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A silver complex of *N*,*N*′-disubstituted cyclic thiourea as an anti-inflammatory inhibitor of IκB kinase†

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A silver complex of *N*,*N*′-disubstituted cyclic thiourea inhibits inflammatory cytokine-stimulated NF-κB activity *via* IκB kinase inactivation.

The prominent biomedical applications of silver are owing to its extraordinary antimicrobial properties and its relatively low toxicity to humans when compared to that normally associated with many heavy metals.[1] Thus, silver based materials are used as some of the most effective biocidal agents in burn wound treatment (*e.g.* silver sulfadiazine, silver dressing) and antimicrobial coatings of critical biomedical materials (*e.g.* silver impregnated catheters, bone cements and surgical sutures). Associated with the antimicrobial activities of silver compounds are their intriguing anti-inflammatory properties that are favorable for wound healing and topical uses;[2] however, these long claimed properties of silver are not well understood and explored.

The biological activities of silver compounds are mostly attributed to the release of Ag^+ ions which readily bind to thiol and imidazole moieties. While Ag^+ ions are susceptible to precipitation, nucleophilic attack and reduction in physiological media, the stability and activity of Ag^+ ions can be modulated by using appropriate auxiliary ligands. In the literature, phosphines, carboxylates, coumarins, thiolates and *N*-heterocyclic carbenes have been employed to develop bioactive silver complexes that display cytotoxicities to microbial or cancer cells.[3] Some silver carbene complexes have been shown to be active in mice models of cancer[3c] and bacterial infection.[3b] We have recently developed bioactive homoleptic coinage metal thiourea complexes ($[Au^I(TU)_2]Cl$ (**AuTU**), $[Ag^I(TU)_2]OTf$ (**AgTU**) and $[Cu^I(TU)_2]PF_6$ (**CuTU**), where TU = *N*,*N*′-disubstituted imidazolidine-2-thione) with monodentate S-donor ligands.[4] The TU ligand serves as a lipophilic carrier of the metal ion to cells and does not exert cytotoxicity. We have demonstrated that the **AuTU** complex exhibits potent tight-binding inhibition of the anti-cancer drug target thioredoxin reductase with an inhibitory constant at the nanomolar level, and was found to show moderate *in vivo* anti-tumour activities in mice. In this work, we report that the silver thiourea complex **AgTU** (Fig. 1) displays anti-inflammatory properties by effective and potent inhibition of the inflammatory cytokine-stimulated activity of the transcription factors NF-κB, which control the expression of diversified genes involved in inflammatory response and cancer progression.[5] In many tumor cells, NF-κB is active and blockade of NF-κB leads to inhibition of cell proliferation, cell death or sensitization to cytotoxic agents. In the literature, studies of the effects of metal compounds on NF-κB activities are sparse.[6] Auranofin[6a] and cyclometalated platinum(II) complexes[6b] have been shown to inhibit NF-κB activities. In this work, we have demonstrated for the first time the inhibition of NF-κB *via* inactivation of the cellular IκB kinase by silver ions which are delivered to cells *via* coordination ligands.

We have previously demonstrated that cellular uptake of silver in HeLa cancer cells mediated by **AgTU** is five times higher than that by $AgNO_3$ solution,[4] suggesting that the TU ligand serves as a lipophilic carrier of the metal ion to the cells. Once inside the cells, one of the fates of Ag^+ ions is determined by their susceptibility to nucleophilic attack by reactive thiols such as reduced glutathione (GSH). The reactivity of **AgTU** in the presence of GSH was investigated by ESI-MS. In a 50 mM ammonium bicarbonate solution (pH 8.0), mixing of 100 μM **AgTU** (m/z = 737.0 for $Ag(TU)_2^+$ and m/z = 423.2 for $Ag(TU)^+$) with a five-fold excess of GSH (m/z = 308.1) did not result in appearance of new species in the mass spectrum up to 3 h, suggesting that **AgTU** is reasonably stable toward GSH (Fig. S1A and S1B, ESI†). In comparison, mixing of the gold thiourea complex **AuTU** with GSH under similar conditions resulted in the ready formation of $Au(GS)_2^-$ (m/z = 809.3) (Fig. S1C, ESI†).

