Supporting Information

Ren et al. 10.1073/pnas.1014275108

SI Materials and Methods

Cell Culture, Chemicals, Antibodies, Transfection, and Immunoblot Analysis. A549, HeLa, MDA-MB-231, and Ishikawa cell lines were purchased from the American Type Culture Collection. The SPEC-2 cell line was reported previously (1). A549-V and A549-K were stably transfected with an empty vector or a vector containing Keap1-CBD, respectively, which were established and characterized by our laboratory (2). All of the chemicals used in this study, including cisplatin, pacilitaxel, carboplatin, and etoposide were purchased from Sigma. An Nrf2 antibody from Abcam or from Santa Cruz Biotechnology was used for immunohistochemistry or for immunoblot analysis, respectively. The antibodies for Keap1, NQO1, MRP1, MRP2, γ-GCS, Keap1, IκBα, c-Myc, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were purchased from Santa Cruz Biotechnology. The CBD antibody (New England Biolabs), the Ub antibody (Sigma), p65, P-p65, and Stat-3 antibodies (Cell Signaling), the β-catenin antibody (BD Biosciences), and the Ki67 antibody (Vector Lab) were all purchased from commercial sources. Transfection of cDNA was performed using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. For immunoblot analysis, cells were lysed in sample buffer (50 mM Tris, 2% SDS, 10% glycerol, 100 mM DTT, and 0.1% bromophenol blue). For tissue immunoblot analysis, frozen tissues were homogenized in lysis butter [0.1 M Tris buffer (pH7.4), 0.1 mM EDTA] in the presence of 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture. Protein concentration was determined using the Bio-Rad DC protein assay kit. Proteins were denatured by heating for 10 min before being eletrophoresed through SDS polyacrylamide gels, transferred to nitrocellulose membranes, and subjected to immunoblot analysis.

Animal Treatment. Athymic nude mice were purchased from Harlan Laboratories. Mice at age of 4–6 wk were injected with A549 (200 μL, 1×10^7 cells) in the subdermal space on the medial side of the thigh. Once tumors reached the desired size, mice were randomly allocated into four groups and treated i.p. with DMSO, cisplatin (2 mg/kg), brusatol (2 mg/kg), or in combination every other day for a total of five times. For another experiment, the five treatments were repeated with a 1-wk interval between the two treatments. Tumor volume (mm^3) was measured semiweekly and calculated using the formula $(a \times a \times b)/2$ (a, the smallest diameter; b, the largest diameter). At the end of the experiments, mice were killed and tumors were dissected and weighed. Formalin-fixed, paraffin-embedded tumor tissue sections were used for IHC and TUNEL analysis, whereas snap-frozen tissues were subjected to immunoblot analysis.

Reporter Gene Assay. MDA-MB-231-ARE-Luc, a stable cell line established in our laboratory, was used for initial screening for Nrf2 inhibitors (3). Cells were treated with brusatol for 16 h and lysed in passive lysis buffer (Promega). Luciferase activity was measured using an assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 500 μM ATP, 250 μM luciferin, and 250 μM CoA) and the Synergy 2 plate reader (BioTek). The reporter gene assay was carried out in triplicate and mean ± SD was calculated.

For the dual luciferase reporter gene assay, A549 cells were transfected with a vector for ARE- or κB-dependent firefly luciferase, along with a vector for TK renilla luciferase as a control for transfection efficiency. At 32 h posttransfection, cells were treated with different doses of brusatol for 16 h. Cell lysates were used to measure firefly and renilla luciferase activity using the Promega dual-luciferase reporter gene assay system according to the manufacturer's instructions. Results are presented as firefly luciferase activity normalized to renilla luciferase activity. Experiments were repeated three times, each in triplicate. Results are expressed as mean ± SD.

