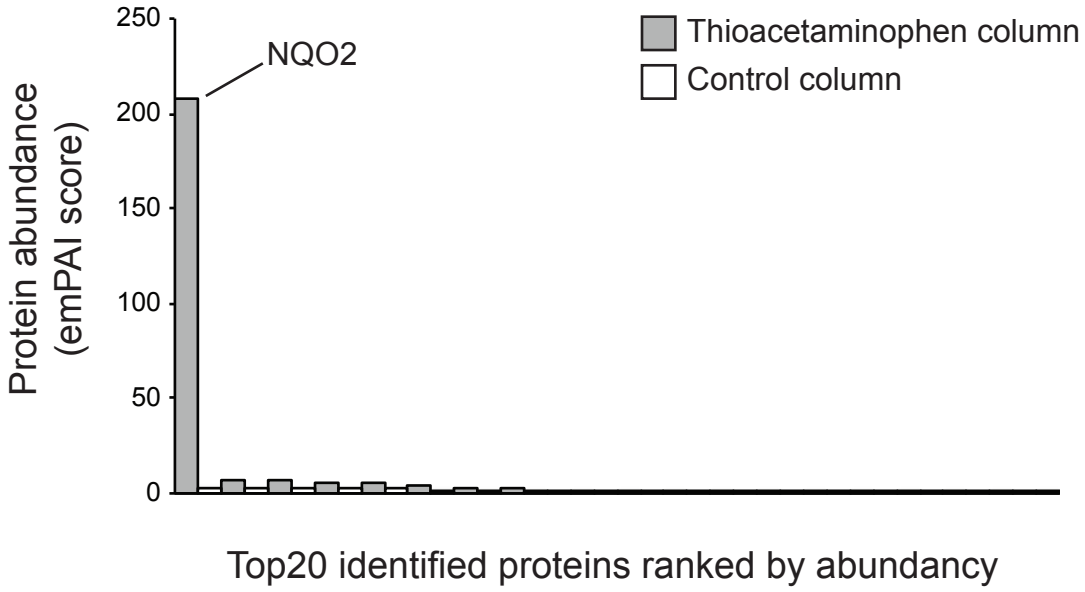
Supporting information figure S1. Miettinen and Björklund.

a

>mNQO2

MAGKKVLIVYAHQEPKSFNGSLKKVAVEELSKQGCTVTVSDLYSMNFEPR
ATRNDITGAPSNPDVFSYGIETHEAYKKKALTSDIFEEQRKVQEADLVIF
QFPLYWFSVPAILKGWMDRVLCRGFAFDIPGFYDSGFLKGKLALLSLTTG
GTAEMYTKDGVSGDFRYFLWPLQHGTLHFCGFKVLAPQISFGLDVSSEEE
RKVMLASWAQRLKSIWKEEPIHCTPPWYFQE

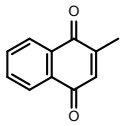
b



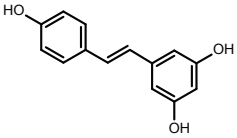
Supporting information figure S2. Miettinen and Björklund.

NQO2 substrates and inhibitors:

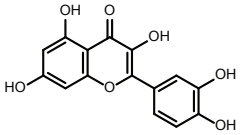
Menadione



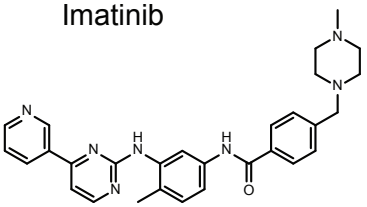
Resveratrol



Quercetin

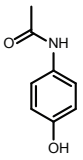


Imatinib

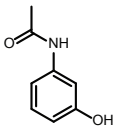


Small chemical library of acetaminophen analogues:

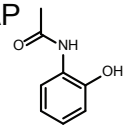
4-Acetaminophen
APAP



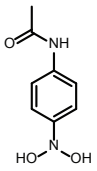
3-acetaminophenol
AMAP



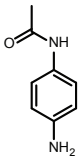
2-acetaminophenol
AOAP



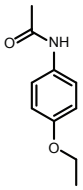
4-Nitroacetanilide



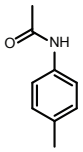
4-Aminoacetanilide



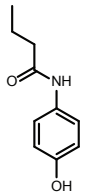
4-Ethoxyacetanilide



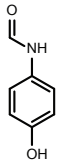
4-Methylacetanilide



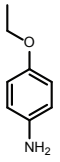
4-Hydroxybutyranilide



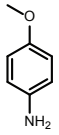
4-Hydroxyformanilide



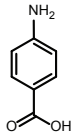
4-Ethoxyaniline



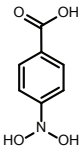
4-Anisidine



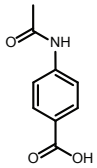
4-Aminobenzoic acid



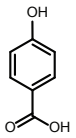
4-Nitrobenzoic acid



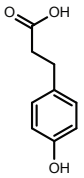
4-Acetamidobenzoic acid



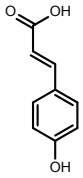
4-Hydroxybenzoic acid



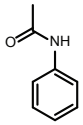
3-(4-Hydroxyphenyl)-
propionic acid



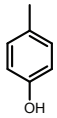
4-Coumaric acid



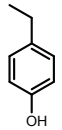
