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Platinated Nucleotides are Substrates for the Human Mitochondrial Deoxynucleotide Carrier (DNC) and DNA Polymerase γ: Relevance for the Development of New Platinum-Based Drugs.

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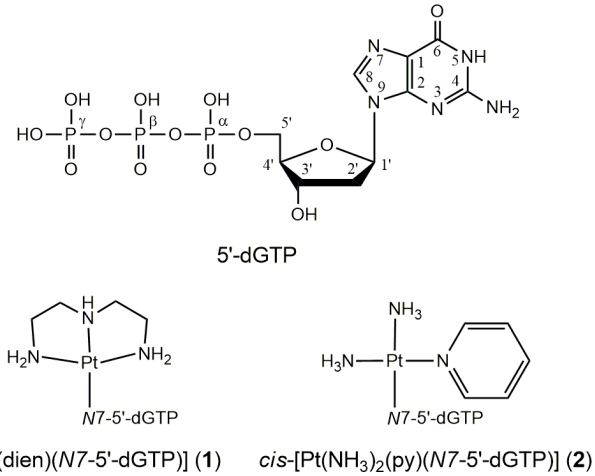
cis-[$PtCl_2(NH_3)_2$] (*cisplatin*) is among the highest effective antitumor drugs used for the chemotherapeutic treatment of a broad range of malignancies. Recently, alongside with the classical direct bond to DNA, an alternative mechanism of action mediated by *N7* platinated nucleotides has been suggested for *cisplatin*. Considering that mitochondria play an important role in cell death activation and in a significant portion of the clinical activity and pharmacological properties associated with *cisplatin*, aim of this research was to evaluate the possibility that platinated deoxynucleotides, as the model complex [Pt(dien)(*N7*-5′-dGTP)] (**1**), dien = diethylenetriamine, could be transported into mitochondria and then incorporated into mtDNA. The kinetic characterization has revealed that the mitochondrial deoxynucleotide carrier (DNC) transports complex **1** with high affinity. Finally, a highly efficient *in organello* DNA synthesis system, followed by ICP-AES, has demonstrated that [Pt(dien)(*N7*-5′-dGTP)] is incorporated in the mitochondrial DNA by DNA polymerase γ. These results may have critical implications in the development of new generations of anticancer and/or antiviral nucleotide analogues with more specific cellular targets and fewer side effects.

After almost fifty years from discovery, platinum based antitumor drugs remain among the most successful for chemotherapy, where their central role remain out of discussion.[1–4] It is noteworthy that in the case of antitumor therapy, most of the tested drug mixtures can benefit of the presence of *cis*-[$PtCl_2(NH_3)_2$] (*cisplatin*), generally resulting in an increase of the patient's life expectancy.[2] As a consequence the range of tumors treatable by *cisplatin* analogues is today considerably expanded.[1–5] It is known that *cisplatin* crosses the cell membrane, mainly by passive diffusion.[2–4] Aquation inside cytosol increases the reactivity of *cisplatin* toward DNA, where it preferentially binds the *N7* of purines, considered the main pharmacological target, Scheme 1.[2–7] Nonetheless, the cascade of events leading to cell death, is still not clear. The picture is complicated by the fact that other substrates and free simple nucleotides, could also bind the *cisplatin* inside cells.[2,5,8,9] We retain that higher accessibility of the *N7* lone pairs in free pu-



Scheme 1. Schematized structures of the considered model Pt(II) complexes.

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rines, with respect to the more crowded DNA and RNA molecules, could enhance the formation of Pt-N bonds. This suggests the possibility to exploit a parallel mechanism of platination of DNA, mediated by interaction of platinum based drugs with single nucleotides.[8] Our interest on this subject is enhanced by relevant pharmacological activity previously observed in the case of some platinum-nucleotide complexes.[10] Consistently, in a previous paper we demonstrated that model *N7*-platinated nucleotides could work as substrates for the synthesis of DNA, catalyzed by DNA polymerases.[8] This indirect process seemed relevant, in experiments where it was compared the incorporation of natural and platinated dGTP's into a plasmidic DNA, by using the model complex [Pt(dien)(*N7*-5'-dGTP)] (**1**).[8]

It is noteworthy that the trafficking of nucleotides is implicated in many essential metabolic functions, such as the synthesis of nucleic acids and the energetic functions of mitochondria.[11] Consistently exposure of cells to *cisplatin* causes relevant mitochondrial alterations leading to the activation of intrinsic apoptotic pathways and finally to cell death.[12]

