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Cytotoxic effects of metal complexes of biogenic polyamines. I. Platinum(II) spermidine compounds: prediction of their antitumour activity

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Abstract

Cytotoxicity and cell growth inhibition studies were performed for three distinct polynuclear platinum(II) complexes of spermidine, which showed to have significant cytotoxic and antiproliferative properties on the HeLa cancer cell line. The chemical environment of the metal centres in the drugs, as well as the coordination pattern of the ligand, were found to be strongly determinant of their cytotoxic ability. In the light of the results gathered, the most effective anticancer compound among the ones tested ($IC_{50} = 5$ μM) was found to be the one displaying three difunctional ($PtCl_2N_2$) moieties ($(PtCl_2)_3(spd)_2$). Both the cytotoxic activity and the antiproliferative properties of the complexes studied showed to be irreversible for all the concentrations tested.

Keywords: Platinum complex; Spermidine; HeLa cell; Cytotoxicity; Antitumor activity

1. Introduction

Biogenic polyamines are low molecular weight polycations, present in all cells of higher organisms as well as in those of many microorganisms, where they perform vital biological functions, namely in cell proliferation and differentiation [1–5]. Due to their positive charge at physiological pH, these polyamines interact with both DNA and RNA [6], being responsible for the regulation and stabilization of the structure and activity of these biomolecules (in the case of DNA, this probably occurs through neutralization of the phosphate groups of the nucleic acid). The most common biogenic polyamines are putrescine, 1,4-diaminobutane ($H_2N(CH_2)_4NH_2$), which is the first amine in the polyamine pathway (biosynthesized from arginine), and a precursor of spermidine ($H_2N(CH_2)_3NH(CH_2)_4NH_2$) and spermine ($H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) (Fig. 1). Because of the absolute polyamine requirement for cell growth, interference with polyamine biosynthesis

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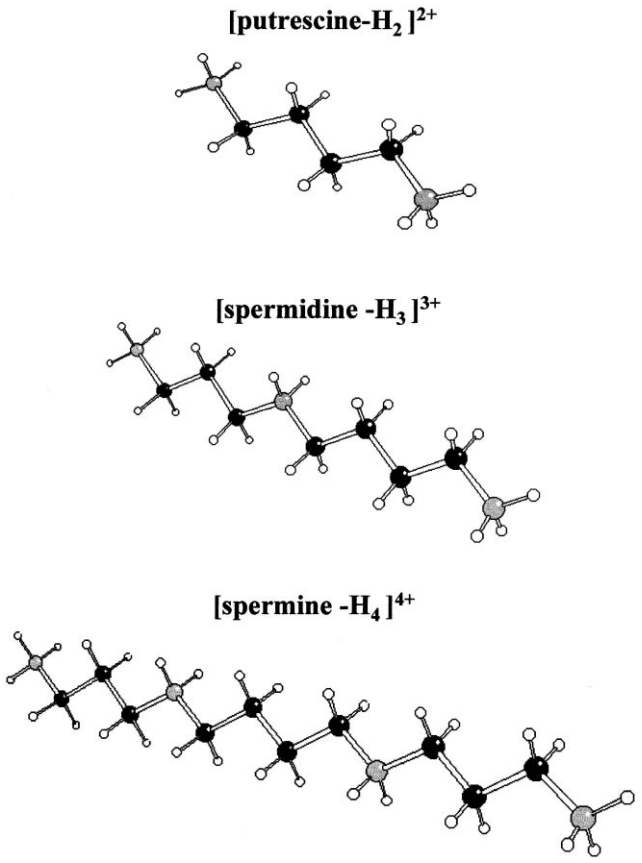


Fig. 1. Schematic representation of the protonated (cationic) forms of the biogenic polyamines putrescine (1,4-diaminobutane), spermidine and spermine. (Data obtained from theoretical calculations ([37]; Marques et al., unpublished data)).

can be a rather promising therapeutic approach against neoplastic diseases. In fact, an increased rate of those biosynthetic reactions has been unequivocally demonstrated in malignant processes [7–10]. The enzymes ornithine decarboxylase, arginine decarboxylase and polyamine oxidase, involved in the biosynthesis and metabolism of these polyamines, are also possible targets for chemotherapy.

