Abstract. Background/Aim: Platinum-based drugs are a very potent class of anticancer drugs commonly used in anticancer therapy. However, resistance development and severe adverse effects make further research on new platinum derivatives necessary. In this study, cytotoxic activity of a new platinum(II) compound containing tris(2-carboxyethyl)phosphine (TCEP) ligand cis-[PtCl₂(TCEP)₂] was tested against canine lymphoma and leukemia cell lines and its activity was compared to that of cisplatin. Materials and Methods: Cells were exposed for 24 h to increasing concentrations of the studied compounds and cell viability was assessed by propidium iodide staining. Disturbances in apoptosis rate and cell cycle were also examined. Results: Cytotoxic activity of the new platinum complex cis-[PtCl₂(TCEP)₂] was higher compared to that of cisplatin and cell death was associated with apoptosis. However, the tested compound differently affected the cell-cycle progression than cisplatin. Conclusion: The cis-[PtCl₂(TCEP)₂] seems to be a potent anticancer agent, although further investigations are necessary to elucidate its biological activity.

Platinum-based drugs constitute a very active class of anticancer agents broadly used in human medicine against a wide spectrum of cancers (1). The first such a drug introduced into medicine was cisplatin (cis-[PtCl₂(NH₃)₂] cis-diamininedichloroplatium CDDP), approved by Food and Drug Administration in 1978 (2). In human medicine, cisplatin is used against ovarian, testicular, bladder, colorectal, small-cell lung, esophageal, cervical, head and neck cancers, lymphomas, melanomas, and many others (1, 3, 4). Cisplatin has been also introduced in veterinary medicine, where it is used in systemic or local chemotherapy, as a single agent or as a part of a combination therapy (5). Its usefulness in treatment of canine osteosarcoma as an adjuvant therapy to amputation was strongly confirmed, and cisplatin was proved to significantly prolong survival time after surgery (6-9). Cisplatin is also used as a radio-sensitizer in dogs (10) and in intralesional therapy of cutaneous tumors such as squamous cell papillomas (SCC) and sarcoïds in horses (11, 12). Nevertheless, cisplatin usage is limited by inherent or acquired resistance and strong toxicity including nephrotoxicity, peripheral neuropathy, ototoxicity, nausea and vomiting (13, 14).

Many efforts have been made to develop new analogs of cisplatin with other mechanisms of action and with activity against cancers resistant to cisplatin (2, 15). Advances in understanding the mechanism of action and resistance to cisplatin enabled a design of more efficient platinum-based drugs to benefit cancer patients (2). To date, two such drugs have gained worldwide approval: carboplatin with similar to cisplatin mode of action and oxaliplatin that forms adducts with DNA strand different than both, cisplatin and carboplatin and thus has a different spectrum of activity (1, 2, 4). Nevertheless, it is still necessary to develop new platinum-based drugs that show other modes of action and activate different pathways leading to an induction of apoptosis. This effort seems to be worth making due to high anticancer potential of this class of drugs and the resistance and strong side-effects of existing platinum-based agents. Recently, a new platinum-based compounds containing a phosphine ligand tris(2-carboxyethyl)phosphine [Pt(CH₂CH₂COOH)₃] (TCEP) instead of ammonia group and two chlorine atoms in cis and in trans conformation with potential anticancer activity has been introduced (16). TCEP is a fast and strong
sulphhydryl reducing agent cleaving disulfide linkage in proteins, peptides and other compounds containing S-S bond (16, 17). Since 1991, when a direct and convenient method of TCEP synthesis was manufactured (17), it has been commercially available and widely used in biochemistry as a reducing agent to mimic natural, reducing environment, so that sulphhydryl groups of proteins, including enzymes, could remain in their reduced form and preserve their activity in solutions (18). It was also observed that TCEP can reduce disulfide bonds between cysteine residues in proteins and peptides and subsequently cleave cysteine-containing proteins (19). TCEP is often used as a reducing agent in the study of interactions of proteins with metal complexes (16) and recently it has been discovered that it promotes the reaction of cisplatin with zinc finger protein Sp1 (20). Specificity protein (Sp) family is a large family of transcription factors belonging to zinc finger proteins, that may regulate an expression of multiple genes involved in the cell cycle, proliferation, differentiation, apoptosis and tumorigenesis (21). Specifically, over-expression of Sp proteins, including Sp1, is involved in cancer development and hence may be used as targets for cancer chemotherapy (21, 22).

