Supporting Information for

Original article

Schisandrol A protects AGEs-induced neuronal cells death by allosterically targeting ATP6V0d1 subunit of V-ATPase

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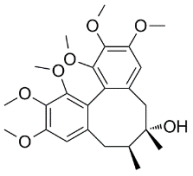
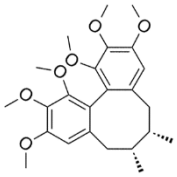
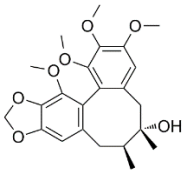
1. Supporting table

Table S1 Name abbreviation of three structurally similar compounds.

Compound name	Schisandrol A	Schisandrin A	Schisandrol B/Gomisin A
CAS number	7432-28-2	61281-38-7	58546-54-6
Molecular formula	$C_{24}H_{32}O_7$	$C_{24}H_{32}O_6$	$C_{23}H_{28}O_7$
Molecular weight	432.507	416.507	416.464
Chemical structure			
Abbreviation	SolA	SchA	SolB/GomA

2. Supporting figures

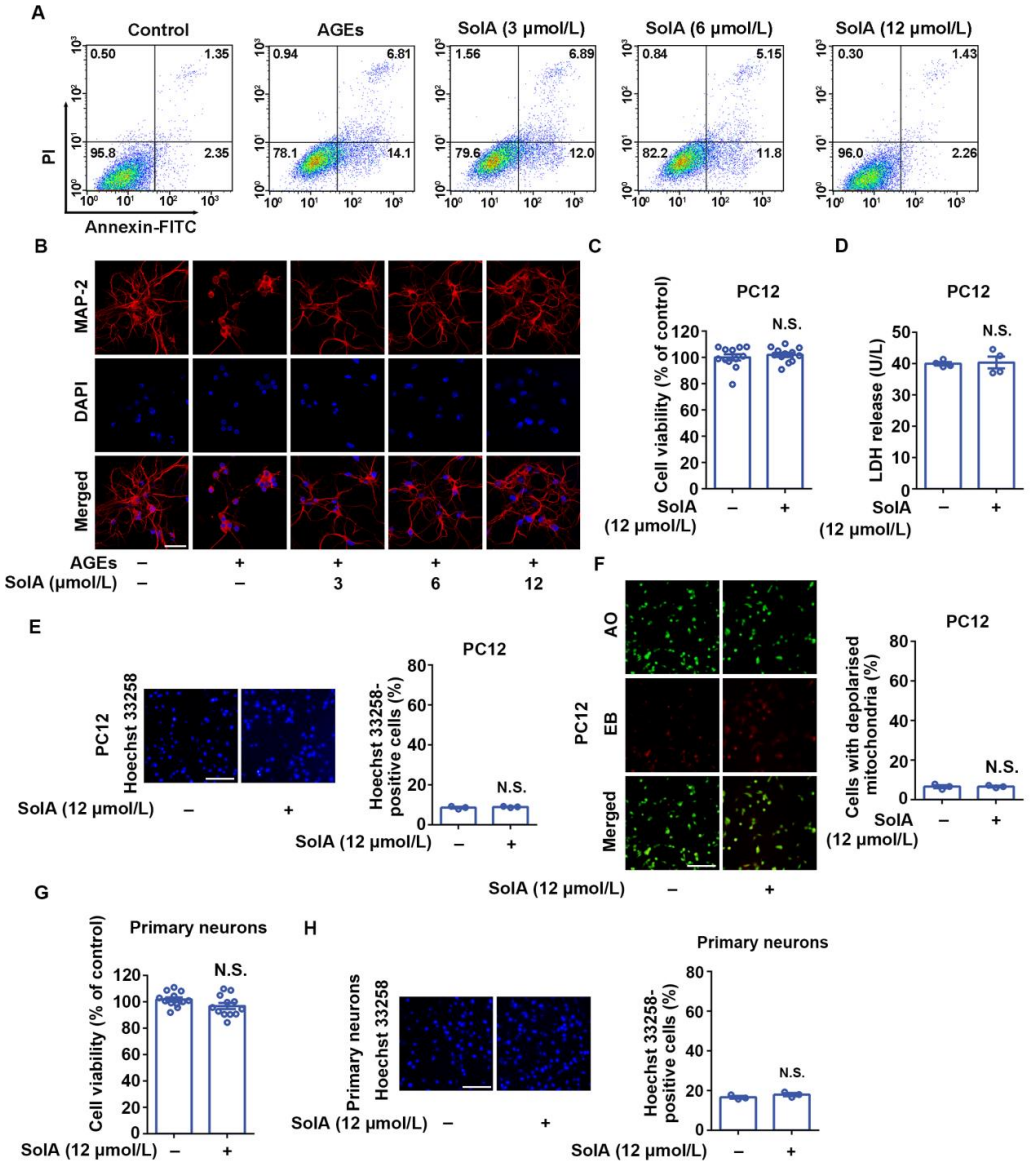


Figure S1 (Related to Fig. 1). Discovery of SolA as a neuroprotective small-molecule against AGEs-induced injury. (A) Sol A inhibited PC12 cells apoptosis by Annexin V-FITC/PI assay. (B) Sol A protected primary neurons from morphological damage by immunofluorescence staining of MAP-2 (scale bar: 40 μm). (C) SolA (12 μmol/L) alone had no effect on the cell viability in AGEs-induced PC12 cells (n = 12). (D) SolA (12 μmol/L) alone had no effect on LDH release in AGEs-induced PC12 cells (n = 4). (E) SolA (12 μmol/L) alone had no effect on PC12 cells apoptosis by Hoechst 33258 staining (scale bar: 100 μm, n = 3). (F) SolA (12 μmol/L) alone had no effect on PC12 cells apoptosis by double staining with AO and EB (scale bar: 100 μm, n = 3). (G) SolA (12 μmol/L) alone had no effect on the cell viability in AGEs-induced rat primary neurons (n = 12). (H) SolA (12 μmol/L) alone had no effect on rat primary neurons apoptosis by Hoechst 33258 staining (scale bar: 100 μm, n = 3). Data are expressed as the mean ± SEM. N.S. not significant (Student's t-test).

