Supplementary Information

Small molecules enhance autophagy and reduce toxicity in Huntington's disease models

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Supplementary Methods

Yeast strains and media

BY4742 (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) and BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) were obtained from American Tissue Culture Collection (ATCC). RM11-1a (MATa leu2Δ ura3Δ) was a generous gift of B. Garvik (Fred Hutchison Cancer Research Center, USA). We thank Dr. Ian Roberts (National Collection of Yeast Cultures, UK) for kindly providing *S. paradoxus*, *S. bayanus* and *S. mikatae*. Rich media (YPD) is 2 % yeast extract, 2 % peptone and 2 % glucose. Complete synthetic media (CSM) is 6.7 g/L yeast nitrogen base (YNB), 0.05 % ammonium sulfate (AS) and 2 % glucose; 0.05 % urea is substituted for AS where appropriate.

Primary screen

Culturing of yeast as well as media formulation was done as previously described[1]. We screened the Chembridge Microformat library and a custom collection of bioactive compounds in duplicate. An overnight culture of BY4742 was appropriately diluted in rich media; 25 μL were dispensed into NUNC 384-well, clear-bottom, untreated, sterile plates (VWR, #62409-604) using the Microfill liquid handler (Biotek); compound from library stock plates was robotically pinned (Seiko Instruments) into assay plates; an additional 15 μL of media containing enough rapamycin (acquired by prescription) to yield a 50 nM final concentration in each well were dispensed into assay plates. Inoculated assay plates were grown without agitation on the bench top at ambient temperature conditions for 48-96 h and visually inspected for primary assay positives. Primary assay positives were ordered either from Chembridge Corporation (www.hit2lead.com) or from Biomol in 5 mg quantities and resuspended in dimethyl sulfoxide (DMSO).

Dose responses and selectivity profiling

SMIRs and SMERs were manually arrayed into plastic 384-well plates as two-fold dilution series. EC_{50} values were determined using GraphPad Prism v. 4.01 (GraphPad Software, Inc.). Yeast were dispensed into 384-well plates and compound was pinned into plates as described above, substituting synthetic media for rich media where appropriate. The following SMPs were used in modifier profiling at the listed concentrations: 555 nM cycloheximide (GR-310); 18.9 μM anisomycin (Biomol, #ST-102); 595 nM tunicamycin (Biomol, #CC-104); 29 μM and 14.5 μM menadione (Sigma-Aldrich, #M5625); 16.6 μM nocodazole (Biomol, T-101).

Characterisation, potency and selectivity of SMIRs and SMERs in yeast

Characterisation of SMIRs and SMERs in yeast: The 21 SMIRs (**1-21**) comprise 18 distinct structural classes; the 12 SMERs (**22-33**) comprise 11 structural classes. Interestingly, two SMIRs are known bioactive compounds: D609 (**1**) is a potassium xanthate derivative and a potential glutathione mimetic[2]; LY-83583 (**2**) has been historically described as a guanylate cyclase inhibitor[3], and more recently, as a modulator of the yeast mitochondrial GTPase, Guf1p[4]. We determined the half-maximal effective concentration (EC_{50}) of suppression and enhancement of the cytostatic effects of rapamycin by each SMIR and SMER, respectively (**Supplementary Fig. 1b** online). The EC_{50} of suppression spans two orders of magnitude, from >50 μM to as low as 0.37 μM. Four SMIRs displayed sub-micromolar suppression of rapamycin (described, where appropriate, by their core heterocycle): D609; SMIR28 (**16**), a thiourea; SMIR30 (**19**), a dihydroquinoline; SMIR32 (**20**), a quinazoline. The EC_{50} of enhancement spanned a smaller range from 50 μM to 1.4 μM, with SMER17 (**26**), a piperazine, being the strongest enhancer. The

overall suppression and enhancement profile was neither strain- nor species-specific, as all tested SMIRs and SMERs exhibited comparable activity in another *S. cerevisiae* strain (RM11-1a), as well as in laboratory strains of *Saccharomyces bayanus*, *Saccharomyces mikatae*, and *Saccharomyces paradoxus* (data not shown). Dose responses were performed in rich media (YPD), and in almost every instance, suppression or enhancement is insensitive to changes in carbon or nitrogen source in the culture media, with a few exceptions (**Supplementary Fig. 1b** online).

Potency and selectivity of the small-molecule modifiers of the cytostatic effects of rapamycin: Most modifiers displayed modest activity (10 μM – 50 μM) (**Supplementary Fig. 1b** online), which may be explained either by weak to modest small-molecule modulation of TOR-relevant targets, or by modest to strong small-molecule modulation of TOR-irrelevant targets, e.g., xenobiotic-response genes. In order to distinguish between these two possibilities, we assessed the selectivity of each small-molecule modifier against small-molecule perturbagens (SMPs) other than rapamycin, including ones that either target processes related or unrelated to those affected by rapamycin. Our goal was to eliminate SMIRs and SMERs that exhibit a lack of selectivity towards the cellular actions of other SMPs; however, a useful by-product of this analysis is the discovery of potentially selective small-molecule modifiers of the growth inhibition induced by SMPs other than rapamycin. We included the protein-synthesis inhibitors cycloheximide (CHX) and anisomycin[5]; the microtubule depolymerizer nocodazole; the protein-glycosylation inhibitor tunicamycin; the oxidant menadione.

