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Substitution-inert polynuclear platinum complexes inhibit activity of DNA polymerase in triplex-forming templates

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Abstract

The formation of triple-helical DNA is implicated in the regulation of gene expression. The triplexes are, however, unstable under physiological conditions so that effective stabilizers for the triplex formation are needed. Here, we describe a new strategy for stabilization of the triplexes based on the antitumor substitution-inert polynuclear platinum complexes (SI-PPCs). These compounds were shown previously to bind to DNA through the phosphate clamp - a discrete mode of DNA-ligand recognition distinct from the canonical intercalation and minor-groove binding. We have found that SI-PPCs efficiently inhibit DNA synthesis by DNA polymerase in sequences prone to the formation of pyrimidine- and purine motif triplex DNAs. Moreover, the results suggest that SI-PPCs are able to induce formation of triple-helical DNA between duplexes and strands which are not completely complementary to each other. Collectively, these data provide evidence that SI-PPCs are very efficient stabilizers of triple-stranded DNA which might exert their action by stabilizing higher order structures such as the triple-helical DNA.

COMMUNICATION

Substitution inert trinuclear platinum complexes are very efficient stabilizers of triple-stranded DNA which might exert their action by stabilizing higher order structures such as the triple-helical DNA.

Keywords

Platinum complexes; DNA triplex; DNA polymerase; anticancer agents

The substitution-inert polynuclear platinum complexes (SI-PPCs) represent a unique class of antitumor agents that bind with high affinity to DNA and RNA.^[1] Their binding to nucleic acids is mediated exclusively by noncovalent (hydrogen bonding, electrostatic) interactions. Recently, it has been revealed that SI-PPCs achieve DNA recognition through use of ‘phosphate clamp’ - a discrete mode of DNA-ligand binding distinct from canonical intercalation and minor-groove binding.^[2] SI-PPCs display interesting biological activities including *in vitro* cytotoxicity in a wide range of cell lines, an antitumor activity that is independent of p53 status and high cellular accumulation.^[1, 3] Rapid cellular entry is mediated through interaction with cell surface heparan sulfate proteoglycans (HSPG), which is a unique mechanism to SI-PPCs allowing for possibilities of tumor selectivity as well as disruption of HSPG function.

The mechanism of SI-PPCs cytotoxic and antitumor activity is not fully understood yet. However, it has been suggested that it may be associated with high-affinity noncovalent binding to DNA resulting in very efficient nuclear condensation.^[1, 3] This unique effect is mirrored by the efficient condensation of DNA and tRNA observed *in vitro*.^[4] Previous studies also showed that SI-PPCs are potent inducers of B→A and B→Z conformational transitions in canonical sequences of DNA, they are even significantly more efficient than spermine.^[5]

The formation of a triple-helical nucleic acid structure was first reported 60 years ago by Felsenfeld and co-workers in synthetic polyribonucleotides consisting of purine and pyrimidine strands.^[6] Triple helix DNA formation occurs when a triplex-forming oligonucleotide (TFO) binds in the major groove to a homopurine region of DNA. This sequence-specific interaction is mediated by the formation of Hoogsteen or reverse Hoogsteen hydrogen bonds between TFO bases and bases in the purine-rich strand. There are two types of DNA triplex depending on the orientation of TFO and the nature of the base triplets.^[7] Pyrimidine-rich TFOs bind in parallel to the duplex purine strand forming C⁺·GC and T·AT triplets and purine-rich TFOs bind in an antiparallel orientation forming G·GC,

A·AT, and T·AT triplets. DNA triplexes attract much interest as potential agents capable of inhibiting the expression of particular genes in a sequence-specific manner.^[8] Notably, DNA triplexes have been also proved useful in the development of DNA-based nanostructures and materials.^[9]