In Vivo Ubiquitination and Pulse-Chase Analysis. To detect ubiquitin-conjugated endogenous Nrf2 or Keap1, cells were left untreated or treated with 40 nM of brusatol for 4 h. Cells were lysed and boiled in a buffer containing 2% SDS, 150 mM NaCl, 10 mM Tris-HCl, and 1 mM DTT. After dilution of the lysate to 0.5% SDS, the lysate was incubated with an Nrf2 or Keap1 antibody for 30 min. Protein A beads (Invitrogen) were added to allow binding overnight at 4 °C. Immunoprecipitated proteins were analyzed by immunoblot analysis with an ubiquitin antibody (Sigma). The band intensities of ubiquitinated Nrf2, ubiquitinated Keap1, Nrf2, and Keap1 were quantified using Quantity One (Bio-Rad). Data shown is ubiquitinated Nrf2 normalized to total amount of Nrf2, or ubiquitinated Keap1 normalized to total amount of Keap1.

To measure the half-life of Nrf2, pulse-chase analysis was used. A549 cells were left untreated or treated with brusatol for 4 h. Cells were labeled with DMEM containing 100 Ci of [^{35}S]methionine and [^{35}S]cysteine supplemented with 10% dialyzed FBS for 45 min. The labeling medium was replaced with complete growth medium. Cell lysates were collected in RIPA buffer following the indicated chase periods and subjected to immunoprecipitation with an Nrf2 antibody. The immunoprecipitated proteins were electrophoresed through a 7.5% SDS-polyacrylamide gel and visualized by fluorography. The band intensity was quantified using Quantity One (Bio-Rad) and plotted on a semilog graph.

Cell Viability Assay, Colony Formation Assay, Apoptotic Cell Death, and Cell Cycle Analysis. Cell viability was measured by the xCELLigence system (Roche), which monitors cell growth in response to treatment in real-time. Cells grow on top of electrodes so that the impedance varies on the basis of the number of cells attached and the quality of cell–electrode interaction. Electrode impedance, which is displayed as cell index, can be used to monitor cell viability. A549 cells (8,000 per well) were seeded overnight and then treated with each chemical alone or in combination, and cell growth was monitored.

To assess colony formation, complete DMEM containing 0.6% low-melting-point (LMP) agarose was added to a 6-well culture plate as a base agar. A549 (2,000 per well) cells in DMEM containing 0.3% LMP were placed over base agar and allowed to harden at room temperature. Media containing PBS, brusatol, cisplatin, or combination were added on top of the agar. Media was changed every week. The cells were allowed to grow for 4 wk before staining with crystal violet. Visible colonies were counted. The results represent the average from three independent experiments with duplicate samples.

For measurement of apoptotic cells in tissue, an in situ cell death detection kit (Roche) was used for detecting apoptotic cell death in tumor tissue according to the manufacturer's instructions and analyzed under a fluorescence microscope (Zeiss Observer Z1, Marianas digital microscopy workstation). For cultured cells, A549 cells were treated with brusatol and apoptotic cells were detected using Annexin V-FITC apoptosis detection kit (Sigma) and analyzed by flow cytometry. For cell cycle analysis, A549 cells were treated with brusatol. A total of 1×10^6 cells were incubated with RNase A and PI before analysis using flow cytometry.

Glutathione Level and Intracellular Cisplatin Concentration. The intracellular glutathione concentration was measured using a QuantiChrom glutathione assay kit from BioAssay Systems and all of the procedures were followed according to the manufacturer's instructions (4). All of the experiments were done in triplicate and results are presented as mean ± SD.