In a previous work,[9] we studied the uptake of model Pt-purine complexes [Pt(dien)(*N7*-5'-dGTP)] and *cis*-[Pt(NH_3)$_2$(py)(*N7*-5'-dGTP)] (**2**) by using phospholipid vesicles (liposomes) reconstituted with the recombinant *Dm*Tpc1 (*Drosophila melanogaster* thiamine pyrophosphate carrier protein). *Dm*Tpc1 is a protein of the inner mitochondrial membrane that catalyzes the mitochondrial uptake of thiamine pyrophosphate or nucleotides. In this work, we decided to study the mitochondrial uptake of model platinated nucleotides and their possible insertion in the newly synthesized mitochondrial DNA.

On this basis, we carried out a series of experiments in which the **1** or **2** model complexes were added to suspensions of freshly isolated rat liver mitochondria. In Figure 1 it is shown

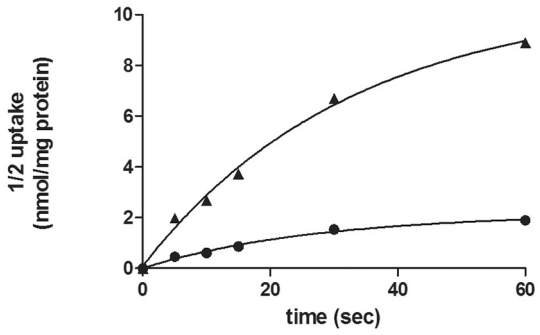


Figure 1. Transport of [Pt(dien)(*N7*-5'-dGTP)] (1) and *cis*-[Pt(NH_3)$_2$(py)(*N7*-dGTP)] (2) into rat liver mitochondria. Freshly isolated rat liver mitochondria were incubated with 0.5 mM **1** (▲) or **2** (●) for the indicated times. The uptake of both **1** and **2** was assessed by determining the platinum amount into mitochondria (ICP-AES). Consistently it was found no **1** and **2** uptake in the presence of 100 μM *p*-chloromercuribenzene sulfonate (*p*-CMBS) inhibitor. Similar results were obtained in at least four experiments.

the time-course of complexes **1** and **2** uptake, operated by rat liver mitochondria. In these experiments, the incubation mixtures contained equal amounts of mitochondrial proteins and the same 0.5 mM external concentration of **1** or **2.** The uptake of **1** was found to increase with time (initial rate 0.327 ± 0.016 nmol/min x mg protein) reaching a maximum after about 60 sec incubation (10.62 ± 0.531 nmol/mg protein). These results clearly show that complex **1** accumulates into isolated mitochondria and that the uptake is time dependent (Figure 1). Instead, complex **2** is transported less efficiently, given probably its higher steric hindrance. No transport activity was measured in the control experiments, when mitochondria were incubated with 0.5 mM [PtCl(dien)]Cl or *cis*-[PtCl(NH_3)$_2$(py)]Cl (*pyriplatin*). These results suggest for complex **1** a more efficient carrier-mediated transport across the inner mitochondrial membrane.

The inner mitochondrial membrane contains several transporters for nucleotides, nucleotide analogs or deoxynucleotides.[11,13,14] Moreover, preliminary studies have shown that the *Dm*Tpc1 is actively involved in the specific uptake of **1**[9] and it has a 53% sequence similarity with respect to the DNC (also named hTpc).[9,13] The ability of DNC to transport complex **1** was tested by using phospholipid vesicles reconstituted with the purified recombinant protein (proteoliposomes).[9]

The kinetics of the uptake of 0.5 mM complex **1**, by proteoliposomes in exchange for internal 5 mM ADP, is shown in Figure 2. The transport, catalyzed by the recombinant DNC, fol-

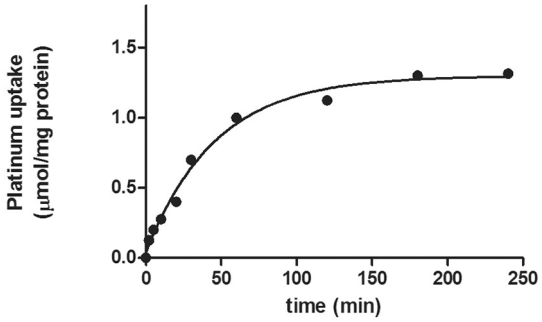


Figure 2. Time course of the [Pt(dien)(*N7*-5'-dGTP)]/ADP exchange in proteoliposomes reconstituted with the recombinant DNC. The assay of transport was initiated by the addition of 0.5 mM [Pt(dien)(*N7*-5'-dGTP)] (**1**) to proteoliposomes containing 5 mM ADP. The uptake of **1** was terminated, after the indicated times, by 100 μM *p*-chloromercuribenzene sulfonate (*p*-CMBS). The uptake of **1** was assessed by determining the platinum amount into proteoliposomes (ICP-AES). The data represent the means of four different experiments.

