SUPPORTING INFORMATION

The natural product cucurbitacin E inhibits depolymerization of actin filaments

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SUPPORTING FIGURES

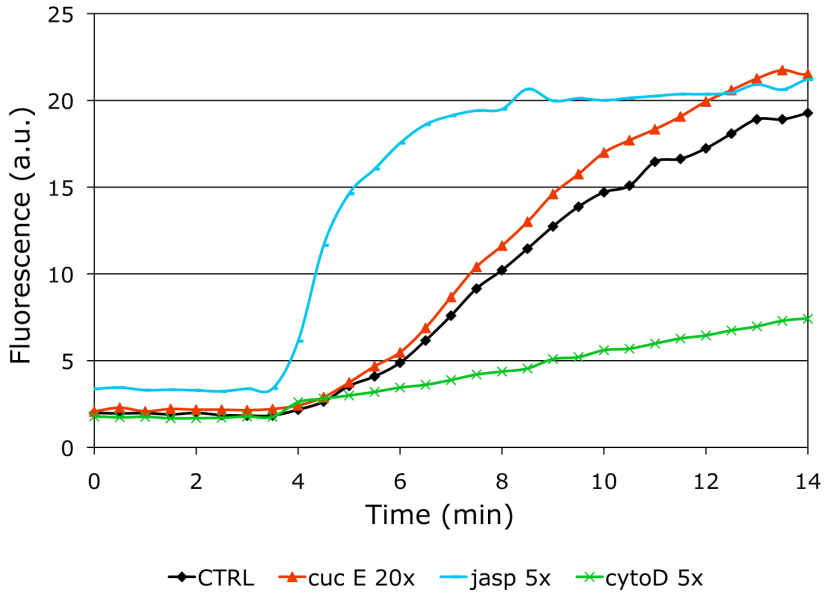
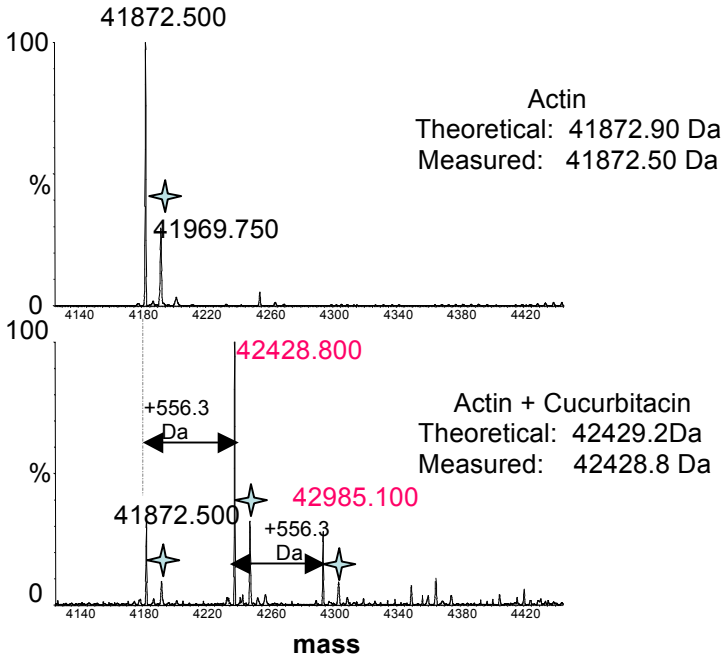


Fig. S1. Pyrene-actin polymerization assay with cucurbitacin E, jasplakinolide, cytochalasin D and DMSO control. G-actin spiked with 10% pyrene labeled actin was incubated with small molecules at 5x the actin concentration (i.e. 15μM jasplakinolide and cytochalasin D) or 20x the actin concentration (i.e. 60 μM cucurbitacin E). The control sample was incubated with an equivalent amount of DMSO. The higher fluorescence at time = 0 of the trace representing actin samples with jasplakinolide is due to jasplakinolide's capacity to nucleate actin.