Here we present the results on a group of SI-PPCs (Figure 1A) and their ability to inhibit DNA synthesis by DNA polymerase in sequences prone to the formation of pyrimidine- and purine motif triplex DNAs. The results were compared to those obtained with spermine, which is regarded as an excellent DNA triplex stabilizer.^[10]

We employed *Taq* DNA polymerase to assay for primer extension on the triplex-forming mirror repeat pyrimidine- or purine-rich templates (**Py-Py_template** or **Pu-Pu_template**, respectively) and the complementary primers (**Pu_primer** or **Py_primer**, respectively). As controls, we used templates incapable of DNA triplex-formation containing just one of the two mirror-repeat sequences complementary to the primer followed either by a random sequence (**Py-random_template** or **Pu-random_template**, respectively) or by a sequence in which the base content was kept the same but the positions of bases were randomly shuffled (**Py-shuffled_template** or **Pu-shuffled_template**, respectively) (for their sequences see Figure 1B).

We first started with the triplex-forming DNA construct composed of a 45 nucleotides (nts) long template containing two pyrimidine-rich mirror-repeat sequences connected by five thymines (**Py-Py_template**) and a 20 nts long purine-rich primer complementary to the 3' side of the former (**Pu_primer**). The long template strand folds back like a hairpin conformation when a pyrimidine-motif (parallel) triplex is formed. As it can be seen in Figure 2, the **Py-Py_template** in the presence of **I-V** did not allow primer extension. It suggests that SI-PPCs induce the formation or stabilization of a DNA conformation incompatible with DNA polymerization. Alternative explanation that SI-PPCs interact with *Taq* DNA polymerase and affect its activity can be excluded because the same primer hybridized with a control template containing a pyrimidine-rich sequence flanked by a random sequence (**Py-random_template**) was efficiently extended despite the presence of SI-PPCs (except for **V**) (Figure S1).

Closer inspection of the gels in Figure 2B reveals that the elongation of the primer is paused after synthesis of 4 and 5 nucleotides. These pausing sites correspond to the 4th and 5th thymine in the five-thymine linker connecting the two mirror-repeat sequences in the **Py-Py_template**. If the template folds back to form a triplex, thymine residues in the loop remain unpaired (see scheme in Figure 2A). In order to probe the formation of triple-helical DNA in the absence of **I-V** and spermine, the oligonucleotide mixtures used for primer extension studies were subject to DNase I digestion. The buffer conditions and the temperature (37 °C) were the same as in primer extension experiments. Indeed, the autoradiogram of the gel in Figure S2 in the Supplementary data confirms that the radioactively labeled **Pu_primer** was protected from the cleavage by DNase I when it was annealed with the triplex-forming **Py-Py_template**. Hybridization of the primer with templates incapable of DNA triplex-formation (**Py-random_template** or **Py-shuffled_template**) did not prevent degradation by DNase I (Figure S2, lanes 7 and 8). It

means that the formation of DNA triple-helix in the absence of SI-PPCs or in the presence of spermine (up to 1000 μM) cannot stop DNA polymerase from the elongation of the primer.

To further corroborate our view that SI-PPCs interact with triple-helical DNA structures, we performed the experiments employing highly fluorescent Thiazole Orange (TO) which binds with much higher affinity to triple-stranded DNA compared to double-stranded (ds) DNA [11]. We measured a fluorescence decrease resulting from displacement of TO from the triple-stranded DNA [represented by the triplex-forming templates (**Py-Py_template** and **Pu-Pu_template**, respectively) hybridized with their complementary primers (**Pu_primer** and **Py_primer**, respectively)] by the competitors SI-PPCs and spermine. We found (Figure S3 and Table S1) that all SI-PPCs (**I-V**) effectively decreased fluorescence of TO. The trend was **V** > **IV** $\approx$ **III** > **II** > **I** > spermine which correlated with the efficiency of SI-PPCs and spermine to inhibit DNA synthesis. Overall, the results of these experiments afford an evidence that SI-PPCs form complexes with triple-helical DNA structures.