The most non-selective SMIRs are 19a (**11**) and 19b (**12**), two structurally related thiophenes; these compounds suppressed 6 of 6 assayed compounds (**Supplementary Fig. 1c** online) but enhanced the ergosterol-biosynthesis inhibitors

ketoconazole and flutrimazole (data not shown), which suggests that SMIR19a and SMIR19b promote xenobiotic efflux by altering membrane permeability. No other SMIRs and none of the SMERs suppressed the antiproliferative effects of menadione, an inducer of oxidative stress, which is a pathway not directly controlled by TOR. Interestingly, four SMIRs (7, 15, 16 and 18) enhanced the antiproliferative effects of anisomycin, while seven of twelve SMERs (3, 6, 10, 14, 20, 22 and 23) suppressed the antiproliferative effects of both anisomycin and CHX (**Supplementary Fig. 1c,d** online). We hypothesize that the subset of SMIRs that enhances the growth inhibition induced by protein-synthesis inhibitors does so by modulating regulatory targets upstream of ribosomes. This hypothesis is consistent with our observation that neither CHX nor anisomycin, which both inhibit protein synthesis at the ribosome, is a SMIR, i.e., suppresses the cytostatic effects of rapamycin in yeast at sub-lethal concentrations (**Supplementary Fig. 1e,f** online).

Plasmid constructs

HD gene exon 1 fragment with 74 polyQ repeats in pEGFP-C1 (Clontech) (EGFP-HDQ74) construct was characterized previously[6]. EGFP-LC3 (kind gift from T. Yoshimori), Atg5 and HA-Atg12 (kind gifts from N. Mizushima) constructs were obtained.

Mammalian cell culture and transfection

African green monkey kidney cells (COS-7), human cervical carcinoma cells (HeLa), stable HeLa cells expressing EGFP-LC3[7] (kind gift from A.M. Tolkovsky), and wild-type Atg5 ($Atg5^{+/+}$) and Atg5-deficient ($Atg5^{-/-}$) mouse embryonic fibroblasts[8] (MEFs) (kind gift from N. Mizushima) were maintained in DMEM supplemented

with 10 % FBS, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine (Sigma) at 37°C, 5 % CO_2. HeLa cells stably expressing Ub^{G76V}-GFP reporter[9] (kind gift from N. P. Dantuma) were grown in the same media used for COS-7 cells supplemented with 0.5 mg/ml G418.

Inducible PC12 stable cell line expressing HA-tagged A53T α-synuclein mutant, previously characterized[10], was maintained at 75 μg/ml hygromycin B (Calbiochem) in DMEM with 10 % horse serum, 5 % FBS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine and 100 μg/ml G418 (GIBCO) at 37°C, 10 % CO_2.

Cells were transfected with the constructs for 4 h using Lipofectamine or Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol, fixed with 4 % paraformaldehyde (Sigma) after 24 h or 48 h (EGFP-HDQ74), or 24 h (EGFP-LC3) post-transfection and mounted in citifluor (Citifluor Ltd.) containing 4',6-diamidino-2-phenylindole (DAPI; 3 μg/ml; Sigma-Aldrich).

Microscopy

Transfected cells were analysed by Nikon Eclipse E600 fluorescence microscope (plan-apo 60x/1.4 oil immersion lens at room temperature) (Nikon, Inc.). Images of EGFP-LC3 HeLa stable cells were acquired on a Zeiss LSM510 META confocal microscope (63x 1.4NA plan-apochromat oil immersion lens) at room temperature using Zeiss LSM510 v3.2 software (Carl Zeiss, Inc.), and Adobe Photoshop 6.0 (Adobe Systems, Inc.) was used for subsequent image processing.

Statistical Analysis for counting aggregation, cell death and EGFP-LC3 vesicles

We have counted approximately 200 EGFP-positive cells per sample for the proportion of EGFP-positive cells with green fluorescent EGFP-HDQ74 aggregates, as described previously[6]. If an EGFP-positive cell has one or many aggregates, the aggregate score is 'one'. If an EGFP-positive cell does not have any aggregate, the aggregate score is 'zero'. For example, the statement 'SMERs significantly reduced EGFP-HDQ74 aggregates' means that the SMERs significantly reduced the proportion of EGFP-positive cells with EGFP-HDQ74 aggregates. Nuclei were stained with DAPI and those showing apoptotic morphology (fragmentation or pyknosis) were considered abnormal. These criteria are specific for cell death, which highly correlate with propidium iodide staining in live cells[11]. Only EGFP-positive cells were counted so that we count only the transfected cells. Analysis was performed with the observer blinded to the identity of slides. Slides were coded and the code was broken after completion of experiment. All experiments were done in triplicate at least twice.

