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Cis-[RuCl(BzCN)(N–N)(P–P)]PF_6 complexes: Synthesis and in vitro antitumor activity (BzCN = benzonitrile; N–N = 2,2′-bipyridine; 1,10-phenanthroline; P–P = 1,4-bis(diphenylphosphino) butane, 1,2-bis(diphenylphosphino)ethane, or 1,1′-(diphenylphosphino)ferrocene)

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ABSTRACT

The motivation to use ruthenium complexes in cancer treatment has led our research group to synthesize complexes with this metal and test them against several types of tumor cells, yielding promising results. In this paper the results of biological tests, assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, were carried out on the complexes *cis*-[RuCl(BzCN)(bipy)(dppe)]PF_6 (**1**), *cis*-[RuCl(BzCN)(bipy)(dppb)]PF_6 (**2**), *cis*-[RuCl(BzCN)(bipy)(dppf)]PF_6 (**3**) and *cis*-[RuCl(BzCN)(phen)(dppb)]PF_6 (**4**) which are described [BzCN = benzonitrile; bipy = 2,2′-bipyridine; phen = 1,10-phenanthroline; dppe = 1,2-bis(diphenylphosphino) ethane; dppb = 1,4-bis-(diphenylphosphino)butane; dppf = 1,1′-bis(diphenylphosphino)ferrocene]. The present study is focused on the cytotoxic activity of complexes (**1**)–(**4**) against four tumor cell lines and on the apoptosis and changes in the cell cycle and gene expression observed in the sarcoma 180 (S180) tumor cell line treated with complex (**1**). The results demonstrated that this complex inhibits S180 cell growth, with an IC_{50} of 17.02 ± 8.21 μM, while exhibiting lower cytotoxicity (IC_{50} = 53.73 ± 5.71 μM) towards lymphocytes (normal cells). Flow cytometry revealed that the complex inhibits the growth of tumor cells by inducing apoptosis as evidenced by an increase in the proportion of cells positive for annexin V staining and G0/G1 phase cell-cycle arrest. Further investigation showed that complex (**1**) induces a drop in the mitochondrial membrane potential and provokes a decrease in Bcl-2 protein expression and increase in caspase 3 activation, while the increased activation of caspase 8 caused a decrease in the gene expression in caspases 3 and 9. Increases in Tp53 and Bax expressions were also observed.

1. Introduction

Chemotherapy based on transition metal compounds became a promising research area after the discovery of cisplatin by Barnett Rosenberg [1]. Cisplatin is a chemotherapeutic drug used to treat carcinomas of the ovary, testis, bladder, head and neck [2]. However, because of the high toxicity, tumor resistance and other side effects of cisplatin and its derivatives, a search for more effective and less toxic antitumor metallodrugs began. In this context, ruthenium complexes are considered very promising, primarily because of their relatively low toxicity and high antitumor activity [3,4]. Ruthenium has the ability to mimic iron in its binding and transferring properties, and this is the most likely reason for its low toxicity. Such low toxicity allows the drug to be administered to the patient at a higher dose for a longer period, leading to more efficient antineoplastic chemotherapy.

NAMI, ImH[*trans*-$RuCl_4$(DMSO)(Im)] (Im = imidazole) and InH [*trans*-$RuCl_4(In)_2$] (In = indazole) have successfully completed phase II clinical trials for the treatment of metastatic tumors and colon cancers, respectively, but both have limitations as antitumor drugs [5]. Few studies have been performed on the anticancer activity of ruthenium (II) polypyridyl complexes [6–12]. Therefore, the potential use of ruthenium complexes for cancer treatment has motivated our research group to synthesize new ruthenium complexes of this class and to test them against a range of tumor cells, yielding promising results [13–19]. In

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this paper, we describe the results of biological studies of the complexes *cis*-[RuCl(BzCN)(bipy)(dppe)]PF_6 (**1**), *cis*-[RuCl(BzCN)(bipy)(dppb)]PF_6 (**2**), *cis*-[RuCl(BzCN)(bipy)(dppf)]PF_6 (**3**) and *cis*-[RuCl(BzCN)(phen)(dppb)]PF_6 (**4**) [BzCN = benzonitrile; bipy = 2,2′-bipyridine; phen = 1,10-phenanthroline; dppe = 1,2-bis(diphenylphosphino)ethane, dppb = 1,4-bis-(diphenylphosphino)butane; dppf = 1,1′-bis(diphenylphosphino)ferrocene]. Diphosphines were chosen as ligands due to their ability to stabilize Ru(II) complexes, as well as their π acceptor properties, and the diimines can assist complex intercalation with DNA. The benzonitrile ligand was introduced into the complexes to make them anionic, and thus more soluble in the cell culture medium.

