Transition Metal Complex Gold (III) Inhibits Potential Anticancer Target Human DNA Topoisomerase IB

PRAFULLA KATKAR[1] RAYMOND WAI-YIN SUN[2] AND ALESSANDRO DESIDERI[3]

[1] Department of Microbiology, Guru Nanak College of Science, Ballarpur Dist-Chandrapur (MS)
[2] Department of Chemistry for Drug Discovery and Synthesis,
The University of Hong Kong, Hong Kong, China.
[3] Deaprtment of Bology, University of Rome"Tor Vergata" Rome Italy

Introduction

Topoisomerases are key enzymes that control the topological state of DNA through the breaking and rejoining of DNA strands. There are two classes of topoisomerase: type I enzymes, which act by transiently nicking one of the two DNA strands, and type II enzymes which nick both DNA strands and whose activity is dependent on the presence of ATP. These enzymes are involved in many vital cellular processes that influence DNA replication, transcription, recombination, integration, and chromosomal segregation [1–2]. A number of antitumor agents have topoisomerases as their target and they act through different mechanisms that are related to different catalytic steps [6-8]. In particular, the drugs inhibiting topoisomerase I are been divided into two classes: poisons and catalytic inhibitors [9, 10].

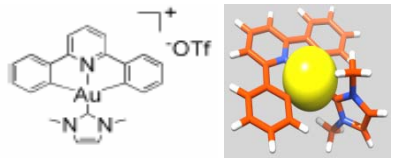
In this contest, square-planar d8 metal complexes have long been known to exhibit promising anticancer activities [4], by covalent crosslink of d8 metal ions to DNA, by intercalation of the planar metal complexes between the DNA base pairs, and/or by inhibition of enzyme activities [6, 8].

During previous analysis, a panel of sTable anticancer gold(III) complexes was prepared and some of these identified to be promising classes of anticancer drugs [1,5]. The promising biological activities of gold(III) complexes supported by dianionic $[C^N^C]^{2-}$ and neutral auxiliary N-heterocyclic carbene (NHC) ligands was then reported [11]. This class of ligands is relatively non-toxic and easily modified. Furthermore, the strong Au–C(carbene) bond is able to stabilize gold(III) in solutions [9].

In case of human topoisomerase IB, Gold (III) [Au(C^N^C)(IMe)]CF3SO3 was reported to inhibit supercoiled DNA relaxation [13] but the mechanism behind the inhibitory effect have to be refined and shown at all. In this work, in order to understand how the drug inhibits the enzyme, we have analyzed the relaxation activity and each different step of the catalytic cycle of topoisomerase IB and showed that Gold (III) inhibits the topoisomerase I cleavage step, probably by a direct interaction with the enzyme.

Materials and methods

1. Gold (III) [Au(C^N^C)(IMe)]CF3SO3 molecule was sent by R.W.Y.Sun and co-authors.



2. DNA Relaxation Assays

To check the protein specific activity, topoisomerase IB was incubated in 20 µl reaction volume containing 0.5 µg of negatively supercoiled pBlueScript KSII(+) DNA and Reaction Buffer (20 mM Tris–HCl, 0.1 mM Na2EDTA, 10 mM MgCl2, 50 µg/ml acetylated BSA and 150 mM KCl, pH 7.5).

The effects of Gold (III) on enzyme activity were measured by adding different concentrations of the compound or the same concentration, at different times. Reaction were stopped with a final concentration of 0.5% SDS after 30 min or after each time-course point at 37°C. The samples were electrophoresed in a horizontal 1% agarose gel in 50 mM Tris, 45 mM boric acid, 1 mM EDTA). The gel was stained with ethidium bromide (5µg/ml), destained with water and photographed under UV illumination. Where indicated, enzyme and inhibitor were preincubated at 37°C, prior to the addition of the substrate. Assay was performed at least three times but only representative gels are shown.

3. Kinetics Of Cleavage

In this case, the oligonucleotides substrate CL14 (5'-GAAAAAAGACTTAG-3') was radiolabelled with [γ-^{32}P]ATP at its 5' end. The CP25 complementary strand (5'-TAAAAATTTTTCTAAGTCTTTTTTC-3'), that is identical to the previous used for equilibrium assay, was phosphorylated at its 5' end with unlabeled ATP. The two strands were annealed at a 2-fold molar excess of CP25 over CL14, creating the so called "suicide substrate", that contains only a partial duplex.

