Chemical Genetics Reveals a Kinase-Independent Role For Protein Kinase R In Pyroptosis

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SUPPLEMENTARY INFORMATION

Supplementary Results

Supplementary Table 1

Compounds tested in standard LT-mediated macrophage cell death assay, mechanism of action, and max concentration of a serial dilution tested.

Compound	Mechanism	Protection in LT assay	Max concentration tested
Actinomycin D	Inhibits transcription	No	160 μM
Cycloheximide	Inhibits translation	No	160 μM
2-AP	Inhibits PKR kinase activity	No	6400 μM
C16	Inhibits PKR kinase activity	No	40 μM
Geldanamycin	Inhibits HSP90	No	160 μM
17-AAG	Inhibits HSP90	No	160 μM
17-DMAG	Inhibits HSP90	No	160 μM
Radicicol	Inhibits HSP90	Yes, but also inhibits ET and LFn-DTA assays	160 μM

Supplementary Table 2

Proteins detected from the pulldown experiment with **19**. Known MW of protein is indicated as well as the MW of the band sent for sequencing.

Protein	MW (kDa)	Band (kDa)	Function
Ddx5	68	60	DEAD box protein, putative RNA helicase, alteration of RNA secondary structure
Eif2s3x	52	55	Subunit of GTP-binding protein, recruitment of methionyl-tRNA(i) to 40S ribosome
Hdac1	60	60	Histone Deacetylase, removal of acetyl groups from lysine, transcriptional repression
Lactb	55	60	Large 39S subunit of the mitochondrial ribosome
Rps4x	30	30	Ribosomal protein S4, a component of the 40S subunit
Sgpl1	63	60	Cleaves sphingoid bases, stress-induced ceramide production and apoptosis
PKR	64	60	Interferon-induced, double-stranded RNA-activated protein kinase, inhibits translation
Prmt1	42	45	Arginine methyltransferase, histone methyltransferase
Ptbp1	57	60	Pre-mRNA splicing, regulation of alternative splicing, binds to intron polypyrimidine tract
Ptbp2	57	60	Pre-mRNA splicing, regulation of alternative splicing, binds to intron polypyrimidine tract
Stk3	56	60	Stress-activated, pro-apoptotic kinase, induces chromatin condensation

Supplementary Table 3 Small molecule screening data

Category	Parameter	Description
Assay	Type of assay	Cell-based (J774A.1 macrophages)
	Target	Phenotypic
	Primary measurement	Survival, measured with Cell Titer Glo
	Key reagents	Cell Titer Glo (Promega), LF and PA (LIST biological)
	Assay protocol	In Methods: Primary LT Assay Screen
	Additional comments	
Library	Library size	31,350 entities
	Library composition	Known bioactives, natural products, DOS compounds, commercially available drug-like compounds
	Source	Broad Institute
	Additional comments	
Screen	Format	384 well plates
	Concentration(s) tested	33 m3 centration
	Plate controls	LFn positive control
	Reagent/ compound dispensing system	nl pin robot
	Detection instrument and software	Envision luminescence plate reader
	Assay validation/QC	PosCon plate at beginning and ending of each screening day, check Z' factor
	Correction factors	None
	Normalization	None, but analyzed each day separately due to potential variation in signal strength between screening days (screened 3 separate days)
	Additional comments	
Post-HTS analysis	Hit criteria	>95% survival of cells, high reproducibility between replicates
	Hit rate	0.5%
	Additional assay(s)	Retested 8-point dose-response of each hit, tested against LFn-DTA, tested against ET, and many other assays described in the paper
	Confirmation of hit purity and structure	Purchased or re-ordered compounds and retested in primary assay
	Additional comments	

Supplementary Figure 1

7DG does not inhibit LT entry or activity

a) EF entry was measured by taking J774 cells pretreated with compound for 2 hours before treating with ET (3 nM EF and 11 nM PA) for 5 hours. Lysate was then used in a competitive cAMP ELISA. High amounts of cAMP indicate effective EF cell entry (ConA 0.32 μM and 7DG 40 μM). b) LFn-DTA entry was determined by incubating J774 cells with indicated compounds and 0.1 nM LFn-DTA and 11nM PA for 24 hours. Cell survival was measured with CTG (7DG 5 μM, Boc-D-CMK 5 μM, ConA 0.04 μM). c) Recombinant LF was diluted in buffer (100 nM final concentration), compound was added, then 10 μM substrate (Ac-Gly-Tyr-bAla-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Val-Leu-Arg-AMC, LF Substrate 3) was added and the amount of cleaved substrate was determined by measuring fluorescent output. Galardin is a known metalloprotease inhibitor. d) J774 macrophages were incubated with compound for 2 hours (7DG 20 μM and ConA 1 μM), then LT (LF 2 nM and PA 11 nM) was added and cells were incubated for 2 hours. Total cell lysates were made and probed with indicated antibodies by immunoblotting. e) J774 cell lysate was incubated with 7DG (40 μM) or a known proteasome inhibitor control (Epoxo at 4 μM), followed by the addition of synthetic proteasome substrates that fluoresce when cleaved by the specific protease *in vitro*. f) J774 cells were treated with different concentrations of compound for 2 hours and then incubated with the cell-permeable protease substrate: chymotrypsin-like: Suc-LLVY-aminoluciferin, caspase-like: Z-nLPnLD-aminoluciferin. After 30 minutes, luminescence was measured. A LT assay was conducted in parallel to determine the amount of protection from LT (cell survival measured using CTG). g) Active, recombinant mouse

caspase-1 was diluted in the provided buffer, followed by addition of compound and finally addition of CaspACE fluorometric substrate. The amount of substrate cleaved was determined by measuring fluorescent output (7DG 100 μM and Z-YVAD-FMK 20 μM). For all assays, data are representative of at least 3 independent experiments, each done in triplicate. Data in graphs are shown as mean +/- StDev. Note that some error bars are too small to see on graphs.

Supplementary Figure 2

SAR reveals critical regions of 7DG, and sites for biotinylation

Synthesized and commercially available small molecules similar to 7DG were tested in the standard LT macrophage survival assay. IC_{50}: concentration for 50% survival (**1** 7DG, **6** gedunin, **9** anthothecol, **19** biotinylated-analog).