A



B

356 375

WITKQEYDEAGPSIVHRKCF

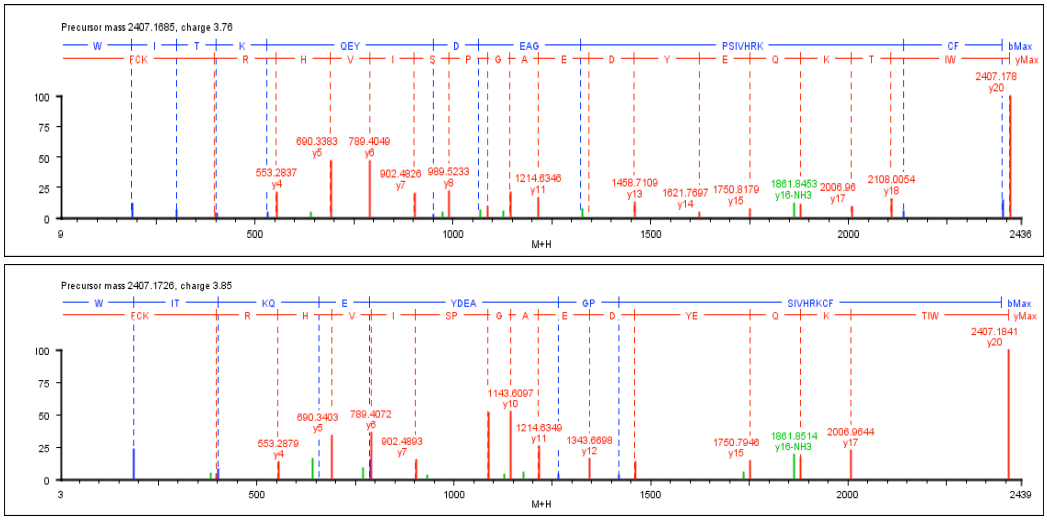


Fig. S2. Mass spectrometric analysis shows that cucurbitacin E binds covalently to actin but does not bind to Cys374. (*A*) Intact ESI mass spectra of chicken smooth muscle actin treated with DMSO (top) or cucurbitacin E (bottom). The transformed mass spectra are shown, and the measured and theoretical molecular weights of unmodified and modified actin are indicated. Addition of one cucurbitacin E molecule increases the molecular weight of the protein by 556.30 Da. Peaks corresponding to a phosphate adduct are indicated with a star. The relative quantification of the modification was as follows: one modification occurred in 50% of the actin molecules, two modifications were found in 15%, and 35% of the actin molecules remained unmodified. As shown in Fig. 4*B*, one of the modifications occurs at Cys257. Assignment of the second addition site was difficult due to the low labeling ratio. (*B*) Tandem MS analysis of a pepsin digest of cucurbitacin E treated actin. Although Cys374 was initially thought to be a site of modification due to its solvent exposure, no labeling at Cys374 was found. The figure shows ESI-MS/MS spectra of the peptic peptide 356-375 ($[M+H]^+ = 2407.17$ Da) containing Cys374. As shown, the spectra are identical, showing that no cucurbitacin E modification occurred at Cys374 (lower panel).

