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First evidence for *N7*-Platinated Guanosine derivatives cell uptake mediated by plasma membrane transport processes

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ABSTRACT

Nucleos(*t*)ide analogues (NA) belong to a family of compounds widely used in anticancer/antiviral treatments. They generally exhibit a cell toxicity limited by cellular uptake levels and the resulting nucleos(*t*)ides metabolism modifications, interfering with the cell machinery for nucleic acids synthesis. We previously synthesized purine nucleos(*t*)ide analogues *N7*-coordinated to a platinum centre with unaltered sugar moieties of the type: [Pt(dien)(*N7*-dGuo)]$^{2+}$ (**1**; dien = diethylenetriamine; dGuo = 2′-deoxy-guanosine), [Pt(dien)(*N7*-dGMP)] (**2**; dGMP = 5′-(2′-deoxy)-guanosine monophosphate), and [Pt(dien)(*N7*-dGTP)]$^{2-}$ (**3**; dGTP = 5′-(2′-deoxy)-guanosine triphosphate), where the indicated electric charge is calculated at physiological pH (7.4). In this work, we specifically investigated the uptake of these complexes (**1–3**) at the plasma membrane level. Specific experiments on HeLa cervical cancer cells indicated a relevant cellular uptake of the model platinated deoxynucleos(*t*)ide **1** and **3** while complex **2** appeared unable to cross the cell plasma membrane. Obtained data buttress an uptake mechanism involving Na^{+}-dependent concentrative transporters localized at the plasma membrane level. Consistently, **1** and **3** showed higher cytotoxicity with respect to complex **2** also suggesting selective possible applications as antiviral/antitumor drugs among the used model compounds.

1. Introduction

Despite the advances in medicine and science, cancer remains worldwide a major cause of death [1] and the most common treatments for cancer (consisting in the administration of chemotherapeutic agents) are often associated with serious adverse side effects and/or resistance phenomena [2,3]. For these reasons, the search for new chemotherapeutic agents is highly desired and nowadays still remains an open field of research [4]. Among the important classes of chemotherapeutic agents used in cancer treatment there are platinum-based drugs and alkylating agents, both acting in a similar way with alteration of the DNA structure [5], and the important class of nucleos(*t*)ide analogues (NA), working as antimetabolites [6]. These drugs are generally used alone or in combined therapeutic protocols involving two or more of them in a synergistic way [7,8].

Since its approval as anticancer drug in 1978, operated by the US Food and Drug Administration (FDA), cisplatin (*cis*-[$PtCl_2(NH_3)_2$]) is considered one of the most-successful inorganic compounds for anticancer chemotherapy [9]. Despite its high effectiveness, in many cases both the intrinsic or acquired resistance of some tumours to cisplatin and its severe side effects strongly limit its clinical use [10]. For these reasons, many platinum compounds have been synthesized after cisplatin in order to improve anticancer and/or pharmaceutical properties [11]. After cisplatin discovery, more than thirty new platinum complexes have entered clinical trials, but unfortunately only few of them (carboplatin, oxaliplatin, etc.) have finally been approved, and are currently used for the clinical treatment of cancer diseases [2].

Nucleos(*t*)ide analogues (NA) constitute a class of compounds currently used both as antiviral and anticancer drugs [6,12–18]. Generally, NA work as antimetabolites able to interfere with the nucleic

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acids synthesis or modify the nucleos(*t*)ides metabolism [19,20]. Indeed, anticancer NA are widely used in the treatment of different types of tumours, ranging from low-grade malignant blood disorders to highly invasive solid tumours [10,12]. However, the toxicity and lack of specificity in the cancer treatment, observed for this class of antimetabolites, highlighted the importance of understanding the complexity of NA metabolism [21–23].

New strategies concerning the merging of the chemotherapeutic properties of platinum drugs and NA were also developed. Some of the previously reported platinum complexes containing a single coordinated nucleobase showed an interesting anticancer activity both *in vitro* and *in vivo* [24,25]. Relevant examples are the platinum(II) antitumor agents, of the type *cis*-$[Pt(Am)Cl(NH_3)_2]^+$ (Am = heterocyclic *N*-donor, i.e. pyridine, pyrimidine, purine or piperidine substituent), first studied by Hollis et al. and exhibiting a good antitumor activity against Sl80, P388, and L1210 cancer cells in mice [26]. Further studies involving the Lippard's group showed that one of the action mechanisms attributable to the Hollis' compounds is related to interference with the cells metabolism consequent to the interaction with purines at the DNA level [27–29].

