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ARTICLE TYPE

Targeting Cytotoxicity and Tubulin Polymerization by Metal-Carbene Complexes on a Purine Tautomer Platform

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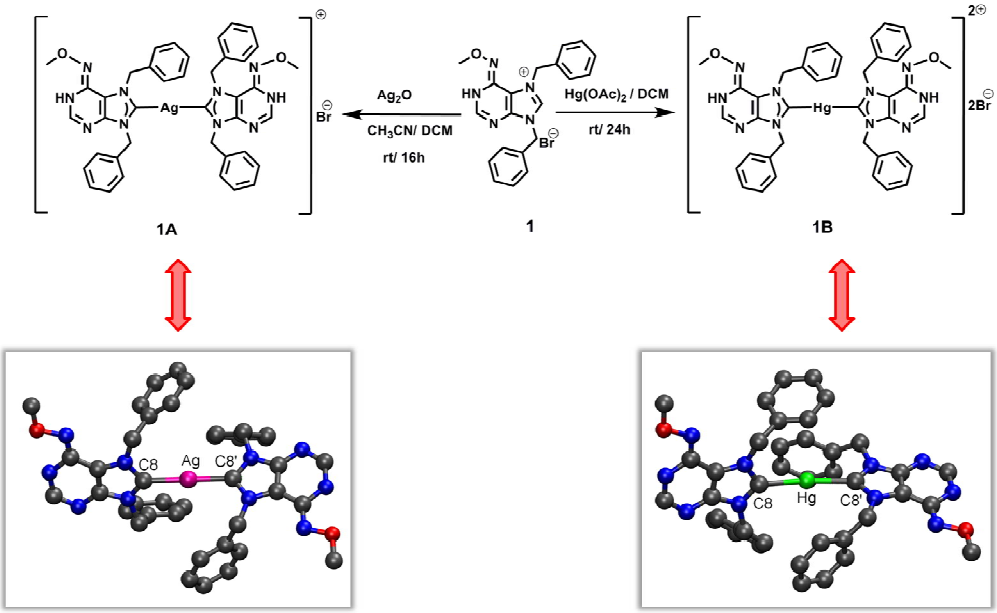
This article describes synthesis, structural investigations and tubulin binding of purine rare imino-tautomer based Ag(I) and Hg(II)-carbene complexes. These complexes exhibit cytotoxicity through tubulin interaction by binding to a site close to the GTP binding site. The structural similarity between guanine and N^6-methoxy adenine imino tautomer is ascribed for the site selectivity.

Biological action of organometallic compounds has elicited considerable interest in the past and it continues to attract close scrutiny due to diverse mechanisms of action, specially in anticancer chemotherapy.[1] In addition to conventional nucleic acid target, organometallic anticancer compounds are being designed to seek alternate macromolecular targets and pathways. Compounds built around cyclopentadienyl core, metal-arene interaction, polynuclear clusters and N-heterocyclic carbenes (NHCs), are promising candidates against a variety of cancer cell lines. Their promise is centered around predictable three dimensional structure, fine-tuning of properties via choice of metal ions and transport to desired sites using conventional drug delivery strategies.[2]

In particular, metal-NHCs offer a versatile platform for discovering novel metallodrugs as their synthesis is relatively simple and it can support different metal ions such as Ag, Au, Pt, Pd, Cu, Ni, and Ru, thus allowing faster optimization of structural requirements and biological activity, in a divergent fashion.[3] Anticancer action of metal-NHCs is of contemporary interest as these complexes exert favourable biological action through a number of mechanisms such as activation of apoptosis, depolarization of mitochondria inner membrane potential, nuclear translocation of apoptosis-inducing factors and caspase-12, inhibition of cysteine-dependent protein tyrosine phosphatases and thioredoxin reductase, to name a few.[2]



Scheme 1. Synthesis of silver carbene (**1A**) and mercury carbene (**1B**) complexes from ligand **1**. Inset: DFT-optimized geometries of **1A** and **1B**.