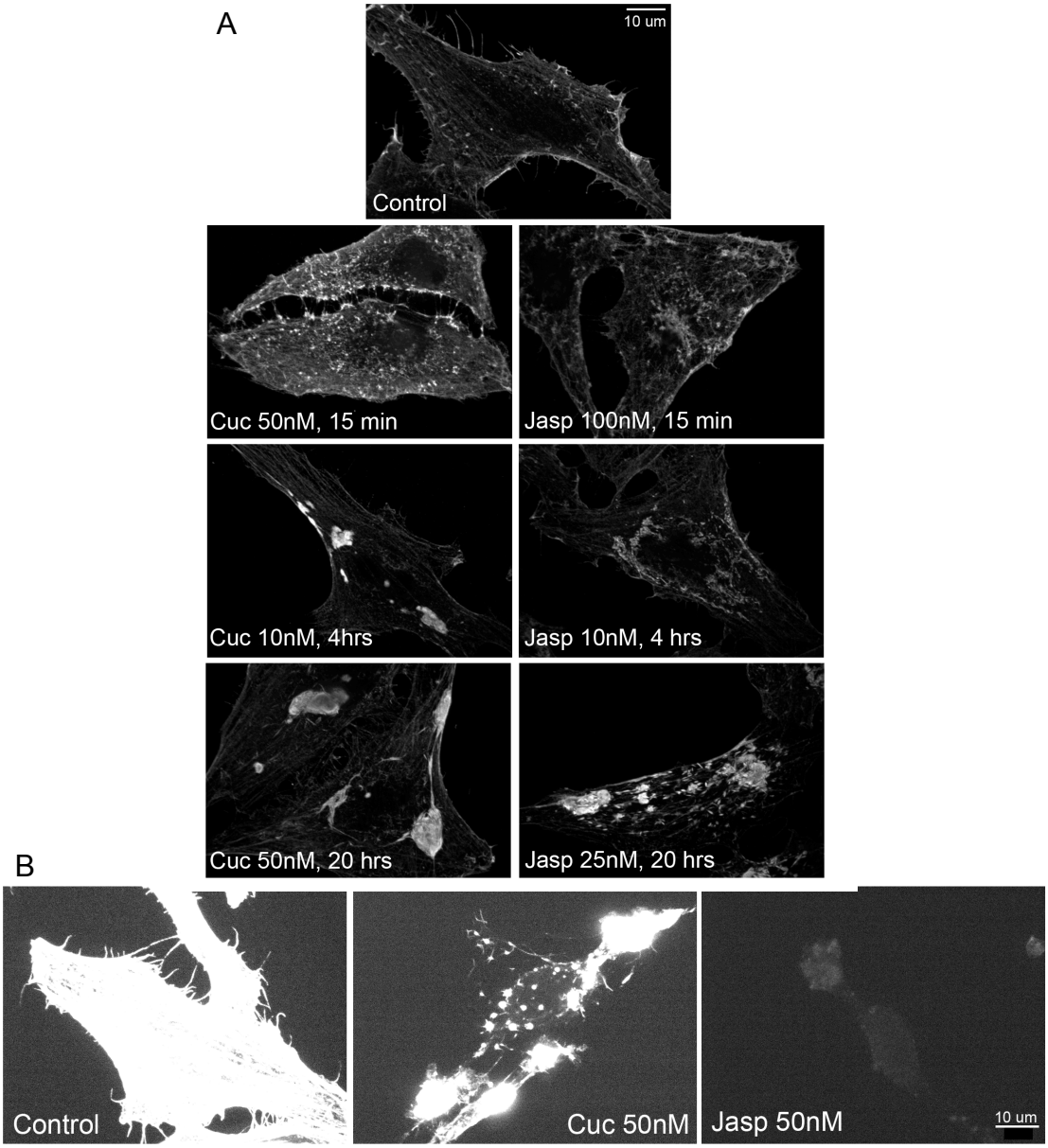


Fig. S3. The effect of cucurbitacin E and jasplakinolide on HeLa cells at short and long time points and adjusted brightness to visualize phalloidin staining in jasplakinolide–treated cells. (*A*) HeLa cells were treated with cucurbitacin E or jasplakinolide for 15 min (top panel), 4hrs (middle panel), and for 20 hrs (bottom panel). Actin was imaged with anti-actin antibody. (*B*) Phalloidin staining of cucurbitacin E and jasplakinolide treated cells with the brightness levels adjusted to illustrate the limited ability of phalloidin

to bind to actin in the presence of jasplakinolide. These are the same images shown in Fig. 6, but the brightness level was increased 10 fold. All three images were taken at the same exposure and are shown at the same brightness level. N=10 cells for all concentrations and time points. Scale bar = 10mm

