Supporting Information

Flavonoid-derived human phenyl-γ-valerolactone metabolites selectively detoxify amyloid-β oligomers and prevent memory impairment in a mouse model of Alzheimer's disease

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Supporting Information Experimental Section

Chemicals

Clioquinol (CQ), (–)-epigallocatechin gallate (EGCG), 3-hydroxybenzoic acid (3-HBA), 3-hydroxyphenylpropionic acid (3-HPP), caffeic acid, ferulic acid, dihydrocaffeic acid and isoferulic acid were purchased from Sigma-Aldrich. Dihydroferulic acid, caffeic acid 3-O-glucuronide, caffeic acid 4-O-glucuronide, dihydrocaffeic acid 3-O-sulfate, dihydrocaffeic acid 3-O-glucuronide, ferulic acid-4-O-sulfate, isoferulic acid 3-O-glucuronide were purchased from Toronto Research Chemicals Inc. Urolithin A, urolithin B, and urolithin B-glucuronide were kindly provided by Dr. Olivier Dangles (INRA, Avignon; France), while urolithin C and urolithin D were purchased from Dalton Pharma Services. All tested compounds were dissolved in DMSO as either 10 or 20 mM stock solutions.

Yeast strains expressing the human $A\beta_{42}$ peptide

A previously constructed pET28-based plasmid expressing human $A\beta_{42}$ fused to *E. coli* thioredoxin (Trx)[1] was utilized as template for the PCR-assisted construction of yeast vectors expressing the unfused human $A\beta_{42}$ peptide or the $A\beta_{42}$-Trx fusion polypeptide using, respectively, the oligonucleotide pairs #A-#B and #C-#D as amplification primers (Supporting Information Table S2). The resulting amplicons were cloned into the *Hind*III-*Xba*I restriction sites of the multicopy yeast expression vector pYES2 (*URA3* selectable marker) under the control of a galactose-inducible (*GAL1*) promoter to produce the pYES2-$A\beta_{42}$ and pYES2-$A\beta_{42}$-Trx plasmids. A pYES2-based plasmid only containing the Trx coding-sequence (PCR-amplified from pET28-Trx[1] using oligonucleotides #C and #D as primers) was also constructed and used as a control.

To produce the EGFP-$A\beta_{42}$ fusion protein, a variant EGFP sequence lacking a terminal stop-codon and containing an extra-sequence coding for a four-amino acids spacer to be interposed between the C-terminus of EGFP and the N-terminus of $A\beta_{42}$, was PCR-amplified using plasmid pYX212-EGFP as template[2] and oligonucleotides #E and #F as primers (Supporting Information Table S2). The resulting amplicon was then cloned into the *Hind*III restriction site of pYES2-$A\beta_{42}$ to generate the plasmid pYES2-EGFP-$A\beta_{42}$.

Yeast codon-optimized sequence coding for the aggregation-prone, E22G 'arctic' variant of the human $A\beta_{42}$ peptide, N-terminally fused to the c-myc tag sequence, was produced by gene synthesis (Eurofins Genomics) and cloned into the *Hind*III-*Xba*I restriction sites of the pYES2 vector to generate the plasmid pYES2-arc-$A\beta_{42}$.

Overlap extension PCR[3] was used to produce an additional $A\beta_{42}$ fusion derivative in which the human $A\beta_{42}$ peptide is preceded by an ER-targeting signal (HDEL) peptide. To this end, the HDEL sequence of the gene coding for the ER chaperone Kar2 was first retrieved by PCR using genomic DNA from the BY4742 strain (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) as template and the oligonucleotide pair #G-#H (Supporting Information Table S2) as amplification primers. The sequence coding for the human $A\beta_{42}$ peptide was amplified from the pYES2-$A\beta_{42}$ plasmid using a pair of oligonucleotide primers (#I and #L in Supporting Information Table S2), one of which (#I) bears a 3'-end complementary to 3'-end of the #H primer previously employed for HDEL amplification. The HDEL- and $A\beta_{42}$-coding amplicons were then mixed and after annealing of the complementary ends corresponding to oligonucleotides #I and #H and addition of the far-end primers (#G and #L), a third amplification reaction was performed to generate the HDEL-$A\beta_{42}$ fusion product. The latter amplicon was then cloned into the *Hind*III-*Xba*I sites of the pYES2 vector to generate the plasmid pYES2-HDEL-$A\beta_{42}$.

