Possible Incorporation of Free *N7*-Platinated Guanines in DNA by DNA Polymerases, Relevance for the Cisplatin Mechanism of Action

Michele Benedetti, Cosimo Ducani, Danilo Migoni, Daniela Antonucci, Vita M. Vecchio, Alessandro Romano, Tiziano Verri, and Francesco P. Fanizzi

Abstract Cisplatin, *cis*-diamminedichloroplatinum (II), is one of the most widely used anticancer drugs. The main cellular target of cisplatin is DNA, where the platinum atom is able to form covalent bonds with the *N7* of purines. It is commonly accepted that there is a direct attack of cisplatin on DNA. But it should be noted that, inside cells, free purine bases, which can react with cisplatin, are also available. Free bases have many functional roles, not least the constitution of building blocks for the synthesis of new DNA and RNA molecules. For this reason, under physiological conditions, the erroneous insertion of platinated bases in the synthesized nucleic acids could compete with direct DNA/RNA platination. Moreover, due to the lower sterical hindrance offered by single nucleobases with respect to nucleic acids, platination is expected to be even easier for free purines with respect to DNA and RNA. We have recently shown, for the first time, that platinated DNA can be formed in vitro by Taq DNA polymerase promoted incorporation of platinated purines. Cytotoxicity tests with [Pt(dien)(*N7*-G)], dien = diethylenetriamine, G = 5'-dGTP, 5'-dGDP, 5'-GMP, 5'-dGMP, GUO, dGUO, complexes on HeLa cancer cells support this hypothesis of the relative cytotoxicity of [Pt(dien)(*N7*-G)] derivatives being clearly related to their bioavailability. In vivo platination of free purines before their incorporation in nucleic acids therefore opens new perspectives in platinum based antitumour drugs, for a better understanding of both the action mechanism and the new molecular design.

Keywords Cisplatin; Platinum; Purine base; DNA; RNA; Cancer; Antitumor drug

Cisplatin and other platinum-based drugs have a central role in cancer chemotherapy (1–9), especially for testicular and ovarian cancer (9, 10). However, the cisplatin

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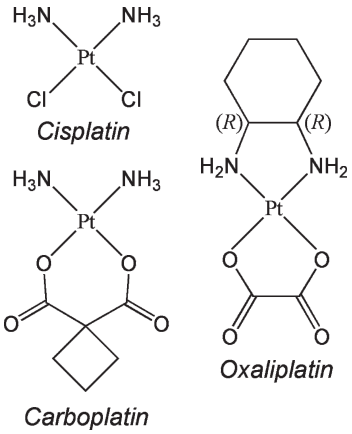

Fig. 1 Schematic representation of cisplatin, carboplatin and oxaliplatin molecular structures