The aim of the study was to compare the anticancer effect of platinum(II) complex with tris(2-carboxyethyl)phosphine, cis-[PtCl₂(TCEP)₂] (Pt-TCEP) and cisplatin on canine lymphoma (CLBL-1, CL-1) and leukemia (GL-1) cell lines.

Materials and Methods

Cell lines and culture. Different canine lymphoma/leukemia cell lines CLBL-1 (B-cell lymphoma cell line), GL-1 (B-cell leukemia) and CL-1 (T-cell lymphoma) were used in this study. CLBL-1 cells were obtained from Barbara C. Ruetgen, Department of Pathobiology, Institute of Immunology, at the University of Veterinary Medicine in Vienna (23) and GL-1 and CL-1 were obtained from Yasuhiro Fujino and Hajime Tsujimoto, respectively, from the Department of Veterinary Internal Medicine at the University of Tokyo (24, 25). CLBL-1, GL-1, and CL-1 cell lines were maintained in RPMI 1640 culture medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and 10% heat-inactivated fetal bovine serum (FBS). The culture was maintained in the atmosphere of 5% CO₂ and 95% humidified air at 37°C. The cells were cultured in 75 cm² cell culture flasks (Corning, USA) and subcultivated every other day to keep at optimal density (i.e. 60-70% of confluency).

Chemicals and reagents. The cis-[PtCl₂(TCEP)₂] was prepared by procedures reported earlier (16). TCEP [Pt(CH₂CH₂COOH)₂], CDDP [cis-[PtCl₂(NH₃)₂]], fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin solution, ribonuclease A and propidium iodide were purchased from Sigma-Aldrich (Steinheim, Germany). Dimethyl sulphoxide (DMSO) and ethanol were acquired from POCh (Gliwice, Poland). Annexin V FITC was bought from Immunostep (Salamanca, Spain). Phosphate buffered saline (PBS) and RPMI 1640 culture medium were obtained from the Institute of Immunology and Experimental Therapy (Wroclaw, Poland).

Drug preparation. Pt-TCEP and TCEP were dissolved in DMSO to prepare 5 mM stock solutions and CDDP was dissolved in 0.9% sterile saline to prepare 2.5 mM stock solution. The stock solutions were stored at ~20°C until they were added to the culture medium to obtain target experimental solutions.

Determination of cell viability. To evaluate and compare the effects exerted by Pt-TCEP and CDDP on the cultures of canine lymphoma (CLBL-1, CL-1) and leukemia (GL-1) cell lines, cells were treated with increasing concentrations of Pt-TCEP (0.078, 0.156, 0.3125, 0.625, 1.25, 2.5, and 5 μM) and CDDP (0.625, 1.25, 2.5, 5, 10, 20, and 40 μM) for 24 h. Additionally, TCEP alone, as a substrate used in the synthesis of the studied compound, was examined at the concentrations similar to those of Pt-TCEP. The cells were seeded in concentration of 1×10⁴ cells per well in a 96-well-plate (TPP, Trasadingen, Switzerland). The compounds were prepared at indicated dilutions in the culture medium (DMSO concentration was less than 0.1% in each dilution, which is considered harmless to the cells) and added to the plates with cells. The cells were incubated in the medium alone or in the medium containing either the vehicle control or increasing concentration of the tested substance for 24 h. After the incubation, the cells were harvested and washed twice in PBS, transferred into cytometric tubes and stained with propidium iodide (PI, final concentration 1 μg/mL). Flow cytometry analysis was performed immediately after, using the flow cytometer FACS Calibur (Becton Dickinson, Biosciences, San Jose, USA). CellQuest 3.1f software (Becton Dickinson, San Jose, USA) was used for data analysis on the basis of histograms of FL2-H showing the population of live (PI negative) and dead (PI positive) cells. The values presented as means with standard deviation were obtained from three independent experiments and showed as a concentration-dependent curve. IC₅₀ for Pt-TCEP and CDDP was calculated as a mean concentration inhibiting cell viability by 50% in three independent experiments.

