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Inhibition of human DNA topoisomerase IB by a Cyclometalated Gold III compound: Analysis on the different steps of the enzyme catalytic cycle

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ABSTRACT

A gold(III) compound [Au(C^N^C)(IMe)]CF_3SO_3 (Gold III) has been reported to have anticancer properties as it is able to reduce topoisomerase IB activity *in vitro* and suppress tumor growth in nude mice model. Here we have investigated the mechanism of inhibition of human topoisomerase IB activity by this compound, analyzing the various steps of the catalytic cycle. DNA supercoiled relaxation and the cleavage reaction are inhibited, but Gold III does not perturb the religation reaction, in contrast to what has been observed for camptothecin. Pre-incubation of enzyme with the inhibitor before adding DNA substrate increases the inhibitory effect. In addition, when Gold III is preincubated with the enzyme it prevents the stabilization of the cleavable complex by camptothecin. The analysis of the DNA-topoisomerase binding reaction indicates that the compound acts as a topoisomerase I inhibitor by preventing the enzyme–DNA interaction.

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 topoisomerases: 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]. All the topoisomerases act introducing transient strand breaks in a DNA double strand molecule. In particular, human topoisomerase IB forms a covalent bond with the 3′-phosphate end of the cleaved strand [3]. During this state, the broken strand can rotate around the uncleaved strand leading to DNA relaxation [4–6]. To restore the correct DNA double strand structure, topoisomerase I catalyzes the religation of the 5′-hydroxyl termini.

A number of antitumor agents have topoisomerases as their target and they act through different mechanisms that are related to the different catalytic steps [7,8]. In particular, the drugs inhibiting topoisomerase I can be divided into two classes: poisons and catalytic inhibitors [9,10]. Topoisomerase I poisons, such as camptothecin (CPT) and its analogs, reversibly stabilize the topoI-DNA complex by inhibiting DNA religation [11–13]. Collision of the stabilized complexes with advancing replication forks leads to the formation of irreversible strand breaks that are ultimately responsible for cell death [14,15]. Conversely, catalytic inhibitors exert their cytotoxicity by preventing topoisomerase I binding to the DNA and/or the cleavage activity of the enzyme, resulting in the inhibition of DNA relaxation [16–19]. Recently a natural compound has been found to be able to inhibit both the cleavage and the religation reaction [20].

Square-planar d8 metal complexes have long been known to exhibit promising anticancer activities [21], by covalent crosslinking of d8 metal ions to DNA, by intercalation of the planar metal complexes between the DNA base pairs, or by inhibition of the topoisomerase I activity [22–24]. A panel of stable anticancer gold(III) complexes has been prepared and some of these were identified as possible new anticancer drugs [25,26]. In detail, gold(III) complexes containing dianionic $[C^N^C]^{2-}$ and neutral auxiliary N-heterocyclic carbene (NHC)[2] ligands have been suggested to be promising candidates since they are more cytotoxic to cancerous

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[2] *Abbreviations used:* NHC, N-heterocyclic carbene; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility-shift assay.

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cells than to normal cells and can be easily modified. Furthermore, the strong Au–C (carbene) bond is able to stabilize gold(III) in solution [27].

The compound [Au(C^N^C)(IMe)]CF_3SO_3 (here abbreviated Gold III) has been reported to inhibit supercoiled DNA relaxation by human topoisomerase IB [27] but the mechanism behind the inhibitory effect has not been described. In this work the effect of Gold III on the relaxation activity and each different step of the catalytic cycle of topoisomerase IB has been analyzed. The results show that the gold(III) compound belongs to the class of the catalytic inhibitors, since it inhibits the topoisomerase I cleavage reaction by not permitting the binding of the DNA substrate.

Materials and methods

The Gold III compound [Au(C^N^C)(IMe)]CF_3SO_3 was synthesized as previously described by Yan and co-authors [27].

Purification of human topoisomerase IB

The human topoisomerase IB was expressed under the galactose inducible promoter in a multi-copy plasmid, YEpGAL1-e-wild type and YEpGAL1-e-Y723F, used for transformation of EKY3 cells, as described previously [28]. The epitope-tagged constructs contain the N-terminal sequence FLAG: DYKDDDY (indicated with "e"), recognized by the M2 monoclonal antibody.

