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Original article

Synthesis, characterization and biological activity of Pt(II) and Pt(IV) complexes with 5-methyl-5(4-pyridyl)-2,4-imidazolidenedione

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Abstract

New platinum(II) and platinum(IV) complexes with 5-methyl-5(4-pyridyl)-2,4-imidazolidenedione and various halogen ions with general formula $[PtL_2X_2]$ and $[PtL_2Cl_4]$, where L is the organic ligand and X is Cl^-, Br^-, J^-, were synthesized. The molecular formulae of all the complexes were confirmed by elemental analysis, IR, ^1H, ^{13}C NMR spectral analyses and molar conductivity. The cytotoxic effects of these complexes were examined on some human tumor cell lines. The newly synthesized *cis*-$[PtL_2Cl_2]$ exerted cytotoxic activity against SKW-3, MCF-7, EJ, U-266 tumor cell lines, while *cis*-$[PtL_2Br_2]$, *trans*-$[PtL_2I_2]$ were less active. The higher oxidation state complex *cis*-$[PtL_2Cl_4]$ was inactive in all cell lines but in SKW-3 some augmentation of the cytotoxicity was seen after co-administration of ascorbic acid but not when treated in combination with reduced glutathione or *N*-acetylcysteine. A DNA-fragmentation analysis revealed that the cytotoxicity of the dichloro analogue, characterized with superior activity compared to the other complexes, is mediated by induction of apoptotic cell death.

Keywords: *cis*/*trans* Pt(II) and Pt(IV) complexes; Cytotoxicity; 2,4-Imidazolidenedione

1. Introduction

Cisplatin is one of the most potent antitumor drugs available for the therapeutic management of solid tumors, such as germ cell tumors, ovarian, lung, head and neck, bladder cancers, etc. Despite its wide application as a chemotherapeutic agent, cisplatin exhibits severe side effects, such as nephrotoxicity, neurotoxicity, ototoxicity, nausea and emetogenicity, etc. which limit the possibilities for gaining therapeutic benefits from dose intensification [1–3]. Thus, a plethora of platinum(II) and platinum(IV) complexes with nitrogen-containing ligands has been the subject of intensive biological evaluation aimed at developing less toxic and more selective anticancer therapeutics [4,5]. Among these hydantoin-containing complexes have been reported to possess cytotoxic/antitumor activity [6,7]. Some hydantoin derivatives such as dithienylhydantoin, 5,5-dipyridylhydantoin, spirohydantoins and 3,5-disubstituted hydantoins exhibit antiviral, anticonvulsive and cytotoxic activities [8].

The classical structure–activity relationship rules established for cisplatin and its structurally related congeners postulate that the leaving groups (generally chlorine) and the two ammine ligands in platinum complexes must be in *cis*-orientation and that the corresponding *trans* compounds are inactive [9,10]. Nevertheless, during the 1990s, several scientific groups have reported *trans*-platinum compounds with *in vitro* growth inhibitory and *in vivo* antitumor properties [11–13]. More importantly some of these complexes have been found to retain considerable efficacy against tumor cells

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resistant to cisplatin. Moreover, recent advances in the cellular and biochemical pharmacology of *trans*-complexes indicate that they are characterized with some important cisplatin-dissimilar pharmacodynamic properties, especially with respect to the spectrum of DNA adducts, and the cellular processing thereof. The discovery of *trans*-platinum isomers with antitumor properties urged for the re-evaluation of structure–activity relationship of platinum complexes [14]. *Trans*-coordination complexes with bulky spectator ligands pose special interest as they tend to be superior vs. their *cis*-analogues against cisplatin-refractory cell lines [15].

A parallel chemical approach aimed at ameliorating the toxicity of Pt(II) complexes is based on their conversion to the corresponding Pt(IV) analogues and thus modifying the substitution reactivity and the overall physicochemical properties thereof by changing axial ligands. Several Pt(IV) complexes have been prepared [16] and subsequently introduced into clinical trials such as Tetraplatin (tetrachloro-*trans*-DL-1,2-DACH-platinum(IV) [17] and JM216 (*cis*-dichloro-*trans*-diacetatoaminecyclohexylamine-platinum(IV)) [18].

In our previous investigations we have synthesized and characterized series of non-classical cationic Pt(II) complexes with some substituted hydantoin ligands, whereby the complexing metal ion is bound to four nitrogen donors, and hence these compounds lacked the classical halogenide or carboxylate leaving groups. The pharmacological evaluation of these cationic species revealed that they exerted cytotoxic activity *in vitro* [8,19]. Now our aim is to modify chemically the structure of cisplatin by replacing the stable ammine ligands with 5-methyl-5(4-pyridyl)-1,2-imidazolidenedione as a carrier ligand and to obtain new neutral platinum complexes.

The present study represents the synthesis, physicochemical evaluation and pharmacological investigation of Pt(II) and Pt(IV) complexes with 5-methyl-5-(4-pyridyl)-2,4-imidazolidenedione as compared to the clinically applied drug cisplatin.

2. Chemistry

The ligand 5-methyl-5(4-pyridyl)-2,4-imidazolidenedione (L) was prepared by previously published method [20].