Supplementary Figure 3

19 identifies PKR, which is confirmed with siRNA assays

a) Biotin tethered 7-DG analog **19** was prepared from anthothecol (**9**) in the following 3 steps: *O*-methylation of enol with iodomethane followed by methanolysis with potassium carbonate in methanol and lastly esterification of the release alcohol with biotin (more details in supplementary notes section). b) J774 macrophages were incubated with compound for 2 hours, then LT (LF 2 nM and PA 11 nM) was added and cells were incubated for 2 hours. Total cell lysates were made and probed with indicated antibodies by immunoblotting. Concentrations: ConA 4 μM, 7DG 10 μM, **18** 20 μM, **19** 5 μM. c) Coomassie-stained SDS-PAGE gel containing proteins eluted from the pulldown with **19**.

Numbers indicate some of the bands that were cut and sent for sequencing. d) Full gel immunoblot from Fig. 3c. e) Full gel immunoblot from Fig. 3e. f) Three unique siRNA oligos targeting PKR were tested along with the upstream vATPase target as a positive control and J774 cells were tested for sensitivity to LT. g) Increasing amounts of siRNA targeting PKR were tested in J774 cells in the LT assay. h) Increasing amounts of siRNA (nM) targeting PKR were tested in J774 cells and caspase-1 activation was determined using FLICA reagent. Scale bar represents 75 μm. i) J774 cells transfected with indicated siRNA (targeting PKR, nontargeting (NT) control, or vATPase) after 3 days were incubated with LT for 2 hours. Total cell lysates were made and probed with indicated antibodies by immunoblotting. For all assays, data are representative of at least 3 independent experiments, each done in triplicate. Data are shown as mean +/- StDev.

Supplementary Figure 4

a) J774 cells were treated with compound and LT for the indicated times, lysates were collected and probed by immunoblot (ConA 4 μM, Boc-D-CMK 20 μM, MG-132 10 μM). MG-132 treatment results in cleavage of PKR into a smaller fragment and phosphorylation of uncleaved full-length PKR. b) J774 cells were pretreated with DMSO or 7DG (40 μM) followed by incubation for 2 hours with a combination of PA and His-tagged catalytically inactive LF and then lysates were generated and LF-His was pulled down using cobalt resin and probed by immunoblot for PKR co-precipitation. Duplicates of this assay are shown. 'Sup' is the lysate supernatant before LF-His was incubated with the cobalt resin and represents the total amount of PKR that was available for co-precipitation. c) J774 cells were treated with compounds for 2 hours and treated with 10

ng/ml LPS for the indicated period of time. Lysates were probed by immunoblot for IκBβ and actin for loading control (7DG 20 μM and Epoxo 4 μM). d) J774 cells were treated with media alone, 2nM LT, or 1μg/ml LPS for 2 hours, supernatants were collected, and amounts of TNFα were determined using an ELISA assay. e) Immortalized mouse macrophages expressing NLRP3-FLAG and ASC-mCerulean were incubated with C16 inhibitor and specific stimuli, fixed with paraformaledehyde, counterstained with the nucleic acid stain DRAQ5, and visualized using microscopy to determine the percentage of cells that contain ASC specks. For all assays, data are representative of at least 3 independent experiments, each done in triplicate. Data are shown as mean +/- StDev.

Supplementary Figure 5

Synthesis of various analogs of 7DG. Details in Supplementary Notes 1.

Supplementary Notes 1

Synthesis of small molecules

Anthothecol analogs **11**~**19** were synthesized from anthothecol **9** in one to three steps. Acetylation of anthothecol **9** provided acetate **16**, and methylation of **9** gave methyl ether **15**. Methanolysis of anthothecol **9** provided alcohol **11** along with methanol adduct **12**. Similarly, methanolysis of **15** gave alcohol **13** and methanol adduct **14**. Ethylation of alcohol **13** gave ethyl ether **17**. Esterificaton of alcohol **13** with valeric acid and *d*-biotin provided ester **18** and biotinylated analog **19**, respectively.

Synthesis of anthothecol analogs **11~19** (SFig4):

General (synthesis): Unless otherwise noted, reagents and solvents were obtained from commercial suppliers (Sigma-Aldrich, VWR, and Fisher Thermo Scientific) and were used as received. Compounds **1**, **3**, **4**, **6**, **7** and **9** were purchased from Gaia Chemical Co. Compounds **2**, **5** and **10** were purchased from Spectrum. Compound **8** was purchased from Analyticon. Column chromatography was performed using EMD silica gel 60 (230-400 mesh). NMR spectra were recorded on Bruker 300 system, ^{1}H-NMR at 300MHz and ^{13}C-NMR at 75 MHz. Chemical shifts are reported in parts per million (ppm, δ) downfield from tetramethylsilane (TMS). Coupling constants (*J*) are reported in Hz. Spin multiplicities are described as s (singlet), brs (broad singlet), t (triplet), q (quartet), and m (multiplet). LC/MS analysis was conducted on a Agilent Poroshell 120 EC-C18 column (3.0 x 30mm, 2.7 μm), eluting with 0.01% formic acid in water (solvent A) and 0.01% formic acid in acetonitrile (solvent B), using the following elution gradient: 0.0 min (5% B)-1.75 min (95% B)-2.25 min (95% B)-2.27 min (5% B)-2.5 min (5% B) at a flow rate of 1.75 mL/min. HPLC purity was assessed by integration of peak areas at 254nm. The mass spectra were recorded in electrospray positive ion modes (ESI+) on a Waters ZQ mass spectrometer.

