Electonic Supplementary Information

Silver Complex of *N,N'*-disubstituted Cyclic Thiourea as Anti-Inflammatory Inhibitor of IκB Kinase

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Materials and Methods

1. Chemical Synthesis

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Chemical Co. Au(THT)Cl was synthesized according to literature procedures.[1] Silver triflate was purchased from Apollo Scientific Co. $[Cu(CH_3CN)_4]PF_6$ was from Strem Chemicals. Analytical grade organic solvents and MiniQ water were used throughout the experiments. Thiourea ligand and metal thiourea complexes were prepared according to literature procedures with modifications.[2]

Synthetic procedures and characterization of the thiourea ligand[2]

Glyoxal-bis-(4-methoxyphenyl)imine

A 40% aqueous solution of glyoxal (3.15 g, 0.02 mol) in a mixture of 4 mL EtOH and 4 mL H_2O was added to a solution of p-anisidine (5.15 g, 0.04 mol) in EtOH (20 mL). Yellow precipitate could be observed after 1 min stirring and the mixture was stirred for 1 h. The yellow precipitate was collected, washed by EtOH and dried *in vacuo*. Yield: 3.80 g (71 %). EI-MS: 268.1 $[M]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.42 (s, 2H), 7.33 (d, *J* = 8.7 Hz, 4H), 6.95 (d, *J* = 8.8 Hz, 4H), 3.85 (s, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 159.8, 157.6, 143.0, 123.1, 114.6, 55.5.

N,N'-Bis-(4-methoxyphenylamino)ethane

Glyoxal-bis-(4-methoxyphenyl)imine (1.40 g, 5.23 mmol) was added to a mixture of 30 mL THF and 3 mL MeOH to form a yellow suspension. Sodium borohydride (0.80 g, 0.02 mol) was added to the suspension at 0 ^{o}C. The mixture was stirred at room temperature for overnight followed by 2 hr reflux. After the addition of water and $CHCl_3$, the pH of aqueous layer was adjusted to slightly basic by 1M HCl and Na_2CO_3. The product was extracted, concentrated and dried *in vacuo*. Yield: 1.36 g (95 %). EI-MS: 272.2 $[M]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.79 (d, *J* = 8.9 Hz, 4H),

6.63 (d, *J* = 8.9 Hz, 4H), 3.75 (s, 6H), 3.34 (s, 4H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 152.5, 142.4, 115.1, 114.6, 56.0, 44.6.

1,3-bis(4-methoxyphenyl)imidazolidine-2-thione (Thiourea, TU)

N,N'-Bis-(4-methoxyphenylamino)ethane (0.65 g, 2.38 mmol) was firstly dissolved in dry THF. 1,1'-thiocarbonyl diimidazole (0.5 g, 2.83 mmol) was then added, the mixture was stirred at room temperature for overnight followed by 2 hr reflux. The resulting mixture was extracted with water and ethyl acetate. The organic layer was then washed with dilute HCl followed by brine, concentrated and dried *in vacuo*. Yield: 0.70 g (93.6 %). The crude product could be further purified through recrystallization from 95 % EtOH. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.41 (d, *J* = 9.0 Hz, 4H), 6.94 (d, *J* = 9.0 Hz, 4H), 4.10 (s, 4H), 3.81 (s, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.3, 158.2, 133.9, 127.2, 114.3, 55.5, 49.9.

Synthetic procedures and characterization of the metal thiourea complexes[2]

$[Ag(TU)_2]OTf$

Silver triflate (0.14 g, 0.54 mmol) was added to a suspension of thiourea **TU** (0.32 g, 1.02 mmol) in 10 mL EtOH. The mixture was stirred for 3 hr at room temperature under argon atmosphere in dark. The unreacted starting material was filtered with 0.2 μm filter and removed. The remaining filtrate was stand overnight. Colorless crystals were collected, filtered and dried *in vacuo.* Yield: 0.39 g (86 %).

$[Au(TU)_2]Cl$

TU (0.50 g, 1.58 mmol) was first dissolved in 5 mL CH_2Cl_2, and was then added to a solution of Au(THT)Cl (0.27 g, 0.83 mmol) in 5 mL distilled MeOH under argon atmosphere. The mixture was stirred overnight at room temperature, filtered and left stand overnight. White solid was collected upon addition of Et_2O to the filtrate, filtered and dried *in vacuo.* Yield: 0.31 g (45 %).

$[Cu(TU)_2]PF_6$

To solution of $[Cu(CH_3CN)_4]PF_6$ (0.19 g, 0.5 mmol) in distilled 10 mL MeOH was added solution of thiourea **TU** (0.31 g, 1 mmol) in 10 mL CH_2Cl_2. The mixture was stirred at room temperature for 2 h under argon atmosphere. The product was washed with MeOH and then Et_2O during filtration, dried *in vacuo* and collected as white solid. Yield: 0.32 g (76 %).

2. Biological Experiments

Chemicals and antibodies.

All starting chemicals for synthesis, unless otherwise stated, were purchased from Sigma-Aldrich Chemical Co. IKKα, IKKβ, P-IKKα/β (Ser$^{176/180}$), NF-κB p65, P-NF-κB p65 (Ser ^{536}), IκBα, P-IκBα (Ser $^{32/36}$) were purchased as NF-κB pathway sampler kit (9936) from Cell Signaling Technology. DYKDDDDK tag antibodies (2368S) were from Cell Signaling Technology. Protein A/G Plus-agarose (sc-2003), IκBα (sc-4094), anti-IKKα/β (sc-7607) and anti-IKKγ (sc-8330) were purchased from Santa Cruz Biotechnology. PMA, LPS, TNF-α (ALX-520-002) and (GST)-IκBα (BML-UW9970) were from Enzo Life Sciences. *Taq* polymerase and RNA isolation kit (RNeasy mini kit) were obtained from QIAGEN. Hoechst 33342 (B-2261), monoclonal anti-FLAG M2 affinity beads (F1804) was obtained from Sigma. AlexaFlour 488 secondary antibodies (A11070 & A11017), hygromycin B (10687-010), SuperScript^{TM} II Reverse Transcriptase, Lipofectamine 2000 reagent and biotinylated idoacetamide (BIAM) were from Invitrogen. pGL4.32 [luc2P/ NF-κB-RE/Hygro] plasmid and luciferase assay system (E1501) was obtained from Promega. pcDNA-IKKb-FLAG WT plasmid (#23298) was obtained from Addgene. Stock solutions of metal thiourea complexes and thiourea ligand were made up with DMSO to concentrations of 5 mM. Stock solution of $AgNO_3$ was made up with MiniQ water to concentration of 10 mM.