Potassium tetrachloroplatinate(II) utilized for the synthetic procedures was purchased from Merck – Germany and platinum(IV) chloride – Heraeus GmbH. All of the other chemicals were of analytical grade.

3. Pharmacology

The present study describes a comparative evaluation of the cytotoxic effects of four newly synthesized platinum(II) and platinum(IV) complexes vs. the referent antineoplastic agent cisplatin in a panel of human tumor cell lines, using the standard MTT-dye reduction assay for cell viability. The panel consisted of the following cell lines: (i) HL-60 acute myeloid leukemia, established from the peripheral blood of a patient with acute promyelocyte leukemia; (ii) SKW-3 human T-cell leukemia, established from peripheral blood of a 61-year-old man with T-cell lymphocytic leukemia; (iii) LAMA-84 human chronic myeloid leukemia, established from peripheral blood of a 29-year-old woman with chronic myeloid leukemia; (iv) U-266 human multiple myeloma, established from the peripheral blood of a 53-year-old man with refractory terminal IgE-secreting myeloma; (v) SAOS-2 human osteogenic sarcoma, established from the primary osteogenic sarcoma of a 11-year-old Caucasian female; (vi) MCF-7 human estrogen receptor positive breast adenocarcinoma, established from the pleural effusion of a 69-year-old Caucasian woman with metastatic mammary carcinoma; (vii) EJ human urinary bladder carcinoma.

Considering the well appreciated notion that the higher oxidation state platinum agents are non-toxic unless reduced to the corresponding Pt(II) analogues we sought to evaluate the influence of the co-administration of bio-reductors such as ascorbic acid, *N*-acetylcysteine (NAC) and reduced glutathione (GSH) upon the antiproliferative effects of the newly synthesized Pt(IV) complex with 5-methyl-5(4-pyridyl)-2,4-imidazolidenedione.

The final part of the biological investigations is aimed at unraveling the mechanistic aspects underlying the antiproliferative activity of tested compounds and in particular the propensity of a chosen complex to recruit the apoptotic cell death signaling pathways.

4. Results and discussion

4.1. Chemistry

On the basis of the data from the elemental analysis for the new complexes the following formulae can be derived: *cis*-$[Pt(C_9H_9N_3O_2)_2Cl_2]$ (**1**), *cis*-$[Pt(C_9H_9N_3O_2)_2Br_2]$ (**2**), *trans*-$[Pt(C_9H_9N_3O_2)_2I_2]$ (**3**) and *cis*-$[Pt(C_9H_9N_3O_2)_2Cl_4]$ (**4**). In order to evaluate the mode of coordination of the ligand to the metal ion, the IR, ^1H and ^{13}C NMR spectra of the pure ligand as well as of their Pt(II) and Pt(IV) complexes were recorded.

4.1.1. IR spectra

The comparative analysis of the IR spectra of the complexes **1**–**4** and of the free ligand revealed that the absorption bands characteristic for the stretching vibrations of –C=N– from the pyridine ring were shifted towards the higher frequencies – from 1605.3 cm^{-1} in the spectrum of the ligand to 1619.9, 1618.4, 1615.2 and 1619.3 cm^{-1} in the complexes, respectively. This indicates that the nitrogen atom from the pyridine's ring participates in the coordination to the platinum ion.

The other characteristic bands of the pyridine ring of the free ligand shift to higher frequencies upon complexation, thus confirming the coordination through the heterocyclic N atoms. The new bands in the low-energy region at 350–189 cm^{-1} are assigned to the Pt–X stretching vibrations. In the spectra of the complexes **1**, **2** and **4** two bands for Pt–X stretching vibrations were observed which show that the halogen anions were in *cis*-orientation. Only in complex **3** one band for Pt–I stretching vibration can be observed showing

most probably that the two iodine anions were in *trans*-orientation according to Nakamoto [21]. From the theoretic point of view I^- shows larger *trans*-effect than Cl^- and Br^-, but in this case the *trans*-effect of the organic ligand is more important.

The bands related to the stretching vibrations of the two carbonyl groups at 1776.2 and 1730.2 cm^{-1} remained unchanged in the complexes. This fact is an evidence that these groups are not involved in the complex formation.

4.1.2. NMR spectra

In the ^1H NMR spectra of complexes **1–4**, the signals of the protons for H-2 and H-6 from the pyridine ring were shifted from 8.56 ppm in the spectrum of the ligand to 8.83, 8.84, 8.79 and 8.83 ppm in the spectra of the complexes, respectively. The differences between the chemical shifts of the protons of the ligand and these of the corresponding complexes are shown as $\Delta\delta$ (in ppm). The shifts are noticeable – $\Delta\delta = 0.24$, 0.25, 0.20 and 0.24 ppm. The signals of the H-3 and H-5 protons from the pyridine ring were shifted from 7.44 ppm in the ligand to 7.59, 7.61, 7.60 and 7.83 ppm in the complexes. The chemical shifts are $\Delta\delta = 0.12$, 0.13, 0.12 and 0.35 ppm, and are twice less than those registered for the nearer to nitrogen atom protons of the pyridine ring. This shows that the most probable bounding of the ligand with the platinum ion in all the complexes is realized through the nitrogen atom from the pyridine ring. The signals for the protons at the nitrogen atoms from the hydantoin ring were not shifted. This fact indicates that these atoms are not involved in the complex formation to the platinum.