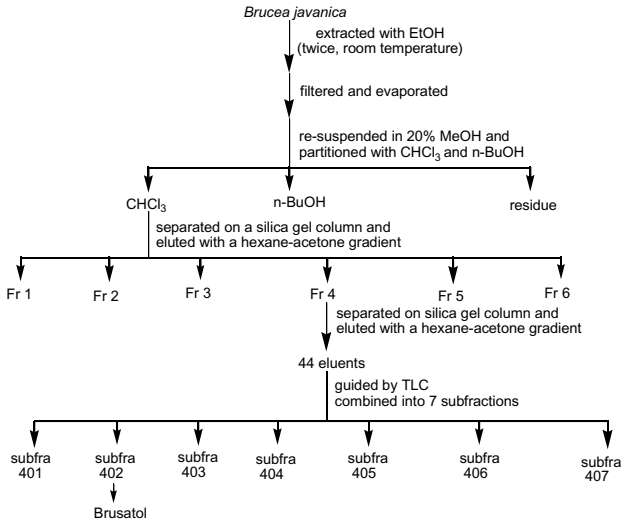
A549 cells were pretreated with brusatol for 16 h. A total of 200 μM of cisplatin was added and cells were further incubated for 2 h. Cells were washed three times and cell pellets were used for the following analysis. Analysis of platinum was carried out using an AAnalyst 600 atomic absorption spectrophotometer with a wavelength of 265.9 nm. The THGA graphite furnace technique was used and the Zeeman-effect background correction was included. The HCL lamp current was set at 30 nm as recommended. Platinum standard was purchased from PerkinElmer (N9300140, 1,000 mg/L in 10% HCl). The standard working solutions were prepared fresh. Experiments have been performed to set up calibration curves for A549 cell pellets analysis. A549 cell pellets were digested in 0.15 mL of concentrated HNO_3 overnight. Then 0.05 mL of 10% H_2O_2 solution was added. After further digestion for 30 min at 70 °C, the samples were cooled and diluted to 1.25 mL with Nanopure water. An aliquot of the diluted sample (0.2 mL) was mixed with an equal volume of water and two volumes of the diluent (containing 0.4% HNO_3 and 0.2% Triton X-100). Twenty microliters of the mixed sample was injected onto the AA system. Triplicate readings were taken for all standards and samples. A calibration curve using A549 cell pellet (blank) was prepared, which was linear from 250 ng to 5,000 ng. The HGA sensitivity check, or the characteristic mass (m_0), was 300 pg for platinum. The amount of platinum determined from the assay was converted to the amount of cisplatin, on the basis of the differences in molecular weight. Results are presented as mean (ng/10^6 cells) ± SD.

Immunohistochemical Analysis. Immunohistochemical (IHC) analysis for Nrf2 or Ki67 was done as previously described (5). Sodium citrate buffer (pH 6) was used as an antigen retrieval solution. Tissue sections were treated with 0.3% peroxidase to quench endogenous peroxidase activity. Next, tissue sections were incubated with 5% normal goat serum for 30 min followed by a 2-h incubation with Nrf2 or Ki67 at 1:100 dilution at room temperature (RT), followed by 1 h sequential incubation with a biotinylated secondary antibody and ABC kit (Vector Lab) at 1:100 dilution at RT. Finally, tissue sections were developed for 30 s using the 3,3′-diaminobenzidine staining kit (DAKO), and counterstained with hematoxylin.

Statistical Analysis. Experiments were conducted in triplicate and data are presented as mean ± SD. Statistical tests were done with SPSS 10.0. Unpaired Student's *t* tests were used to compare the means of two groups. One-way ANOVA was applied to compare the means of three or more groups. $P < 0.05$ was considered to be significant.

1. Jiang T, et al. (2010) High levels of Nrf2 determine chemoresistance in type II endometrial cancer. *Cancer Res* 70:5486–5496.
2. Wang XJ, et al. (2008) Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. *Carcinogenesis* 29:1235–1243.
3. Du Y, et al. (2008) Oridonin confers protection against arsenic-induced toxicity through activation of the Nrf2-mediated defensive response. *Environ Health Perspect* 116: 1154–1161.
4. Chen W, et al. (2009) Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. *Mol Cell* 34:663–673.
5. Jiang T, et al. (2010) The protective role of Nrf2 in streptozotocin-induced diabetic nephropathy. *Diabetes* 59:850–860.