In recent works, we could demonstrate that the complex [Pt(dien)(*N7*-dGTP)]$^{2-}$ (**3**; dien = diethylenetriamine; dGTP = 5′-(2′-deoxy)-guanosine triphosphate), used as model complex, can be recognized by DNA polymerases and consequently incorporated into newly-synthesized DNA [30,31]. We also demonstrated that the [Pt(dien)(*N7*-dGTP)]$^{2-}$ (**3**) can actively be transported, with high affinity, by the mitochondrial membrane deoxynucleotide carrier (DNC) and then incorporated into mtDNA by action of the mitochondrial DNA polymerase ϒ [32,33]. On the other hand, [Pt(dien)(*N7*-GTP)]$^{2-}$ (GTP = 5′-guanosine triphosphate) is not incorporated by RNA polymerases into newly-synthesized RNA [34]. Moreover, there are papers showing both enhanced or decreased antiviral/antitumor activity for platinated nucleotide analogues with respect to the original drugs. Nevertheless, the observed variation in antiviral/antitumor activities resulted strongly influenced by ancillary ligands [35,36].

Drug uptake across the cell membrane is one of the pivotal steps of the mechanisms responsible for both pharmacological activity and induced resistance processes. [37] For this reason, the transport of Pt drugs (e.g. cisplatin, oxaliplatin, etc.) via mechanisms involving different sets of transporters continues to be studied [27,38–42]. The ability of platinated nucleos(*t*)ide derivatives to mimic physiological cell substrates, acting as antimetabolites into target cells, clearly depends on the availability of suitable cell membrane transporters. This work is, therefore, focused on the cellular uptake of a set of platinated deoxynucleos(*t*)ides to be considered as possible substrates for human transporters in the HeLa cervical cancer cell model.

In the present paper we have focused the investigation on platinated nucleos(*t*)ides. In this respect, a specific direct comparison between the cellular uptake of structurally similar [Pt(dien)(dGuo)]$^{2+}$ (dGuo = 2′-deoxy-guanosine), [Pt(dien)(dGMP)] (dGMP = 5′-(2′-deoxy)-guanosine monophosphate) and [Pt(dien)(dGTP)]$^{2-}$ complexes (whose electric charges are determined only by the number of guanosine bonded phosphates) has been studied and is reported. Indeed, the specificity of NA uptake by transmembrane transporters may determine their access to selected cell types and/or intracellular compartments [43]. Therefore, it cannot be excluded the direct involvement in the cell membrane crossing process of transporters suitable for the uptake of platinated nucleos(*t*)ides.

2. Experimental section

2.1. Reagents and methods

All solvents and reagents, unless otherwise stated, were purchased from Aldrich Chemical Company and used as received. [PtCl(dien)]Cl was prepared following the synthesis reported by Annibale and co-workers [44]. NMR spectra were recorded on a Bruker AVANCE III - 400 spectrometer using deuterated solvents.

2.2. Synthesis of [Pt(dien)(N7-G)] complexes, G = dGuo, dGMP, dGTP

The considered [Pt(dien)(*N7*-dGuo)]$^{2+}$ (**1**), [Pt(dien)(*N7*-dGMP)] (**2**) and [Pt(dien)(*N7*-dGTP)]$^{2-}$ (**3**) complexes were synthesized by a previously reported method [30,45]. In a typical reaction to a 10 mM D_2O solution of the [PtCl(dien)]Cl complex it was gradually added the stoichiometric amount of dGuo, dGMP or dGTP (1:1 M ratio). The progress of the reaction was monitored at ambient temperature within completion by ^{1}H NMR spectroscopy. The pH (uncorrected) was maintained in the 3–5 interval and adjusted by DNO_3 or KOD addition, as required. The final products **1**–**3** were lyophilized and redissolved in H_2O before their use in biological assays.