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Fig. 1 Chemical structure of **AgTU**.

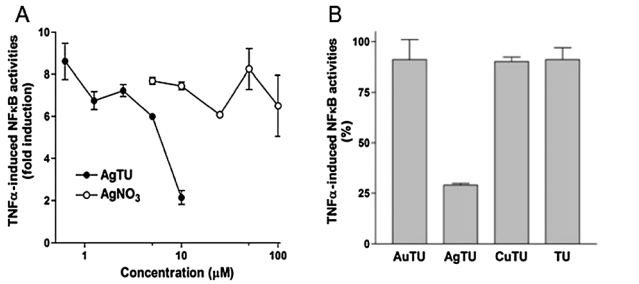

Fig. 2 Inhibition of TNF-α stimulated NF-κB activity by **AgTU**. (A) HeLa cells transfected with the NF-κB-luciferase reporter gene were treated with **AgTU** or $AgNO_3$ for 2 h, followed by TNF-α stimulation for 4 h and the cellular luciferase activities were determined. (B) The cells were treated with 10 μM **AuTU**, **AgTU**, **CuTU** or TU for 2 h, followed by TNF-α stimulation and the cellular luciferase activities were determined.

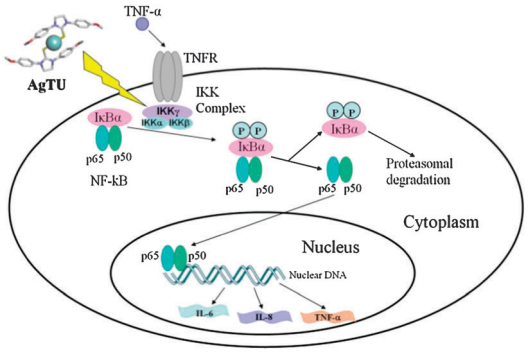

Fig. 3 The NF-κB pathway and inhibition of IKK by **AgTU**.

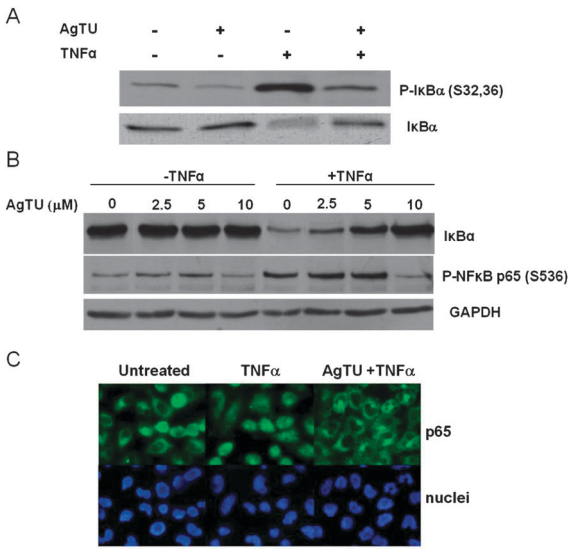

Fig. 4 Inhibition of TNF-α-stimulated IκBα phosphorylation, IκBα degradation and NF-κB p65 phosphorylation by **AgTU**. HeLa cells were treated with or without 10 μM **AgTU** for 2 h followed by TNF-α stimulation for 10 min as indicated. The phosphorylation of IκBα (A) and NF-κB p65 (S536) (B) and expression of IκBα were determined by immunoblot analysis. (C) Nuclear translocation of p65 was determined by immunofluorescence.