The gels in Figure 2B were analyzed by densitometry and obtained data were used to generate plots of the extent of full-length product synthesis on the triplex-forming **Py-Py_template** as a function of **I-V** and spermine concentrations as shown in Figure 2C. The values of concentrations required to cause 50% reduction of full-product synthesis (EC_{50}) are summarized in Table 1.

We observed the following order of effectiveness: **V** > **IV** > **III** > **II** > **I**, which is in agreement with the biological activity of SI-PPCs. Intriguingly, increasing concentrations of spermine lead to partial inhibition of *Taq* DNA polymerase activity with a maximum inhibitory effect at $\sim 500 \mu\text{M}$ concentration and further additions of spermine resulted in an enhancement of DNA polymerization. The least effective of the investigated SI-PPCs, complex **I**, with the EC_{50} value of $90 \pm 21 \mu\text{M}$ was able to completely inhibit DNA synthesis despite having the same charge as spermine (4+). The lowest EC_{50} value of $0.27 \pm 0.02 \mu\text{M}$ was observed for **V**, while compounds **IV**, **III**, and **II** had EC_{50} values of $2.7 \pm 0.8 \mu\text{M}$, $3.8 \pm 1.5 \mu\text{M}$, $7.9 \pm 2.4 \mu\text{M}$, respectively. It means that **V** and **IV** are ~ 330 -fold and ~ 30 -fold more potent inhibitors of *Taq* DNA polymerase than **I**, respectively.

None of the SI-PPCs was able to inhibit primer extension on the template containing a pyrimidine-rich sequence flanked by a random sequence (**Py-random_template**) up to the concentration of $600 \mu\text{M}$ (Figure S1) except for **V** with the EC_{50} of $2.92 \pm 0.08 \mu\text{M}$, which is approximately 10-fold higher than the EC_{50} value obtained for the primer extension on the triplex-forming **Py-Py_template**. The inhibitory effect of **V** on the DNA polymerase may be associated with its outstanding potency to condense/aggregate nucleic acids compared to compounds **I-IV** as it was demonstrated previously.^[4] Nevertheless, the interaction of **V** with the enzyme itself cannot be excluded.

The random sequence flanking the pyrimidine-rich sequence differs in base composition and was deliberately designed to prevent DNA triplex formation. In the next experiment, we used a template which contained the pyrimidine-rich sequence followed by a sequence in which the base content was kept the same, but the positions of bases were randomly shuffled (**Py-shuffled_template**). The results were quite surprising. As it can be seen in the

autoradiograms of the gels in Figure S4, complexes **II-V** were able to inhibit the primer extension although at much higher concentrations. Inspection of the EC₅₀ values in Table 1 reveals that compounds **V**, **IV**, **III**, and **II** were approximately 4-, 10-, 19-, and 17-fold less effective in inhibiting the primer elongation on the **Py-shuffled_template** than on the triplex-forming **Py-Py_template**. DNase I digestion of the **Pu_primer** hybridized with the **Py-shuffled_template** (Figure S4) indicates that the template is unable to fold back to protect the primer from DNase I cleavage by forming DNA triplex. It is also in agreement with the observation that gels in Figure S4A do not contain pausing sites 4 and 5 nucleotides away from the start that are present in gels in Figure 2B with products of DNA synthesis on the template capable of the triplex formation. Taken together, it suggests that the mechanism by which **II-V** inhibit DNA synthesis on the **Py-shuffled_template** is probably different. We can speculate that the **Py-shuffled_template**, having the same base content as the **Py-Py_template** but in randomized-order is still able, in the presence of **II-V**, to form a parallel triple helix which can inhibit the primer extension by DNA polymerase (see the scheme of alternative triplexes formed by the **Py-shuffled_template** in Figure S7 in the Supplementary data).