lowed first-order kinetics (rate constant, 0.0217 min^{-1}; initial rate, 0.0282 μmol/min x mg of protein), isotopic equilibrium being approached exponentially. Similarly, liposomes reconstituted with the recombinant DNC catalyzed the exchange of 0.5 mM dATPαS-[^{35}S] for 5 mM ADP with first-order kinetics and an initial rate of 0.695 μmol/min x mg protein, Figure 1S. Fur-

thermore, in the proteoliposomes reconstituted with the recombinant protein the complex **1** inhibited the uptake of dATPαS-[^{35}S] competitively, with a Ki value of 0.588 ± 0.038 mM. The potency of **1** inhibition on DNC substrate transport activity is 2.6 fold lower than that of dGTP [Ki = 0.224 ± 0.020 mM].[13] Such data confirm that the DNC carrier catalyzes the import of complex **1**, although less efficiently than dGTP, probably for its higher steric hindrance. Even in this case no transport activity was measured in control experiments, when proteoliposomes were incubated with 0.5 mM [PtCl(dien)]Cl or *cis*-[PtCl(NH_3)$_2$(py)]Cl.

Competition experiments were performed to evaluate the effect on **1** uptake of external substrates both in isolated rat liver mitochondria and proteoliposomes reconstituted with recombinant DNC. The assay of transport was initiated by adding complex **1** to mitochondria (Figure 3 A) or proteoliposomes (Figure 3B), together with dATP, dGTP or GMP. dATP and dGTP are known to show high affinity for the human DNC, instead GMP shows a lower affinity.[13] As shown, in Figure 3 the measured platinum amount decreases in the presence of 0.5 mM dGTP and is virtually nothing in the presence of 0.5 mM dATP. On the contrary, a negligible inhibition was observed with GMP. These results demonstrate that dGTP and dATP compete with **1** for the binding to the active site of DNC, confirming that it catalyzes the uptake of complex **1.**

An *in organello* synthesis approach was used to investigate the effects of **1** on mtDNA replication.[15] mtDNA synthesis in intact organelles was assessed by incorporation of the radiolabeled [α-^{32}P]dATP and visualized by gel electrophoresis and autoradiography. The labeled mtDNA was distributed in three different gel areas, the slow moving concatenated/circular mtDNA oligomers, the 16.5 Kb open-circular mtDNA and the fast moving 7S DNA molecules (Figure 4 A). *In organello* mtDNA synthesis was strongly stimulated by the exogenous dNTPs (Figure 4; dGTP vs -dNTPs). The gradual substitution (50% steps) of natural dGTP by **1** decreased progressively the incorporation of radioactivity into mtDNA (Figure 4). However, when natural dGTP was completely replaced by **1**, the mtDNA synthesis remained significantly higher (~2-fold) than that observed in the reaction where the dNTPs were omitted (Figure 4; **1** vs dNTPs).

The effect of platinated dGTP on mtDNA synthesis was evaluated by using a mixture containing 95% of **1** and 5% of [PtCl(dien)]Cl. This excess of [PtCl(dien)]Cl allowed complete consumption of natural dGTP and inhibition of the metal-modified dGTP dissociation during the synthesis of **1** and ensured that **1** was the only exogenous dGTP added to the *in organello* reaction. To control for a nonspecific effect of the [PtCl(dien)]Cl excess, mtDNA synthesis was evaluated in the presence of [PtCl(dien)]Cl (5 μM). The excess of [PtCl(dien)]Cl did not change the incorporation of dNTPs into mtDNA (Figure 4; dGTP vs [PtCl(dien)]Cl). Overall, these results suggest that platinated nucleotides could be incorporated with low efficiency into mtDNA by DNA polymerase γ.

