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Electronic Supplementary Information for

Inhibition of TNF-α stimulated nuclear factor-kappa B (NF-κB) activation by cyclometalated platinum(II) complexes

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Experimental Section

Materials. All starting materials were commercially purchased and used as received. Complexes **1a-d** were synthesized according to literature.[1-2] Camptothecin (CPT) and etoposide were purchased from Sigma-Aldrich (MO, USA). Antibodies were commercially obtained from Cell Signalling Technology (MA, USA) or Santa Cruz Biotechnology (CA, USA) as indicated. siRNA were commercially obtained from Santa Cruz Biotechnology (CA, USA).

Human hepatocellular carcinoma (HepG2) cell line was obtained from American Type Culture Collection (MD, USA). HepG2 cells carrying reporter genes were kindly provided by Prof. Y. C. Cheng (Department of Pharmacology, Yale University). Mutant FLAG-p65 plasmid constructed with Ser276 site mutated to alanine was kindly provided by Prof. James M. Samet (Human Studies Division, National Health and Environmental Effects Research Laboratory, U.S. EPA, Research Triangle Park, NC, USA). To determine the role of Ser276 phosphorylation in the inhibition of the NF-κB dependent gene transcription by **1a**, HepG2 cells carrying NF-kB reporter gene were further transfected with FLAG-p65 S276A plasmid construct according to the procedure suggested by Invitrogen. Cell culture flasks and 96-well microtitre plates were obtained from Nalge Nunc Int. (Rochester, NY, USA). Culture medium, other medium constituents and phosphate-buffered saline (PBS) were obtained from Gibco BRL (Rockville, Maryland, USA). Other reagents include: Tumor necrosis factor-alpha (TNF-α, Calbiochem, San Diego, CA); Phorbol-12-myristate-13-acetate (TPA, Calbiochem, San Diego, CA); Dexamethasone (Calbiochem, San Diego, CA); forskolin (Calbiochem, San Diego, CA); transfection reagent Lipofectamine (Invitrogen, CA, USA); luciferase reporter assay system (Promega, WI, USA).

Instrumentation. The fluorescence images of cells were taken by fluorescence microscope (Zeiss Axiovert 200M). For luciferase reporter assays, the luminescence was quantified by using a PerkinElmer Fusion Reader (Packard BioScience Company).

Luciferase reporter assay. HepG2 cells carrying reporter genes growing in a 48-well plate were pre-incubated with different concentrations of **1a-d** for 2 hr and subsequently activated with forskolin (CREB), TNF-α (NF-κB), serum plus TPA (SRE), TPA (AP1), Dexamethasone (GRE) for 4 hr. The cells were then lysed in a passive lysis buffer. Transcriptional activity was determined by measuring the activities of firefly luciferases using luciferase reporter assay system according to the manufacturer's instructions. All experiments were repeated for at least three times.

Electrophoretic-mobility-shift assay. HepG2 cells grown in 10-cm dishes were incubated with **1a–d** for 2 hr and then activated with TNF-α (25 ng/mL) for 4 hr. Nuclear extracts were obtained by following an established method[3]. Briefly, cells were collected in cold Tris-buffered saline (TBS) and centrifuged at 1,500 rpm for 5 minutes. Cells were then washed in buffer A [10 mmol/L Hepes (pH 7.9), 10 mmol/L

KCl, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L DTT and 0.5 mmol/L PMSF], lysed in the same buffer supplemented with 0.1% Nonidet P40, and centrifuged at 13,000 rpm for 10 minutes. The supernatant was removed as the cytosolic component. After that, the pellet was resuspended in buffer B [20 mmol/L Hepes (pH 7.9), 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 0.5 mmol/L spermidine, 0.15 mmol/L spermine, and 100 μmol/L sodium orthovanadate], and centrifuged at 13,000 rpm for 10 minutes. Then five volumes of buffer C [20 mmol/L Hepes (pH 7.9), 20% glycerol, 0.2 mmol/L EDTA, 50 mmol/L KCl, 0.5 mmol/L DTT, and 0.5 mmol/L PMSF] were mixed with the supernatant solution.

For EMSA, biotin-labeled probe (5'→3', AGTTGAGGGGACTTTCCCAGGC, containing NF-κB binding sites, 1×10^{-8}g per reaction) was incubated with the nuclear extracts (4 μg, excess amount compared with DNA probe) and different concentrations of **1a** (1-10 μM) at room temperature for 30 min in the presence of 50 μg/mL poly dI:dC, and the mixture was separated on a 6% non-denatured polyacrylamide gel. Specificity of protein-DNA interaction was confirmed by competition with excess unlabeled probes. The gel was dried, incubated with HRP-labeled avidin, and then detected by using Amersham's enhanced chemiluminescence (ECL) system. To interpret the results, if the interaction of **1a** with DNA impairs the binding of NF-kB with DNA, less DNA probe will bind to the NF-kB and results in the enhancement in band intensity corresponding to the 'free probe'.