The above plasmids, except for pYES2-arc-$A\beta_{42}$ and pYES2-HDEL-$A\beta_{42}$, were individually transformed into the wild-type *Saccharomyces cerevisiae* strain BY4742 using a standard, lithium acetate-based transformation protocol.[4] Yeast transformants were selected on synthetic defined dextrose (SD) medium containing 2% (w/v) glucose plus histidine, leucine and lysine, but lacking uracil. The pYES2-arc-$A\beta_{42}$ and pYES2-HDEL-$A\beta_{42}$ plasmids were transformed into the wild-type *S. cerevisiae* strain W303α (*MATα can1-100, his3-11,15, leu2-3,112, ade2-1, trp1-1, ura3-1*) and transformants were selected on SD medium lacking uracil.

Yeast strains expressing the β23 polypeptide

A yeast codon-optimized β23 coding sequence[5] was produced by gene synthesis (Eurofins Genomics) and inserted into the *Hind*III-*Xba*I restriction sites of the pYES2 vector under the control

of a galactose-inducible (*GAL1*) promoter. After sequence verification, the resulting construct was transformed into the wild-type *S. cerevisiae* strain W303α and transformants were selected under non-inducing conditions on SD medium containing 2% (w/v) glucose plus histidine, leucine, adenine and tryptophan, but without uracil. The same yeast strain transformed with the empty pYES2 vector served as a control.

For integrative transformant construction, a β23 expression cassette (β23 coding sequence under the control of the *GAL1* promoter and the *CYC1* terminator) was generated by PCR-amplification using the pYES2-β23 plasmid as template and oligonucleotides #M and #N as primers (Supporting Information Table S2). The resulting β23 expression cassette was then inserted into the *Sma*I restriction site of the integration vector pFL26 (*LEU2* selectable marker). Following sequence verification, the one-copy β23 cassette was integrated into the *leu2* locus of the *pdr1Δpdr3Δ* (W303α genetic background; kindly provided by the laboratory of Susan Lindquist[6]), low-efflux mutant strain and the resulting one-copy (1X) β23 transformants were selected on SD medium lacking leucine. The β23 expression cassette was separately cloned into the *Not*I-*Sal*I restriction sites of the pMW#2 vector (*HIS3* selectable marker) and integrated into the *his3* locus of the previously generated one-copy β23 integrative transformant. Two-copy (2X) β23 transformants were thus generated and selected on SD medium lacking leucine and histidine. Integrative yeast transformants harbouring the pFL26 and pMW#2 empty vectors were also produced and used as control strains.

Plasmids for mammalian cell expression

A construct coding for the EGFP-$A\beta_{42}$ fusion protein was produced by a two-step PCR strategy that also allowed to introduce a 12-amino acids spacer between the two polypeptides. The sequence coding for human $A\beta_{42}$ was first amplified using the pET28-$A\beta_{42}$-Trx plasmid as template[1] and the #O-#P oligonucleotides (Supporting Information Table S2) as primers. After clean-up of the reaction mixture, the resulting $A\beta_{42}$ amplicon was used as template for a second PCR employing the #Q-#P oligonucleotides (Supporting Information Table S2) as primers. The product of this second amplification was then cloned into the *Hind*III/*Bam*HI restriction sites of the pEGFP-C1 vector in-

frame with the EGFP coding sequence, under the control of the human cytomegalovirus (CMV) immediate early promoter.

Human codon-optimized sequence coding for the $A\beta_{42}$ peptide with the E22G 'arctic' mutation and the β23 peptide, N-terminally fused to the c-myc tag sequence, were produced by gene synthesis (Eurofins Genomics) and cloned into the pEGFP-C1 vector, pre-digested with *Nhe*I and *Xba*I enzymes in order to remove the EGFP coding unit.