The suicide cleavage reactions were carried out incubating 20nM of this partial duplex substrate with an excess of enzyme in Reaction Buffer at 37°C and in presence of 50µM Gold (III). DMSO was added to no-drug control.

Before the addition of the protein, 5µl sample of the reaction mixture was removed and used as the zero time point (R). At various time points 5µl aliquots were removed and the reaction stopped with 0.5% SDS. After ethanol precipitation samples were resuspended in 6µl of 1 mg/ml trypsin and incubated at 37°C for 1 hour. Samples were analyzed using denaturing urea/polyacrylamide gel electrophoresis. The experiment was replicated at least three times and a representative gel is shown. The percentage of cleavage at the preferential site (CL1) was quantified through PhosphoImager and ImageQuant software, comparing the percentage of the CL1 product obtained in each line to the maximal CL1 percentage obtained at the longest times.

4. Kinetics of religation using oligonucleotide substrate

Suicide CL14/CP25 substrate (20 nM), prepared as above, was incubated with an excess of topoisomerase IB enzyme for 30 min at 37°C in reaction Buffer. A 5µl sample of the reaction mixture was removed and used as the zero time point. Religation reactions were initiated by adding a 200-fold molar excess of R11 oligonucleotide (5'-AGAAAAATTTT-3') over the duplex CL14/CP25 in the presence or absence of 50µM Gold (III). This addition allow the enzyme to perform the religation step restoring a fully duplex oligonucelotide as a final product. DMSO was added to no-drug controls.

At time-course points, 5µl aliquots were removed and the reaction stopped with 0.5% SDS. After ethanol precipitation samples were resuspended in 5µl of 1 mg/ml trypsin and incubated at 37°C for 30 min. Samples were analyzed by denaturing urea/polyacrylamide gel electrophoresis. The experiment was replicated three times and a representative gel is shown. The percentage of religation was determined by PhosphoImager and ImageQuant software, normalized on the total amount of radioactivity in each lane and relatively to the highest amount of substrate converted to reaction product by human topoisomerase IB in the experiments.

5. EMSA (electrophoretic mobility-shift assay)

This assay was done using the same double-stranded 25 bp oligonucleotide CL25/CP25 prepared for the cleavage/equilibrium assay. The reactions were carried out using the catalytically inactive mutant Y723F or the wild type enzyme. Both wild type and mutant enzyme were incubated in standard reaction conditions [20 mMTris/HCl, pH 7.5, 0.1 mM Na2EDTA, 10 mM MgCl2, 50 μg/ml acetylated BSA and 150 mM KCl] in the presence of 1% (v/v) DMSO or 50 μM Gold at 37° C for 30 min in a final volume of 20 μl.

Experimental results and discussion

1. Effect of Gold (III) on topoisomerase IB relaxation

The Gold (III) have an inhibitory effect on the human topoisomerase I relaxation activity, as shown by the plasmid relaxation assay, reported in Fig. 1A. The assay, detecting the different electrophoretic mobility of the DNA supercoiled plasmid, respect to the relaxed form produced by the enzyme, indicates that Gold (III) inhibits the human topoisomerase I in a dose dependent manner (Fig. 1A, lane 3–7). A full inhibition being achieved at 20 µM Gold (III). The complete inhibition also remains after dilution of the pre-incubated Gold (III)–enzyme mixture (Fig. 1C), indicating that the inhibitory effect of gold is irreversible (Fig. 1B, lanes 3-6). As a control it is shown that the enzyme only preincubated with DMSO in the absence of Gold (III) and diluted as the sample maintain its activity (Fig. 1B, lanes 8–11). Fig. 1B shows the time course experiment for the enzyme alone, in presence of 6.5 µM Gold (III) after preincubating the Gold (III) with the substrate or after preincubating the Gold (III) with the enzyme.

