Supplementary Material

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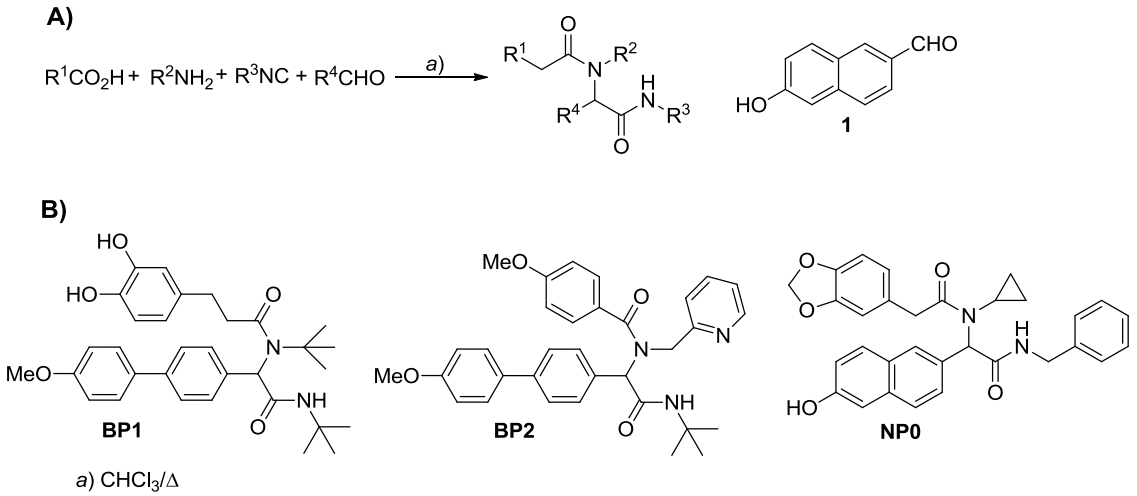
Material and Methods

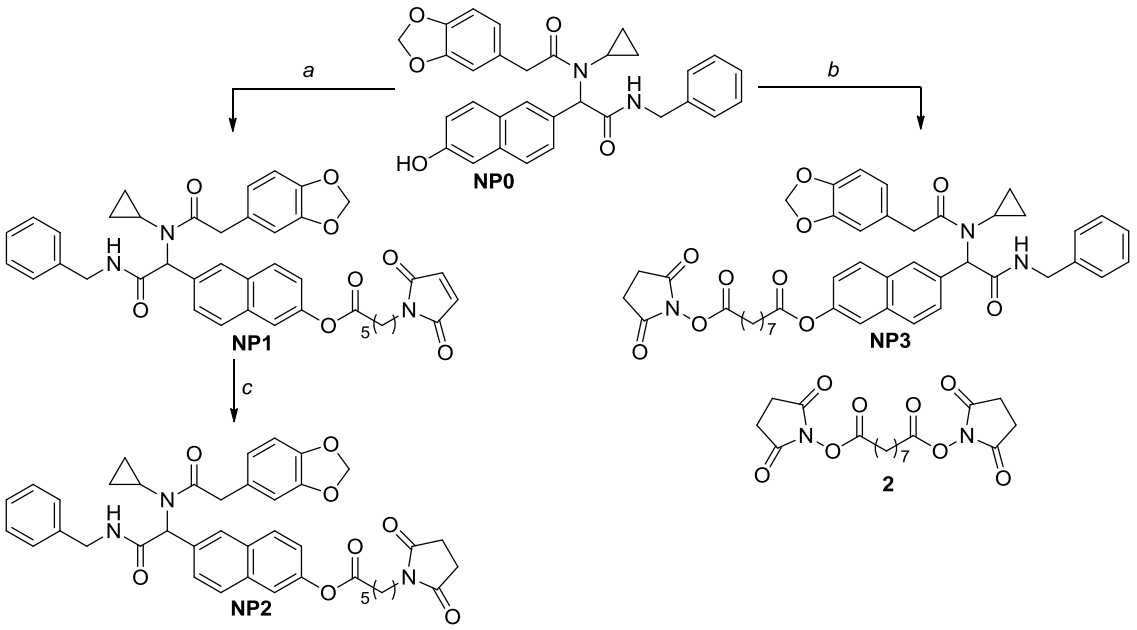
General synthetic methods. Unless otherwise noted, all chemicals were commercial grade and used without further purification. Solvents were purified from the following: THF from sodium/benzophenone, $CHCl_3$ from CaH_2. NMR spectra were recorded on a Bruker 500 and/or 600 MHz spectrometer. Chemical shifts were reported in parts per million (ppm). For ^{1}H NMR spectra ($CDCl_3$) the residual solvent peak was used as the reference (7.26 ppm), while the central solvent peak was used as the reference (77.1 ppm) for ^{13}C NMR spectra. Analytical thin layer chromatography (TLC) was performed with EMD precoated TLC plates, silica gel 60F-254, 0.25 mm layer thickness. TLC plates were visualized by exposure to UV light or submersion in aqueous potassium permanganate followed by brief heating on a hot plate. Flash chromatography separations were performed on Silicycle silica gel (40-63 mesh). Preparative TLC was performed using EMD precoated TLC plates, silica gel 60F-254, 0.5 mm layer thickness. When necessary, reaction vessels were oven dried and cooled in a dessicator prior to reaction performance under an inert atmosphere of argon.

Library synthesis. Members of the library were synthesized using an Ugi multi-component reaction[1,2] previously reported by our group [3]. Briefly, a round bottom flask was charged with amine (1 molar equivalent) and aldehyde (0.5 molar equivalent) in $CHCl_3$ (1 ml) and stirred at room temperature for one hour, followed by the addition of isocyanide (1 molar equivalent) and carboxylic acid (1 molar equivalent) sequentially. The flask was then heated to 75^o C and stirred at the same temperature overnight. The entire reaction was then loaded onto silica gel and eluted first with $CHCl_3$ to remove unreacted isocyanide followed by $CHCl_3$:MeOH (20:1) to elute the Ugi product. All products were analyzed by ^{1}H NMR, ^{13}C NMR and high resolution mass spectrometry (HRMS). The synthesis of several compounds that was not described in our previous reports is described below.