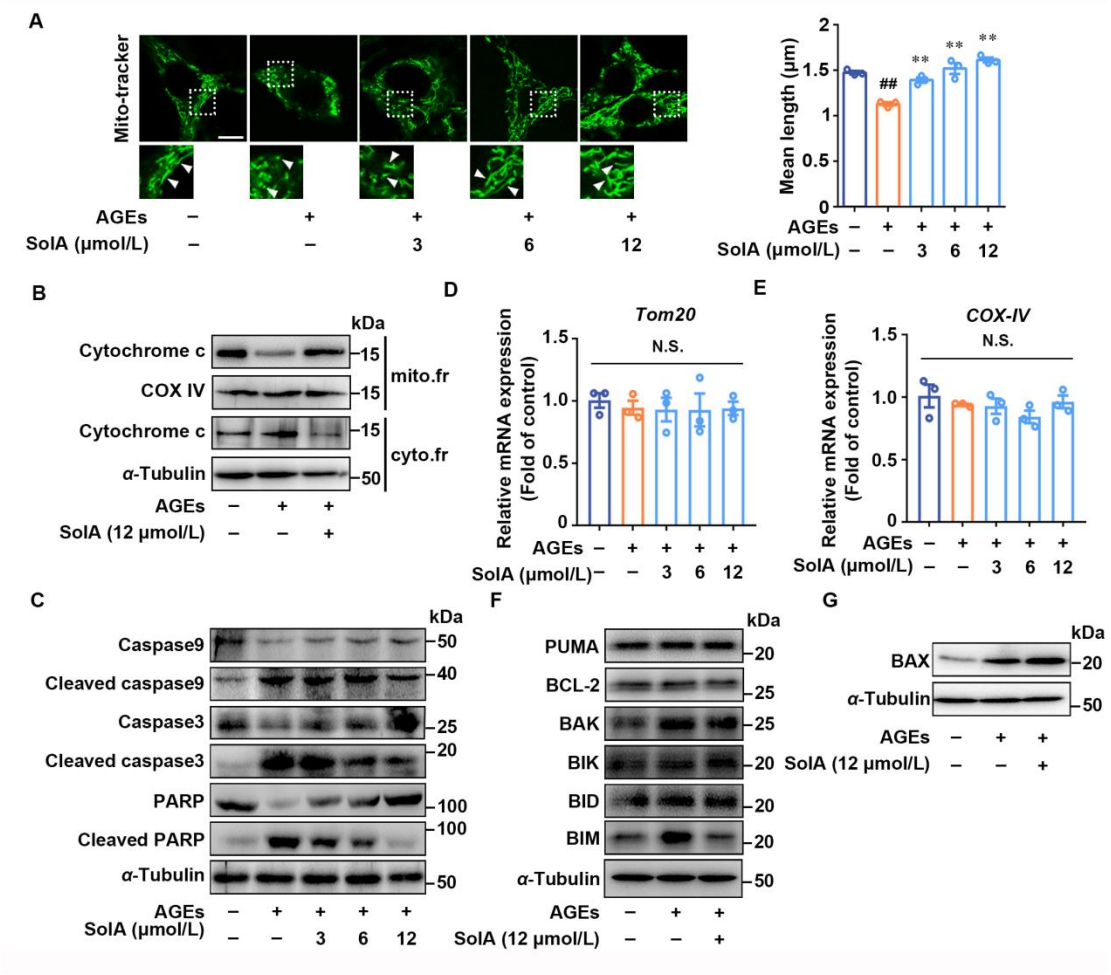


Figure S2 (Related to Fig. 2). SolA maintains mitochondrial homeostasis by promoting BIM degradation. (A) SolA improved AGEs-induced PC12 mitochondrial damage by Mito-tracker staining (scale bar: 10 μm, $n = 3$). (B) SolA had no effect on the mRNA expressions of *Tom20* ($n = 3$). (C) SolA had no effect on the mRNA expressions of *COX IV* ($n = 3$). (D) SolA (12 μmol/L) inhibited cytochrome *c* release from mitochondria into cytosol. (E) SolA inhibited caspase9, caspase3 and PARP cleavage. (F) SolA (12 μmol/L) significantly inhibited BIM expression rather than other mitochondrial proteins. (G) SolA had no effect on BAX expression. Data are expressed as the mean ± SEM. $^{\#\#}P < 0.01$ *vs.* control group, $^{**}P < 0.01$ *vs.* AGEs group.