In order to evaluate the effective ability of DNA polymerase γ to incorporate platinated dGTP during mtDNA synthesis, incorporation of **1** was assessed measuring the platinum content

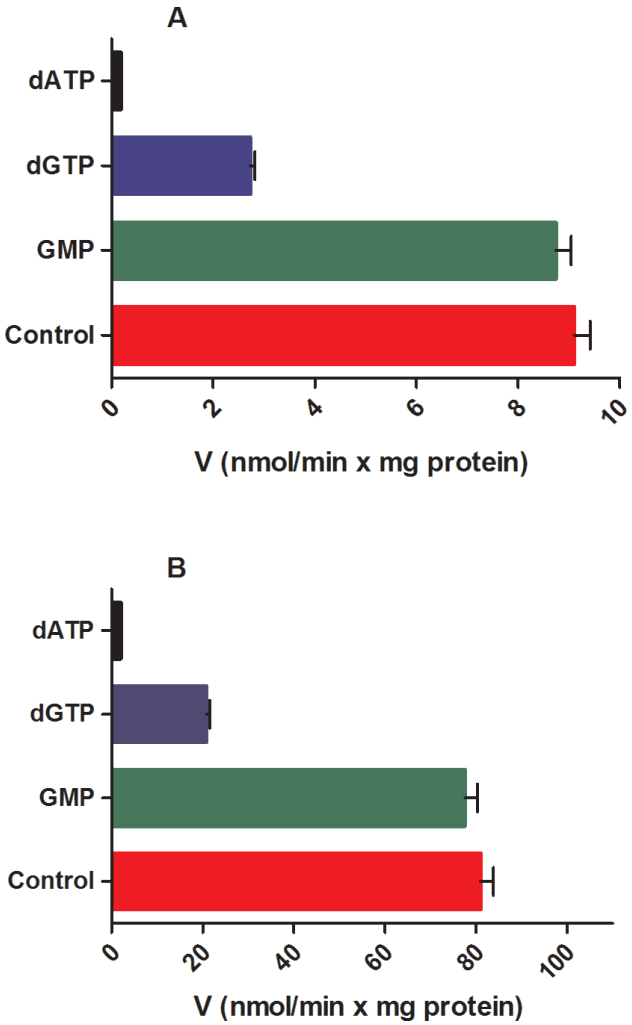
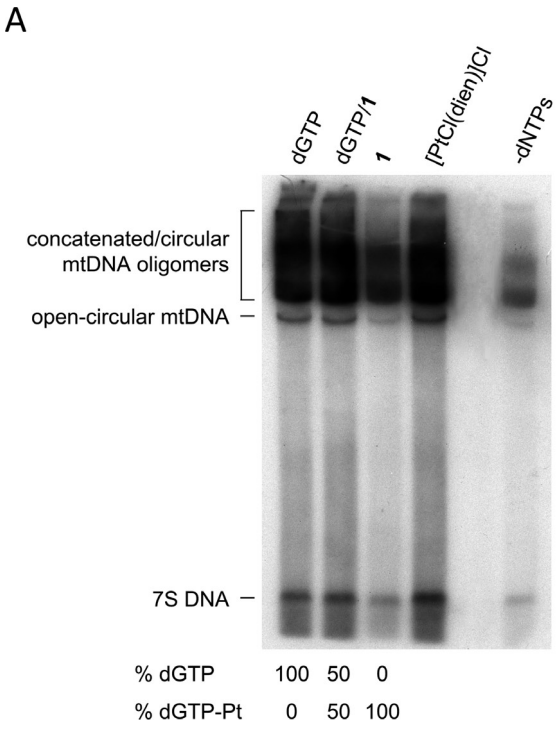


Figure 3. Effect of external substrates on the [Pt(dien)(*N7*-5′-dGTP)], 1, uptake into rat liver mitochondria (A) and in proteoliposomes reconstituited with recombinant DNC (B). (A) Freshly isolated rat liver mitochondria were incubated with 0.8 mM of platinated deoxyguanosine triphosphate together with the external substrates dATP, dGTP or GMP (concentration 1 mM). After 60 sec, reisolated and digested mitochondria were analysed for the presence of platinum by ICP-AES. (B) The assay of transport was initiated by the addition to proteoliposomes of 0.8 mM of **1** together with the external substrates dATP, dGTP or GMP (concentration 1 mM). The uptake of **1** was terminated, after 2 min, by the addition of 0.1 mM *p*-CMBS and the platinum amount into proteoliposomes were analysed by ICP-AES. The control value for uninhibited **1** uptake was: (A) 9.099 ± 0.318 nmol/min x mg protein for intact mitochondria; (B) 81 ± 2.87 nmol/min x mg protein for reconstituted recombinant DNC. Similar results were obtained in at least four independent experiments.

into the mtDNA adhering to DE81 filter paper by ICP-AES. In this assay, a mixture containing 95% of **1** and 5% of dGTP was used. Under these conditions, the introduction of platinum into the newly-synthesized mtDNA is necessarily operated by DNA polymerase γ.