Similar analysis in triplicate was done for counting the proportion of EGFP-positive cells with EGFP-LC3 vesicles[12]. Approximately 200 EGFP-positive cells were counted for the proportions of EGFP-positive cells with >5 LC3-positive vesicles. We considered an EGFP-positive cell as having a score of 'zero' if there were 5 or fewer vesicles (as cells have basal levels of autophagy) and cells scored 'one' if they had >5 LC3-positive vesicles.

Pooled estimates for the changes in aggregate formation, cell death or EGFP-LC3 vesicles, resulting from perturbations assessed in multiple experiments, were calculated as odds ratios with 95% confidence intervals [Odds ratio of aggregation = (percentage of cells expressing construct with aggregates in perturbation

conditions/percentage of cells expressing construct without aggregates in perturbation conditions)/(percentage of cells expressing construct with aggregates in control conditions/percentage of cells expressing construct without aggregates in control conditions)]. Odds ratios were considered to be the most appropriate summary statistic for reporting multiple independent replicate experiments of this type, because the percentage of cells with aggregates under specified conditions can vary between experiments on different days, whereas the relative change in the proportion of cells with aggregates induced by an experimental perturbation is expected to be more consistent. We have used this method frequently in the past to allow analysis of data from multiple independent experiments[11-13]. Odds ratios and p values were determined by unconditional logistical regression analysis, using the general log-linear analysis option of SPSS 9 software (SPSS, Chicago). When EGFP-LC3 vesicle counts were expressed as a percentage of cells, the error bars denote standard error of mean. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; NS, Non-significant.

Statistical Analysis for densitometry on Western blots

Densitometry analysis on the immunoblots was done by Scion Image Beta 4.02 software (Scion Corporation) from three independent experiments (n=3). Significance for the clearance of mutant proteins was determined by factorial ANOVA test using STATVIEW software, version 4.53 (Abacus Concepts). The control condition was set to 100 % and the error bars denote standard error of mean. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; NS, Non-significant.

Toxicity testing: SMERs have no overt toxicity in cells and flies at concentrations used

Mammalian cells: COS-7 cells were treated with 47 μm SMER10, 43 μm SMER18 and 47 μm SMER 28 or DMSO as the carrier for 24 h. Cells were then analysed for apoptotic nuclear morphology. The mean percentage of living cells (+/– standard deviation) from triplicate samples where 10,000 cells were scored by FACS were: DMSO: 99.5 (0.04); SMER10: 99.3 (0.15); SMER18: 98.8 (0.33); SMER28: 97.8 (0.21).

Drosophila: Three virgins of the genotype *y w; gmr-httNterm(1-171)Q120* (*gmrQ120)* were crossed with two isogenised w^{1118} males at 25^0C. The crosses were set up in duplicates in vials containing instant fly food with DMSO or each SMER at different concentrations (100 μM, 200 μM, 500 μM). All the crosses were set up at the same time.

After one week we counted the number of pupae obtained from each vial, looking at the effect of different SMER on fly development. We compared numbers of pupae obtained with SMER and DMSO at different concentrations. For SMERs 10 and 28, we didn't observe any toxic effects at 100 μM and 200 μM. Indeed, pupae hatched and the eclosed flies appeared normal. In particular, the numbers of pupae obtained with SMER 10 and 28 at 100 μM were similar to what was obtained with DMSO (at 100 μM: 35 pupae for SMER10, 47 for SMER28 versus 41 of DMSO); at 200 μM we observed an increase in the number of pupae treated with the SMERs compared to DMSO (at 200 μM: 46 for SMER10, 42 for SMER28 versus 14 of DMSO). Treatment with 500 μM of SMER, as well as the increased amounts of DMSO required to deliver the SMER, had toxic effects, as there were dramatically

reduced numbers of pupae (18 for SMER10, 12 for SMER28, 15 for DMSO) and no flies eclosed.