Methanolysis of anthothecol (Synthesis of alcohol 11 and methanol adduct 12):

To a solution of anthothecol **9** (22.7 mg, 47 μmol) in methanol (10 mL) was added K_2CO_3 (10.6 mg, 77 μmol) and the mixture was stirred at room temperature for 22h. The mixture was evaporated and redissolved with chloroform, washed with water and Brine and dried over Na_2SO_4 and evaporated. The residue was chromatographed on silica gel

(AcOEt:hexanes=1:3) to give alcohol **11** (9 mg, 43% yield) and methanol adduct **12** (9 mg, 41% yiled). Alcohol **11**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.73 (d, *J* = 10.0, 1H), 7.38 (s, 1H), 7.16 (s, 1H), 6.36 (s, 1H), 6.19 (s, 1H), 6.03 (d, *J* = 9.9, 1H), 4.58 – 4.36 (m, 1H), 3.80 (s, 1H), 2.88 (d, *J* = 11.1, 1H), 2.78 (dd, *J* = 11.1, 6.4, 1H), 2.44 (dd, *J* = 12.7, 6.8, 1H), 2.32 (dd, *J* = 13.6, 6.5, 1H), 1.94 (dd, *J* = 13.5, 11.2, 1H), 1.77 (d, *J* = 3.7, 1H), 1.65 (dd, *J* = 12.8, 9.2, 1H), 1.58 (s, 3H), 1.54 (s, 3H), 1.50 (s, 3H), 1.16 (s, 3H), 0.79 (s, 3H). ESIMS *m/z* +43.10. Rt 1.48 min, purity >95%. Compound **12**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38 (s, 1H), 7.16 (s, 1H), 6.27 (s, 1H), 6.19 (s, 1H), 4.51 (dd, *J* = 4.8, 1.4, 1H), 4.47 – 4.31 (m, 1H), 3.79 (s, 1H), 3.41 – 3.28 (m, 4H), 2.81 – 2.74 (m, 1H), 2.71 (t, *J* = 6.3, 2H), 2.34 (ddd, *J* = 19.8, 13.1, 6.6, 2H), 1.95 (dd, *J* = 13.2, 11.5, 1H), 1.67 – 1.48 (m, 5H), 1.42 (s, 3H), 1.31 – 1.18 (m, 3H), 1.13 (s, 3H), 0.82 (s, 3H). ESIMS *m/z* +471.15. Rt 1.47 min, purity >90%.

Acetylation of anthothecol (Synthesis of *O*^{11}-acetyl-anthothecol 16):

A mixture of anthothecol **9** (8.8 mg, 18 μmol), acetic anhydride (17 μL, 180 μmol) in pyridine (0.2 mL) was stirred at room temperature for 40 h. The mixture was evaporated and the residue was chromatographed on silica gel (AcOEt:hexanes=1:5) to give acetate **16** (7.1 mg, 74% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.49 (d, *J* = 9.6, 1H), 7.36 (d, *J* = 0.8, 1H), 7.14 (s, 1H), 6.16 (s, 1H), 6.14 (d, *J* = 10.2, 1H), 5.48 (dd, *J* = 19.2, 8.2, 1H), 3.74 (s, 1H), 3.13 (d, *J* = 10.5, 1H), 2.81 (dd, *J* = 10.8, 6.5, 1H), 2.61 (dd, *J* = 12.8, 7.1, 1H), 2.37 – 2.23 (m, 4H), 2.12 (s, 3H), 2.00 – 1.84 (m, 1H), 1.52 (t, *J* = 10.7, 4H), 1.41 (s, 6H), 1.28 (s, 3H), 0.81 (s, 3H). ^{13}C NMR (75 MHz, CDCl3) δ 201.96, 169.51, 168.77,

154.27, 143.24, 139.68, 127.00, 122.89, 110.80, 69.85, 56.22, 49.43, 43.40, 41.86, 41.63, 31.92, 28.20, 24.30, 22.97, 21.58, 20.83. ESIMS *m/z* +523.17. Rt 1.62 min, purity >95%.

Methylation of anthothecol (Synthesis of *O*^{6}-methyl-anthothecol 15):

A mixture of anthothecol **9** (35 mg, 73 μmol), K_2CO_3 (16.9 mg, 122 μmol) and iodomethane (91 uL, 1.46 mmol) in acetone (2 mL) was stirred at 50°C for 32 h. The mixture was cooled to room temperature and diluted with AcOEt, washed with brine, dried over Na_2SO_4 and evaporated. The residue was chromatographed on silica gel (AcOEt:hexanes=1:5) to give methyl ether **15** (44 mg, quantitative). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42 (d, *J* = 9.9, 1H), 7.36 (s, 1H), 7.15 (s, 1H), 6.17 (s, 1H), 6.10 (d, *J* = 9.9, 1H), 5.45 (ddd, *J* = 16.3, 12.2, 6.7, 1H), 3.69 (s, 3H), 3.08 (d, *J* = 11.6, 1H), 2.80 (dd, *J* = 10.8, 6.8, 1H), 2.60 (dd, *J* = 12.9, 7.2, 1H), 2.31 (dd, *J* = 13.6, 6.4, 1H), 2.10 (s, 4H), 1.96 (dd, *J* = 13.1, 11.3, 1H), 1.58 (d, *J* = 2.4, 1H), 1.53 (s, 3H), 1.51 (s, 3H), 1.33 (s, 3H), 1.20 (s, 3H), 0.83 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 195.88, 169.54, 154.30, 147.48, 146.22, 143.18, 139.63, 126.82, 122.91, 110.76, 69.90, 68.70, 60.49, 59.03, 56.32, 49.86, 48.22, 45.19, 43.31, 41.79, 41.61, 41.10, 31.90, 28.22, 24.28, 22.98, 22.67, 21.53, 20.03. ESIMS *m/z* +495.16. Rt 1.64 min, purity >95%.

Methanolysis of *O*^{6}-methyl-anthothecol 15 (Synthesis of deacetyl *O*^{6}-methyl-anthothecol 13 and methanol adduct 14):

To a solution of methyl ether **15** (9.5 mg, 19 μmol) in methanol (2 mL) was added 50 mM K_2CO_3 in methanol (0.8 mL) and the whole was stirred at room temperature for 3 h. Acetic acid (3 μL) was added and the mixture was evaporated to dryness. The residue