In the second part, we used the triplex-forming DNA construct composed of a 41 nts long template with two purine-rich mirror-repeat sequences (**Pu-Pu_template**) and a 20 nts long homo-pyrimidine sequence containing primer (**Py_primer**) complementary to the 3' side of the former (for their sequences see Figure 1B). When the single-stranded part of the template strand folds back like a hairpin, it forms a purine-motif (antiparallel) triplex DNA (see scheme in Figure 3A). The results in Figure 3 show that, similarly to the previously used pyrimidine-motif forming template, the purine-rich template (**Pu-Pu_template**) also prevented primer (**Py_primer**) extension in the presence of **I-V**, whereas a control template with a purine-rich sequence flanked by a randomly compiled sequence (**Pu-random_template**) and hybridized with the **Py_primer** was efficiently elongated (except for **V**) (Figure S5). Gels in Figure 3B show that the primer extension in the presence of **I-V** was paused after addition of just one nucleotide. This base corresponds to adenine which remains unpaired connecting two mirror-repeat sequences when the template folds back to form triplex (see scheme in Figure 3A). To probe the DNA triplex formation the oligonucleotide mixtures used in primer extension assays were allowed to react with DNase I in the absence of SI-PPCs and spermine. The results in Figure S3 in the Supplementary data demonstrate that cutting of the **Py_primer** by DNase I was prevented only when it was annealed with the triplex-forming template. Hybridization of the **Py_primer** with two other templates **Pu-random_template** or **Pu-shuffled_template**, respectively did not have any protective effect.

Plots of the extent of full-length product synthesis on the triplex-forming template as a function of **I-V** and spermine concentrations are shown in Figure 3C, and the EC₅₀ values derived from these data are presented in Table 2. The order of effectiveness of individual complexes: **V** > **IV** > **III** > **II** > **I**, was the same as in the previous experiments. Spermine was unable to inhibit DNA polymerization up to the highest concentration tested (1000 μM). The EC₅₀ value of the least effective complex **I** was 570 ± 30 μM whereas the compounds **V**, **IV**, **III**, and **II** had EC₅₀ values of 1.07 ± 0.05 μM, 10.1 ± 0.6 μM, 31.3 ± 1.7 μM, and

56.9 ± 3.4 μM, respectively. It implies that **V**, as the most potent inhibitor of DNA polymerization, was ~530-fold more active than **I**.

Results in Figure S5 and Table 2 show that complex **V** was again the only one of the investigated SI-PPCs capable of inhibiting DNA synthesis on the template containing a pyrimidine-rich sequence flanked by a random sequence (**Pu-random_template**) with EC₅₀ value of 5.2 ± 0.3 μM which is approximately 5-fold higher concentration than on the triplex-forming **Pu-Pu_template**. Other complexes **I-IV** and spermine did not exhibit any inhibitory effect up to the highest concentration tested (600 μM).

When the primer extension was performed on the template composed of a purine-rich sequence flanked by a sequence in which the base content was kept the same but the positions of bases were randomly shuffled (**Pu-shuffled_template**), inhibition activity of **II-V** on *Taq* DNA polymerase was observed (Figure S6). The EC₅₀ values in Table 2 show that concentrations of **V**, **IV**, **III**, and **II** required to achieve 50% reduction of full-product synthesis were 2-, 5-, 5-, and 5-fold higher, respectively, compared to the primer extension on the triplex-forming template. Also in this case we can hypothesize that **II-V** might induce formation of the purine-motif triplex DNA in the **Pu-shuffled_template**. The scheme of prospective triplexes formed by the **Pu-shuffled_template** can be seen in Figure S8 in the Supplementary data.

