Anticancer kiteplatin pyrophosphate derivatives show unexpected target selectivity for DNA

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Highlights

- Kiteplatin {[$PtCl_2$(*cis*-1,4-diaminocyclohexane)} is a potent antitumor drug.
- Derivatization of kiteplatin with the pyrophosphate ligand potentiates its activity.
- DNA was previously excluded as the target of phosphaplatins in contrast to cisplatin.
- Intriguingly, DNA is a target for the activity of kiteplatin pyrophosphate analogs.

Abstract

Background: One of the promising new antitumor platinum complexes is a large-ring chelate complex [$PtCl_2$(*cis*-1,4-DACH)] (DACH = diaminocyclohexane) (kiteplatin). Recently, new platinum(II) derivatives of kiteplatin with pyrophosphate as carrier ligand were synthesized and tested on a panel of human cancer cell lines. These derivatives of kiteplatin were found to be more effective than clinically used anticancer platinum drugs. The design of kiteplatin pyrophosphate derivatives came out from the concept of pyrophosphate coordinated platinum(II), phosphaplatin. Phosphaplatins were shown to function without binding to DNA so that DNA has been excluded as the target of phosphaplatins in contrast to conventional antitumor platinum drugs.
Methods: Cytotoxicity, major cellular target and DNA interactions of the new anticancer platinum drug were characterized by the standard biochemical methods and methods of molecular and cellular biology.
Results: We demonstrate that, in contrast to what has been reported on closely related phosphaplatins, the derivatives of kiteplatin with the pyrophosphate carrier ligand are activated in the cellular environment. This activation, which yields species capable of platination of DNA, very likely comprises the hydrolytic release of the pyrophosphate ligand(s) that could be enzymatically catalyzed.
Conclusions: Collectively, these data provide convincing evidence that DNA is an important target for the biological activity of the kiteplatin pyrophosphate derivatives, although the overall mechanism of action might be different from those of conventional platinum drugs.
General Significance: We demonstrate that unexpectedly DNA appears to be an important target of the biological action of new anticancer derivatives of kiteplatin with the pyrophosphate carrier ligand.

Keywords

Cytotoxicity; Platinum pyrophosphate derivatives; DNA; Real-time cell electronic sensing; Comet assay; Mutagenicity

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Abbreviations

1, [Pt(dihydrogen pyrophosphate)(*cis*-1,4-DACH)]; **2**, [{Pt(*cis*-1,4-DACH)}$_2$(pyrophosphate)]; 6-TG, 6-thioguanine; bp, base pair; cisplatin, *cis*-diamminedichloridoplatinum(II); CT, calf thymus; DMEM, Dulbecco's Modified Eagle's Medium; FAAS, flameless atomic absorption spectrometry; FBS, fetal bovine serum; HPRT, hypoxanthine phosphoribosyl transferase; IC_{50}, concentration of compound which causes death in 50% of cells; ICP-MS, inductively coupled plasma mass spectrometry; LB, luria broth; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NER, nucleotide excision repair; r_b, number of molecules of platinum complex bound per nucleotide residue; r_i, molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA; RTCA, real-time cell electronic sensing

1. Introduction

The conventional platinum anticancer agents, cisplatin and its derivatives, are widely used in the clinic [1]. The mechanism of action of these platinum drugs is connected mainly with binding to and damaging DNA, forming intrastrand and interstrand cross-links [2, 3]. Platinum adducts distort the conformation of DNA [4, 5] inhibiting DNA and RNA synthesis and if not repaired, induce programmed cell death [6]. However, the conventional platinum drugs are not effective in many types of human cancer due to inherent or acquired resistance to the chemotherapy by these drugs. These drawbacks along with side effects limit the clinical use of platinum-based therapies.

The search for more effective platinum anticancer drugs led to design, synthesis and preclinical testing of hundreds of new platinum complexes. One of the promising new platinum complexes is the large-ring chelate complex [$PtCl_2$(*cis*-1,4-DACH)] (DACH = diaminocyclohexane) (kiteplatin) which has been tested for cytotoxicity in tumor cell lines and antitumor activity in solid human tumors [7, 8]. Notably, this complex containing *cis*-1,4-DACH ligand, an isomeric form of the carrier ligand present in oxaliplatin, exhibits a different and broader spectrum of anticancer activity as compared to cisplatin and oxaliplatin being able to overcome resistance to these platinum cytostatics. Interestingly, this different biological activity occurs despite the fact that the DNA binding mode of kiteplatin is similar to that of cisplatin, although the DNA adducts of [$PtCl_2$(*cis*-1,4-DACH)] inhibit more markedly the DNA polymerase and lower slightly more the efficiency of DNA repair systems [9, 10]. In pursuit of improving the antitumor properties of kiteplatin, new platinum(II) derivatives of kiteplatin with pyrophosphate as carrier ligand, one mononuclear (**1**) and one dinuclear (**2**) (Fig. 1), have been synthesized and tested on a panel of human cancer cell lines [11]. These derivatives, in particular the dinuclear complex, were found to be more effective than cisplatin and oxaliplatin.

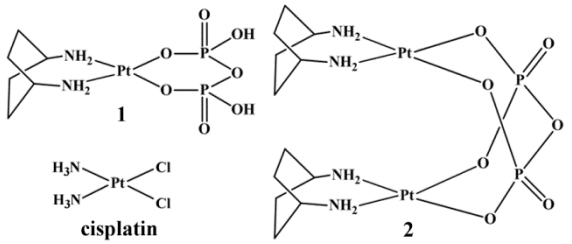

Fig. 1. Molecular structures of [Pt(dihydrogen pyrophosphate)(*cis*-1,4-DACH)] (**1**), [{Pt(*cis*-1,4-DACH)}$_2$(pyrophosphate)] (**2**) and cisplatin.