The nuclear factor kappa B (NF-κB) transcription factors control the expression of genes involved in cell survival, proliferation and inflammation.[5] Thus, NF-κB is an important anti-inflammatory and anticancer drug target. In this work, we have investigated the effects of **AgTU** on NF-κB transcriptional activity using HeLa cells stably transfected with the luciferase reporter gene of NF-κB (experimental procedures described in ESI†). Upon stimulation by TNF-α, the NF-κB transcriptional activity as measured by luciferase assay was increased by at least ~8 fold (Fig. 2A). Pretreatment of cells with **AgTU** for 2 h markedly inhibited the TNF-α stimulated NF-κB activity with an IC_{50} of about 5 μM and exerted maximal inhibition of 80% at 10 μM (Fig. 2A). For comparison, several metal complexes and ligands were also tested. Under identical assay conditions, the free TU ligand, **AuTU** and **CuTU** added at 10 μM did not show a significant effect on the TNF-α-stimulated NF-κB activity (Fig. 2B). A homoleptic Ag complex of the *N*-heterocyclic carbene complex ($[Ag^{I}(NHC)_2]OTf$, where NHC = 1,3-dimethylimidazol-2-ylidene) shows a weak inhibitory effect on the NF-κB activity (Fig. S2A, ESI†). It is notable that treatment of cells with $AgNO_3$ only moderately inhibited the stimulated NF-κB activity by at most 50% at concentration up to 100 μM (Fig. 2A). Thus, **AgTU** potently inhibits the cytokine-stimulated NF-κB activity, presumably *via* the TU ligand-mediated delivery of Ag^+. NF-κB activity can be stimulated by a number of cytokines and tumor promoters *via* different upstream cell receptor signaling. In addition to inhibition of the NF-κB activity stimulated by TNF-α, **AgTU** treatment also effectively inhibited the NF-κB activity induced by the tumor promoter phorbol ester (Fig. S3, ESI†). This implies that **AgTU** may act on a converging molecular component downstream to the receptor stimulation in the NF-κB pathway.

A scheme of the NF-κB pathway is depicted in Fig. 3.[5c] A critical step in the activation of NF-κB is the phosphorylation of IκB by the IκB kinase (IKK) complex. This process results in proteasomal degradation of IκB and release of NF-κB to the nucleus, thereby inducing inflammatory gene transcription. In this work, the effect of **AgTU** on the IKK activation has been investigated. As depicted in Fig. 4A, TNF-α markedly stimulated the phosphorylation of IκB, which is in concomitance with decrease of IκB levels. Treatment of cells with **AgTU** markedly inhibited the stimulated IκB phosphorylation and prevented the reduction of IκB levels (Fig. 4A). The observed concentration dependence of the **AgTU** blockade of IκB degradation (Fig. 4B) is also in accord with that of inhibition of stimulated NF-κB activity (Fig. 2). **AgTU** treatment also resulted in decreased p65 phosphorylation at S536, which could also be mediated by IKK (Fig. 4B).[5c] Thus these data altogether suggest that **AgTU** effectively blocks the NF-κB activity mainly by inhibiting IKK. A role of direct inhibition of proteasomal degradation of IκB seems less likely, as **AgTU** was found to be a relatively weak inhibitor of the ubiquitin–proteasome system[7] (Fig. S4, ESI†) and it inhibited the IκB phosphorylation even in the presence of a proteasome inhibitor (Fig. S5, ESI†).

The consequence of **AgTU** inhibited IKK-mediated IκB degradation has also been revealed by the following experiments: treatment of cells with **AgTU** blocked the translocation of p65 from the cytoplasm to the nucleus triggered by TNF-α (Fig. 4C). **AgTU** also suppressed the TNF-α-stimulated transcriptional upregulation of inflammatory cytokines such as IL-6, IL-8 and TNF-α (Fig. S6, ESI†). It was noted that treatment of cells with $AgNO_3$ (50–100 μM) did not result in significant inhibition of TNF-α stimulated responses including IκB phosphorylation and degradation, p65 phosphorylation and nuclear translocation, and cytokine induction (Fig. S6–S8, ESI†).

