slides (Thermo) and treated on the following day, as indicated in the figure legends. Cells were washed twice with PBS and fixed in 4% PFA for30 min at room temperature followed by three washes with PBS and incubation in SuperBlock buffer (Thermo) for 30 min at room temperature. Primary antibodies were 1:200 diluted in PBS and incubated with the cells at 4°C overnight. Cells were then washed three times with PBS for 30 min, followed by incubation with secondary antibodies 1:1000 diluted in PBS at room temperature for 2 hr. After another three washes with PBS, the slides were covered and sealed, and were then examined with a Nikon A1-R confocal microscope.

Oxygen Consumption Rate:

30,000 Cells/well were seeded in Agilent Seahorse 96-well XF Cell Culture Microplate and treated on the following day as indicated in the figure legends. The oxygen consumption rate was measured using a Seahorse XF Cell Mito Stress Kit and an XFe96Analyzer, according to the instructions from Agilent.

References

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2. Jiang X, Jiang H, Shen Z, & Wang X (2014) Activation of mitochondrial protease OMA1 by Bax and Bak promotes cytochrome c release during apoptosis. Proceedings of the National Academy of Sciences 111(41):14782-14787.
3. Jiang X, et al. (2016) A small molecule that protects the integrity of the electron transfer chain blocks the mitochondrial apoptotic pathway. Molecular cell 63(2):229-239.