Despite the large number of reported studies on the different functions of polyamines in living cells, the exact nature of the biochemical mechanisms through which they act is still unknown, and has lately been the target of numerous research works. Over the last decade several studies of the cytotoxic properties of platinum(II) and palladium(II) complexes of distinct polyamines have been carried out, aiming at obtaining new anticancer, third-generation drugs, displaying enhanced efficacy and tissue specificity relative to the nowadays clinically used compounds [11–16]. In fact, DNA attack by metal complexes depends strongly on the chemical nature of the compound. For instance, the widely used drug cisplatin (*cis*-diamminedichloroplatinum(II), CDDP [17]), despite its well established anticancer properties, is associated to both severe toxic side effects and the development of resistance in certain cancer cells [18], its effectiveness being thus limited to a somewhat narrow range of human tumours.

In the last few years, a new class of compounds comprising two CDDP-like Pd(II) or Pt(II) containing moieties (e.g. $[PtCl(NH_3)_2]$ or $[Pt(NH_3)Cl_2]$) linked by a variable length alkanediamine chain, have proven to display novel antitumour and DNA binding properties (inducing irreversible $B \rightarrow Z$ transitions) both in vitro and in vivo. In some cases they were even found to be active in cisplatin-resistant cell lines [19–21], and have since been the object of intense research [22–35]. In fact, such agents yield different types of DNA adducts, such as long distance intra- and interstrand cross-links not available to conventional, mononuclear metal complexes. In particular, the antiproliferative properties of compounds with spermidine and spermine as bridging units between the metal ions have been studied by several research groups, including that of Navarro-Ranninger et al. [32–35]. The group of Farrell et al., in turn, has lately reported the antitumour efficacy of polynuclear Pt(II) complexes containing diamine ($H_2N(CH_2)_nNH_2$), putrescine-like molecules acting as linkers (e.g. triplatinum BBR3464, now under preclinical development in cisplatin-resistant cell lines [16,30,31]).

In the present work, the analysis of the cytotoxic and antiproliferative properties of different polynuclear Pt(II) complexes of spermidine as a bridging linker, in a human cancer cell line was carried out. This was an interactive study, the structural characteristics of those compounds being determined through vibrational spectroscopy methods and their synthesis being adapted – namely in the number of Pt(II) ions, the length of the polyamine linker(s) between metal centres and the flexibility of these ligands – according to the results of their cytotoxicity and antiproliferative evaluation. Hopefully, in combination with further spectroscopic (Raman and FTIR) and theoretical (ab initio) studies performed

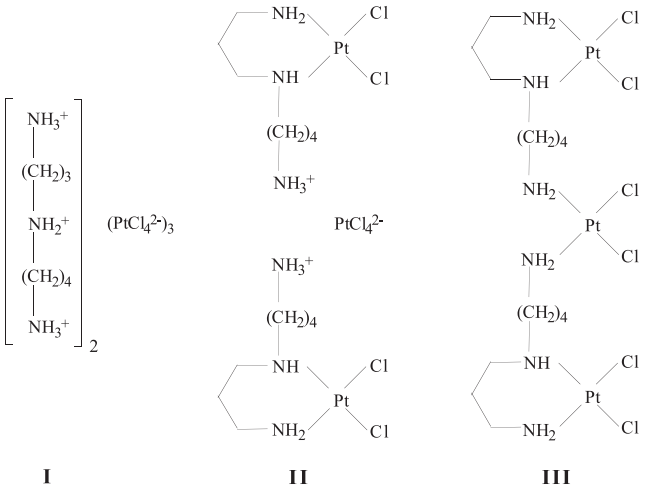
simultaneously on the same systems [36,37], new light may be shed on their structure–activity relationships at the molecular level, so that a better understanding of the biochemical mechanisms underlying their toxicity can be achieved.