In the ^{13}C NMR spectra of the complexes, the signals for the carbon atoms C-2 and C-6 from the pyridine ring were shifted from 149.97 ppm in the spectrum of the ligand to 153.02, 153.61, 153.54, and 154.97 ppm for the complexes. The downfield chemical shifts of the signals are $\Delta\delta = 3.07$, 3.64, 3.57, and 5.00 ppm for the complexes **1–4** and indicate that probably the nitrogen atom from the pyridine ring participates in the coordination with the metal ion. The signals of the C-3 and C-5 were shifted from 120.51 ppm in the spectrum of the ligand to 123.27, 123.32, 122.83, and 124.09 ppm for the complexes. The chemical shifts are smaller, $\Delta\delta = 2.76$, 2.51, 2.32 and 3.58 ppm, for the complexes, which can be explained by the fact that the carbon atoms, which are nearer to the nitrogen atom from the pyridine ring are less shielded because of the participation of the nitrogen atom in binding with platinum.

The signals of the two C=O groups from the hydantoin ring in all complexes were not changed. This finding is an indication that these groups are not involved in the binding to the metal ion.

An additional investigation of the free ligand and the complexes of Pt(II) and Pt(IV) in solution was carried out. Solutions (1×10^{-3} mol/dm^3) in DMSO were prepared and their molar conductance was evaluated. For the ligand a λ_M value of 1.85 S cm^2 mol^{-1} was encountered. According to the referent literature data, the newly synthesized complexes could be assigned to the non-electrolytes [22].

On the basis of the results from the physicochemical investigations, the following, most probable schematic structures of the Pt(II) and Pt(IV) complexes with L could be proposed (Fig. 1).

4.2. Pharmacology

4.2.1. In vitro cytotoxicity

Evident from the cytotoxicity data summarized in Table 1, the newly synthesized Pt(II) complexes exerted concentration dependent antiproliferative activity against the human tumor cell lines. The Pt(IV) complex, however, demonstrated only marginal activity and failed to cause 50% inhibition of malignant growth with the only exception of the lymphoid leukemia SKW-3. The comparison of biological data amongst the newly synthesized complexes unambiguously indicates that *cis*-$[PtL_2Cl_2]$ is superior in terms of potency, causing 50% inhibition of cellular viability at lower concentrations as compared to *trans*-$[PtL_2I_2]$ and the *cis*-$[PtL_2Br_2]$. These findings, however, could not be ascribed solely to the nature of leaving groups, having into consideration the *trans*-configuration of $[PtL_2I_2]$. The cytotoxicity data indicate the latter complex as biologically active in concordance with numerous data pointing out the possibilities to confer cytotoxicity to *trans*-configuration compounds by means of introducing bulky spectator ligands [23].

In order to allow juxtaposition of the biological activity spectra between the novel compounds and cisplatin a midgraph analysis of cytotoxicity data was carried out. Considering the poor biological activity of *cis*-$[PtL_2Cl_4]$ it was excluded from these evaluations. The midgraph plots depicted in Fig. 2 showed that the dichloro analogue exerts a cisplatin-similar spectrum of activity, except for the lower sensitivity of the novel complex against MCF-7, EJ and especially LAMA-84. The modification of leaving groups in the other complexes is consistent with a significant alteration of the relative sensitivity of cell lines vs. both the referent drug cisplatin and the dichloro analogue. The only common feature shared by all novel compounds was the relatively high sensitivity of the chromic myeloid SKW-3 and the low responsiveness of the osteogenic sarcoma SAOS-2.

4.2.2. Cytotoxicity of cis-$[PtL_2Cl_4]$ after co-administration of bio-reductors

Pt(IV) compounds are considered as inert prodrugs which are capable of interacting with DNA and hence killing cells only upon reduction to square planar Pt(II) species [24]. On this ground we aimed at evaluating whether the presence of certain biological antioxidants, known to reduce Pt(IV) compounds, would condition an augmentation of the biological activity of *cis*-$[PtL_2Cl_4]$. To meet this objective SKW-3 cells were treated for 72 h with *cis*-$[PtL_2Cl_4]$ at 25, 50, 100 or 200 μM alone or in combination with either ascorbic acid, NAC or GSH. The biological reductors were applied at non-cytotoxic concentrations – 12.5 or 50 μM.

