Synthesis and evaluation of platinum complexes with potential antitumor activity

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A novel series of platinum (II) complexes was synthesized and the complexes were evaluated for their in vitro cytotoxicity against four human cancer cells lines. Five platinum complexes showed activity against at least one tumor cell line. Complexes 3 and 6 were promising, being active, at micromolar concentrations, against all the assayed tumor cell lines. Compound 3 was selected for further studies in mice with Ehrlich solid tumors and it was able to reduce the rate of tumor growth significantly during the first seven days. However, at the end of the experiments, there was no significant difference between the group of animals treated with 3 and the control group. The low solubility of the compound in the assay conditions can explain, at least in part, these results.


INTRODUCTION

Considered as a public health problem, cancer affects people of all ages, races, gender and nationality. It is the second major cause of death by disease worldwide. According to World Health Organization, 8.2 million people worldwide died from cancer in 2012 and this number is expected to rise to 13.1 million deaths in 2030 (WHO, 2015).

Cisplatin [cis-diamminedichloroplatinum (II)] is an antitumor drug used to treat different types of cancer, including testicular cancer, germ cell cancer, lymphoma, and ovarian cancer. It is one of the most widely used and effective antineoplastic agents (Gómez-Ruiz et al., 2012). However, its clinical usefulness is often limited by severe side effects, such as nephrotoxicity, ototoxicity and neurotoxicity, and development of resistance by cancer cells. The mechanism of action of cisplatin involves its interaction with DNA to form DNA adducts, primarily intrastrand crosslink adducts (Gómez-Ruiz et al., 2012; Florea, Büsselberg, 2011).

Extensive research efforts have been directed towards the development of novel platinum compounds with a broader spectrum of antitumor activity, reduced side effects and having improved pharmacokinetic properties. Besides, the design of new platinum-based anti-cancer agents might help to overcome cisplatin resistance. The search for new platinum complex is an ever present issue because of the potential of this class of compounds as antitumor agents (Hildebrandt et al., 2016; Lakomska et al., 2016; Pollak, Goddard, Porschke, 2016; Popova et al., 2016; Rehm et al., 2016; Wang et al., 2016).

Many platinum complexes have been shown to inhibit the growth of cancer cells. These compounds have, in general, at least one amino group, which is important for hydrogen-bonding to DNA (Ahmad, Isab, Ali, 2006). Furthermore, the variation of the ligands on the metal centre is a valuable strategy for modeling the physicochemical properties, increasing the uptake of platinum compounds (Graf, Lippard, 2012).

Based on the potential of platinum complexes as anticancer agents and in order to obtain new, more potent and less toxic cisplatin analogues, we set out to synthesize novel cis-diammine platinum complexes, derived from epichlohydrin or pentaerythritol (Figure 1).
The compounds were screened for their ability to inhibit the proliferation of four human cell lines representing breast (MCF-7), colorectal (HCT116), leukaemia (HL-60) and T lymphocytic (Jurkat) cancers. The effects of the different ligands on the cytotoxic activity of platinum complexes have been investigated. Among the synthesized compounds, one was selected for evaluation on mice bearing solid Ehrlich tumor.

MATERIAL AND METHODS

General procedures

Melting points were determined on a Microquimica MQAPF 301 apparatus and are uncorrected FT-IR spectra were recorded using a Perkin Elmer Spectrum One infrared spectrometer and absorptions are reported as wave numbers (cm⁻¹). NMR spectra were recorded on a Bruker Avance DPX 200 SPECTROMETER (200 MHz). Chemical shifts are given in δ (ppm) scale and J values are given in Hz. All reagents of analytical grade were obtained from commercial suppliers and used without previous purification. Reactions were monitored by TLC using silica gel coated plates and different solvent solutions as the mobile phase. The diazide derivatives 7 (Lopes et al., 2015) and 8 (Bitha et al., 1989) were synthesized according to the published procedure.

General procedure for the preparation of the diazide derivatives 9-14

A 50% w/v aqueous solution of sodium hydroxide was placed into a 50 mL round bottom flask. Then, a solution of the appropriate diazide (1 equivalent) in ethyl ether (double volume regarding the aqueous solution of sodium hydroxide) and n-tetrabutylammonium bromide (0.3 equivalent) were added to the flask. Finally, the alkyl halide (5 equivalent for the preparation of 9-12 and 10 equivalent for 13-14) was added drop-wise and the reaction mixture was stirred at room temperature until complete consumption of the starting material, as judged by TLC analysis. The reaction mixture was transferred to a separatory funnel and the organic layer was collected. The aqueous layer was extracted with ethyl ether (4 x 50 mL). The ethereal phases were combined, washed with water to pH 5, dried (anhydrous Na₂SO₄), filtered and concentrated to dryness. The crude compound was purified by column chromatography.