All Ugi reaction components were commercially available with the exception of aldehyde **1**, which was synthesized using a previously reported procedure[3] shown in Supplementary Scheme 1. Maleimide **NP1** was synthesized by EDC coupling of Ugi product **NP0** with 6-maleimidohexanoic acid, and could be subsequently reduced to succinimide **NP2** by hydrogenation with Pd/C. N-hydroxysuccinimide **NP3** was constructed by reaction of Ugi product **NP0** with di-N-hydroxysuccinimide **2** (Supplementary Scheme 2).



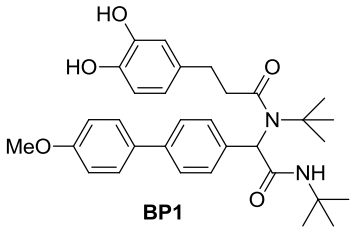
Supplementary Scheme 1. A) General Ugi synthetic protocol and aldehyde **1**. B) Ugi products.



Supplementary Scheme 2. Synthetic protocol for the modification of Ugi product **NP0**.

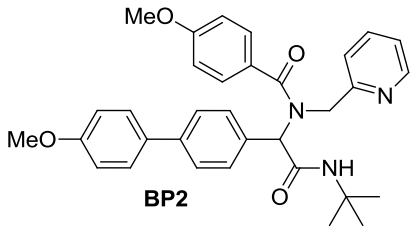
N-(tert-butyl)-N-(2-(tert-butylamino)-1-(4'-methoxy-[1,1'-biphenyl]-4-yl)-2-oxoethyl)-3-(3,4-dihydroxyphenyl)propanamide/BP1: 65% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.46 (dd, *J* = 7.5, 1.5 Hz, 1H), 7.92 (dd, *J* = 7.5, 1.5 Hz, 2H), 7.68 (dd, *J* = 7.5,

1.5 Hz, 2H), 7.33 (dd, *J* = 7.5, 1.5 Hz, 2H), 7.31 (dd, *J* = 7.5, 1.5 Hz, 2H), 7.29 (dd, *J* = 7.5, 1.5 Hz, 2H), 7.17 (dd, *J* = 7.5, 1.5 Hz, 2H), 7.05 (dd, *J* = 7.5, 1.5 Hz, 2H), 5.82 (s, 1H), 4.79 (s, 2H), 3.83 (s, 6H), 1.37 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.5, 168.6, 161.6, 159.5, 156.1, 148.6, 139.7, 139.6, 135.7, 133.1, 130.1, 130.1, 130.1, 130.1, 128.4, 128.4, 128.2, 127.5, 124.1, 120.9, 114.8, 114.8, 114.1, 114.1, 70.8, 60.0, 55.8, 55.8, 52.7, 29.2, 29.2, 29.2; HRMS (ESI) *m/z* calculated for $C_{33}H_{35}N_3O_4$ [M+H]+ 538.270, found 538.269.



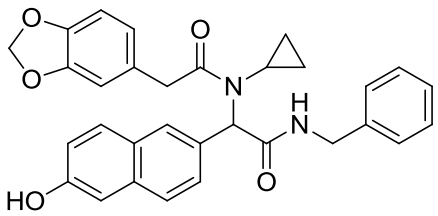
BP1

N-(2-(tert-butylamino)-1-(4'-methoxy-[1,1'-biphenyl]-4-yl)-2-oxoethyl)-4-methoxy-N-(pyridin-2-ylmethyl)benzamide/BP2: 70% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.68 (dd, *J* = 7.5, 1.5 Hz, 2H), 7.33 (dd, *J* = 7.5, 1.5 Hz, 2H), 7.29 (dd, *J* = 7.5, 1.5 Hz, 2H), 7.05 (dd, *J* = 7.5, 1.5 Hz, 2H), 6.86 (s, 1H), 6.73 (d, *J* = 7.5 Hz, 1H), 6.68 (d, *J* = 7.5 Hz, 1H), 5.82 (s, 1H), 3.83 (s, 3H), 2.80–2.51 (m, 4H), 1.47 (s, 9H), 1.37 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.4, 168.6, 159.5, 145.6, 144.5, 139.7, 135.7, 133.4, 133.1, 130.1, 130.1, 130.1, 130.1, 128.4, 128.4, 122.8, 116.4, 115.9, 114.8, 114.8, 65.4, 62.7, 60.0, 55.8, 33.6, 31.7, 29.2, 29.2, 29.2, 27.8, 27.8, 27.8; HRMS (ESI) *m/z* calculated for $C_{32}H_{40}N_2O_5$ [M+H]+ 533.301, found 533.300.



BP2

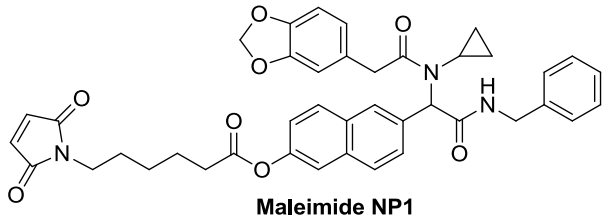
6-(1-(2-(benzo[*d*][1,3]dioxol-5-yl)-*N*-cyclopropylacetamido)-2-(benzylamino)-2-oxoethyl)nahpthalen-2-yl 6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl) hexanoate/ NP0: 75% yield.^{1}H NMR (500 MHz, $CDCl_3$) δ 7.80 (br s, 1 H), 7.70 (s, 1 H), 7.58 (d, *J* = 8.5 Hz, 1 H), 7.55 (d, *J* = 8.6 Hz, 1 H), 7.32 (m, 8 H), 7.12 (s, 1 H), 6.86 (s, 1 H), 6.77 (s, 1 H), 6.53 (t, *J* = 5.4 Hz, 1 H), 5.92 (s, 2 H), 5.75 (s, 1 H), 4.56 (m, 2 H), 4.0 (m, 2 H), 2.71 (m, 1 H), 1.15 (m, 1 H), 0.92 (m, 2 H), 0.74 (m, 1 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.2, 170.8, 147.3, 146.0, 138.0, 134.5, 129.9, 129.7, 128.7, 128.4, 128.3, 128.2, 127.9, 127.7, 127.5, 127.1, 126.9, 122.5, 119.0, 109.9, 109.3, 108.3, 101.3, 68.4, 44.3, 41.7, 32.2, 11.1, 10.0. HRMS found 509.2049 [calculated for $C_{31}H_{29}N_2O_5$ (M + H^+) 509.2049].