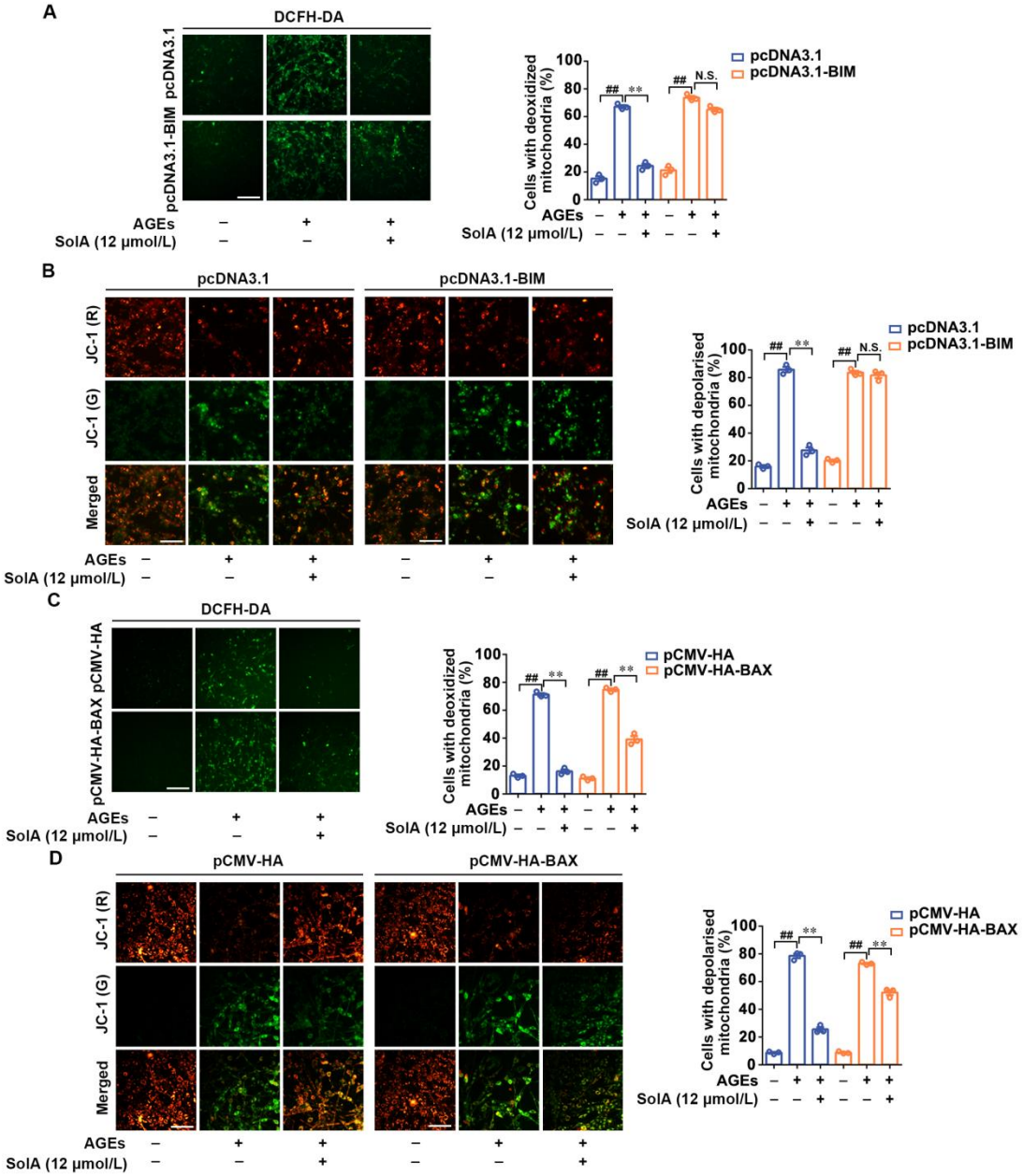


Figure S3 (Related to Fig. 2). SolA maintains mitochondrial homeostasis by promoting BIM degradation. (A) BIM overexpression reversed SolA (12 μmol/L)-mediated ROS decrease (scale bar: 200 μm, $n = 3$). (B) BIM overexpression reversed SolA (12 μmol/L)-mediated mitochondrial depolarization decrease by JC-1 staining (scale bar: 200 μm, $n = 3$). (C) BAX overexpression reversed SolA (12 μmol/L)-mediated ROS decrease (scale bar: 200 μm, $n = 3$). (D) BAX overexpression reversed SolA (12 μmol/L)-mediated mitochondrial depolarization decrease by JC-1 staining (scale bar: 200 μm, $n = 3$). Data are expressed as the mean ± SEM. $^{\#\#}P < 0.01$ *vs.* control group, $^{**}P < 0.01$ *vs.* AGEs group.