2. Materials and methods

2.1. Synthesis of the Pt(II) complexes

The compounds tested were $(H_3spd)_2(PtCl_4)_3$ (I), $[PtCl_2(Hspd)]_2(PtCl_4)$ (II) and $(PtCl_2)_3(spd)_2$ (III) (spd = spermidine) (Fig. 2), synthesized according to reported methods [34]. (Anal. found for $Pt_3C_{14}Cl_{12}H_{44}N_6$: C, 12.72; H, 3.49; N, 6.37; Cl, 32.95. Anal. found for $Pt_3C_{14}Cl_8H_{40}N_6$: C, 14.27; H, 3.50; N, 7.10; Cl, 23.94. Anal. found for $Pt_3C_{14}Cl_6H_{38}N_6$: C, 15.38; H, 3.57; N, 7.80; Cl, 17.63.) (The elemental analyses were carried out at the Microanalysis Laboratory, Chemistry Department, University of Manchester and at the Butterworth Laboratory, Middlesex, UK.) Raman experiments were also carried out, aiming at obtaining further structural information on the complexes.

All compounds studied are water soluble. Solutions were prepared in concentrations ranging from 1.0×10^{-5} M to 1.0×10^{-4} M in phosphate buffered saline solution (PBS): 132.0×10^{-3} M NaCl, 4.0×10^{-3} M KCl; 1.2×10^{-3} M NaH_2PO_4; 1.4×10^{-3} M $MgCl_2$; 6.0×10^{-3} M glucose; 1.0×10^{-2} M HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[4-butanesulphonic acid]). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was prepared in a concentration of 0.5 mg/ml in PBS solution containing 1.0×10^{-3} M $CaCl_2$.



I II III

Fig. 2. Schematic representation of the Pt(II) complexes studied.

2.2. Chemicals

The HeLa cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Culture media, antibiotics, Trypan blue, phenol red, MTT, inorganic salts and acids (of analytical grade) were purchased from Sigma-Aldrich (Madrid, Spain). Fetal calf serum was obtained from Biochrom (Berlin, Germany). Cisplatin was a gift from Bristol-Myers Squibb (Portugal).

2.3. Cell culture

HeLa cells – an epithelial-like adherent cell line from human cervix adenocarcinoma – were grown in monolayers and maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagle's high glucose (4500 mg/l) medium (DMEM-HG), supplemented with 10% heat-inactivated fetal calf serum, glutamine (1.168 g/l) and antibiotics (100 units of penicillin and 100 mg streptomycin). The cells were subcultured twice a week, using a dissociation medium composed of 136.9×10^{-3} M NaCl, 2.7×10^{-3} M KCl, 8.2×10^{-3} M Na_2HPO_4, 1.5×10^{-3} M KH_2PO_4, 4.0×10^{-4} M EDTA (ethylenediaminetetraacetic acid, disodium salt, dihydrate) (pH 7.4) and containing 400 μl of a 1% (w/v) phenol red solution.

2.4. Toxicity and cell growth inhibition studies

Cytotoxicity and cell density evaluation after drug exposure – for drug concentrations between 5 and 100 μM – were assessed by cell growth inhibition assays. Cells were plated at $1–5\times10^5$ cells/ml on 12-well dishes. Twenty-four hours after seeding, drug solutions were added to the medium and the cultures were incubated at 37°C. Cells were harvested and analysed (both in controls and in drug-treated cultures) every 24 h for a total period of 3–5 days (depending on the compound used). Reversibility of the drug effect was tested by removing the drug and adding fresh culture medium on the last day of in-

cubation with the drug, and assessing the cell viability following 3 more days of incubation. Cell density and viability were determined by Trypan blue exclusion on single-cell suspensions obtained from the monolayer cultures. Cell viability was further assessed by mitochondrial dehydrogenase activity – MTT colorimetric assay. All experiments were performed in triplicate.