Incorporation of **1** into mtDNA (measured as pmol of platinum) increased as the natural dGTP was substituted by **1** in the reaction (Figure 5). In particular, the amount of platinum incorporated into mtDNA by DNA polymerase γ was 1.0 ± 0.2



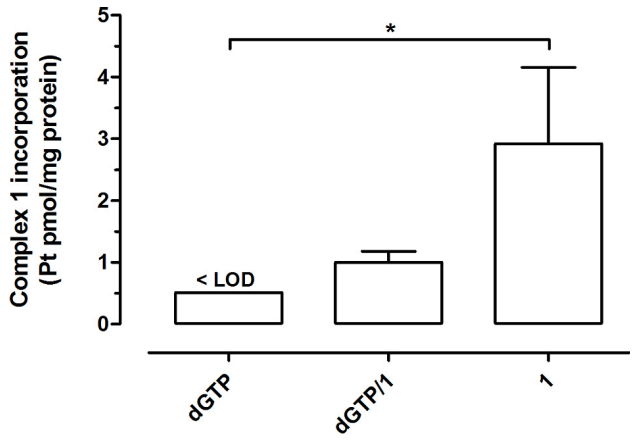


Figure 5. Incorporation of the modified nucleotide [Pt(dien)(*N7*-5′-dGTP)], 1, into DNA during in organello mtDNA replication. Platinum incorporation into mtDNA was assayed in the presence of natural dGTP, an equimolar mixture of dGTP and **1**, (dGTP/**1**) or the modified nucleotide **1**, under standard conditions. The high-molecular weight polynucleotide reaction products were captured on DE81 anion-exchanger paper, determining platinum content by ICP-AES. Modified nucleotide **1** incorporation was reported as pmol Pt/mg mitochondrial proteins. The bar graph is expressed as mean ± SEM of three independent experiments. Statistical analysis was done by one-way ANOVA followed by Bonferroni post hoc test (*$p < 0.05$). LOD: Limit Of Detection.

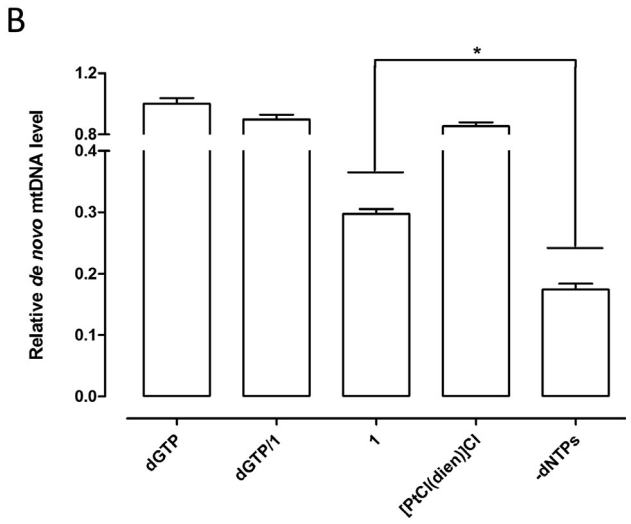


Figure 4. Effect of the modified nucleotide [Pt(dien)(*N7*-5′-dGTP)], 1, on DNA synthesis in isolate rat liver mitochondria. (A) Autoradiogram after electrophoresis through agarose slab gel of mtDNA synthesized in isolated rat liver mitochondria incubated under standard conditions. *In organello* reactions were performed in the presence of natural dGTP (positive control), a 1:1 mixture of natural dGTP/**1** or the modified nucleotide **1** (lanes dGTP, dGTP/**1** and **1**, respectively). A control reaction, was performed in the presence of [PtCl(dien)]Cl, to check its eventual nonspecific effects (lane [PtCl (dien)]Cl) on the *in organello* mtDNA synthesis. The eventual role of endogenous dNTPs on the mtDNA replication was evaluated omitting all the dNTPs, except the radioactive labeled nucleotide precursor (lane -dNTPs). (B) Densitometric quantification from the autoradiography of the radioactivity incorporated into mtDNA synthesized in intact isolated mitochondria. The labelled mtDNA intensities were quantified by using the Image-J software, available free of charge at https://imagej.nih.gov/ij/. The bar graph is expressed as mean ± SEM of three independent experiments and normalized to the control group. Statistic analysis was done by one-way ANOVA followed by Bonferroni post hoc test (*$p < 0.05$).