The relationship of the inhibition of DNA synthesis by SI-PPC stabilizers of triple-stranded DNA to the toxicity of SI-PPCs in tumor cells deserves further discussion. DNA is the established and important pharmacological target of anticancer platinum complexes.^[12] Hence, the inhibited DNA synthesis by triple-stranded structures stabilized by SI-PPCs could contribute to cytotoxicity of these platinum complexes, the phenomenon seen in the case of conventional antitumor platinum drugs, such as cisplatin and its derivatives. The relevance of the inhibition of DNA synthesis by stabilizers of triple-stranded DNA to the cytotoxicity of these stabilizers is also given by the fact that the nucleotide sequences prone to form triple-stranded structures are present in natural DNA. Polypyrimidine-polypurine sequences with mirror symmetry capable of forming intramolecular triple-helical structures in DNA molecules are found in several eukaryotic genomes preferentially near regulatory regions.^[13] Moreover, the existence of triple-stranded nucleic acid structures *in vivo* and their potential participation in a variety of biological processes such as chromatin organization, DNA repair, transcriptional regulation and RNA processing have been also reported.^[14] In this connection it is important to emphasize that our results demonstrate a very effective termination of DNA synthesis by triple-stranded DNA stabilized by SI-PPCs; and because inhibition of DNA synthesis in tumor cells undoubtedly contributes to programmed cell death and triple-helical structures in DNA molecules are relatively abundant in eukaryotic genomes, it is reasonable to assume that antitumor effects of stabilizers of triple-stranded DNA may be also connected with their capability to stabilize triple-helical structures in DNA. Moreover, our thesis that SI-PPCs inhibit DNA synthesis is further corroborated by the results published earlier^[3] demonstrating that population of tumor cells treated with SI-PPCs in S phase markedly increased (in the S phase DNA synthesis replicates the genetic material) and thus correlated with cytotoxicity of these platinum complexes. Another widely used protocol to monitor rates of DNA synthesis is

based on metabolic incorporation of tritiated thymidine (^3H -TdR) into cellular DNA. We found that treatment of exponentially proliferating tumor HeLa cell line with SI-PPCs resulted in a marked inhibition of ^3H -thymidine incorporation, which is also consistent with the ability of SI-PPCs to effectively inhibit DNA synthesis (Figure S9). Importantly, the effectivity of highly cytotoxic Triplatin-NC (complex **V**), which also efficiently stabilizes triple-stranded DNA, to inhibit ^3H -thymidine incorporation was markedly higher than that of considerably less cytotoxic and less effective in stabilizing triple-stranded DNA, complex **I**. Notably, inhibition of DNA synthesis by clinically used platinum complexes has been associated with the formation of covalent adducts on DNA by these platinum drugs. However, SI-PPCs are substitution-inert complexes which cannot bind DNA coordinatively and form DNA adducts so that other mechanism different from that associated with the formation of bulky DNA adducts is responsible for inhibition of DNA synthesis by SI-PPCs. Thus, the results presented in this article appear to support the hypothesis that the mechanism based on the stabilization of triple-stranded structures by SI-PPCs in relatively abundant triplex-forming sequences in eukaryotic cells could also contribute to antitumor effects of SI-PPCs.

We demonstrate in this study that SI-PPCs display a remarkable effectivity in stabilizing triple--stranded DNA. Moreover, our results suggest that stabilization of triple-stranded DNA leading to disruption of DNA-related enzymatic activities might contribute to the mechanism of biological activity of SI-PPCs, particularly at lower concentrations before condensation/aggregation of nucleic acids by SI-PPCs can occur. Collectively, SI-PPCs may now be seen as multi-function DNA binding agents, the DNA binding mode of which is unique and distinctly different from DNA binding modes of DNA triplex stabilizers hitherto investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. (A) The chemical structures of SI polynuclear platinum complexes. **I**, $[\{\text{Pt}(\text{NH}_3)_3\}_2\text{-}\mu\text{-NH}_2(\text{CH}_2)_6\text{NH}_2\}]^{4+}$. **II**, $[\{\text{Pt}(\text{NH}_3)_3\}_2\text{-}\mu\text{-spermidine}]^{5+}$. **III**, $[\{\text{Pt}(\text{NH}_3)_3\}_2\text{-}\mu\text{-spermine}]^{6+}$. **IV**, TriplatinNC-A, $[\{\text{Pt}(\text{NH}_3)_3\}_2\text{-}\mu\text{-}\{trans\text{-Pt}(\text{NH}_3)_2(\text{NH}_2(\text{CH}_2)_6\text{NH}_2)_2\}]^{6+}$. **V**, TriplatinNC, $[\{\text{trans-Pt}(\text{NH}_3)_2(\text{NH}_2(\text{CH}_2)_6\text{NH}_3^+)\}_2\text{-}\mu\text{-}\{trans\text{-Pt}(\text{NH}_3)_2(\text{NH}_2(\text{CH}_2)_6\text{NH}_2)_2\}]^{8+}$. (B) The sequences of oligonucleotides used in primer extension study.

