Effect of the Metal Center on the Antitumor Activity of the Analogous Dinuclear Spermine Chelates $(PdCl_2)_2$(Spermine) and $(PtCl_2)_2$(Spermine)

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Abstract: The cytotoxic activity of $(PdCl_2)_2$(spermine) against a human cancer cell line (HSC-3) was evaluated. The results show an activity higher than that of the analogous Pt(II) chelate and, for 24 h incubations, identical to that of cisplatin. They also suggest a faster intracellular uptake and DNA binding relative to cisplatin.

Keywords: Multinuclear polyamine complexes, Pt(II), Pd(II), Spermine, Cytotoxic Activity, Anticancer potential.

INTRODUCTION

Platinum coordination compounds have been playing a critical role in the chemotherapy of cancer since 1978, when cisplatin (*cis*-diamminedichloroplatinum(II)) was introduced clinically as a chemotherapeutic agent [1]. Although cisplatin is effective against different types of cancer, it presents important drawbacks, namely severe toxicity and development of resistance after continued treatment [1,2]. A tremendous amount of research effort has been dedicated to the development of cisplatin analogues with an improved therapeutic effectiveness and safety index and/or a broader spectrum of activity. However, of the thousands of mononuclear analogues that were synthesized as potential anticancer agents, only two, carboplatin and oxaliplatin, are presently widely used in the treatment of cancer, as all others failed to demonstrate improved properties [1,2]. As the anticancer properties of Pt(II)-based compounds result, at least in part, from a selective interaction of the compound with DNA, which inhibits essential cellular processes and triggers cell death [2], failure to expand the clinical spectrum of this type of agents is probably related to the high structural homology that they share with cisplatin, therefore triggering similar molecular and cellular mechanisms.

Polynuclear Pt(II) polyamine compounds comprising cisplatin-like moieties linked by alkanediamine chains have been designed to circumvent cellular resistance to cisplatin [3], and one of them, the trinuclear Pt(II) compound BBR3464, has already undergone Phase II clinical trials [4]. The presence of the linkers allows for innovative mechanisms of DNA interaction, such as “long-distance” inter- and intrastrand crosslinks within the DNA helix [5], which probably contribute to the improved antitumor efficacy exhibited by some of these complexes, namely a clear pattern of responses in cancers not normally treatable with cisplatin [6,7].

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In order to overcome cisplatin limitations, several other routes have been pursued, such as the synthesis of drugs based on palladium. A series of Pd(II) analogues of active Pt(II) complexes were tested at an early stage of the platinum structure-activity studies, but none seemed promising in view of their discrete antitumor activity, which was attributed to deactivation as a consequence of the high lability of the compounds tested [8]. However, evidence of increases in antitumor activity upon substitution of Pd(II) for Pt(II) as the metal center have been available in the literature for some time [9-11], showing that the effect of this metal center substitution depends strongly upon the system under study. Considering the significant potential of polynuclear Pt(II) polyamine compounds as anticancer drugs, it seemed interesting to evaluate the effect of substituting Pd(II) for Pt(II) on their antitumor activity. In this manuscript, we describe the cytotoxic effects towards a human cancer cell line (HSC-3) of the dinuclear Pd(II) spermine (sp) chelate $(PdCl_2)_2$(sp) (Fig. (**1**)) and compare it to that previously reported by the authors for the analogous Pt(II) chelate [12]. The results obtained confirm and extend those previously obtained with another polynuclear *cis*-dichloro(chelating diamine) system [13], *i.e.*, an increase in cytotoxic activity upon substitution of Pt(II) with Pd(II).

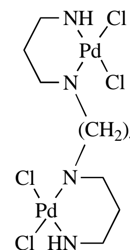

Fig. (1). Structure of complex $(PdCl_2)_2$(sp), a dinuclear complex of Pd(II) containing two *cis*-dichloropalladium(II) units bridged by the biogenic polyamine spermine (sp, $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$).