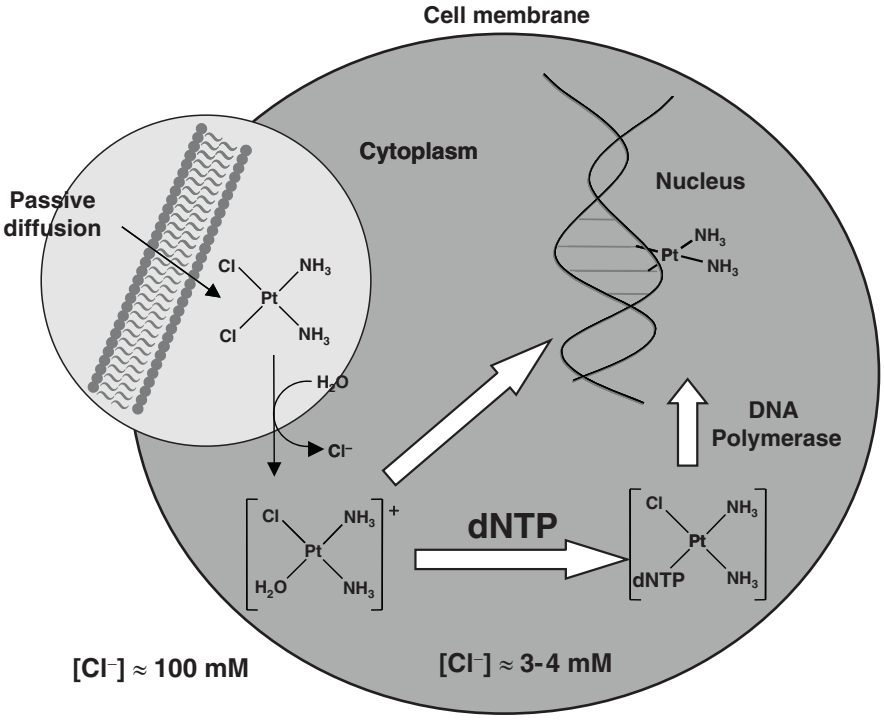

Fig. 2 Schematic representation of the mechanism of action of the cisplatin antitumor drug. Both, the mechanism of direct platination of DNA by cisplatin aquated species *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ and the newly proposed mechanism of DNA platination, mediated by platinated nucleotides and DNA polymerases, are schematized

chemotherapic use is strongly limited by serious side effects, e.g., nephrotoxicity, emetogenesis and neurotoxicity and/or acquired or intrinsic tumor resistance. In order to overcome these problems, research activity has pointed, in the last decades, to the synthesis of thousands of novel platinum compounds as potential antitumor drugs alternative to cisplatin. Unfortunately, only a few were approved for clinical use and just one, oxaliplatin, [(*R,R*)-1,2-diaminocyclohexane(oxalato-*O,O'*)platinum(II)] (Fig. 1), was found able to overcome resistance of some tumors to cisplatin (11).

Since the beginning of cisplatin related research, strong efforts have been made to rationalize the mechanism of action and the drug design. Early studies suggested that cisplatin crosses the cell membrane mainly by passive diffusion (12) and that once inside the cell, it undergoes aquation to form *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ because of the low (~3 mM) intracellular chloride concentration. The reactive aquated species could interact with DNA, which was recognized to be the primary biological target for the drug (6–8, 10, 13–22). Adducts formed with DNA are considered to be responsible for the pharmacological activity of the drug (Fig. 2).

It is known that inside the cell there are free purine bases with disparate functions, not least the constitution of building blocks for the synthesis of new DNA and RNA molecules (23). Our present working hypothesis is that there could be a mechanism of action for cisplatin, according to which free purines can be targeted by the platinum drug as well as nucleic acids. In agreement with this hypothesis, aquated cisplatin, *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$, could form a covalent bond with the *N7* of free nucleosides or nucleotides, with the formation of mono-adducts of the type *cis*-$[Pt(NH_3)_2Cl(N7\text{-Purine})]$. Because of the lower sterical hindrance around *N7*, free purines platination is expected to be even easier than platination of DNA and RNA. According to this hypothesis we thought that *N7* platinated purines could be used as a substrate for nucleic acids synthesis by DNA polymerases. If this occurs under physiological conditions, the erroneous insertion of platinated bases in the synthesized nucleic acids should compete with the direct platination process (24). Recently we demonstrated (25), for the first time, that platinated purines can be inserted into DNA, by DNA polymerases, using an in vitro synthetic process (Fig. 3).

Our experimental system was constituted by a model DNA polymerase, i.e., Taq DNA polymerase and a model platinated purine, i.e., the complex [Pt(dien)(*N7*-dGTP)] (1), dien = diethylenetriamine; dGTP = 5'-dGTP = 5'-(2'-deoxy)-guanosine triphosphate, (Fig. 4). Because of the lack of labile chloride ligands, complex 1 is unable to bind other vicinal purine nucleotides.

The competition between dGTP and [Pt(dien)(*N7*-dGTP)] (1) for incorporation into a plasmidic DNA (pUC19), by Taq DNA polymerase, was evaluated by standard PCR-based assays (25, 26). As a result we observed a lower Taq DNA polymerase efficiency in the presence of increasing amounts of complex 1, in agreement with the reported insertion of other types of *N7* modified nucleobases (26). In fact we observed that when only complex 1 is available, the Taq DNA polymerase activity is strongly reduced but not completely quenched. Our results were also consistent with the well-known concept that platinated DNA templates are able to severely repress DNA polymerase activity (27–29). The limits for the extension of our findings to other polymerases (including eukaryotic polymerases) and various platinum complexes, bearing purine bases, have still to be defined.