The design of kiteplatin pyrophosphate derivatives **1** and **2** came out from the concept, already introduced by Bose et al. [12, 13], of improving the activity of conventional cisplatin by substitution of pyrophosphate for the two chlorido ligands (pyrophosphate derivatives were collectively named phosphaplatins). In fact mononuclear complex **1** is an isomer of Bose's [Pt(dihydrogen pyrophosphato)(1*R*,2*R*-DACH)] (pyrodach-2) which contains 1,4-DACH instead of 1,2-DACH in pyrodach-2. Phoshaplatin and its derivatives exhibit antitumor properties against a variety of cancers [12] and strikingly, the results of the studies aimed at understanding the mechanism of action were interpreted to mean that phosphaplatins function without binding to DNA [12, 13]. Unlike phosphaplatins, complexes **1** and **2** were shown to undergo slow hydrolysis at physiological pH which becomes faster in more acidic media typical for cancerous cells, releasing active [$PtCl_2$(cis-1,4-DACH)] (or its solvato species) capable of binding to a monomeric constituent of RNA, GMP [11]. An important step in

understanding of the mechanism of action of antitumor agents is the determination of their biological target(s). In the present work we applied several experimental criteria [3] to demonstrate that in contrast to what has been reported on phosphaplatins, complexes **1** and **2** bind to polymeric DNA in cell-free media and even in the cells treated with these kiteplatin pyrophosphate derivatives and DNA appears to be an important target of their biological action.

2. Experimental

2.1. Chemicals

Commercial reagent grade chemicals, solvents and reagents for the preparation of solutions for biological work were used as received from commercial suppliers without further purification. Calf thymus (CT) DNA (42% G + C, mean molecular mass ca. 2×10^7) was prepared and characterized as previously described [14, 15]. Plasmid pPUC19 [2686 base pairs (bp)] was isolated according to standard procedures. Restriction endonuclease EcoRI was purchased from New England Biolabs. Agarose was obtained from Merck KgaA (Darmstadt, Germany). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was from Calbiochem (Darmstadt, Germany). [α-^{32}P]ATP was obtained from MP Biomedicals, LLC (Irvine, CA). RNase A was from Qiagen (USA). DNazol (genomic DNA isolation reagent) was obtained from MRC (Cincinnati, OH). Cisplatin was from Sigma-Aldrich (Prague, Czech Republic). Complexes **1** and **2** were synthesized and characterized as described in the previously published article [11].

2.2. Cell lines

The human ovarian carcinoma cells A2780 (ECACC) were cultured in RPMI-1640 medium supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Biosera LTD, Ringmer, U. K.) and gentamycin (50 μg mL^{-1}, Serva). The CHO-K1 and MMC-2 (Chinese hamster ovary cells) were grown in DMEM medium (high glucose, 4.5 g L^{-1}, Biosera) supplemented with gentamycin (50 μg mL^{-1}, Serva) and 10% heat-inactivated fetal bovine serum (Biosera). The A2780 cells were kindly supplied by prof. Keppler (Vienna, Austria), the CHO-K1 cell line (wild type) and its mutant cell line MMC-2 were kindly supplied by Dr. M. Pirsel, Cancer Research Institute, Slovak Academy of Sciences (Bratislava, Slovakia). The cells were cultured in a humidified incubator at 37 °C in a 5% CO_2 atmosphere and subcultured 2-3 times a week with an appropriate plating density. *Escherichia coli* (CCM 7929) was obtained from the Czech Collection of Microorganisms (CCM), Masaryk University, Faculty of Science, Brno, Czech Republic as a freeze-dried pellet. The cells were rehydrated and propagated on Luria broth (LB) agar plates according to the supplier protocol. Microbiological experiments were performed under standard sterile conditions.

2.3. Interaction of platinum complexes with cells monitored by real-time cell electronic sensing (RTCA)

RTCA equipment (xCELLigence RTCA SP, Roche) was calibrated for the background impedance with 100 μL of fresh RPMI-1640 medium. A2780 cells were added at the density of 8000 cells/well and incubated for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. Subsequently, the cells were treated with the platinum compounds, controls were treated with an adequate amount of solvent (MilliQ water) used to dissolve platinum compounds. Cell-sensor impedance called as cell index is defined as $(R_t - R_b)/15$, where R_t is the impedance of the cells at defined time points and R_b is the background impedance of the culture medium. Impedance was monitored every 5 min for all the time during the experiment. Cell index was

normalized at the time of treatment (24 h). Each sample was plated on the E-plate as quadruplicate. The concentrations of platinum in the media used for treatment were verified by flameless atomic absorption spectrometry (FAAS).

2.4. Cytotoxicity in DNA repair proficient and deficient cells

Chinese hamster ovary cells CHO-K1 (wild type) and its derivative MMC-2 carrying ERCC3/XPB mutation (NER-deficient) were plated out 16 h prior to testing in 96-well tissue culture plates at a density of $6x10^3$ cells per well in 100 µL of DMEM medium supplemented with 10% FBS and 50 µg L^{-1} gentamycin. The cells were then treated for 72 h with the compounds at the final concentrations in the range of 0 to 128 µM in a final volume of 200 µL per well (37 °C, 5% CO_2, humidified). After the treatment period, the viability of the cells was tested using MTT assay as already described [16]. Briefly, after the incubation period, 10 µL of a freshly diluted MTT solution (2.5 mg mL^{-1}) was added to each well, and the plate was incubated at 37 °C in a humidified 5% CO_2 atmosphere for 4 h. The medium was then removed and the resulting formazan product was dissolved in 100 µL of DMSO. Cell viability was evaluated by measurement of the absorbance at 570 nm (reference wavelength at 630 nm), using an Absorbance Reader Sunrise Tecan Schoeller. The reading values were converted to the percentage of control (% cell survival). IC_{50} values (compound concentrations that produce 50% of cell growth inhibition) were calculated from curves constructed by plotting cell survival (%) versus drug concentration (µM). All experiments were done in triplicate.