TNF-α-TNFR-1 binding ELISA. The experiment is conducted following literature.[3] Brifely, microtitre plates were coated overnight with TNF-α (0.625 μg/mL) in PBS. After being washed three times with PBS/0.05% Tween 20 (PBST), the wells are blocked with 200 μL PBST containing 1% BSA for 60 min. Serial dilutions of **1a** in 50 μL PBS containing 2% DMSO were added to the wells and the microtitre plates were incubated for 20 min. TNFR-1 (0.2 μg/mL) in 50 μL PBS was added to the wells and the plates were incubated for a further 2 h. The plates were washed as before and incubated for 2 h with TNFR-1 antibody (1:1000) in 100 μL PBST containing 1% BSA. The plates were washed three times with PBST and incubated for 2 h with HRP-conjugated secondary antibody. The detection was achieved by incubation of wells with TMB solution for 30 min. The absorbance at λ = 450 nm reflects the binding amount of TNFR1 to the coated TNF-α. If the drug has no interference in the binding reaction of TNFR1 with TNF-α or does not affect the activity of TNF-α, the absorbance at 450 nm will show little difference with that coming from the vehicle control (no drug treatment).

Immunocytochemistry. After incubations with **1a**, TNF-α, or both TNF-α and **1a** for indicated time, HepG2 cells were fixed with 3% paraformaldehyde and were permeabilized with ice-cold 80% methanol. After incubation with 10% bovine serum albumin (BSA) in phosphate buffered saline solution with 0.5% tween 20 (PBST) for 60 min, the preparation was incubated for 1 h at room temperature with the anti-p65

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antibody diluted in 5% BSA. The preparation was next washed three times with PBST and was then exposed to the secondary antibody (fluorescein isothiocyanate-conjugated anti-rabbit IgG at 1:1000) and 10% BSA/PBST for 60 min. The fluorescent image was captured with a fluorescence microscope (Zeiss Axiovert 200M).

siRNA knockdown of ATM and MSK1/2. Knockdown of the expression of ATM and MSK1/2 in HepG2 cells containing NF-κB reporter gene was achieved by transfection of siRNA. The siRNAs of human ATM and MSK1/2 were purchased from Santa Cruz Biotechnology. Transfection of siRNA was performed by using Lipofectamine (Invitrogen, CA, USA). Briefly, cells were plated in 6-well dishes at a density of 2×10^5 cells/well. The next day, cells were treated with the siRNA transfection mixtures following the Invitrogen's protocol.

Western blot analysis. Total cell lysates were obtained by direct lysis in RIPA buffer (1% Triton X-100, 10% deoxycholate, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 0.1% PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin). The samples were fractionated in a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane (Amersham Biosciences), and incubated with specific antibodies (anti-p65, anti-phosphorylated p65, anti-ATM, and anti-pATM, from cell signaling; anti-pMSK1/2$^{Ser376/360}$ from R&D Systems; anti-MSK1 and anti-MSK2 from Santa Cruz) and the corresponding horseradish peroxidase-conjugated secondary antibodies. Proteins of interest were visualized by the chemiluminescent detection.

Synthesis of platinum(II) complexes. Complexes **1a–c** were prepared according to the procedures reported in the literature.[1,2]

Complex 1d (ClO_4^-): The methodology for the complex **1a** was adopted except that 2-naphthyl isocyanide was used. Anal. Calcd for $C_{27}H_{18}ClN_3O_4Pt$: C, 47.76; H, 2.67; N, 6.19. Found: C, 47.78; H, 2.71; N, 6.15. FAB-MS: *m*/*z* 579 [M+]. ^{1}H NMR (CD_3CN): 6.96 (m, 2H), 7.12 (m, 2H), 7.46(m, 2H), 7.51(t, 1H), 7.58 (m, 3H), 7.87 (m, 5H), 8.03 (d, 2H), 8.50 (d, 1H). IR (cm^{-1}): 2183.3 (N≡C).

References

1. S.-W. Lai, M. C.-W. Chan, K.-K. Cheung and C.-M. Che, *Organometallics*, 1999, **18**, 3327.
2. S.-W. Lai, H.-W. Lam, W. Lu, K.-K. Cheung and C.-M. Che, *Organometallics*, 2002, **21**, 226.
3. D. S.-H. Chan, H.-M. Lee, F. Yang, C.-M. Che, C. C. L.Wong, R. Abagyan, C.-H. Leung and D.-L. Ma, *Angew. Chem. Int. Ed.* 2010, **49**, 2860.