Cell culture and transfection.

The human cervix epithelial carcinoma cells (HeLa) were maintained in minimal essential medium supplemented with 10 % fetal bovine serum. The cell cultures were incubated at 37 ^{o}C in a 5 % CO_2/95 % air humidified atmosphere and sub-cultured twice weekly. HeLa cells stably expressing the NF-κB-luciferase reporters (pGL4.32[luc2P/NF-kB-RE/Hygro]) were prepared by transfection with the use of Lipofectamine 2000 reagent followed by hygromycin selection.

RT-PCR analysis.

RT-PCR was performed with 30 ng RNA using SuperScript III one-step RT-PCR system (Invitrogen). The cDNA was synthesized at 55 ^{o}C for 30 min. The PCR profile was denatured at 94 ^{o}C for 1 min, 1 min annealing at 55 ^{o}C, and 68 ^{o}C extension for 1.5 min for 28 cycles (C1000 Thermal Cycler, BioRad).

The sequences of primers used in the RT-PCR experiments are:

Gene symbol	Primer sequence	Product size (bp)
IL-6	Left: GGGAACGAAAGAGAAGCTCT Right: ACCAGAAGAAGGAATGCCCA	730
IL-8	Left: ACCGGAAGGAACCATCTCACTG Right: GCATCTGGCAACCCTACAACA	444
TNF-α	Left: GAGCACTGAAAGCATGATCCGGGAC Right: TTGGTCTGGTAGGAGACGGCGATGC	495

Immunoblot analysis.

Cells were lysed with radioimmunoprecipitation assay buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate) supplemented with protease inhibitor. Equal amount of proteins (50 μg) was resolved by SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked with Tris-buffered saline containing 0.1 % Tween 20 and 5 % BSA, and then incubated with the primary antibodies at 4 ^{o}C overnight, followed with appropriate horseradish peroxidase conjugated secondary antibodies for 2 h. The immunoreactivities were detected using enhanced chemiluminescence detection kit (GE Healthcare).

Luciferase reporter assay.

HeLa or HepG2 cells stably transfected with pGL4 NFκB-Luc plasmid were seeded in 48-well plates and grown for 24 h. Cells were drug-treated for 2 h followed by stimulation of 25 ng/ml of TNF-α for 4 h. Cells were lysed in 1x reporter lysis buffer. Luciferase activity was measured with luciferase assay system using plate reader.

IKK kinase assay.

Cells were lysed in lysis buffer supplemented with protease inhibitor (20 mM HEPES pH 7.3, 150 mM NaCl, 1 mM EDTA, 0.1 % NP-40, 10 % glycerol, 1 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate) for 15 min at 4 ºC. The cell lysate was centrifuged at 13,000 g for 19 min at 4 ºC. The supernatant was collected, 150 μg protein was incubated with 1 μg anti-IKKγ for 2 h at 4 ºC followed by 30 μl of Protein A/G Plus agarose for 2 h at 4 ºC on a shaker. Beads coated with IKKγ were washed with lysis buffer for 3 times and HEPES saline (50 mM HEPES, pH 7.3, 150 mM NaCl) for 1 time. Kinase reaction was started by incubating the beads with 0.5 μg GST-IκBα substrate, 50 μM ATP in 20 μl kinase buffer (20 mM HEPES, pH 7.3, 20

mM $MgCl_2$, 10 mM β-glycerophosphate) at room temperature for 30 min on a shaker. Proteins were resolved by 8 % SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membrane. The membrane was blocked with Tris-buffered saline containing 0.1 % Tween 20 and 5 % BSA. The membrane was probed with anti-P-IκBα (Ser $^{32/36}$) antibody (1:1000).

Immunofluorescence staining.

HeLa cells were drug-treated for 2 h followed by TNF-α stimulation (25 ng/ml). The pretreated cells were fixed with 3 % formaldehyde in PBS, permeabilized with 0.2 % Triton X-100 in PBS, and blocked with 1 % BSA in PBS. The cells were incubated with NFκB-p65 primary antibodies for 2 h followed by AlexaFlour 488 secondary antibodies for 1 h. The nuclei were stained by incubation of 1 μM Hoechst 33342 for 5 min. The stained cells were visualized under fluorescence microscope.