2.5. DNA platination in cells

The level of platinum from **1**, **2** and cisplatin associated with DNA was measured in CHO-K1, MMC-2 and A2780 cells. The cells were seeded in 150 cm^2 culture flasks at a density $1x10^7$ cells/flask (67 000 cells/cm^2). After 48-h incubation, the cells were treated with the compounds (10 µM) for 24 h. The attached cells were washed twice with PBS (4 °C). Cells were then lysed in DNAzol (DNAzol® genomic DNA isolation reagent, MRC) supplemented with RNAse A (100 µg mL^{-1}). The genomic DNA was precipitated from the lysate with ethanol, dried and resuspended in water. The DNA content in each sample was determined by UV spectrophotometry. The DNA samples were digested in the presence of hydrochloric acid (11 M) using high-pressure microwave mineralization system (MARS5, CEM). The platinum content was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Three independent experiments were performed in triplicate and the values are the means ± SD.

2.6. Effect of platinum complexes on bacterial morphology

The ability of platinum complexes **1, 2** and cisplatin to induce filamentous growth of bacteria has been studied as already described [17, 18]. Briefly, a colony of *E. coli* (CCM 7929) was picked and grown to saturation in LB following overnight incubation at 37 °C. A 100 µL aliquot of this suspension was added to 6 mL of LB which was incubated at 37 °C until the absorbance of a sample measured at a wavelength of 600 nm (OD_{600}) of the solution reached 0.5. Aliquots of this suspension, 5 µL each, were added to 1 mL of LB containing either 30 µM of compound **1**, **2** or cisplatin or no platinum. The mixtures were incubated at 37 °C. After 5 h, a 5 µL drop of each cell suspension was mounted on a separate microscope slide and imaged using microscope Olympus CXK 41 equipped with digital camera and photographed.

2.7. DNA modifications in cell-free media

CT DNA (64 µg mL^{-1}) was incubated with the platinum complexes in $NaClO_4$ (10 mM, pH 6) at 37 °C in the dark so that r_i was 0.05 (r_i is defined as the molar ratio of free platinum

complex to nucleotide phosphates at the onset of incubation with DNA). Aliquots were withdrawn at various time intervals, quickly cooled to -20°C, and exhaustively dialyzed against water. The platinum content in these samples was determined by FAAS, while the concentration of DNA was determined by absorption spectrophotometry.

2.8. Preparation of cell extract

A2780 cells were grown to confluence on 100 mm Petri dishes, washed three times with ice-cold PBS, scraped and centrifuged for 10 min at 0 °C (1000 g). The pellet was resuspended in the lysis buffer [Tris·HCl (10 mM, pH 8.0), KCl (60 mM), EDTA (1.2 mM), DTT (1 mM), PMSF (0.1 mM), NP-40 (0.05%)] for 10 min on ice and centrifuged for 10 min at 0 °C (1200g). The protein concentration of the cytosolic extract was determined by the Bradford assay, and stored at −80 °C until used.

2.9. DNA interstrand cross-linking in cell-free media

0.5 μg of a linearized plasmid DNA pUC19/EcoRI (2686 bp) was incubated with varying concentrations of platinum complexes for 24 h at 37 °C. The linear DNA was first 3′-end labeled by means of the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dATP. The resulting samples were analyzed for DNA interstrand cross-links by previously published procedures [19, 20]. The number of interstrand cross-links was analyzed by electrophoresis under denaturing conditions on an alkaline agarose gel (1%). After the electrophoresis had been completed, the intensities of the bands corresponding to single strands of DNA and interstrand cross-linked duplex were quantified. The frequency of interstrand cross-links was calculated as % ICL/Pt = XL/5372rb (the DNA fragment contained 5372 nucleotide residues), where % ICL/Pt is the number of interstrand cross-links per adduct multiplied by 100, and XL is the number of interstrand cross-links per molecule of the linearized DNA duplex and was calculated assuming a Poisson distribution of the interstrand cross-links as XL = −ln A, where A is the fraction of molecules running as a band corresponding to the single-stranded, non-cross-linked DNA.

2.10. Single cell gel electrophoresis

The A2780 cells were treated with **1**, **2** or cisplatin for 2 h. After the treatment, cells were subjected to 50 μM H_2O_2 (4 °C, 5 min) and immediately processed for the comet assay [21, 22]. Nuclei were stained with ethidium bromide (ex. λ_{max} = 285 nm, em. λ_{max} = 605 nm) and visualized using IX81 motorized inverted research microscope CellR (Olympus) equipped with DSU (Disk Scanning Unit) and digital monochrome CCD camera CCD-ORCA/ER. Fifty randomly selected nuclei from each slide were analyzed. Slides were coded, and blinded analysis was performed. The percent of DNA in the comet tail was evaluated as a measure of DNA migration. Experiments were performed two times independently (each in duplicate), and data points represent mean values ± SD.

2.11. Mutagenicity testing

The HPRT/V79 mutation assay was used for detection of 6-thioguanin (6-TG) resistant colonies in V79 cells in accordance with Robichova et al. with some modifications [23]. Briefly, V79 cells were seeded on 100 mm Petri dishes at a density of $5x10^5$ cells/dish in duplicate for each sample and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 48 h. Cells were exposed to platinum complexes at the concentration of $5x10^{-6}$ M for 2 h. After treatment, the cells were trypsinized and plated ($3.5x10^5$ cells, in triplicate per sample) for further cultivation. For colony forming ability (CFA) the cells were plated on 60 mm Petri dishes ($3 x10^2$ cells, in triplicate per sample). On the seventh day after treatment, the cells were stained with methylene blue and the number of colonies was counted. From the ratio of

the number of colonies and the number of cells plated, the percentage of CFA was calculated. The cells for further cultivation were kept by regular subculture at a certain cell density to avoid overcrowding. Each sample was processed as follows: the cells were plated: (i) on five Petri dishes (10 mm) at a density of $2x10^5$ cells/dish in pentaplicate per sample for detection of 6-TG resistant mutations; after the cells were attached, the selective agent 6-TG was added in the final concentration of 37 x 10^{-6} M; (ii) on three Petri dishes (60 mm) at a density of 3 x 10^2 cells in triplicate per sample for estimation of viability of the cells. On the seventh day of expression, the percentage of CFA was calculated. Colonies of mutations were stained with methylene blue and counted 10 days after adding 6-TG. The yield of 6-TG resistant mutations was calculated. The results were statistically evaluated by Student t-test