Apoptosis analysis. At the next stage we checked whether the inhibition of cell viability was associated with apoptosis. For this purpose, flow cytometry analysis of Annexin-V binding to phosphatidylserine residues was performed. For comparison of the apoptosis rate after 24 h exposure to Pt-TCEP in three studied cell lines, the concentration of 1 μM was chosen based on the results of cell viability assay. Additionally, the cells of three canine cancer cell lines were treated with 15 μM of CDDP. Apoptotic events were detected using Annexin V-FITC/PI staining. Briefly, the cells were treated with the tested compounds at the indicated concentrations for 24 h. After harvesting, the cells were washed twice with cold PBS, suspended in a binding buffer and stained with Annexin V-FITC for 10 min at room temperature. After the incubation, propidium iodide (PI) was added and flow cytometric analysis was immediately performed using a flow cytometer (FACS Calibur; Becton Dickinson, Biosciences, San Jose, USA). CellQuest 3.1f software (Becton Dickinson, San Jose, USA) was used for data analysis.

Cell-cycle analysis. GL-1 cell line that appeared to be moderately sensitive to Pt-TCEP in cell viability assay was used for cell cycle analysis. Briefly, GL-1 cells were seeded in 24-well plates (TPP, Trasadingen, Switzerland) for 24 h with the tested compounds at a concentration of 1 μM (Pt-TCEP) and 15 μM (CDDP). After incubation, the cells were harvested and washed twice in PBS.
Then, they were fixed in cold 70% ethanol and incubated for at least one hour at 4°C. After the second cycle of washing, the cells were treated with ribonuclease (final RNAse concentration 0.5 mg/mL), incubated for 1 h and stained with propidium iodide (PI) (final concentration 10 μg/mL). The sample analysis was performed using a flow cytometer (FACS Calibur; Becton Dickinson, Biosciences, San Jose, CA) and CellQuest 3.1f software. Based on FL-2H histograms, percentage of the cells in sub-G0, G0/G1, S and G2/M phase was compared with the results for untreated controls. The presented results were obtained from three independent experiments.

**Statistical analysis.** All data were shown as means with standard deviations (SD). Statistical difference was analyzed using Student’s t-test for normally distributed values and one-way ANOVA followed by Tukey’s post hoc test was used to test statistical differences among treatment groups. The results were considered significant when \( p<0.05 \).

**Results**

**Determination of cell viability.** The study demonstrated that incubation with Pt-TCEP for 24 h induced cell death in canine cancer cell lines in a concentration-dependent manner (Figure 1). The efficacy of the new compound was much higher than that of CDDP (\( p<0.05 \)). Furthermore, in CLBL-1 and GL-1 cell lines treated with 2.5 μM Pt-TCEP nearly 100% of the cells were killed (cell viability at this concentration was 5.33±2.21% and 1.68±0.47% for GL-1 and CLBL-1 cell line, respectively), while viability of the cells exposed to cisplatin at this concentration was not statistically different from the control (\( p>0.05 \)). Viability of CL-1 cell line at the highest concentration of Pt-TCEP (5 μM) was reduced to 26.50±6.1% and at the same concentration of CDDP it was reduced to 79.70±1.32%. TCEP alone did not exhibit any cytotoxic activity at the concentrations analogous to those of Pt-TCEP.

The IC\(_{50}\) for Pt-TCEP after 24 h treatment is shown in Figure 2. CLBL-1 line was the most sensitive to Pt-TCEP with IC\(_{50}\) of 0.92±0.05 μM, while CL-1 cell line was the most resistant and its viability was inhibited by 50% at Pt-TCEP concentration of 2.59±0.03 μM (\( p<0.05 \)). IC\(_{50}\) for Pt-TCEP in GL-1 cell line was determined at 1.35±0.07 μM. IC\(_{50}\) of CDDP was also calculated and it was 25.98±3.74 μM and 22.99±2.42 μM for GL-1 and CLBL-1 cell line, respectively. As shown in Figure 3, viability of CL-1 cell line treated with 10 μM CDDP decreased to 67.13±1.58% but at higher CDDP concentrations it increased to over 70% and finally IC\(_{50}\) was not achieved at the highest concentration studied (40 μM). Moreover, cell viability assay showed that although at 5 and 10 μM CDDP the sensitivity of CL-1 cell line was higher than that of the other two cell lines, at higher CDDP concentrations the sensitivity of CL-1 cell line decreased and at the highest concentration studied (40 μM) it was lower than in GL-1 and CLBL-1 cell lines (\( p<0.05 \)).

