On the mechanism of gold NHC compounds binding to DNA G-quadruplexes elucidated by combined metadynamics and biophysical methods

Darren Wragg,[a] Andreia de Almeida,[a] Riccardo Bonsignore,[a] Fritz E. Kühn,[b] Stefano Leoni*[a] and Angela Casini*[a,c]

Abstract: The binding modes and free-energy landscape of two Au(I) N-heterocyclic carbene complexes interacting with G-quadruplexes, namely a human telomeric (hTel) and a promoter (C-KIT) sequence, are studied here for the first time by metadynamics. The theoretical results are validated by FRET DNA melting assays and provide an accurate estimate of the absolute gold complex/DNA binding free energy. This advanced in silico approach is valuable to achieve rational drug design of selective G4s binders.

DNA can adopt different structures other than the canonical right-handed double helix (B-DNA), and numerous structural studies have revealed that guanine-rich DNA sequences can form secondary structures termed G-quadruplexes (G4s).[1] To form G4s, four guanine bases assemble into a pseudoplanar tetrad (G-quartet) are held together by one or more nucleotide strands and stabilized by metal ions. Recent bioinformatics studies have shown that there are ca. 716,000 DNA sequences in the human genome that can potentially form G4 structures.[2] These non-canonical DNA structures are present in telomeres and promoter regions of oncogenes and have been the subject of intense study over the past 10 years, being associated with a number of biological processes such as telomere maintenance, gene regulation, and replication.[3] It has been proposed that formation of the quadruplex structure in promoter regions can control transcription and, as a consequence, the expression of the corresponding oncogenes.[4] Moreover, stabilizing G4s in telomeres indirectly inhibits telomerase activity, thus affecting cancer mortality.[5]

Within this context, G4s emerge as promising targets for anticancer drug discovery, while their roles in cancer biology have yet to be completely elucidated. A number of studies report on the efficient G4 stabilization by small molecules with associated anticancer effects.[6] For example, the tri-substituted acridine derivative, BRACO-19, a telomeric G4 stabilizer, has shown in vitro anticancer activity in prostate cancer.[7] Of note, two quinolone molecules, CX-3543 and CX-5461, selectively stabilize G4s structures, and are now in clinical trials.[8]

In addition to organic molecules, metal-based compounds have also been developed as promising experimental G4 stabilizers, including several Schiff-base metal complexes (mainly Ni2+, [9] Cu2+, [8c-e] Zn2+, [8a-c] Pt2+ [8] and Pt4+, [9a]) as well as some metallo-supramolecular DNA-binders.[10] Despite the great advances in the development of G4 stabilizers, still important challenges remain to be tackled, including achieving selective binding of small molecules to a specific quadruplex over duplex DNA and other G4s.

Our pioneering work in this area identified small-molecule organometallic Au(I) compounds, featuring N-heterocyclic carbenes (NHCs) ligands, as potent and selective stabilizers of telomeric G4s.[11] Including the bis-NHC gold(I) complex - [Au(9-methylcaffein-8-ylidene)2]2+ (AuTMX2, Figure 1),[11a] X-ray diffraction analysis of the adduct formed by AuTMX2 and a 23-nucleotide telomere repeat sequence (Tel23) indicated that the compound binds non-covalently between neighbouring G4s.[11b]

Based on these promising results, our research in an unmet medical need involves developing new organometallic Au(I) NHC complexes targeting specific G4 structures for possible applications in therapy and/or imaging. In order to rationally achieve selectivity, computational methods, including molecular dynamics (MD) approaches, are highly valuable in elucidating both the structural and energetics requisites underlying the ligand/target recognition process. In fact, a number of classical MD studies on the adducts of G4s structures with different stabilizers have been performed providing atomistic support for the interpretation of the binding mechanism to G4-DNA.[8a-c, 12]

Recently, funnel-metadynamics has been shown to be successful at calculating the free energy surface for organic ligands and their interactions with G4s.[13] Thus, we applied
metadynamics to evaluate the binding of AuTMX₂ to two different G4 structures, namely the human telomeric sequence hTel0 (pdb 2HY9[14]) and the C-KIT1 oncogene promoter sequence (pdb 4W02[15]). The results have been compared with those obtained for the neutral mono-carbene complex AuTMX-I (Figure 1). Furthermore, we have validated the accuracy of our calculations performing gold complexes/G4 binding assays using FRET (fluorescence resonance energy transfer) DNA melting.