1,3-Diazido-2-ethoxypropane (9)

Oil, 52% yield. IR (υ/cm⁻¹): 2979, 2931 and 2877 (sp³ CH stretching), 2089 (N, stretching), 1109 (C-O stretching). ¹HNMR (200 MHz, CDCl₃), δ/ppm: 3.71-3.54 (3H, m, CH₂O and CH₂O); 3.34 (4H, d, 3J = 7 Hz, 2x CH₂N); 1.25 (3H, t, 3J = 7 Hz, CH₃). ¹³CNMR (50 MHz, CDCl₃), δ/ppm: 77.6 (CHO); 66.0 (CH₂O); 51.6 (CH₂N); 15.3 (CH₃).

1,3-Diazido-2-n-butyloxypropane (10)

Oil, 50% yield. IR (υ/cm⁻¹): 2960, 2934 and 2872 (sp³ CH stretching), 2091 (N, stretching), 1111 (C-O stretching). ¹HNMR (200 MHz, CDCl₃), δ/ppm: 3.62-3.51 (3H, m, CH₂O and CH₂O); 3.34 (4H, d, 3J = 5 Hz, 2x CH₂N); 1.67-1.49 (2H, m, CH₂); 1.46-1.31 (2H, m, CH₂); 0.93 (3H, t, 3J = 7 Hz, CH₃). ¹³CNMR (50 MHz, CDCl₃), δ/ppm: 77.8 (CHO); 70.5 (CH₂O); 52.0 (CH₂N); 31.9 (CH₃); 19.1 (CH₂); 13.8 (CH₃).

1,3-Diazido-2-octyloxypropane (11)

Oil, 63% yield. IR (υ/cm⁻¹): 2926 and 2856 (sp³ CH stretching), 2093 (N, stretching), 1111 (C-O stretching); 723 (CH₃ rocking). ¹HNMR (200 MHz, CDCl₃), δ/ppm: 3.61-3.51 (3H, m, CH₂O and CH₂O); 3.34 (4H, d, 3J = 7 Hz, 2x CH₂N); 1.65-1.56 (2H, m, CH₂); 1.45-1.15 (10H, m, CH₃); 0.88 (3H, t, 3J = 6 Hz, CH₃). ¹³CNMR (50 MHz, CDCl₃), δ/ppm: 77.8 (CHO); 70.6 (CH₂O); 51.7 (CH₂N); 31.7 (CH₂); 29.8 (CH₂); 29.3 (CH₂); 29.1 (CH₂); 25.9 (CH₂); 22.5 (CH₂); 15.3 (CH₃).

1,3-Diazido-2-benzyloxypropane (12)

Oil, 45% yield. IR (υ/cm⁻¹): 3032 (arom CH stretching), 2930 and 2869 (sp³ CH stretching), 2090 (N, stretching), 1496 and 1494 (arom C=C stretching), 1095 (C-O stretching), 737, 696 (CH bending, phenyl). ¹HNMR (200 MHz, CDCl₃), δ/ppm: 7.43 (5H, broad singlet, CH₃); 4.70 (2H, s, CH₂O); 3.71 (1H, q, 3J = 5.8, CH₂O); 3.39 (4H, d, 3J = 5.8 Hz, 2x CH₂N). ¹³CNMR (50 MHz, CDCl₃), δ/ppm: 137.2 (C ipso); 128.5 (C meta); 127.9 (C ortho); 76.9 (CHO); 72.4 (CH₂O); 51.6 (CH₂N).
2,2-Bis(2-azidomethyl)-1,3-diaminopropane (13)
Oil, 43 % yield. IR (υ/cm⁻¹): 2977, 2934, 2871 and 2804 (sp³ CH stretching), 2939 (N-H stretching), 1105 (C=O stretching). ¹HNMR (200 MHz, CDCl₃), δ/ppm: 3.46 (4H, q, J= 7Hz, CH₂N₂); 3.33 (4H, s, 2 x CH₂N₂); 3.30 (4H, s, 2 x CH₂N₂); 1.17 (6H, t, J= 7 Hz, 2 x CH₃). ¹³CNMR (50 MHz, CDCl₃), δ/ppm: 68.9 (CH₂O); 66.7 (CH₂O); 67.3 (CH₂N₂); 45.1 (quaternary C); 14.9 (CH₃).