was chromatographed on silica gel (AcOEt:hexanes=1:3) to give deacetylated compound **13** (4.5 mg, 52% yield) and methanol adduct **14** (1.6 mg, 17% yield). Compound **13**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.64 (d, *J* = 9.9, 1H), 7.38 (s, 1H), 7.16 (s, 1H), 6.19 (s, 1H), 6.04 (d, *J* = 9.9, 1H), 4.53 – 4.38 (m, 1H), 3.68 (s, 3H), 2.88 (d, *J* = 11.0, 1H), 2.77 (dd, *J* = 11.2, 6.3, 1H), 2.44 (dd, *J* = 12.7, 6.9, 1H), 2.31 (dd, *J* = 13.6, 6.3, 1H), 2.06 – 1.89 (m, 1H), 1.68 (d, *J* = 3.6, 1H), 1.61 (dd, *J* = 12.0, 2.9, 1H), 1.55 (s, 3H), 1.53 (s, 3H), 1.51 (s, 3H), 1.17 (s, 3H), 0.81 (s, 3H). ESIMS *m/z* +453.17. Rt 1.51 min, purity 88%. Compound **14**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37 (s, 1H), 7.15 (s, 1H), 6.19 (s, 1H), 4.46 (dd, *J* = 4.8, 2.0, 1H), 4.42 – 4.31 (m, 1H), 3.71 (s, 1H), 3.66 (s, 3H), 3.37 (s, 1H), 3.33 (s, 3H), 2.76 (dd, *J* = 10.9, 6.7, 1H), 2.71 – 2.65 (m, 2H), 2.42 – 2.34 (m, 1H), 2.28 (dd, *J* = 9.7, 4.0, 1H), 1.98 (dd, *J* = 13.1, 11.4, 1H), 1.54 – 1.47 (m, 5H), 1.41 (s, 3H), 1.23 (s, 3H), 1.14 (s, 3H), 0.85 (s, 3H). ESIMS *m/z* +485.15. Rt 1.52 min, purity >95%.

Synthesis of O^{11}-ethyl-deacetyl-O^6-methyl-anthothecol 17:

To a suspension of NaH (1.7 mg, 60% oil dispersion, 49 μmol) in THF (0.2 mL) was added a solution of alcohol **13** (6.6 mg, 15 μmol) in THF (0.3 mL) and the mixture was stirred at room temperature for 5 min. Iodoethane (2.5 μL, 31 μmol) was added and the whole was stirred at room temperature for 10 h and then stirred at 30°C. After 11 h and 18 h at 30°C, additional iodoethane (2.5 μL and 5 μL, respectively) was added. After stirred 61 h at 30°C, the mixture was evaporated. The residue was chromatographed on silica gel (AcOEt:hexanes=1:10) to give ethyl ether **17** (1.9 mg, 27% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.61 (d, *J* = 9.9, 1H), 7.38 (s, 1H), 7.16 (s, 1H), 6.21 (s, 1H), 6.00 (d, *J* = 9.9, 1H), 3.94 (ddd, *J* = 10.9, 8.9, 6.9, 1H), 3.83 – 3.71 (m, 2H), 3.68 (s, 3H), 3.38

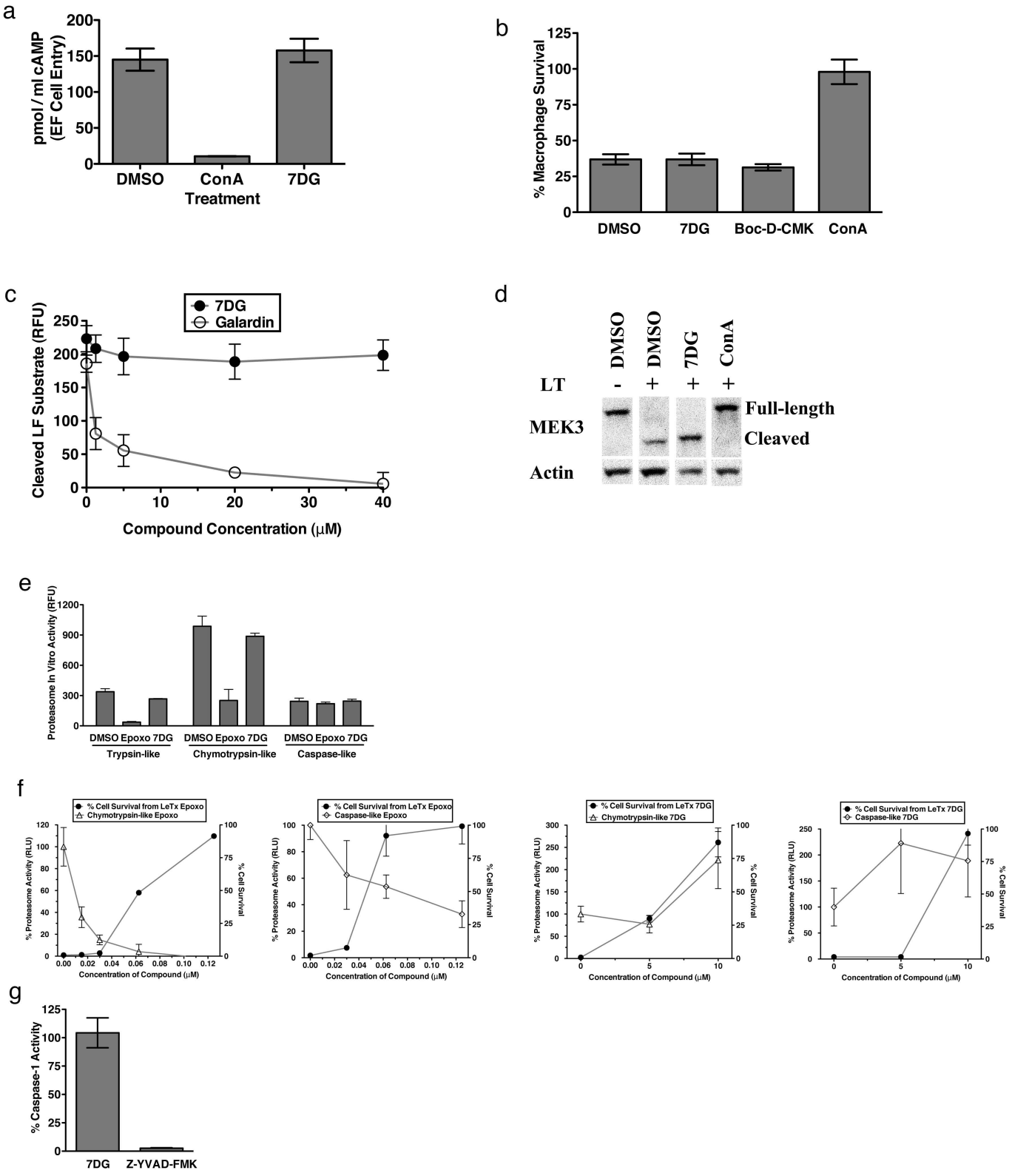
(dt, *J* = 15.7, 7.1, 1H), 2.91 (d, *J* = 11.0, 1H), 2.75 (dd, *J* = 11.0, 6.3, 1H), 2.57 (dd, *J* = 12.8, 6.7, 1H), 2.30 (dd, *J* = 13.6, 6.4, 1H), 1.97 (dd, *J* = 13.4, 11.2, 1H), 1.53 (s, 3H), 1.50 (s, 3H), 1.45 (s, 3H), 1.35 – 1.27 (m, 2H), 1.21 (t, *J* = 6.9, 3H), 1.16 (s, 3H), 0.92 – 0.82 (m, 2H), 0.80 (s, 3H). ESIMS m/z +479.19. Rt 1.86 min, purity >90%.