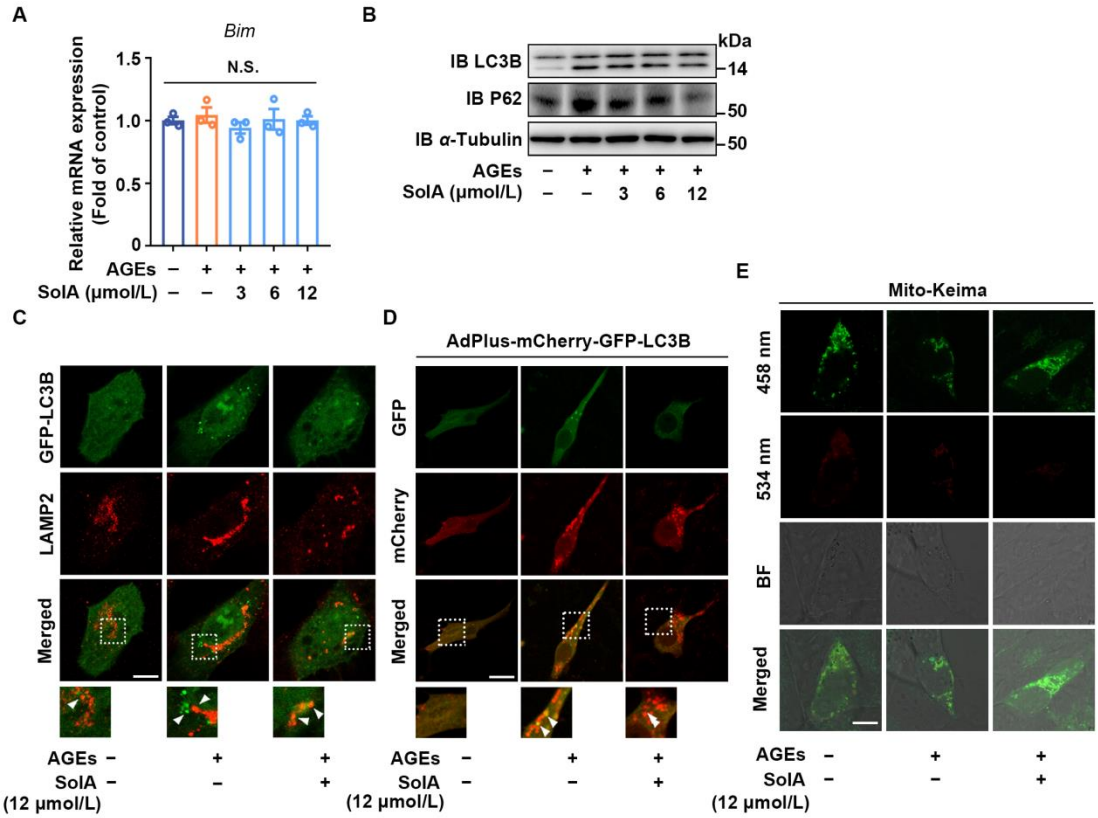


Figure S4 (Related to Fig. 2). SolA maintains mitochondrial homeostasis by promoting BIM degradation. (A) SolA did not regulate *Bim* mRNA expression. (B) SolA slightly decreased P62 and LC3B level (n = 3). (C) SolA (12 μmol/L) increased the co-localization of LC3B and lysosomes (scale bar: 10 μm). (D) SolA (12 μmol/L) reactivated autophagy flux by mCherry-eGFP-LC3B dual fluorescence system (scale bar: 20 μm). (E) SolA showed no obvious effect on the mitophagy in PC12 cells (scale bar: 10 μm). Data are expressed as the mean ± SEM. N.S. not significant.