NP0

6-(1-(2-benzo[*d*][1,3]dioxol-5-yl)-*N*-cycloproylacetamido-2-(benzylamino)-2-oxoethyl) naphtahalen-2-yl 6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl) hexanoate/NP1: 6-maleimidohexanoic acid (114 mg/0.52 mmol) and EDC (99 mg/0.52 mmol) were combined in 2 ml of CH_2Cl_2 at 0^{o} C followed by the addition of NEt_3 (180 μl/1.3 mmol).

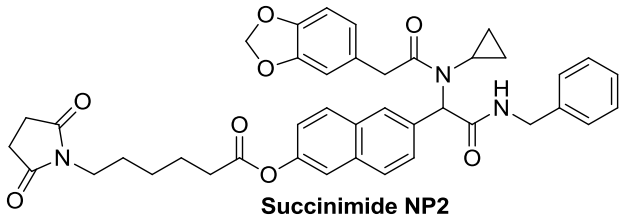
The resulting solution was stirred at 0^{o} C for 15 minutes, followed by the addition of Ugi product **NP0** (70 mg/0.13 mmol) in one portion. The resulting solution was allowed to warm to room temperature overnight. The reaction was transferred to a separatory funnel and the organic phase was washed with water (1 x 4 ml). The aqueous phase was extracted with CH_2Cl_2 (2 x 4 ml), the combined organic phases dried with $MgSO_4$, and the solvent removed under reduced pressure. The resulting crude material was purified by flash chromatography (2:1 Hexanes:EtOAc to 2:1 EtOAc:Hexanes) to give the pure product as an amorphous solid (75 mg/77% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.85 (s, 1 H), 7.81 (d, *J* =8.7 Hz, 1 H), 7.60 (s, 1 H), 7.47 (d, *J* = 8.5 Hz, 1 H), 7.34 (m, 7 H), 6.89 (s, 1 H), 6.80 (m, 2 H), 6.52 (t, *J* = 5.7 Hz, 1 H), 6.02 (s, 2 H), 5.83 (s, 1 H), 5.37 (s, 2 H), 4.55 (m, 2 H), 4.06 (m, 2 H), 3.65 (t, *J* = 7.2 Hz, 2 H), 2.69 (t, *J* = 7.4 Hz, 2 H), 2.65 (m, 1 H), 1.90 (m, 2 H), 1.76 (m, 2 H), 1.52 (m, 2 H), 1.16 (m, 1 H), 0.93 (m, 2 H), 0.73 (m, 1 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.4, 172.4, 171.2, 170.3, 149.4, 148.1, 146.7, 138.5, 134.5, 133.6, 133.4, 131.5, 130.1, 129.0, 128.8, 128.5, 128.4, 128.0, 127.8, 127.7, 122.7, 122.1, 118.5, 110.2, 108.7, 101.3, 67.3, 53.8, 44.2, 41.7, 38.0, 34.5, 28.6, 26.6, 24.6, 11.2, 9.9. HRMS found 702.2797 [calculated for $C_{41}H_{40}N_3O_8$ ($M+H^+$) 702.2815].



Maleimide NP1

6-(1-(2-(benzo[d][1,3]dioxol-5-yl)-N-cyclopropylacetamido)-2-(benzylamino)-2-oxoethyl)naphthalen-2-yl 6-(2,5-dioxopyrrolidin-1-yl)hexanoate/Succinimide NP2:

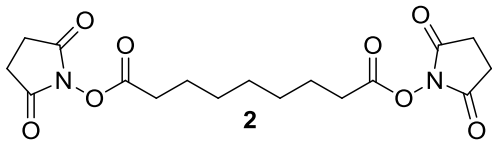
Maleimide NP1 (40 mg/0.06 mmol) was dissolved in EtOAc (2 ml) followed by the addition of a spatula tip of Pd/C. The resulting solution was stirred under a balloon of hydrogen (1 atm) for 24 hours, followed by filtration over Celite. The solvents were removed under reduced pressure and the product purified by flash chromatography (6:1 EtOAc:Hexanes) (35 mg/88%yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77 (s, 1 H), 7.73 (d, *J* = 8.7 Hz, 1 H), 7.51 (d, *J* = 2.0 Hz, 1 H), 7.39 (d, *J* = 10.0 Hz, 1 H), 7.25 (m, 7 H), 6.80 (s, 1 H), 6.73 (m, 2 H), 6.45 (t, *J* = 5.7 Hz, 1 H), 5.94 (s, 2 H), 5.75 (s, 1 H), 4.46 (m, 2 H), 3.93 (m, 2 H), 3.55 (t, *J* = 7.3 Hz, 2 H), 2.70 (s, 4 H), 2.61 (t, *J* = 7.4 Hz, 2H), 2.57 (m, 1 H), 1.80 (m, 2 H), 1.65 (m, 2 H), 1.45 (m, 2 H), 1.08 (m, 1 H), 0.85 (m, 2 H), 0.66 (m, 1 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ177.6, 175.5, 172.4, 170.8, 149.2, 148.2, 146.9, 138.2, 133.5, 133.2, 131.3, 129.8, 128.8, 128.5, 128.3, 128.2, 127.8, 127.5, 127.4, 122.5, 121.9, 118.5, 109.9, 108.4, 101.1, 67.1, 53.6, 43.9, 41.5, 38.7, 34.3, 28.3, 27.5, 26.5, 24.6, 10.9, 9.8. HRMS found 704.2964 [calculated for $C_{41}H_{42}N_3O_8$ ($M+H^+$) 704.2964].