A

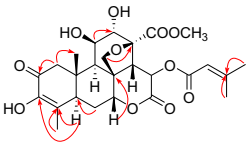


B

The structure of brusatol was elucidated based on spectroscopic methods, including NMR and MS. Brusatol was obtained as a colorless crystal. The molecular formula was determined as $C_{26}H_{33}O_{11}$ based on the HR-ESI-MS ($C_{26}H_{33}O_{11}H$ requires m/z 521.20174, found 521.20126). ^{1}H NMR (600 MHz, DMSO-d_6, TMS) δ1.22 (3H, s, H-18), 1.72 (3H, d, J = 1.8 Hz, H-19), 1.69 (1H, m, H-6α), 1.90 (3H, d, J = 1.2 Hz, H-5'), 2.07 (1H, m, H-6β), 2.10 (3H, d, J = 1.2 Hz, H-4'), 2.15 (1H, d, J = 4.2 Hz, H- 9), 2.62 (2H, d, J = 15.6 Hz, H-1), 2.88 (1H, brd, J = 13.2 Hz, H-5), 3.59 (1H, d, J = 7.8 Hz, H-20α), 3.60 (3H, s, H-23), 3.96 (1H, t, J = 4.8 Hz, H-11), 4.48 (1H, d, J = 7.8Hz, H-20β), 4.86 (1H, d, J = 4.8 Hz, H-12), 4.92 (1H, brs, H-7), 5.61 (1H, s, H-2').

^{13}C NMR (150 MHz, DMSO-d_6, TMS) δ 13.2 (C-19), 15.0 (C-18), 19.9 (C-4'), 26.9 (C-5'), 28.6 (C-6), 40.5 (C-5), 40.5 (C-9), 41.1 (C-10), 44.7 (C-8), 48.7 (C-1), 52.0 (C-23), 71.4 (C-11), 72.2 (C-20), 74.5 (C-12), 81.2 (C-13), 82.5 (C-7), 114.8 (C-2'), 128.3 (C-4), 144.2 (C-3), 158.2 (C-3'), 164.1 (C-1'), 169.9 (C-16), 169.9 (C-22), 192.7 (C-2).

The key HMBC correlations between H and C are shown below.



Key HMBC (H→C) for Brusatol

Fig. S1. (*A*) A schematic procedure for brusatol isolation. (*B*) Structure elucidation of brusatol by NMR and high-resolution mass spectrometry.

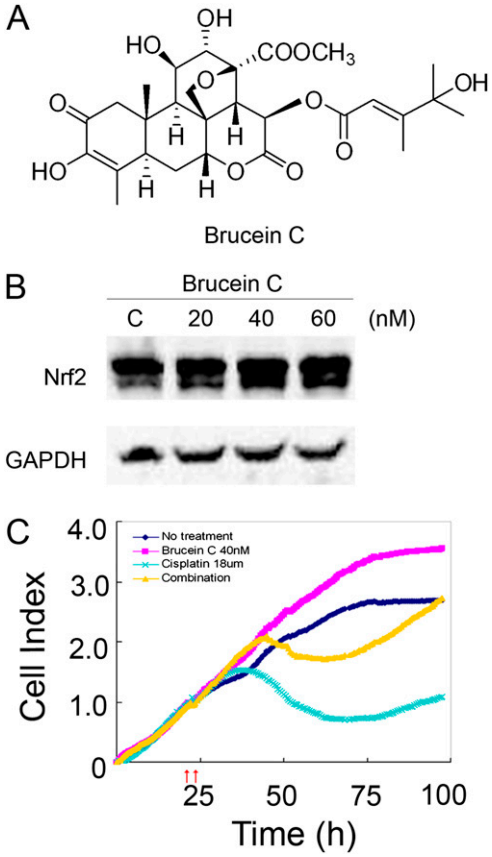


Fig. S2. Brucein C had no effect on the protein level of Nrf2 and did not sensitize A549 cells to cisplatin. (*A*) Structure of brucein C. (*B*) Brucein C did not reduce the protein level of Nrf2. A549 cells were treated with the indicated doses of brucein C for 16 h. (*C*) Brucein C did not sensitize A549 cells to cisplatin treatment. A549 cells were pretreated with 40 nM of brucein for 4 h before the addition of DMSO or 18 μM of cisplatin.