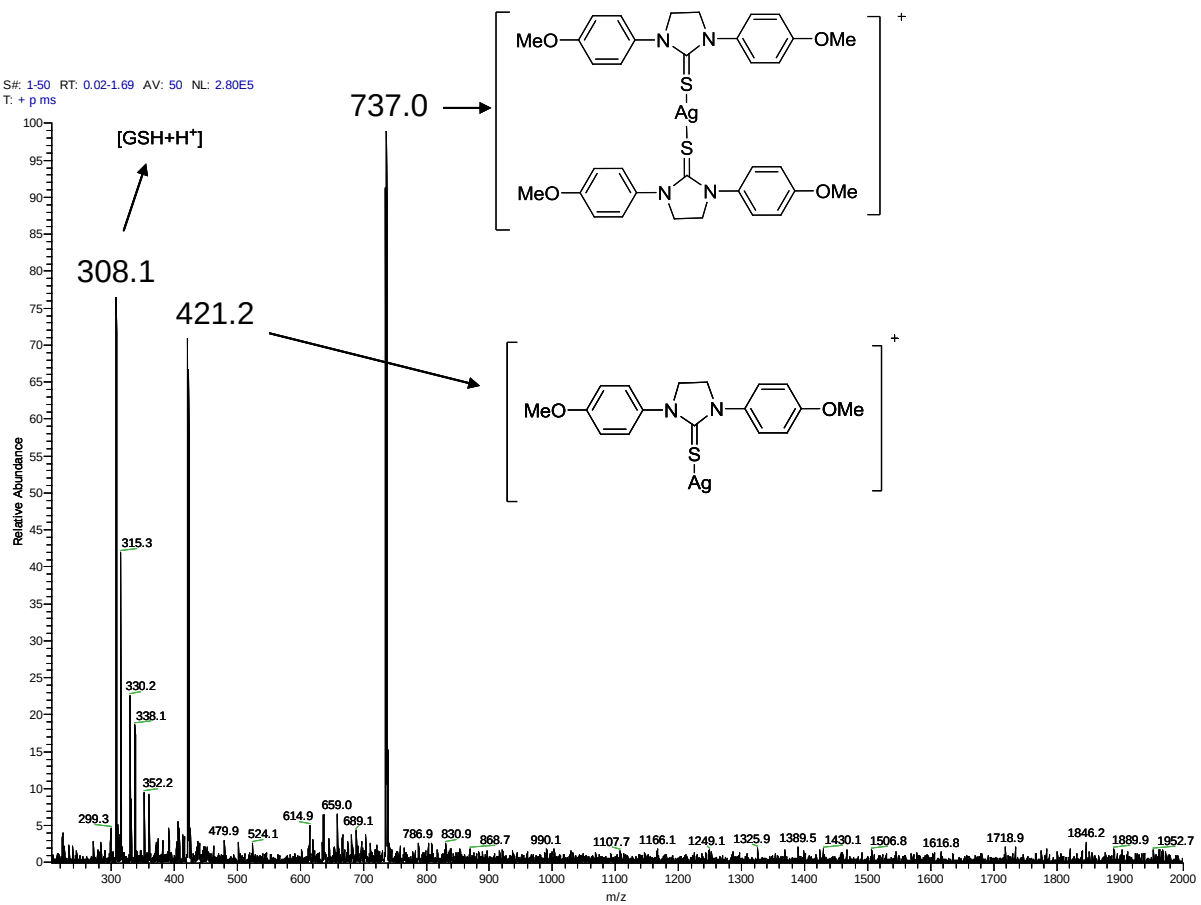
Assessment of cysteine modification[2,3]

The TNF-α-stimulated cells were lysed in lysis buffer (20 mM HEPES pH 7.3, 150 mM NaCl, 1 mM EDTA, 0.1 % NP-40, 10 % glycerol, 1 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, protease inhibitors and 1 mM DTT). The cell extracts were immunoprecipitated with anti-IKKγ antibody. The immunopreciptate was suspended in 10 mM Tris-Cl, pH 7.4 and 10 μM of **AgTU** or **$AgNO_3$** was added and incubated for 10 min. 50 μM of BIAM in 100 mM Tris-Cl buffered at pH 6.5 or pH 8.5 was added and the labeling reaction was carried out at 37 ^{o}C for 30 min. The reaction mixtures were mixed with loading buffer and subjected to SDS-PAGE. The separated proteins were transferred to PVDF membrane. The BIAM labelled proteins were detected with horseradish peroxidase conjugated streptavidin and enhanced chemiluminescence detection. The IKK subunits were detected as loading control with anti-IKKα/β or IKKγ antibody.

Supplementary Figures and Tables

Figure S1. ESI-MS spectrum of **AgTU** in the presence of GSH. (A) Positive mode spectrum of a mixture of **AgTU** (100 μM) and GSH (500 μM) in a solution of 50 mM ammonium bicarbonate (pH 8.0) containing 10% MeOH, recorded immediately after mixing and dilution of the samples. (B) As in (A), recorded at 3h after mixing. (C) Negative mode spectrum of a mixture of **AuTU** (100 μM) and GSH (500 μM) in a solution of 50 mM ammonium bicarbonate (pH 8.0) containing 10% MeOH, recorded immediately after mixing and dilution. All the mass spectra were recorded using Finnigan LCQ mass spectrometer.