Synthesis of O^{11}-valeroyl-deacetyl-O^{6}-methyl-anthothecol 18:

To a solution of valeric acid (1 μL, 9.2 μmol) in DMF (0.1 mL) was added a solution of alcohol **13** (3.3 mg, 7.3 μmol) in CH_2Cl_2 (0.1 mL) and a solution of ethyl 3-dimethylaminopropyl-carbodiimide hydrochloride (EDCI) in CH_2Cl_2 (0.2 M, 46 μL, 9.2 μmol of EDCI) and the mixture was stirred at room temperature for 5 min. A solution of 4-dimethylamino-pyridine (DMAP) in CH_2Cl_2 (0.2 M, 5 μL, 1 μmol of DMAP) was added and the whole was stirred at room temperature. After 20 h, additional valeric acid (2 μL, 18.4 μmol) and 0.2 M EDCI in CH_2Cl_2 (92 μL, 18.4 μmol of EDCI) were added. After stirred another 50 h, the mixture was evaporated. The residue was chromatographed on silica gel (AcOEt:hexanes=1:9~1:4) to give valeroyl ester **18** (2.5 mg, 64% yield) as colorless caramel. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43 (d, *J* = 9.9, 1H), 7.36 (s, 1H), 7.15 (s, 1H), 6.17 (s, 1H), 6.10 (d, *J* = 9.9, 1H), 5.45 (ddd, *J* = 11.4, 8.7, 7.2, 1H), 3.75 (s, 1H), 3.69 (s, 3H), 3.08 (d, *J* = 11.7, 1H), 2.81 (dd, *J* = 10.6, 6.6, 1H), 2.60 (dd, *J* = 13.0, 7.1, 1H), 2.43 – 2.23 (m, 3H), 1.96 (dd, *J* = 13.1, 11.2, 1H), 1.64 (dt, *J* = 13.8, 5.3, 2H), 1.54 (s, 3H), 1.52 – 1.43 (m, 4H), 1.41 – 1.34 (m, 2H), 1.33 (s, 3H), 1.20 (s, 3H), 0.98 – 0.85 (m, 3H), 0.83 (s, 3H). ESIMS *m/z* +537.24. Rt 1.88 min, purity >90%.

Synthesis of O^{11}-*d*-biotinyl-deacetyl-O^{6}-methyl-anthothecol 19:

To a mixture of alcohol **13** (12.9 mg, 28.5 μmol) and *d*-biotin (16.9 mg, 69 μmol) in DMF (0.5 mL) was added a 0.5 M solution of EDCI in DMF (140 μl, 70 μmol of EDCI) and the mixture was stirred at room temperature for 5 min. A 0.2 M solution of DMAP in CH_2Cl_2 (70 μL, 14 μmol of DMAP) was added and the whole was stirred at room temperature. After stirred for 68 h, the mixture was evaporated. The residue was chromatographed on silica gel (AcOEt:hexanes=1:3 then $CHCl_3$:MeOH=49:1~48:2) to give biotinyl ester **19** (8.9 mg, 46% yield, colorless film) and recovered alcohol **13** (6.6 mg, 51% recovery). Compound **19**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42 (d, *J* = 9.9, 1H), 7.36 (s, 1H), 7.16 (s, 1H), 6.18 (s, 1H), 6.14 (d, *J* = 9.9, 1H), 5.78 (s, 1H), 5.45 (dt, *J* = 11.3, 8.0, 1H), 5.17 (s, 1H), 4.59 – 4.46 (m, 1H), 4.38 – 4.25 (m, 1H), 3.75 (s, 1H), 3.69 (s, 3H), 3.16 (dd, *J* = 12.4, 6.7, 1H), 3.09 (d, *J* = 11.6, 1H), 2.96 (dd, *J* = 12.9, 5.0, 1H), 2.85 – 2.78 (m, 1H), 2.75 (d, *J* = 13.0, 1H), 2.59 (dd, *J* = 13.0, 7.2, 1H), 2.47 – 2.21 (m, 3H), 2.03 – 1.89 (m, 1H), 1.83 – 1.58 (m, 6H), 1.52 (d, *J* = 8.3, 7H), 1.32 (s, 3H), 1.20 (s, 3H), 0.83 (s, 3H). ESIMS *m/z* +679.37. Rt 1.52 min, purity >95%.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DMSO
ConA
7DG
Treatment
b
% Macrophage Survival
DMSO
7DG
Boc-D-CMK
ConA
c
7DG
Galardin
Cleaved LF Substrate (RFU)
Compound Concentration (μM)
d
DMSO
DMSO
7DG
ConA
LT
MEK3
Actin
Full-length
Cleaved
e
Proteasome In Vitro Activity (RFU)
DMSO Epoxo 7DG
Trypsin-like
Chymotrypsin-like
Caspase-like
f
% Cell Survival from LeTx Epoxo
Chymotrypsin-like Epoxo
Caspase-like Epoxo
% Cell Survival from LeTx 7DG
Chymotrypsin-like 7DG
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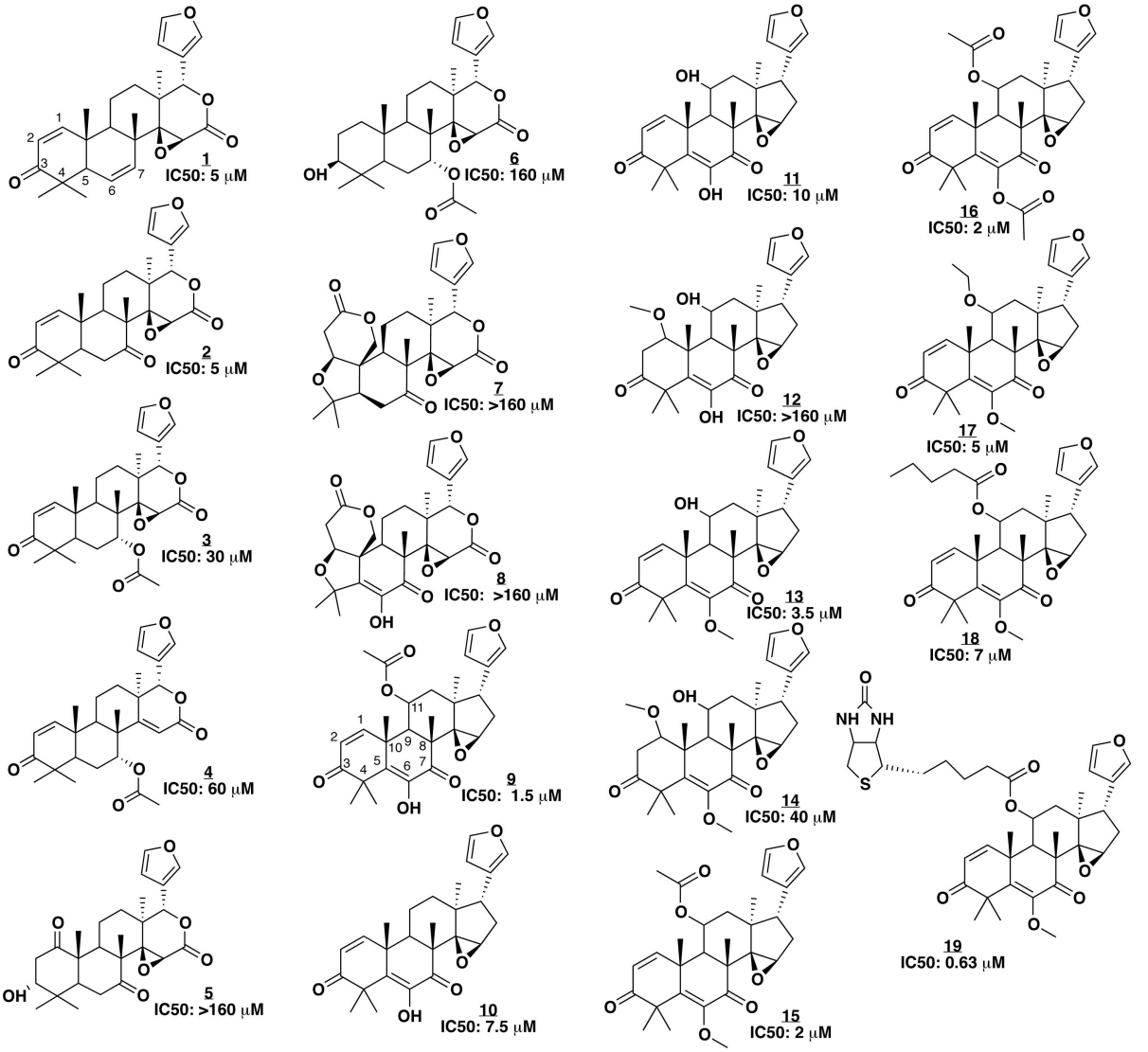
% Cell Survival
Concentration of Compound (μM)
g
% Caspase-1 Activity
7DG
Z-YVAD-FMK