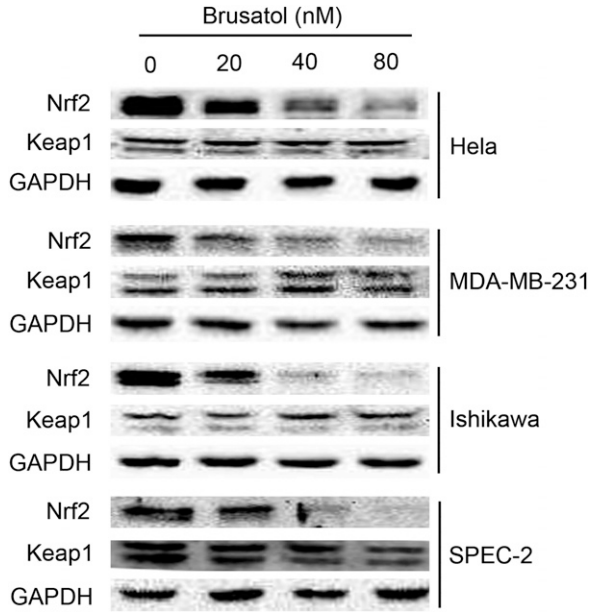


Fig. S3. Brusatol reduced Nrf2 protein levels in HeLa, MDA-MB-231, Ishikawa, and Spec-2 cancer cell lines. Cells were treated with the indicated doses of brusatol for 4 h and subjected to immunoblot analysis.