![AuTMX₂ and AuTMX-I](image)

**Figure 1.** Chemical structures of the two Au(I) NHCs investigated in this study.

Initially, the X-ray structure of the telomeric-G4 adduct with AuTMX₂ (pdb SCW[16]) was used as reference to run a first set of metadynamics simulations, to calculate the compound’s thermodynamically most stable positions, providing a starting point for the free energy calculations with hTel0 and C-KIT1 (see Experimental for details). This allowed the validation and positioning of the interaction of AuTMX₂ with the selected G4 models (Figure S1). In our study, the Gibbs-free energy (at 300 K, ΔGMD) was determined for all the seven compound’s poses (Table S1) and showed that each one has different binding energy. The position corresponding to the AuTMX₂’s interaction with the topmost tetrad (pose 1, Figure S1), was chosen for the further calculations of the interactions with hTel0 and C-KIT1.

In the work of Moraca et al., funnel metadynamics was used to constrain the ligand within a specific area determined to be the top tetrad surface.[13] However, in our study, the gold-complexes were not constrained and were allowed to find the most energetically favourable interactions with the entire G4 models, including loops and top and bottom tetrads, allowing possible further interactions to be identified. Thus, five 50 ns trajectories were calculated for each combination of compound and G4 model (for a total of 4 experimental conditions and a total of 20 simulations). This was performed using a simple distance collective variable (CV) between the Au⁺ centre of the complex and the K⁺ at the centre of the uppermost tetrad (see experimental section for details), resulting in a free-energy (ΔGMD) profile output, based on the Au⁺-K⁺ distance (Figure S2, Table 1). Moreover, to closely investigate the molecular mechanism of interaction of the gold complexes, multi-CV calculations were run on the same systems. This involved adding a second CV for the torsion angle between the complexes and the uppermost tetrads (Figure 2).

As metadynamics explores the whole energy surface of an interaction, rather than just one minimum, further possible meta-stable positions can also be observed. In fact, hTel0's trajectories with AuTMX₂ show two possible binding sites (state I and II) with the first one (state I) having the lowest energy (ca. -37 KJ/mol, Table 1). Figures 2 shows the multiple collective variable (CV) plot of free energy surface of AuTMX₂ interactions with hTel0. Interestingly, state I shows two minima (a and b), corresponding to the same Au-K⁺ distance (ca. 0.8 nm) but with different torsion angles. The latter are related to AuTMX₂ being virtually in the same position but with the gold complex rotating around its centre, resulting in the same pose with two different torsion angles (see position of the caffeine ligands in Figure 2).

In state I, AuTMX₂ is interacting with both an adenine (A13) in the loop b region, and two guanine bases of the tetrad (G4 and G22), with strong π-stacking between the NHCs of the gold complex and the aromatic rings of G22 (Figure 2). Instead, the higher energy state II (ca. -14 KJ/mol, Table 1) corresponds to a position where the gold complex does not interact with the guanine bases, but exclusively with the loop thymine (T11) (Figure 2). In this second state, the loop covers the top of the G4-tetrad, hindering possible interactions between the gold complex and the G-tetrad.

Interestingly, a similar behaviour was observed for AuTMX₂ binding to C-KIT1, with two states I and II (Figure S3, Table 1). C-KIT1 has a very different structure and surface from hTel0: while the former has a prominent flanking loop that may cover the top of the tetrad, C-KIT1 top surface is virtually flat, leading the gold complex to interact with the top of the tetrad and the rings of flanking bases (A1) (Figure S3). Thus, state I corresponds to AuTMX₂ stabilized by π-stacking with the guanine rings (lowest energy), while in state II, it interacts with both A1 and G6 via π-stacking (Figure S3).

