

Effect of the PARP inhibitor veliparib on germ cell tumor cell lines

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Abstract. Germ cell tumors (GCTs) usually represent efficiently curable neoplasms due to their chemosensitivity to platinum-based therapeutic regimen. However, some patients develop therapeutic resistance and succumb to their disease. Novel therapeutic approaches are therefore needed for these patients. It has previously been demonstrated that poly (ADP-ribose) polymerase (PARP) expression is upregulated in GCTs compared with normal testis tissue. Therefore, PARP expression was analyzed in GCT cell lines and xenografts and it was examined whether its inhibition by veliparib can reverse cisplatin-resistance. Its expression was analyzed in sensitive and cisplatin-resistant variants (referred to as CisR throughout the manuscript) GCT cell lines and xenografts using quantitative PCR, western blotting and immunohistochemistry. The present study investigated whether the combination of cisplatin with the PARP inhibitor veliparib increased the cytotoxic effect of cisplatin *in vitro* using a luminescent viability assay and an immunodeficient mouse model *in vivo*. PARP expression was observed in all tested cell lines, with the highest expression in embryonal carcinoma (EC) cell lines and xenografts. Low or no expression was detected in the JEG-3 choriocarcinoma cell line pairs and xenografts. The combination of veliparib and

cisplatin or carboplatin was examined in the cisplatin-resistant NTERA-2 CisR and NCCIT CisR EC cell lines and synergistic effects were observed in NTERA-2 CisR cells. However, *in vivo* analysis did not confirm this synergy. The present data indicated PARP expression in GCT cell lines and xenografts. However, veliparib failed to increase the cytotoxicity of platinum-based drugs. Therefore, further research is warranted to effectively inhibit PARP using different PARP inhibitors or other drug combinations.

Introduction

Germ cell tumors (GCTs) are a heterogeneous group of neoplasms arising in the gonads, both the ovaries and the testes. Due to the migration of primordial germ cells along the midline of the body, GCTs can also arise in extragonadal sites and the brain (1). Testicular germ cell tumors (TGCTs) are rare solid tumors that account for 1% of cancers in men. However, in young males between the ages of 15 and 44 years, TGCTs represent the most frequent solid malignancy and have the highest mortality (2). The incidence rates of TGCTs have been steadily rising in young men (3). In general, TGCT treatment by cisplatin-based therapy is highly successful, even when the disease is highly metastatic (4). Unfortunately, acquired resistance to chemotherapy is the major barrier to curing patients with refractory disease and results in poor outcomes. Approximately 50% of these patients die from progressive disease (5). The mechanism of cisplatin resistance in TGCTs remains unknown, although some mechanisms have been proposed (6-9).

It has been previously shown that polyadenosine diphosphate-ribose polymerase (PARP) is overexpressed in TGCTs compared to normal testis (10). PARPs represent a family of 17 enzymes associated with several cellular processes, such as DNA repair, genome maintenance and cell death (11). The most well-studied member of the PARP family is PARP1, which

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has a key role in the detection of single-strand DNA breaks and repair initiation (12). Moreover, more than 80% of overall PARP activity is constituted by PARP1, which has also been identified as a platinum-DNA damage response protein (13). Therefore, there is a strong rationale to target the enzymatic activity of PARPs and use PARP inhibitors (PARPi) as a new therapeutic strategy in the treatment of cancer.