2,2-Bis(2-azidomethyl)-1,3-bis(ethoxy)propane (14)
Oil, 35 % yield. IR (υ/cm⁻¹): 2959, 2934, 2867 and 2801 (sp³ CH stretching), 2904 (N-H stretching), 1100 (C=O stretching). ¹HNMR (200 MHz, CDCl₃), δ/ppm: 3.43-3.29 (12H, m, 4 x CH₂O, 2 x CH₂N₃); 1.58-1.29 (18H, m, 6 x CH₃N₃); 1.01 (3H, t, J= 7 Hz, 2 x CH₂O). ¹³CNMR (50 MHz, CDCl₃), δ/ppm: 71.2 (CH₂O); 69.2 (CH₂O); 66.7 (CH₂O); 51.8 (CH₂N₃); 45.1 (quaternary C); 31.4 (CH₃); 19.4 (CH₂); 13.8 (CH₃).

General procedures for the preparation of diamines 15-17 and 19-20: catalytic hydrogenation
A solution of the he appropriately alkylated diazide (1 equivalent) in methanol was placed into a 50 mL round bottom flask. Palladium 10% on carbon (40% of the weight of the diazide) was added and the heterogeneous mixture was stirred at room temperature until the complete consumption of the starting material, as judged by TLC analysis. The catalyst was removed by filtration and the solvent distilled off using a rotary evaporator. The crude compound was used in the next step without further purification.

Procedure for the preparation of diamin 18: Staudinger reduction with triphenylphosphine
A solution of diazide 12 (1 eq) and triphenylphosphine (2 eq) in tetrahydrofuran (5 mL) was added to a 50 mL round bottom flask. The reaction mixture was set aside until the complete consumption of the starting material, as judged by TLC analysis. The solvent was removed with a stream of nitrogen and the residue dissolved in HCl 1 mol L⁻¹. This solution was transferred to a separatory funnel and extracted with ethyl acetate (5 x 30 mL) to remove impurities. The acidic solution was then evaporated to dryness to furnish the diamine 18 in the form of a hygroscopic dihydrochloride.

2-Ethoxy-1,3-diaminopropane (15)
Oil, 86 % yield. IR (υ/cm⁻¹): 3400, 3290 (NH stretching), 2973, 2928, 2872 (sp³ CH stretching), 1571 (NH bending), 1082 (C=O stretching).

2-Butyloxy-1,3-diaminopropane (16)
Oil, 74 % yield. IR (υ/cm⁻¹): 3364, 3294 (NH stretching), 2957, 2932, 2868 (sp³ CH stretching), 1587 (NH bending), 1100 (C=O stretching).

2-Octyloxy-1,3-diaminopropane (17)
Oil, 95 % yield. IR (υ/cm⁻¹): 3241, 3190 (NH stretching), 2922, 2852 (sp³ CH stretching), 1585 (NH bending), 1103 (C=O stretching), 723 (CH₃ rocking).

2-Benzylxy-1,3-diaminopropane (18)
Oil, 80 % yield. IR (υ/cm⁻¹): 3400, 3290 (NH stretching), 2973, 2928, 2872 (sp³ CH stretching), 1571 (NH bending), 1082 (C=O stretching), 744, 700 (CH bending, phenyl).

2,2-Bis(2-aminoethyl)-1,3-bis(ethoxy)propane (19)
Oil, 86 % yield. IR (υ/cm⁻¹): 3373, 3270 (NH stretching), 2974, 2932, 2867 (sp³ CH stretching), 1571 (NH bending), 1102 (C=O stretching).

2,2-Bis(2-aminoethyl)-1,3-bis(butoxy)propane (20)
Oil, 89 % yield. IR (υ/cm⁻¹): 3430, 3292 (NH stretching), 2957, 2931, 2863 (sp³ CH stretching), 1579 (NH bending), 1103 (C=O stretching).

General procedure: synthesis of platinum complexes
To an aqueous solution of K₂PtCl₄ was added dropwise an equimolar amount of the appropriate diamine derivative dissolved in water. The reaction mixture was stirred for 90 minutes at room temperature, the complex derivative dissolved in water. The reaction mixture was then refluxed for 90 minutes, the complex was filtered, washed with distilled water an dried.