Imidazolium moiety carrying synthetic ligands are preferred scaffolds to stabilize carbenoid species, thus a majority of reports focus on modification of this heterocycle for NHCs synthesis.[3a,4] Notably, despite the presence of imidazole-like ring in purine heterocycles, reports of carbene generation on natural purine nucleobases are rare, and most reports concern xanthine and caffeine.[5] Recent advances in organometallic anticancer drugs and possibility of using natural purines, such as adenine and guanine, gave us an impetus to design adenine-based carbenes as antitumor agents.[6]

We have investigated metalated adenine analogues for the generation of higher order supramolecular frameworks and surface patterning, synthesis of polymeric adenine templates, and their use in catalysis and DNA cleavage.[7] The present study describes a novel methoxyadenine rare tautomer platform for stabilizing silver- and mercury-based carbenoid species, for possible cytotoxicity and interaction with the microtubules. Structural tunability of ligand and metal ion stabilizing carbene were considered as beneficial feature to exploit this framework in these studies.

It was envisaged that two benzyl rings in the imidazole ring of ligand **1** will render C8 carbon susceptible for carbene generation, which in turn could react with Ag or Hg to create desired organometallic species. Consequently, C8-H abstraction was achieved using Ag_2O and $Hg(OAc)_2$, where metal oxide or acetate acted as base as well as metal center, stabilizing formation of N-heterocyclic carbene. The proposed structure of C8 centered metallodimers, **1A** and **1B** are shown in Scheme 1. The synthesis of ligand **1** and complexes **1A** and **1B** are described in the supporting information. The formation of these complexes was confirmed by spectroscopic methods. ^{1}H NMR spectra of **1A** and **1B** clearly revealed complete disappearance of C8-H signals present at δ 9.26 in ligand **1** (Fig S2). This was further confirmed by ^{13}C NMR where a peak corresponding to C8 at δ 136.37 is shifted to δ 178.52 for **1A** and 175.80 for **1B**, respectively, a characteristic of corresponding metal carbene signal (Fig S3). HRMS data also confirmed formation of carbene complexes **1A** and **1B** (Fig S4).

Complexes **1A** and **1B** did not crystallize despite trying multiple crystallization conditions. Thus, we resorted to Density Functional Theoretical (DFT) calculations to gain insight into the structural features of these carbene complexes. DFT calculations for both complexes were performed by employing B3LYP exchange-correlation functional,[8] with 6-31G(d,p) basis set for all non-metallic atoms and LANL2DZ basis set for both Ag and Hg ions[9] (SI for geometry optimizations). The normal mode analysis of vibrational frequencies was performed and the absence of any negative frequency confirmed the structure to be at minima (*at least local*). These calculations considered basis set superposition errors (BSSE) in order to calculate accurate energies in the counterpoise approximation.[10] Geometry optimization of **1A** and **1B** afforded nearly linear carbene complexes, with C8–Ag–C8′ bond angle of 178.63° and C8–Hg–C8′ bond angle of 170.62° (Scheme 1). A slight elongation in C8–N bond was observed, while N7–C8–N9 bond angle is decreased by 3.13° in **1A** and by 1.9° in **1B** (Table S2). All the DFT calculations were performed using Gaussian 09 (see SI).

Silver and other metal complexes have been studied for multiple biological effects such as antiseptic activity, inhibition of inflammation, antibacterial and antitumor action.[11] We became interested in assessing possible anticancer action of **1A** and **1B**, as recent studies reported effect of silver complexes on caspase-independent cancer cell apoptosis, through mitochondrial apoptosis inducing factor pathway.[12] Mitochondrial targeting and lack of genotoxicity further augmented the scope of their biological action.

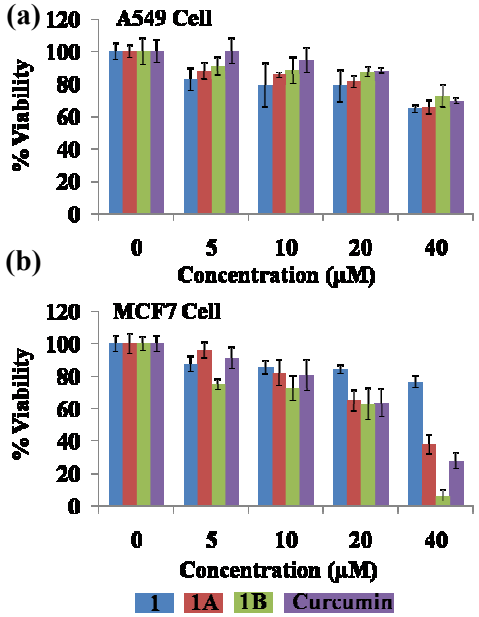


Figure 1. Percentage viability of cancer cell lines after treatment with **1**, its metal complexes (**1A** and **1B**) and curcumin (as a control) was assessed by MTT assay in: (a) A549 and (b) MCF7 cell lines for 24 h.