2.3. Cell culture and treatments

HeLa cells (human cervical adenocarcinoma epithelial cells) derived from frozen stocks purchased from the American Type Cell Culture (ATCC, Manassas, MA; ATCC® CCL-2™). In brief, cells were cultured in standard conditions according to ATCC protocols, in Dulbecco's Modified Eagle's Medium (D-MEM; Sigma-Aldrich), supplemented with FBS (fetal bovine serum; 10% *v*/v), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 ng/ml), in a water-saturated atmosphere of 5% CO_2 and 95% air at 37 °C. Cells were routinely propagated and transferred to new flasks or petri dishes, when reaching 70–80% confluence (~ every 3 days). The culture medium was replaced with fresh medium 24 h prior to experiments. For propagation, cells were detached and harvested with a 0.3% (*v*/v) trypsin solution in D-PBS (Dulbecco's Phosphate Buffer Saline). All experiments with cells were conducted between passage 3 and 6 of propagation.

2.4. MTT proliferation assay

The MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to evaluate the effects of the investigated complexes on mitochondrial activity as reference of cellular viability. Cells were seeded in 96-well plates (20×10^3 cells per well) and incubated for 24 h at 37 °C. After incubation, the medium was removed and replaced with fresh medium containing complexes at concentrations ranging from 1 to 1000 μM, obtained by serial dilutions. After 48 h treatment, MTT solution (in sterile filtered PBS, pH 7.4) was added to each well at final concentration of 0.5 mg/mL MTT, and plates were incubated at 37 °C for 3 h. The dark-blue formazan crystals were then solubilized, by cell lysis with 200 μL/well of 2-propanol/HCl 4 N, and the absorbance was measured at 550 nm with a microplate reader. Data were reported as % of control and were the mean (± SD) of eight sample replicates of each treatment, for each of three independent experiments.

2.5. Uptake studies

Uptake experiments were performed in HeLa cells 2–3 days after plating, i.e. at 80–90% confluence, on 35-mm diameter dishes. The following standard uptake procedures were adapted according to literature reported protocols [46–50].

2.5.1. Time- and dose-dependent uptake studies

Growth medium was removed, and dishes were washed three times in 1 ml of prewarmed (37 °C) sodium or choline medium (137 mM NaCl or 137 mM choline chloride, respectively; 5.4 mM KCl; 2.8 mM $CaCl_2$; 1.2 mM $MgSO_4$; 10 mM HEPES/Tris, pH 7.4, where HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and Tris = tris-hydroxymethyl- aminomethane). Uptake media (sodium or choline) were obtained by adding the platinated nucleotides and nucleosides (50 μM final concentration). Uptake was started by adding 1 ml of uptake medium (at

37 °C) to the plate and incubating for different times (t = 0, 5, 15, 30 min); finally, the uptake was blocked by removing uptake medium from the plate and by three washings in a cold (4 °C) stop solution (132 mM NaCl, 14 mM Tris/HCl, pH 7.4). Nonspecific binding was assessed by measuring the uptake at t = 0 (that is adding the uptake medium and removing it immediately, then stopping the uptake with stop solution). Cell lysates were obtained by adding 1 ml of 0.5% Triton X-100 in D-PBS (Dulbecco's phosphate-buffered saline) per dish and subsequent orbital agitation overnight. 100 μl of the final lysates were removed for protein determination according to the method of Lowry et al. [51]. The remaining lysate volume was used for platinum amount determination by ICP-AES. Pt uptake was finally expressed as picomoles per milligram of cellular protein content.

For dose-dependent uptake studies, uptake media were prepared by adding the platinated nucleotides and nucleosides with increasing concentrations of the synthesized complexes (ranging between 0.5 and 500 μM, obtained by serial dilutions for each respective sample). Uptake was started by adding 1 ml of uptake medium (at 37 °C) to each plate and incubating for 10 min, and finally terminated by removing uptake medium from the plate and by washing it three times in cold stop solution.

2.5.2. Analysis by ICP-AES

For the platinum amount determination, each sample was previously treated with 0.5 ml of 67% Suprapur® nitric acid. The samples were then diluted with Suprapur® water to a 5 ml final volume in order to obtain a suitable dilution of acid used for the mineralization process and to avoid damage to the system. Before injection into the instrument, each sample was filtered (0.45 μm) to avoid the insertion of any remaining suspension inside. The platinum concentration in analyzed samples was determined by a Thermo Scientific iCAP 6300 Duo ICP-OES spectrometer. The spectrophotometer was calibrated with a calibration line consisting of four points each one corresponding to Pt concentration of 1 μg/L, 10 μg/L, 100 μg/L and 1000 μg/L.