Supp Fig 2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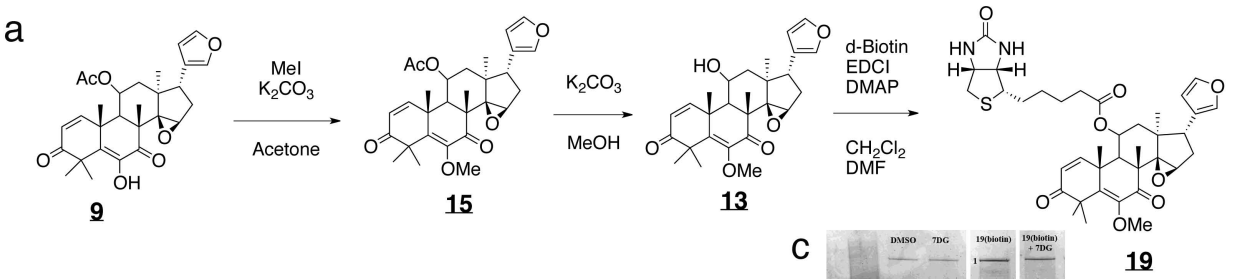
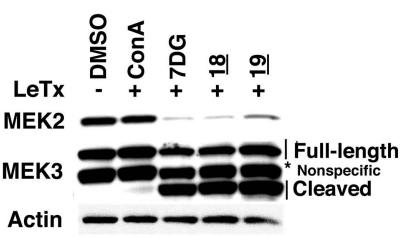
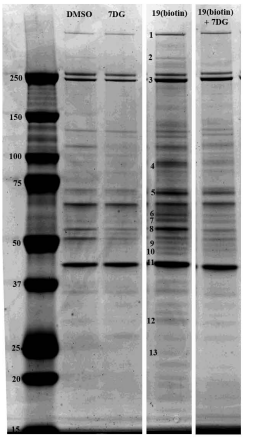
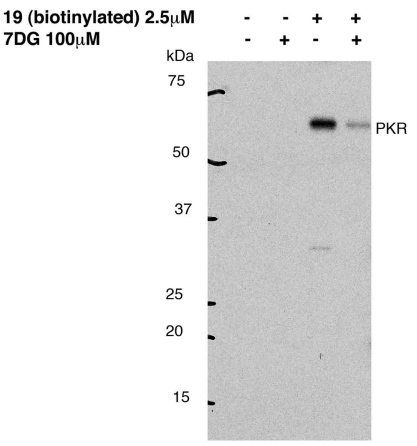
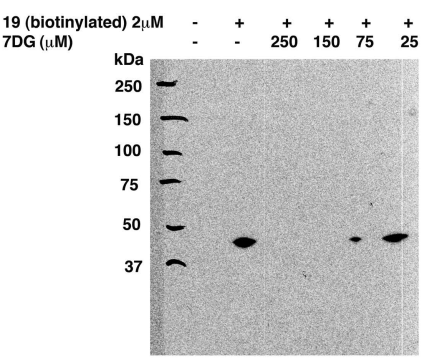
IC50: 5 μM
2
IC50: 5 μM
3
IC50: 30 μM
4
IC50: 60 μM
5
IC50: >160 μM
6
IC50: 160 μM
7
IC50: >160 μM
8
IC50: >160 μM
9
IC50: 1.5 μM
10
IC50: 7.5 μM
11
IC50: 10 μM
12
IC50: >160 μM
13
IC50: 3.5 μM
14
IC50: 40 μM
15
IC50: 2 μM
16
IC50: 2 μM
17
IC50: 5 μM
18
IC50: 7 μM
19
IC50: 0.63 μM