We assessed anticancer properties of ligand (**1**) and its metal complexes (**1A** and **1B**) by MTT assay, which uses cytosolic redox enzymes for reduction of tetrazolium dye as an indicator of cell viability, in A549 lung cancer and MCF7 breast cancer cell lines, for 24 h. Curcumin, a known anticancer compound, was used as a control.[13] **1**, **1A** and **1B** (40 μM) exhibited similar cytotoxicity (loss of viable cell) against A549 adenocarcinoma cell line , which was comparable to curcumin control (40 μM) (Figure 1a). However, for MCF7 cell line **1, 1A** and **1B** (40 μM) show ~20%, ~60% and ~90% cytotoxicity, respectively, when compared to ~70% cytotoxicity exhibited by curcumin (40 μM) i. e., MCF7 cell line is 80%, 40%, 10% and 30% viable after treatment with **1, 1A, 1B** and curcumin, respectively, (Figure 1b). These results suggest that while **1** is least cytotoxic to MCF7 cell line, cytotoxicity of **1A** is comparable to that of curcumin with **1B** exhibiting excellent cytotoxicity. At this time, it can be proposed that this difference in cytotoxicity could be attributed to specificity for a particular cell line. After determining the cytotoxicity of these compounds, changes in the morphology of cancer cell lines were studied after 24 h of conjugates uptake and ensuing changes in cellular morphology was compared as evident from differential interference contrast images (Figure 2, S5 and S6). Cell deformation alludes to potential anticancer activity of

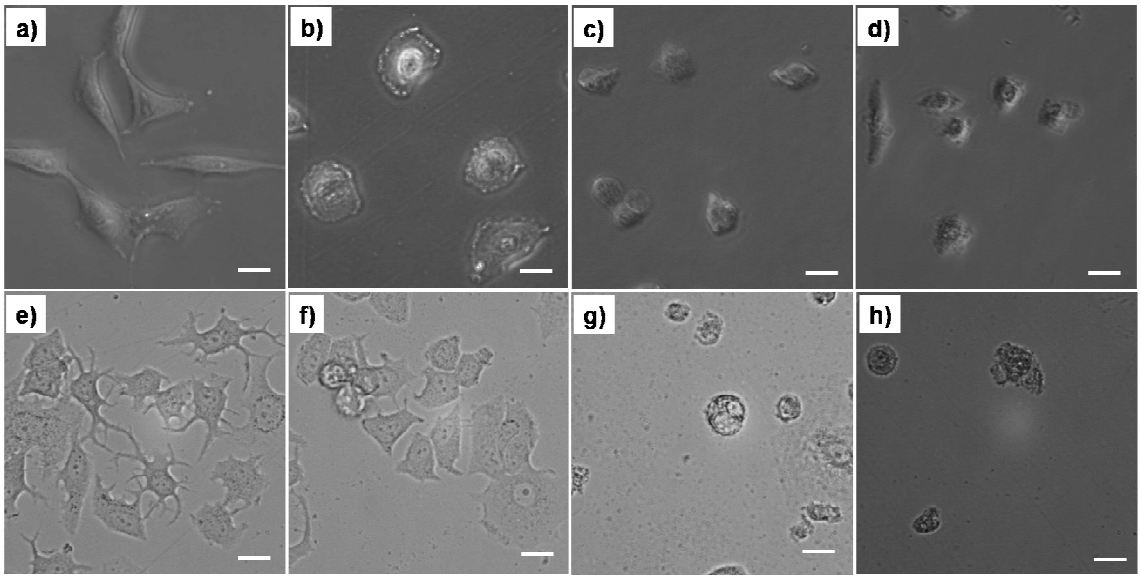


Figure 2. Changes in cell morphology after treatment with **1** and its complexes for 24 h. Upper panel: (a) Untreated A549 cells; (b) with **1**; (c) with **1A**; (d) with **1B**. Lower panel: (e) Untreated MCF7 cells; (f) with **1**; (g) with **1A**; (h) with **1B**. [Scale bar = 20 μm].

1A and **1B**.