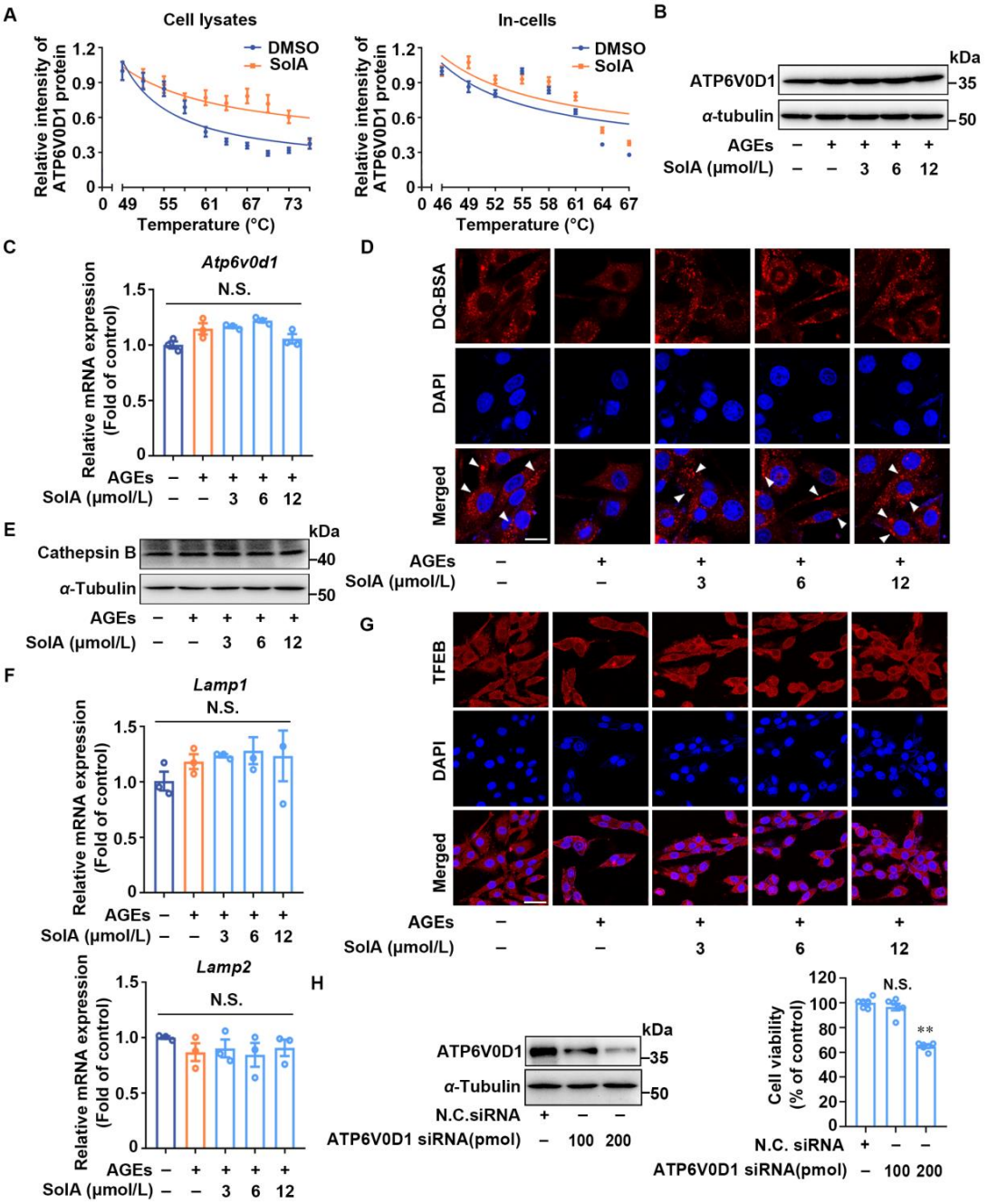


Figure S5 (Related to Fig. 3). Identification of V-ATPase subunit ATP6V0D1 as a cellular target of SolA. (A) SolA promoted ATP6V0D1 resistant to different temperature gradients (n = 3). (B) SolA had no effect on ATP6V0D1 protein expression. (C) SolA did not regulate *Atp6v0d1* mRNA expression in PC12 cells (n = 3). (D) SolA promoted lysosomal proteolytic activity in a concentration-dependent manner (scale bar: 20 μm). Arrows indicate brightly stained lysosomes. (E) SolA had no effect on cathepsin B expression. (F) SolA did not regulate the transcriptions of *Lamp1* and *Lamp2* (n = 3). (G) SolA showed no obvious effect on the nuclear translocation of TFEB in AGEs-induced PC12 cells (scale bar: 40 μm). (H) Excessive knockdown of ATP6V0D1 led to serious injury of neuronal cells (n = 6). Data are expressed as the mean ± SEM. $^{**}P < 0.01$ *vs.* AGEs group. N.S. not significant.