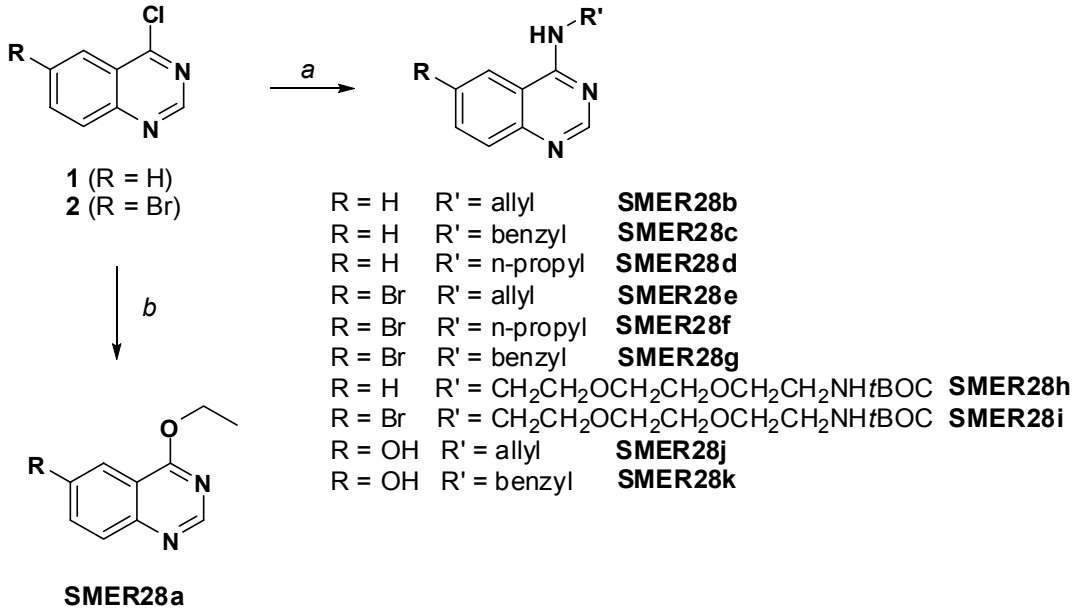
Due to differences in SMER solubilities, the dosage of DMSO used to solubilise SMER18 was slightly different to the dosage of DMSO used for SMER10 and 28. We tested different concentrations of SMER18 and we compared its effects to the appropriate DMSO control. Results obtained with SMER18 were similar to those obtained using SMER10 and SMER28. We didn't observe any toxic effect at 100 μM and 200 μM and we observed a decrease in the number of pupae at 500 μM. The adult flies at 100 μM and 200 μM looked normal. In particular SMER18 at 100 μM gave 99 pupae versus 66 of DMSO; at 200 μM gave 99 pupae versus 109 of DMSO; at 500 μM gave 26 pupae versus 65 of DMSO.

To test the effect of different SMERs on rhabdomere neurodegeneration, we scored two day old male progeny of the above cross using the sensitive pseudopupil technique. We collected progeny 0-4 h post-eclosion and fed them with food of the same composition of DMSO or SMER that they were reared on. We scored 8-10 males (120-150 ommatidia) from each SMER at 100 μM and 200 μM and we compared the frequency of rhabdomeres scored using SMERs with DMSO control. Even using the pseudopupil technique, we could confirm that SMERs 10, 18 and 28 had no toxic effects at 100 μM and 200 μM, compared to DMSO-treated flies.

Synthesis

General Procedures. Starting materials and reagents were purchased from commercial suppliers and used without further purification. All products were determined to be >95% pure based upon HPLC analysis with UV detection and tandem mass spectral detection. Purification by flash chromatography was performed

using E. Merck silica gel 60 (230-400 mesh). Analytical thin layer chromatography was performed on E. Merck 0.25 mm silica gel 60-F plates. Visualization was accomplished with UV light (254 nm). Analytical LC/MS chromatography was performed on Waters Alliance 2690 HPLC system using a Waters Symmetry C18 column (3.5 μm, 4.6 × 100 mm) with a gradient of 20-80% CH_3CN in water with constant 0.1% formic acid, with UV detection at 214 and 280 nm and a Micromass LCZ (ESI) spectrometer. Melting points are uncorrected. ^1H NMR spectra were recorded on a Varian 500 MHz spectrometer and are reported in ppm and referenced to residual protons in the NMR solvent. Data are reported as shift, splitting (s = singlet, d = doublet, t = triplet, m = multiplet; br = broad), coupling constant in Hz; integration. ^{13}C NMR spectra were recorded at 125 MHz on a Varian spectrometer, ^{13}C shifts are reported in ppm and referenced to carbon resonances in the NMR solvent. Microwave assisted reactions were performed at a power level of 60 W using an Emrys Optimizer (Personal Chemistry AB). High resolution mass spectroscopy was performed by the Harvard University Mass Spectrometry facility on a JEOL AX-505H instrument with EI ionization.



key: *a*) amine, microwave (60 W) , 100 °C; *b*) EtOH, 50 °C.

SMER28a. Compound **2**[15] (170 mg, 0.68 mmol) was dissolved in 20 mL of refluxing EtOH and stirred overnight at 50 °C. The reaction mixture was concentrated, dissolved in CH_2Cl_2 and rinsed with water. After drying (Na_2SO_4), the CH_2Cl_2 was filtered, concentrated and chromatographed in 1:1 EtOAc/hexane to yield 70 mg of **SMER28a** (39%): mp 115-116 ºC; ^{1}H NMR (DMSO-d_6) δ 8.82 (d, *J*=1.0 Hz, 1H), 8.26 (d, *J*=2.2 Hz, 1H), 8.08 (ddd, *J*=1, 2.2, 8.9 Hz, 1H), 7.86 (d, *J*=8.9 Hz, 1H), 4.60 (q, *J*=7.1 Hz, 2H), 1.45 (t, *J*=7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6) δ 164.9, 154.5, 148.9, 136.9, 129.6, 125.1, 119.7, 116.9, 63.1, 13.9; HRMS (m/z): $[M+H]^+$ calcd for $C_{10}H_9BrN_2O$, 252.9976; found, 252.9985.