RESULTS AND DISCUSSION

The results obtained in the present study (Fig. (**2**)) show that the cytotoxic effects of $(PdCl_2)_2$(sp) against HSC-3 cul-

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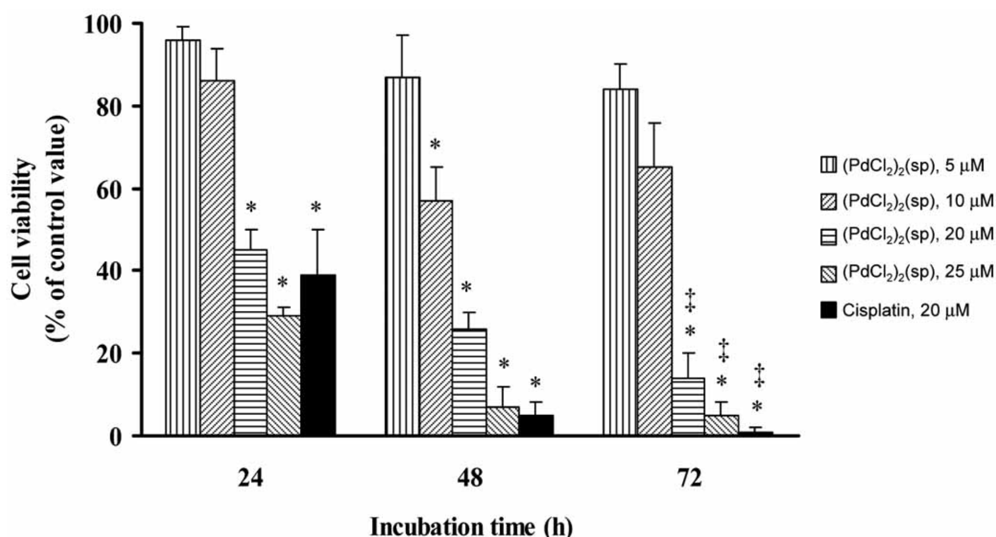

Fig. (2). Time- and dose-dependence of the effects of $(PdCl_2)_2$(sp) on the cell viability of HSC-3 cultures. For comparison purposes, the effect of 20 μM cisplatin was evaluated in parallel experiments. Values are the means of at least three independent experiments. Bars represent standard deviations. All statistical analyses were carried out using ANOVA with Tukey's Multiple Comparison test. For each incubation time, $^*P<0.05$ for values statistically different from the control value. For each treatment, $^{\ddagger}P<0.05$ for values at 24 h statistically different from those at 72 h. No statistically significant differences were observed when cultures were exposed for the same period of time to 20 μM of either cisplatin or $(PdCl_2)_2$(sp).

tures are both time- and dose-dependent. However, the observed increase in cytotoxicity over time was not very pronounced, as can be inferred from the calculated IC_{50} values for exposures to the drug of 24, 48 and 72 h (19, 14 and 13 μM, respectively). These IC_{50} values suggest an improved antitumor activity for $(PdCl_2)_2$(sp) compared to the analogous Pt(II) complex, whose evaluation was carried out by the authors in a previous study [12]. In fact, for the Pt(II) complex, and using the same cell line, the calculated IC_{50} value for 72 h exposures was 20 μM, a value identical to the one obtained in the present study for $(PdCl_2)_2$(sp) for a much shorter exposure (19 μM; 24 h). These results are in line with what we had previously found for the trinuclear $(MCl_2)_3(\text{spermidine})_2$ (M = Pd, Pt) system, i.e., an increase in cytotoxic activity upon substitution of Pd(II) for Pt(II) [13].

The significant cytotoxic potency observed for $(PdCl_2)_2$(sp) is not compatible with an extensive deactivation under physiological conditions, as was probably the case with the systems where substituting Pd(II) for Pt(II) resulted in a decrease in cytotoxic activity. In fact, although Pd(II) complexes are usually very labile, deactivation *via* *cis→trans* isomerization is unlikely. Spermine is a linear *N*-donor polydentate ligand, a type of ligand known to form extremely strong chelates with Pd(II), due precisely to a large chelate effect [14]. By remaining effectively bound to Pd(II), spermine imposes the *cis*-coordination to the labile Cl^- ligands, preventing this type of deactivation. On the contrary, deactivation due to interactions with cellular components other than DNA was a possibility, due to the high lability generally observed with Pd(II) complexes (much higher rates of ligand exchange are usually observed for Pd(II) complexes than for their Pt(II) counterparts). Indeed, if once inside the cell $(PdCl_2)_2$(sp) underwent fast hydrolysis of the chloride moiety, this would enable the resulting aquated species to interact extensively with other cellular targets (*e.g.*, thiols) before they could interact with DNA. However, it must be stressed that the rates of ligand exchange for square-planar Pd(II) complexes can span several orders of magnitude, as they are highly influenced by the interplay of the metal center and its ligands [15]. For instance, although the water exchange rate for $Pd(H_2O)_4^{2+}$ is 10^6 times higher than that of $Pt(H_2O)_4^{2+}$ [15], for other systems the Pd(II) and Pt(II) complexes can exhibit identical rates of ligand exchange [15,16]. In the case of $(PdCl_2)_2$(sp), the presence of a bulky ligand may have resulted in a significant decrease in the rate of hydrolysis for the labile chloro ligands, as it has been demonstrated for Pd(II) complexes that steric hindrance can have a remarkable effect on substitution processes [15,17].