Thus, considering that complex (**1**) presented better selectivity indexes, among all four complexes, against all the tumor cells studied here, it was selected for other additional biological experiments.

2. Material and methods

2.1. Chemicals

The reactions were carried out under an atmosphere of purified argon using the standard Schlenk technique. Reagent grade solvents were appropriately distilled and dried before use. All chemicals used in this study were purchased from Aldrich. The starting complexes, *cis*-[$RuCl_2$(dppb)(bipy)], *cis*-[$RuCl_2$(dppf)(bipy)], *cis*-[$RuCl_2$(dppb)(phen)] and *cis*-[RuCl(BzCN)(bipy)(dppe)]PF_6, were prepared using the methods described in the literature [19]. The syntheses of the complexes *cis*-[RuCl(BzCN)(bipy)(dppb)]PF_6, *cis*-[RuCl(BzCN)(bipy)(dppf)]PF_6 and *cis*-[RuCl(BzCN)(phen)(dppb)]PF_6 were published elsewhere [20,21].

2.2. Synthesis

The syntheses of complexes *cis*-[RuCl(BzCN)(P–P)(N–N)]PF_6 (N–N = bipy or phen, P–P = dppb or dppf, and BzCN) were performed as described in reference [21], by allowing the corresponding *cis*-[$RuCl_2$(P–P)(N–N)] precursor, dissolved in CH_2Cl_2, to react with a threefold excess of BzCN, using KPF_6, in methanol, under an argon atmosphere. Typically, *cis*-[RuCl(BzCN)(dppe)(bipy)]PF_6, was synthesized from *cis*-[$RuCl_2$(bipy)(dppe)]PF_6 (0.10 mmol), which was dissolved in 40 mL CH_2Cl_2, with an excess of benzonitrile (21 μL, 0.20 mmol). KPF_6 (27.70 mg, 0.15 mmol) was dissolved in ca. 5 mL methanol, and added to the ruthenium solution and then stirred for 24 h under an argon atmosphere. The volume of the solution was reduced to approximately 2 mL and diethyl ether (15 mL) was added to precipitate the product, which was filtered off and washed with water (2 × 10 mL) and diethyl ether (2 × 5 mL). The yield was 90%. Crystals suitable for determining the X-ray structure of the species *cis*-[RuCl(BzCN)(bipy)(dppe)]PF_6 were produced from the CH_2Cl_2/Et_2O solution, by slow evaporation. Microanalysis of *cis*-[RuCl(BzCN)(bipy)(dppe)]$PF_6 \cdot 2H_2O$: Found/calc. (%): C, 52.98/52.96; H, 4.53/4.23; N, 4.19/4.30; ^{1}H NMR data [400 MHz, $(CD_3)_2CO$]: δ 9.40 ppm (1 H, m; bipy); 8.63 ppm (1 H, d, J = 8.4 Hz; bipy); 8.49 ppm (2 H, t, J = 9.2 Hz; Ph); 8.36–8.28 ppm (2 H, m; bipy); 8.25–8.18 ppm (2 H, m; Ph); 7.86 ppm (1 H, t, J = 6.4 Hz; bipy); 7.81–7.71 ppm (1 H, bipy; 3 H, Ph, m); 7.65 ppm (1 Hp, t, J = 7.3 Hz; BzCN); 7.50–7.37 ppm (8 H, m, Ph); 7.31 ppm (2 Ho, dd, J = 8.0 and 2.4 Hz, BzCN); 7.20 ppm (1 H, m, bipy); 7.12 ppm (1 H, m, bipy); 6.97 ppm (2 Hm, dt, J = 7.8, 1.6 Hz BzCN); 6.82–6.75 ppm (2 H, t, J = 8.8; Ph); 6.68–6.62 ppm (1 H, m; Ph); and 2.95–2.79 ppm (br m, 4 H, CH_2CH_2).