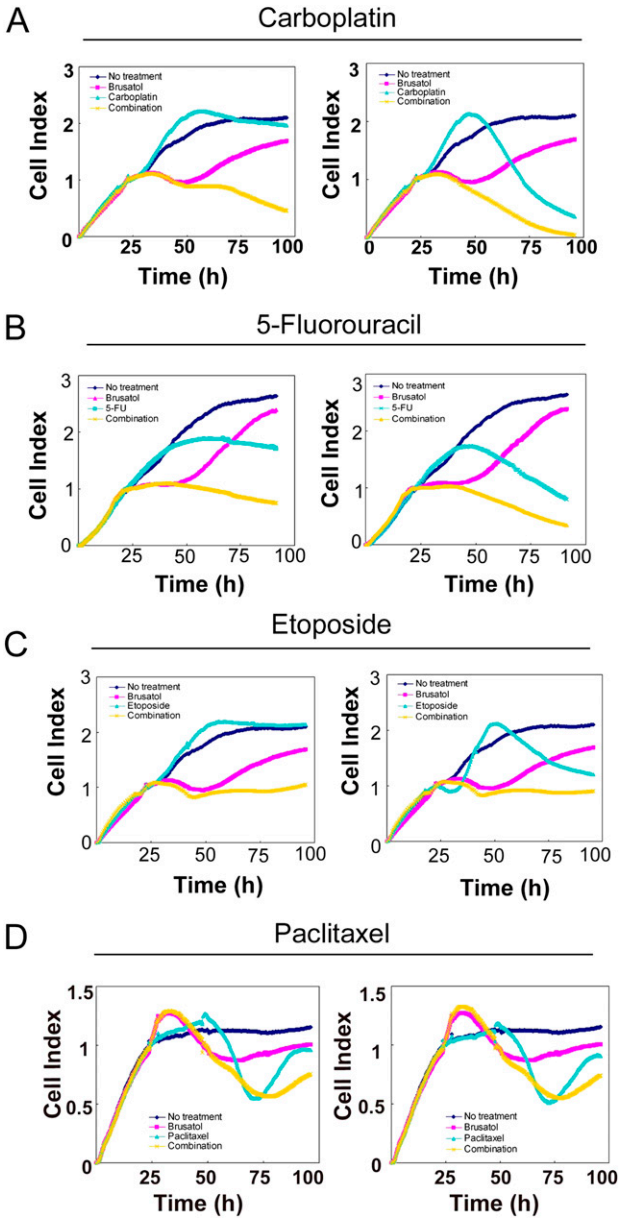


Fig. S4. Brusatol sensitized A549 cells to other chemotherapeutic drugs such as carboplatin, 5-fluorouracil, etoposide, and pacilitaxel. The xCELLigence system (Roche) was used to monitor cell growth in real time. A549 cells (8,000 per well) were seeded overnight. (*A*) A549 cells were treated with 40 nM of brusatol for 4 h before the addition of DMSO or carboplatin (80 μM, *Left* and 160 μM, *Right*). (*B*) A549 cells were treated with 40 nM of brusatol for 4 h before the addition of DMSO or 5-fluorouracil (50 μM, *Left* and 100 μM, *Right*). (*C*) A549 cells were treated with 40 nM of brusatol for 24 h before the addition of DMSO or etoposide (28 μM, *Left* and 56 μM, *Right*). (*D*) A549 cells were treated with 40 nM brusatol for 24 h before the addition of DMSO or pacilitaxel (100 nM, *Left* and 200 nM, *Right*).