Fig. S1. Suppression of the TNF-α activated transcriptional activity of NF-κB by **1a**. HepG2 cells containing reporter genes of NF-κB-Luc, c-fos-Luc, GR-Luc, AP-1-Luc, CREB-Luc were treated with **1a** for 2 hr at the indicated concentrations and subsequently activated with forskolin (CREB), TNF-α (NF-κB), serum plus TPA (SRE), TPA (AP1), Dexamethasone (GRE) for 4 hr. After that, cells were collected and assayed for luciferase reporter activities. [SRE: Serum response factor (c-fos serum response element-binding transcription factor), also known as SRF. GRE: Glucocorticoid responsive elements. AP-1: Activator protein 1. CRE: cAMP response elements, also known as CREB, cAMP response element-binding].

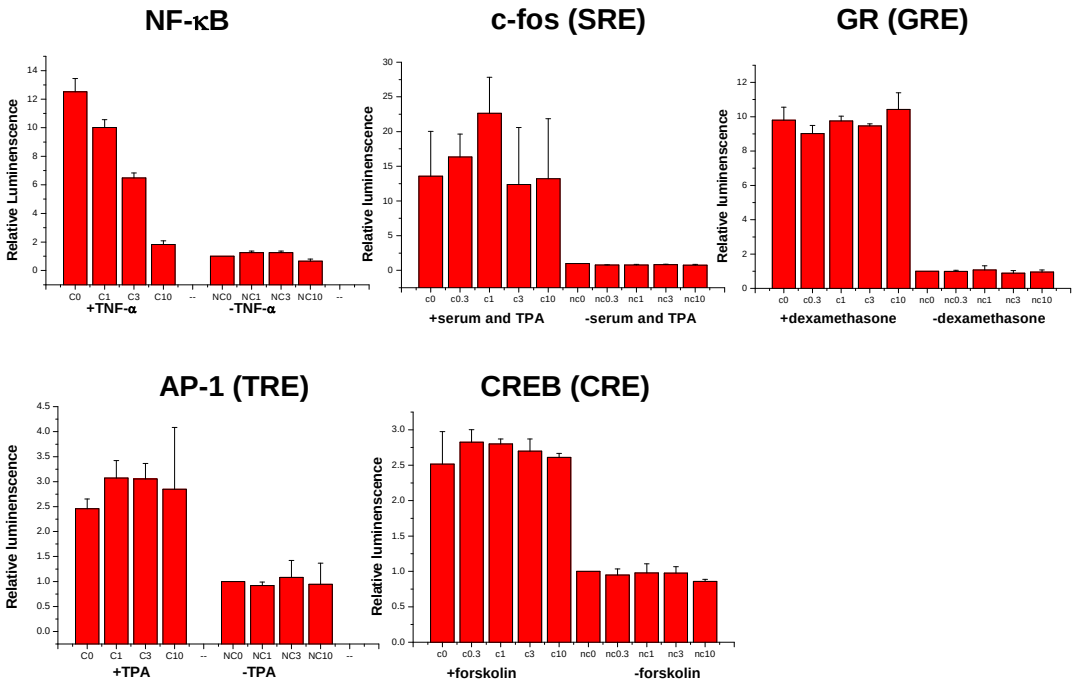

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Fig. S2. The effect of **1a** and a positive control indoloquinolizidine on the binding of TNFR-1 to immobilized TNF-alpha (ELISA).