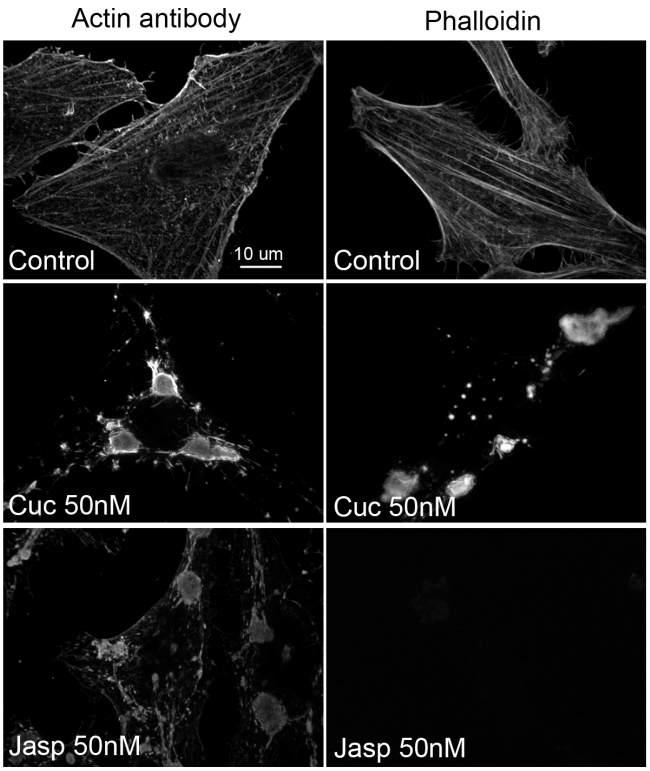


Figure S4. Cucurbitacin E and jasplakinolide aggregate actin in HeLa cells. Actin was imaged with actin antibody (left column) and Alexa Fluor 555 phalloidin (right column). Cells were treated with 50 nM cucurbitacin E and jasplakinolide for 4 hrs. Note the absence of phalloidin staining at saturating jasplakinolide concentrations. Representative images are shown, N=10 cells for each concentration and time point. Scale bar = 10μm

MATERIALS AND METHODS

Reagents. Rabbit skeletal muscle actin (Cytoskeleton AKL99) and pyrene muscle actin (Cytoskeleton AP05) were diluted, snap frozen, and stored in a buffer containing 5mM Tris-HCl (pH 8.0), 0.2mM $CaCl_2$, 0.2mM ATP, 5% (w/v) sucrose and 1% (w/v) dextran at -70°C. Vitamin D binding protein (Calbiochem 345802) was diluted to 0.2 mM, snap frozen and stored in an assay buffer containing 2mM $MgCl_2$, 50mM KCl, 100mM beta-mercaptoethanol, and 100mM Hepes (pH 7.7). Stock solutions of cucurbitacin E (APIN Chemicals N07056c) and jasplakinolide (Calbiochem 420127) were prepared in DMSO.

Pyrene-actin assays. For all pyrene-actin assays, rabbit skeletal muscle actin was mixed with pyrene actin at a 1:10 ratio. The mix was diluted in G-buffer (0.2mM $CaCl_2$, 0.2mM ATP, 5mM TrisCl (pH 8.0)) to a final actin concentration of 3μM and incubated for 40 min on ice. One of three routes was then followed: for the substoichiometric pyrene-actin assays (Fig. 2), G-actin was incubated on ice with cucurbitacin E or jasplakinolide at varying substoichiometric concentrations for 2.5 hrs. Polymerization was induced by addition of 10x polymerization salts (20mM $MgCl_2$, 500mM KCl, 20mM ATP, and 1M Hepes (pH 7.8)), followed by incubation at room temperature for 50 min. Depolymerization was induced by addition of vitamin D binding protein at a 1:20 ratio. The kinetics of depolymerization were monitored at room temperature by measuring emission at 365 nm with a fluorimeter (Cary eclipse, Varian).