A



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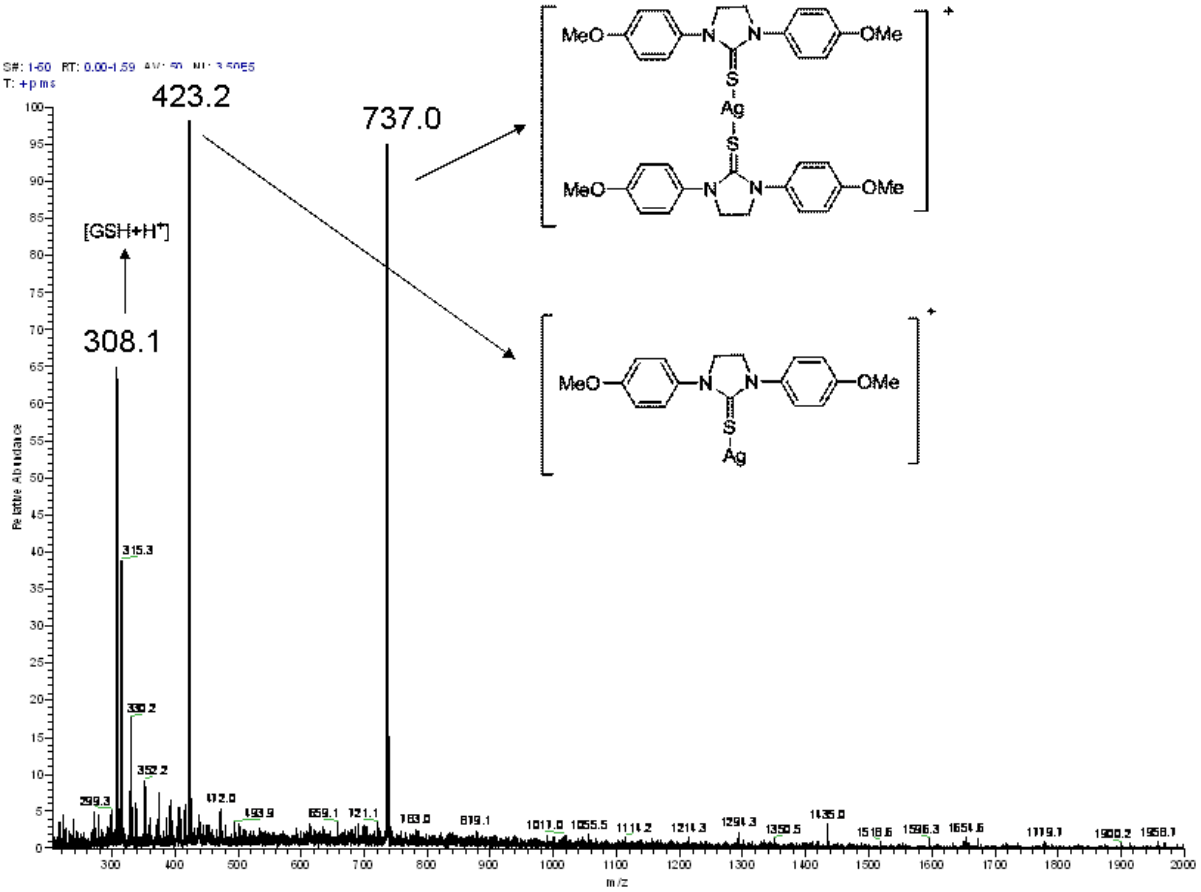

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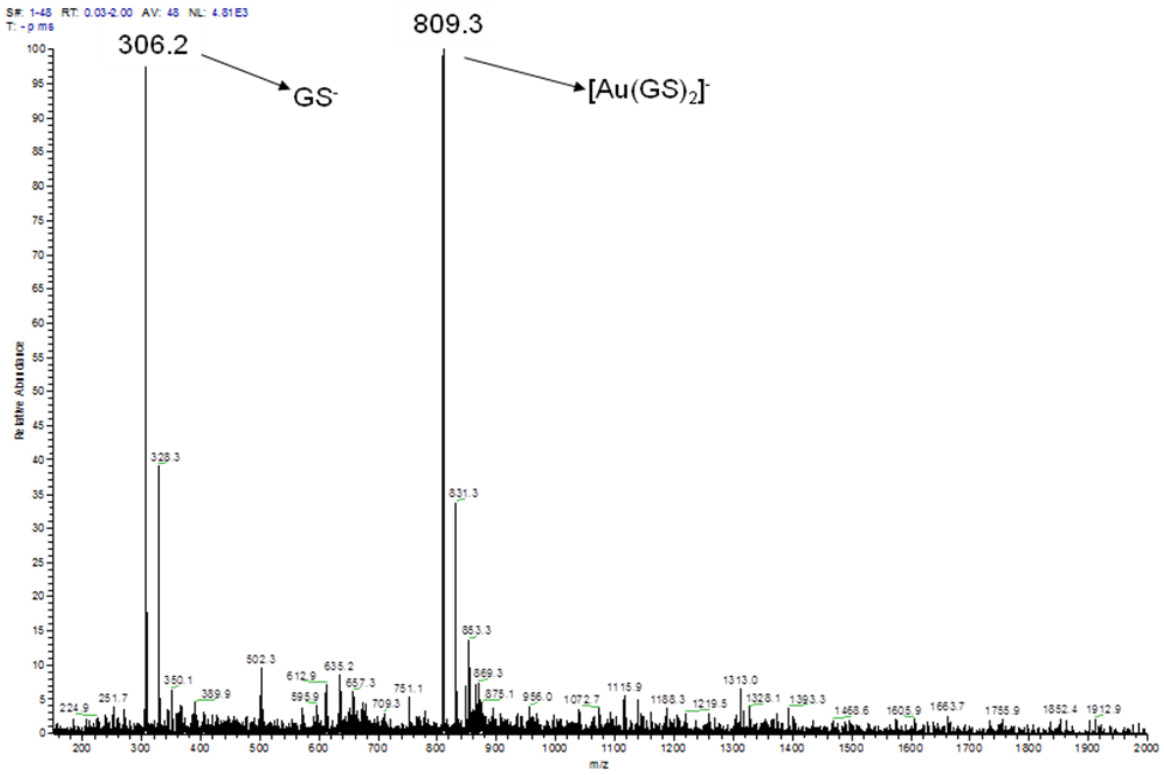


Figure S2. (A) Inhibition of TNF-α-stimulated NF-κB activity by Ag complex of *N*-heterocyclic carbene complex ($[Ag^{I}(NHC)_2]$ OTf where NHC= 1,3-dimethylimidazol-2-ylidene). HeLa cells transfected with NF-κB-luciferase reporter gene were treated with the Ag complex for 2 h, followed by TNF-α stimulation for 4 h and the cellular luciferase activities were determined. (B) Chemical structure of $[Ag^{I}(NHC)_2]$ OTf.