PARPi are a class of anticancer drugs that compete with nicotinamide for the catalytically active sites of PARP molecules. PARP inhibition proved to be a successful strategy in the treatment of homologous recombination repair (HRR)-deficient tumors, especially tumors with mutations in the essential HR genes *BRCA1* and *BRCA2* (14-17). This synthetic lethal interaction between PARP inhibition and mutations in *BRCA1* or *BRCA2* was discovered by two independent research groups in 2005 (18,19). Olaparib, rucaparib, niraparib, talazoparib, pamiparib, and veliparib are PARPi used in the clinic or under investigation in several trials (20). The initial clinical development of PARPi was based on potentiating tumor cell killing by DNA-damaging agents such as platinum-based chemotherapeutics. A synergistic effect of cisplatin and PARP inhibition was shown in three human esophageal cancer cell lines (21). Another drug combination experiment revealed that the PARPi olaparib and veliparib potentiated the killing of non-small cell lung cancer cells by cisplatin (22). The combination of veliparib with cisplatin or carboplatin increased the recurrence-free and overall survival of a genetically engineered mouse model for BRCA1-associated breast cancer (23). Another study showed synergistic cytotoxicity of olaparib and cisplatin against BRCA2-deficient mammary tumor cells (24). Many clinical trials have been confirmed that the effect of PARP inhibition observed preclinically could be recapitulated in patients (25-32).

In this study, we hypothesized that the PARPi veliparib would synergistically increase the cytotoxicity of platinum-based drugs and reverse cisplatin resistance in refractory GCTs. We used a series of cisplatin-resistant variants (referred to as CisR throughout the manuscript) cell line models and analyzed the expression of PARP in these cell lines and derived xenografts as well as their sensitivity to veliparib, and we performed combined treatment with cisplatin and carboplatin.

Materials and methods

Chemicals. Chemicals were purchased from Sigma-Aldrich if not stated otherwise.

Cell lines. NTERA-2 (human embryonal carcinoma, ATCC® CRL-1973™) and JEG-3 (choriocarcinoma, ATCC® HTB-36™) cell lines were maintained in high-glucose (4.5 g/l) DMEM (PAA Laboratories GmbH) containing 10% FBS (GIBCO® Invitrogen), 10,000 IU/ml penicillin (Biotica, Part. Lupca, Slovakia), 5 µg/ml streptomycin, 2.5 µg/ml amphotericin and 2 mM glutamine (PAA Laboratories GmbH). TCam-2 (human testicular seminoma, kindly provided by Dr Kitazawa, Ehime University Hospital, Shitsukawa, Japan), NCCIT (embryonal carcinoma, ATCC® CRL-2073™) and NOY-1 (ovarian yolk sac tumor, cat. no. ENGI01, FA; Kerafast) cell lines were cultivated in RPMI (GIBCO® Invitrogen)

containing 10% FBS, 10,000 IU/ml penicillin, 5 µg/ml streptomycin, 2.5 µg/ml amphotericin and 2 mM glutamine. Cisplatin-resistant variants of parental cell lines, designated CisR, were all derived by propagating the cells in increasing concentrations of cisplatin (Hospira UK Ltd.) for 6 months as described previously (33-35). All cell lines were cultivated at 37°C in a humidified atmosphere and 5% CO₂.

RNA extraction, cDNA synthesis and RT-qPCR. Total RNA was extracted using a NucleoSpin® RNA II kit (Macherey-Nagel) and treated with RNase-free DNase (Qiagen). A RevertAid™ H minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) was used for cDNA synthesis. RT-qPCR was run in the AriaMx Real-time PCR System (Agilent) using commercially available PARP1 and PARP2 gene expression assays (TaqMan™ gene expression assay IDs: Hs00911369_g1, cat. no. 4331182, Dye FAM-MGB and Hs00193931_m1, cat. no. 4331182, Dye FAM-MGB, Applied Biosystems®). HPRT1 was used as housekeeping gene (TaqMan™ gene expression, assay ID Hs03929098_m1, cat. no. 4331182, Dye FAM-MGB, Applied Biosystems®).

TaqMan™ Fast Advanced Master Mix (Applied Biosystems®) was used according to the manufacturer's protocol, thermocycling conditions as follows: 50°C 2 mins, 95°C 20 sec, followed by 45 cycles of 95°C 3 sec and 60°C 30 sec. The obtained data were analyzed by Agilent Aria software version 1.5. Relative gene expression changes were examined using the 2^{-ΔΔC_q} method (36). All samples were analyzed in triplicate, and the data are expressed as the means ± SEMs. The significance of fold changes in gene expression between groups was analyzed using the software tool REST (REST 2009-RG Mode, Qiagen) for groupwise comparison and statistical analysis of relative expression results in quantitative PCR (37).