2.12. Other physical methods

Absorption spectra were measured with a Beckman 7400 DU spectrophotometer equipped with a thermoelectrically controlled cell holder. The FAAS measurements were carried out on a Varian AA240Z Zeeman atomic absorption spectrometer equipped with a GTA 120 graphite tube atomizer. The analysis with the aid of ICP-MS was performed using Agilent 7500 (Agilent, Japan). The gels were visualized on a BAS 2500 FUJIFILM bioimaging analyzer, and the radioactivity associated with bands was quantified with the AIDA image analyzer software (Raytest, Germany). Statistical evaluation of the experiments was carried out using Student's t-test.

3. Results and discussion

3.1. Cytotoxicity

The cytotoxic activity of **1**, **2** and cisplatin was determined against human ovarian carcinoma cell line A2780 (cisplatin sensitive). The cells were incubated and treated with the compounds as described in Materials and methods and treated with the compounds at 37 °C for additional 24 or 72 h. Cell viability was evaluated after the treatment time of 24 or 72 h and cytotoxic effects were expressed as IC_{50}. The data in Table 1 confirm that in the early phases of the treatment [after short (24 h) incubation of A2780 cells with the platinum complexes], **1** and **2** are markedly more effective than cisplatin, whereas the cytotoxic effect of cisplatin is manifested mostly after long period (72 h) of incubation.

Table 1
Toxicity of **1**, **2** and cisplatin [IC_{50} Mean Values (μM)] in A2780 cells[a]

	24 h	**72 h**
cisplatin	41 ± 2	4.27 ± 0.09
1	5.92 ± 0.08	2.66 ± 0.06
2	3.34 ± 0.06	1.296 ± 0.003

[a]The experiments were performed in triplicate. The results are expressed as mean values ± SD of three independent experiments, each of them made in quadruplicate.

3.2. Impedance-based time-dependent cell response profiling

It has been shown [24-27] that impedance-based monitoring of cellular responses to biologically active small molecule compounds produces TCRPs (time-dependent cell response profiles), which can be predictive of the mechanism of action of small molecules. Therefore, this method has been used to characterize cellular responses to **1** and **2** and the results were compared with those obtained for cisplatin. In agreement with the previously published data, the TCRPs obtained for cisplatin were characterized by an initial slight

increase in the cell index above the level of control followed by a decrease below the control level [26, 27] (Fig. 2). Interestingly, the shape of TCRPs determined for compounds **1** and **2** at equitoxic concentrations was identical to that obtained for cisplatin (Fig. 2A) suggesting that the character of cellular responses to **1** and **2** is similar to that to cisplatin. The TCRPs were also recorded for the three platinum complexes at equimolar concentrations (Fig. 2B) which apparently reflect their different cytotoxic efficiency, **2** being more cytotoxic than **1** (Table 1 and Ref. [11]). The TCRPs of **1**, **2** and cisplatin (Fig. 2) coclustered with the TCRPs of DNA-damaging compounds interfering with DNA synthesis, replication and transcription [24, 26]. These results underscore the ability of **1** and **2** to damage DNA in the tumor cells treated with these complexes and involvement of DNA damage in their mechanism of action.

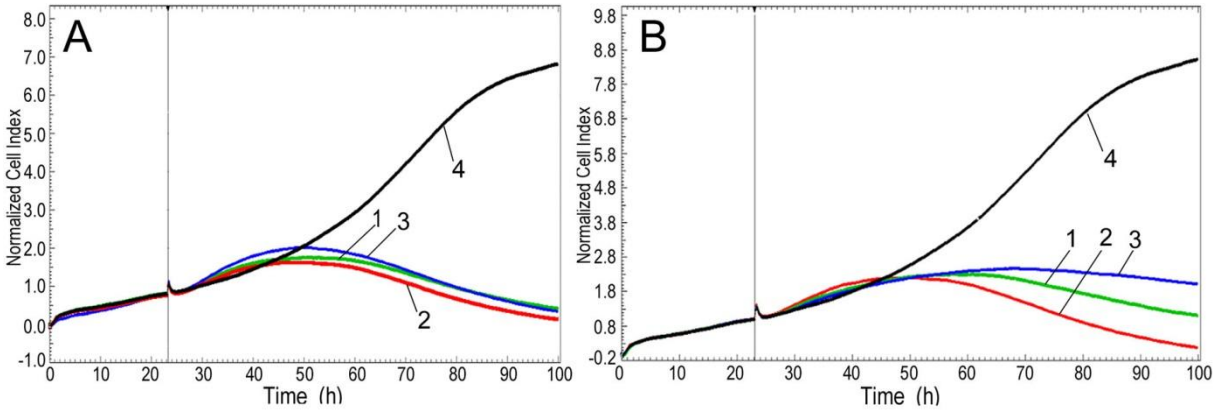

Fig. 2. TRCPs of A2780 cells treated with: A. Equitoxic concentrations of **1** (5 μM, curve 1), **2** (3 μM; curve 2), and cisplatin (10 μM; curve 3). B. Equimolar (3 μM) concentration of **1** (curve 1), **2** (curve 2), and cisplatin (curve 3). Curves 4, untreated cells (control).