Succinimide NP2

Bis(2,5-dioxopyrrolidin-1-yl) nonanedioate/Di-N-hydroxysuccinimide 2: N-hydroxysuccinimide (0.52 g /4.5 mmol) was dissolved in THF (10 ml) and the resulting solution cooled to 0^{o} C before the addition of NEt_3 (652 μl/4.5 mmol) and azelaoyl chloride (438 μl/2.2 mmol). The solution was allowed to warm slowly to room

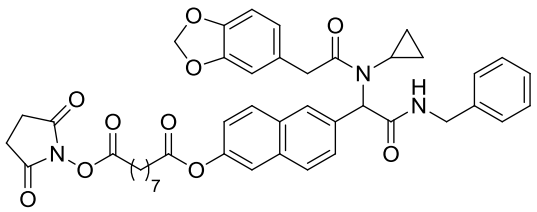
temperature overnight before removing THF under reduced pressure. The resulting residue was dissolved in CH_2Cl_2 (20 ml), washed with water (3 x 10 ml), and the organic phase dried with $MgSO_4$. The solvent was removed under reduced pressure to yield a white solid which was used without further purification (756 mg/90% crude yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 2.85 (s, 8 H), 2.60 (t, *J* = 7.4 Hz, 4 H), 1.76 (m, 4 H), 1.43 (m, 4 H), 1.37 (m, 2 H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.7, 168.8, 31.0, 28.6, 28.5, 25.8, 24.6. HRMS found 383.1433 [calculated for $C_{17}H_{23}N_2O_8$ ($M+H^+$) 383.1449].



2

1-(6-(1-(2-(benzo[d][1,3]dioxol-5-yl)-N-cyclopropylacetamido)-2-(benzylamino)-2-oxoethyl)naphthalen-2-yl) 9-(2,5-dioxopyrrolidin-1-yl) nonanedioate/N-hydroxysuccinimide NP3: A solution of Ugi product **NP0** (22 mg/0.05 mmol) and di-NHS ester **2** (20 mg/0.05 mmol) in CH_2Cl_2 (4 ml) was cooled to 0^{o}C before the dropwise addition of NEt_3 (7.6 μl /0.05 mmol) at the same temperature. The solution was allowed to warm slowly to room temperature overnight, then transferred to a separatory funnel and washed with water (1 x 5 ml). The aqueous phase was extracted with CH_2Cl_2 (2 x 5 ml), the combined organic layers dried with $MgSO_4$ and the solvent removed under reduced pressure to give the crude product which was purified by preparative TLC (15:1 $CHCl_3$:MeOH) (20 mg/48% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.76 (s, 1 H), 7.72 (m, 1 H), 7.51 (s, 1 H), 7.39 (t, *J* = 9.3 Hz, 1 H), 7.25 (m, 7 H), 6.78 (s, 1 H), 6.72 (m, 2 H), 6.45 (m, 1 H), 5.93 (s, 2 H), 5.72 (s, 1 H), 4.49 (m, 2 H), 3.91 (m, 2 H), 2.81 (s, 4 H),

2.62 (m, 4 H), 2.59 (s, 1 H), 1.79 (m, 2 H), 1.45 (m, 4 H), 1.25 (m, 4 H), 1.08 (m, 1 H), 0.85 (m, 2 H), 0.67 (m, 1 H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 175.9, 173.2, 170.7, 170.0, 169.5, 149.7, 148.6, 147.4, 138.2, 134.6, 133.7, 131.1, 130.5, 129.5, 128.9, 128.88, 128.85, 128.5, 128.2, 128.0, 123.1, 122.6, 119.1, 110.6, 109.1, 101.8, 68.0, 44.6, 42.2, 35.7, 31.4, 30.6, 29.6, 29.5, 29.4, 26.4, 25.8, 25.6, 11.6, 10.5. HRMS found 776.3168 [calculated for $C_{44}H_{46}N_3O_{10}$ ($M+H^+$) 776.3178].



N-Hydroxysuccinimide NP3

Reagents. The members of "credit library", including compounds BP1, BP2, NP1 and derivatives of NP1 (NP2, NP3 and NP8), were synthesized and purified as described above. The purity was greater than 99% and was confirmed by HPLC/mass spectrometry analysis. All synthetic molecules were dissolved in DMSO at 200x of the desired concentration and aliquots were stored at -20 °C. In addition, a quantitative QCL-1,000 chromogenic Limulus amoebocyte lysate assay (BioWhittaker) demonstrated that preparations of compounds BP1, BP2 and NP1 were endotoxin free. *Salmonella minnesota* Re595 LPS and heat-killed *Staphylococcus aureus* (HKSA) were prepared as described [4], polyI: polyC (pI:pC) was purchased from Amersham Pharmacia Biotech, and unmethylated cytosine-phosphate-guanine-containing DNA (CpG) was purchased from InvivoGen (San Diego, CA).

Mice, bone marrow-derived macrophages and other cells. C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and used for the preparation of bone marrow-derived macrophages (BMDM) and murine embryonic fibroblasts (MEFs) by using standard protocols. L929/NCTC clone 929 (connective tissue, mouse) cell lines were purchased from ATCC (Manassas, VA). MEFs and cell lines were maintained in growth medium (GM): DMEM medium (4.5 g/l glucose) supplemented with 10% FBS (HyClone, Logan, UT), *L*-glutamine, penicillin/streptomycin and nonessential amino acids (Invitrogen, Carlsbad, CA). BMDM were cultured in 70% GM and 30% L929 conditioned medium. THP-1 blue CD14 (THP-1) cells were purchased from InvivoGen and cultured according to supplier's protocol. In general, cells were incubated in corresponding fresh medium for 12-14 h before stimulation. Confluent MEFs were incubated in 0.5% serum-containing medium for 40-48 hours before stimulation.

Cell stimulation. In all experiments, the cells were stimulated with LPS (100 ng/ml), TNF (40 ng/ml), HKSA (~10^8 cfu/ml), poliI:polyC (50 μg/ml), CpG (10 μg/ml), a compound (usually appropriate aliquot from 200x stock solution was added directly to cultured cells to final concentration 0.1-50 μM) or a combination of stimuli as indicated in the figure legends; MEFs were stimulated in similar manner, but a higher dose of LPS (500 ng/ml) was used. In general, cells were incubated in corresponding fresh medium for 12-14 h before stimulation. Confluent MEF were incubated in 0.5% serum-containing medium for 40-48 hours before stimulation.