**Apoptosis analysis.** The percentage of apoptotic cells after 24 h treatment with 1 μM Pt-TCEP increased to 31.25±1.16%, 65.53±2.89% and 12.35±0.44% for GL-1, CLBL-1 and CL-1 cell line, respectively (\( p<0.05 \)) (Figure 4). CDDP at 15 μM enhanced the percentage of apoptotic...
cells to 37.80±3.9%, 45.67±5.58% and 37.80±5.76% for GL-1, CLBL-1 and CL-1 cell lines, respectively (p<0.05). This indicated that the apoptosis rate following the exposure to 15 μM CDDP was similar in all cell lines investigated in the study (p>0.05). However, taking into account the results of cell viability assay (Figure 3), it was also examined whether the percentage of apoptotic cells in CL-1 cell line increased at higher concentrations of CDDP. It turned out that the percentage of apoptotic cells did not increase in a concentration-dependent manner and at 40 μM of CDDP was the same as at 15 μM (p>0.05), reflecting the results of the viability assay (data not shown). Representative dot plots of Annexin V-FITC/PI staining of GL-1, CLBL-1 and CL-1 cell lines after incubation with 1 μM Pt-TCEP and 15 μM CDDP are shown in Figure 5.

Cell-cycle analysis. Incubation with 1 μM of Pt-TCEP increased the percentage of cells in sub-G0 phase from 2.96±0.75% in control to 31.50±5.73% and decreased the percentage of cells in G0/G1 phase from 65.03±2.36% in control to 53.20±3.08%, in S phase from 14.95±1.59% in control to 8.11±1.99%, and in G2/M phase from 17.03±1.99% in control to 7.21±1.38% (p<0.05) (Figure 6). In CDDP variant, the increase in the percentage of cells in sub-G0 reached 48.27±5.66% after the incubation with 15 μM CDDP, as compared with 2.96±0.75% in control. CDDP decreased the amount of cells in the G0/G1 phase from 65.03±2.36% in control to 25±2.54% and the cells in G2/M phase from 17.03±1.99% in control to 7.67±0.46% (p<0.05). Contrary to Pt-TCEP, the decrease in amount of cells in S phase was not observed and was calculated to be 19±3.05% (p>0.05).

Discussion

Due to high activity of platinum-based compounds, research on new derivatives with intensified cytotoxic activity to enhance the antineoplastic efficacy of platinum drugs are constantly being undertaken. Many new analogs of cisplatin have been designed and tested. Some of them were successful in clinical trials and were introduced in medical practice. Other are still under scrutiny (1, 4, 26). Platinum complexes with phosphine derivatives were also investigated for their anticancer properties and some of them exhibited higher cytotoxic activity than cisplatin. They were also active against cancer cell lines resistant to...
cisplatin (27-31). Phosphine ligands combined with other metals such as gold or palladium also showed a cytotoxic potential (32-35).

The study clearly indicated that Pt-TCEP was more potent in inducing cell death than CDDP in all three canine cancer cell lines. As shown in cell viability assay, Pt-TCEP reduced cell viability at lower concentrations than CDDP. Additionally, CDDP was not able to reduce cell viability in concentrations similar to those, which indicated cell viability inhibition in the case of Pt-TCEP. CLBL-1 cell line was the most sensitive and CL-1 the most resistant to Pt-TCEP. These results are similar to the results of previous studies, in which CL-1 cell line was the most resistant and CLBL-1 the most sensitive to various anticancer drugs such as cytosine arabinose, chlorambucil, 4-HO-cyclophosphamide, etoposide, and vincristine (36). The results for CDDP were not that obvious. There was no significant difference in the sensitivity of GL-1 and CLBL-1 cell line, while cell viability of CL-1 cell line decreased to 67.13±1.58% at 10 μM CDDP but increased to over 70% at higher concentrations. However, Pt-TCEP at 5 μM was capable of decreasing cell viability in this cell line to 26.50±6.1%, while for CDDP at this concentration the cell viability decreased only to 79.70±1.32%.