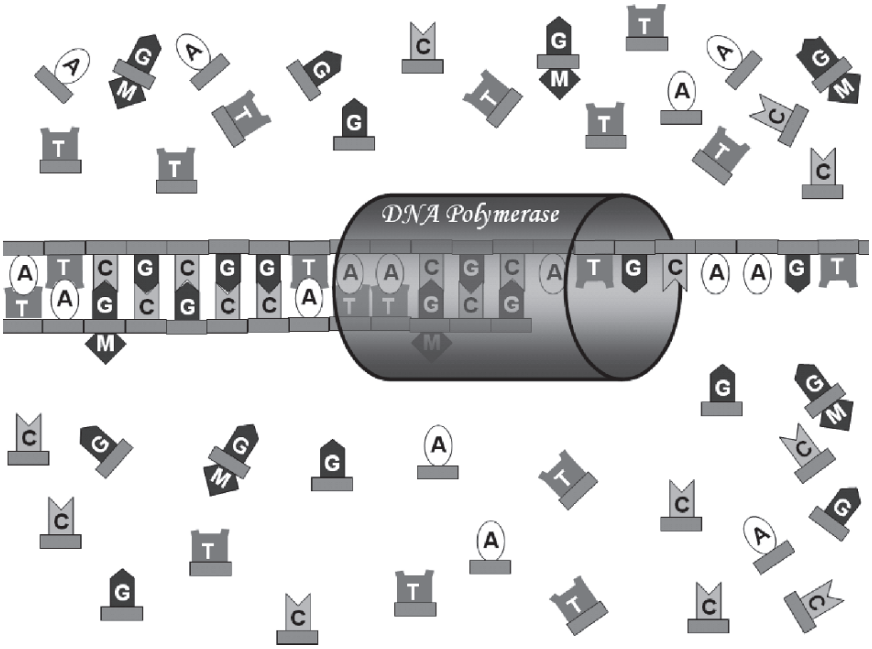

Fig. 3 Schematic representation of the insertion mechanism of platinated nucleotides operated by DNA polymerases, during the synthesis of the complementary DNA chain, in the presence of metallated guanines

We focused on the insertion of single platinated dGTP's in the newly-synthesized complementary DNA chains operated by the enzymatic activity of DNA polymerases. In this particular case, the overall yield of platination observed in *in vitro* experiments was of about 60%, showing that the insertion rate of dGTP by Taq DNA polymerase, with respect to the corresponding platinated derivative 1, is about 15 times faster. Considering that the reported minimum amount of DNA platination in human cells necessary to induce apoptosis with cisplatin, is of about 9–10 platinated nucleobases/DNA (10), even the misinsertion of very few platinated guanines could in principle, promote apoptotic pathways.

The previous findings suggested to look for a possible apoptotic pathway related to the presence of metallated primers as complex 1, in living model cells. Therefore we preliminarily evaluated the toxicological consequences of the possible incorporation of platinated purines. For this purpose, we evaluated the in vitro cytotoxicity (30), on HeLa human tumor cells, of [Pt(dien)(*N7*-5′-dGTP)] (1), [Pt(dien)(*N7*-5′-dGDP)] (2), [Pt(dien)(*N7*-5′-GMP)] (3), [Pt(dien)(*N7*-5′-dGMP)] (4), [Pt(dien)(*N7*-Guo)] (5) and [Pt(dien)(*N7*-dGuo)] (6) coordination compounds. Complexes 1–6 were prepared with a method similar to that previously reported (25). HeLa cells were grown in DMEM (Euroclone). The culture medium was supplemented with 10% heat-inactivated fetal bovine serum (Euroclone), 0.1 mg/mL streptomycin, 200 IU/mL penicillin. Cells were cultured routinely at 37°C and 5%

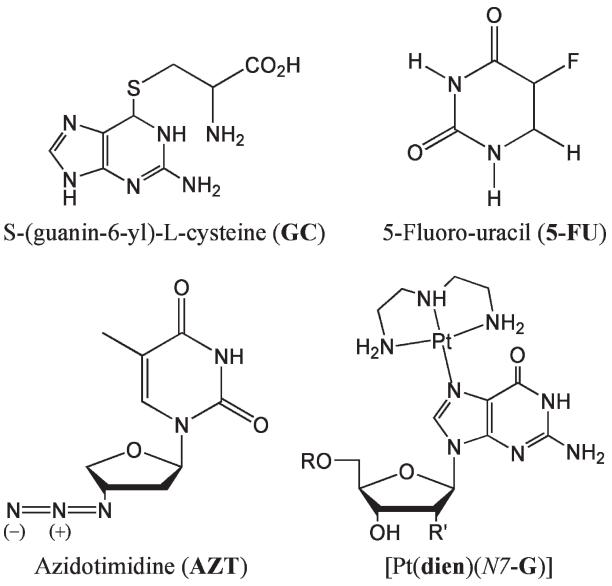

Fig. 4 Structure of antitumor and antiviral drugs or pro-drugs: *S*-(guanin-6-yl)-L-cysteine (GC), 5-fluoro-1*H*-pyrimidine-2,4-dione(5-fluorouracil,5-FU),3'-azido-2',3'-dideoxitimidine(Azidotimidine, AZT) and the complexes tested here, [Pt(dien)(*N7*-G)], G = 5'-dGTP, 5'-dGDP, 5'-GMP, 5'-dGMP, GUO, dGUO