SMER28b. To a small microwave vial was added 4-chloroquinazoline **1**[15] (50 mg, 0.30 mmol) and 1 mL of allylamine. The mixture was heated at 100 °C for 5 min in a microwave reactor, concentrated, dissolved in CH_2Cl_2 and rinsed with water. The CH_2Cl_2 layer was dried (Na_2SO_4), filtered and concentrated to give 29 mg of

SMER28b as a light yellow powder (94%): mp 130-133 ºC; ^{1}H NMR (DMSO-d_6) δ 8.45 (s, 1H), 8.25 (d, *J*=8.3 Hz, 1H), 7.75 (t, *J*=8.3 Hz, 1H), 7.67 (d, *J*=8.3 Hz, 1H), 7.50 (t, *J*=8.3 Hz, 1H), 5.98 (m, 1H), 5.19 (dd, *J*=17.2, 1.7 Hz, 1H), 5.09 (dd, *J*=10.3, 1.7 Hz, 1H), 4.19 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 159.9, 155.8, 149.8, 135.8, 133.1, 128.9, 126.3, 123.3, 116.1, 115.6, 43.3; HRMS (m/z): $[M+H]^+$ calcd for $C_{11}H_1N_3$, 186.1031; found, 186.1040.

SMER28c. To a small microwave vial was added 4-chloroquinazoline **1**[15] (50 mg, 0.30 mmol) and 1 mL of benzylamine. The mixture was heated at 100 °C for 5 min in a microwave reactor, concentrated, dissolved in ether and washed with 1N HCl until the water layer was acidic. The ether layer was washed with brine, dried (Na_2SO_4), filtered and concentrated to give 62 mg of **SMER28c** (87%): ^{1}H NMR (DMSO-d_6) δ 8.85 (t, *J*=5.6 Hz, 1H), 8.44 (s, 1H), 8.30 (d, *J*=8.1 Hz, 1H), 7.78 (t, *J*=7.3 Hz, 1H), 7.69 (d, *J*=8.1 Hz, 1H), 7.53 (t, *J*=7.3 Hz, 1H), 7.33 (m, 4H), 7.23 (t, *J*=7.2 Hz, 1H), 4.80 (d, *J*=5.9 Hz, 2H); ^{13}C NMR (DMSO-d_6) δ 160.1, 155.8, 149.9, 140.1, 133.3, 128.9, 128.2, 127.9, 127.4, 126.4, 123.3, 115.36, 44.2; HRMS (m/z): $[M+H]^+$ calcd for $C_{15}H_{13}N_3$, 236.1187; found, 236.1198

SMER28d. To a small microwave vial was added 4-chloroquinazoline **1**[15] (50 mg, 0.30 mmol) and 1 mL of *n*-propylamine. The mixture was heated at 100 °C for 5 min in a microwave reactor, concentrated, dissolved in CH_2Cl_2 and rinsed with water. The CH_2Cl_2 layer was dried (Na_2SO_4), filtered and concentrated to give 46 mg of **SMER28d** as a light yellow powder (82%): ^{1}H NMR (DMSO-d_6) δ 8.44 (s, 1H), 8.28 (t, *J*=5.5 Hz, 1H), 8.23 (d, *J*=8.2 Hz, 1H), 7.74 (t, *J*=7.0 Hz, 1H), 7.66 (d, *J*=8.2 Hz, 1H), 7.49 (t, *J*=8.2 Hz, 1H), 3.48 (m, 2H), 1.65 (sextet, *J*=7.4 Hz, 2H), 0.93 (t, *J*=7.4 Hz, 3H); ^{13}C NMR (DMSO-d_6) δ 159.3, 155.1, 148.9, 132.3, 127.4, 125.4,

122.6, 114.9, 42.2, 21.8, 11.4; HRMS (m/z): $[M+H]^+$ calcd for $C_{11}H_{13}N_3$, 188.1188; found, 188.1185.

SMER28e. To a small microwave vial was added 4-chloroquinazoline **2**[15] (50 mg, 0.21 mmol) and 1 mL of allylamine. The mixture was heated at 100 °C for 5 min in a microwave reactor, concentrated, partitioned between ether and 1N HCl. The ether layer was dried (Na_2SO_4), filtered and concentrated to give 50 mg of **SMER28e** as a light yellow powder (92%): ^{1}H NMR (DMSO-d_6) δ 8.59 (d, *J*=2.1 Hz, 1H), 8.48 (s, 1H), 7.89 (dd, *J*=8.8, 2.1 Hz, 1H), 7.63 (d, *J*=8.8 Hz, 1H), 5.97 (m, 1H), 5.21 (dd, *J*=17.2, 1.7 Hz, 1H), 5.11 (dd, *J*=10.3, 1.7 Hz, 1H), 4.17 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 158.2, 155.3, 147.9, 135.3, 134.5, 129.6, 125.1, 117.7, 116.1, 115.5, 42.6; HRMS (m/z): $[M+H]^+$ calcd for $C_{11}H_{10}BrN_3$, 264.0136; found, 264.0134.