It is also noteworthy that, although for 24 h exposures the effect of $(PdCl_2)_2$(sp) on the viability of HSC-3 cultures was analogous to that of cisplatin, for longer exposures the cytotoxicity of cisplatin was clearly superior (Fig. (**2**)). A similar finding was reported by Manzotti and co-workers [6], who observed a much less pronounced decrease in cell viability over time for cultures treated with complex BBR3464 than for those treated with cisplatin. According to these authors, this different cytotoxicity pattern may result from a faster intracellular accumulation and DNA binding for this trinuclear Pt(II) complex, compared to cisplatin. Therefore, it is possible that the mechanisms of cytotoxic activity of $(PdCl_2)_2$(sp) are distinct from those of cisplatin, an important pre-requisite to overcome the major limitations associated with cisplatin.

In order to further characterize the antitumor properties of $(PdCl_2)_2$(sp), the persistence of its cytotoxic effects on withdrawal was assessed. To this end, after a 24 h incubation period, the drug-containing medium was removed and re-

Table 1. Cell Viability of Cultures of HSC-3 Cells Exposed to Either $(PdCl_2)_2$(sp) or Cisplatin, Immediately and 48 h After the End of the Exposure

compound	cell viability of cultures exposed for 24 h to 20 µM of the compound[a] (% of control value)	
	immediately after the exposure	48 h after the exposure[b]
$(PdCl_2)_2$(sp)	44 ± 6	18 ± 10
cisplatin	24 ± 2	0 ± 1

[a]For each culture, cell viability was determined in terms of its capacity to reduce the MTT dye. Values represent means ± standard error of the means for triplicate cultures of a single experiment.
[b]At the end of the 24 h incubation period, the drug-containing medium was removed and, after washing once with PBS, cultures were grown for a further 48 h in the absence of drug.

placed with fresh medium without drug. Cultures were then incubated for a further 48 h period (hereafter referred to as the reversibility period) before their cell viability was evaluated. The same procedure was applied to control cultures and to cultures exposed to cisplatin. The results obtained (Table **1**) evidence that the viability of cultures exposed to either $(PdCl_2)_2$(sp) or cisplatin (expressed in terms of percent of control value) further decreased after withdrawal of the drug, suggesting that their cytotoxic actions were not reversible. However, as the cell density of the drug-treated cultures at the start of the reversibility period was considerably lower than that of the control cultures, a possibility existed that the further decrease in viability detected for the drug-treated cultures compared to that of the controls was the result of some type of growth disadvantage. To elucidate this point, new cultures of both control and drug-treated cells with identical cell densities were established at the end of the 24 h exposure. Again, these cultures were incubated for a further 48 h period in fresh medium before assessment of their viability. The results obtained (Table **2**) indicate, for both complex $(PdCl_2)_2$(sp) and cisplatin, that the viability of the exposed cultures further decreased after withdrawal of the drugs, confirming that their cytotoxic actions were not reversible.

Table 2. Persistence on Withdrawal of the Cytotoxic Effects of Complex $(PdCl_2)_2$(sp) and Cisplatin Against Cultures of HSC-3 Cells[a]

compound	cell viability (% of control value)
$(PdCl_2)_2$(sp) (20 µM)	68 ± 7
Cisplatin (20 µM)	0 ± 2

[a]Cell viability was determined in cultures that were established from cells exposed for 24 h to either $(PdCl_2)_2$(sp), cisplatin or the addition vehicle (control cultures). These cultures, of identical initial densities, were grown for a further 48 h in the absence of the drug (reversibility period) before their viability was evaluated using the MTT assay. Values are from a single experiment and represent means ± standard error of the means for triplicate cultures.