The MTT assay for cell injury is based on the ability of mitochondrial dehydrogenases of viable cells to reduce MTT to a purple formazan product (insoluble in water) [38] which can be quantified spectrophotometrically [39,40] (after solubilization in isopropyl alcohol containing 40 mM HCl). For each time point, the culture medium was aspirated and 1 ml of MTT solution (5 mg/ml in PBS) was added to the cells (both control and drug-treated cells) which were incubated for 4 h at 37°C. After this period, the formazan crystals formed were dissolved upon addition of 1 ml of 0.04 M HCl in isopropanol and the absorbance of these solutions was measured at 570 nm (against a blank containing MTT and HCl/isopropanol in a 1:1 ratio). The results were expressed as the percentage of MTT reduction, assuming the absorbance of control cells as 100%.

The 50% inhibitory concentration (concentration of drug required to inhibit cell growth by 50%, IC_{50}) was calculated for each drug tested from dose–response curves (for incubation periods of 48 and 72 h).

3. Results and discussion

The distinct complexes tested for both their cytotoxic and antitumour activity differ mainly in the chemical environment of the Pt(II) centres, their flex-

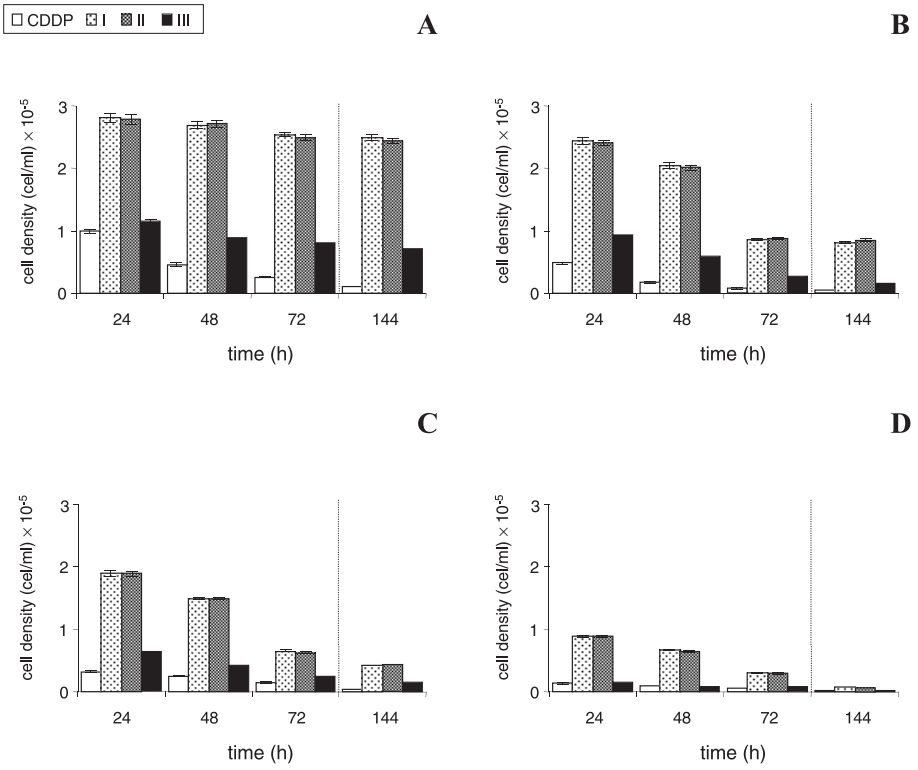


Fig. 3. Time- and dose-dependent antiproliferative effects of complexes I, II and III on HeLa cells. 3.0×10^5 cells/ml were incubated with the drugs for 24–72 h. Every 24 h, aliquots of the cell suspensions were removed and the cell density was evaluated by the Trypan blue exclusion method (as described in Section 2). In addition, the drug was removed 72 h after seeding and the cell density was assessed following a further incubation of 72 h. The data represent the average ± mean standard deviation from experiments carried out in triplicate. The following drug concentrations are represented: (A) 25 μM; (B) 50 μM; (C) 75 μM; (D) 100 μM. (Values for cisplatin are included for comparison. Cell density for the control: 24 h, 6.3×10^5 cells/ml; 48 h, 9.5×10^5 cells/ml; 72 h, 14.0×10^5 cells/ml; 144 h, 23.2×10^5 cells/ml.)