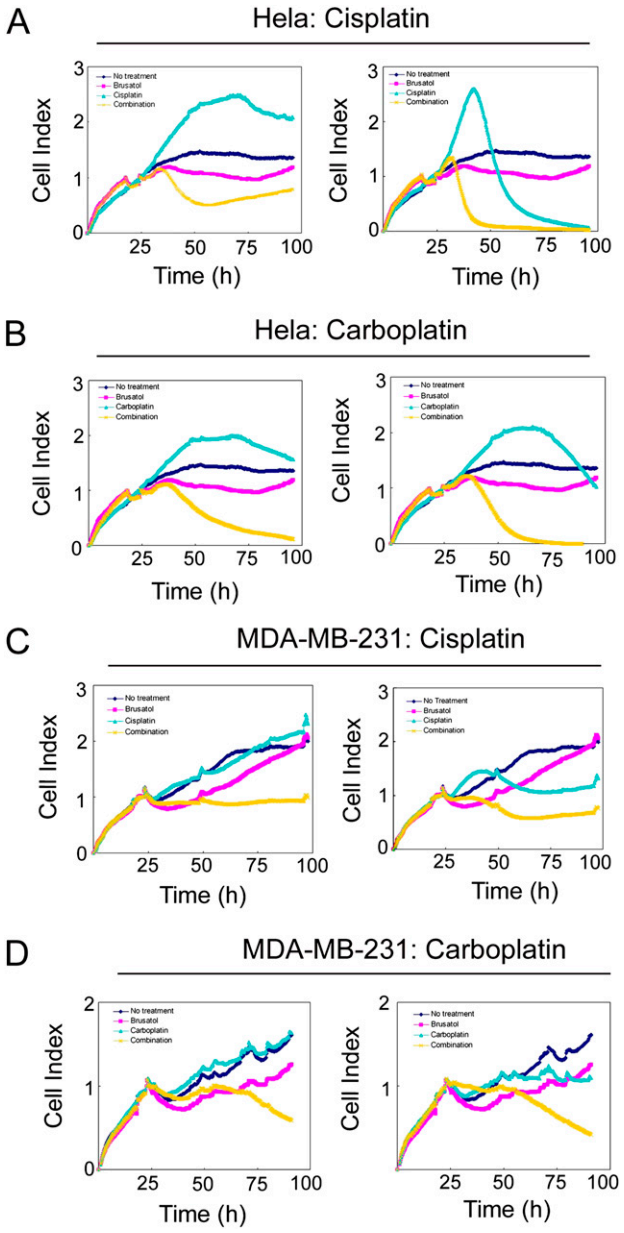


Fig. S5. Brusatol-mediated sensitization to chemotherapeutic drugs was observed in other cancer cell lines. (*A*) HeLa cells were treated with 40 nM of brusatol for 4 h before the addition of DMSO or cisplatin (6 μM, *Left* and 18 μM, *Right*). (*B*) HeLa cells were pretreated with 40 nM of brusatol for 4 h before the addition of DMSO or carboplatin (80 μM, *Left* and 160 μM, *Right*). (*C*) MDA-MB-231 cells were treated with 40 nM of brusatol for 4 h before the addition of DMSO or cisplatin (6 μM, *Left* and 18 μM, *Right*). (*D*) MDA-MB-231 cells were treated with 40 nM of brusatol for 4 h before the addition of DMSO or carboplatin (80 μM, *Left* and 160 μM, *Right*).

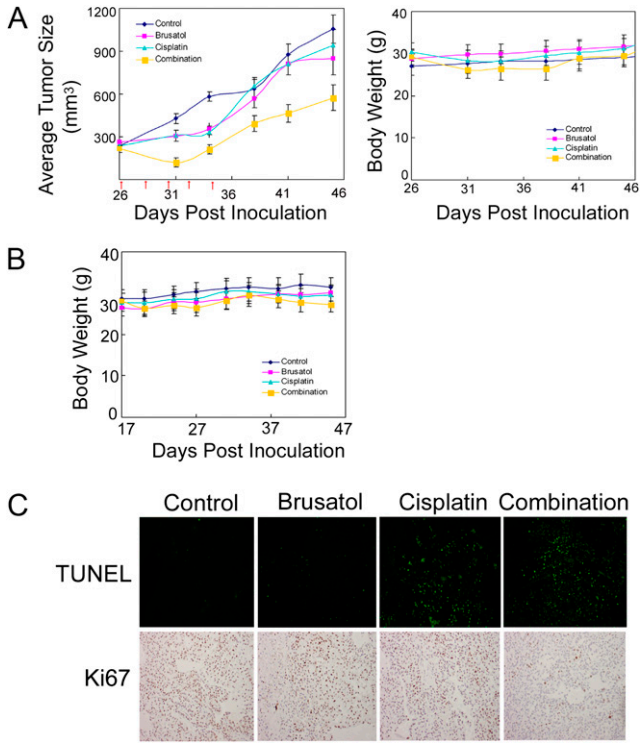


Fig. S6. Brusatol sensitized A549 xenografts to cisplatin treatment by enhancing cisplatin-induced apoptosis. (*A*) Brusatol sensitized xenografts to cisplatin treatment. Thirty-two nude mice 4–6 wk old were injected with A549 (1×10^7 cells). Once tumor size reached 230 mm^3, mice were randomly allocated into four groups (eight mice per group) and were treated i.p. with DMSO, cisplatin (2 mg/kg), brusatol (2 mg/kg), or in combination every other day for a total of five times. Tumor size (*Left*) and body weight (*Right*) were measured semiweekly. (*B*) Treatment did not have significant effects on body weight. Mice were treated with five-time cisplatin treatment regimen twice, as described in Fig. 3*D*. (*C*) Brusatol itself did not cause cell death or significantly inhibit cell proliferation in tumors. The isolated tumors from mice treated as described in Fig. 3*D* were fixed and subjected to TUNEL analysis and IHC-Ki67 staining.