Finally, it was also observed that cell viability 72 h after drug addition (at a final concentration of 20 µM) was very similar for cultures that were exposed to the drugs for the whole 72 h period and for those exposed for only 24 h, followed by a 48 h incubation in the absence of the drug (Fig. (**2**) and Table **1**. For $(PdCl_2)_2$(sp): 14% and 18% of control value, respectively; for cisplatin: 0% of control value in both cases). These observations suggest that after a 24 h exposure to either drug, most cells in culture were already committed to death. However, results are from a single experiment and need confirmation.

Taken together, the results obtained in this and in our previous studies [12,13] make this type of stable polynuclear *cis*-dichloro(chelating diamine) Pd(II) complexes promising candidates for a more detailed analysis of their antitumor potential.

EXPERIMENTAL

Chemicals

$(PdCl_2)_2$(sp) was synthesized according to Codina *et al.* [18], with slight modifications. Briefly, 2 mmol of K_2PdCl_4 were dissolved in a minimal amount of water, and an aqueous solution containing 1 mmol of spermine was added dropwise under continuous stirring (stirring was kept for about 24 h). This reaction yielded a yellow powder of $(PdCl_2)_2$(sp). This compound was fully characterized by elemental analysis (including Cl and Pd), conductivity and vibrational spectroscopy (both Raman and Inelastic Neutron Scattering, coupled to theoretical calculations) [19,20]. The elemental analysis was carried out at the Microanalysis Laboratory, Chemistry Department, University of Manchester, U.K. Anal. $Pd_2C_{10}Cl_4H_{26}N_4$ (C,H,N,Cl,Pd). The conductivity results were compatible with a neutral complex and the vibrational analysis clearly evidenced the presence of the vibrational bands characteristic of this particular metal-amine chelate. All other chemicals and biochemicals were obtained from commercial suppliers. Before use, cisplatin was dissolved in saline (0.9% NaCl) and $(PdCl_2)_2$(sp) was dissolved in PBS. It was not possible to use the same addition vehicle for the two drugs, as cisplatin is very unstable in PBS, whereas $(PdCl_2)_2$(sp) is not soluble in saline.

Cell Line and Growth Conditions

HSC-3, an epithelial-like adherent human cell line from a squamous tongue epithelioma, was obtained from the Japanese Collection of Research Bioresources (JCRB 0623). Cells were cultured as monolayers, at 37 °C, in a humidified atmosphere of 5% CO_2, in DMEM-HG medium supplemented with L-glutamine (2 mM), fetal calf serum (10%)

and a mixture of penicillin (100 U/mL) and streptomycin (100 μg/mL). Under these growth conditions, the duplication time was 38 h. Cells were always in the logarithmic phase of growth.

Cytotoxicity Assays

Cytotoxic activity was evaluated in terms of capacity to inhibit the dehydrogenase activity of exposed cultures, using the MTT assay [21]. The linearity between the assay response (A_{570}) and the number of viable cells in culture was confirmed under the experimental conditions employed. For comparison purposes, control cultures (exposed to the addition vehicle only) and cultures exposed to the reference drug cisplatin were also established and evaluated in parallel. IC_{50} values, defined as the drug concentration causing a 50% reduction in dehydrogenase activity over that of untreated controls, were calculated from dose-response curves. Briefly, cultures were established in 24 well-plates in 0.5 mL of growth medium. Twenty four hours after seeding, triplicate cultures were treated for different time periods with either $(PdCl_2)_2$(sp), cisplatin or the addition vehicle. Cell viability was determined at the end of each exposure. Reversibility studies were carried out similarly, except that after a 24 h exposure to the drug, the drug-containing medium was removed and, after washing once with PBS to remove any traces of the drug, cultures were incubated for a further 48 h in the absence of the drug, before assessment of cell viability. Alternatively, the 24 h incubation was carried out in cultures grown in 25 cm^2 flasks. Afterwards, new cultures of identical densities of the control and of the drug treated cells were prepared in 24-well plates which were incubated for a further 48 h in the absence of the drug.

Statistical Analysis

The statistical significance of differences was assessed using ANOVA with Tukey's Multiple Comparison test.

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