Complex 1
Solid, m.p. 215 °C (decomposition), 19% yield. IR (υ/cm⁻¹): 3600-3100, (NH stretching), 2970, 2850 (sp³ CH stretching), 1572 (NH bending), 1040 (C=O stretching), 485, 415 (Pt-N stretching), 347, 303 (Pt-Cl stretching).

Complex 2
Solid, m.p. 199 °C (decomposition), 55% yield. IR (υ/cm⁻¹): 3600-3100, (NH stretching), 2959, 2872 (sp³ CH stretching), 1587 (NH bending), 1106 (C=O stretching), 531, 429 (Pt-N stretching), 329, 312 (Pt-Cl stretching).

Complex 3
Solid, m.p. 194 °C (decomposition), 50% yield. IR (υ/cm⁻¹): 3600-3100, (NH stretching), 2928, 2856 (sp³ CH stretching), 1578 (NH bending), 1105 (C=O stretching), 468 (Pt-N stretching), 312 (Pt-Cl stretching).
Complex 4
Solid, m.p. 281 °C (decomposition), 12% yield. IR (ʋ/cm⁻¹): 3600-3100, (NH stretching), 2936, 2856 (sp³ CH stretching), 1571 (NH bending), 1091 (C-O stretching), 740, 698 (CH bending, phenyl), 470 (Pt-N stretching), 328, 318 (Pt-Cl stretching).

Complex 5
Solid, m.p. 211 °C (decomposition), 30% yield. IR (ʋ/cm⁻¹): 3600-3100, (NH stretching), 2974, 2932, 2871 (sp³ CH stretching), 1587 (NH bending), 1112 (C-O stretching), 472 (Pt-N stretching), 324, 312 (Pt-Cl stretching).

Complex 6
Solid, m.p. 257 °C (decomposition), 41% yield. IR (ʋ/cm⁻¹): 3600-3100, (NH stretching), 2958, 2872 (sp³ CH stretching), 1584 (NH bending), 1120 (C-O stretching), 457 (Pt-N stretching), 323 (Pt-Cl stretching).

Cytotoxicity assays. The screening assays were performed in 96 well plates pre-incubated for 24 h at 37 °C to allow adaptation of cells prior to the addition of the compounds at 50 µM. Treated and untreated cells (DMSO 0.5%) were incubated for 48 hours and compounds that inhibited the cell viability greater than 50% had the IC₅₀ values obtained graphically from concentration-effect curves using Prism 5.0 (GraphPad Software Inc.). Cisplatin was used as positive control (Mosmann, 1983).

In vivo experiments. Female Swiss albino mice weighing 25–30 g, 6 weeks old, were obtained from Faculdade de Farmácia da Universidade Federal de Minas Gerais. The animals were housed in plastic cages with free access to food and water. The protocol was approved by the Ethics Committee for Animal Experiments at the Universidade Federal de Minas Gerais (protocol 236/2013) and are in compliance with the guide for the care and use of laboratory animals recommended by the Institute of Laboratory Animal Resources.

Ehrlich solid tumor
Ehrlich ascites tumor (EAT) cells from the peritoneal cavity of Swiss mice were cultured and solid tumors were induced as previously described by us (Lages et al., 2013). Briefly, solid tumors were induced in mice by subcutaneous inoculation of 0.2 mL containing 3 × 10⁶ viable tumor cells on the left flank. After the inoculation of Ehrlich ascites tumor cells, over 90 per cent of the mice developed solid tumors. Tumor growth was determined by caliper measurement of the largest diameter and its perpendicular. Tumor size was calculated as: tumor size (mm³) = a x (b)²/2, where a: the largest diameter and; b is its perpendicular.

When the primary tumor reached a size of approximately 290 mm³ (day 0), animals were divided into three groups, each composed of 6 mice. Groups 1: control group treated only with a mixture of dimethylacetamide (DMA): PEG-300:Tween 80:saline (6:9:5:80 v/v); Group 2: group treated with complex 3 (4 mg/kg), twice a week for a period of 2 weeks; and Group 3: group treated with cisplatin (4 mg/kg). The compounds was dissolved in a mixture of DMA:PEG-300:Tween 80:saline (6:9:5:80 v/v) so that the volume to be administered to each animal was 0.1 ml. The mice were treated by intratumoral injection. At the end of the experiments, the animals were euthanized by cervical dislocation under anesthesia with a ketamine-xylazine mixture.