To investigate cucurbitacin E's or jasplakinolide's effects on G-actin (Fig. 3*A* and *C*), monomeric actin was first prepared as described above. It was then centrifuged in a TLA 100.3 rotor at 80,000 rpm for 30 min to remove aggregates. The remaining monomeric actin was incubated with either cucurbitacin E or jasplakinolide on ice for 2.5 hrs. Unbound compound was washed away by dilution in 10 ml F-buffer (made by addition of 10x polymerization salts to G-buffer) and spinning at 4,000 rpm through a centrifugal filter unit with a membrane cut-off of 30 kDa (Millipore UFC903024). This was repeated 3 times. Polymerization and depolymerization were induced and monitored as described above.

To investigate cucurbitacin E's or jasplakinolide's effects on F-actin (Fig. 3*B* and *D*), monomeric actin was first prepared as described above. It was then polymerized with 10x polymerization salts for 50 min on ice. Resulting actin filaments were incubated on ice with cucurbitacin E or jasplakinolide for 2.5 hrs and subsequently centrifuged with a TLA 100.3 rotor at 80,000 rpm for 30 min to isolate filamentous actin in the pellet from remaining monomeric actin in the supernatant. Filamentous actin was resuspended in F-buffer and unbound compound was washed away as described above. Depolymerization was induced with vitamin D binding protein and monitored as above.

Cell staining. HeLa cells were grown in polystyrene cell culture dishes (Corning) at 37°C, in 5% CO_2 in air, with DMEM medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen) and penicillin/streptavidin (Cellgro). Prior to the experiment, the cells were trypsinized and plated onto 12 mm diameter glass coverslips (Fisher 12-545-81) and cultured overnight. The cells were then incubated with cucurbitacin E or jasplakinolide at varying concentrations and lengths of time. For phalloidin staining, cells were fixed and permeabilized with 3.7% formaldehyde, 0.2% TritonX-100, 1mM $MgCl_2$, 10mM EGTA, 0.2% TritonX-100, and 100mM Pipes/KOH (pH 6.8) for 15 min, washed with PBS, and then incubated for 10 min with 5U/ml Alexa Fluor 555 phalloidin (Invitrogen A34055) diluted in PBS. For actin antibody staining, cells were fixed and permeabilized with formaldehyde fix followed by chilled methanol for 15 min, blocked with AbDil (TBS with 1% TritonX-100 and 2% BSA) for 40 min, and then incubated with primary monoclonal mouse anti-actin antibody (Abcam ab40864), 1:500 dilution for 2hrs at RT. After washing 6-8 times with AbDil, cells were incubated with anti-mouse IgG Alexa Fluor 488 conjugated secondary antibody, 1:1000 dilution for 30 min at RT. The DNA of both phalloidin and actin antibody stained cells was stained with 300nM DAPI (Invitrogen D1306) diluted in PBS for 5 min. Finally, after washing three times with PBS, cells were mounted on glass slides using Prolong Gold antifade reagent (Invitrogen P36930).

Imaging. The stained cells were examined at the Nikon Imaging Center at Harvard Medical School with a Yokogawa CSU-10 spinning disk confocal on a Nikon Ti-E inverted microscope using a 100x Nikon 1.4 NA objective lens. Alexa Fluor 555 phalloidin was excited with a 100mW Melles Griot Argon-Krypton laser at 568nm (using a 568/10 filter) and collected with a triple band pass dichroic mirror (488/568/647) and a 620/60 emission filter. Alexa Fluor 488 conjugated secondary antibody was excited at 488nm (using a 488/10 filter) and collected with the same dichroic mirror and a 525/50 emission filter. DAPI fluorescence was excited at 355 nm (using a 355/50 filter) and collected with a 400 dichroic mirror and 460/25 emission filter. All filters and mirrors were from Chroma Technologies.

Images were acquired with a Hamamatsu ORCA-AG cooled CCD camera using MetaMorph v7.7 software. For each image 14 z-series were collected at a step size of 0.2 microns using a Prior Proscan II focus motor. Z-series are shown as maximum z-projections generated with AutoQuant blind deconvolution software.