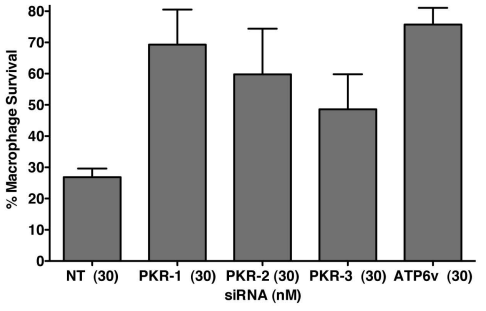

a
AcO
OH
9
MeI
K_2CO_3
Acetone
OMe
15
K_2CO_3
MeOH
HO
13
d-Biotin
EDCI
DMAP
CH_2Cl_2
DMF
HN
NH
S
19

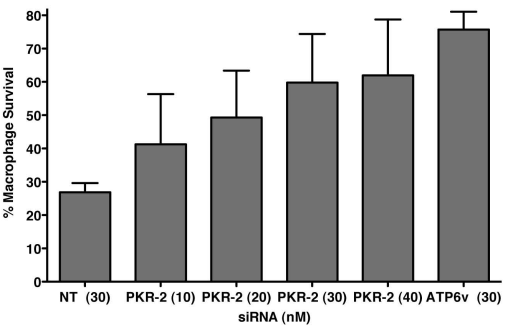

b
LeTx
- DMSO
+ ConA
+ 7DG
+ 18
+ 19
MEK2
MEK3
Actin
Full-length
* Nonspecific
Cleaved

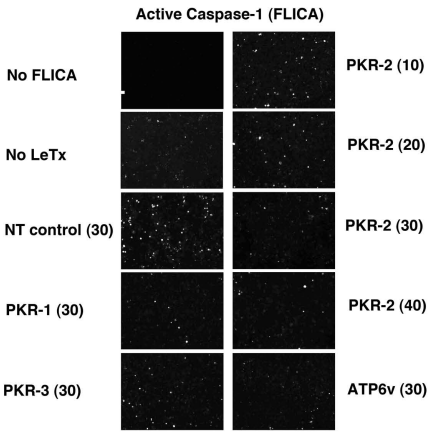

c
DMSO
7DG
19(biotin)
19(biotin) + 7DG
250
150
100
75
50
37
25
20

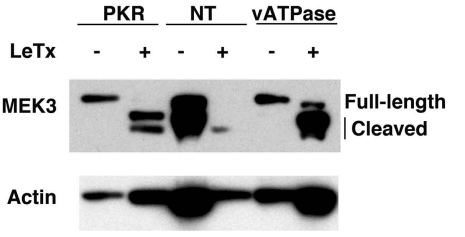

d
19 (biotinylated) 2.5μM
7DG 100μM
kDa
75
50
37
25
20
15
PKR


e
19 (biotinylated) 2μM
7DG (μM)
250
150
75
25
kDa
250
150
100
75
50
37

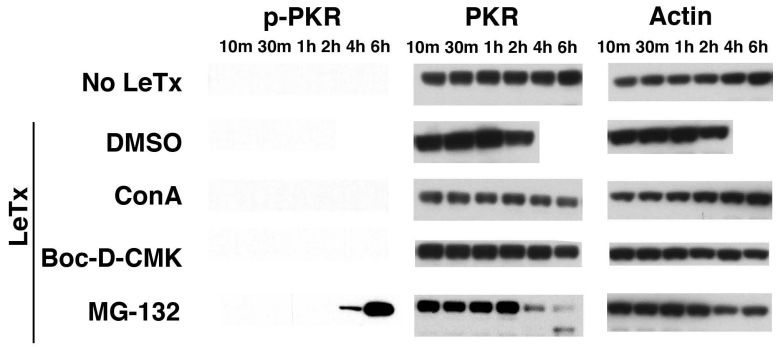

f
% Macrophage Survival
NT (30)
PKR-1 (30)
PKR-2 (30)
PKR-3 (30)
ATP6v (30)
siRNA (nM)


g
% Macrophage Survival
NT (30)
PKR-2 (10)
PKR-2 (20)
PKR-2 (30)
PKR-2 (40)
ATP6v (30)
siRNA (nM)

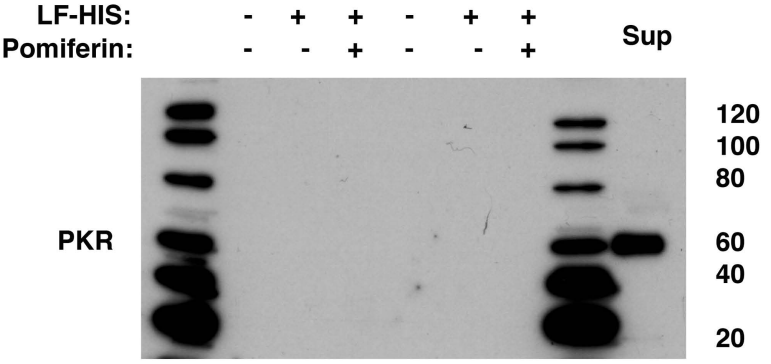

h
Active Caspase-1 (FLICA)
No FLICA
No LeTx
NT control (30)
PKR-1 (30)
PKR-3 (30)
PKR-2 (10)
PKR-2 (20)
PKR-2 (30)
PKR-2 (40)
ATP6v (30)


i
PKR
NT
vATPase
LeTx
MEK3
Actin
Full-length
Cleaved

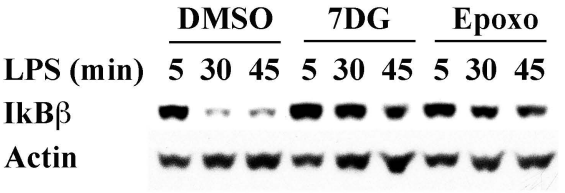
a


p-PKR
PKR
Actin
10m 30m 1h 2h 4h 6h
10m 30m 1h 2h 4h 6h
10m 30m 1h 2h 4h 6h
No LeTx
LeTx
DMSO
ConA
Boc-D-CMK
MG-132