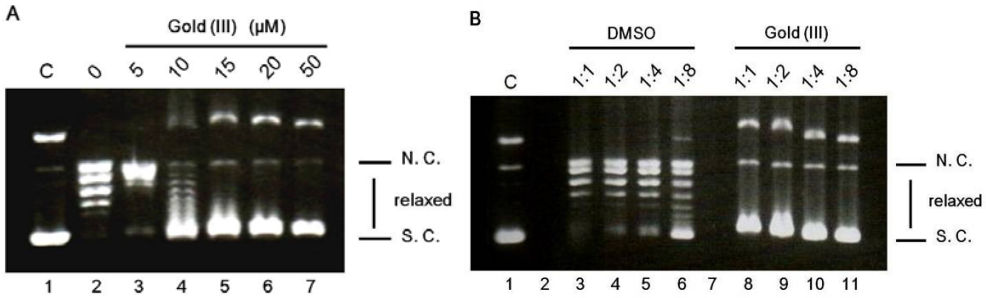


Fig.1- A) Relaxation of negative supercoiled plasmid DNA by topoisomerase IB in the presence of increasing concentrations of Gold III (lanes 3–7).

The reaction products were resolved in an agarose gel and visualized with ethidium bromide. Lane 1, no protein added. Lane 2, control reaction with DNA and enzyme, in absence of Gold III. NC, nicked circular plasmid DNA. SC, supercoiled plasmid DNA. (B) Relaxation assay of topoisomerase IB in DMSO diluted 2–4- or 8-fold (lanes 2–5) or in presence of Gold III (20 μM) diluted 2–4- or 8-fold (lanes 6–9) for 30 min at 37 C. Lane 1, no protein added.

Analysis of cleavage reaction

A time course experiment of a suicide cleavage substrate incu- bated with the enzyme alone, with the enzyme in the presence of 50 lM Gold III, or with the enzyme pre-incubated with 50 lM Gold III is shown in Fig. 2, where the band corresponding to the cleaved DNA fragment is indicated as CL1. The cleavage reaction is fast in absence of the inhibitor, since 90% of the cleavage product is produced within the first 30 sec. (Fig. 2, lanes 2–7) and in less than 5 min the maximum quantity of cleaved substrate is obtained. In the presence of Gold III, the cleavage reaction is clearly inhibited, since the band of the cleaved substrate corresponds to not more than 30% even after 1 h incubation (Fig. 2, lanes 8–13), and cleavage inhibition is almost complete when the enzyme is preincubated with 50 l M Gold III (Fig. 2, lanes 14–19). These experiments demonstrate that after pre-incubation Gold III produces a full inhibition of the enzyme cleavage activity.

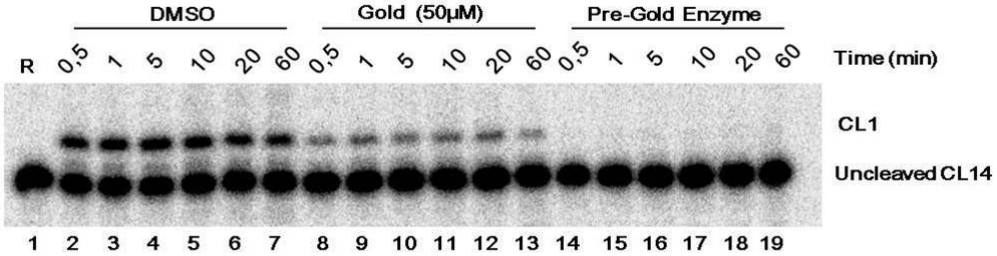


Fig. 2. Suicide cleavage kinetics with the CL14/CP25 substrate for topoisomerase IB alone (lanes 2–7), in presence of 50 μM Gold III (lanes 8–13), or after 5 min enzyme- Gold III pre-incubation (lanes 14–19). Lane 1, no protein added. CL1 represents the DNA fragment cleaved at the preferred enzyme site.