Mass spectrometry. Samples were prepared for mass spectrometry by diluting either Bovine Cardiac Actin (Cytoskeleton AD99), Chicken Smooth Muscle Actin (Cytoskeleton AS99), or Rabbit Skeletal Muscle Actin (Cytoskeleton AKL99) in G-buffer (0.2mM $CaCl_2$, 0.2mM ATP, 5mM TrisCl (pH 8.0)) to a final actin concentration of 3μM. After incubation on ice for 40 min, cucurbitacin E was added at 100 equivalents (i.e. 300μM) to one of the samples, and both the control sample and the drug treated sample were incubated overnight. For intact protein mass spectral analysis, control and drug treated sample were separately injected onto a POROS 20 R2 protein trap and desalted with 0.05% trifluroacetic acid (TFA) at a flow rate of 100 μL/min. The proteins were eluted into an LCT-Premier instrument (Waters Corp., Milford, MA, USA) with a linear 15%–75% (v/v) acetonitrile gradient over 4 min at 50 μL/min.

Pepsin digestion and peptide analysis. For identification of the modification site, actin and cucurbitacin E–bound actin (100 pmol each) were digested with pepsin (enzyme:substrate ratio was 1:1) in a potassium phosphate buffer (75 mM KH_2PO_4/75 mM K_2HPO_4) at pH 2.5 for 5 min on ice. The resulting

peptides were injected into a Waters nanoAcquity UPLC system (Waters Corp, Milford, MA), trapped, and desalted for 3 min at 100 μL/min and then separated for 60 min by an 8%–40% acetonitrile:water gradient of 40 μL/min. The separation column was a 1.0×100.0 mm ACQUITY UPLC C18 BEH (Waters) containing 1.7 μm particles. Mass spectra were obtained with a Waters QTOF Premier equipped with standard ESI source (Waters Corp., Milford, MA, USA). Mass spectra were acquired over an *m/z* range of 100 to 2000. Mass accuracy was ensured by calibration with 100 fmol/μL Glu-fibrinopeptide, and was less than 10 ppm throughout all experiments. Identification of the peptic fragments was accomplished through a combination of exact mass analysis and MS^E using custom Identity^E Software from the Waters Corporation (51). MS^E was performed by a series of low-high collision energies ramping from 5-25 V, therefore ensuring proper fragmentation of all the peptic peptides eluting from the LC system.

Photoactivation protocol. HeLa cells, cultured in DEM (Gibco Life Technologies, UK) with penicillin/streptomycin, glutamine and 10% of an 80:20 mix of DCS:FCS, were trypsinized, plated onto 25 mm diameter glass coverslips, and cultured overnight. The cells were then transfected with actin-PAGFP-mRFP plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The cDNA content was 1 μg per well of a 6-well plate (Invitrogen) and cells were examined the following day. Cucurbitacin E was added to the culture medium at 10 nM and the cells were left to incubate for 30 min. Cucurbitacin E and jasplakinolide were also present at the same concentration in the imaging medium (Leibovitz L15 medium from Invitrogen, supplemented with 10% FBS). Fluorophores were activated at 405 nm wavelength and excited at 488 nm wave-length GFP-tagged proteins and 543 nm wavelength for RFP-tagged proteins. Laser intensities of 5% of a 20 mW laser were utilized to obtain both a strong signal and minimize photobleaching. Images were acquired at 1 s intervals to minimize the general loss of fluorescence due to imaging while still sampling recovery sufficiently fast. Fluorescent imaging was done using a 1.3 NA100x oil-immersion objective on a scanning laser confocal microscope (Olympus Fluoview FV1000; Olympus). In the photoactivation experiments, fluorescence decay was

monitored over a small circular region (r = 2 μm) and activation was effected over a circular bleach spot (r = 1 μm) in the center of the imaging region. This helped to minimize imaging-induced bleaching of GFP-tagged proteins by not exposing the whole cell volume to light. Activation was performed by scanning the 405 nm beam, operating at 30% laser power, line by line over the circular bleach region. Experiments were carried out as follows: two frames were acquired for normalization of the fluorescence signal before bleaching with a single iteration of the bleach pulse at 8 μs/pixel. Recovery was imaged over 200 frames. To test pairwise differences between treatments, student's t-test was performed. Values of $p < 0.01$ compared to control were considered significant.