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online data

SUPPLEMENTARY ONLINE DATA

Pleiotropic mechanisms facilitated by resveratrol and its metabolites

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METHODS

Expression and purification of hSIRT1

Wild-type hSIRT1 from the SIRT1-pHex vector was purified from 4 litres of Rosetta pLysS *Escherichia coli* cells. Cells were grown at 37 °C in LB (Luria–Bertani) broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Once the D_{600} reached 0.7–0.8, protein expression was induced with 1 mM IPTG (isopropyl β-D-thiogalactopyranoside) for 16–20 h at 16 °C. Cells were collected by centrifugation at 4300 rev./min for 15 min in a Sorvall Super T21 centrifuge (ST-H750 rotor) and resuspended in 60 ml of Buffer A [25 mM Tris/HCl, pH 8.0, 250 mM NaCl and 1 mM DTT (dithiothreitol)] containing EDTA-free Complete™ mini protease inhibitor cocktail tablets (Roche Applied Science), a pinch of solid DNase I, lysozyme (1 mg/ml) and 0.5 mM PMSF. The cell suspension was sonicated at 600 W for a total of 9 min using a high intensity ultrasonic processor Sonic processor VCX600, with a pulse of 6.6 s on and 9.9 s off. The lysed cells were pelleted by centrifugation at 18000 rev./min for 45 min in a Sorvall Super T21 centrifuge (SL-50T rotor) at 4 °C. The cleared lysate was then loaded on to a Hitrap chelating HP affinity column (GE Healthcare) charged with cobalt and equilibrated with 25 mM Tris/HCl, pH 7.5, 500 mM NaCl, 10 mM imidazole and 1 mM DTT. The protein was eluted using a linear gradient from 5 to 50 % buffer B (25 mM Tris/HCl, pH 8.0, 500 mM NaCl, 500 mM imidazole and 1 mM DTT). Fractions containing the enzyme were pooled and dialysed overnight at 4 °C in 25 mM Tris/HCl, pH 8.0, 10 mM NaCl and 1 mM DTT. The resulting enzyme pool was then applied to a MonoQ (GE Healthcare) anion-exchange column. The protein was eluted using a linear gradient ranging from 5 % to 50 % of 25 mM Tris/HCl, pH 8.0, 1M NaCl and 1 mM DTT. Fractions containing the enzyme of interest were pooled and finally applied to a Superdex 200 gel filtration column (GE Healthcare), and eluted with 50 mM Tris/HCl, pH 7.5, 300 mM NaCl and 1 mM DTT. The purest fractions were pooled, buffer-exchanged to 50 mM Tris/HCl, pH 8.0, 150 mM NaCl and 10 % (v/v) glycerol, and concentrated up to 5 mg/ml using the Centricon Plus-20 device (Millipore). SIRT1 was then stored at − 80 °C until needed. During the purification, the protein concentration was measured using the determined absorption coefficient ($\varepsilon = 43810$ $M^{-1} \cdot cm^{-1}$).

Cloning, expression and purification of human QR2 (quinone reductase 2)

Human QR2 cDNA (MGC-12729) was purchased from ATCC. The primers used in cloning were: 5′-AACCATGGCAGGTAAGAAAGTACTC (the added NcoI site is underlined) and 5′-AACTCGAGTTATTGCCCGAAGTGCCAGTG (the added XhoI site is underlined). The PCR product was digested with NcoI and XhoI restriction enzymes, purified and then ligated into a pET-23d expression vector (Novagen). The parental vector was digested with the methylation-sensitive restriction enzyme DpnI, whereas the unmethylated PCR-amplified vector remains intact. The ligation product was transformed into *E. coli* XL1 Blue and several colonies were screened for positive gene insertion in the vector by restriction digestion. The entire insert containing the human *QR2* gene was sequenced via the Research Resources Center DNA core facility at University of Illinois at Chicago. Human QR2 was purified from 3 litres of *E. coli* BL21(DE3) in LB broth containing 100 μg/ml ampicillin grown at 37 °C. The expression of human QR2 was induced by the addition of 1 mM IPTG when the culture attenuance at 600 nm reached 0.7–0.8. Cells were then transferred at 16 °C for 12 h. The enzyme expression was evaluated using SDS/PAGE and enzyme activity

Table S1 X-ray data collection and refinement statistics for the COX-1–resveratrol complex

*Value corresponding to the overall resolution range of 20.0 to 3.0 Å. †Value corresponding to the last resolution shell. RMSD, root mean square deviation.