The effect of TCEP was also evaluated. It showed no inhibitory activity towards canine lymphoma (CLBL-1, CL-1) and leukemia (GL-1) cell lines at the applied
concentrations. Other studies also indicated that it was not only harmless to the cells but also that its reductive properties could protect cells from deleterious effects of reactive oxygen species (ROS). In a rat optic nerve crush model of axotomy TCEP was found to be neuroprotective in in vivo model of retinal ganglion cell (RGC) axonal damage (37). In vitro studies of RGC survival under reduced conditions showed that incubation of the retinal ganglion cells with TCEP resulted in a long-term survival equivalent to or better than the incubation with neurotrophic factors (38). However, higher concentration of the agent was used to achieve this (100 μM), and at lower concentrations TCEP did not show any neuroprotective activity (37, 38).

The study demonstrated the degree to which the cell death related to apoptosis, the main mechanism by which DNA-binding anticancer drugs, such as platinum based drugs, cause death of cancer cells (39). We showed that similar concentrations of Pt-TCEP were responsible for the inhibition of cell viability and apoptosis. As demonstrated in cell viability assay, at 1 μM Pt-TCEP cell viability in GL-1 cell line were reduced to approximately 70%, while the apoptosis rate was 31.25±1.16%. CLBL-1 viability was inhibited by ca. 50%, and the percentage of apoptotic cells was 65.53±2.89% at this concentration. In CL-1 cell line, the inhibition of viability was not detected, while the apoptosis rate was estimated to be 12.35±0.44%. These results showed that the cells exposing phosphatidylserine on the outer leaflet of the membrane also lost their membrane integrity and apoptosis was responsible for cytotoxicity of Pt-TCEP. Apoptosis rate for CDDP was similar in all cell lines and amounted to 37.80±3.9% for GL-1, 45.67±5.58% for CLBL-1, and 37.80±5.76% for CL-1. Although apoptosis rate in CL-1 cell line at 15 μM CDDP was similar to that in GL-1 and CLBL-1 cell lines, and in Pt-TCEP variant the percentage of apoptotic cells was much lower than in the other cell lines, further investigation revealed that at higher concentrations of CDDP the percentage of apoptotic cells did not increase and matched the results for cell viability assay in this cell line. Nevertheless, apoptotic events caused by Pt-TCEP were detected at the concentration 15-times lower than in the case of CDDP.

The most common cell cycle perturbations caused by cisplatin and other platinum-based drugs is a transient slowing of cell cycle progression in S phase and its arrest in G2/M phase (40-43). Cell cycle arrest in G2/M phase is required to repair the damage and prevent mitosis of damaged cells (44). The cells with cisplatin-damaged DNA slowdown in the transit through S phase and when they reach G2 phase they remain there for some time and, depending on the level of DNA damage, may return to the cycle and divide or undergo apoptosis (40, 45). However, the referenced studies were conducted at lower concentration of CDDP applied for only two hours and these observations were made during several days after the drug exposure (40, 41, 43, 45). With increasing dose of the drug and longer periods of exposure this influence could not be showed so clearly due to high concentration of cells in sub-G0 phase indicating that the cells might undergo apoptosis (46). Similar results were achieved in this study, where, due to high concentrations of CDDP used for a long period of time, the percentage of cells in sub-G0 phase significantly increased, and the percentage of cells in G0/G1 significantly decreased. A decrease in the percentage of G2/M cells was also observed but not in S phase, where the percentage of cells was not statistically different from the control. Nevertheless, the impact of Pt-TCEP on the cell cycle was different than that of CDDP. The increase in the percentage of the sub-G0 cells was smaller, and the decrease in the percentage of the G0/G1 cells was not evident. Contrary to cisplatin, Pt-TCEP reduced the number of cells in the S phase, while in the G2/M phase the effect of both compounds was similar. This may indicate that Pt-TCEP did not arrest the cell cycle in the S phase before inducing apoptosis and that the mechanism involved in cell death triggered by the studied compound was not similar to that of CDDP, but further studies at various Pt-TCEP concentrations and exposure times are needed to confirm this.

This study clearly showed that Pt-TCEP was more potent in inducing cell death than CDDP in canine cancer cell lines. However, further investigations are needed to elucidate its biological activity.

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References