When the simulation was repeated for the neutral mono-NHC complex AuTMX-I, the compound was shown to interact via π-π and π-alkyl interactions with the guanine tetrad (G22) and the loop (A13) in hTel0 (Figure S4), as observed for AuTMX₂ (state I). However, as expected, the calculated ΔGMD was lower with respect to the one for AuTMX₂, due to the lack of the second caffeine ligand (Table 1). The enhanced efficiency of multiple collective variable simulations proved extremely valuable in identifying a second, unexpected binding mode of AuTMX-I (state II), similar in energy to state I. Therein, the complex interacts within a groove in loop c (Figure S4) by π-π stacking the caffeine moiety with T18. This interaction was only observed when using the multiple collective variable simulations.

With C-KIT1, AuTMX-I shows a single binding mode due to its π-stacking with G6 of the uppermost tetrad of the G4 (Figure S5). Moreover, the iodido ligand tends to be positioned outside the G4 structure, in both hTel0 and C-KIT1 adducts.
Figure 2. Multiple collective variable (CV) plot of free energy surface of AuTMX\textsubscript{2} interactions with hTel0 (centre). CVs correspond to distance (nm) between Au\textsuperscript{+} in AuTMX\textsubscript{2} and K\textsuperscript{+} in upper tetrad and torsion angle (rad). Two states are highlighted (I and II) and two poses for state I are shown as a and b. States I-a, b and II are shown in translucent molecular surface, coloured according to lipophilicity (green: lipophilic, pink: hydrophilic). G4 structure is shown as sticks and ribbon, with hidden backbone for clarity. AuTMX\textsubscript{2} is shown in ball and stick, with each caffeine ligand coloured differently (black and grey). C2 and C4 highlight the carbon atom positions in AuTMX\textsubscript{2} in each of the related poses I-a or I-b.

Table 1. Gibbs-free energy values, experimental ($\Delta G_{\text{exp}}$) and calculated by metadynamics ($\Delta G_{\text{MD}}$), for AuTMX\textsubscript{2} and AuTMX-I interactions with hTel0 and C-KIT1. $\Delta G$ values are expressed in kJ/mol and obtained considering $T = 300$K. Experimental binding constants ($K_b$) are reported in Table S2.

<table>
<thead>
<tr>
<th>G4 model</th>
<th>hTel0</th>
<th>C-KIT1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta G_{\text{MD}}$</td>
<td>$\Delta G_{\text{exp}}$</td>
</tr>
<tr>
<td>AuTMX\textsubscript{2} (state I)</td>
<td>-37 ± 7</td>
<td>-39 ± 2</td>
</tr>
<tr>
<td>AuTMX\textsubscript{2} (state II)</td>
<td>-14 ± 3</td>
<td>-12.1 ± 0.4</td>
</tr>
<tr>
<td>AuTMX-I</td>
<td>-28 ± 3</td>
<td>-23.2 ± 0.4</td>
</tr>
</tbody>
</table>

$\Delta G_{\text{MD}}$ are obtained from simulations using a simple distance collective variable.

Following the interesting observations of multiple binding modes of AuTMX\textsubscript{2} and to further investigate the stabilization properties of the two gold-based complexes, determination of the Gibbs-free energy ($\Delta G_{\text{exp}}$) from the DNA FRET melting profiles was performed. Thus, AuTMX\textsubscript{2} and AuTMX-I were synthesized by adapting published protocols,\cite{11a, 16} starting from their methylated precursors (Scheme S1 and S2). The difference in DNA melting temperature ($\Delta T_m$, in °C) of hTel0 and C-KIT1 induced by the binding of the two Au(I) NHC complexes was readily monitored through the modification of the FRET phenomenon and enabled an easy quantification of the compounds’ stabilization properties of G4-DNA. Afterwards, the compounds were incubated with fixed amounts of each G4 for 10 min and the DNA melting profile recorded. As shown in Figure 3, both complexes stabilize the hTel0 and C-KIT1 structures, with the strongest effects observed for AuTMX\textsubscript{2}.