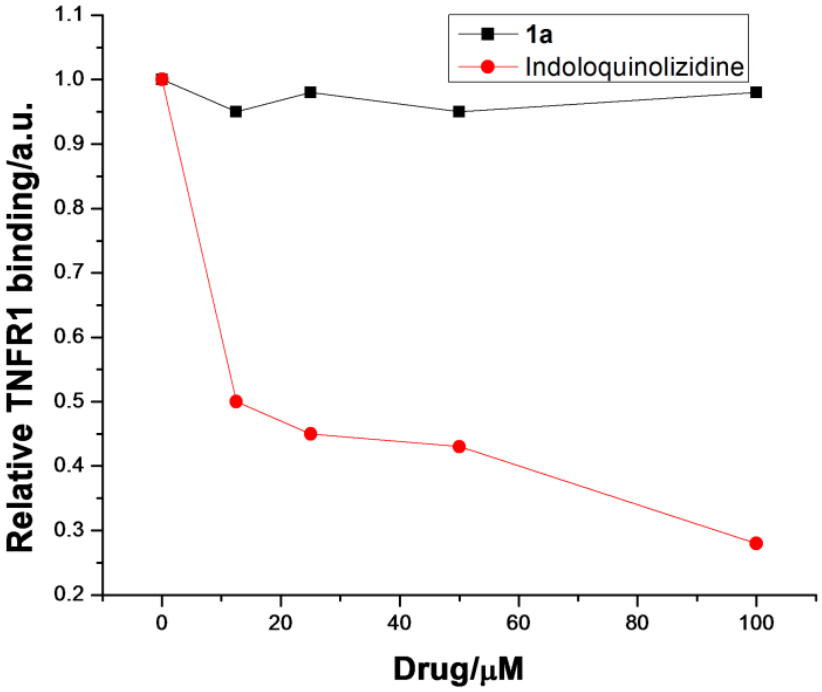

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Fig. S3. UV-vis absorption titration of **1a** in Tris buffered solution at 298 K with increasing ratio of [DNA]/[**1a**] showing the binding constant of 1.0×10^{6} $mol^{-1}dm^{3}$.

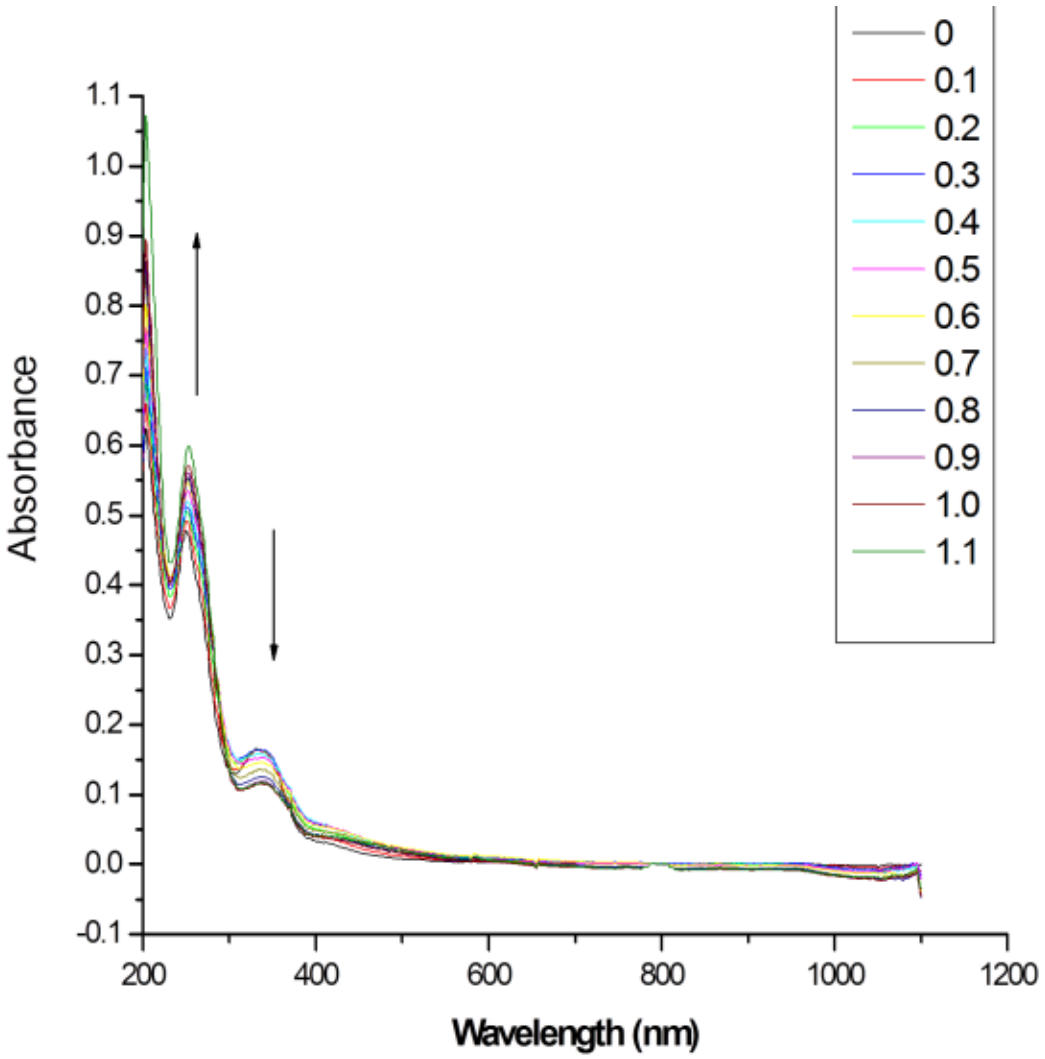

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Fig. S4. Relative specific viscosity (relative increase in contour length, L/L_0) of ctDNA in the presence of ethidium bromide (EB, ●), Hoechst 33342 (H33342, ■), and **1a** (▲) shown as a function of the molar ratio of added compound to DNA nucleotides (r).