N-Acetanilide



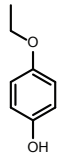
4-Methylphenol



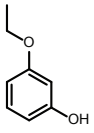
4-Ethylphenol



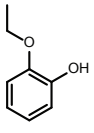
4-Ethoxyphenol



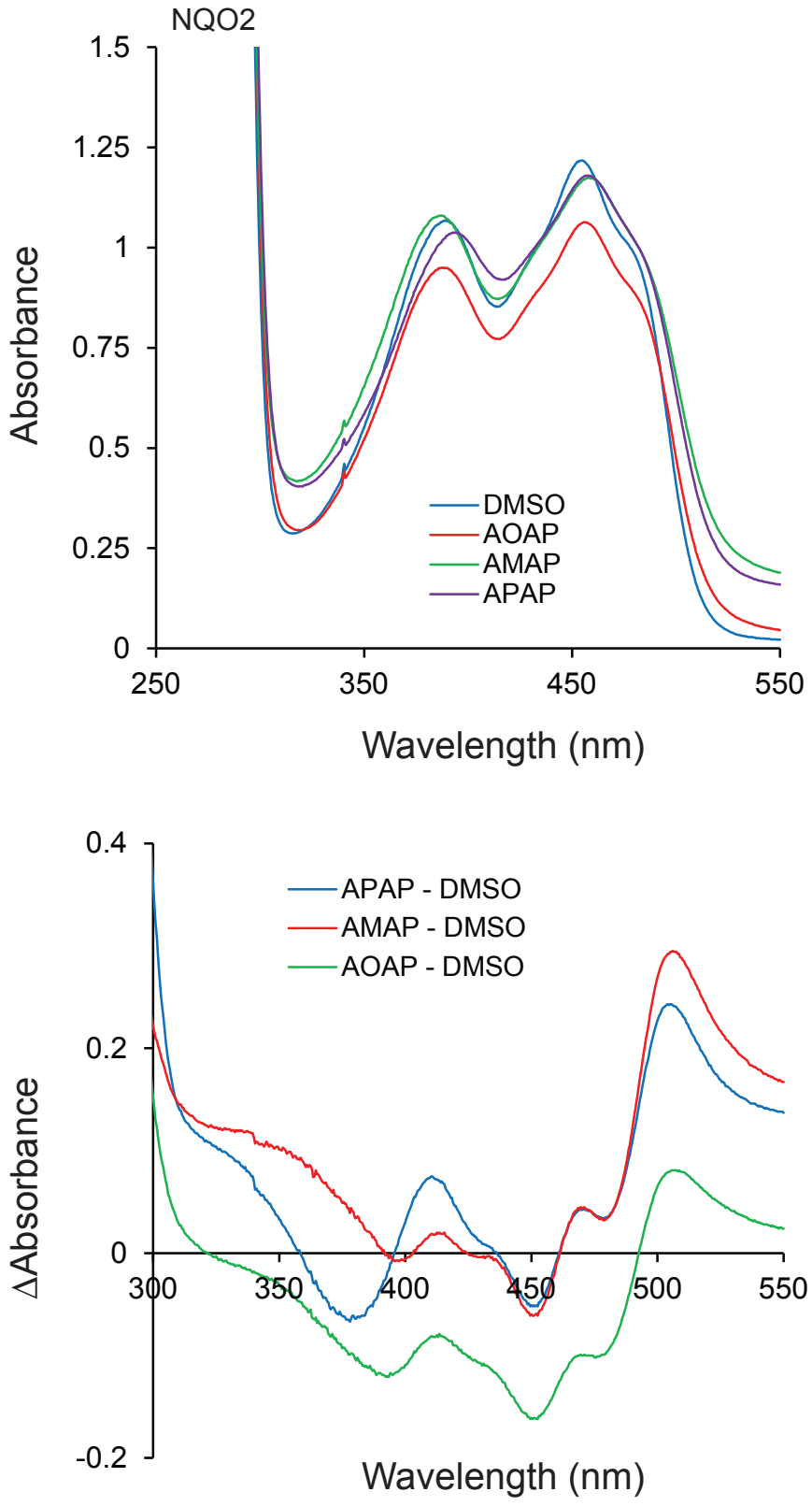
3-Ethoxyphenol



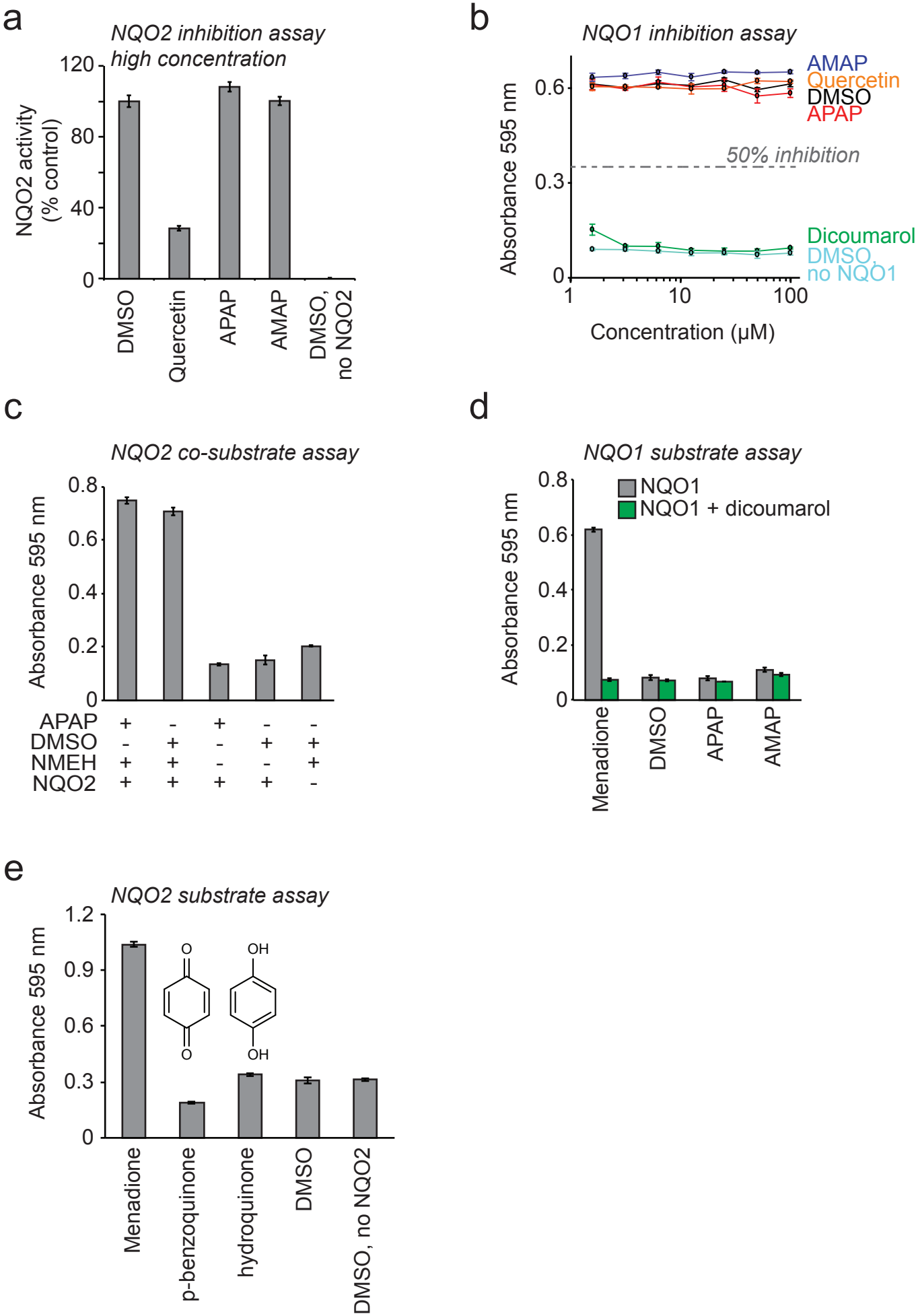
2-Ethoxyphenol



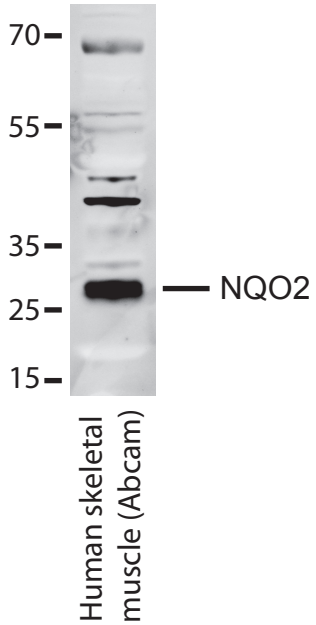
Supporting information figure S3. Miettinen and Björklund.



Supporting information figure S4. Miettinen and Björklund.

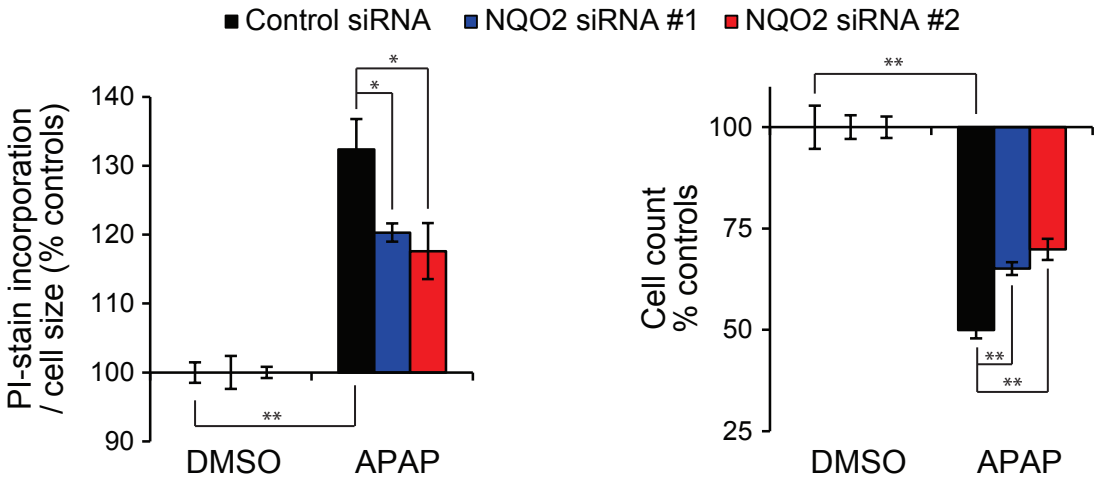


Supporting information figure S5. Miettinen and Björklund.