Yeast cell extracts

Cell extracts for immuno-dot blot analysis were prepared by inoculating a pre-culture of the two-copy β23 strain into 'inducing' (2% galactose) selective synthetic medium at an OD_{600} of 0.3, followed by 8 h at 30°C. Cells were harvested by centrifugation, resuspended in ice-cold lysis buffer (25 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100, 10% glycerol, plus protease inhibitors) supplemented with an equal volume of glass beads (0.5 mm diameter), and lysed by five rounds of vigorous vortexing (1 minute stroke followed by 1 minute incubation on ice) with a Mini-Beadbeater-16 (Bio Spec Products Inc.). The resulting lysates were clarified by centrifugation (10000 rpm, 30 minutes at 4°C) and the total protein concentration of supernatant fractions was determined with the Bradford reagent (Bio-Rad). Aliquots of the above supernatants (10 µg total protein each in a final volume of 100 µl) were used for immuno-dot blot analysis (see 'Experimental Section' for details).

Animals

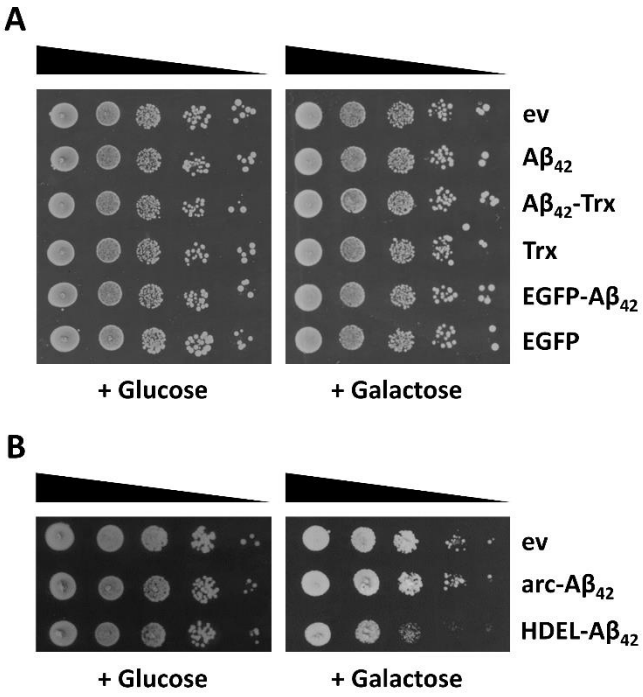
Eight-weeks-old C57BL6/N male mice (Charles River Laboratories) were used for *in vivo* studies. Mice were all drug and behavioural test naïve and experiments were conducted during the light cycle. Prior to brain surgery, animals were housed in a SPF facility in groups of four in standard mouse cages containing sawdust plus food (2018S Harlan diet) and water ad libitum, under conventional laboratory conditions (20±2°C; 60% humidity) with a 12/12 hour light/dark cycle (7:00 am – 7:00 pm). The IRFMN (Istituto di Ricerche Farmacologiche Mario Negri) adheres to the principles set out in the following laws, regulations, and policies governing the Care and Use of

Laboratory Animals: Italian Government Law (D. lgs 26/2014; Authorization n.19/2008-A issued by the Ministry of Health on March 6, 2008); Mario Negri Institutional Regulations and Policies providing internal authorization for persons conducting animal experiments (Quality Management System Certificate – UNI EN ISO 9001:2015 – Reg. N° 6121); the NIH Guide for Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). The statement of Compliance (Assurance) with the Public Health Service (PHS) Policy on Human Care and Use of Laboratory Animals has been reviewed (9/9/2014) and will expire on September 30, 2019 (Animal Welfare Assurance #A5023-01).