Evident from the results summarized in Fig. 3, the thiols NAC and GSH failed to produce any significant modulation

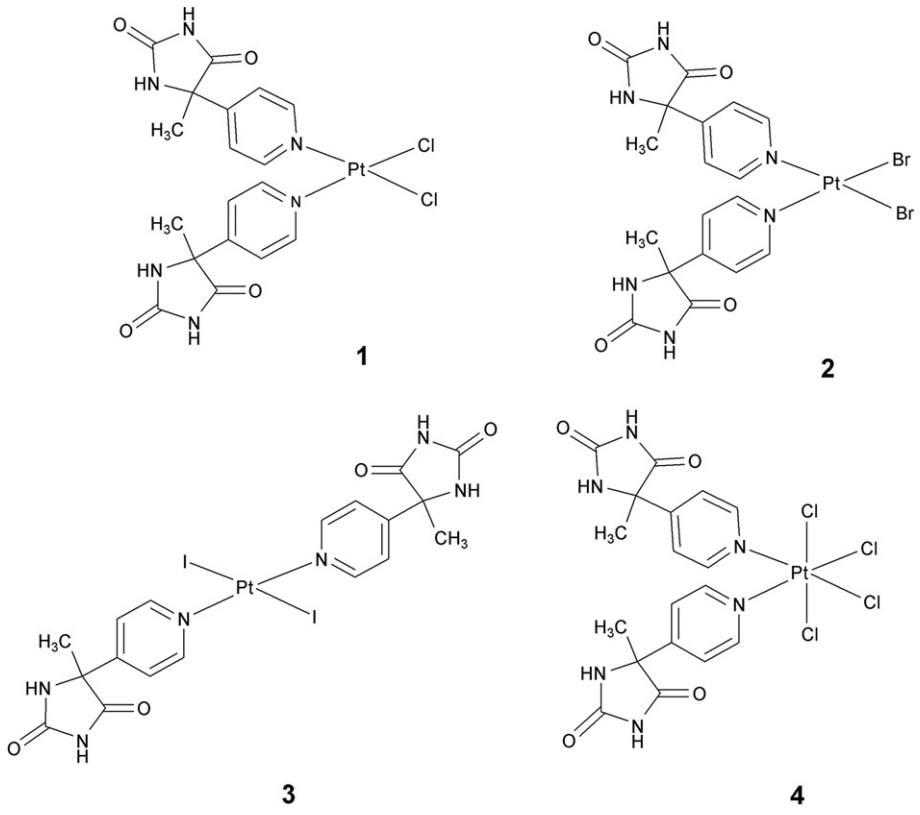

Fig. 1. Schematic structures of the investigated Pt(II) complexes *cis*-[PtL_2Cl_2] (**1**), *cis*-[PtL_2Br_2] (**2**), *trans*-[PtL_2I_2] (**3**) and *cis*-[PtL_2Cl_4] (**4**).

of the antiproliferative activity of *cis*-[PtL_2Cl_4]. This could be ascribed to their propensity to form Pt-adducts and hence even though certain reduction of *cis*-[PtL_2Cl_4] to more cytotoxic Pt(II) species could not be ruled out, these could in turn be neutralized via interactions with the thiol moieties of NAC and GSH. In a dissimilar fashion ascorbic acid caused a frank augmentation of the antiproliferative activity of *cis*-[PtL_2Cl_4], which was more pronounced at the lower concentrations of the complex. These data indicate that the cytotoxic effects of the Pt(IV) complex in SKW-3 cells are most probably mediated by its reduced biotransformation products.

Table 1
Cytotoxic activity of the new platinum complexes vs. cisplatin in a panel of human malignant cell lines as determined by the MTT-dye reduction assay following a 72 h continuous exposure

Cell line	IC_{50} value[a] (μM)				
	[PtL_2Cl_2]	[PtL_2Br_2]	[PtL_2I_2]	[PtL_2Cl_4]	Cisplatin
SKW-3[b]	39.7 ± 2.9	146.7 ± 4.6	89.6 ± 3.5	183.1 ± 5.9	11.4 ± 1.7
LAMA-84[c]	150.1 ± 4.4	159.2 ± 5.0	111.4 ± 4.6	>200	16.9 ± 1.7
U-266[d]	77.2 ± 4.0	150.0 ± 4.8	135.2 ± 4.9	>200	11.2 ± 1.2
SAOS-2[e]	>200	>200	>200	>200	66.2 ± 3.1
MCF-7[f]	64.5 ± 3.7	190.5 ± 6.1	140.2 ± 4.7	>200	9.1 ± 0.9
EJ[g]	71.2 ± 3.5	151.3 ± 5.1	152.0 ± 5.0	>200	10.2 ± 1.3

[a] Means ± sd of 8 separate wells, run in triplicate.
[b] T-cell leukemia.
[c] Chronic myeloid leukemia.
[d] Multiple myeloma.
[e] Osteogenic sarcoma.
[f] Breast adenocarcinoma.
[g] Urinary bladder carcinoma.

4.2.3. Proapoptotic effect of cis-[PtL_2Cl_2]

The proapoptotic activity of the most active compound *cis*-[PtL_2Cl_2] was monitored using a commercial ELISA kit for genomic fragmentation which has been repeatedly reported as a hallmark feature of apoptosis. Fig. 4 demonstrates the concentration dependent enrichment of the cytosolic fractions of SKW-3 with mono- and oligonucleosomal DNA fragments, after 24 h *cis*-[PtL_2Cl_2] exposure. These findings indicate that the cytotoxic mode of action of *cis*-[PtL_2Cl_2] involves activation of programmed cell death signaling pathways.