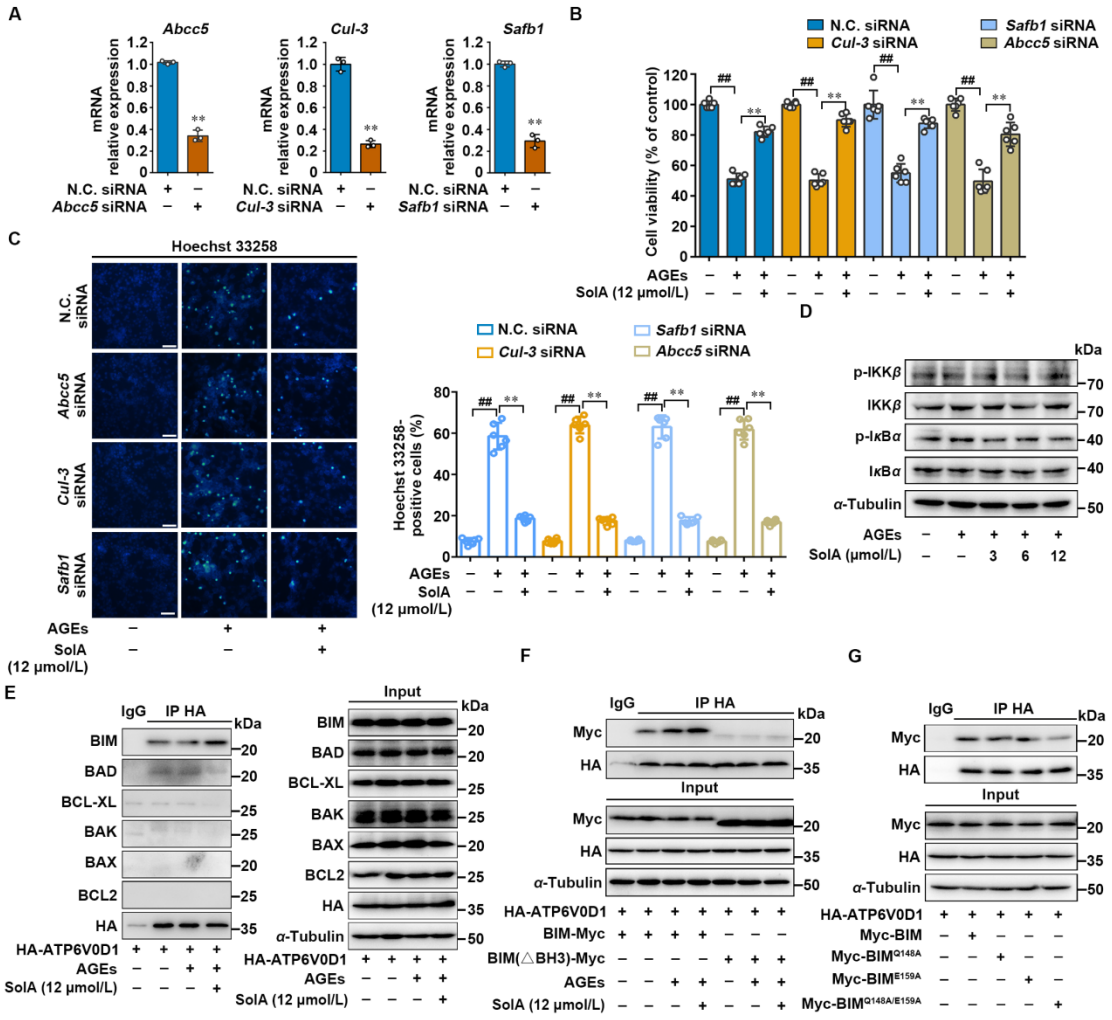


Figure S6 (Related to Fig. 4). SolA protects against cell death through lysosome-mitochondria crosstalk. (A) *Abcc5*, *Cul-3* and *Safb1* were knocked down by siRNA in PC12 cells ($n = 3$). $^{**}P < 0.01$ (Student's *t*-test). (B) *Abcc5*, *Cul-3* and *Safb1* deletion had no effect on SolA (12 μmol/L)-mediated PC12 cell viability by MTT ($n = 6$). (C) *Abcc5*, *Cul-3* and *Safb1* deletion had no effect on SolA (12 μmol/L)-mediated PC12 cell survival by Hoechst 33258 staining (scale bar: 100 μm, $n = 6$). (D) SolA (12 μmol/L) did not regulate IKK cascade in PC12 cells. (E) SolA (12 μmol/L) failed to induce BAD, BCL-XL, BAK, BAX and BCL-2 binding to ATP6V0D1 in AGEs-induced PC12 cells. (F) BH3-deletion BIM (ΔBH3-BIM) failed to bind with ATP6V0D1 in AGEs-induced PC12 cells. (G) Glu159 rather than Gln148 mediated BIM–ATP6V0D1 interaction. Data are expressed as the mean ± SEM. $^{\#\#}P < 0.01$ *vs.* control group, $^{**}P < 0.01$ *vs.* AGEs group.