The synthesis of *cis*-[RuCl(BzCN)(dppb)(bipy)]PF_6 was performed in the same way as the complex with the dppe ligand. In this case, the yield was 92%. Microanalysis of *cis*-[RuCl(BzCN)(bipy)(dppb)]$PF_6 \cdot H_2O$: Found/calc. (%): C, 54.39/54.86; H, 4.73/4.40; N, 4.45/4.26; RMN ^{1}H (400 MHz, $(CD_3)_2CO$): δ 9.05 ppm (1 H, m; bipy); 8.87 ppm (1 H, d, J = 6.0 Hz; bipy); 8.40 ppm (2 H, t, J = 8.4 Hz; Ph); 8.31 ppm (1 H, d, J = 8.0 Hz; bipy); 8.12 ppm (2 H, m; Ph); 8.00–7.85 ppm (2 H, bipy; 3 H, Ph, m); 7.73 ppm (1 H, t, J = 6.0 Hz, bipy); 7.54 ppm (1 Hp, t, J = 7.4 Hz, BzCN); 7.40–7.31 ppm (5 H, m, Ph); 7.20 ppm (2 Ho, dd, J = 7.9 Hz, BzCN); 7.08 ppm (1 H, t, J = 7.2 Hz, bipy); 7.00 ppm (1 H, t, J = 7.2 Hz, bipy); 6.77 ppm (2 Hm, dt, J = 7.6, 1.5 Hz BzCN); 6.32 ppm (2 H, t, J = 8.8; Ph); 2.40–2.00 ppm (br, m, 4 H, $CH_2(CH_2)_2CH_2$); and 1.43–1.54 ppm (br m, 4 H, $CH_2(CH_2)_2CH_2$).

2.3. Apparatus

C, H and N contents were recorded in a Fisons EA1108 Instrument CHNS/O elemental analyzer at the Microanalytical Laboratory at the Federal University of São Carlos (São Carlos, Brazil). The IR spectra of the complexes were recorded in a FTIR Bomem-Michelson 102 spectrometer in the 4000–200 cm^{-1} region, using solid samples pressed in CsI pellets. The electronic spectra were recorded in a Hewlett-Packard diode array model 8452A spectrophotometer. $^{31}P\{^1H\}$ NMR experiments were performed using a BRUKER 9.4 T spectrometer (400 MHz for hydrogen frequency), in CH_2Cl_2, using a capillary containing D_2O. The circular dichroism (CD) spectra were recorded using a JASCO 720 spectropolarimeter.

2.4. X-ray crystallography

The crystals of the isolated complexes were grown by slow evaporation of dichloromethane/diethyl ether solutions. One of these crystals was mounted in an Enraf-Nonius Kappa-CCD diffractometer with graphite monochromated Mo Kα (λ = 0.71073 Å) radiation. The final unit cell parameters were based on all reflections. Data were collected with the COLLECT program [22]. The integration and scaling of the reflections were performed using the HKL Denzo-Scalepack computer package [23]. Gaussian absorption corrections were carried out [24]. The structures were solved by direct methods with SHELXS-97 [25]. The models were refined by full-matrix least squares on F^2 by means of SHELXL-97 [25]. All hydrogen atoms were stereochemically positioned and refined with the riding model. The ORTEP molecular structure shown in Fig. 1 was prepared with ORTEP-3 for Windows [26]. The data were collected and some experimental details are summarized in Table 1.

2.5. Cell culture

S180 (mouse sarcoma), DU145 (prostate cancer), K562 (chronic myeloid leukemia) and A549 (lung cancer) cells were obtained from the Rio de Janeiro Cell Bank (RJ, Brazil). The cells were cultured in RPMI 1640 or DMEM medium (pH 7.2–7.4), supplemented with 100 μm L^{-1} penicillin G, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine, 1.5 g L^{-1} sodium bicarbonate, 10 mM HEPES [4-(2-hydroxyethyl)1-piperazineethanesulfonic acid] and 10% fetal bovine serum (FBS) (all reagents were obtained from Gibco, Grand Island, NY) at 37 °C under a 5% CO_2, humidified atmosphere. Human peripheral blood mononuclear cells (PBMCs) were collected from healthy volunteers aged 20–30 years with no history of smoking, drinking or chronic drug use. The PBMCs were isolated by density gradient centrifugation of heparinized blood on Lymphoprep (Nycomed, Oslo, Norway), washed three times with Hank's balanced salt solution (Sigma Chemical, St. Louis, MO, USA), counted, suspended in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Gibco), and incubated (37 °C, 5% CO_2) for 24 h before the drug treatment [27].

The protocol (043/2007) for these experiments was approved by the Ethics Committee of the Federal University of Goiás and, prior to joining the study, all blood donors signed an informed consent form.

2.6. Cytotoxicity assays

The effects of the complexes on the viability of S180, DU145, K562, A549 and lymphocyte cells were studied using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described previously [28]. In brief, 1.0×10^5 S180, DU145, K562, A549 or lymphocyte cells were plated in 96-well tissue culture plates and treated with