5. Conclusion

Four new Pt(II) and Pt(IV) complexes with 5-methyl-5-(4-pyridyl)-2,4-imidazolidenedione and various halogen anions were synthesized. The molecular formulae of the complexes were confirmed by elemental and spectral analyses such as IR, ^1H, ^{13}C NMR spectra and molar conductivity. Three of the complexes are in *cis*-position and most probably only one is in *trans*-position. The biological evaluation thereof unambiguously indicates that the dichloro analogue is the most active compound, whereas the substitution of the leaving groups as well as the change in the oxidation state is associated with decrease in cytotoxicity. The cytotoxic effects of [PtL_2Cl_2] are mediated by induction of cell death through apoptosis as evidenced by the genomic DNA fragmentation triggered by 24 h exposure of SKW-3 cells with this compound.

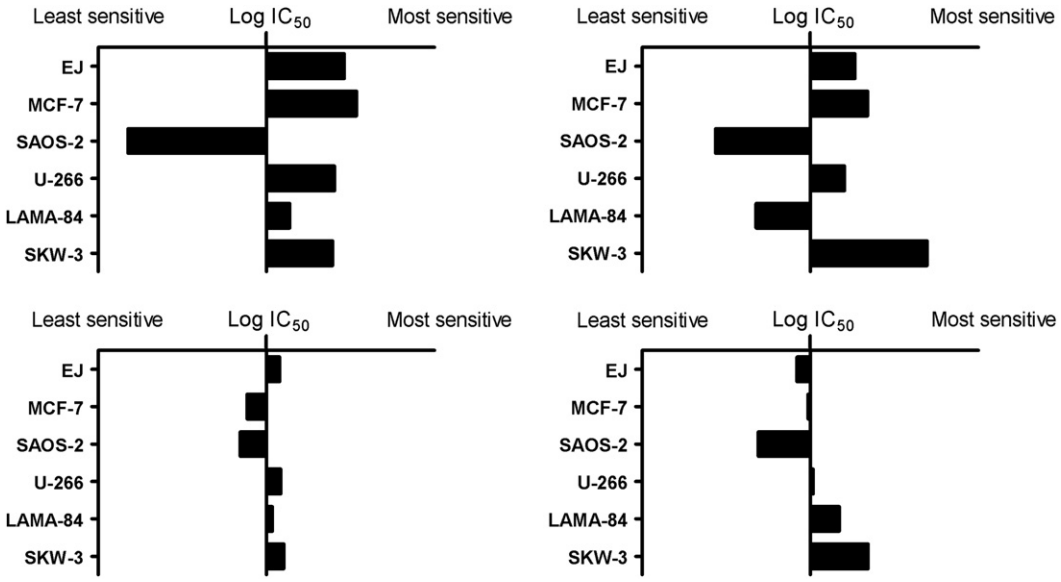

Fig. 2. Mean graph analysis of cytotoxicity data for cisplatin (left panel, upper plot), *cis*-[PtL_2Cl_2] (right panel, upper plot), *trans*-[PtL_2I_2] (left panel, lower plot) and *cis*-[PtL_2Br_2] (right panel, lower plot). Bars to the left of the mean IC_{50} line represent the least sensitive cell lines, whereas those on the right indicate the most sensitive ones, on a logarithmic scale.

6. Experimental protocols

6.1. Chemistry

6.1.1. General methods

The newly synthesized Pt(II) and Pt(IV) complexes were characterized by elemental analysis, IR, ^1H and ^{13}C NMR spectra. The carbon, nitrogen and hydrogen contents of the compounds were determined by elemental analysis. The elemental analysis was carried out on a 'Carlo Erba' apparatus.

6.1.2. Spectral measurements

The IR spectra were recorded on IFS 113 v Bruker FTIR spectrophotometer in the range of 4000–400 and 400–150 cm^{-1} as tablets CsI. The ^1H and ^{13}C NMR spectra were registered on a Bruker WM 250 (250 MHz) spectrometer in DMSO-d_6. Corrected melting points were determined, using a Bushi 535 apparatus. The molar conductivity of 1×10^{-3} mol/l solutions of the complexes in DMSO was measured by means of a Metrohm conductometer 660 (cell constant – 0.82 cm^{-1}).

6.1.3. Synthesis of the complexes

6.1.3.1. Synthesis of cis-dichloro-bis(5-methyl-5(4-pyridyl)-2,4-imidazolidenedione)platinum(II) [PtL_2Cl_2]. Two water solutions of $K_2[PtCl_4]$ and of the ligand (L) were prepared for the synthesis of the complex *cis*-[PtL_2Cl_2] (**1**). The solution of L (0.2766 g, 1.4482 mmol) was added dropwise to the water solution of $K_2[PtCl_4]$ (0.3012 g, 0.7258 mmol) at constant stirring and at temperature 50 °C. After the addition of the ligand the homogenous solution was stirred for 5–6 h. The solution was concentrated and cooled to 0 °C. A bright-yellow product was obtained, which was filtered, washed several times with ethyl ether and dried in a vacuum desiccator. The substance is soluble in DMSO and weakly soluble in water and ethanol. The purity is checked up by thin layer chromatography with the eluent $CH_3COOC_2H_5/C_2H_5OH = 2{:}1$. Yield: ca. 52%, m.p.: >271 °C (dec.). Anal. Calcd. (%) for [$Pt(C_9H_9N_3O_2)_2Cl_2$]: Pt, 31.10; C, 32.34; H, 2.78; N, 12.97. Found (%): Pt, 31.18; C, 31.78; H, 2.58; N, 12.18: $\lambda_M = 6.44$ S cm^2 mol^{-1}.