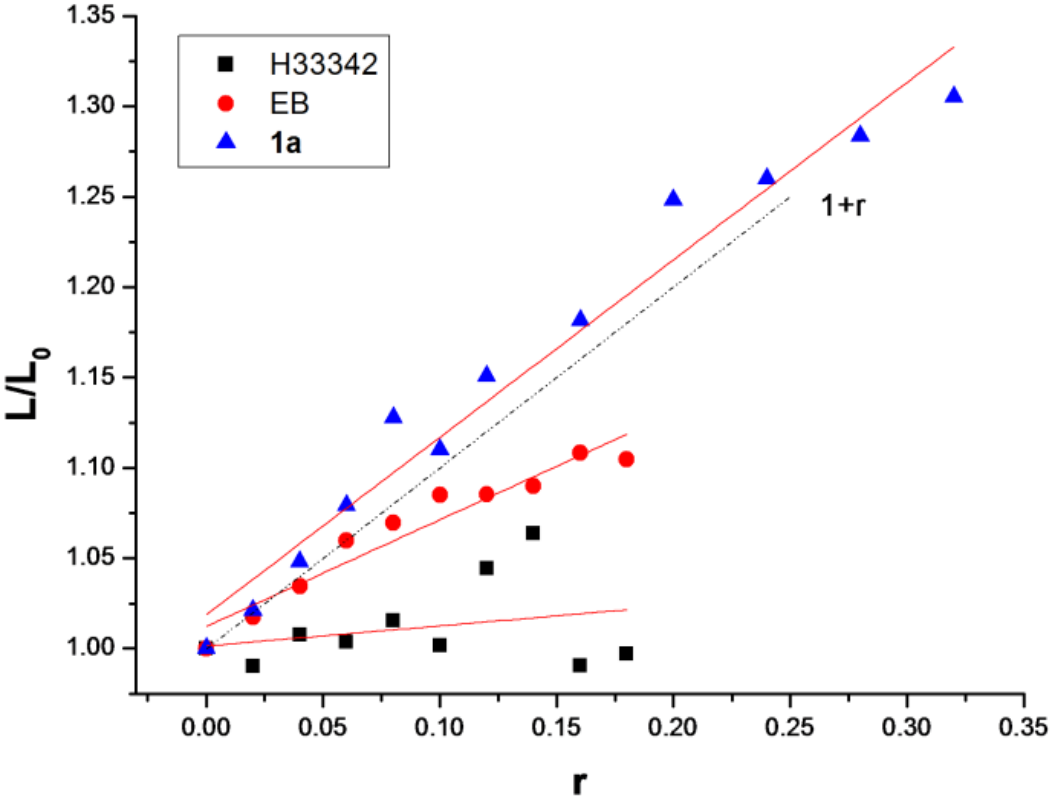
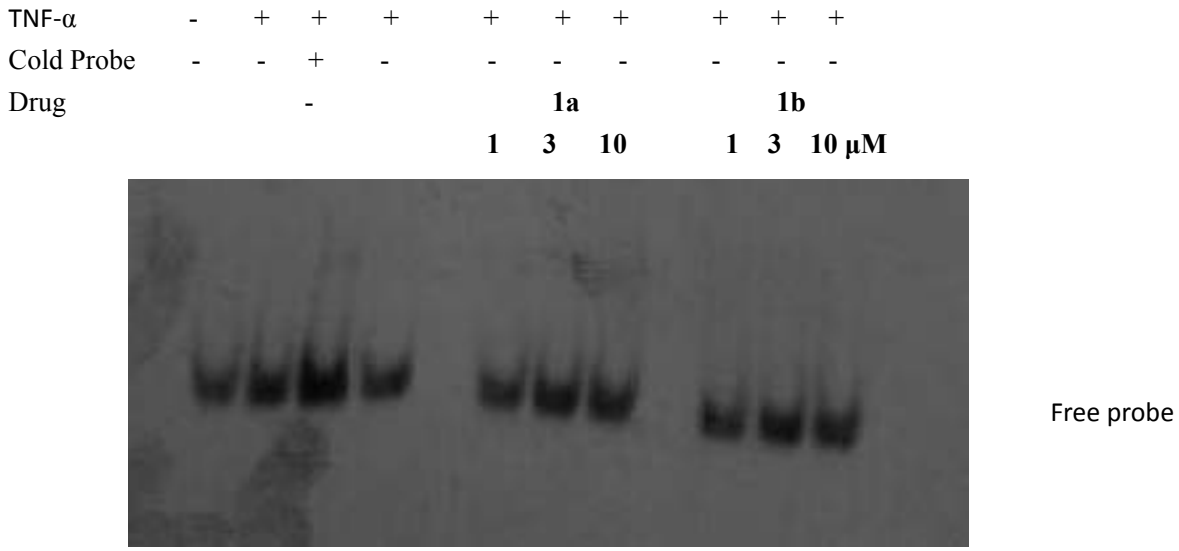


