Electronic Supplementary Information

Deciphering intracellular targets of organochalcogen based redox catalysts

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Synthesis of compounds 1 and 2

Compounds **1** and **2** have previously been prepared by us. Synthetic procedures have been published in the literature. **1** and **2** were synthesized according to the given procedures.[1,2,3]

Biological studies

All biological studies were done in triplicates except otherwise stated. Data are given as means ± standard deviation (SD).

Cell culture

Cell lines were purchased from DSMZ (Braunschweig, Germany) and grown at 37 °C and 10 % CO_2 in the following media: A-431 cells in RPMI 1640 (Gibco), L-929 cells in DMEM medium (Lonza), PtK2 cells in MEM medium (Gibco), KB-3-1 cells in DMEM medium (Lonza) and HCT-116 cells in McCoy's 5A + GlutaMAX[TM] (Gibco). All media were supplemented with 10 % fetal calf serum (Lonza or Gibco). In all experiments, exponentially growing cells were used.

Cell viability measurement using standard MTT assay

MTT dye was used to measure the metabolic activity of cells which are capable of reducing this dye via active dehydrogenases to a violet formazan product. Briefly, 120 μL aliquots of a cell suspension (100,000 cells/mL) in 96-well microplates were incubated at 37 °C and 10 % CO_2 and allowed to grow for two days. Then 60 μL of serial dilutions of the test compounds were added and incubated for further 18 h. After that, 20 μL MTT in PBS were added to a final concentration of 0.5 mg/mL. After 2 h the precipitate of formazan crystals was centrifuged, and the supernatant discarded. The precipitate was washed two times with 100 μl

PBS and dissolved in 100 μl isopropanol containing 0.4 % hydrochloric acid. The resulting color was measured at 595 nm using a multiplate reader (Synergy H4 Hybrid Multi-Mode Reader, BioTek, Germany).

Cell staining

PtK2 cells were grown on glass cover slips (13 mm diameter) in 4-well plates and incubated with the compounds **1** and **2** (at IC_{90} = 10.5 and 9 μg/mL, resp.) for 18 h. Cells were fixed with 3.7 % paraformaldehyde for 10 min, followed by incubation with 0.1% Triton-X100 for 5 min. Primary and secondary antibodies were incubated for 45 min. Nuclei were stained using DAPI. Cells were washed with phosphate-buffered saline (PBS) between each step.

Cover slips were mounted in anti fade mounting medium (Molecular Probes) and images taken with a Zeiss Axiophot fluorescence microscope using appropriate filter sets. The following antibodies were used for cell staining: rat anti-GRP94, mouse anti-α-tubulin, anti-mouse Alexa Fluor 488 and anti-rat Alexa Fluor 488. The actin filaments were stained with phalloidin Alexa Fluor 594.

DCF assay

This assay is based on the oxidation of the non-fluorescent 2′,7′-dichlorodihydrofluorescein (DCFH) to the highly fluorescent DCF,[4] which is considered to give a general indication of ROS levels, since DCFH reacts with H_2O_2, $ONOO^-$ and lipid hydroperoxides.

Preparation DCFH diacetate stock solution (DCFH-DA). 30 mM DCFH-DA stock solution was prepared by dissolving 50 mg (97 %, Sigma Aldrich) in 3.4 mL DMSO.

Assay procedure. A-431 cells and HUVECs were seeded in black 96-well plates with a density of 10^6 cells/mL in RPMI 1640/EBM-2 medium and treated with different concentrations of test compounds for 1 h. Menadione (Fig. S1) was used as reference compound. The cells were then incubated with DCFH-DA (20 μL, 10 μM) for further 30 min in the dark. The intensity of the resulting fluorescence was read in a multiplate reader (DCF, λ_{ex} = 485 ± 20 nm; λ_{em} = 528 ± 20 nm).

DHE assay

The intracellular ROS levels could also be measured fluorimetrically via oxidation of dihydroethidium (DHE). This assay is used for detection of $O_2^{\bullet-}$ in cells in the presence of other ROS.

DHE stock solution. 30 mM DHE stock solution was prepared by dissolving 10 mg (95 %, Sigma Aldrich) in 1 mL DMSO.