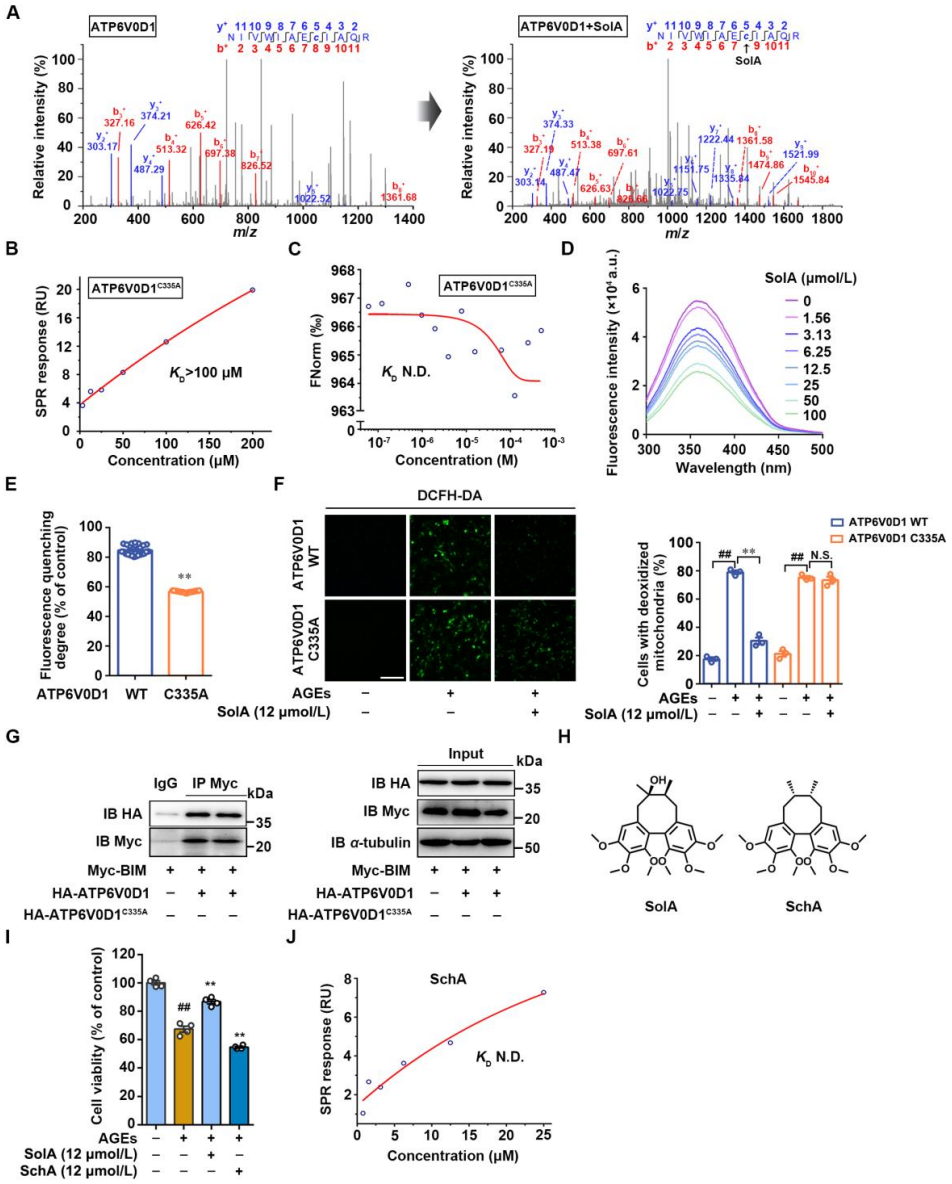


Figure S7 (Related to Fig. 5). Cys335 serves as a pharmacological allosteric site of ATP6V0D1. (A) Trypsin-digest LC–MS/MS analysis revealed Cys335 as a binding site of SolA. Recombinant ATP6V0D1 was incubated with (bottom) or without (top) SolA. (B) Binding affinity of SolA with ATP6V0D1^{C335A} was determined by SPR. (C) Binding affinity of SolA with ATP6V0D1^{C335A} was determined by MST. (D) Tryptophan fluorescence quenching assay for SolA-mediated ATP6V0D1^{C335A} conformational change. (E) Representative comparison of SolA-mediated ATP6V0D1^{WT} and ATP6V0D1^{C335A} conformational change on fluorescence quenching capacity (n = 119). (F) ATP6V0D1 C335A mutation reversed SolA (12 μmol/L)-mediated ROS decrease by DCFH-DA detection (scale bar: 200 μm, n = 3). (G) Cys335 mutation of ATP6V0D1 had no effect on the interaction of ATP6V0D1 and BIM. (H) Chemical structures of SolA and SchA. (I) SolA (12 μmol/L) rather than SchA (12 μmol/L) increased the cell viability in AGEs-induced PC12 cells (n = 4). (J) Binding affinity analysis of SchA with ATP6V0D1 determined by SPR. Data are expressed as the mean ± SEM. $^{\#\#}P < 0.01$ *vs.* control group, $^{**}P < 0.01$ *vs.* AGEs group. N.S. not significant.