We decided to probe possible mechanism of morphological changes by these conjugates. It is well known that microtubule, an important constituent of cytoskeleton, is a key target for anticancer drug action. Moreover, microtubule is also one of the vital cytoskeleton filaments, which maintains cell structure. Therefore, ability of these conjugates in perturbing microtubule networks inside cancer cells was assessed. As imagined, microtubule networks in A549 and MCF7 cell lines were dramatically affected after treatment with the conjugates for 24 h, which resulted in drastic morphological changes suggesting this as a possible mechanism in eliciting anticancer response (Figure 3, S7). Based on these results, we surmise that anticancer activity of our compounds is perhaps a consequence of disruption of the microtubule networks leading to deformation of cellular morphology.

The effect of these conjugates on microtubule dynamics was further investigated by following *in vitro* microtubule assembly/polymerization, via turbidity assay, in presence of **1**, **1A**, **1B** and curcumin, at 37 ^{o}C for 40 min. Curcumin is known to induce significant depolymerization of microtubules.[14] Significant increase in turbidity, when measured at 350 nm, is observed during *in vitro* microtubule polymerization.[15] Interference with this process was inferred from the turbidity data (Figure 4a), where **1A** offered greatest inhibition and relative inhibition was assessed as **1A**> curcumin> **1**> **1B**. Thus, anticancer activity of **1A** and **1B** could be ascribed to their ability to target microtubule polymerization.

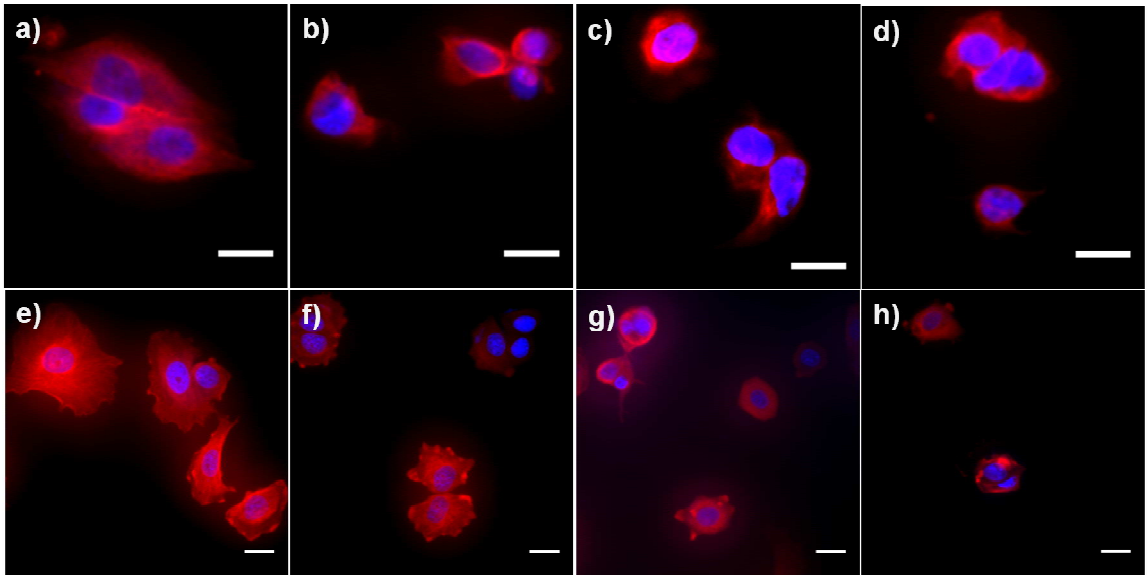


Figure 3. Changes in cellular microtubule network on treatment with **1** and its complexes for 24 h. Upper panel: (a) Untreated A549 cells; (b) with **1**; (c) with **1A**; (d) with **1B**. Lower panel: (e) Untreated MCF7 cells; (f) with **1**; (g) with **1A**; (h) with **1B**. [Scale bar = 20 μm; tubulin labelled by antibody (red) and nucleus stained by DAPI (blue)].