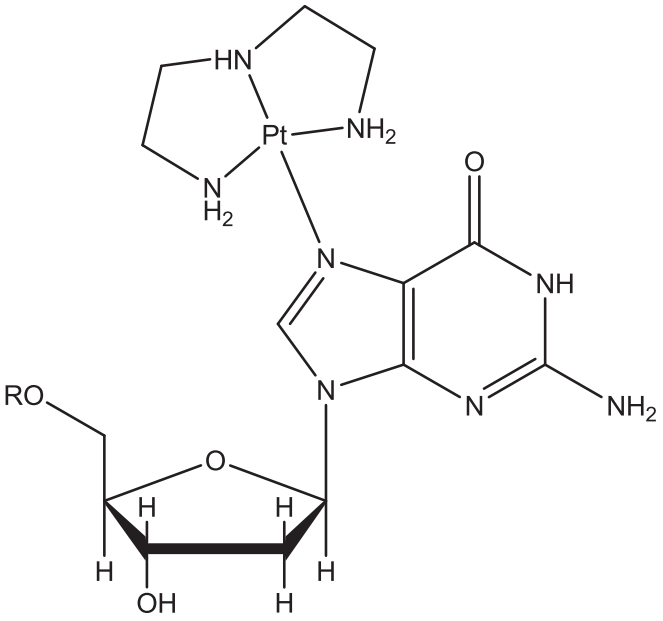
3. Results and discussion

In this work, we investigated the possible transport of three types of platinum deoxynucleos(*t*)ides: [Pt(dien)(*N7*-dGuo)]$^{2+}$ (**1**), [Pt(dien)(*N7*-dGMP)] (**2**), [Pt(dien)(*N7*-dGTP)]$^{2-}$ (**3**). The electric charge of the considered platinum complexes is pH-dependent; for this reason, we considered (and report) the electric charge expected for the species prevailing at physiological pH ≈ 7.4 in water solution, on the basis of the pK_a values known for the free nucleos(*t*)ides [52–54] (7.4 is also the pH at which our uptake experiments were performed), Scheme 1.

The uptake experiments were performed by incubating (at four increasing times) cells in sodium medium (NaCl 137 mM) or choline medium (choline chloride 137 mM), both supplemented with the synthesized [Pt(dien)(*N7*-dGuo)]$^{2+}$ (**1**), [Pt(dien)(*N7*-dGMP)] (**2**) and [Pt(dien)(*N7*-dGTP)]$^{2-}$ (**3**) complexes (50 μM). The accumulation of *N7*-platinated purines was therefore analyzed in the treated HeLa cells (by ICP-AES measure of the Pt accumulation).

The quantitative analysis of the Pt content in HeLa cells lysates assessed a strong prevalence of a Na^+-dependent component of the uptake process for the *N7*-platinated deoxynucleos(*t*)ides: [Pt(dien)(*N7*-dGuo)]$^{2+}$ (**1**), and [Pt(dien)(*N7*-dGTP)]$^{2-}$ (**3**), Fig. 1. Interestingly, the amount of transport detected for the first complex (**1**) is about ten times more pronounced than that for the second one (**3**). Moreover, in the presence of choline, no statistically significant uptake could be revealed for all the investigated complexes. Instead, the platinated nucleotide [Pt(dien)(*N7*-dGMP)] (**2**) seems to be faintly transported by HeLa cells plasma membranes, without any difference either in sodium or choline containing uptake medium.

The time course analyses for the two transported platinated complexes, [Pt(dien)(*N7*-dGuo)]$^{2+}$ (**1**) and [Pt(dien)(*N7*-dGTP)]$^{2-}$ (**3**), confirm that the uptake in HeLa cells in both cases depends on the presence of sodium in the experimental medium also showing a time-



Scheme 1. General structure of the considered platinated nucleos(*t*)ides: [Pt(dien)(*N7*-dGuo)]$^{2+}$ (**1**; R = H), [Pt(dien)(*N7*-dGMP)] (**2**; R = PO_3^{2-}) and [Pt(dien)(*N7*-dGTP)]$^{2-}$ (**3**; R = $P_3O_9^{4-}$). The electric charge of the phosphates is referred to a water solution at the 7.4 physiologic pH.