ibility and their total electric charge. Thus, while complex I is an ionic species (with a zero net charge), II and III are cationic covalent compounds, closely related except for the number of metal centres directly bound to the spermidine molecules (Fig. 2). In fact, the triamine acts as a bidentate *N*,*N*′-donor ligand – forming six-membered rings through coordination to Pt(II) – its terminal protonated amino groups being free in complex II, and bound through a third metal centre in III (which reduces significantly the motional freedom of this compound). Although both complexes II and III are difunctional in each metal centre, the former has two CDDP-like structures as opposed to III which has three, yielding (upon hydrolysis) four and six coordination sites to the DNA helix, respectively (Fig. 2). Moreover, compound III displays a longer, stretched geometry with six protonated, positively charged nitrogen atoms. It should be noted that, apart from covalent binding, both electrostatic interactions and formation of hydrogen bonds between the polyamine chain and the nucleic acid backbone play a very important role in the mechanism of complex–DNA interaction [41], being thus determinant of the cytotoxic and antiproliferative action of these compounds. Factors such as the differential transport mechanisms of the drug (e.g. across cellular membranes) are also closely associated to its cytotoxic activity and are strongly dependent on the charge of the complex as well as on the conformational characteristics of the ligand(s).

Fig. 3 represents the cell density variation as a function of the incubation time of the cells with the three distinct Pt(II) complexes tested, for different drug concentrations. Fig. 4 displays the dose-dependent cytotoxic effects on HeLa cancer cells of those compounds. By analysing these data, it is clear that only for drug concentrations of approx. 50 μM or higher may compounds I and II be considered to