SMER28f. To a small microwave vial was added 4-chloroquinazoline **2**[15] (50 mg, 0.21 mmol) and 1 mL of *n*-propylamine. The mixture was heated at 100 °C for 5 min in a microwave reactor, concentrated, partitioned between ether and 1N HCl. The ether layer was dried (Na_2SO_4), filtered and concentrated to give 46 mg of **SMER28f** as a light yellow powder (84%): mp 201-204 ºC; ^{1}H NMR (DMSO-d_6) δ 8.56 (d, *J*=2.2 Hz, 1H), 8.47 (s, 1H), 8.38 (t, *J*=5.1 Hz, 1H), 7.87 (dd, *J*=8.8, 2.2 Hz, 1H), 7.61 (d, *J*=8.8 Hz, 1H), 3.47 (m, 2H), 1.64 (sextet, *J*=7.4, 2H), 0.93 (t, *J*=7.4, 3H); ^{13}C NMR (DMSO-d_6) δ 158.4, 155.5, 147.9, 135.3, 129.7, 125.1, 117.6, 116.2, 42.3, 21.5, 11.4; HRMS (m/z): $[M+H]^+$ calcd for $C_{11}H_{12}BrN_3$, 266.0293; found, 266.0284.

SMER28g. To a small microwave vial was added 4-chloroquinazoline **2**[15] (50 mg, 0.21 mmol) and 1 mL of benzylamine. The mixture was heated at 100 °C for 5 min in a microwave reactor, concentrated, dissolved in ether and washed with 1N HCl until the water layer was acidic. The ether layer was washed with brine, dried

(Na_2SO_4), filtered and concentrated to give 61 mg of **SMER28g** as a light yellow powder (95%): ^{1}H NMR (DMSO-d_6) δ 8.94 (t, *J*=5.4 Hz, 1H), 8.62 (d, *J*=2.0 Hz, 1H), 8.49 (s, 1H), 7.90 (dd, *J*=8.8, 2.0 Hz, 1H), 7.64 (d, *J*=8.8 Hz, 1h), 7.34 (m, 5H), 4.70 (d, *J*=5.4 Hz, 2H); ^{13}C NMR (DMSO-d_6) δ 158.3, 155.3, 147.9, 138.9, 135.4, 129.7, 127.2, 126.7, 125.1, 117.8, 116.1, 94.1, 43.5; HRMS (m/z): $[M+H]^+$ calcd for $C_{15}H_{12}BrN_3$, 314.0293; found, 314.0281.

SMER28h. To a small microwave vial was added 4-chloroquinazoline **1**[15] (50 mg, 0.30 mmol), 150 mg (0.61 mmol) of *N*-tBOC-2,2'-(ethylenedioxy)bis(ethylamine)[2] and 1 mL of isopropanol. The mixture was heated at 100 °C for 5 min in a microwave reactor before concentration and chromatography (5% MeOH in EtOAc) yielded 94 mg of **SMER28h** (82%): ^{1}H NMR (DMSO-d_6) δ 8.46 (s, 1H), 8.32 (t, *J*=5.2 Hz, 1H), 8.24 (d, *J*=8.2, 1H), 7.75 (t, *J*=8.2 Hz, 1H), 7.67 (d, *J*=8.2 Hz, 1H), 7.50 (t, *J*=7.2 Hz, 1H), 7.36 (s, 1H), 6.74 (t, *J*=5.2 Hz, 1H), 3.70 (m, 2H), 3.65 (m, 2H), 3.54 (m, 2H), 3.50 (m, 2H), 3.35 (m, 2H), 3.04 (m, 2H), 1.35 (s, 9H); ^{13}C NMR (DMSO-d_6) δ 159.3, 155.5, 154.9, 149.0, 132.4, 127.4, 125.5, 122.6, 114.9, 77.5, 69.6, 69.4, 69.1, 68.3, 40.3, 39.6, 28.1; HRMS (m/z): $[M+H]^+$ calcd for $C_{19}H_{28}N_4O_4$, 377.2189; found, 377.2190.