3.3. Effect of nucleotide excision repair (NER) on the sensitivity of cells

The impedance-based monitoring of cellular responses to **1** and **2** suggested that their cytotoxicity could result from mechanisms associated with nuclear DNA damage as in the case of cisplatin. Therefore, we further investigated the involvement of DNA damage in mediating cellular sensitivity to **1** and **2**. First, we performed an experiment aiming to determine the cellular (pharmacological) target of **1** and **2**. The cellular (pharmacological) target is defined [3] as a site within the cell (mostly a biomacromolecule) which is altered by the drug and whose modification triggers processes leading to tumor cell death. Several experimental criteria have been applied to support the thesis that binding of cisplatin to DNA is responsible for the cytotoxicity of this drug [3, 28]. One of these criteria is based on the observation that the drug exhibits a higher toxicity in cells which are deficient in DNA repair. It is so because the abilty of DNA lesions to trigger cell death is inversely dependent on the capacity of cells to repair the damage. The assay used in the present work is based on introducing a point mutation in a gene coding one of the proteins necessary for the repair of DNA [29]. Thus, mutant Chinese hamster ovary cells MMC-2 deficient in NER and parental CHO-K1 cells proficient in NER afforded us a powerful biological tool to explore our hypothesis that DNA is the potential target of biological action of **1** and **2**. We observed that MMC-2 cells were significantly more sensitive to killing than wild-type CHO-K1cells when treated with **1**, **2** and cisplatin (Fig. 3). This result indicates that unrepaired DNA lesions formed by **1** and **2** contribute significantly to their cytotoxicity, similarly to cisplatin for which it is generally accepted that DNA is the major pharmacological target of its cytotoxic effects [3, 30].

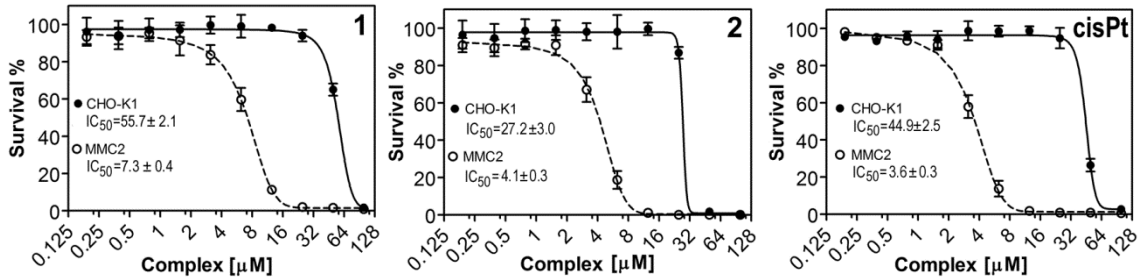

Fig. 3. Toxic effects of **1**, **2** and cisplatin in CHO cells proficient (CHO-K1) or deficient (MMC-2) in nucleotide excision repair. The IC_{50} values are related to the concentration of the complex (not to the concentration of platinum).

To further support the involvement of DNA damage in mediating cellular sensitivity to **1** and **2**, we determined whether nuclear DNA was damaged (platinated) when living cells were exposed to **1** or **2**. Thus, the CHO-K1 and MMC-2 cells were exposed to a 10 μM concentration of **1**, **2** or cisplatin for 24 h, harvested, washed exhaustively and divided into two portions. The first portion was mineralized and total amount of platinum accumulated in cells was determined by ICP-MS. The other portion of the cells was used to isolate nuclear DNA as described in Material and methods and the total amount of platinum associated with DNA was also assessed. As shown in Table 2, DNA was platinated to a similar extent when the cells were treated with **1**, **2** and cisplatin. Interestingly, although the cellular content of platinum was very similar in the two cell lines tested in this work, the levels of DNA platination in DNA repair deficient MMC-2 cells was significantly (approximately 2-times) higher than those in the parent repair-proficient CHO-K1 cells suggesting that DNA lesions induced by the treatment of the cells with **1** and **2** are reparable similarly to cisplatin lesions and DNA repair may modulate the overall biological effects not only of cisplatin, but also of **1** and **2**.

Table 2
Cellular accumulation of Pt from **1**, **2,** and cisplatin and nuclear DNA platination

	Cell uptake (pmol Pt/10^6 cell)[a]		Platinum content of DNA (pmol Pt/μg DNA)[a]	
	CHO-K1	MMC-2	CHO-K1	MMC-2
cisplatin	65 ± 1	69 ± 7	0.11 ± 0.02	0.23 ± 0.02
1	66 ± 1	66 ± 6	0.11 ± 0.01	0.191 ± 0.008
2	82 ± 2	106 ± 4	0.20 ± 0.02	0.34 ± 0.08

[a] Cells were treated for 24 h with **1**, **2**, or cisplatin at a concentration of 10 μM (this concentration is related to the concentration of the complex (not to the concentration of platinum).

3.4. DNA binding in cell free media

The NMR investigation showed [11] that complexes **1** and **2** did not react with the monomeric constituent of RNA, 5'-GMP, but their hydrolysis products readily did so. These hydrolysis products were identified as [$PtCl_2$(*cis*-1,4-DACH)] (or its solvato derivatives). However, the hydrolysis was very slow at physiological pH so that fifty percent decomposition of **1** and **2** was observed after 26 and 6 days, respectively [11]. This result is similar to the result reported by Bose et al. on the related [Pt(dihydrogenpyrophosphate)(1*R*,2*R*-DACH)], which exhibited no detectable binding to polymeric DNA after 7 days of incubation [12]. However, the data summarized in Table 2

clearly show that a significant portion of platinum was bound to nuclear DNA in cells treated with **1** or **2** for 24 h. To contribute to understanding these inconsistencies, the kinetics of binding of platinum from **1** (and **2**) to mammalian CT DNA in a cell-free medium was assessed.