Fig. S5. Electrophoretic-mobility-shift assay shows that **1a–c** displayed little effect on the DNA binding reaction of NF-kB.



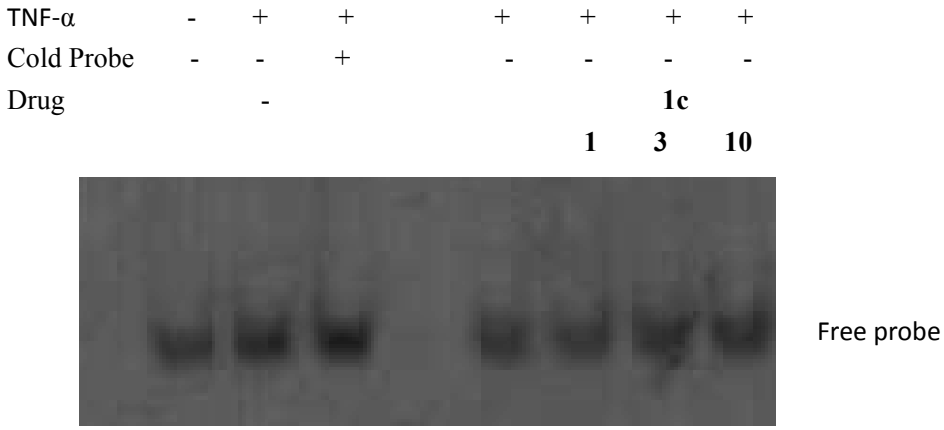
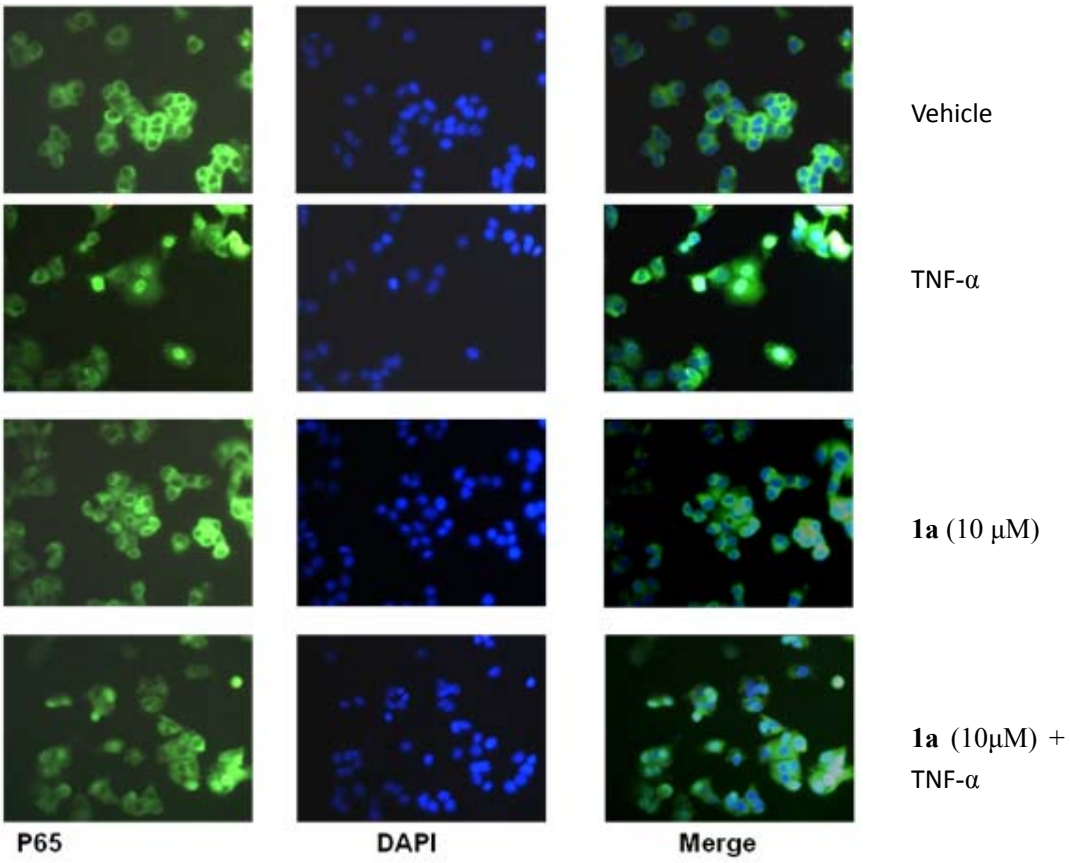