SMER28i. To a small microwave vial was added 4-chloroquinazoline **2**[15] (50 mg, 0.30 mmol), 127 mg (0.51 mmol) of *N*-tBOC-2,2'-(ethylenedioxy)bis(ethylamine)[2] and 1 mL of isopropanol. The mixture was heated at 100 °C for 5 min in a microwave reactor before concentration and chromatography (5%MeOH in EtOAc) yielded 82 mg of **SMER28h** (88%): mp 104-106 ºC; ^{1}H NMR (DMSO-d_6) δ 8.56 (d, *J*=2.0 Hz, 1H), 8.48 (s, 1H), 4.45 (t, *J*=5.2 Hz, 1h), 7.86 (dd, *J*=8.9, 2.0 Hz, 1H), 7.61 (d, *J*=8.9 Hz, 1H), 6.73 (t, *J*=5.2 Hz, 1H), 3.67 (m, 2H), 3.64 (m, 2H), 3.54 (m, 2H), 3.50 (m, 2H), 3.36 (m, 2H), 3.04 (m, 2H), 1.35 (s, 9H); ^{13}C

NMR (DMSO-d_6) δ 159.3, 156.2, 156.1, 148.7, 136.2, 130.5, 125.9, 118.5, 116.9, 78.2, 70.3, 70.1, 69.9, 68.9, 41.2, 40.3, 28.9; HRMS (m/z): $[M+H]^+$ calcd for $C_{19}H_{27}BrN_4O_4$, 455.1294; found, 455.1278.

SMER28j. To a small microwave vial was added 4-chloroquinazoline **3**[17] (100 mg, 0.45 mmol) and 1 mL of allylamine. The mixture was heated at 100 °C for 5 min in a microwave reactor, concentrated, and chromatographed (5% MeOH in CH_2Cl_2) to give 34 mg of **SMER28j** (38%): ^{1}H NMR (DMSO-d_6) δ 9.89 (s, 1H), 8.30 (s, 1H), 8.14 (t, *J*=5.4 Hz, 1H), 7.55 (d, *J*=8.9 Hz, 1H), 7.47 (d, *J*=2.5 Hz, 1H), 7.31 (dd, *J*=8.9, 2.5 Hz, 1H), 5.98 (m, 1H), 5.17 (dd, *J*=17.2, 1.7 Hz, 1H), 5.08 (dd, *J*=10.3, 1.7 Hz, 1H), 4.15 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 158.3, 154.9, 152.1, 142.9, 135.3, 128.7, 123.4, 115.8, 115.1, 104.8, 42.5; HRMS (m/z): $[M+H]^+$ calcd for $C_{11}H_{11}N_3O$, 202.0980; found, 202.0983.

SMER28k. To a small microwave vial was added 4-chloroquinazoline **3**[17] (100 mg, 0.45 mmol) and 1 mL of benzylamine. The mixture was heated at 100 °C for 5 min in a microwave reactor. The excess benzylamine was removed with heating under a high vacuum before chromatography (5% MeOH in CH_2Cl_2) to give 83 mg of **SMER28k** (74%): mp 259 ºC (dec.); ^{1}H NMR (DMSO-d_6) δ 9.90 (s, 1H), 8.49 (t, *J*=5.8 Hz, 1H), 8.28 (s, 1H), 7.57 (d, *J*=8.0 Hz, 1H), 7.51 (d, *J*=1.6 Hz, 1H), 7.31 (m, 5H), 7.21 (t, *J*=8.0, 1H), 4.75 (d, *J*=5.5 Hz, 2H); ^{13}C NMR (DMSO-d_6) δ 158.4, 155.0, 152.1, 143.2, 139.7, 128.9, 128.1, 127.0, 126.5, 123.4, 115.8, 104.8, 43.4; HRMS (m/z): $[M]^+$ calcd for $C_{15}H_{13}N_3O$, 252.1137; found, 252.1131.

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Supplementary Figure Legends

Supplementary Figure 1. Results of a small-molecule screen for suppressors (SMIRs) and enhancers (SMERs) of the cytostatic effects of rapamycin in yeast, and characterisation, potency and selectivity of the identified SMIRs and SMERs.

(**a**) Of 50,729 compounds screened in duplicate in yeast BY4742 strain, 52 (0.001 %) suppressors and 20 (0.0004 %) enhancers were initially identified, of which 21 suppressors and 12 enhancers retested positively. (In cases where multiple structural analogs scored as primary assay positives, a single representative was chosen; exceptions are compounds with a lower-case letter in their name, e.g. SMIR19a). 427 candidate enhancers were subsequently found to be growth-inhibitory as single agents, and were eliminated from further consideration. Library compounds were assessed at approximately 75 μM. The exact concentration varies depending on the molecular weight of each compound.

(**b**) Table summarizing EC_{50} values (listed in descending order of potency) of 21 suppressors of rapamycin (SMIRs) (shown in green) and 12 enhancers of rapamycin (SMERs) (shown in red). Concentrations are listed in micromolar (μM). The EC_{50} of suppression was determined in 50 nM rapamycin; the EC_{50} of enhancement was determined in 20 nM rapamycin. EC_{50} values of asterisked compounds were determined in synthetic media; all other EC_{50} values were determined in rich media.

(**c,d**) Potency and selectivity of 33 small-molecule modifiers of the cytostatic effects of rapamycin (rows) against a panel of 6 assay compounds (columns). Two-dimensional (2D-) heatmaps display negative log-transformed (green) and positive log-transformed (red) EC_{50} values derived from averaged duplicate OD_{600} absorbance

measurements of a 2-fold dilution series of SMIRs (**c**) and of SMERs (**d**) treated with either 50 nM (used in **c**) and 20 nM (used in **d**) rapamycin or 555 nM cycloheximide (CHX) or 18.9 μM anisomycin or 16.6 μM nocodazole or 595 nM tunicamycin or 29 μM (used in **c**) and 14.5 μM (used in **d**) menadione. Black indicates no interaction between small-molecule modifiers and assay compounds; intense green corresponds to low half-maximal suppression; intense red corresponds to low half-maximal enhancement.