Parameter	Value
Data collection	
Space group	$P6_522$
Unit cell (Å)	$a = b = 181.89$, $c = 104.1$
Resolution (Å)	3.0
Total number of reflections	694551
Number of averaged reflections	20765
R_{merge} (%)	15.2
$I/\sigma I$	25.1* (2.2)†
χ^2	1.74* (1.69)†
Completeness (%)	99.9* (99.9)†
Refinement	
Resolution range (Å)	20.0–3.0
Number of reflections in working set	19710
Number of reflections in test set	1021
R_{cryst} (%)	21.5
R_{free} (%)	29.9
FOM (figure of merit)	0.77
Average B-factor (Å^2)	68.7
RMSD from ideal geometry	
Bond lengths (Å)	0.009
Bond angles (°)	1.51
Ramachandran plot	
Allowed (%)	85.6
Generous (%)	96.7
Disallowed (%)	3.3

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The atomic co-ordinates for the crystal structure of the cyclo-oxygenase-1–resveratrol complex will appear in the Protein Data Bank under accession code 3BGN.

Table S2 Comparison of the steady-state kinetic parameters for various inhibition models for the inhibition of QR2 by resveratrol

(a) Fixed concentration of menadione (30 μM) with variable concentrations of NMeH as given in Figure 6 of the main text.

Equation	Competitive*	Noncompetitive	Uncompetitive
V_{max}	27.7 ± 0.6 μM/min	34.0 ± 0.8 μM/min	35.1 ± 1.8 μM/min
K_m	6.1 ± 1.8 μM	29.1 ± 2.0 μM	35 ± 5.6 μM
K_i	0.08 ± 0.02 μM	1.4 ± 0.09 μM	1.0 ± 0.09 μM
R^2	**0.94**	0.89	0.85
Test	Pass	Pass	Pass
Convergence	Yes	Yes	Yes
AICc	**77.4**	117.8	149.6

(b) Fixed concentration of NMeH (100 μM) with variable concentrations of menadione as given in Figure 6 of the main text.

Equation	Competitive	Noncompetitive	Uncompetitive*
V_{max}	28.5 ± 1.8 μM/min	31.9 ± 1.2 μM/min	35.4 ± 1.4 μM/min
K_m	4.2 ± 1.8 μM	6.5 ± 1.02 μM	10.1 ± 1.3 μM
K_i	0.4 ± 0.17 μM	1.37 ± 0.3 μM	0.9 ± 0.1 μM
R^2	0.79	0.90	**0.95**
Test	Pass	Pass	Pass
Convergence	Yes	Yes	Yes
AICc	105.2	52.7	**34.0**

*The final kinetic inhibition models chosen (bold type) were chosen based upon the higher values for R^2 values and the lower AIC (Akaike's information criterion; AICc) values. The correct model was easily discriminated. All data were fitted using the Enzyme Kinetics module of SigmaPlot 9.0.

assay. The cells were collected by centrifugation at 4300 rev./min for 15 min in a Sorvall Super T21 centrifuge (ST-H750 rotor) and resuspended in 60 ml of Buffer A_1 (25 mM Tris/HCl, pH 8.0, 10 mM NaCl and 1 mM DTT) containing 2 tablets of EDTA-free Complete™ mini protease inhibitor cocktail tablets and 0.5 mM PMSF. The cell suspension was sonicated at 600 W for a total of 6 min using a high intensity ultrasonic processor Sonic processor VCX600, with a pulse of 6.6 s on and 9.9 s off. The lysed cells were pelleted by centrifugation at 18000 rev./min for 45 min in a Sorvall Super T21 centrifuge (SL-50T rotor) at 4 °C. The supernatant was checked for human QR2 activity using the enzyme activity assay described in the Experimental section of the main text, and then was applied to a DEAE-Sepharose column (Amersham Pharmacia Biotech) equilibrated with buffer A_1. The enzyme was eluted with a linear gradient of increasing buffer B_1 (25 mM Tris/HCl, pH 8.0, 1 M NaCl and 1 mM DTT). Fractions containing the enzyme (detected by SDS/PAGE) were pooled and concentrated using an 80 ml Centricon Plus-20 concentration device from Millipore (molecular mass cut-off 10000 Da). The enzyme was then applied to a Superdex 75 (Amersham Pharmacia Biotech) column equilibrated with buffer A. The enzyme was eluted with buffer A and the fractions containing pure enzyme were pooled and finally applied to a MonoQ (GE Healthcare) anion-exchange column equilibrated with buffer A. The protein was eluted using a gradient ranging from 5 % to 50 % buffer B. Protein was collected in 3 ml fractions. The purest fractions were pooled, the buffer was exchanged to 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10 % (v/v) glycerol, and concentrated using the Centricon Plus-20 device. QR2 was then stored at −80 °C. During the purification, the protein concentration was measured using the Bradford assay [1].