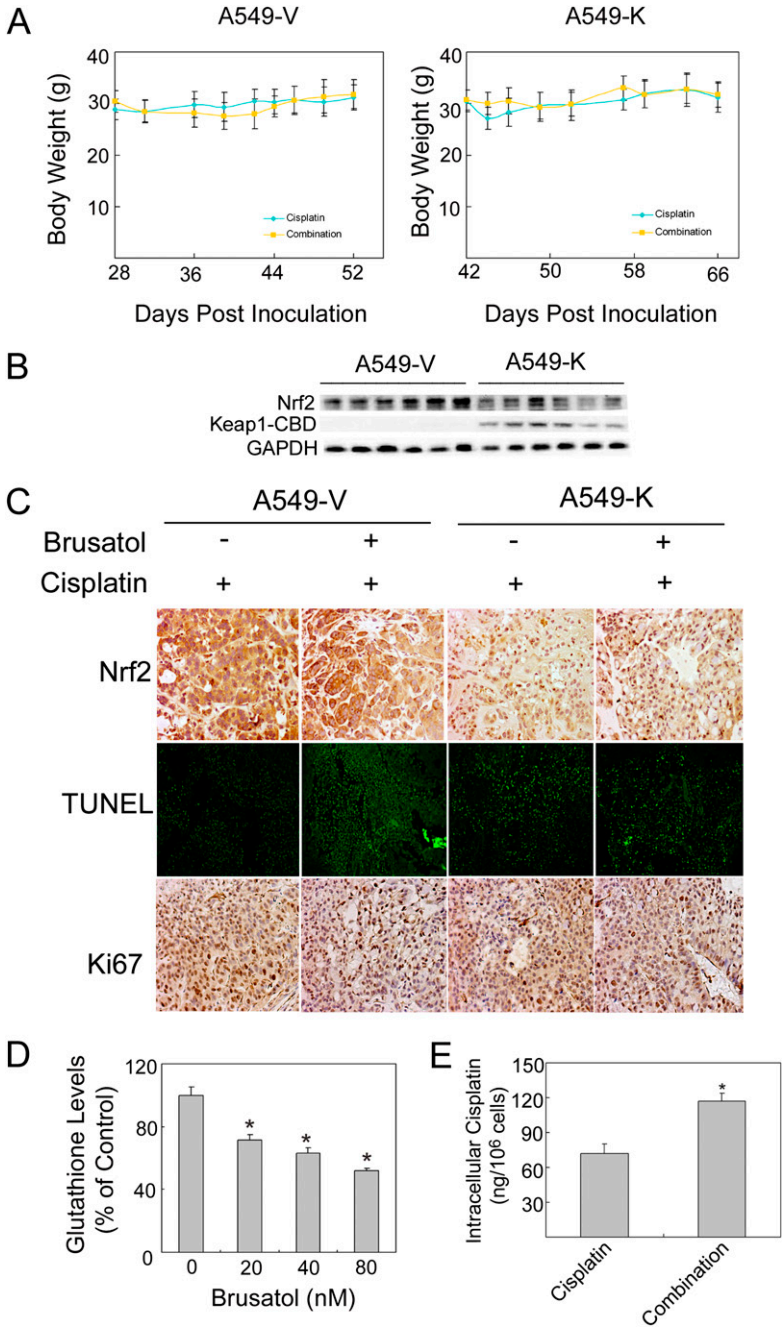


Fig. S7. Brusatol sensitized xenografts to cisplatin treatment in an Nrf2-dependent manner. In addition, brusatol treatment decreased glutathione and increased the intracellular concentration of cisplatin. (*A*) Brusatol had no effect on body weight of mice. Mice were treated as described in Fig. 3*F*. (*B*) Nrf2 levels were lower in the A549-K xenografts. Tumors were excised at the end of the experiment (52 d for A549-V groups; 66 d for A549-K groups) and subjected to immunoblot analysis. (*C*) Nrf2 levels were lower in A549-K xenografts. In addition, combined treatment enhanced apoptosis and reduced cell proliferation compared with cisplatin alone. Tumor tissues were subjected to IHC-Nrf2, TUNEL, and IHC-Ki67 staining. (*D*) Brusatol reduced intracellular glutathione levels. A549 cells were treated with the indicated doses of brusatol for 16 h before measurement of glutathione concentration. (*E*) Brusatol increased intracellular concentrations of cisplatin. A549 cells were pretreated with 40 nM of brusatol for 16 h, then 200 μM of cisplatin was added and incubated for an additional 2 h. The intracellular concentration of cisplatin was measured by atomic absorption spectrometry.