Western blotting. Cell lysates were prepared using RIPA buffer (cat. no. 9806; Cell Signaling Technology, Inc.) supplemented with PhosSTOP phosphatase inhibitor (Roche) and cOmplete protease inhibitor (Roche) and centrifuged for 10 min at 14,000 x g at 4°C, and protein concentrations were determined by a Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Protein extracts were resolved to a final concentration of 30 µg protein per sample. Protein electrophoretic separation was performed on a 7.5% dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred onto a nitrocellulose membrane (Thermo Fisher Scientific, Inc.). Subsequently, the membrane was blocked overnight at 4°C in 5% nonfat dry milk in Tris-buffered saline (TBS). The next day, the membrane was incubated for 1 h at room temperature with primary anti-PARP1 antibody (ab137653; Abcam; dilution 1:1,000, 113 kDa). The membrane was washed in Tris buffer saline with Tween-20 (TBS-T) and reacted for 1 h at room temperature with horse peroxidase secondary anti-rabbit antibody (cat. no. 7074S; Cell Signaling Technology, Inc.; dilution 1:1,000). The bands were viewed by Super Signal™ West Dura Extended Duration chemiluminescence detection substrate (Thermo Fisher Scientific, Inc.). Finally, signals were visualized by a Li-Cor scanner (Image Studio™ Lite Software). The same membrane was washed in TBS-T and incubated for 1 h at room temperature with β-actin primary antibody (A1978;

Sigma-Aldrich; dilution 1:4,000, 42 kDa). Subsequently, the membrane was washed again and reacted for 1 h at room temperature with horse peroxidase secondary anti-mouse antibody (cat. no. 7076S; Cell Signaling Technology, Inc.; dilution 1:2,000). The signals were visualized in the same way. Western blotting analysis was repeated three times. The ImageJ 1.46r program (NIH) was used to measure PARP1 and β -actin densities for densitometric analysis, and the obtained data were statistically processed.

Viability assay. Cells were plated at 3×10^3 – 5×10^3 cells/100 μ l media per well and seeded in 96-well white-walled plates (Corning Costar Life Sciences) overnight. The cells were treated with veliparib (SelleckChem, 25–175 μ M) or in combination with cisplatin (Hospira UK Ltd, 0.1–0.8 μ g/ml), carboplatin (Fresenius Kabi Oncology Plc., 1–20 μ g/ml) or novobiocin (Carl Roth, 50–250 μ M). The relative viability of the cells was determined by the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega Corporation) and evaluated by the GloMax Discover System reader (Promega Corporation) after 3 days of treatment. Experiments were performed in biological quadruplicates, each with experimental triplicates. Values are expressed as the means \pm SDs. The combined effect of drugs was calculated according to Chou (38) using Calcsyn software (Biosoft) (39). The combination index (CI) was computed for every affected fraction (fa, proportion of dead cells): CI <1 represents synergism, CI=1 additivity and CI >1 antagonism.

In vivo experiments. Six- to 8-week-old SCID beige mice (CD17 Cg-Prkdcscid Lystbg/Crl, Charles River) or NSG mice (The Jackson Laboratory) were used in accordance with institutional guidelines under approved protocols. Project was performed in the Animal Facility for Immunodeficient Mice of the Biomedical Research Center SAS Bratislava (license no. SK UCH 02017). Project was approved by the Institutional Ethic Committee of the Biomedical Research Center SAS Bratislava and by State Veterinary and Food Administration of the Slovak Republic as the national competence authority under the registration no. Ro 1030/18-221 in compliance with the Directive 2010/63/EU and the Regulation 377/2012 on the protection of animals used for scientific purposes. Sacrifice of the animals at the experiment endpoint was done by induction of anaesthesia by 3.0–3.5% isoflurane for 7–10 min. Signs of muscle relaxation and loss of consciousness were observed. The deep anaesthesia was followed by cervical dislocation and the death of animals was determined as the absence of a corneal reflex, failure to detect respiration, and the absence of a heartbeat for a period of more than 5 min to confirm death. The largest long diameter of the xenograft in this study reached 14.6 mm and the largest short diameter reached 10.4 mm (for details see the respective Results subsection). Total number of animals used for this study was n=22.