Screening the Library. THP-1 cells were distributed into 96-well plates to a final density of $5x10^5$cells/ml and stimulated with LPS or its combination with 0.5% DMSO (a vehicle control) or a compound (50 μM). After overnight (~ 17 h) incubation, the aliquot (20 μl) of supernatants were withdrawn and dispensed into 200 μl QuantiBlue (InvivoGen) detection reagent on a new 96-well plate. After 3 h incubation, an absorbance was read at 630 nm to test for reporter gene activity according to manufacturer's protocol. Mean values and standard deviations were determined and normalized to a vehicle control. Compounds yielding 60 % decrease of reporter gene activity were further evaluated. Data analysis was performed using GraphPad Prism (version 5.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Dose response experiments were undertaken under the same conditions as for screening, except for variation in compound concentrations.

Experiments with human peripheral blood mononuclear cells (PBMC). Human blood was collected using heparin anticoagulant (~10 U/ml) by the TSRI Normal Blood Donor Service. For purification of PBMC, human blood was diluted with an equal volume of Dulbecco's phosphate buffered saline (DPBS) and layered over Ficoll Paque Premium (GE Healthcare) and centrifuged 400 x G, 35 minutes at 20ºC. The mononuclear cell layer was harvested, washed in DPBS and suspended in RPMI-1640 with 10% FBS. Cells were plated in 96-well plates (~$5x10^6$ cells/well) overnight (~15-19 hr), and then stimulated with LPS (100 ng/ml) alone or in the combination with BP1, BP2 or NP1 (50

μM of each) for 3-4 h. Supernatant levels of TNF in the samples were measured by ELISA (BD Biosciences Pharmingen) and normalized to those for a vehicle control. Cells viability was estimated in the presence of different doses of a compound by using XTT-based toxicology assay kit (Sigma); under our experimental conditions viability of NP-1-treated cell was indistinguishable from control cells, as was determined by using a cytotoxicity assay kit as well as Western blot analysis for integrity of poly(ADP-ribose) polymerase (PARP) with anti-PARP antibodies (Cell Signaling; 9542).

Antibodies and Western blot assays. The following commercial antibodies were used: HRP-conjugated anti-rabbit IgG and anti-mouse IgG (Bio-Rad); anti-IκBα (Santa-Cruz; sc-371), anti-phosphorylated IκBα (Cell Signaling; 9246), anti-phosphorylated p-38 (Cell Signaling; 9211), anti-p38 (Cell Signaling; 9212), anti-phosphorylated IKKα/IKKβ (Cell Signaling; 2694), anti-phosphorylated RelA/p65 (Cell Signaling; 3031), anti-IKKβ (Cell Signaling; 2370); anti-RelA/p65 (Santa-Cruz; sc-372), actin (Sigma), Western blot analysis was performed according to protocol recommended by the manufacturer (Cell Signaling). Each Western blot shown is representative of at least three experiments.

Northern blot. Total RNA was isolated by using TRizol reagent (Invitrogen). Total RNA (10 μg/lane) were analyzed by Northern blot as previously described [5] Blots were hybridized with specific anti-sense oligonucleotides end-labeled by T4 polynucleotide kinase using γ-[^{32}P]-ATP with specific activity 6000 Ci/mmol.

Complementation assay. DNA constructs encoding fusion proteins MyD88-Bla(a) and TLR4-Bla(b) were used for β-lactamase complementation assay with CCF2/AM (PanVera LLC, Madison, WI) as previously described[6]. Briefly, CHO cells co-expressing TLR4-Bla(a) and MyD88-Bla(b) (TLR4/MyD88) or empty vector transfected cells (Control) were plated in 6-well plates with growth medium (GM). After 24 hours, cells were left untreated or treated for 30 min with 10 μg/ml of a generic lactamase inhibitor, clavulanate, 10 μM of 50-F12, an inhibitor of TLR4-MyD88 interactions[7], or various concentrations of NP1 as indicated (see Supplementary Fig. 4). Finally, the cells were loaded with 1 μM of the lactamase substrate, CCF2/AM, for 1 h at room temperature and analyzed by fluorescence microscopy.

Data Presentation. Data depicted in main text and Supplementary Figures represent one of three or more experiments with each graph reflecting findings of multiple studies.

Supplementary References

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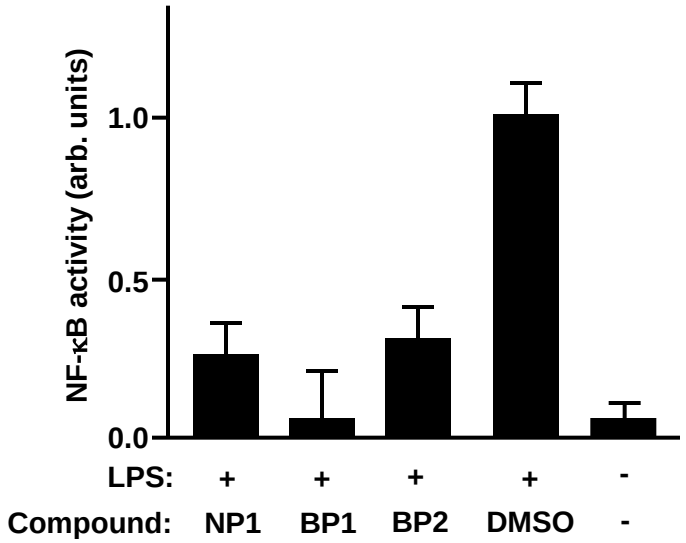
a