Effect of resveratrol on QR1 activity

This assay was modified from a previously described method [2]. Cultured Hepa 1c1c7 mouse hepatoma cells were plated at a density of 2×10^4 cells/ml in 96-well plates and incubated for 24 h. The medium was then changed and resveratrol, dissolved in 10 % (v/v) DMSO, was added to each well. The cells were incubated for an additional 48 h. QR1 activity was measured by the NADPH-dependent menadiol-mediated reduction of MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] to a blue formazan. Enzyme activity was expressed as a 'CD' value, the concentration of test material needed to double the specific activity of QR1.

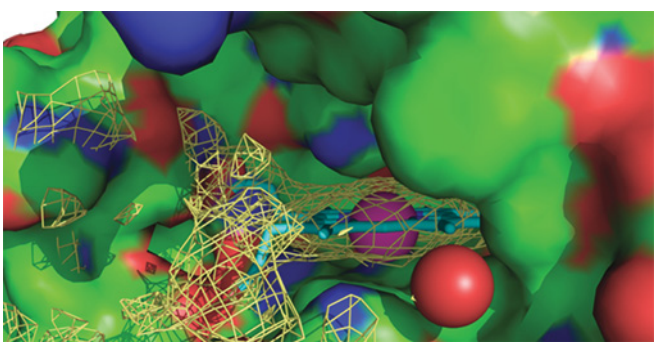

Figure S1 Resveratrol does not bind to the peroxidase site of COX-1

Electron density map ($2F_o-F_c$ contoured at 1σ surrounding the haem cofactor in the peroxidase site of COX-1. Residual electron density for resveratrol is not observed. The haem cofactor (light blue) is shown as stick representation. COX-1 is represented by the surface, and the metal and water molecules are represented as pink and red spheres respectively.

Table S3 Effect of resveratrol on the activity of hQR1

CD, concentration of resveratrol required to double the enzyme activity.

Compound	CD (μM)	IC_{50} (μM)
Resveratrol	190	>250

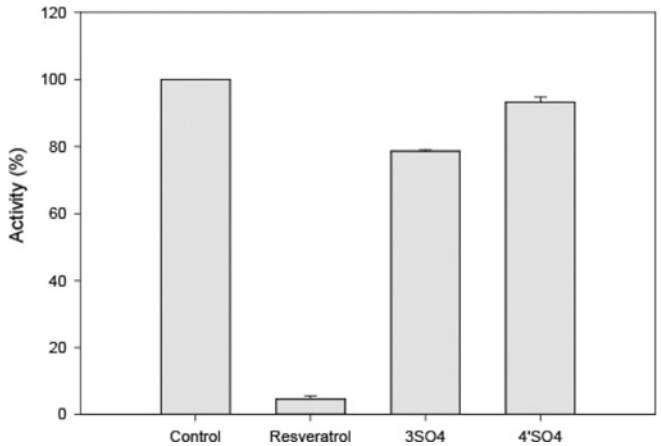

Figure S2 Residual activity of QR2 in the absence and presence of resveratrol and resveratrol-sulfate metabolites

The assays were measured using 5 nM of QR2, 100 μM NMeH (*N*-methylnicotinamide) and 30 μM menadione. The compounds were tested at final concentrations of 100 μM. 3SO4, resveratrol-3-*O*-sulfate; 4′SO4, resveratrol-4′-*O*-sulfate.

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