IR (tabl. CsI)): 3211, 3194, 1777.2, 1725.5, 1619.9, 329.9, 322.2 cm^{-1}.

^1H NMR (DMSO-d_6): 11.06 (NH-3), 8.83 (2H, pyr.), 8.69 (NH-1), 7.59 (2H, pyr.), 1.66 ppm (3H, s).

^{13}C NMR (DMSO-d_6): 175.10 (C=O-4′), 156.18 (C=O-2′), 153.02 (C-2, C-6), 123.27 (C-3, C-5), 63.50 (C-5′), 24.60 ppm (CH_3).

6.1.3.2. Synthesis of cis-dibromo-bis(5-methyl-5(4-pyridyl)-2,4-imidazolidenedione) platinum(II) [PtL_2Br_2]. The complex *cis*-[PtL_2Br_2] (**2**) was prepared according to a reported procedure [25]. $K_2[PtCl_4]$ of 0.1007 g (0.2427 mmol) was mixed with a saturated solution of potassium bromide (in excess) (0.1435 g) and heated on a water bath for 5 min, thus $K_2[PtCl_4]$ was quantitatively converted into a solution of $K_2[PtBr_4]$. To this mixture 0.0930 g (0.4869 mmol) of L were added. The solution was stirred for 1 h at 50 °C. Orange-yellow crystals were obtained and filtered. The complex is soluble in DMSO. The purity is checked up by thin layer chromatography with the eluent $CH_3COOC_2H_5/C_2H_5OH = 2{:}1$. Yield: ca. 91%, m.p.: >260 °C (dec.). Anal. Calcd. (%) for [$Pt(C_9H_9N_3O_2)_2Br_2$]: Pt, 26.47; C, 29.32; H, 2.44; N, 11.35. Found (%): Pt, 26.14; C, 29.05; H, 2.05; N, 11.37: $\lambda_M = 7.16$ S cm^2 mol^{-1}.

IR (tabl. CsI): 3482.0, 3241.0, 1777.2, 1724.8, 1618.4, 235.2, 232.4 cm^{-1}.

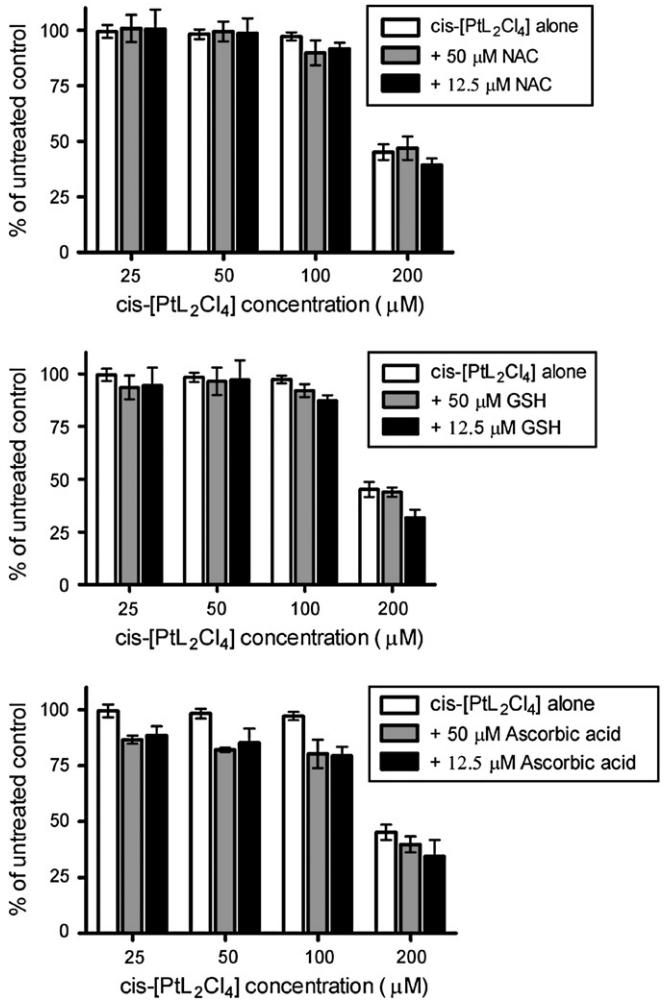

Fig. 3. Cytotoxic effects of compound **4** applied alone or in combination with either *N*-acetylcysteine (NAC) (upper plot), reduced glutathione (GSH) (middle plot) or ascorbic acid (lower plot) against the human T-cell leukemia SKW-3 (MTT-dye reduction assay, 72 h continuous exposure). Each column represents the arithmetic mean of 8 separate wells (run in triplicate), error bars represent the corresponding sd.