Statistical analysis
All data are presented as mean ± sd. Data were analyzed using the Minitab software (version 14.0). Statistical significance of differences between data was evaluated by General Linear Model ANOVA using the t student test. A value of p<0.05 was considered significant.

RESULTS AND DISCUSSION
The platinum complexes 1-6 were synthesized using the corresponding diazide derivatives 7 and 8 as key intermediates. The azido-alcohols 7 and 8 were used in the synthesis of the azido-ethers 9-14 by phase-transfer catalyzed alkylations, which, except for 12, were reduced under hydrogenation conditions in presence of Pd/C to afford the corresponding diamine derivative 15-20 in 74-95% yield. Chemoselective reduction of compound 12 was achieved following the method described by Staudinger, Meyer (1919), using triphenylphosphine. The platinum complexes 1-6 were synthesized by reaction of equimolar amount of the appropriate diamine with K₂PtCl₆ in 12-55% (Scheme 1).

The platinum complexes 1-6 were assayed in vitro at 50 µM against four human cancer cells lines MCF-7 (breast), HCT116 (colorectal), HL-60 (leukaemia) and Jurkat (T lymphocytic), using cisplatin as standard control drug. The concentrations that cause 50% inhibition of the tumor cell growth (GI₅₀) were evaluated for the more potent compounds (those presenting more than
The results are summarized in Table I.

Based on the results shown in Table I, platinum complex 1 was inactive against all of the tested tumor cell lines. All other complexes were able to inhibit more than 50% of the growth of at least one of the cancer cell lines and then their GI_{50} values were determined (IC_{50} = 4-51 µM). The human acute myeloid leukemia cell line HL60 was more sensitive to the action of the compounds. Complexes 3 and 6 showed activity comparable to cisplatin (CI_{50} = 2.1 µM) against HL-60 cells, with CI_{50} values in the low-micromolar range (6 and 4 µM, respectively). Complexes 3 and 6 can be considered the most promising compounds since they were active against all the assayed tumor cell lines, with emphasis on their cytotoxic activity against the human breast cancer cell lines MCF-7, which was resistant to the action of cisplatin. These results suggest that the presence of longer alkyl chain is favorable for the activity, perhaps by facilitating the penetration of these complexes into the tumor cells.

Based on the results of in vitro cytotoxicity assay, the compound 3 was selected for further in vivo evaluation in a mouse model bearing solid Ehrlich tumor. Solid tumor was induced in female Swiss albino mice by inoculation of Ehrlich ascites tumor cells as previously described by us (Lages et al., 2013). Due to its low aqueous solubility,
3 was dispersed in a mixture of dimethylacetamide (DMA), polyethylene glycol (PEG-300), Tween 80 and saline (6:9:5:80 v/v) and administered by intratumoral injection at a dose of 4 mg/Kg. A group of animals treated with vehicle alone (n = 6) and a group of animals treated with cisplatin at a dose of 4 mg/Kg (n = 6) were included as negative and positive controls, respectively. The mice were treated twice per week for 2 weeks.

In the first week of treatment, a marked delay in the rate of tumor growth, compared with that of the control group (p < 0.05), was observed in the groups treated with complex 3 and cisplatin. However, tumor growth increased significantly after the eighth day of treatment with 3, reaching a size similar to the control group (p > 0.05) (Figure 2). In contrast, the tumor volume of cisplatin-treated group continued to decrease with time and two animals showed complete regression of the tumor. It is noteworthy that only mice treated with cisplatin exhibited signs of toxicity, such as decreased activity, piloerection and weight loss, and two animals died before the end of the experiments.

**CONCLUSION**

In conclusion, six platinum (II) complexes have been synthesized and tested as potential antiproliferative agents against four cancer cell lines: breast (MCF-7), colorectal (HCT116), leukaemia (HL-60) and T lymphocytic (Jurkat). Five of them were found to be effective against at least one tumor cell line, at micromolar concentrations. Based on the results of in vitro cytotoxicity assay, the platinum complex 3, having an alkyl chain of 8 carbon atoms, was selected for evaluation on mice bearing solid Ehrlich tumor. This compound showed antitumor activity only in the first half of the treatment. Further studies aimed at enhancing the solubility of compound 3 in the assay conditions are needed to confirm its antitumor potential.

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