The purification was carried out using the ANTI-FLAG M2 Affinity Gel (Sigma) column. The FLAG-fusion topoisomerase IB was eluted by competition with five column volumes of a solution containing 100 μg/ml FLAG peptide in 50 mM Tris–HCl, 150 mM KCl pH 7.4. Glycerol was added into each fraction collected up to a final concentration of 33%; all the fractions were stored at −20 °C. One aliquot from each single fraction was resolved by SDS–polyacrylamide gel electrophoresis; using the epitope-specific monoclonal antibody M2 protein, concentration and integrity were measured through immunoblot assay.

DNA relaxation assays

The activity of topoisomerase IB was assayed in 20 μl of reaction volume containing 0.5 μg of negatively supercoiled pBlueScript KSII(+) DNA and Reaction Buffer (20 mM Tris–HCl, 0.1 mM Na_2EDTA, 10 mM $MgCl_2$, 50 μg/ml acetylated BSA and 150 mM KCl, pH 7.5).

The effect of Gold III on enzyme activity was measured by adding different concentrations of the compound, at different times. Reactions 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 (5 μg/ml), destained with water and photographed under UV illumination. Where indicated, enzyme and inhibitor were preincubated at 37 °C for 5 min, prior to the addition of the substrate. Assays were performed at least three times but only one representative gel is shown.

Cleavage/religation equilibrium assay

The oligonucleotide DNA substrate CL25 (5′-GAAAAAAGACTTAGAAAAATTTTTA-3′) was radiolabelled with [γ-^{32}P]ATP at its 5′ end, while the CP25 complementary strand (5′-TAAAAATTTTTC TAAGTCTTTTTTC-3′) was phosphorylated at its 5′ end with unlabeled ATP. The two strands were annealed at a 2-fold molar excess of CP25 over CL25 as previously described [29]. An excess of topoisomerase IB enzyme was incubated at 37 °C with a duplex CL25/CP25 (final concentration of 20 nM) in Reaction Buffer in presence or absence of 50 μM gold(III) compound and/or 50 μM CPT. Dimethyl sulfoxide (DMSO) was added in the controls not containing the drug. After 30 min the reaction was stopped by adding 0.5% SDS and digested with trypsin after ethanol precipitation. Reaction products were resolved in 20% acrylamide-7 M urea gel (25 mA for 30 min). The experiments have been repeated at least three times.

Kinetics of cleavage

The oligonucleotide substrate CL14 (5′-GAAAAAAGACTTAG-3′) was radiolabelled with [γ-^{32}P]ATP at its 5′ end. The CP25 complementary strand (5′-TAAAAATTTTTCTAAGTCTTTTTTC-3′) 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 a partial duplex.

The suicide cleavage reactions were carried out incubating 20 nM of this partial duplex substrate with an excess of enzyme in Reaction Buffer at 37 °C and in presence of 50 μM Gold III. DMSO was added to no-drug control. Before the addition of the protein, 5 μl sample of the reaction mixture was removed and used as the zero time point (C). At different time points 5 μl aliquots were removed and the reaction stopped with 0.5% SDS. After ethanol precipitation samples were resuspended in 6 μl of 1 mg/ml trypsin and incubated at 37 °C for 1 h. 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 amount of the CL1 product obtained in each line to the maximal CL1 level obtained at the longest times.

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 5 μl 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 50 μM Gold III. This addition allows the enzyme to perform the religation step restoring a fully duplex oligonucleotide as the final product. DMSO was added to no-drug controls.

At time-course points, 5 μl aliquots were removed and the reaction stopped with 0.5% SDS. After ethanol precipitation samples were resuspended in 5 μl 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 to the total amount of radioactivity in each lane and relative to the highest amount of substrate converted to reaction product by human topoisomerase IB in the experiments.

Electrophoretic mobility-shift assay (EMSA)

The 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. The mutant enzyme was incubated in standard reaction conditions [20 mM Tris–HCl, pH 7.5, 0.1 mM Na_2EDTA, 10 mM $MgCl_2$, 50 μg/ml acetylated BSA and 150 mM KCl] in the presence of 1% (v/v) DMSO, 50 μM Gold III or 100 μM CPT at 37 °C for 30 min in a final volume of 20 μl.