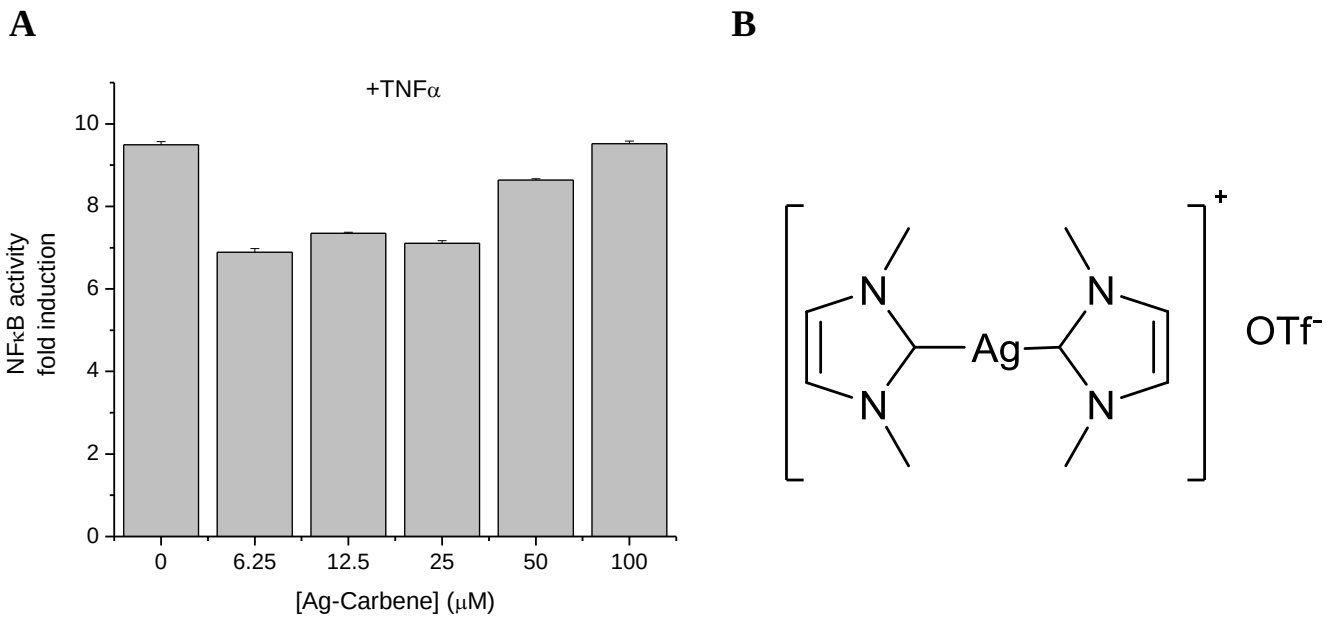


Figure S3 Inhibition of PMA stimulated NF-κB activity by **AgTU**. HeLa cells transfected with NF-κB-luciferase reporter gene were treated with **AgTU** for 2 h, followed by stimulation PMA (100 nM) for 4 h and the cellular luciferase activities were determined.

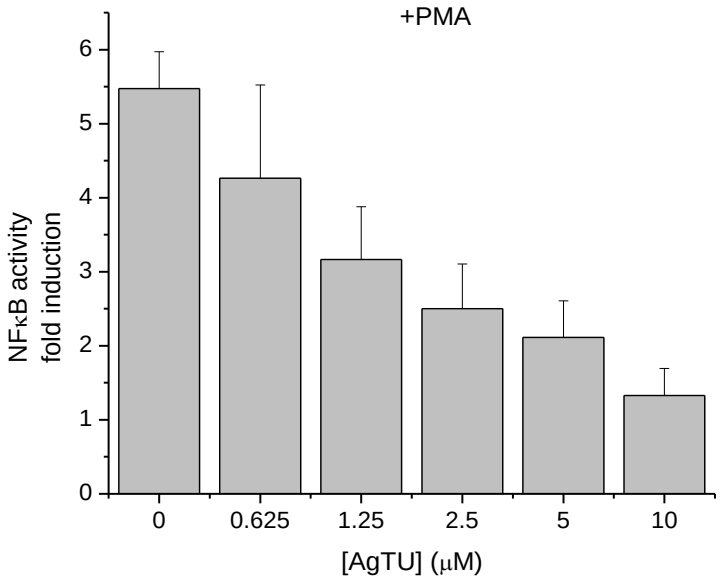


Figure S4 Effects of **AgTU** and $AgNO_3$ on the ubiquitin-proteasomal degradation. The cellular activities of ubiquitin-proteasomal degradation system were assayed using HeLa cells stably transfected with (A) a yellow fluorescence reporter protein (Ub^{G76V}-YFP) with a constitutively active degradation domain (Ub^{G76V}) that targets the proteins for ubiquitination and proteasomal degradation and (B) a green fluorescence reporter protein fused with a degradation domain of ODC (GFP-ODC) that targets the protein for rapid ubiquitin-independent proteasomal degradation. MG132 (5 μM) was used as positive control for proteasome inhibition. [4]