Figure 2.

Inhibition of DNA synthesis catalyzed by *Taq* DNA polymerase due to the pyrimidine-motif triplex formation. The polymerization reaction was performed with the mirror repeat pyrimidine-rich template (**Py-Py_template**) and the complementary primer (**Pu_primer**) at 37 °C in the presence of increasing concentrations of **I-V** and spermine. (A) Scheme of the triplex formation (for the nucleotide sequences of **Pu_primer** and **Py-Py_template**, see Figure 1B and Materials and methods). (B) Autoradiograms of 12% PAA sequencing gels. *p* and *fp* correspond to primer and full-product, respectively. (C) Graphical representation of full-product synthesis using the **Py-Py_template** in the presence of increasing concentrations of **I-V** and spermine.

Figure 3.

Inhibition of DNA synthesis catalyzed by *Taq* DNA polymerase due to the purine-motif triplex formation. The polymerization reaction was performed with the mirror repeat purine-rich template (**Pu-Pu_template**) and the complementary primer (**Py_primer**) at 37 °C in the presence of increasing concentrations of **I-V** and spermine. (A) Scheme of the triplex formation. (B) Autoradiograms of 12% PAA sequencing gels. *p* and *fp* correspond to primer and full-product, respectively. (C) Graphical representation of full-product synthesis using the **Pu-Pu_template** in the presence of increasing concentrations of **I-V**.

Table 1.

Inhibition of DNA synthesis catalyzed by *Taq* DNA polymerase due to the pyrimidine-motif triplex formation.
[a]

Compound	Concentration required for 50% inhibition of full-product synthesis (EC ₅₀ , μM)		
	Py-Py_template	Py-random_template	Py-shuffled_template
V	0.27 ± 0.02	2.92 ± 0.08	1.00 ± 0.08
IV	2.7 ± 0.8	>600	27.7 ± 3.8
III	3.8 ± 1.5	>600	74.1 ± 6.2
II	7.9 ± 2.4	>600	137 ± 14
I	90 ± 21	>600	>600
spermine	~500 ^[b]	>1000	>1000

[a] For other details, see Figure 2.

[b] Further increase of spermine concentration above 500 μM resulted in an enhancement of full-product synthesis (see Figure 2C).

Table 2.Inhibition of DNA synthesis catalyzed by *Taq* DNA polymerase due to the purine-motif triplex formation.^a

Compound	Concentration required for 50% inhibition of full-product synthesis (EC ₅₀ , μ M)		
	Pu-Pu_ template	Pu-random_ template	Pu-shuffled_ template
V	1.07 $\pm$ 0.05	5.2 $\pm$ 0.3	2.4 $\pm$ 0.3
IV	10.1 $\pm$ 0.6	>600	55 $\pm$ 10
III	31.3 $\pm$ 1.7	>600	151 $\pm$ 16
II	56.9 $\pm$ 3.4	>600	297 $\pm$ 11
I	570 $\pm$ 30	>600	>600
spermine	>1000	>1000	>1000

^aFor other details, see Figure 3.