As shown in Fig. 4, the amount of platinum associated with DNA increased with increasing the time of incubation in 10 mM $NaClO_4$ at 37 °C, suggesting that the investigated complexes or their hydrolysis products can bind high-molecular-mass DNA. After 24 h, cisplatin was quantitatively bound, in accordance with previously published results, whereas only ca. 45 or 49 % of platinum from **1** or **2**, respectively, was bound to DNA after this period of incubation. Thus, the DNA binding experiments indicated the irreversible coordination of platinum from **1** and **2** to DNA, which was, however, significantly slower than binding of cisplatin, very likely due to the slow pyrophosphate ligand [11].

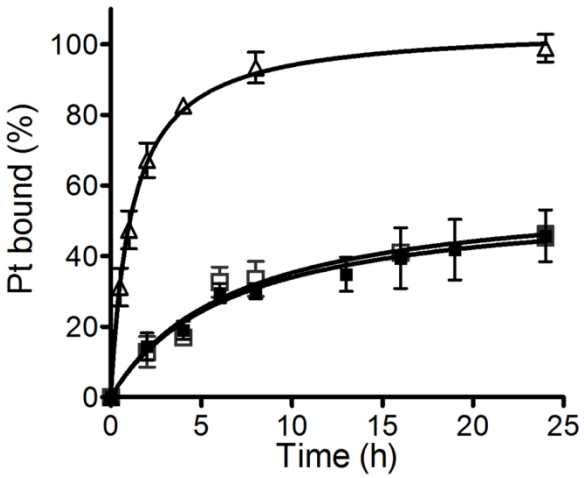

Fig. 4. Kinetics of DNA platination when CT DNA is incubated with **1**(■), **2** (□) and cisplatin (Δ) in 10 mM $NaClO_4$ at 37 °C. The symbols represent the average values of four (**1**, **2**) or two (cisplatin) independent experiments ± SD.

3.5. Activation of DNA-binding of 1 and 2 in cell extracts

The experiments described above reveal that in cell-free medium DNA incubated with **1** and **2** is platinated markedly less than when incubated with cisplatin. However, the degree of platination of nuclear DNA in cells treated with equal concentrations of **1**, **2** and cisplatin was comparable (Table 2). This comparison suggests that there might be an alternative mechanism that facilitates DNA platination in the presence of **1** or **2** inside living cells. To prove this hypothesis, we investigated the platination of DNA in the presence of **1** or **2** in a solution of 10 mM $NaClO_4$ to which cellular extract was added.

Thus, CT DNA was incubated with **1**, **2** or cisplatin at $r_i = 0.05$ in 10 mM $NaClO_4$ in the presence or absence of extract from A2780 cells (0.25 mg of protein mL^{-1}) at 37 °C. After 24 h, the samples were extracted with phenol and chloroform to remove proteins and subsequently exhaustively dialyzed against water. DNA concentrations in these samples were determined by absorption spectrophotometry, whereas concentrations of platinum associated with DNA were measured by FAAS. The results showed (Fig. 5) that cytosolic extract from human ovarian cancer A2780 cells was able to facilitate DNA platination in the presence of **1** and **2**. This result clearly demonstrates that **1** and **2** can be activated in the cellular environment in terms of their interactions with the target DNA.

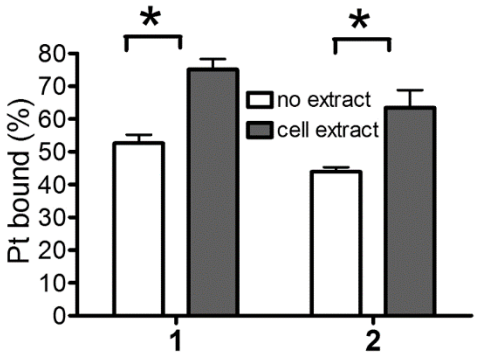

Fig. 5. Platination of CT DNA when incubated with **1** or **2** in 10 mM $NaClO_4$ for 24 h at 37 °C in the presence or absence of cellular extract. The results are means of three independent experiments; error bars indicate SD. The symbol (*) denotes a significant difference ($p < 0.01$).

3.4. Interstrand cross-linking

Bifunctional platinum compounds that covalently bind to DNA form various types of interstrand and intrastrand cross-links. Considerable evidence suggests that the antitumor efficacy of these compounds results from their ability to form these lesions [31]. Therefore, we quantitated DNA interstrand cross-linking efficiency of **1** and **2** and compared it with that of cisplatin. Linearized pUC19 plasmid DNA, radioactively labeled on its 3'-end, was incubated with various concentrations of **1**, **2** or cisplatin for 24 h at 37 °C. The samples were then analyzed by denaturing agarose gel electrophoresis [32]. Upon electrophoresis, the linearized plasmid containing no interstrand cross-link migrated as a 2686-base single strand, whereas the cross-linked strands migrated more slowly as a higher molecular mass species (Fig. 6). The radioactivity associated with the individual bands in each lane was measured and the frequency of interstrand cross-links [number of interstrand cross-links per one molecule of complex bound (%)] was calculated using the Poisson distribution [19]. The DNA interstrand cross-linking efficiency of cisplatin was 6 ± 1%, in agreement with already published data [20]. The interstrand cross-linking efficiency of **1** and **2** was found to be 5.1 ± 0.7 and 11 ± 3 %, respectively. So, the interstrand cross-linking efficiency of **1** is not significantly different from that of cisplatin. In contrast, the frequency of interstrand cross-links formation by **2** is almost 2-times higher, which is consistent with the fact that dinuclear **2**, after 24 h, decomposes to yield two molecules of **1** (or related solvato species) under physiological conditions [11].

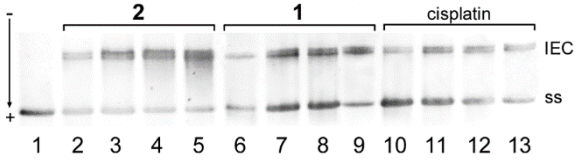

Fig. 6. Autoradiogram of denaturing 1% agarose gels of linearized pSPUC19 DNA which was 3´-end labeled. The interstrand cross-linked DNA appears as the top bands (IEC) migrating more slowly than the single-stranded DNA (ss). The DNA was nonplatinated (control) (lane 1); modified in the presence of **2** at r_b = 0.0005, 0.001, 0.0015 or 0.002 (lanes 2-5, respectively); modified in the presence of **1** at r_b = 0.0005, 0.001, 0.0015 or 0.002 (lanes 6-9, respectively); or modified in the presence of cisplatin at r_b = 0.0005, 0.001, 0.0015 or 0.002 (lanes 10-13, respectively).