Anti-cancer activities of our conjugates prompted us to qualitatively interrogate their interaction with tubulin focusing on probable binding site, nature of binding and amino acids supporting this interaction. As adenine imino tautomer exhibits structural similarity to guanine, we decided to investigate possibility of our tautomer occupying GTP binding site in β-tubulin, through docking studies.[16] It is known that GTP binds to both α and β-tubulin monomers, where β-tubulin bound GTP is hydrolyzed during microtubule assembly.[17] Docking studies performed to assess binding of **1** in proximity to GTP binding site suggested that **1** probably binds close to Asn117 residue of β-tubulin (Figure 4b, 4c), similar to GTP/GDP binding. Incidentally, this site is proximal to GTP/GDP binding pocket of β-tubulin (Figure 4d).[17] Such interaction could be possibly ascribed to structural similarity between GTP and rare purine tautomer, **1**. Thus, it could be proposed that **1** exerts its action through hydrophobic and hydrogen bonding interactions with β-tubulin.

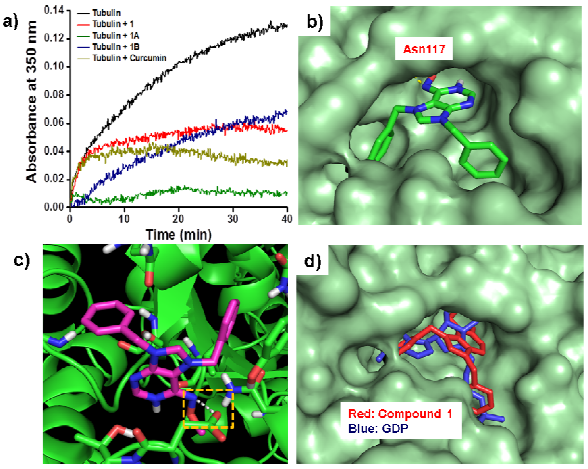


Figure 4. (a) Turbidity assay indicating inhibition of tubulin polymerization by curcumin, **1**, **1A** and **1B**. (b) Docked image depicting interaction of **1** with Asn117 in *β*-tubulin. (c) Binding of **1** with Asn117 residue of *β*-tubulin through H-bonding. (d) Docking studies illustrate similar binding pocket for **1** and guanine nucleotide, in *β*-tubulin subunit.

In conclusion, we have presented carbene generation on a rare purine tautomer, its characterization by analytical methods and structure via DFT calculations, and its potential anticancer activity against lung carcinoma and breast cancer cell lines, A549 and MCF7, respectively. **1**, **1A** and **1B** altered cellular morphology by inhibiting tubulin polymerization via noncovalent interactions in tubulin binding site. Given structural similarity to guanine, **1** and its metal complexes are implicated in blocking the GTP binding site. We surmise that this approach may present new avenue for the discovery of microtubule polymerization inhibitors. Further investigations in this direction are currently in process.

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Notes and references

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† Electronic Supplementary Information (ESI) available: The supplementary crystallographic data for this paper with deposition number of CCDC 965785 has also been deposited with the Cambridge Crystallographic Data Centre. The coordinates can be obtained, upon request, from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, U.K. [fax +44-1223/336-033; e-mail deposit@ccdc.cam.ac.uk. See DOI: 10.1039/b000000x/

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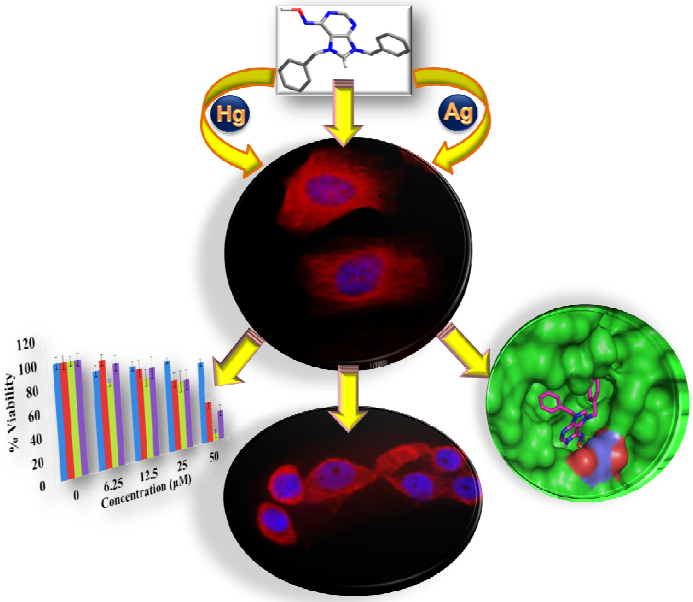
SYNOPSIS TOC



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