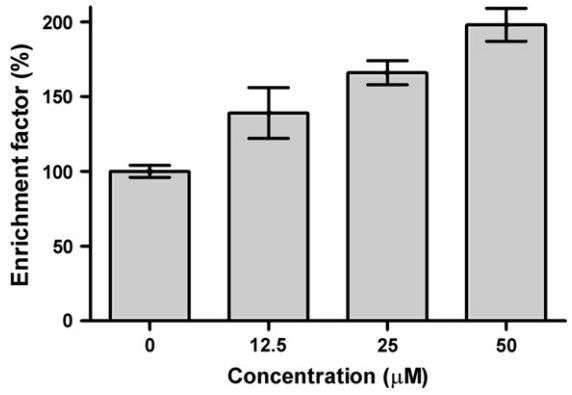

Fig. 4. Cytosolic enrichment of SKW-3 cells with histon-associated mono- and oligonucleosomal DNA fragments after 24 h treatment with *cis*-$[PtL_2Cl_2]$. Each column represents the arithmetic mean ± sd of 3 independent experiments (Cell Death Detection™ ELISA).

^{1}H NMR (DMSO-d_6): 11.05 (NH-3), 8.84 (2H, pyr.), 8.73 (NH-1), 7.61 (2H, pyr.), 1.65 ppm (3H, s).

^{13}C NMR (DMSO-d_6): 175.02 (C═O-4′), 156.03 (C═O-2′), 153.60 (C-2, C-6, pyr.), 123.02 (C-3, C-5, pyr.), 63.53 (C-5′), 24.58 ppm (CH_3).

6.1.3.3. Synthesis of trans-diiodo-bis(5-methyl-5(4-pyridyl)-2,4-imidazolidenedione) platinum(II) [PtL$_2$I$_2$]. The complex *trans*-$[PtL_2I_2]$ (**3**) was prepared according to the same procedure as described above [25]. Orange-yellow crystals were obtained and filtered. The complex is soluble in DMSO. The purity is checked up by thin layer chromatography with the eluent $CH_3COOC_2H_5/C_2H_5OH = 2{:}1$. Yield: ca. 75%, m.p.: >280 °C (dec.). Anal. Calcd. (%) for $[Pt(C_9H_9N_3O_2)_2I_2]$: Pt, 23.47; C, 26.00; H, 2,80; N, 10.11. Found (%): Pt, 23.29; C, 26.01; H, 2.16; N, 10.19: $\lambda_M = 4.41$ S cm^2 mol^{-1}.

IR (tabl. CsI): 3224.4, 3091.4, 1772.7, 1724.4, 1615.2, 189.0 cm^{-1}.

^{1}H NMR (DMSO-d_6): 11.11 (NH-3), 8.84 (2H, pyr.), 8.61 (NH-1), 7.60 (2H, pyr.), 1.65 ppm (3H, s).

^{13}C NMR (DMSO-d_6): 175.92 (C═O-4′), 156.21 (C═O-2′), 153.54 (C-2, C-6, pyr.), 122.83 (C-3, C-5, pyr.), 63.53 (C-5′), 24.57 ppm (CH_3).

6.1.3.4. Synthesis of cis-tetrachloro-bis(5-methyl-5(4-pyridyl)-2,4-imidazolidenedione) platinum(II) [PtL$_2$Cl$_4$]. The complex *cis*-$[PtL_2Cl_4]$ (**4**) was prepared by a method given in literature [26]. Two solutions of the $PtCl_4$ and of the 5-methyl-5(4-pyridyl)-2,4-imidazolidenedione in water were prepared. The water solution of the ligand (0.2255 g, 1.1806 mmol) was added dropwise to the water solution of $PtCl_4$ (0.2020 g, 0.5998 mmol) with stirring magnetically for 5–6 h at 50 °C. The solution was concentrated and cooled to 0 °C. A bright-yellow product was obtained and filtered, washed several times with ethyl ether and dried in a vacuum desiccator. The compound is soluble in DMSO, water and ethanol. The purity is checked up by thin layer chromatography with the eluent $CH_3COOC_2H_5/C_2H_5OH = 2{:}1$. Yield: ca. 52%, m.p.: >260 °C (dec.). Anal. Calcd. (%) for $[Pt(C_9H_9N_3O_2)_2Cl_4]$: Pt, 27.13; C, 30.05; H, 2.50; N, 11.69. Found (%): Pt, 27.04; C, 30.49; H, 2.57; N, 11.69: $\lambda_M = 4.31$ S cm^2 mol^{-1}.

IR (tabl. CsI): 3288.3, 3055.5, 1777.8, 1724.9, 1619.3, 349.0, 333.3 cm^{-1}.

^{1}H NMR (DMSO-d_6): 11.15 (NH-3), 8.83 (2H, pyr.), 8.68 (NH-1), 7.83 (2H, pyr.), 1.71 ppm (3H, s).