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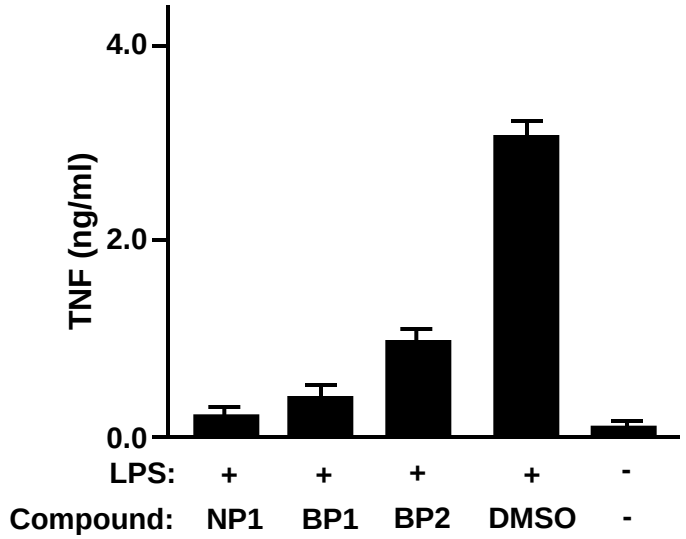
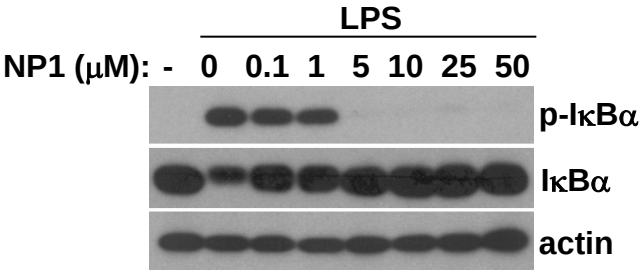
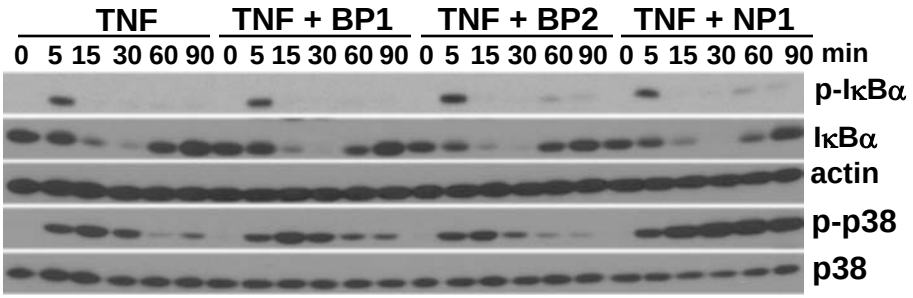


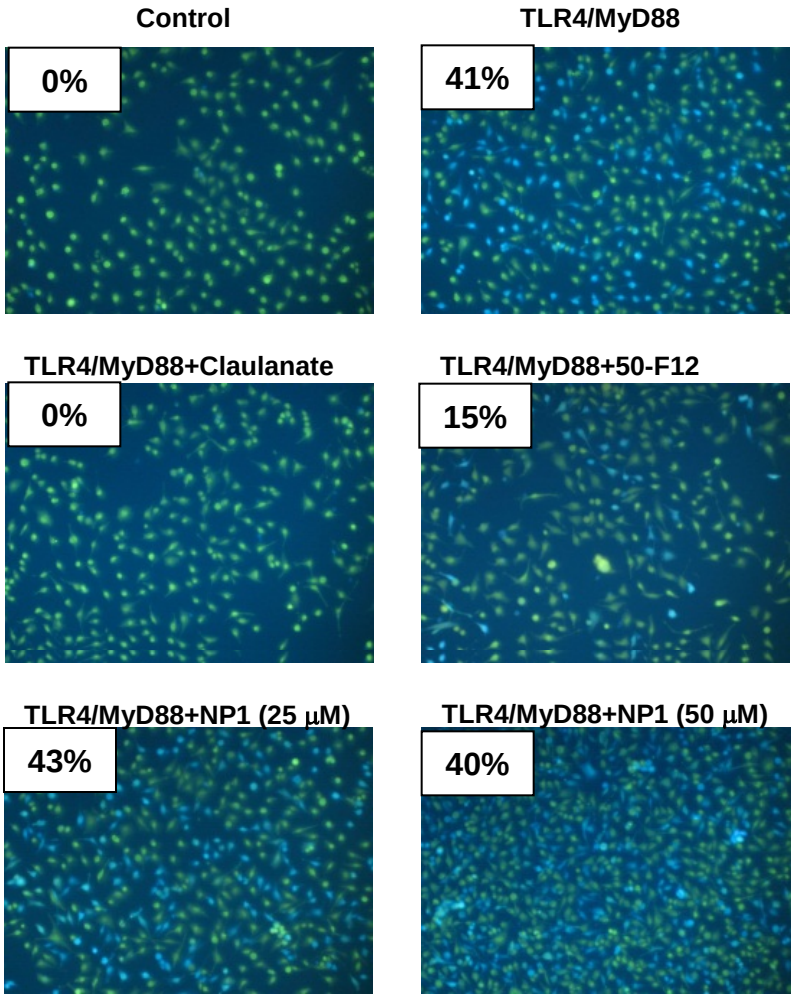
Supplementary Figure 1: Comparison of NP1-, BP1- and BP2-mediated effects on LPS-induced NF-κB activity. **(a)** THP1 cells harboring an alkaline phosphatase (AP) reporter gene driven by a NF-κB-responsive promoter (InVivogen; THP-1-blue-CD14) were stimulated with LPS (100 ng/ml) in the presence of library compounds (NP1, BP1 or BP2; 50 μM of each) or vehicle (DMSO). Conversion of substrate by AP was measured after 24 hr of stimulation and expressed as arbitrary units. **(b)** Human peripheral blood mononuclear cells were stimulated with LPS in the presence or absence of compounds as indicated in (a), and cellular supernatants were analyzed by ELISA assay for production of TNF, gene transcription of which depends on NF-κB activity.