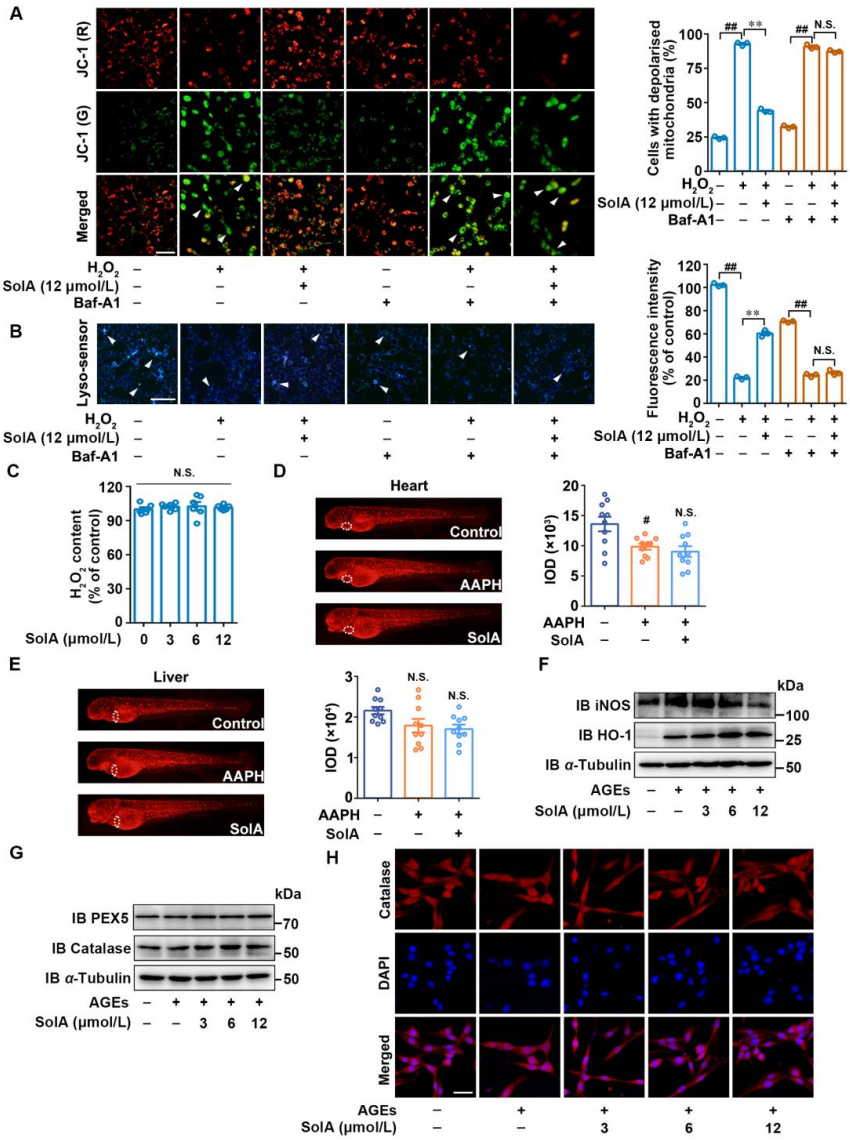


Figure S8 (Related to Fig. 6). ATP6V0D1 is necessary for SolA-mediated mitochondrial protection and lysosomal acidification. (A) Baf-A1 eliminated SolA-mediated mitochondrial depolarization by JC-1 staining in H_2O_2-induced PC12 cells (scale bar: 100 μm, $n = 3$). Arrows indicate the cells with depolarized mitochondria. (B) Baf-A1 reversed SolA-mediated lysosomal acidification by Lyso-sensor detection in H_2O_2-induced PC12 cells (scale bar: 100 μm, $n = 3$). Arrows indicate highly acidic lysosomes. (C) SolA did not directly react with H_2O_2 ($n = 6$). (D) SolA (6 μmol/L) failed to enhance mitochondrial activity by Mito-tracker Red CMXRos staining in 2,2′-azobis(2-methylpropionamidine) (AAPH)-induced zebrafish heart ($n = 10$). AAPH (6.25 mmol/L) was used to induce the oxidative stress state. (E) AAPH or SolA (6 μmol/L) had no effect on mitochondrial activity by Mito-tracker Red CMXRos staining in zebrafish liver ($n = 10$). (F) SolA moderately decreased iNOS level and slightly increased HO-1 level. (G) SolA did not regulate the protein levels of PEX5 and catalase. (H) SolA had no effect on the fluorescence intensity of catalase (scale bar: 40 μm). Data are expressed as the mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* control group, $^{**}P < 0.01$ *vs.* H_2O_2 group. N.S. not significant.