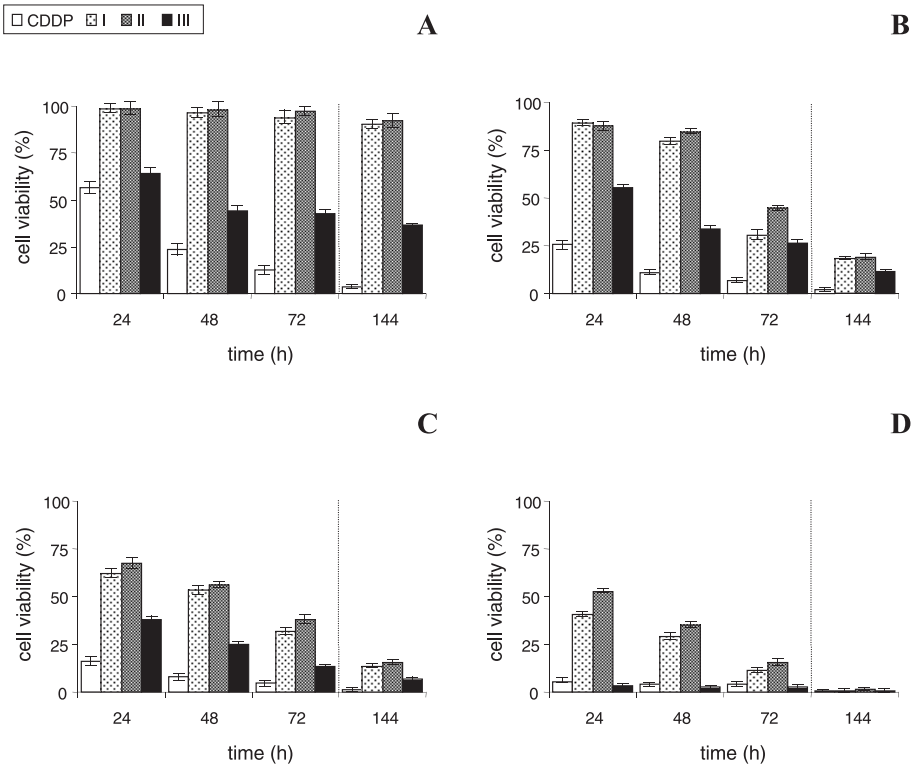


Fig. 4. Time- and dose-dependent cytotoxic effects of complexes I, II and III on HeLa cells. $1–5\times10^5$ cells/ml were incubated with the drugs for 24–72 h. Every 24 h, aliquots of the cell suspensions were removed and cell viability was evaluated by the MTT colorimetric assay (as described in Section 2). In addition, the drug was removed 72 h after seeding and the cell viability was assessed following a further incubation of 72 h. The data are expressed as a percentage of the control MTT reduction (100%) and represent the average ± mean standard deviation from experiments carried out in triplicate. The following drug concentrations are represented: (A) 25 μM; (B) 50 μM; (C) 75 μM; (D) 100 μM. (Values for cisplatin are included for comparison.)

have significant cytotoxic and antiproliferative properties, while complex III displays by far the highest cytotoxic and antitumour activity for concentrations equal to or higher than 25 μM. Experiments to evaluate both cell viability and cell density were also carried out for drug concentrations of 5–20 μM, but no cytotoxic or antiproliferative activity was detected.

These results are most probably due to the structural features of compounds I, II and III, described above, namely the fact that complex III is the only one to display three CDDP-like moieties, which understandably seems to render it much more prone to interact with the DNA helix. In the case of complex II, instead, each Pt(II) ion binds to two hydrolysable chlorine atoms and to two nitrogens of the polyamine ligand, the remaining of the (protonated) spermidine backbone being free to interact – favourably or unfavourably – with neighbouring groups in the double helix. As to compound I, its ionic nature provides it with particular features, reflected as shown in less significant antiproliferative and cytotoxic characteristics. However, despite the different chemical features of complexes I and II they display a very similar activity on HeLa cells, which seems to highlight the fact that the number of metal centres is a most important, if not determinant, factor governing the cytotoxic and antiproliferative properties of this kind of metal complexes.

Table 1 illustrates the IC_{50} values for the compounds tested. As expected from the previously discussed data, complex III displays the lowest IC_{50} value (5 μM), followed by compounds II (13 μM) and I (20 μM). In the light of these results, complexes II and III may be regarded as having potential cytotoxic and antitumour activity [42].

Table 1
Cytotoxic potency (IC_{50}) of the Pt(II) complexes on HeLa cells

Complex	IC_{50} (μM)
I	20.0
II	13.0
III	5.0
Cisplatin	2.0

IC_{50} values were calculated from dose–response curves for a 72 h drug exposure. Data were obtained through independent measurements of cell viability by the MTT assay and the Trypan blue exclusion method.

Following drug treatment, a cell line may or may not be able to restore its activity. In the case of cancer cells, the degree of reversibility of the cytotoxic and antiproliferative effects displayed by compounds aimed for use as antitumour drugs is of the utmost importance. All complexes studied showed an irreversible effect against HeLa cells for concentrations equal to or higher than 50 μM (Fig. 4). In fact, for high drug concentrations (100 μM) a drastic decrease of cell viability was observed – similar to the one detected for cisplatin – even after incubation of the cells with drug-free medium for an extra 72 h period (Fig. 4D).

The results obtained in the present work for Pt(II) complexes II and III are in good accordance with previous cytotoxicity studies carried out for the same compounds in human breast (MDA-MB468) and leukaemia (HL-60) cell lines [34]. On the contrary, compound I was found to behave as a good antiproliferative agent on MDA-MB468 and HL-60 cells [34] but not on HeLa cells – IC_{50} approx. 2.5 and 2.3 μM, respectively, vs. 20 μM obtained in the present work. In the previously reported study, however, the antitumour activity of these complexes was determined by cell density measurements only [34]. Also, no viability evaluation was performed, nor was the reversibility of the drugs tested. Based on their results, those authors proposed that the polyamine complexes induce changes in the secondary as well as in the tertiary structure of the DNA double helix (even leading, in some cases, to a shortening effect) [34].