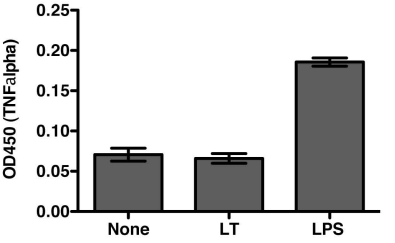
b


LF-HIS: - + + - + +
Pomiferin: - - + - - +
Sup
PKR
120
100
80
60
40
20

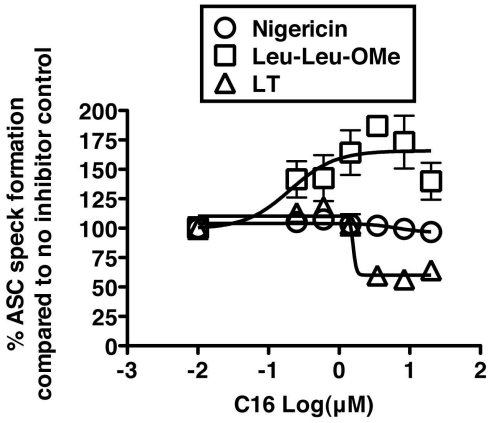
c

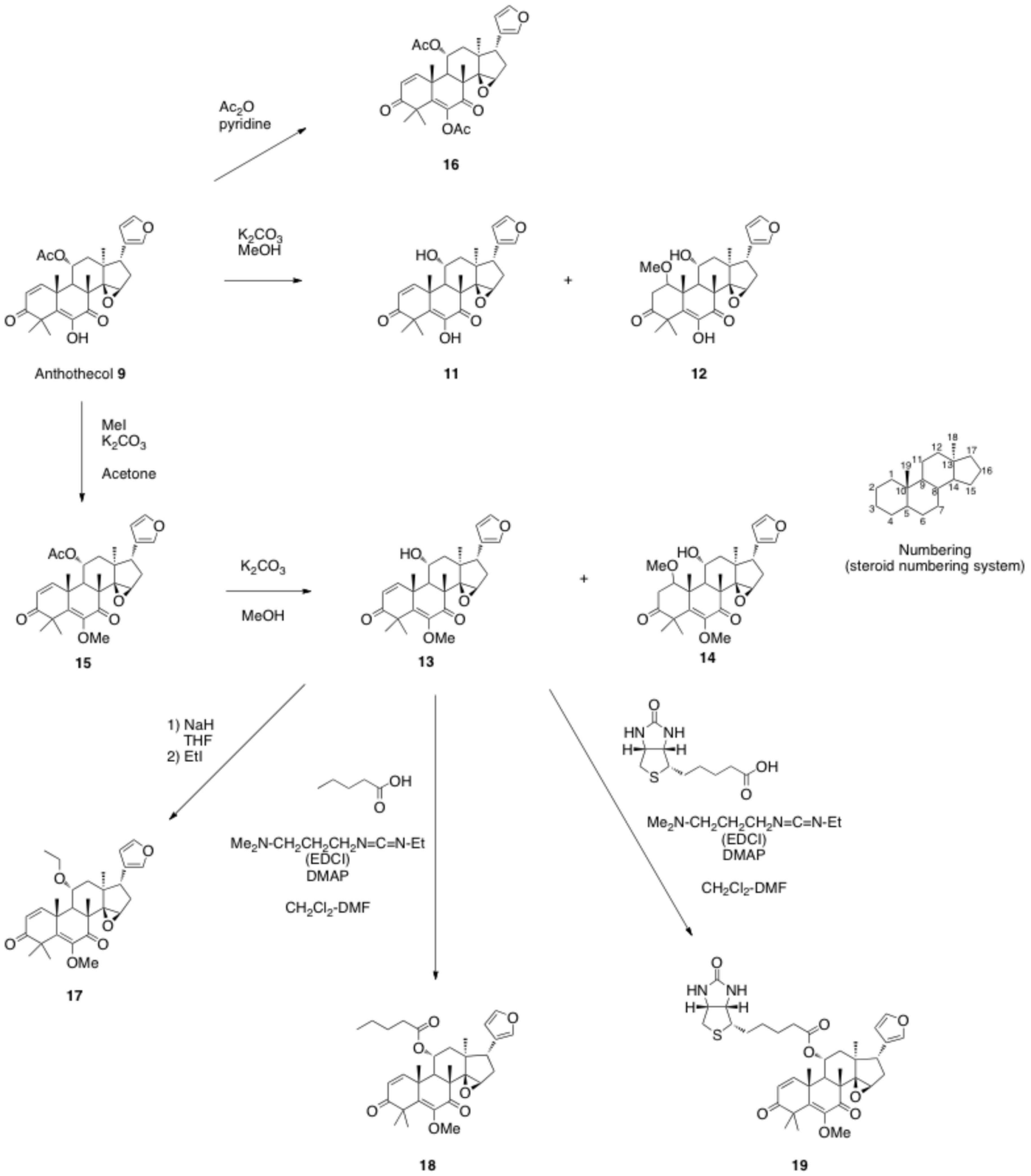

DMSO
7DG
Epoxo
LPS (min) 5 30 45 5 30 45 5 30 45
IkBβ
Actin

d


OD450 (TNFalpha)
0.25
0.20
0.15
0.10
0.05
0.00
None
LT
LPS

e


Nigericin
Leu-Leu-OMe
LT
% ASC speck formation compared to no inhibitor control
200
175
150
125
100
75
50
25
0
-3
-2
-1
0
1
2
C16 Log(μM)


Ac2O
pyridine
16
K2CO3
MeOH
Anthothecol 9
11
12
MeI
K2CO3
Acetone
15
K2CO3
MeOH
13
14
Numbering
(steroid numbering system)
1) NaH
THF
2) EtI
17
Me2N-CH2CH2CH2N=C=N-Et
(EDCI)
DMAP
CH2Cl2-DMF
18
Me2N-CH2CH2CH2N=C=N-Et
(EDCI)
DMAP
CH2Cl2-DMF
19