(**e,f**) Dose-response curves correspond to 2-fold dilutions of either anisomycin (data shown in **e**) or CHX (data shown in **f**) in the presence of 25 nM rapamycin (filled shapes) or vehicle (unfilled shapes).

Supplementary Figure 2. Screen for the autophagy-inhibitory SMIRs and the autophagy-inducing SMERs in mammalian cell line.

(**a,b**) A stable inducible PC12 cell line expressing A53T α-synuclein mutant was induced with doxycycline for 48 h, and expression of the transgene was switched off for 24 h, with DMSO (control), or 1:400 dilution of 5 mg/ml SMIRs 1, 2, 7, 8b, 11, 12, 14-18, 19a, 19b, 20-23, 28, 29a, 29b, 30, 31, added in the switch-off period. The levels of A53T α-synuclein (α-syn) was analysed by immunoblotting with antibody against HA (**a**) and densitometry analysis relative to actin (**b**).

(**c,d**) A stable inducible PC12 cell line expressing A53T α-synuclein mutant was induced with doxycycline for 48 h, and expression of the transgene was switched off for 24 h, with DMSO (control), or 1:400 dilution of 5 mg/ml SMERs 1-3, 6, 9-11, 13, 14, 16-24, 26, 28, added in the switch-off period. The levels of A53T α-synuclein (α-syn) was analysed by immunoblotting with antibody against HA (**c**) and densitometry analysis relative to actin (**d**).

Supplementary Figure 3. The effect of SMERs 10, 18 and 28 on mTOR activity, Beclin-1/Atg6, Atg5, Atg7, Atg12, Atg5-Atg12 conjugation and proteasome activity.

(**a**) Wild-type ($Atg5^{+/+}$) and knock-out ($Atg5^{-/-}$) Atg5 mouse embryonic fibroblasts were transfected with EGFP-HDQ74 construct for 4 h and fixed at 48 h post-transfection. The percentage of EGFP-positive cells with EGFP-HDQ74 aggregates were assessed and expressed as odds ratio. The control (EGFP-HDQ74 aggregation in $Atg5^{+/+}$ cells) was taken as 1. $p<0.0001$.

(**b,c**) COS-7 cells treated with DMSO (control), 47 μM SMER10, 43 μM SMER18, 47 μM SMER28 or 0.2 μM rapamycin (rap) for 24 h, were analysed for mTOR activity by immunoblotting for levels of phospho- and total p70S6K (**b**) and 4E-BP1 (**c**). Note that 4E-BP1 runs as a set of bands on gels, as phosphorylation slows its mobility – the bands with the slowest mobility are decreased with rapamycin.

(**d**) COS-7 cells treated with DMSO (control) or with 47 μM SMER10, 43 μM SMER18 or 47 μM SMER28 for 24 h, were analysed for Beclin-1 levels by immunoblotting with anti-Beclin-1 antibody.

(**e–g**) HeLa cells treated with DMSO (control) or with 47 μM SMER10, 43 μM SMER18 or 47 μM SMER28 for 24 h, were analysed for Atg5 (**e**), Atg7 (**f**) or Atg12 (**g**) levels by immunoblotting with anti-Atg5 (**e**), anti-Atg7 (**f**) or anti-Atg12 (**g**) antibodies.

(**h**) COS-7 cells transfected with HA-Atg12 and either Atg5 or empty vector (1:2 ratio) for 4 h were analysed for Atg5–HA-Atg12 conjugation levels at 24 h post-transfection by immunoblotting with anti-HA antibody (i). Atg5–HA-Atg12 conjugate is only seen when Atg5 is co-transfected, compatible when data reported previously[14] Note that the gel strips are from non-adjacent lanes of the same immunoblot (i). COS-

7 cells transfected with HA-Atg12 and Atg5 (1:2 ratio) for 4 h and then treated with DMSO (control) or with 47 μM SMER10, 43 μM SMER18 or 47 μM SMER28 for 24 h, were analysed for Atg5–HA-Atg12 conjugation levels by immunoblotting with anti-HA antibody (ii) and densitometry analysis of Atg5–HA-Atg12 conjugate to Atg12 (iii). p=0.3638 (SMER10), p=0.742 (SMER18), p=0.4547 (SMER28).

(**i**) HeLa cells stably expressing Ub^{G76V}-EGFP reporter, treated with or without 10 μM lactacystin (lact), 47 μM SMER10, 43 μM SMER18 or 47 μM SMER28 for 24 h, were analysed for inhibition of proteasome activity by immunoblotting with antibody against EGFP.

***, p<0.0001; NS, Non-significant.