Supporting information figure S6. Miettinen and Björklund.

a



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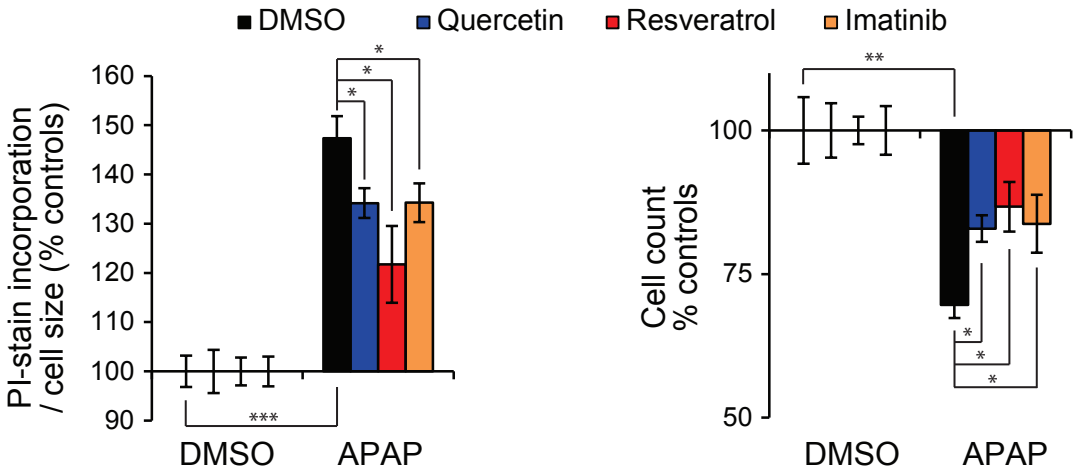

SUPPORTING INFORMATION FIGURE LEGENDS

Supplementary Figure 1. Identification of mouse NQO2 binding to thioacetaminophen column. (a) Coverage of mass spectrometry identified peptides (red) in mouse NQO2. (b) Proteins present in the ~30 kDa gel fragment were analysed by mass spectrometry and label-free quantification of top 20 identified proteins eluted from thioacetaminophen and control columns are shown. Exponentially modified protein abundance index (emPAI score) was used for quantification.

Supplementary Figure 2. Structures of known NQO2 substrate and inhibitors and structures of acetaminophen analogs used. See Table S1 for data on analogs.

Supplementary Figure 3. Electronic absorption spectroscopy of NQO2 (final concentration approximately 2.5 mg/ml) with 0.5 mM acetaminophen regioisomers and DMSO (top). Difference spectrums in comparison to DMSO are shown on the bottom.

Supplementary Figure 4. (a) NQO2 inhibition assay with 10 mM APAP and AMAP or 100 µM quercetin. (b) NQO1 *in vitro* inhibition assay. NQO1 was incubated in the presence of compounds at the concentrations indicated and NQO1 activity measured with a colorimetric assay. Dicoumarol is a known NQO1 inhibitor. (c) NQO2 co-substrate assay. NQO2 activity is shown in the presence of control (DMSO) or APAP (10 mM) with or without the co-substrate NMEH (0.5 mM). (d) NQO1 substrate assay. Substrate activities of menadione (25µM), APAP (2.5mM), AMAP (2.5mM) and control (DMSO) are shown. All substrate activities were abolished by addition of dicoumarol (10µM). (e) Potential contaminations of degradation products do not explain the NQO2 substrate activity of acetaminophen. NQO2 activities of menadione (250µM), 4-benzoquinone (1 mM), 4-hydroquinone (1 mM) and control (DMSO) are shown. (N = 3 in all panels).