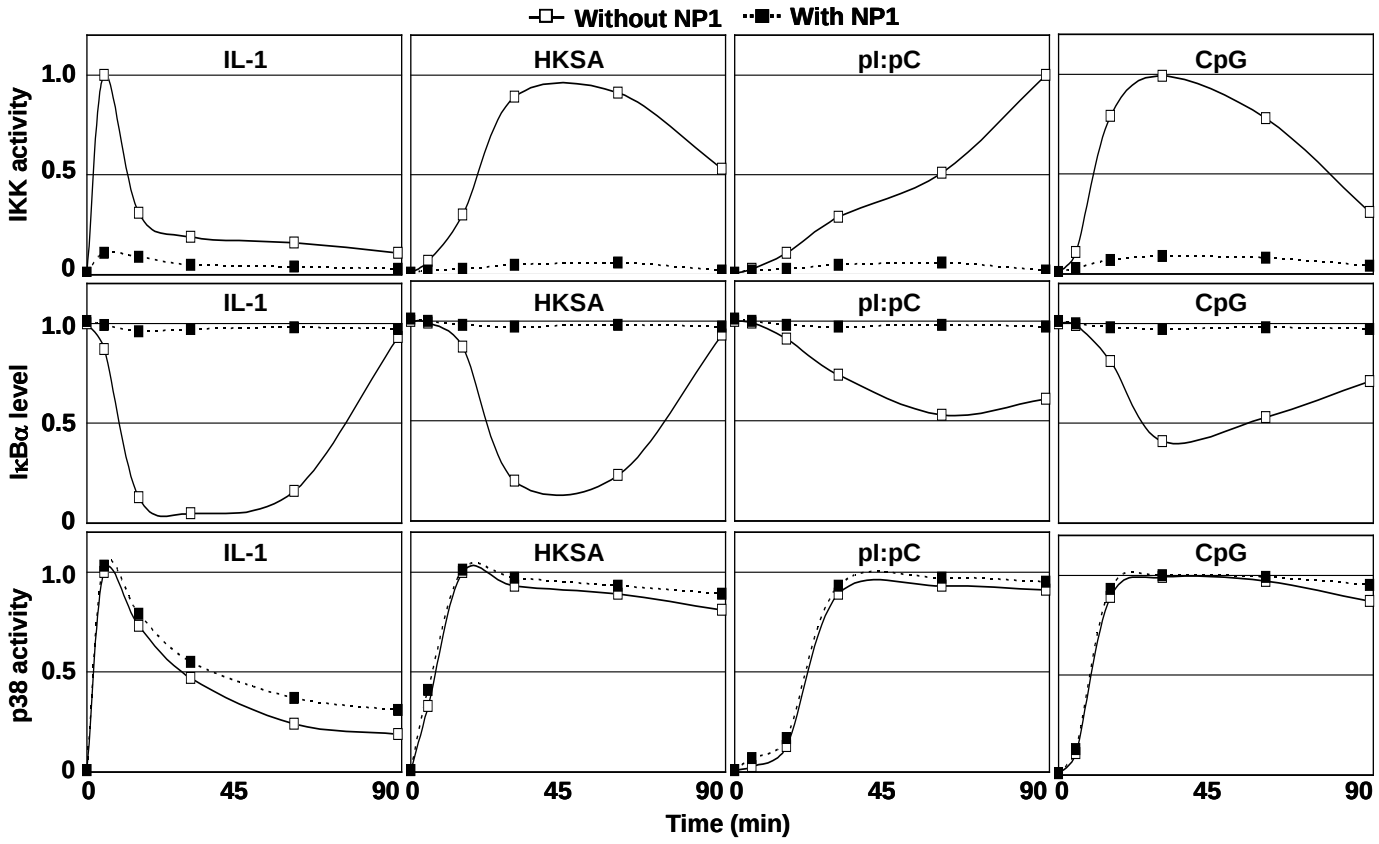
Supplementary Figure 2: Dose titration experiments shows the effect of NP1 on LPS-induced NF-κB signaling measured by Western blot analysis of IκBα phosphorylation and degradation in total protein extracts prepared after a 10 min treatment of macrophages with LPS (100 ng/ml) in the presence of different doses of NP1. Western blot analysis of actin was used as a loading control.



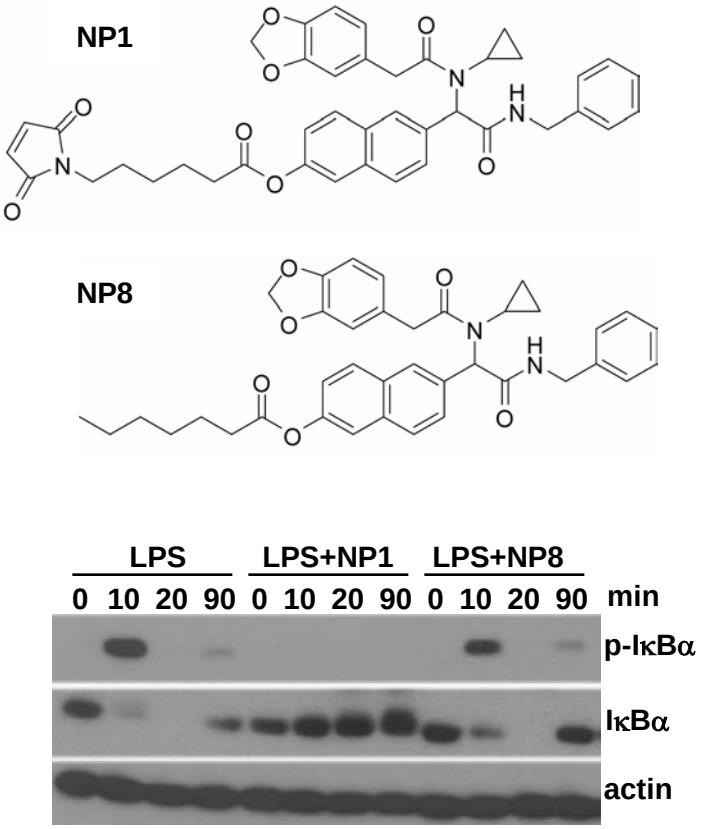
Supplementary Figure 3: TNF-induced NF-κB signaling and activation of the p38 pathway are unaffected in the presence of NP1, BP1 or BP2. Western blot analysis of IκBα, p38 and their phosphorylated forms (p-IκBα and p-p38, respectively) in extracts prepared from bone-marrow derived macrophages after treatment with TNF (40 ng/ml) alone or in combination with BP1, BP2 or NP1 (50 μM of each). Western blotting for actin was used as loading control.