As previously reported,\cite{11c} AuTMX\textsubscript{2} leads to a characteristic melting profile for hTel0, featuring a two-step melting pattern, where a small increase in fluorescence is initially observed before the steep increase after ca. 65 °C (Figure 3A, red trace). Instead, AuTMX\textsubscript{2} with C-KIT1, investigated for the first time, shows a gradually incrementing curve, rather than an initial steep ramp or two-step curve.

In the presence of the mono-caffeine derivative, AuTMX-I, the $\Delta T_m$ of both G4s is approximately 2-fold lower than the one found for AuTMX\textsubscript{2} (6.5 ± 0.2 °C for hTel0 and 11.0 ± 1.8 °C for C-KIT1, respectively). Interestingly, AuTMX-I does not have an effect on the shape of the melting profiles of either DNA sequence, which is similar to the control DNA (Figure 3A-B, black and blue traces).
In order to determine the energy of binding ($\Delta G_{\text{exp}}$) of the gold compounds to each G4, the experimental data were normalized to folded fraction ($\theta$) of G4-DNA and fitted according to Eq. 1 and 2, where the enthalpy ($\Delta H$) for the process was derived from the resulting fit (see Experimental section). In order to fit a two-step melting profile of AuTMX$_2$ with hTelo, another equation (Eq. 4, Experimental section) was used, taking into account the upper and lower limits of the sigmoid curve used for fitting. This allowed us to treat the data fits as two independent melting curves. Thus, $\Delta G_{\text{exp}}$ was calculated for both compounds vs each G4 structure, and also for hTelo’s two-step melting curve (Table 1). The resulting fits are shown in Figure S6. From the reported results a trend could also be identified in which the greater the stabilization of the G4 structure by the compound and lower is the energy of binding (at least considering the most stable mode, state I for hTelo).

Most importantly, the $\Delta G_{\text{exp}}$ are in perfect accordance with the $\Delta G_{\text{MD}}$ values obtained using metadynamics (Table 1). The analysis of the melting curves and $\Delta G_{\text{exp}}$ values of AuTMX$_2$ with the two G4 models clearly suggest the existence of two distinct modes of binding, possibly mutually exclusive, in line with the computational results. Thus, considering hTelo, the first binding mode corresponds to the lower energy state I (Figures 2 and S6), exclusively featuring compound’s interaction with the guanines in the tetrad. The second binding mode at higher energy (state II) involves loop/flanking base interactions and/or interactions with part of the tetrad (Figures 2 and S6). Instead, as observed before, the flatness of the top tetrad of C-KIT1 allows the compound to probe the whole topsurface of the G4. Since the stacking of the complex with the guanines is more favourable (state I), it may be expected that this is the interaction most likely to occur in vitro. Notably, our metadynamics results also point towards the existence of a second binding site for AuTMX-I on hTelo, involving the loop C. This interaction may be for further optimization of selective hTelo stabilizers.

Overall, the in silico results confirm and complement the experimental data revealing two ligand binding modes of AuTMX$_2$ on the two G4s structures, and providing further structural and energetics information on ligand binding mechanism, including a quantitatively well-characterized free-energy landscape. The experimental validation of the binding energy of Au(I) NHC to G4s complexes calculated by metadynamics methods was also achieved. This advanced approach can be extended to other types of molecules as G4 stabilizers, highlighting selectivity features essential to orient the drug design.

Acknowledgements

A.C. acknowledges support from Cardiff University and the Hans Fischer Senior Fellowship of the Technical University of Munich – Institute for Advanced Study, funded by the German Excellence Initiative and the European Union Seventh Framework Program, under grant agreement n° 291763. R.B. acknowledges funding from the European Union’s Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant agreement nº 663830. This work has been performed using resources provided by the “Cambridge Service for Data Driven Discovery” (CSD3, http://csd3.cam.ac.uk) system operated by the University of Cambridge Research Computing Service (http://www.hpc.cam.ac.uk) funded by EPSRC Tier-2 capital grant EP/P020259/1. We gratefully acknowledge the support of NVIDIA Corporation with the donation of a Quadro P5000 GPU used for this research.

Keywords: gold N-heterocyclic carbenes • G-quadruplexes • metadynamics • FRET DNA melting • cancer
References