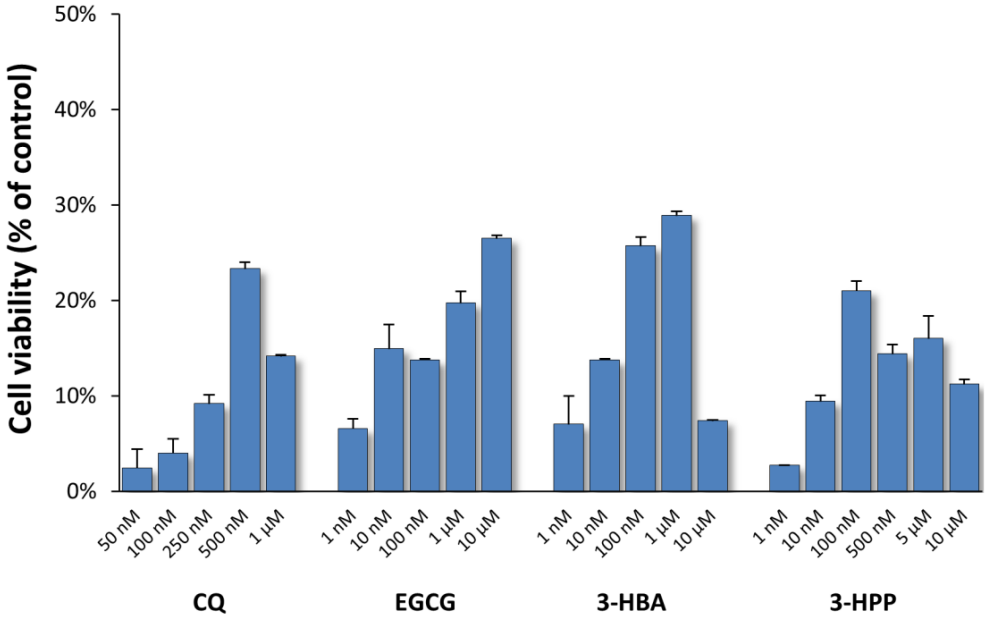
Neuro-histochemical analysis

Brain slices were incubated 1 h at 4°C in blocking solution (3% normal goat serum plus 0.3% Triton X-100 for GFAP immunodetection; 3% normal goat serum plus 0.4% Triton X-100 for Iba1 immunodetection) prior to overnight incubation with anti-GFAP and anti-Iba1 primary antibodies. Following incubation with the appropriate biotinylated secondary antibodies (Vector Laboratories; 1:200 dilution), immunostaining was developed and visualized using an avidin/biotin blocking kit (Vector Laboratories) and diaminobenzidine as chromogen. Diaminobenzidine tissue analysis and image acquisition were performed with an Olympus image analyser (VS-ASW 2.8 v.). Quantitative analysis was conducted with the use of an operator-blind-to-treatment, Fiji software, with normalization on the quantified area.