pmol, using a 1:1 mixture of dGTP/**1** and 2.9 ± 1.2 pmol using only platinated dGTP in the reaction (Figure 5). The platinum amount measured in mtDNA from reactions performed in the presence of only natural dGTP was below the limit of detection (LOD < 0.5 pmol; Figure 5). To verify that incorporation of **1** was mediated by DNA polymerase γ activity, the effect of temperature and inhibitors was analyzed. Incorporation of platinated nucleotides was strongly inhibited by inhibitors of DNA polymerases, such as EDTA, ethidium bromide and cold temperatures (Table 1). Overall, these results confirm the ability of DNA polymerase γ to incorporate platinated nucleotides into mtDNA.

Table 1. Effect of inhibitors and temperature on [Pt(dien)(*N7*-5′-dGTP)] (**1**), incorporation by mtDNA polymerase γ. Standard reaction conditions were 10 mM Tris-HCl, pH 7.4, 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM K_2HPO_4, 0.05 mM EDTA, 5 mM $MgCl_2$, 1 mM ADP, 10 mM glutamate, 2.5 mM malate, 1 mg of fatty acid-free bovine serum albumin (BSA) per ml, 50 μM of dATP, dGTP, dTTP and **1** at 37 °C for 4 h in a rotary shaker.

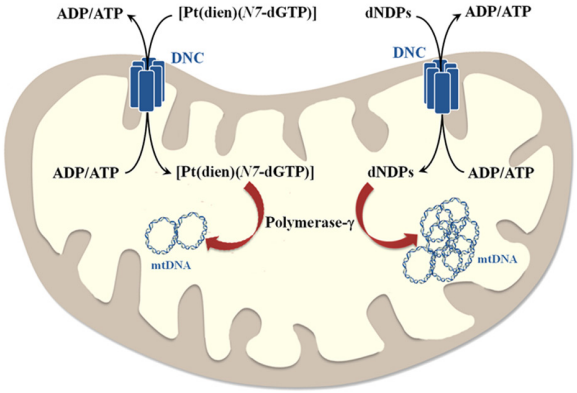
Condition	Incorporation of complex 1 pmol/mg protein	%
Standard	2.9	100
EDTA (10 mmol/L)	1.0	36.8
Ethidium bromide (100 mg/L)	1.1	38.6
4 °C	< 0.5 (LOD)[a]	< 17.5 (LOD)[a]

[a] LOD = Limit of detection

Cisplatin is among the most important antitumor agents ever developed. Nonetheless, more than a generation after its clinical introduction, the exact action mechanism of this drug is

not fully defined. The preponderance of research over the last three decades has focused on *cisplatin* direct bonds with nuclear DNA. Recently, an alternative mode of action for *cisplatin*, according to which it could also form covalent bonds with the *N7* of free cytoplasmic nucleobases, nucleosides or nucleotides, has been demonstrated.

In the present study, we report that the model complex **1** (Scheme 1) can be transported into mitochondria by DNC and incorporated into mtDNA by polymerase γ (Scheme 2). In particular, time course experiments provided evidence for a rapid and highly selective uptake of **1** into freshly isolated rat liver mitochondria and in proteoliposomes reconstituted with the purified recombinant DNC. Moreover, the competition assays proved that the import of **1** is specifically catalyzed by DNC. Finally, *in organello* mtDNA synthesis assays revealed that **1** is incorporated into mtDNA by DNA polymerase γ activity.



Scheme 2. Interaction of mitochondria with platinated nucleotides. Mitochondrial uptake of the model complex [Pt(dien)(*N7*-5′-dGTP)], **1**, operated by DNC (deoxynucleotide carrier) and incorporation of **1** into mtDNA operated by DNA polymerase γ.

The optimization of the platinated (metalated) nucleobase for therapeutic purposes, although envisaged in this paper, it is clearly far behind the scope of the present study. However, these results might have a wide range of pharmacological implications (design of new platinum based antitumor drugs), as it was suggested that the DNC and the mitochondrial polymerase γ could be directly involved in the cytotoxicity of nucleoside analogs based drugs.[16]

Supporting Information

Experimental and methodological details and Figure 1S.

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