CO_2 in a humidified incubator. Platinum containing compounds were administered to each well in appropriate concentrations ranging from 1 to 1,000 µM. The toxicity of these compounds was tested for 48 h of incubation. It should be pointed out that due to the lack of labile chlorides, all tested compounds (1–6), were not expected to exhibit high cisplatin cytotoxicity. Indeed the mono-adducts formed with DNA, after the insertion by DNA polymerases of a platinated guanine (complex 1), are unable to give bis-adducts lesions (including the well known 1, 2-intrastrand) generated by cisplatin, due to a lack of *cis* coordinating sites. For the latter reasons we focused on the evaluation of the relative toxicity of complexes 1–6 since differences among them could give new useful hints, although they were expected to behave differently than cisplatin. Results of the in vitro cytotoxicity tests are reported in Fig. 5. As expected, all tested complexes were less cytotoxic with respect to cisplatin. However their cytotoxicity, which could be observed only at the highest tested concentrations (500–1,000 µM), seems to be strongly related to the expected relative bioavailability. In particular, the transport across cell membranes (31, 32) of the possible GUO and dGUO derivatives and the direct incorporation of the dGTP derivatives into synthesized DNA could account for the higher cytotoxicity of complexes 1, 5 and 6 with respect to 2, 3 and 4.

In perspective, our results suggest a possible alternative mechanism for DNA platination in living cells, which may parallel the direct DNA platination process operated by cisplatin and its derivatives. Such a novel approach might open the possibility of

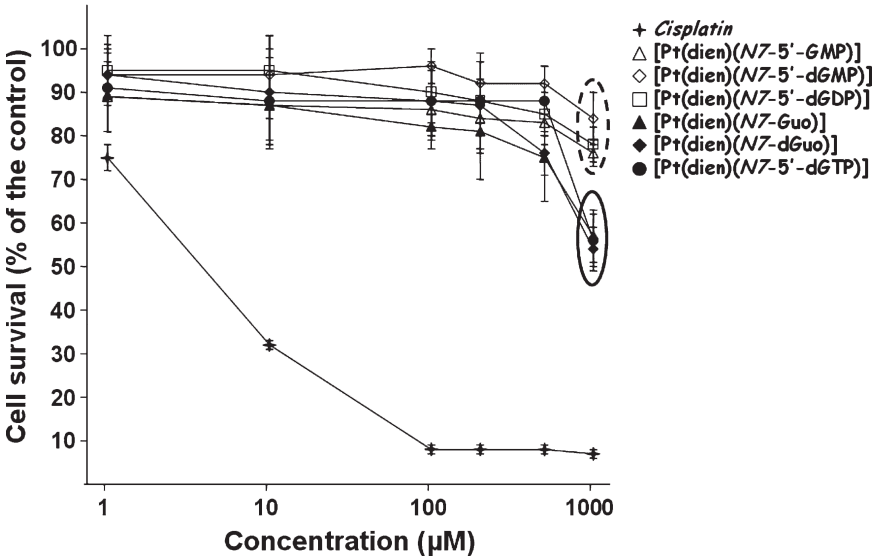

Fig. 5 HeLa cell survival measured by MTT test after 48 h of incubation. Tested complexes were administered in concentrations ranging from 1 to 1,000 μM. The data were the results of three different experiments presented as means ±*SD*

designing and developing, on a different rationale, a new generation of metal based drugs. Finally our hypothesis for the cisplatin (and analogues) mechanism of action allows the conceptual merging of these compounds (as *pro*-drugs) in the general drug/*pro*-drug class of modified DNA and RNA nucleobases (5-fluoro-uracil, 5-FU; azidotimidine, AZT; S-(guanin-6-yl)-L-cysteine, GC; ganciclovir, etc.) (23, 33) (see Fig. 4).

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