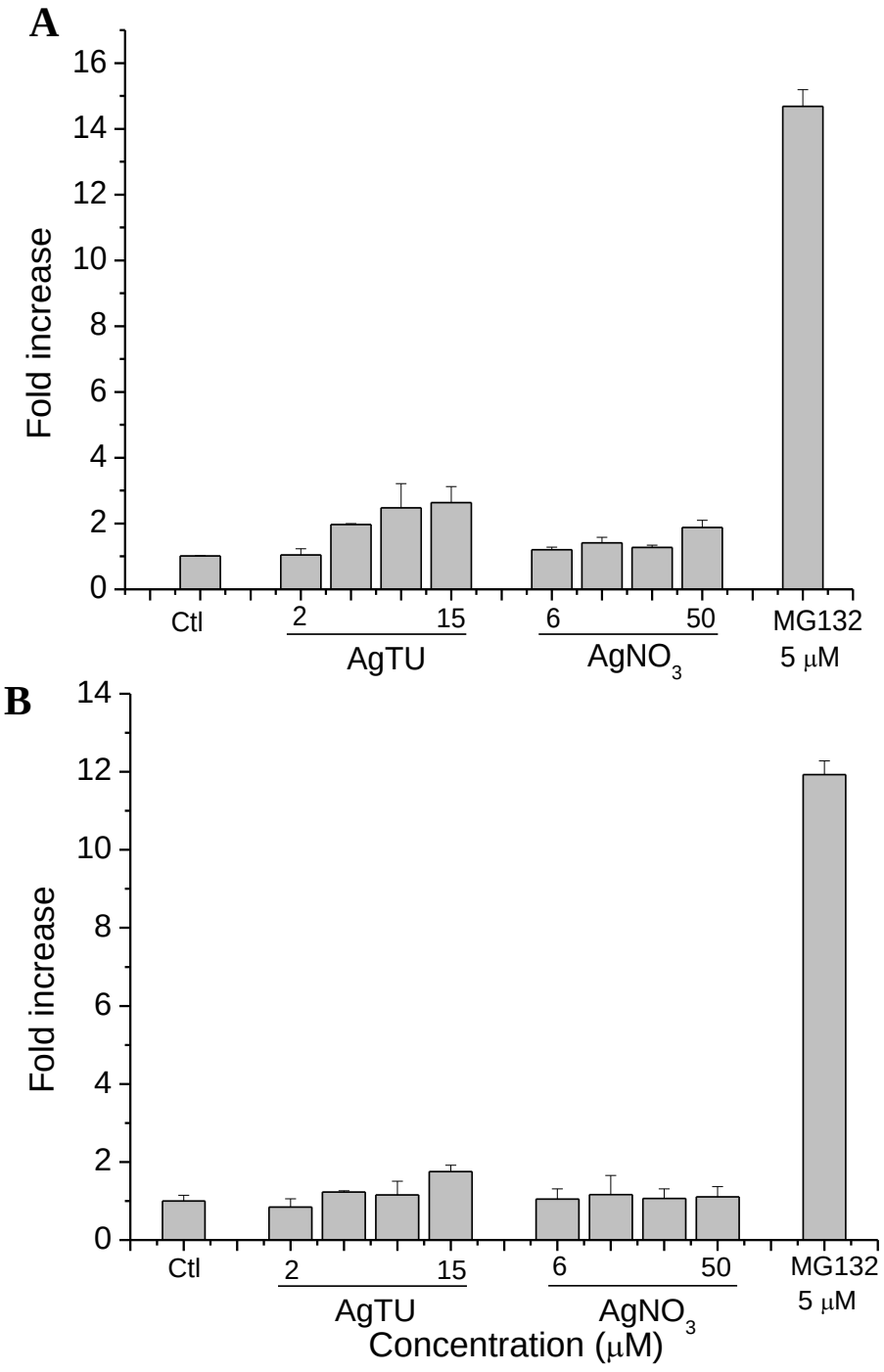


Figure S5. Inhibition of TNF-α-stimulated IκBα phosphorylation and IκBα degradation by **AgTU** in the presence of proteasome inhibitor MG-132. HeLa cells were treated with 10 μM **AgTU** or 50 μM **$AgNO_3$** for 2 h, 10 μM MG-132 for 30 min, followed by the TNF-α stimulation for 10 min. The phosphorylation of IκBα and expression of IκBα were determined by immunoblot analysis.

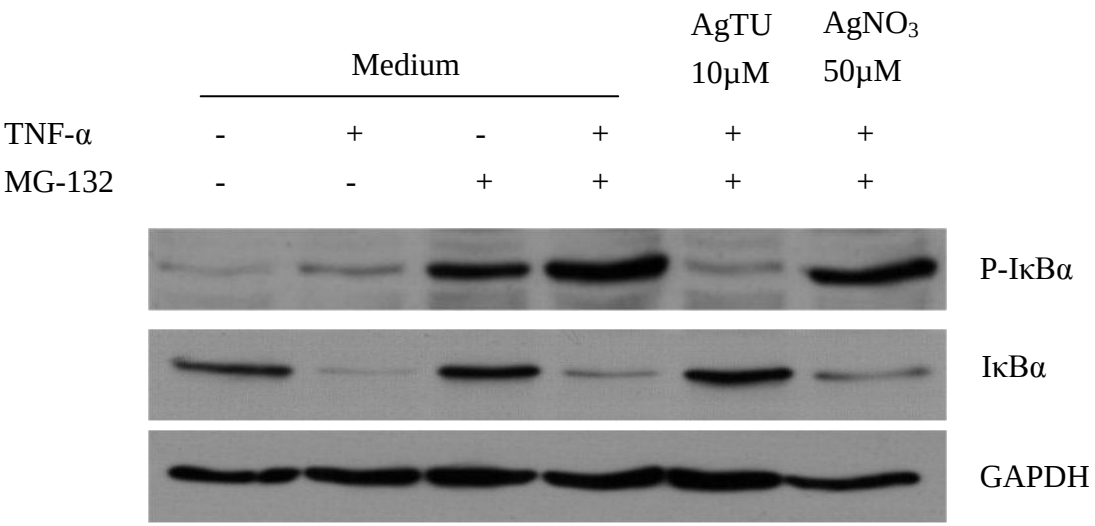


Figure S6. Suppression of TNF-α-stimulated expression of inflammatory cytokines (IL-6, IL-8 and TNF-α) by **AgTU**. HeLa cells were pretreated with 10 μM **AgTU** or 50 μM **$AgNO_3$** for 2 hr followed by TNF-α stimulation for 2 hr. The mRNA expression levels were examined by RT-PCR assay.