Assay procedure. A-431 cells and HUVECs were seeded in a black-walled 96-well plate at a density of 10^6 cells/mL in RPMI 1640/EBM-2 medium and treated with different concentrations of test compounds for 1 h. The cells were then incubated with 20 μL of DHE for 30 min in the dark. The intensity of fluorescence was read afterwards in a multiplate reader (DHE, $\lambda_{ex} = 540 \pm 25$ nm; $\lambda_{em} = 600 \pm 40$ nm).
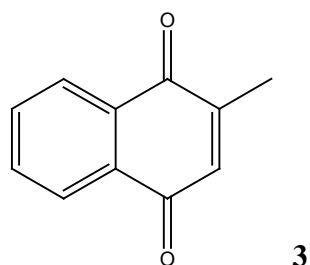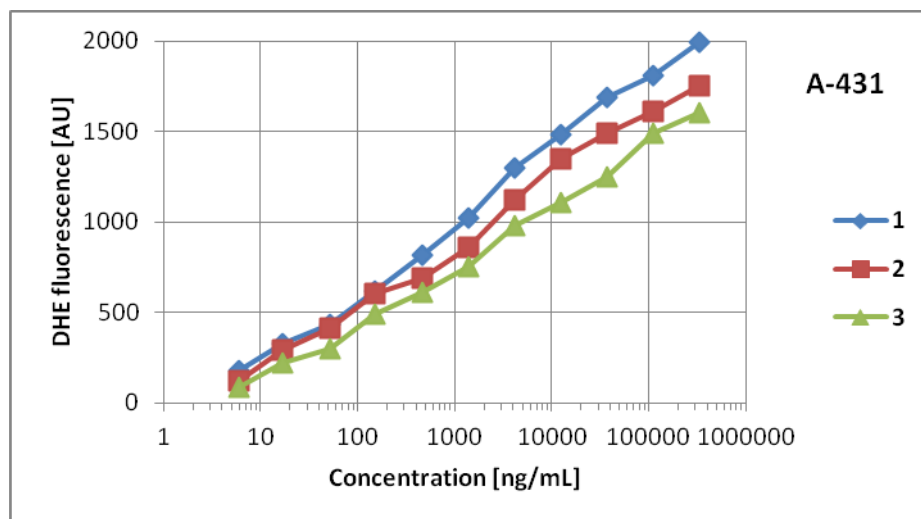

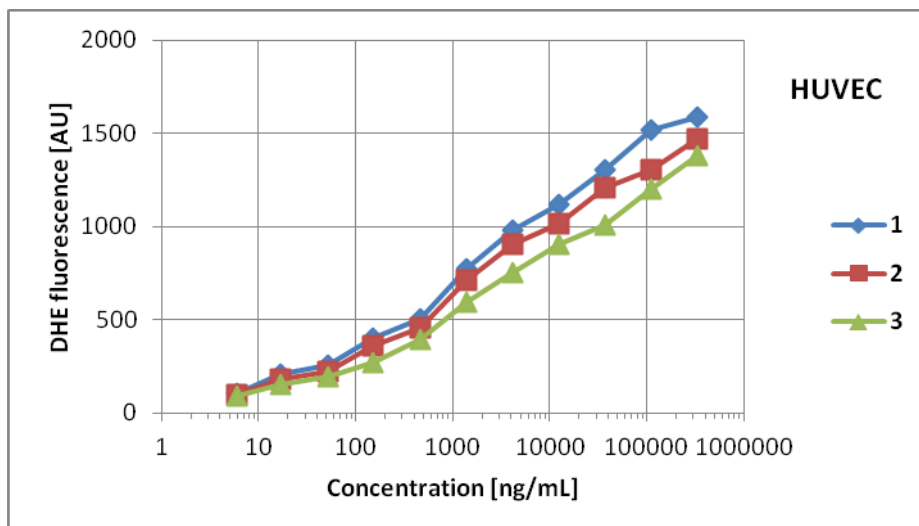**Fig. S1**: Menadione (2-methyl-naphthoquinone)

Fig. S2: Estimation of $O_2^{\bullet -}$ levels in A-431 cells (top) and HUVECs (bottom) incubated with **1**, **2** and **3** by a DHE assay. $O_2^{\bullet -}$ levels increased with increasing concentration of compounds. Curves represent means of triplicates. The SD was < 7 %.

DTNB (5,5'-dithiobis(2-nitrobenzoate)) assay

Ellman's reagent (DTNB, 98 %, Sigma Aldrich) was used to estimate the intracellular levels of thiols (primarily glutathione).[5]

Preparation of stock solutions:

a) Miller's phosphate buffer solution: NaH_2PO_4 solution (5.3 mL, 0.2 M) and Na_2HPO_4 solution (94.7 mL, 0.2 M) were mixed together. The resulting buffer was then diluted to 0.1 M (pH = 8) by adding an equal volume of distilled H_2O.

b) L-Glutathione stock solution (GSH): A 10 mM stock solution of GSH was prepared by dissolving 50 mg L-glutathione (98 %, Sigma Aldrich) in 16 mL DMSO.

c) DTNB stock solution: A 0.04 mM stock solution of DTNB was prepared by dissolving 50 mg of 5,5'-dithiobis(2-nitrobenzoate) (98 %, Sigma Aldrich) in 3 mL DMSO.