3.5. Single cell electrophoresis in denaturing gel

The DNA cross-linking ability of **1** and **2** was further assessed in living cells using single cell gel electrophoresis and compared with that of cisplatin. The A2780 cells were treated with **1**, **2** or cisplatin at the concentrations indicated in Fig. 7. The short treatment time (2 h) was chosen in order to avoid DNA damage due to downstream processes, such as apoptotic fragmentation. Immediately after the treatment, the cells were subjected to H_2O_2 which causes DNA fragmentation so that a large comet tail appeared in the absence of the crosslinking agent. Crosslinking of DNA has been shown to reduce the amount of DNA fragmentation and hence reduces the size of the tail. As seen in Fig. 7, treatment of the cells with cisplatin significantly affected (reduced) migration of DNA out of the nuclei, resulting in a significant decrease of comet tails. This observation is in agreement with already published data [33, 34] and the well-known fact that cisplatin binds cellular DNA effectively forming intra- and interstrand cross-links. Similarly to cisplatin, a significant reduction in comet tail was also found for the cells treated with **1** or **2**, which is consistent with **1**- or **2**-induced formation of cross-links that retard DNA migration. Complex **2** was slightly more effective, which could be connected with the fact that this dinuclear complex can dissociate into two molecules of **1** (or related solvato species) before binding DNA [11]. However, the overall crosslinking efficiency was not significantly different from that of cisplatin in two out of the three concentrations of platinum complexes tested in this experiment. Thus, these results establish that complexes **1** and **2** platinate DNA and form DNA cross-links even in living cells, in accord with the results described above.

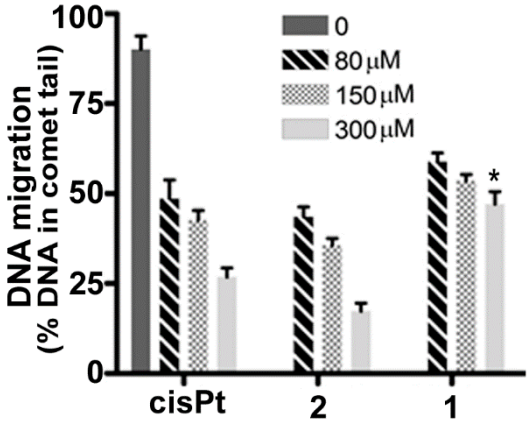

Fig. 7. Single-cell gel electrophoresis data (comet assay). The production of crosslinked DNA in A2780 cells treated with **1**, **2** and cisplatin (cisPt). The results represent the mean ± SE of three independent experiments, each performed in duplicate. All samples related to the cell treated with platinum complexes were significantly ($p < 0.01$) different from control, untreated cells. The symbol (*) denotes a significant difference ($p < 0.05$) from the corresponding concentration of cisplatin.

3.6. Mutagenicity testing

Mutagens are those agents (chemical or physical) that can cause changes in the genetic material, i.e. DNA, thus increasing the frequency of mutations above the natural background level. All mutagens are DNA-damaging agents. Therefore, the potential of **1** to induce mutations at the hprt locus of V79 cells (as evidence of DNA targeting) was assessed by the HPRT-forward-mutation-assay, which is frequently used for assessment of the genotoxicity of the tested agents [35]. V79 cells were exposed to **1** or cisplatin (5 μM) for 2 h and subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection [31]. The treated cells were kept by regular subculture for 7 days and mutant

frequency was determined by seeding $2x10^5$ cells in medium containing the 6-thioguanine (6-TG) as a selective agent to detect mutant cells. To determine the cloning efficiency (viability), the same number of cells ($2x10^5$) was further incubated in the medium without 6-TG. Treatment of V79 cells with **1** and cisplatin caused a decrease in cloning efficiency resulting from the increased lethality of cells (Table 3); the cytotoxicity of **1** in V79 cells was higher than that of cisplatin. This is consistent with the results of cytotoxicity testing, which indicated that compounds **1** and **2** were significantly more cytotoxic after a short time of incubation in contrast to cisplatin (Table 1). The results of the HPRT mutation assays (Table 3) also revealed that cisplatin, in accordance with previously published results [31, 36], produced a significant increase in mutation frequency (MF). Interestingly, **1** was also found to be mutagenic, even slightly more than cisplatin; the number of 6-TG resistant mutations per 10^5 viable cells treated with **1** was 1.3-fold higher than that of cisplatin. These results are consistent and support the view that **1** affects nuclear DNA and causes alterations (distortions) in the DNA molecule.

Table 3
Mutant frequency of **1** and cisplatin in V79 cells[a]

	Expression time: 0 min		Expression time: day 7	
	Cloning efficiency [%]	R[b]	ML[c]	MF[d]
Control	69.9 ± 5.2	1	2.4 ± 0.2	1
Cisplatin	40.3 ± 3.4	0.58	11.9 ± 0.8[e]	4.96
1	24.9 ± 1.0	0.36	15.7 ± 0.7[e,f]	6.54

[a]The cells were exposed to the investigated compounds (5 μM) for 2 h. [b]Ratio of cloning efficiency in treated vs. untreated cells. [c]The number of 6-TG resistant mutations per 10^5 viable cells treated with **1** or cisplatin. [d]Mutation frequency (ratio of induced to spontaneous mutations). [e]Significantly different from control, $p < 0.01$. [f]Significantly different from cisplatin, $p < 0.05$.