^{13}C NMR (DMSO-d_6): 174.75 (CO-4′), 156.04 (CO-2′), 154.97 (C-2, C-6, pyr.), 124.09 (C-3, C-5, pyr.), 63.75 (C-5′), 25.16 ppm (CH_3).

6.2. Pharmacology

6.2.1. Cell lines and culture conditions

The following cell lines were used in the experiments: SKW-3 (DSMZ No.: ACC 53, cell type: human T-cell leukemia, established from peripheral blood of a 61-year-old man with T-cell lymphocytic leukemia); LAMA-84 (DSMZ No.: ACC 168, cell type: human chronic myeloid leukemia,

established from peripheral blood of a 29-year-old woman with chronic myeloid leukemia); U-266 (DSMZ No.: ACC 9, cell type: human multiple myeloma, established from the peripheral blood of a 53-year-old man with refractory terminal IgE-secreting myeloma), SAOS-2 (DSMZ No.: ACC 243, cell type: human osteogenic sarcoma, established from the primary osteogenic sarcoma of a 11-year-old Caucasian female), MCF-7 (DSMZ No.: ACC 115, cell type human estrogen receptor positive breast adenocarcinoma, established from the pleural effusion of a 69-year-old Caucasian woman with metastatic mammary carcinoma); EJ (ATCC, cell type: human urinary bladder carcinoma).

SKW-3, LAMA-84 and U-266 as suspension and EJ as adherent cultures were grown in RPMI-1640 liquid medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. MCF-7 cells were grown as monolayer adherent cultures in 90% RPMI-1640 supplemented with 10% FBS, non-essential amino acids, 1 mM sodium pyruvate and 10 μg/ml human insulin. SAOS-2 cells were grown as monolayer adherent cultures in 85% McCoy's 5A supplemented with 15% FBS. All cell lines were maintained in tissue culture flasks in a humidified atmosphere at 37 °C and 5% CO_2. Cells were kept in log phase by supplementation with fresh medium 2–3 times per week. Adherent cells were reset by trypsinization twice weekly.

6.2.2. Cytotoxicity assessment

Cytotoxicity of the compounds was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay as described by Mossman [27] with some modifications [18]. Exponentially growing cells were seeded in 96-well microplates (100 μl/well at a density of 3.5×10^5 cells/ml for the adherent and 1×10^5 cells/ml for the suspension cell lines) and allowed to grow for 24 h prior to the exposure to the studied compounds. Stock solutions of the platinum complexes were freshly prepared in DMSO and then diluted with corresponding growth medium. At the final dilutions the solvent concentration never exceeded 0.5%. Cells were exposed to the tested agents for 72 h, whereby for each concentration a set of 8 separate wells were used. Every test was run in triplicate, i.e. in three separate microplates. After incubation with the tested compounds MTT solution (10 mg/ml in PBS) aliquots were added to each well. The plates were further incubated for 4 h at 37 °C and the formazan crystals formed were dissolved by adding 110 μl of 5% HCOOH in 2-propanol. Absorption of the samples was measured by an ELISA reader (Uniscan Titertec) at 580 nm. Survival fraction was calculated as percentage of the untreated control. The experimental data were processed using GraphPad Prizm software and were fitted to sigmoidal concentration/response curves.

6.2.3. Mean graph analysis

The mean graph analysis allows the relation of the magnitude of activity (IC_{50}) for a given cell line to the mean IC_{50} for all cell lines, indicating superior or lower activity against a particular tumor type. The procedure was carried out as described elsewhere [28]. Briefly, the IC_{50} values of tested compounds were converted to log values and thereafter averaged for every compound. The magnitude of the log values for a given cell line was then plotted as a bar diagram to the right or to the left of a vertical line, corresponding to the average IC_{50} values. In the mean graph the bars appearing on the left or on the right side of the averaged IC_{50} value indicate cell lines with lower or higher than average activity.

The oligonucleosomal DNA fragmentation was detected using a commercially available Cell Death Detection® ELISA kit (Roche Diagnostics GmbH, Germany). Briefly, cells were exposed to varying concentrations of *cis*-$[PtL_2Cl_2]$ for 24 h and then cytosolic fractions of 1×10^4 cells per group (treated or untreated) served as antigen source in a sandwich ELISA, utilizing primary anti-histone antibody-coated microplate and a secondary peroxidase-conjugated anti-DNA antibody. The photometric immunoassay for histone-associated DNA fragments was executed according to the manufacturers' instructions at 405 nm, using ELISA reader (Uniscan Titertec). The results are expressed as the oligonucleosome enrichment factor (representing a ratio between the absorption in the treated vs. the untreated control samples).

6.2.4. Data processing and statistics

Cytotoxicity data were processed using commercially available software packages (Microsoft Excel and GraphPad Prizm for PC). The MTT survival plots were fitted to sigmoidal dose–response curves and the corresponding IC_{50} values were calculated on the basis of 8 separate wells, run in triplicate. The statistical procedures included Student's *t*-test with $p \leq 0.05$ set as significance level.

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