Assay procedure. A-431 cells (10^6 /mL) were treated with different concentrations of test compounds for 1 h at 37 °C. The cells were removed by mild trypsinization, centrifuged at 800 rpm for 5 min, washed twice with cold PBS and lysed using 5 % w/v chilled metaphosphoric acid at 4 °C for 2 h to extract cellular GSH. The suspension was then centrifuged at 13,000 rpm. The supernatant was mixed with 0.2 M sodium phosphate buffer (pH 8.0) and 0.04 mM DTNB and kept at room temperature for 10 min. The absorbance of the samples was measured in a multiplate reader at 412 nm, and the GSH levels caculated using a standard curve.
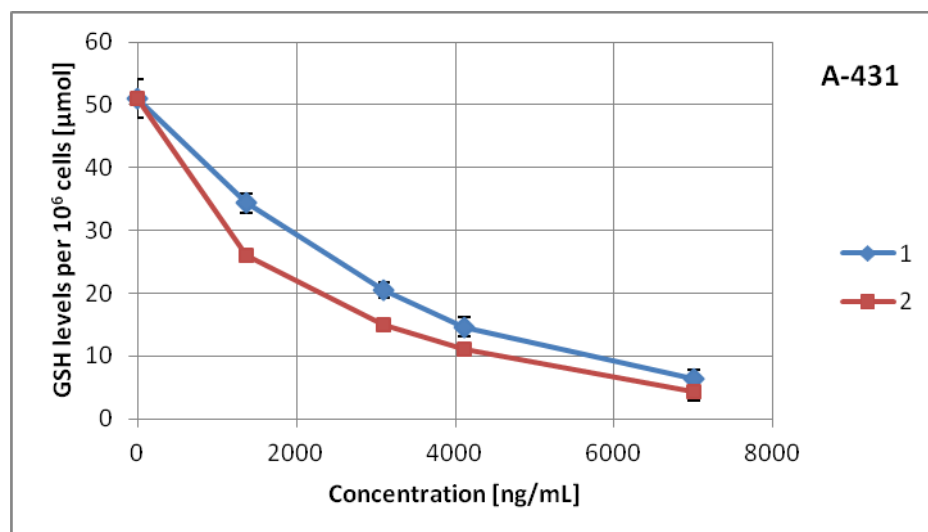

Fig. S3: Assessment of intracellular GSH in A-431 cells. Intracellular levels of reduced GSH in presence of **1** and **2** decreased in a dose-dependent manner. The symbols represent means of triplicates ± SD.

Assessment of metabolic activity (ATP-quantification) as a measure of cell viability

The toxicity of the test compounds was determined using the Cell Titer Glo Luminescent Cell Viability Assay Kit (Promega, Germany). Assays were performed according to the manufacturer's instructions.

Briefly, the assay measures the number of viable cells, based on the quantification of ATP as an indicator of metabolic activity. Lypophilized enzyme/substrate mixture was reconstituted with the provided buffer. Then, an equal volume of the reaction buffer was added to the medium containing A-431 cells (untreated or treated with the indicated compounds). The mixture was shaken on a rocking platform for 2 min, incubated for further 10 min in the dark at RT. The luminescence signal, proportional to the amount of ATP present, was quantified by using a multiplate reader (Synergy H4 Hybrid Multi-Mode Reader, BioTek, Germany). Data were normalized to the control and reported as percentage of viable cells.
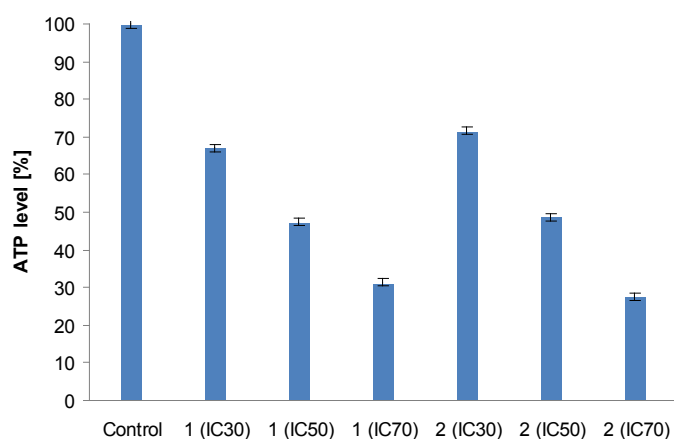

Fig. S4: Influence of compound **1** and **2** on cell viability. ATP-level of A-431 cells decreased with increasing concentration of compound (IC_{30} = 0.5 and 1.4 µg/mL; IC_{50} = 1.9 and 2.5 µg/mL; IC_{70} = 4.1 and 3.9 µg/mL). Bars show means of triplicates ± SD.