The effect of **AgTU** on the IKK was also investigated by an *in vitro* kinase assay (Fig. 5). The IKK complex was immunoprecipitated

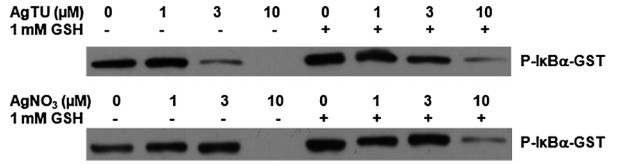

Fig. 5 Inhibition of *in vitro* IKK activities by **AgTU** and $AgNO_3$. The immunoprecipitated IKK complex from HeLa cells was treated with **AgTU** or $AgNO_3$ in the presence or absence of 1 mM GSH, and the kinase activities were assayed using IκBα–GST as a substrate.

with IKKγ antibody and the kinase activity was assayed with IκB–GST fusion protein as the phosphorylation substrate. Incubation of the kinase with 1–10 μM **AgTU** resulted in concentration-dependent inhibition of the phosphorylation of the IκB substrate. To examine the effect of the thiol moiety, which is abundant in the intracellular environment, the assays were performed in the presence of 1 mM GSH buffered in 10 mM Tris–HCl buffer (pH 8.0). The presence of GSH partially reduced the inhibition of the IKK activity by **AgTU** but significant inhibition was still observed when the GSH was 100-fold excess than **AgTU** (10 μM). Under the same assay conditions, $AgNO_3$ also inhibited the *in vitro* IKK activity and the inhibition was partially blocked by excess GSH (Fig. 5). As $AgNO_3$ did not show inhibition of cellular IKK activity (Fig. S7, ESI†), it is likely that **AgTU** delivers otherwise less cell-permeable Ag^+ ions to mediate specific inhibition of IKK.

AgTU may inhibit the IKK presumably *via* inactivation of functionally important cysteine residues of IKK.[8] To examine whether cysteine residues are involved in the **AgTU** mediated inhibition of IKK, the free cysteines of the IKK complex after treatment with or without **AgTU** were probed with biotinylated iodoacetamide (BIAM) using previously described procedures (Fig. 6).[4,8b,c] The results show that the IKK subunits (α, β, γ) can be efficiently labelled by BIAM. The labelling was more efficient at pH 8.5 than at pH 6.5, owing to the fact that typical pK_a values of most protein cysteine sulfhydryl residues are near the alkaline range.[8c] Upon treatment of IKK with **AgTU** or $AgNO_3$, a marked inhibition of the BIAM labelling of the IKKβ subunit was observed (Fig. 6). These results suggest that Ag^+ ion modifies the sensitive cysteine residues of IKK.

The functional IKK complex is composed of two catalytic subunits (α and β) and one regulatory subunit (γ).[5] We have also investigated whether additional mechanisms of IKK inhibition by **AgTU** treatment may operate at the IKK complex assembly stage (Fig. S9, ESI†). In IKKβ transfected cells, **AgTU** treatment resulted in distinct appearance of a high-molecular-weight protein band (200 kDa) that could be attributed to an IKKβ dimer as indicated by its mobility on the SDS–polyacrylamide gel (Fig. S9A, ESI†). Furthermore, when cells were treated with **AgTU** and the IKK complexes were immunoprecipitated followed by immunoblot detection of the IKK subunits, a loss of the heteromeric assembly of the three IKK subunits was observed (Fig. S9B, ESI†). Taken together, **AgTU** inhibits IKK kinase activity *via* intervention of the IKK complexation.

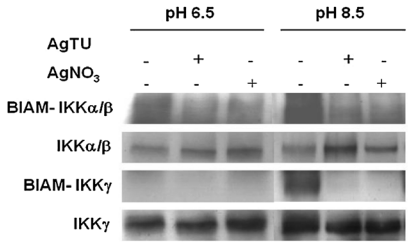

Fig. 6 Probing the sensitive cysteines of IKK. The IKK complex isolated by immunoprecipitation was treated with 10 μM **AgTU** or 10 μM $AgNO_3$ and the sensitive cysteine residues were detected by BIAM labeling at pH 6.5 and pH 8.5. The IKK subunits were detected as loading control by immunoblot.

In summary, we have identified **AgTU** to be a potent NF-κB inhibitor acting through suppression of IKK activity *via* thiol modifications and interference of the IKK complex assembly. Our study demonstrated that the inhibitory effect of Ag^+ ions on an anti-inflammatory and anti-cancer drug target could be effectively delivered *via* a non-toxic thiourea ligand.

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