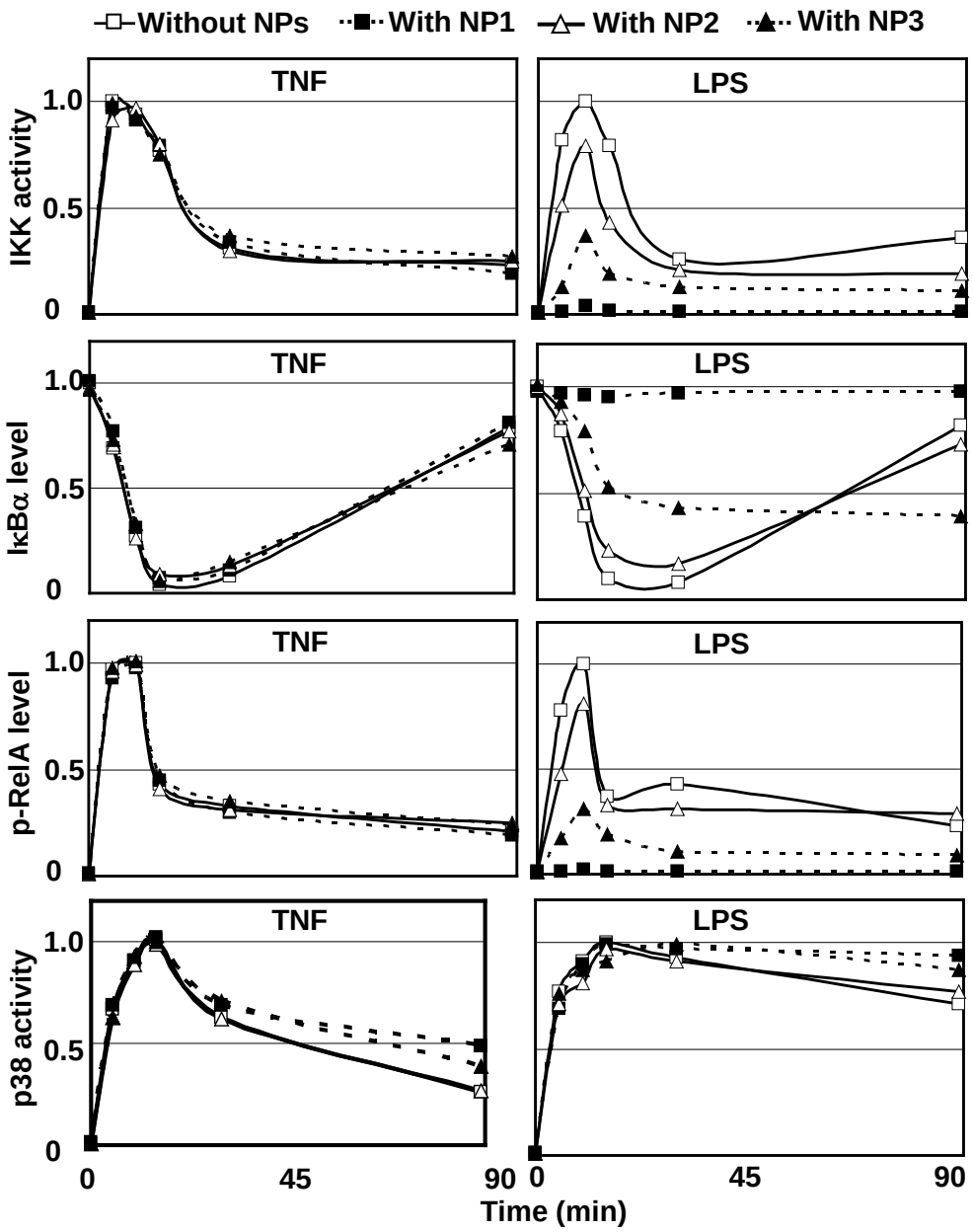
Supplementary Figure 4: The cell-based β-lactamase complementation assay shows the effect of inhibitors on the TLR4-MyD88 interaction. TLR4/MyD88 and control CHO cells were left untreated or treated for 30 min with 10 μg/ml of a generic lactamase inhibitor, clavulanate, 10 μM of 50-F12, an inhibitor of TLR4-MyD88 interactions, or 25 or 50 μM of NP1, as indicated. Finally, the cells were loaded with 1 μM of the lactamase substrate, CCF2/AM, for 1 h at room temperature and analyzed by fluorescence microscopy using a filter set with 405 nm excitation, 420 nm dichroic mirror, and 435 nm long-pass emission. TLR4/MyD88 cells co-expressed fusion proteins, MyD88-Bla(a) and TLR4-Bla(b) (see section **Supplementary Material and Methods**); the TLR4-MyD88 interaction brings two β-lactamase fragments into proximity resulting in an active form of β-lactamase. This β-lactamase further hydrolyzes CCF2/AM producing the coumarin donor that emits blue fluorescence. In contract, the intact CCF2/AM emits green fluorescence. The percent population of β-lactamase positive cells (blue) was estimated and showed in each panel.



Supplementary Figure 5: The profiles of IKK and p38 activities and IκBα protein concentration in bone-marrow derived macrophages stimulated with IL-1 (40 ng/ml), HKSA (~10^8 cfu/ml) , pI:pC (50 μg/ml) and CpG (10 μg/ml) in the absence or in the presence of NP1 (10 μM) as indicated. Quantitated and normalized results from three independent experiments are shown.



Supplementary Figure 6: The maliemide moiety of NP1 is required for NP1-mediated effect on LPS-induced NF-κB signaling. Bone marrow-derived macrophages were treated with LPS (100 ng/ml) in the absence and in the presence of NP1 or NP8 (a derivative of NP1 without maliemide group) for indicated period of time, and the prepared cellular extracts were analyzed by Western blot for IκBα and its phosphorylated form (p-IκBα). The chemical structures of compounds NP1 and NP8 are shown on the top. The final concentration of both compounds was 50 μM.



Supplementary Figure 7: The profiles of IKK and p38 activities as well as IκBα protein concentration and levels of RelA phosphorylation (p-RelA) in bone-marrow derived macrophages stimulated with TNF (40 ng/ml) and LPS (100 ng/ml) in the absence or in the presence of NP1, NP2 and NP3 as indicated. The concentration of all compounds was 25 μM. Quantitated and normalized results from three independent experiments are shown.