Apoptosis

Caspase 3/7 assay. Caspase activities were determined using the homogeneous, luminescent caspase-Glo 3/7 kit (Promega, Germany) which measures caspase 3 and 7 activity. A-431 cells were seeded in white 384 well plates at a density of 20,000 cells per well and incubated with 2.6 µM of the compounds at 37 °C. After different time points the Caspase-Glo 3/7 reagent was added and the cells were incubated at room temperature for a further 30 min in the dark. The luminescence was measured in a multiplate reader then (Synergy H4 Hybrid Multi-Mode Reader, BioTek, Germany).
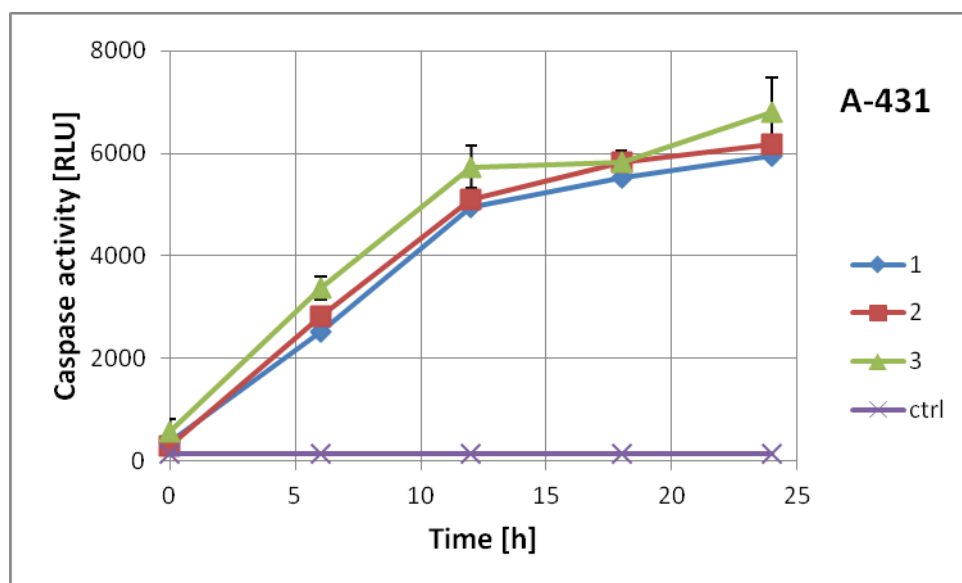

Fig. S5: Caspase 3/7 activity in A-431 cells increased significantly in a time dependent manner when incubated with **1**, **2**, and **3**. The symbols represent means of triplicates ± SD.

Annexin V measurements. Apoptosis was assessed by flow cytometric analysis of A-431 cells treated with different concentrations of test compounds for 16 h using the FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen, Germany).

A-431 cells (10^6 cells/mL) were treated with IC_{90} concentrations (**1**: 10 µg/mL; **2**: 12 µg/mL) of test compounds for 16 h. After incubation cells were suspended in 1 mL binding buffer and washed two times with PBS buffer. After washing 100 µL of the suspension was stained with a mixture of 5 µL FITC annexin V and 5 µL PI. After 15 min of incubation, 400 µL of binding buffer was added and the samples were analysed by flow cytometry after a further 30 min.