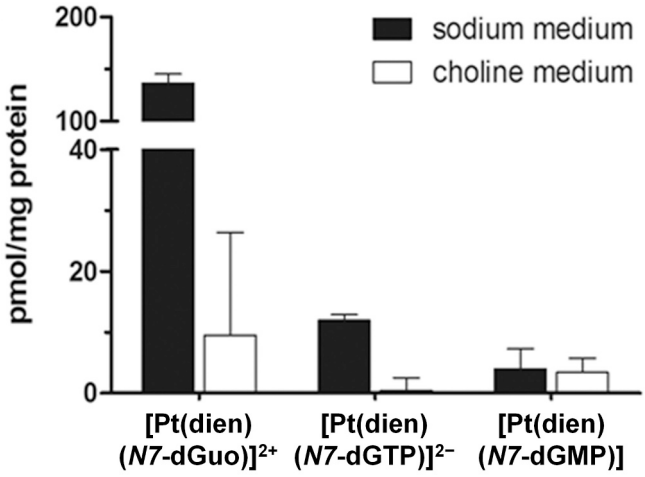


Fig. 1. Transport into HeLa cells of the newly-synthesized complexes [Pt(dien)(*N7*-dGuo)]$^{2+}$ (**1**), [Pt(dien)(*N7*-dGMP)] (**2**) and [Pt(dien)(*N7*-dGTP)]$^{2-}$ (**3**) in the presence of sodium or choline (50 μM concentration and 15 min incubation). Data points are means ± SEM (Standard Error of the Mean) of three independent determinations. Uptake in HeLa cells is expressed as picomoles of substrate/mg of cellular protein content (pmol/mg protein).

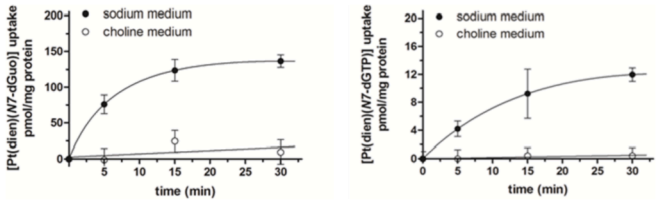


Fig. 2. Time course of [Pt(dien)(*N7*-dGuo)]$^{2+}$ (**1**), and [Pt(dien)(*N7*-dGTP)]$^{2-}$ (**3**). Data points are means ± SEM (Standard Error of the Mean) of three independent determinations.

dependent saturation kinetic trend, Fig. 2.

Dose-dependent uptake experiments were further performed. At this scope the sodium medium (NaCl 137 mM) was supplemented with increasing concentrations of the studied Pt-complexes (concentrations ranging between 0.5 and 500 μM, incubation time 5 min). The accumulation of *N*7-platinated purines was analyzed in HeLa cells by measuring Pt accumulation (by ICP-AES) after incubation of the cells with each single tested platinated deoxynucleos(*t*)ide. As shown in Fig. 3, the platinated deoxynucleoside [Pt(dien)(*N*7-dGuo)]$^{2+}$ (**1**) seems to be more abundantly accumulated vs. the respective triphosphate form [Pt(dien)(*N*7-dGTP)]$^{2-}$ (**3**) up to a concentration of 50 μM. At higher concentration (i.e. at 500 μM), higher uptake occurs for [Pt(dien)(*N*7-dGTP)]$^{2-}$ (**3**) with respect to [Pt(dien)(*N*7-dGuo)]$^{2+}$ (**1**). Nevertheless, for both complexes, data suggest an overall dose-dependent trend of uptake, as in Fig. 3. Although the uptake of platinated deoxynucleos(*t*)ides appeared to be based on a Na^+-dependent transport component, the dose-dependence data suggest that a non-saturable (diffusion) and/or Na^+-independent component might also be contributing, becoming prevalent at high concentrations (500 μM) and favorable to complex **3** over **1**, Fig. 3.

The reported results confirm that the uptake of platinated nucleotides can be mediated by specific transporters [52–59]. All cells are generally characterized by the presence of multiple transport systems where each transmembrane transporter often exhibits broad substrate specificity. Specific secondary active Na^+-dependent transporters seem to be involved in the uptake of complex **1** although, according to our observations, a secondary contribution from other kinds of (Na^+-independent) transporters might also play a role. In the case of complex **3** we observe a Na^+-dependent transport with a saturation pattern suggesting secondary active transport processes. The negligible cellular uptake observed for complex **2** suggests that the lack of charge results in the loss of involvement of the transporter-mediated mechanisms observed for complexes **1** and **3**.