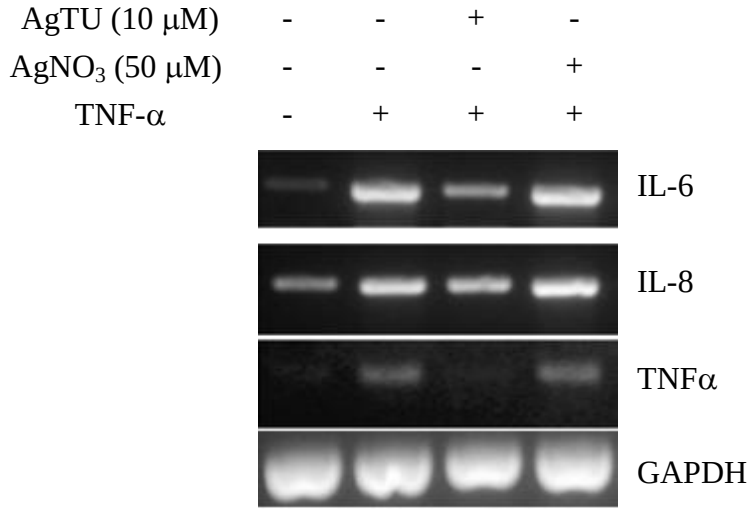


Figure S7 Inhibition of TNF-α-stimulated IκBα degradation and NF-κB p65 phosphorylation by **AgTU** or $AgNO_3$. HeLa cells were treated with 10 μM **AgTU** (A) or $AgNO_3$ (B) for 2 h followed by stimulation with TNFα (25 ng/mL) for 10 min. The expression of IκBα and NF-κB p65 (S536) were determined by immunoblot analysis.

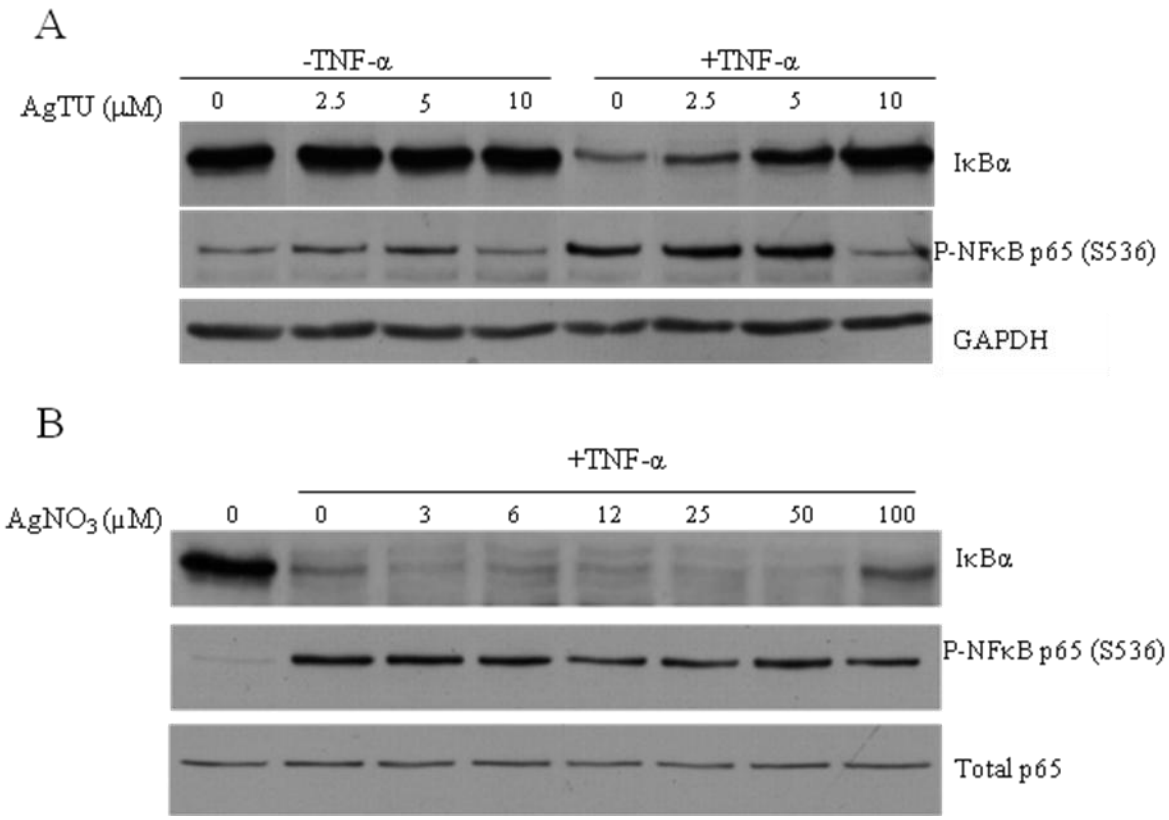


Figure S8 Immunofluorescence microscopic examination of nuclear translocation of p65 in HeLa cells treated with **AgTU** (10 μM) or **$AgNO_3$** (50 μM) for 2 h followed by stimulation with TNF-α (25 ng/mL) for 30 min.