Fig. S6. Complex **1a** has no apparent effect on the translocation of p65. (a) Immunocytochemistry of HepG2 cells with four different kinds of treatment, including (i) vehicle control, (ii) stimulation with TNF-α for 4 hr, (iii) incubation with **1a** for 2 hr, and (iv) co-treatment with both TNF-α and **1a** as indicated above. (b) The nuclear extracts of HepG2 cells were analyzed for p65 by Western blot after either treatment with **1a** alone with indicated concentration or co-treatment with both TNF-α and **1a** with indicated concentration.

(a)



(b)

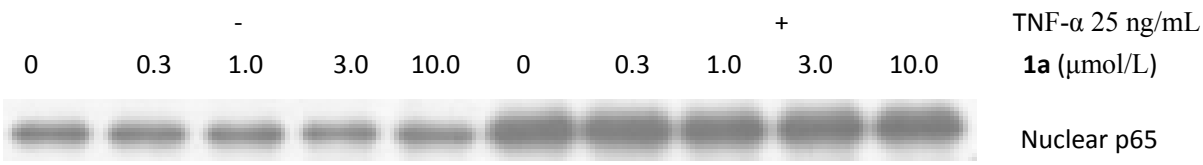
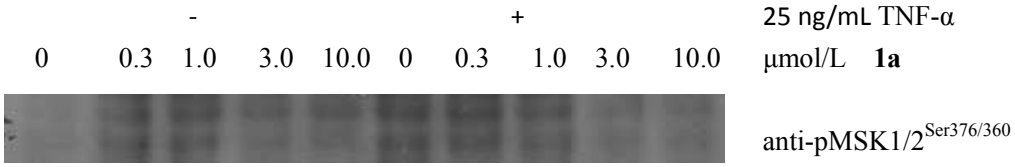


Fig. S7. The upstream kinase MSK2 was found to be involved in the inhibition of NF-κB activation by **1a** in HepG2 cells. (a) Phosphorylation of MSK1/2 at Ser376/360 was inhibited by co-treatment of **1a** and TNF-α. (b) MSK2 enhanced the inhibition of NF-κB by **1a**, which was assessed from the luciferase reporter gene assay. "____" represents siRNA silence; "____" represents untreated control. (c) Western bloting shows the decreased expression of MSK1 and MSK2 after siRNA transfection.

(a)



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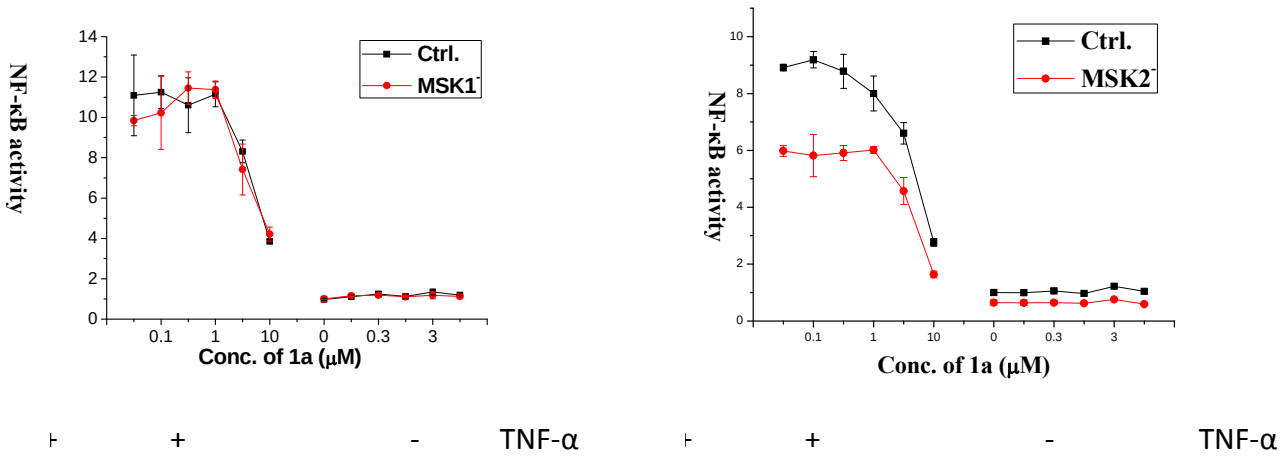