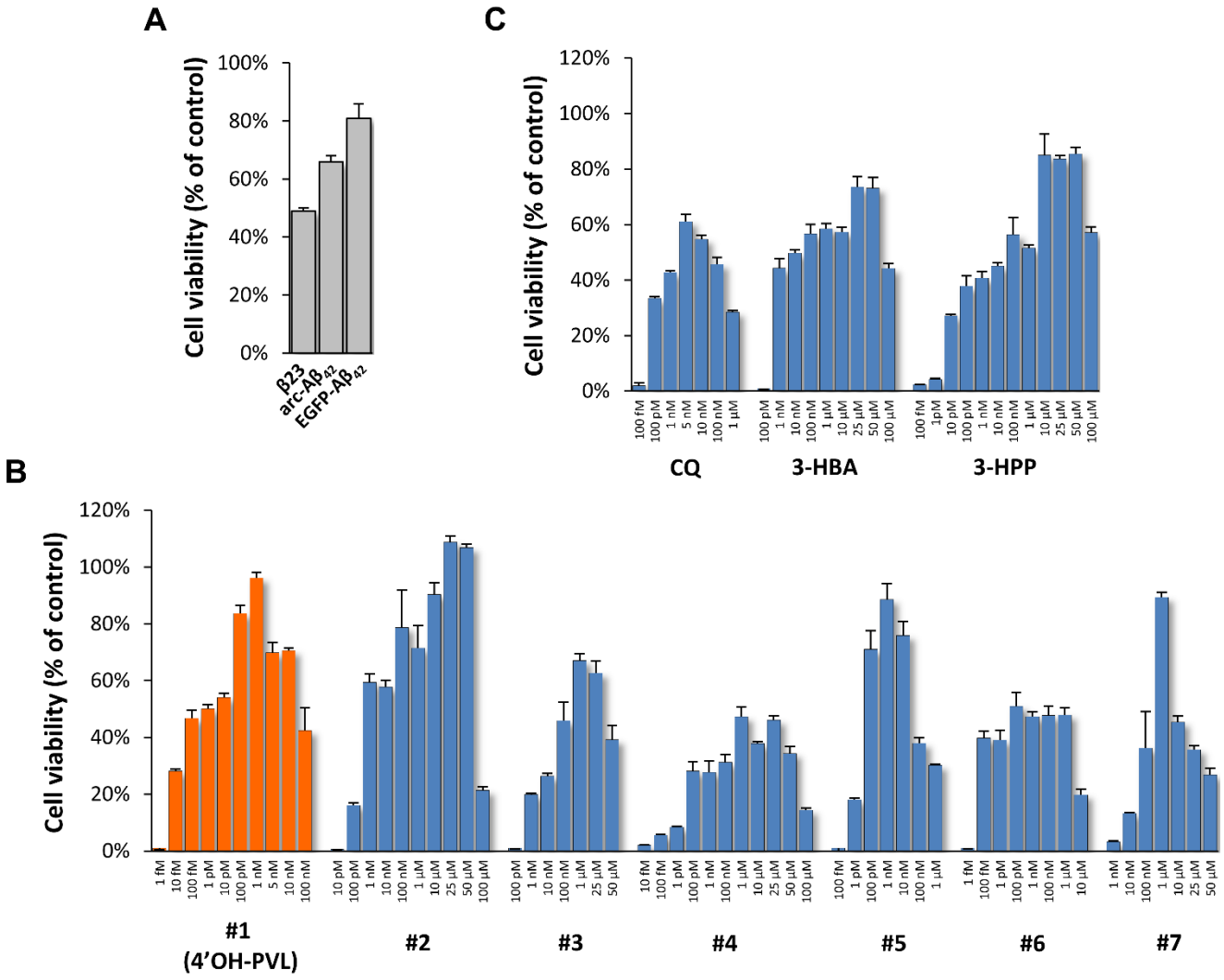
Supporting Information Figures



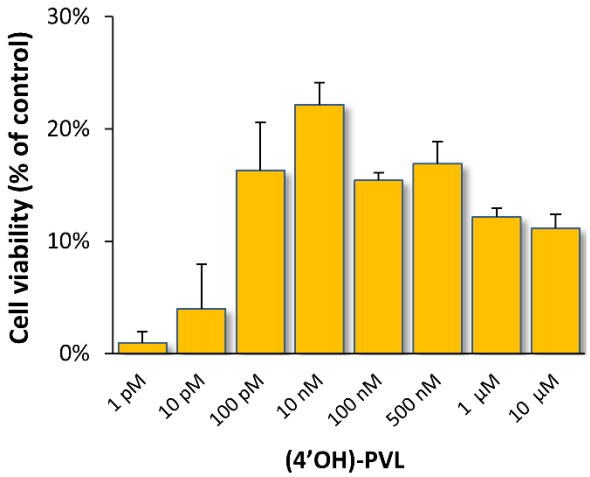
Supporting Information Figure S1. Cytosolic expression of $A\beta_{42}$ does not affect cell viability in yeast. A) Yeast cells (BY4742 strain) were transformed with multicopy expression plasmids harboring galactose-inducible genes coding for the human $A\beta_{42}$ peptide alone or the same peptide fused with the indicated scaffold proteins (thioredoxin, Trx; enhanced green fluorescent protein, EGFP). Yeast cells transformed with the empty vector (pYES2; ev) or with constructs for the expression of the $A\beta_{42}$-lacking forms of Trx and EGFP served as controls. Serial dilutions (ten-fold increments) of the indicated transformants were spotted on agar plates under repressive (+ *Glucose*) or inducing (+ *Galactose*) conditions and cell growth was assessed after 2 days at 30°C. B) Yeast cells (W303α strain) were transformed with pYES2 plasmids expressing an E22G arctic variant of the human amyloid peptide (*arc-$A\beta_{42}$*) and a fusion derivative of $A\beta_{42}$ that due to the presence of an ER-retention peptide is forced into the secretory pathway (*HDEL-$A\beta_{42}$*). HDEL-$A\beta_{42}$ expression causes a detectable reduction of the yeast cell viability under inducing (+ *Galactose*) conditions. Serial dilution spot assay conditions were the same as in panel (A).