Analysis of religation reaction

To clarify whether Gold III affects the cleavage/religation equilibrium perturbing the religation or the cleavage reaction, these two processes were evaluated in separate experiments. To measure the religation rate the oligonucleotides substrate CL14, 5'end radiolabelled and containing a preferred cleavage site for the enzyme has been annealed to the CP25 complementary strand to pro- duce the suicide substrate, i.e. a duplex with an 11-base pair.single strand extension (Fig. 3A). With this substrate the enzyme is not able to carry out the religation step, because the dinucleotide, generated during cleavage, cannot be religated . I n order to mea- sure the religation rate the suicide cleavage substrate has been incubated with an excess of native enzyme to allow suicide cleavage to proceed to completion, then a 200-fold molar excess of the complementary R11 oligonucleotides (see Fig. 3A) has been added in the absence or presence of 50 μM Gold III, or after incubating the suicide DNA-complex with 50 μM Gold III. The urea–polyacrylamide gel of different aliquots, analyzed as a function of time, indicates that an identical religation rate is observed in all three conditions (Fig. 3B and C). The opposite is observed in the presence of CPT which reduces the religation rate (data not shown), indicating that Gold III must have an inhibition mechanism different from CPT and must involve an event connected with the cleavage reaction.

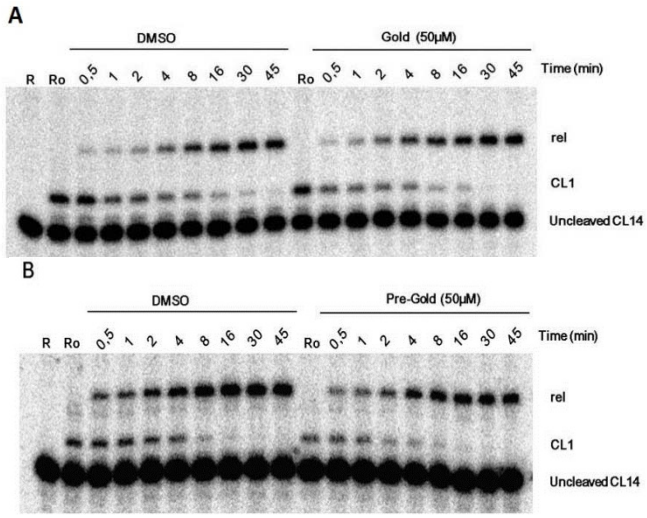


Fig. 3. (A) The CL14/CP25 suicide substrate and the R11 complementary oligonucleotides used to measure the religation kinetics of the enzyme. (B) Gel analysis of the religation kinetics for topoisomerase IB in absence (lanes 2–10) or presence of 50 μM Gold III (lanes 11–19). CL1 represents the DNA fragment cleaved at the preferred enzyme site. Rel represents the religation product. (C) Religation kinetics for topoisomerase IB in absence of Gold III (lanes 2–10) or after 5 min pre- incubation with 50 μM Gold III (lanes 11–19).

Binding Analysis by EMSA

The inactive Y723F human topoisomerase IB mutant has been incubated with the radiolabelled CL25/CP25 DNA substrate in presence of DMSO, 50 l M Gold III, 100 μM CPT, or pre-incubated with

50 μM Gold III for 5 m in (Fig.5, lanes 2–5). In the absence of protein no DNA shift was observed (Fig. 5 lane 1). In the presence of the mutant a tiny slowly migrating band, corresponding to a DNA-topoisomerase IB complex, is formed (Fig. 5, lane 2). The band has a similar intensity in presence of CPT (Fig. 5, lane 5) whilst it is not observed when Gold III is present (Fig.5, lane 3) or is preincubated with the enzyme (Fig.5, lane 4). These results demonstrate that Gold III acts by preventing the topoisomerase IB–DNA complex formation.

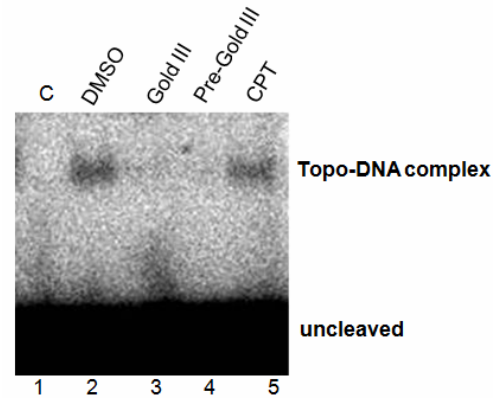


Fig.4. Electrophoretic mobility-shift assay (EMSA) of the radiolabelled CL25/CP25 DNA substrate alone (lane 1); with the inactive Y723F mutant enzyme (lane 2); in presence of 50 μM Gold III (lane 3); after 5 min enzyme pre-incubation with 50 μM Gold III (lane 4); 100 l M CPT (lane 5). The entire samples were analyzed after 30 min. The asterisk indicates the DNA–protein complex.