(c)

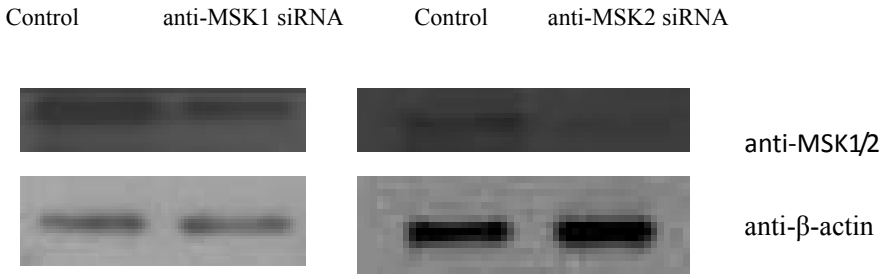
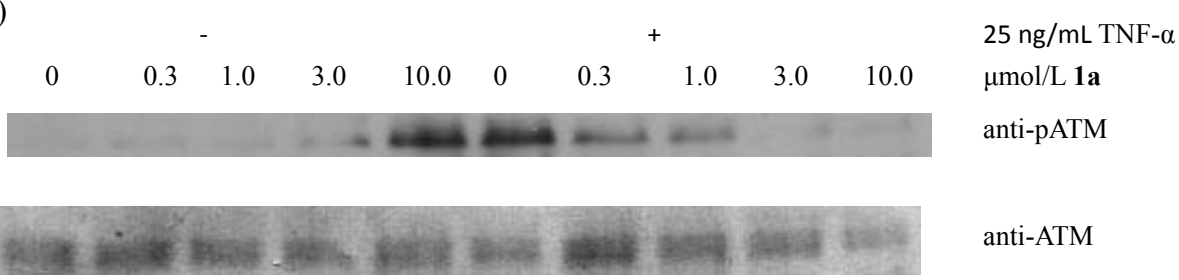
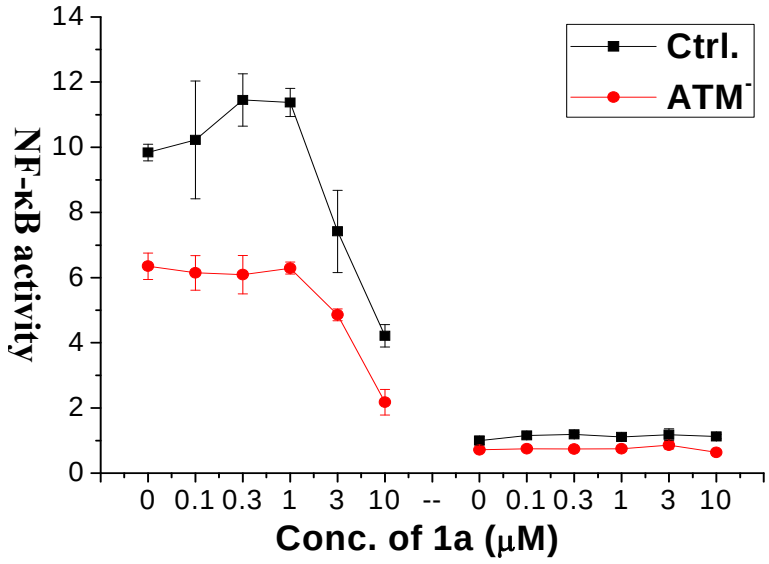


Fig. S8. The upstream kinase ATM was found to be involved in the inhibition of TNF-α stimulated NF-κB activation by **1a**. (a) Western blot assay of ATM and its phosphorylated form. (b) Luciferase gene reporter assay was used to assess the role of ATM. (c) Western bloting shows the decreased expression of ATM after siRNA transfection.

(a)



(b)



(c)

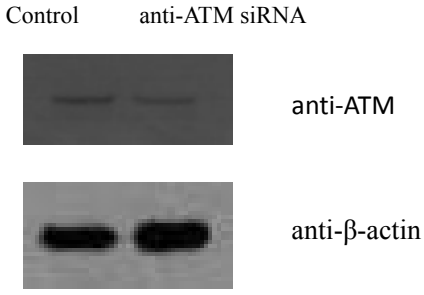


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Table S1. The half maximal inhibitory concentration (IC_{50}) of CPT and platinum complexes **1a-d** towards TNF-α stimulated NF-κB dependent gene transcription.

compound	IC_{50}/μM
1a	3.8±0.8
1b	2.5±1.0
1c	9.8±1.5
1d	>20
CPT	3.3±1.3