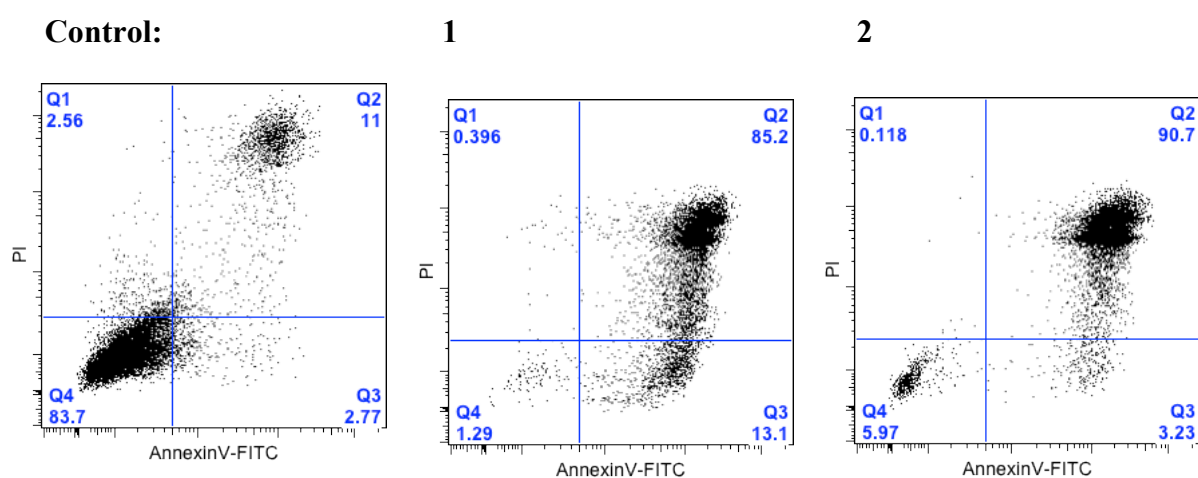**Control:**   **1**   **2**

Fig. S6: Flow cytometric analysis of apoptosis induction by **1** and **2** in A-431 cells. Quadrant 1 (Q1) shows the percentage of dead cells, quadrant 2 (Q2) the percentage of late apoptotic cells, quadrant 3 (Q3) the percentage of early apoptotic cells and quadrant 4 (Q4) the percentage of viable cells.

Microbiological activity spectrum

The potential antibacterial and antifungal activity of the compounds were tested by agar diffusion (halo) assays.

Growing bacteria: Bacteria were grown in EBS medium: A mixture of proteose peptone (8 g, Roth), bacto peptone (5 g, BD), dry meat extract (1 g, Merck), HEPES buffer (10 g, Roth) and bacto yeast extract (1 g, BD) were suspended in 1 L deionized H_2O. The pH was adjusted to 7.

Growing fungi: We used medium 90 for all fungi. A mixture of malt extract (30 g, BD) and bacto peptone (3 g, BD) was suspended in 1 L deionized H_2O. The pH was adjusted to 5.6 using acetic acid. Hyphal fungi were grown on medium 90 agar plates for formation of spores, which were harvested by rinsing and seeded into liquid agar medium for halo assays according to experience.

Assay procedure: The bacteria or yeasts were grown on standard growth media and seeded into liquid agar medium (containing 15g/L bacto agar; BD) to a final optical density of 0.01 (bacteria) or 0.1 (yeasts). Paper discs of 6 mm diameter soaked with 20 μL of compounds dissolved in DMSO (stock concentration: 1000 μg/mL) were added to the agar plates and incubated at 30 °C. The bacteria growth was observed after 1 and 2 days. The diameter of the resulting inhibition zones were used as a measure for antibacterial activity.

Bacteria	E. coli tolC	E. coli A1	Klebsiella pneumoniae	Pseudomonas aeruginosa	Staph. aureus	Micrococcus luteus	Mycobacterium phlei
DMSO	<7	<7	<7	<7	<7	<7	<7
1	9±1.1	11±1.2	10±1.0	<7	13±1.4	8±0.7	13±1.2
2	8±0.7	11±1.4	8±0.6	<7	<7	n.d.	11±1.0

Fungi	Hansenula. anomala	Botrytis cinerea	Pythium. debaryanum	Aspergillus niger
DMSO	<7	<7	<7	<7
1	7±0.5	11±1.2	15±1.4	11±0.9
2	<7	<7	7±0.6	<7

Table S1: Diameters of inhibition zones [mm] of agar diffusion assays with a variety of bacteria and fungi. Data represent means of triplicates ± SD.

Impedance measurements

The impedance measurements were performed with small modifications on a RT-CES system (xCelligence) from Acea Biosciences (Roche), which has been described previously.[6,7,8] For time-dependent cell response profiling, 60 μL of media was added to 96-well E-Plate to obtain background readings followed by the addition of 120 μL cell suspension of L-929 cells. After each step, the E-Plates were incubated for 30 min at room temperature and then placed on the reader in the incubator for continuous recording of impedance as reflected by cell index. After 24 h of incubation the cells were treated with the compounds. To prepare the compounds for screening each stock solution (10 mM in DMSO) was diluted with cell media to get a final test concentration of the IC_{90} and less than 0.1 % DMSO. 1 μL of each prepared solution was then transferred into the 96-well E-Plate. Each E-plate contained also wells with DMSO only as a solvent control. All measurements were performed in triplicates and run for 5 d. The time-dependent cellular response profiles (TCRP) were recorded by the Roche RTCA Software, Version 1.2. Data processing and mining workflow was implemented in the statistical programming language R, Version 2.12.2 (R Development Core Team, 2011). The following additional R packages were used in addition: class[9], gplots[10] and MASS[9]. For the development of the R code the integrated development environment R Studio, Version 0.94.92, was used. The workflow starts by importing the raw impedance data which is provided by the RTCA software as cell index (CI) data. The CI is already background corrected and is calculated as follows (Eq. 1):[11]

Eq. 1:
$$CI(t) = \frac{R_t - R_b}{Z_n}$$

where CI(t) is the cell index at time point t, R_t is defined as measured electrode impedance of the well with the cells in the medium at a certain time point and R_b as measured background impedance of the well with the cell medium alone. Z_n is a frequency factor which corrects for different frequencies of the alternating voltage the xCelligence system can use, the standard setting is $Z_n=15$. The raw data were imported into R and normalized as suggested by Abassi and colleagues by dividing the cell indices for each time point after compound addition by the cell index at a reference time point (Eq. 2).[12] As reference time point the last measurement before compound addition was taken. For the further analysis, only the measurements starting at the reference time point (with normalized cell index = 1) and later were considered.