Supporting Information Figure S2. Anti-proteotoxic effect of a subset of $A\beta_{42}$-active compounds in the yeast β23 system. Dose-response analysis of a subset of reference anti-Aβ compounds performed on the 2X-β23 transformant strain under 'inducing' conditions [2% galactose plus a non-repressing glucose concentration (0.005% w/v)]. The results are expressed as percentage of cell viability relative to the DMSO vehicle control (arbitrarily set to 0%); data are the mean ± SD of three replicates. The tested compounds are the metal chelator clioquinol (CQ), the plant polyphenol metabolite (–)-epigallocatechin gallate (EGCG), and the human flavonoid metabolites 3-hydroxyphenylpropionic acid (3-HBA) and 3-hydroxyphenylpropionic acid (3-HPP).



Supporting Information Figure S3. Cytoprotective activity of PVLs in a β23-expressing human cell line. A) HEK293 cells were transiently transfected with pC1 plasmids expressing the β23 peptide, an E22G arctic variant of the human amyloid peptide (*arc-Aβ$_{42}$*) or the EGFP-Aβ$_{42}$ fusion protein under the control of the constitutive CMV promoter. 72 h after transfection, the MTT assay was used to assess cell viability; the results are expressed as percentage of cell viability relative to control cells transfected with the empty pC1 vector (arbitrarily set to 100%). **B**) The MTT cell viability assay was used to assess the ability of the indicated PVLs to protect against β23 cytotoxicity in HEK293 cells. Cell viability assays were performed 72 h after transfection with the pC1-β23 plasmid (see 'Experimental Section' for details). Results are expressed as percentage of cell viability relative to the DMSO vehicle control (arbitrarily set to 0%); data are the mean ± SD of three replicates. **C**) Dose-response analysis (as described in panel B) of a subset of reference anti-Aβ compounds performed in pC1-β23 transfected HEK293 cells.



Supporting Information Figure S4. Cytoprotective effect of (4'-OH)-PVL in the HDEL-Aβ_{42} yeast model of amyloid toxicity. Dose-response analysis of the effect of (4'-OH)-PVL in the secretory model of HDEL-Aβ_{42}-induced cytotoxicity. Yeast cells transformed with a multicopy plasmid expressing HDEL-Aβ_{42} under the control of a galactose-inducible promoter were cultured under 'inducing' conditions [2% galactose plus a non-repressing glucose concentration (0.005% w/v)] in the presence of the indicated concentrations of (4'-OH)-PVL**.** The results are expressed as percentage of cell viability relative to the DMSO vehicle control (arbitrarily set to 0%); data are the mean ± SD of three replicates.

Supporting Information Tables

Supporting Information Table S1. Polyphenolic compounds used in this work.