Farrell and coworkers have also recently reported the synthesis and antitumour properties of several di- and trinuclear Pt(II) complexes with bridging diamine linkers (of varying chain lengths) [16,26,29–31], that displayed high activity both in vitro and in vivo, namely against cancer cell lines resistant to cisplatin. These covalent compounds, however, are distinct from the ones tested in the present study, since they contain two $PtCl(NH_3)_2$ moieties linked by linear polyamine groups, as opposed to complexes II and III, in which the metal ion is coordinated to two or more nitrogens from spermidine molecules. This chelate formation leads to a significant flexibility decrease and consequently a different mechanism will be involved in the interaction with DNA.

The antitumour properties of Pt(II) complexes is believed to be strongly dependent on the nature of the bonding at the metal centre, namely on the electronic character of the Pt–X (X = halogen) and Pt–ligand bonds, which underlies the relevance of the structural knowledge that can be gathered through the use of spectroscopic techniques, namely vibrational spectroscopy. The Raman pattern now observed for complexes I, II and III is fully compatible with their anticipated structures.

4. Conclusion

The spermidine Pt(II) complexes studied in the present work revealed themselves as rather efficient cytotoxic and antiproliferative drugs on the human cancer cell line HeLa. All the data presented related to these properties were gathered from two independent methods: Trypan blue exclusion for determining cell density and viability, and the MTT colorimetric assay for assessing cell viability. It was verified that compound III displayed the highest cytotoxic and antitumour efficacy on HeLa cells, with an IC_{50} value of 5 μM.

It is well known that the DNA binding profiles of the polyamine polynuclear Pt(II) complexes recently studied [28–31] are distinctly different from that of cisplatin, namely in the high percentage of long-range (Pt,Pt) interstrand cross-links (which depend on the polyamine chain length). Moreover, compounds displaying novel mechanisms of action may eventually circumvent resistance to drugs currently in clinical use (being active against cisplatin-resistant tumours).

The results obtained in this work allow us to conclude that the conformational preferences of the Pt(II) complexes, under physiological conditions, are determinant of their antitumour activity through induction of DNA structural rearrangements, namely: (i) the nature (e.g. flexibility) of the polyamine ligand(s); (ii) the characteristics of the leaving groups at the platinum (usually chloride atoms); (iii) the number and coordination mode of the metal ion(s); (iv) the distance between the metal centres within the molecule and their chemical environment (which is basically constituted by a di- or triamine and a di- or monochloro moiety). Thus, the design of new, more effective anticancer drugs should be ruled by these crucial factors, since slight changes in either the polyamine conformation or the metal coordination are sufficient to significantly modify both the cytotoxic and the antiproliferative activity of this kind of compounds on tumour cell lines. Moreover, some relevant criteria for future, third-generation, Pt(II) drugs should be considered, namely: (i) good water solubility and stability under physiological conditions; (ii) absence of undesired parallel reactions and optimized hydrolysis processes within the cell. Furthermore, tissue specificity and low toxicity towards healthy cells are also goals hopefully to be achieved in the near future. In order to obtain such kind of information regarding the complexes presently reported, comparative data are already being gathered with different human cancer cell lines as well as with normal fibroblasts.

Overall, in polynuclear Pt(II) complexes containing linear amines acting as amplifying linkers, such as the ones studied in the present work, combination of the polyamine effects – electrostatic, hydrophobic and hydrogen bond-type interactions – with the coordination abilities of the metal centre leads to a greater efficiency at inducing DNA conformational changes (e.g. B → Z transitions). The biological activity of this kind of compounds may then be systematically varied through either the ligand characteristics or the geometry of the Pt(II) coordination sphere. These studies are currently in progress in our laboratory.

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