Eq. 2:
$$NCI = \frac{CI_t}{CI_{t(reference)}}$$

The reference compounds and the test compounds with unknown mode of action were measured as triplicates which were randomly distributed over the microtiter plates (using sampling without replacement in R) to avoid batch effects.

Detection and removal of outliers was carried out using the median polishing procedure. The central idea of the data mining concept is to use cubic smoothing splines for the approximation of the impedance data and as dimension reduction technique. This approach has the benefit of avoiding the curse of dimensionality and the Runge phenomenon that occurs for high polynomials, while keeping the complexity of the data set. The smooth.spline function of R was used for TCRP approximation. As set of descriptors the spline basis coefficients were extracted to construct a distance matrix that was used for hierarchical cluster analysis. A heatmap was constructed that displays the Z-transformed values of the 22 descriptors (= basis spline coefficients). Hierarchical cluster analysis of the reference compounds together with the compound of unknown mode of action was carried out. Co-clustering of the compound of unknown mode of action with reference compounds with known activity class label is used to predict the mode of action.

Chemogenetic assay

Mutants of *S. cerevisiae* generated by the European Archive for Functional Analysis (EUROSCARF) were grown on standard YPD medium and seeded into liquid agar medium 90 to get a final optical density of 0.1 (measured at 548 nm). Paper discs of 6 mm diameter soaked with 20 μl of compounds dissolved in DMSO (stock concentration: 1000 μg/mL) were added to the agar plates and incubated at 30 °C. The yeast growth was observed after 1 and 2 days. The diameter of resulting inhibition zones is given as a measure of anti-microbial activity.

ROS related mutants:

	BY4741 wild type	YHR008C	YDR032C	YHL028W	YLL060C
DMSO	<7	<7	<7	<7	<7
1	<7	16±1.2	13±1.1	17±1.2	18±1.3
2	<7	18±1.4	15±1.3	15±0.9	13±0.7

	YPL188W	YGL158W	YOL049W	YLL009C
DMSO	<7	<7	<7	<7
1	15±1.1	14±0.8	13±1.2	12±0.7
2	14±0.8	12±1.2	11±0.7	14±0.9

Microtubule cytoskeleton related mutants:

	BY4741 wild type	YEL003W	YER007W	YOR349W	YNL153C
DMSO	<7	<7	<7	<7	<7
1	<7	8±0.6	<7	<7	8±0.5
2	<7	<7	<7	<7	<7

	YBL007C	YCL029C	YDR150W	YLR200W	YGR078C
DMSO	<7	<7	<7	<7	<7
1	<7	9±0.6	6±0.5	<7	<7
2	<7	<7	<7	<7	<7

	YPR141C	YML094W	YMR138W	YOR265W
DMSO	<7	<7	<7	<7
1	<7	7±0.6	<7	<7
2	<7	<7	<7	<7

Actin cytoskeleton related mutants:

	BY4741 wild type	YNL271C	YIL034C	YHR129C	YOR129C	YOR239W
DMSO	<7	<7	<7	<7	<7	<7
1	<7	<7	<7	<7	7±0.5	<7
2	<7	<7	<7	<7	10±0.9	<7

	YLR085C	YLR227C	YKL007W	YKL212W	YLR337C
DMSO	<7	<7	<7	<7	<7
1	<7	8±0.5	<7	7±0.6	<7
2	<7	9±0.6	<7	8±0.5	<7

	YNL020C	YPR124W	YFL031W
DMSO	<7	<7	<7
1	<7	6±0.5	<7
2	<7	8±0.5	<7

Table S2: Activity of compounds **1** and **2** against *S. cerevisiae* wild type and mutant strains. Diameters of inhibition zones of agar diffusion assays are given [mm]. The data represent means of triplicates ± SD.

Polymerisation assays *in vitro*

Tubulin purification. Microtubule proteins were purified from porcine brain homogenates by using standard procedures that comprised two to three cycles of temperature-dependent polymerisation and depolymerisation.[13] Microtubule proteins are composed of tubulin and microtubule-associated proteins (MAPs).