FLAVONOIDS	
Flavan-3-ols	
Phenyl-γ-valerolactones (PVL)	
(–)-5-(4'-Hydroxyphenyl)-γ-valerolactone [#1; (4'-OH)-PVL]	Gut microbial metabolite
(–)-5-(3',4'-Dihydroxyphenyl)-γ-valerolactone (#2)	Gut microbial metabolite
(–)-5-(3'-Hydroxyphenyl)-γ-valerolactone (#3)	Gut microbial metabolite
(–)-5-Phenyl-γ-valerolactone-4'-sulfate (#4)	Gut microbial + human host phase II metabolite
(–)-5-(4'-Hydroxyphenyl)-γ-valerolactone-3'-sulfate (#5)	Gut microbial + human host phase II metabolite
(–)-5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-sulfate (#6)	Gut microbial + human host phase II metabolite
(–)-5-Phenyl-γ-valerolactone-3',4'-disulphate (#7)	Gut microbial + human host phase II metabolite
(–)-Epigallocatechin gallate (EGCG)	Plant metabolite
3-(3′-Hydroxyphenyl)propionic acid (3-HPP)	Gut microbial metabolite
NON-FLAVONOIDS	
Hydroxycinnamic acids	
Caffeic acid	Plant metabolite
Ferulic acid	Plant metabolite
Dihydrocaffeic acid	Gut microbial metabolite
Dihydroferulic acid	Gut microbial metabolite
Isoferulic acid	Gut microbial metabolite
Caffeic acid 3-O-glucuronide	Human phase II metabolite
Caffeic acid 4-O-glucuronide	Human phase II metabolite
Dihydrocaffeic acid 3-O-sulfate	Gut microbial + human host phase II metabolite
Dihydrocaffeic acid 3-O-glucuronide	Gut microbial + human host phase II metabolite
Ferulic acid 4-O-sulfate	Human phase II metabolite
Isoferulic acid 3-O-glucuronide	Human phase II metabolite
Hydroxybenzoic acids	
3-Hydroxybenzoic acid (3-HBA)	Gut microbial metabolite
Ellagitannins	
Urolithin A	Gut microbial metabolite
Urolithin B	Gut microbial metabolite
Urolithin C	Gut microbial metabolite
Urolithin D	Gut microbial metabolite
Urolithin B 3-O-glucuronide	Gut microbial + human host phase II metabolite

Supporting Information Table S2. Oligonucleotides used in this work.

Gene construct	Primer name	Primer sequence[a]
pYES2-Aβ$_{42}$	**#A**	FW: 5'-TAAATATAAAAAGCTTGCCACCATGGATGCAGAATTCCGACATGAC-3'
	#B	RE: 5'-AATTATTTTATCTAGATTACGCTATGACAACACCGCCCACC-3'
pYES2-Aβ$_{42}$-Trx; pYES2-Trx	**#C**	FW: 5'-TAAATATAAAAAGCTTGCCACCATGAGCGATAAAATTATTCACCTGAC-3'
	#D	RE: 5'-AATTATTTTATCTAGATTACGCCAGGTTAGCGTCGAGGAACTC-3'
pYES2-EGFP-Aβ$_{42}$	**#E**	FW: TAAATATAAAAAGCTTGCCACCATGAGTAAAGGAGAAGAACTTTTC-3'
	#F	RE: 5'-AATTATTTTAAAGCTTTTTGTATAGTTCATCCATGCCATG-3'
pYES2-HDEL-Aβ$_{42}$	**#G**	FW: 5'-TAAATATAAAAAGCTTGCTAGCGCCACCATGTTTTTCAACAGACTAAGCGCTG-3'
	#H	RE: 5'-ATCCAGAGTCATGTCTAAACTCAGCATCACCTCTAACTAAAACATTGGAGGAG-3'
	#I	FW: 5'-TTCCACTCCTCCAATGTTTTAGTTAGAGGTGATGCTGAGTTTAGACATGACTC-3'
	#L	RE: 5'-ATTTTATTAATCTAGATTATCAGGCAATGACCAC-3'
β23 expression cassette	**#M**	FW: 5'-GATGATCCACTAGTACGGATTAGAAG-3'
	#N	RE: 5'-ATAAATAGGGACCTAGACTTCAGGTTG-3'
pC1-EGFP-Aβ$_{42}$	**#O**	FW: 5'-TGCCGGCTCTGCCGGCTCCGATGCAGAATTCCGACATGACTC-3'
	#P	RE: 5'-AATTATTTTAGGATCCTCACGCTATGACAACACCGCCCAC-3'
	#Q	FW: 5'-TAAATATAAAAAGCTTCCGGCTCCGCCGGCTCCGCTGCCGGCTCTGCCGGCTCC-3'

[a]FW, forward primer; RE, reverse primer.