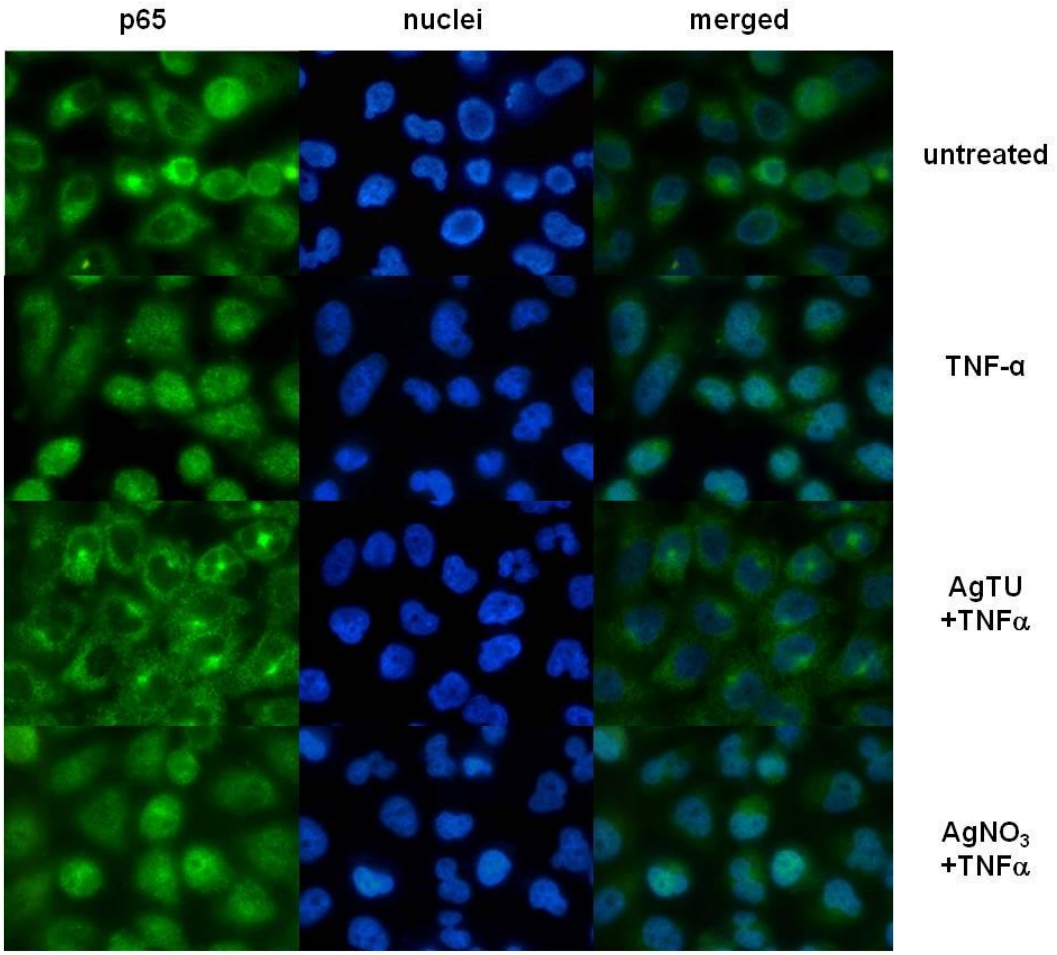
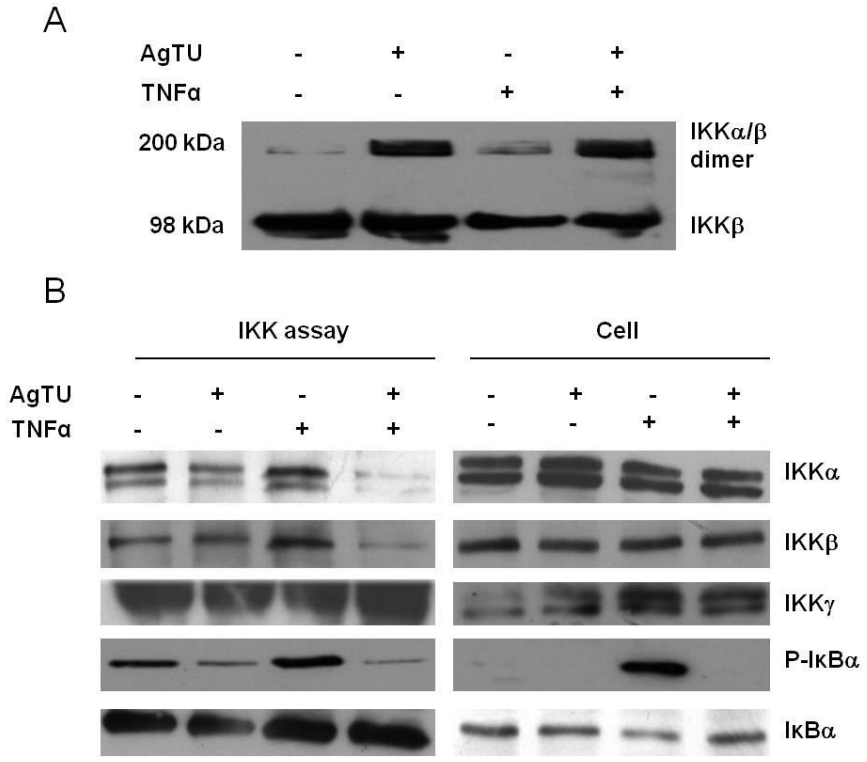


Figure S9 Interference of IKK complex by **AgTU**. (A) HeLa cells transfected with FLAG-tagged IKKβ were treated with 10 μM **AgTU** and the expression of IKKβ was detected by immunoblot using anti-FLAG antibody. (B) HeLa cells were treated with 10 μM **AgTU** for 2 h and then TNF-α stimulation for 5 min as indicated. IKK was immunoprecipitated with IKKγ antibody and subjected to *in vitro* kinase assay using IκBα as substrate. The expression of IKK subunits, IκBα and its phosphorylated form in the assay mixtures and input cell lystes were detected by immunoblot.



Notes and references

(1) Uson, R.; Laguna, A.; Laguna, M.; Briggs, D. A.; Murray, H. H.; Fackler, J. P. *Inorganic Syntheses* **1989**, *26*, 85-91.

(2) Yan, K.; Lok, C. N.; Bierla, K.; Che, C. M. *Chem. Commun.* **2010**, *46*, 7691-7693.

(3) Kim, J. R., Yoon, H. W., Kwon, K. S., Lee, S. R. and Rhee, S. G. *Anal Biochem* **2000**, *283*, 214-221.

(4) Siu, F. M., Lin, I. W. S., Yan K., Lok, C. N., Low, K. H., Leung, T. Y. C., Lam, T. L. and Che, C. M., *Chemical Science* **2012**, *3*, 1785-1793.