Tubulin polymerisation assay. Tubulin polymerisation was monitored by using turbidometry.[14] Samples (200 µL) in PEM polymerisation buffer (0.1 M PIPES, pH 6.6, 1 mM EGTA, 1 mM $MgSO_4$, and 1 mM GTP) were rapidly heated to 37 °C in a water-jacketed cuvette holder of a diode array photometer (Beckman Spectrophotometer DU 7500). Absorbance at 350 nm was measured in the absence and presence of the compounds.

Actin polymerisation assay. Actin polymerisation was measured *in vitro* using the Actin Polymerisation Biochem Kit from Cytoskeleton which uses skeletal muscle actin from rabbit. The polymerisation buffer contained KCl (50 mM) and $MgCl_2$ (2 mM).
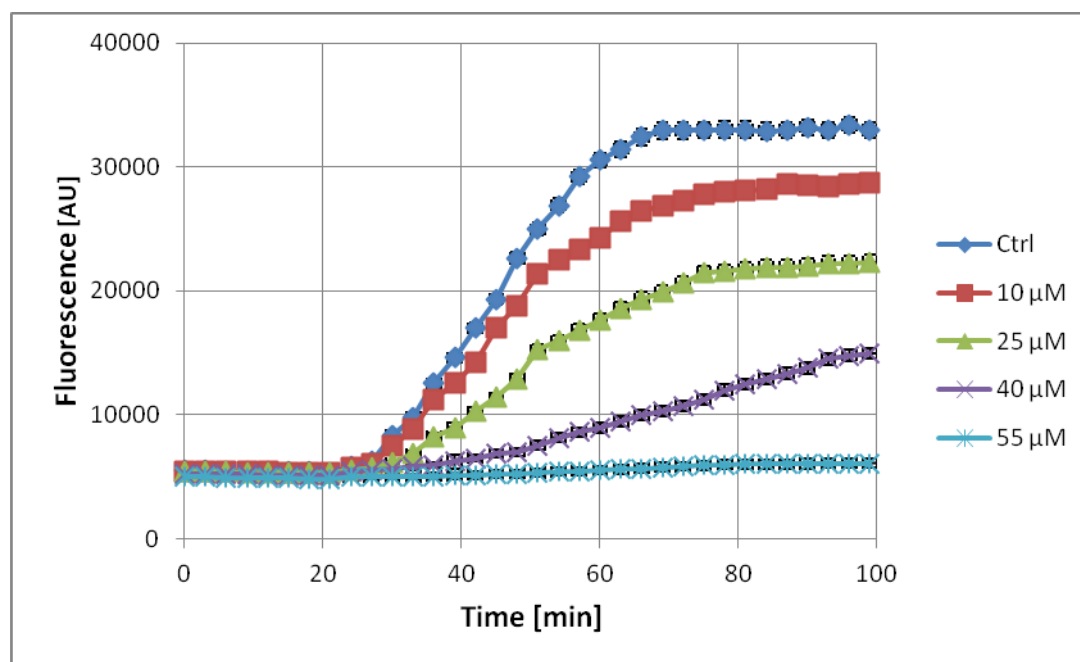

Fig. S7: *In vitro* polymerisation assay with pyrene actin in the presence of different concentrations of **2**. Symbols represent means of triplicates ± SD.

DARTS (drug affinity response target stability) and western blot analysis

For confirming the drug-protein interactions DARTS followed by western blot analysis were carried out.[15] Briefly, KB-3-1 cells were lysed with MPER$^{®}$ lysis buffer (Thermo Scientific, Germany) on ice for 15 min. The lysate was centrifuged at maximum speed (14,000 rpm) for 10 min and the supernatant was collected. Protein was measured using Pierce$^{®}$ BCA Protein Assay Kit (Thermo Scientific, Germany).