Conclusion

The plasmid relaxation assays demonstrates that Gold III is a topoisomerase I inhibitor (Fig. 1A) able to fully inhibits the enzyme and that the effect is enhanced by pre-incubation (Fig. 1B). The inhibition is irreversible since dilution of the Gold III–enzyme mixture does not restore any activity. (Fig. 1C). The inhibitory mechanism of Gold III is different from that of CPT, as demonstrated by cleavage, religation and DNA-binding experiments. I n presence of Gold III it is not observed the band corresponding to the cleavable complex, present with the enzyme alone and enhanced by the presence of CPT in the cleavage/religation equilibrium experiment. Moreover pre-incubation with the Gold III compound pre- vents the formation o f the ternary complex stabilized by CPT. Gold III does not effect the religation rate of topoisomerase I (Fig. 3) at variance on what observed in presence of CPT. On the other hand the Gold III inhibits cleavage (Fig. 2) and this effect is enhanced by pre-incubation. The cleavage inhibition is due to the inability of the enzyme, reacted with Gold III, to bind the substrate, as shown by the binding assay in Fig. 4. Gold compounds are known to strongly react with cysteine and we suggest that this can be the main reason for the inhibition activity of our com- pound. As a matter of fact it has been previously shown that that thiol-reactive compounds such as N - ethylmaleimide and phenyl arsine oxide can impair topoisomerase catalytic activity and it is likely that Gold III acts through a similar mechanism. In conclusion we have characterized at functional level the interaction

of a gold (III) compound with topoisomerase IB demonstrating that it inhibits the cleavage reaction binding to the enzyme and not permitting any more the binding of the DNA substrate.

References

[1] J. Champoux, Annu. NY Rev. Biochem. 70 (2001) 369–413.

[2] J.C. Wang, Ann. Rev. Biochem. 65 (1996) 635–692.

[3] Champoux, J. J. (1981) J. Biol. Chem. 256, 4805–4809.

[4] Stewart, L., Redinbo, M. R., Qiu, X., Hol, W. G., and Champoux, J. J. (1998) Science 279, 1534–1541.

[5] Koster, D. A., Croquette, V., Dekker, C., Shuman, S., and Dekker, N. H. (2005) Nature 434, 671–674.

[6] Champoux, J. J. (1990) in Mechanistic Aspects of Type-I Topoisomerases (Wang, J. C., and Cozarelli, N. R., eds) pp. 217–242, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[7] F. Boege, T. Straub, A. Kehr, C. Bosenberg, K. Christiansen, A. Anderson, F. Jakob, J. Kohrle, J. Biol. Chem. 271 (1996) 2262–2270.

[8] J.M. Bridewell, G.J. Finlay, B.C. Baguley, Oncol. Res. 9 (1997) 535–542.

[9] Burden, D. A. and Osheroff, N. (1998) Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. Biochim. Biophys. Acta 1400, 139–154.

[10] Holden, J. A. (2001) DNA topoisomerase as anticancer drug targets: from the laboratory to the clinic. Curr. Med. Chem. Anticancer Agents 1, 1–25.

[11] Liu, L. F., Desai, S. D., Li, T. K., Mao, Y., Sun, M., and Sim, S. P. (2000) Ann. N. Y. Acad. Sci. 922, 1–10.

[12] Pommier, Y., Pourquier, P., Fan, Y., and Strumberg, D. (1998) Biochim.Biophys. Acta 1400, 83–105.

[13] Pommier, Y. (2009) DNA topoisomerase I inhibitors: chemistry, biology, and interfacial inhibition. Chem. Rev. 109, 2894–2902