In human cells a group of secondary Na^+-dependent organic cation transporters (OCTN, belonging to the SLC22 family) has been shown to be involved in the transport of nucleoside analogues [55] and, in general, both organic cation transporters (OCT) and organic anion transporters (OAT) of the SLC22 family have been claimed to mediate the transport of nucleoside analogues [56]. In addition, a member of the Na^+-dependent ascorbic acid transporters group (SLC23A4, belonging to the SLC23 family) and a member of the Na^+-independent, system-L-like amino acid transporters group (SLC43A3, belonging to the SLC43 family) have been shown to interact with nucleobases [56]. Moreover, two groups of nucleoside transporters have been discovered: the equilibrative nucleoside transporters (ENT, belonging to the SLC29 gene family) and the concentrative nucleoside transporters (CNT, belonging to the SLC28 gene family) [57]. The ENT operate the transport of nucleoside analogues according to the substrate concentration gradient formed across the plasma membrane of the cells. This concentration gradient enables the ENT-mediated Na^+-independent transport of nucleoside analogues to promote concentration equilibration across the membranes [58]. Differently, the CNT operate the uptake of nucleoside analogues against the concentration gradients in a Na^+-dependent manner [59]. It has been reported that permeant specificity, tissue distribution and cellular localization of these transporters contribute to the antineoplastic activity of the purine nucleoside analogues [60]. The uptake of nucleoside analogues is the first key step of tissue-specific nucleoside analogue-induced toxicity. In some cases, nucleoside transporters themselves were found to be directly involved in the tissue-specific nucleoside analogues toxicity [16,43,61]. Interestingly, transport results are consistent with the higher cytotoxicity of complexes **1** and **3**, with respect to complex **2**, suggesting a possible application of type **1** and **3** compounds as antiviral/antitumor drugs, Fig. 4. It should be also considered that NA antitumor/antiviral activity is generally related to the efficiency of their cellular membrane crossing and the subsequent ability to interfere with nucleobases metabolism rather than to a specific massive uptake increase [62,63]. In previous work, we studied the uptake of platinated nucleotides into rat liver mitochondria mediated by the deoxynucleotide carriers. Nucleotide transporter affinity, for [Pt(dien)(*N*7-dGTP)] complexes [32,33] clearly resulted related to the *N*7-bonded group steric hindrance. The overall observed variation (found within a four-fold range) also suggests possible usefulness of these molecular systems for pharmacologic applications. On the other hand, the present work specifically focused on the platinated nucleos(*t*)ides analogue transport, and the overall low cytotoxicity exhibited (with respect to cisplatin) by the most active compounds **1** and

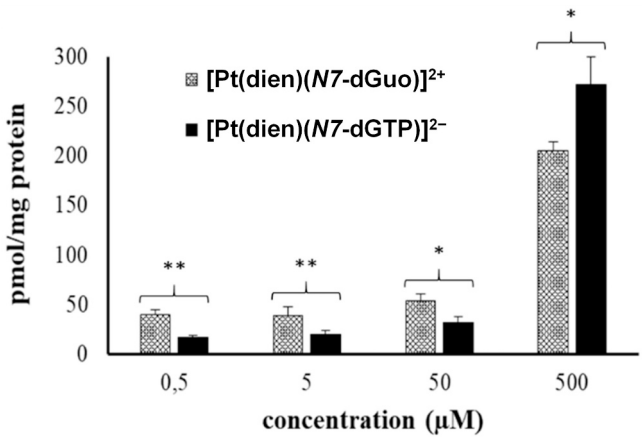


Fig. 3. Dose-dependence (0.5, 5, 50 and 500 μM concentration and 10 min incubation) of [Pt(dien)(*N*7-dGuo)]$^{2+}$ (**1**) and [Pt(dien)(*N*7-dGTP)]$^{2-}$ (**3**). Uptake in HeLa cells is expressed as picomoles of substrate normalized per milligram of cellular protein content (pmol/mg protein). Data points are the mean values ± SEM (Standard Error of the Mean) of three independent biological replicates; statistical analysis: Student *t*-test (* $p < 0,05$; ** p ⟨0,01).