Supporting Information Table S3. Cytoprotective efficacy of compounds antagonizing β23 proteotoxicity in yeast.

Compound[a]		EC_{50}	Maximum response (cell viability rescue)[b]
PVLs	**#1 [(4'-OH)-PVL]**	498 pM	35% ± 1.9%
	#2	250 nM	14% ± 0.7%
	#3	5 nM	23% ± 0.8%
	#4	369 nM	31% ± 1.4%
	#5	2 nM	41% ± 2.4%
	#6	31 nM	30% ± 0.9%
	#7	533 pM	14% ± 1.5%
Caffeic acid		157 nM	12% ± 2%
Ferulic acid		95 nM	14% ± 0.5%
Dihydroferulic acid		5 nM	14% ± 1%
Caffeic acid 4-O-glucuronide		254 nM	15% ± 0.5%
EGCG		5 nM	26% ± 0.3%
3-HBA		18 nM	29% ± 0.5%
3-HPP		10 nM	21% ± 1%
CQ		254 nM	23% ± 0.7%

[a]EGCG, (–)-epigallocatechin gallate; 3-HBA, 3-hydroxybenzoic acid; 3-HPP, 3-hydroxyphenylpropionic acid; CQ, clioquinol; see Figure 1 for the chemical structures of the PVLs.
[b]Cell viability rescue data were normalized with respect to the DMSO vehicle control (arbitrarily set to 0%) and are the mean ± SD of three independent replicates, from which the indicated EC_{50} values were derived.

Supporting Information Table S4. Anti-β23 activity of PVLs and other compounds in HEK293 cells.

Compound		EC_{50}	Maximal response (cell viability rescue)[a]
PVLs	**#1 [(4'-OH)-PVL]**	0.1 pM	96% ± 2%
	#2	3 nM	109% ± 2.2%
	#3	10 nM	67% ± 2.4%
	#4	49 pM	47% ± 3.5%
	#5	5 pM	89% ± 5.5%
	#6	0.6 pM	51% ± 1%
	#7	93 nM	89% ± 1.8%
CQ		57 pM	61% ± 2.6%
3-HBA		823 pM	74% ± 3.7%
3-HPP		661 pM	85% ± 2.4%

[a]Cell viability rescue data were normalized with respect to the DMSO vehicle control (arbitrarily set to 0%) and are the mean ± SD of three independent replicates, from which the indicated EC_{50} values were derived.

References

[1] N. Moretto, A. Bolchi, C. Rivetti, B. P. Imbimbo, G. Villetti, V. Pietrini, L. Polonelli, S. Del Signore, K. M. Smith, R. J. Ferrante, S. Ottonello, *J. Biol. Chem.* **2007**, *282*, 11436.

[2] E. Levati, S. Sartini, A. Bolchi, S. Ottonello, B. Montanini, *Sci. Rep.* **2016**, *6*, 25165.

[3] R. Higuchi, B. Krummel, R. K. Saiki, *Nucleic Acids Res.* **1988**, *16*, 7351.

[4] H. Ito, Y. Fukuda, K. Murata, A. Kimura, *J. Bacteriol.* **1983**, *153*, 163.

[5] H. Olzscha, S. M. Schermann, A. C. Woerner, S. Pinkert, M. H. Hecht, G. G. Tartaglia, M. Vendruscolo, M. Hayer-Hartl, F. U. Hartl, R. M. Vabulas, *Cell* **2011**, *144*, 67.

[6] A. D. Gitler, B. J. Bevis, J. Shorter, K. E. Strathearn, S. Hamamichi, L. J. Su, K. A. Caldwell, G. A. Caldwell, J. C. Rochet, J. M. McCaffery, C. Barlowe, S. Lindquist, *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 145.