The protein lysates (50 µg/mL) were incubated with various concentrations of **1** and **2** (IC$_{50}$ = 0.5 and 0.14, IC$_{90}$ = 3.8 and 3.9 µg/mL, resp.) on ice for 2 h. The following positive and negative controls were used: tubulysin A (inhibitor of tubulin polymerisation), chivosazol A (inhibitor of actin polymerisation) and cycloheximide (protein translation inhibitor; negative control). After incubation the protein solutions were digested with pronase (0.07 %) in pronase buffer (25 mM Tris-HCl, pH 7.5; 10 mM EDTA; 0.5 % SDS) for 30 min at 37 °C. Then loading dye was added to the samples and incubated at 95 °C for 10 min. The protein solutions were separated by SDS-PAGE (10 %) and then transferred to nitrocellulose membranes, which were incubated with the following primary antibodies: mouse anti-alpha-tubulin (Cell Signalling, The Netherlands), rabbit anti-beta-actin (Cell Signalling, The Netherlands), rabbit anti-GAPDH (Cell Signalling, The Netherlands) followed by incubation with the corresponding secondary antibodies: anti-mouse HRP-linked antibody (Cell Signalling, The Netherlands) or anti-rabbit HRP-linked antibody (Cell Signalling, The Netherlands). Specific immunoreactive proteins were visualized using the SuperSignal$^{®}$ West Pico Chemiluminescent Substrate (Thermoscientific, Germany) and recorded on an autoradiography film (Amersham Hyperfilm ECL).
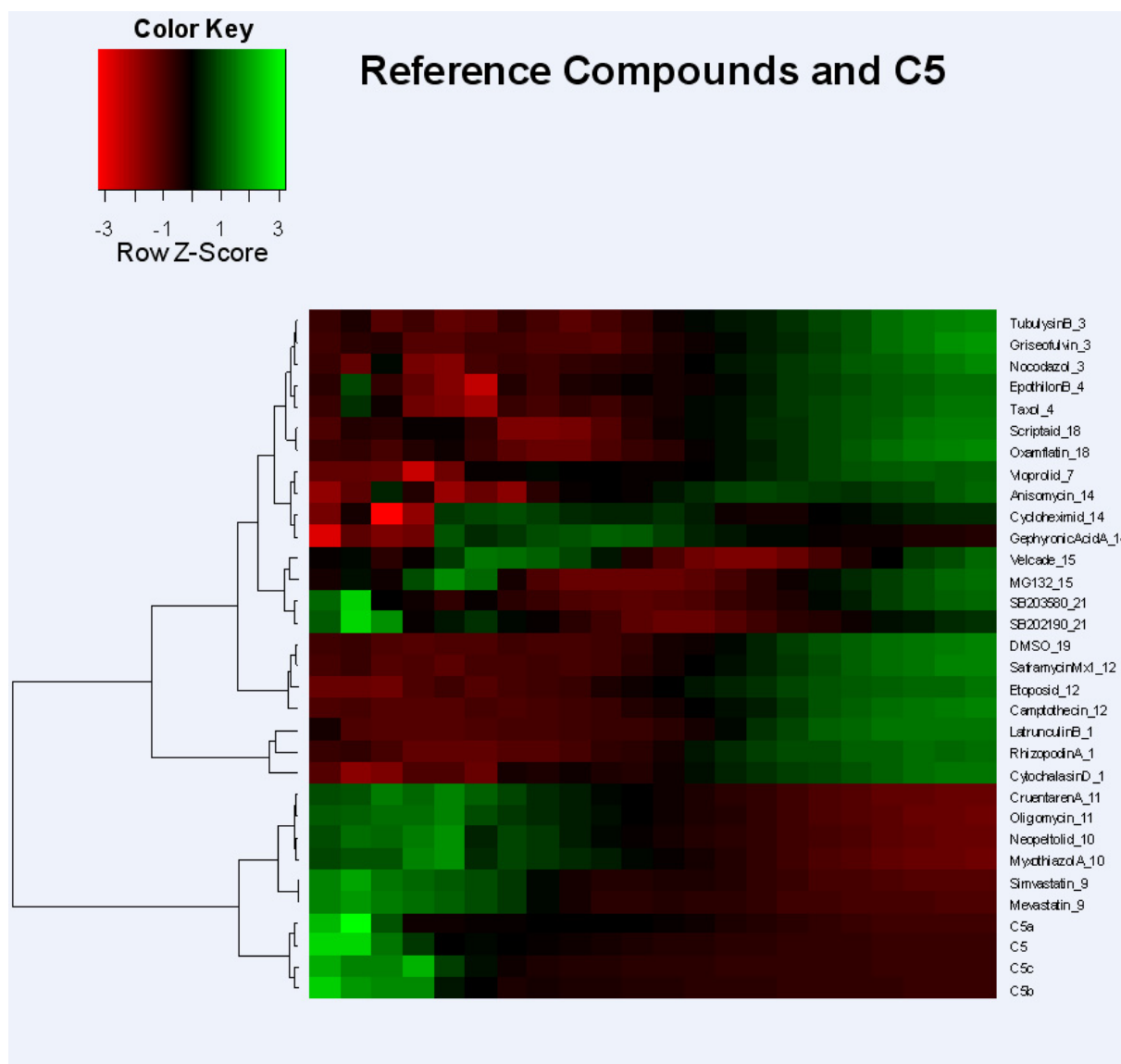
Fig. S8: Hierarchical cluster analysis of data from impedance curves obtained with L-929 cells that were incubated with 4 samples of compound **2** (C5, C5a to c). The basis of the cluster analysis were means of triplicate curves for each sample.

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