3.7. Effect on bacterial morphology

Bacterial filamentation results from bacterial response to various stresses, including DNA damage or inhibition of replication, through the SOS response system. One of the early Rosenberg's experiments demonstrated that antitumor cisplatin induced bacterial filamentation [37]. This result is considered to be one of the key experiment demonstrating that platinum-DNA lesions are the critical lesions responsible for the cell death. Moreover, previous studies suggested that induction of filamentation in *E. coli* by monofunctional platinum complex [17] or antitumor transplatin-derivatives [18] implicated DNA as the cellular target of these metallodrugs. Therefore, we investigated the effect of **1** and **2** on bacteria as well.

E. coli cells were cultured as described in Materials and methods in the presence or absence of one of the investigated platinum complexes. After 5 h of incubation at 37 °C, 2 μL aliquots of each cell culture suspension were mounted on microscope slides and analyzed by microscopy. As shown in Fig. 8, the morphology of cells treated with **1**, **2**, and cisplatin was changed so that bacteria became filamentous and cells markedly longer than those in the control are seen besides bacteria of a normal length (Fig. 8B-D). The lengths of the bacterial filaments induced by **1** or **2** were comparable to those seen following the treatment with cisplatin. In contrast, the control, untreated bacteria display the morphology expected for *E.coli* cells, the short rods 2–3 μm in length (Fig. 8A). Thus, complexes **1** and **2**, like bifunctional cisplatin, can block a division and induce a filamentous phenotype of *E.coli*.

These results further establish that the biological activity of **1** or **2** is elicited through interaction with DNA and that cisplatin and these pyrophosphate derivatives of kiteplatin share the same target, i.e. DNA.

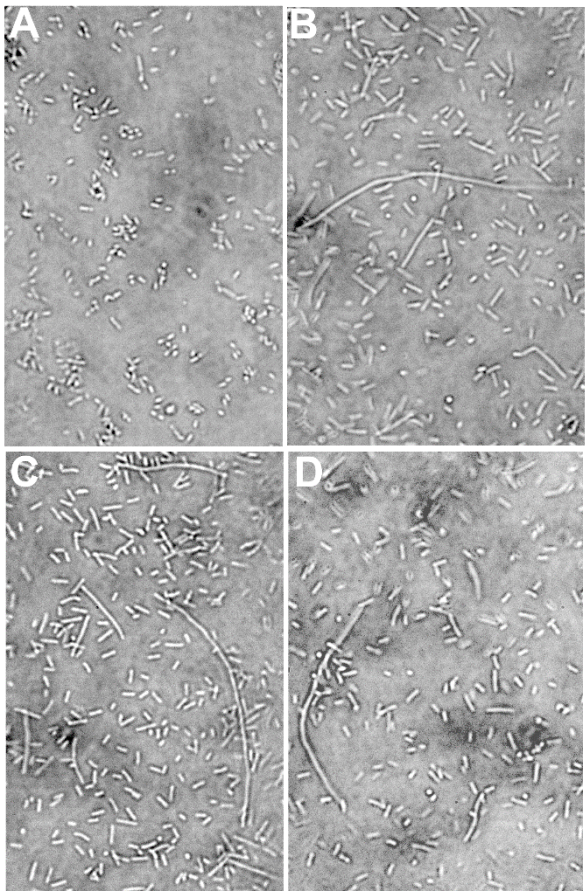

Fig. 8. Effect of **1** (panel B), **2** (panel C) and cisplatin (panel D) on the morphology of *E. coli* K12 bacteria. Control, untreated bacteria are shown in the panel A.

Conclusions

New kiteplatin pyrophosphate derivatives **1** and **2** are direct analogs of phosphaplatins which were highlighted as non-DNA binding platinum anticancer drugs [12]. Thus, phosphaplatins were proposed to act by different cytotoxic mechanisms in comparison with conventional cisplatin and its derivatives representing a clear paradigm shift in expanding the molecular targets for platinum anticancer drugs [12]. We show that **1** and **2**, in contrast with the conclusions proposed for the mechanism of action of phosphaplatins, readily platinate DNA in tumor cells treated with these kiteplatin pyrophosphate derivatives and that DNA is an important target of their cytotoxic action. The hypothesis that **1** and **2** exert biological effects through DNA damage, as in the case of cisplatin, was corroborated by applying several experimental criteria used to demonstrate that binding of an antitumor platinum drug to DNA is responsible for its cytotoxicity.

We demonstrate that platination of DNA during its incubation with **1** or **2** in celll-free media was slow in comparison with binding of cisplatin, apparently due to the slow hydrolysis of the kiteplatin pyrophosphate derivatives affording species capable of coordination to DNA. Notably, cytosolic extract from cancer cells was able to facilitate DNA platination in the presence of **1** and **2**. These data are consistent with activation of **1** and **2** in the cellular environment. Such an activation yielding species capable of platination of DNA

very likely comprises the hydrolytic release of the pyrophosphate ligand which can be enzymatically catalyzed. Examples of the enzymes identified to participate in pyrophosphate metabolism and concentration homeostasis in cells are ectonucleotide pyrophosphatase/phosphodiesterase [38], tissue-nonspecific alkaline phosphatase [39], inorganic pyrophosphatase [40].

The results described in this report are contextualized by comparison with those obtained with conventional cisplatin and corroborate the hypothesis that the biological activity of the kiteplatin pyrophosphate derivatives is mediated by the interaction of these substrates (or their hydrolytic products) with DNA. Of course, the intervention of additional mechanisms cannot be excluded.

Compliance with ethical standards

The authors declare they have no competing financial interests.

Acknowledgements

This work was supported by the Czech Science Foundation (grant 17-05302S). J.P. acknowledges her funding by the National Program of Sustainability I (LO1204). The authors thank the University of Bari and the Consorzio Interuniversitario di Ricerca in Chimica deiMetalli nei Sistemi Biologici (CIRCMSB) for support.

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