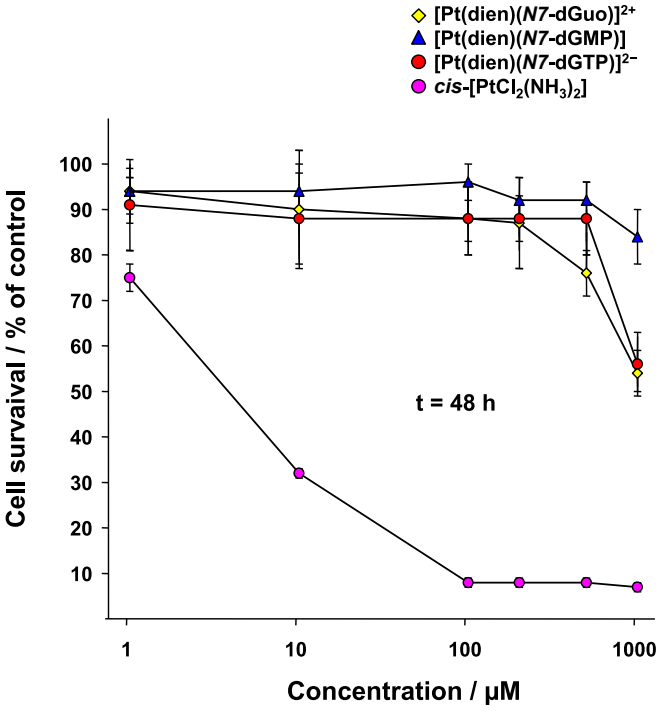


Fig. 4. HeLa cells survival measured by MTT test after 48 h incubation. The tested [Pt(dien)(*N*7-dGuo)]$^{2+}$ (**1**), [Pt(dien)(*N*7-dGMP)] (**2**), and [Pt(dien)(*N*7-dGTP)]$^{2-}$ (**3**) complexes were administered in concentrations ranging from 1 to 1000 μM. The data were the results of three different experiments ($n = 8$) presented as means ± SD.

3 clearly indicates the need of further structure modifications to improve the activity.

The possible involvement of some of the above-mentioned transporters (and possibly others) in the transport of complexes **1** and **3**, could have important implications in the side effects shown by this type of complexes if used as antiviral/antitumor drugs. Indeed, the involvement of both Na^+-dependent and Na^+-independent secondary active transporters in the eukaryotic cells and kidney detoxification subsequent to many drugs administration is well known [64]. The occurrence of carrier-mediated transport mechanisms for our molecules is even more interesting if we consider the generally lower reactivity of the here reported platinum complexes [45] compared to the commonly used platinum drugs (cisplatin, carboplatin, oxaliplatin, etc.) often depleted by unspecific interactions with targets different than DNA.

4. Conclusions

The research results reported in this work suggest that platinum coordination of nucleos(*t*)ides do not necessarily compromise their possible cellular uptake at the plasma membrane level. This is highly relevant since platinum coordinated nucleobases can be thus considered as nucleoside analogues (NA), a family of compounds widely used in anticancer/antiviral treatments.

In this work, we considered platinum *N7*-coordinated model purine nucleos(*t*)ide analogues with unaltered sugar moieties of the type [Pt(dien)(*N7*-dGuo)]$^{2+}$ (**1**), [Pt(dien)(*N7*-dGMP)] (**2**), and [Pt(dien)(*N7*-dGTP)]$^{2-}$ (**3**) where the indicated electric charge is calculated at physiological pH. For these species, which are potential good substrates for DNA polymerases (as already demonstrated for complex **3**), the mechanism of cell membrane crossing has been here investigated. The occurrence of a transporter mediated transfer at the plasma membrane level has been observed on the HeLa cervical cancer cells system, one of the cell models most commonly used for preclinical studies on human cancer diseases. As a result, we observed the most relevant cellular uptake with the electrically charged [Pt(dien)(*N7*-dGuo)]$^{2+}$ (**1**) and [Pt(dien)(*N7*-dGTP)]$^{2-}$ (**3**) platinated nucleos(*t*)ides. In particular, we could show that Na^+-dependent concentrative transporters are involved in the uptake of both type of complexes **1** and **3**, while complex **2** appears unable to cross the plasmatic cell membrane. The possible involvement of more than one carrier system in the transport of complexes **1** and **3**, respectively has been also discussed. Transport results consistency with the higher cytotoxicity exhibited by complexes **1** and **3**, with respect to complex **2**, also suggests a possible application of type **1** and **3** compounds as antiviral/antitumor drugs.

A better comprehension of the molecular mechanisms that allow cellular processing of the *N7*-platinated nucleos(*t*)ide molecules proposed in this study would be pivotal for the correct design of new platinum-based drugs with antitumor/antiviral activity aimed to the development of more effective therapeutic strategies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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