Supplementary Figure 5. NQO2 Western blot of human skeletal muscle tissue lysate.

Supplementary Figure 6. NQO2 inhibition results in modest protection from acetaminophen toxicity. (a) Cellular membrane integrity (left) and cell counts (right) after NQO2 RNAi. HeLa cells were treated with control or NQO2 siRNA for 48h followed by 10h treatment with vehicle (DMSO) or APAP (10mM). (b) Cellular membrane integrity (left) and cell counts (right) after chemical inhibition of NQO2. HeLa cells were treated with control (DMSO), quercetin (10µM), resveratrol (10µM) or imatinib (20µM) for 1h followed by 10h treatment with vehicle (DMSO) or APAP (10mM). Data is normalised to each control for clarity and PI-stain intensity was also normalised to average cell size (N = 3 in all panels).

Table S1. NQO2 Substrate assay, NQO2 Differential Scanning fluorometry assay and Hela cell superoxide production assay results for acetaminophen analogues.
See Supplemental Figure 1 for structures.
All data is presented as values relative to control (DMSO) to ease comparison.
For cell culture experiments (MitoSOX assays), HeLa cells were treated with 2mM analogs for 1h.
For *in vitro* experiments (substrate and thermal shift assays) 2mM analogs were used.

Chemical	**MitoSOX relative to DMSO**		**Substrate assay relative to DMSO**		**Thermal shift**		
	Average (%)	StDev (%)	Average (%)	StDev (%)	Average (ºC)	StDev (ºC)	ΔTm
3-Acetaminophenol	82%	2%	140%	2%	56.24	0.36	4.71
3-(4-Hydroxyphenyl)propionic acid	86%	5%	101%	3%	49.57	0.19	-1.96
4-Aminoacetanilide	91%	12%	124%	3%	54.36	0.58	2.83
4-Aminobenzoic acid	96%	2%	98%	2%	49.89	0.29	-1.64
4-Hydroxyformanilide	100%	7%	148%	2%	55.95	0.40	4.42
DMSO	100%	6%	100%	4%	51.53	0.27	0.00
4-Coumaric acid	108%	7%	105%	2%	53.74	1.16	2.20
4-Hydroxybenzoic acid	122%	6%	101%	0%	49.06	0.95	-2.48
4-Acetamidobenzoic acid	125%	8%	102%	2%	50.08	0.22	-1.45
N-Acetanilide	130%	5%	150%	1%	52.06	0.66	0.52
4-Nitrobenzoic acid	133%	20%	98%	0%	49.70	0.48	-1.84
4-Anisidine	143%	4%	175%	4%	53.14	0.40	1.61
4-Ethoxyacetanilide	144%	20%	155%	4%	55.63	0.62	4.10
4-Ethoxyaniline	162%	2%	187%	7%	53.08	0.11	1.55
4-Methylacetanilide	163%	6%	170%	2%	56.14	0.11	4.61
4-Acetaminophen	166%	4%	123%	3%	54.29	0.27	2.76
2-Acetaminophenol	238%	7%	138%	2%	55.98	0.10	4.44
4-Hydroxybutyranilide	238%	3%	127%	4%	53.52	0.29	1.99
2-Ethoxyphenol	382%	26%	167%	2%	55.06	0.88	3.53
3-Ethoxyphenol	430%	45%	186%	2%	55.28	1.02	3.75
4-Nitroacetanilide	525%	10%	171%	6%	58.95	1.33	7.41
4-Ethoxyphenol	744%	42%	180%	1%	53.27	0.22	1.74
4-Methylphenol	1048%	27%	190%	1%	54.42	0.11	2.89
4-Ethylphenol	1129%	109%	185%	2%	53.29	0.51	2.24

Note, that all acetic derivatives of acetaminophenol show no interaction with NQO2, exept for coumaric acid, which resembles also resveratrol.