To produce GCT cell line xenografts for PARP1 immunohistochemical analysis, a suspension of 2×10^6 of NTERA-2, NTERA-2 CisR, NCCIT, NCCIT CisR, TCam-2, TCam-2 CisR, JEG-3, JEG-3 CisR, NOY-1 and NOY-1 CisR cells in 100 μ l of extracellular matrix (ECM) mixture 1:1 (50 μ l serum-free DMEM or RPMI medium, 50 μ l ECM) was injected bilaterally s.c. into each of the flank of NSG mice

(n=2 animals per cell line pair, total n=10). Xenografts were measured by caliper and animals were sacrificed at the point when the tumors exceeded 1 cm in diameter. One representative tumor xenograft was used for IHC staining, details are listed in the results section.

To test the effect of veliparib *in vivo*, a suspension of 2×10^5 NTERA-2 CisR in 100 μ l of extracellular matrix mixture (1:1, 50 μ l serum-free DMEM, 50 μ l ECM) was injected s.c. into the flanks, for a total of 2 injections per SCID mouse (n=4 per group/treatment). Mice were divided into three groups according to the treatment: cisplatin i.p./veliparib and cisplatin i.p./untreated controls. Tumors were measured by caliper, and volume was calculated according to the formula for the volume of ellipsoid: volume = $0.52 \times ((\text{width} + \text{length})/2)^3$. Animals were sacrificed at the point when the tumors exceeded 1 cm in diameter. The results were evaluated as the mean tumor volume \pm SEM.

Immunohistochemical analysis of PARP1 in GCT cell line xenografts. Slides were deparaffinized, rehydrated and immersed in phosphate buffered saline solution (10 mM, pH 7.2). No epitope retrieval was applied to the slides. The slides were incubated for 2 h with primary anti-PARP1 monoclonal antibody (H-250, dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and immunostained using anti-mouse/anti-rabbit secondary antibody (Nichirei Biosciences, Japan) for 30 min according to the manufacturer's instructions. The reaction was visualized by diaminobenzidine substrate-chromogen solution (DAB, Dako) for 5 min. Ultimately, the slides were counterstained with hematoxylin. We used normal tissue as a positive control, with lymphatic tissue as a positive control for nuclear PARP (40). As a negative control, lymphatic tissue was used, omitting the primary antibody from the staining protocol. Representative images were captured with an Olympus BX40 microscope (Olympus Corporation) and a Canon EOS 1000D (Canon Inc.).

PARP1 expression was scored by the multiplicative quickscore (QS) method, evaluating both the percentage of positive cells and the staining intensity of the nuclei. Briefly, the proportion of cells with nuclear staining was multiplied by the intensity of staining to provide a final quickscore. The average intensity of the positively stained cells was given a score from 1 to 3 (1=weak, 2=intermediate, and 3=strong staining). QS was calculated as follows: QS = (1 x percentage weakly stained) + (2 x percentage moderately stained) + (3 x percentage strongly stained) (10).

Statistical analysis. For the statistical analysis of studies involving comparisons between the two groups, the assumption of normality was tested using the Shapiro-Wilk test, and differences were assessed by Student's t-test or the Mann-Whitney U test depending on the normality of the data. For comparisons involving more than two groups, when the effect of the tested drugs *in vivo* was analyzed, multivariate analysis one-way ANOVA Tukey's Honest Significant Difference test was used. Multiple comparisons were not performed in cases where the overall test did not indicate significant differences across samples. GraphPad Prism® software (GraphPad Inc.) was used. P-values <0.05 were considered statistically significant.

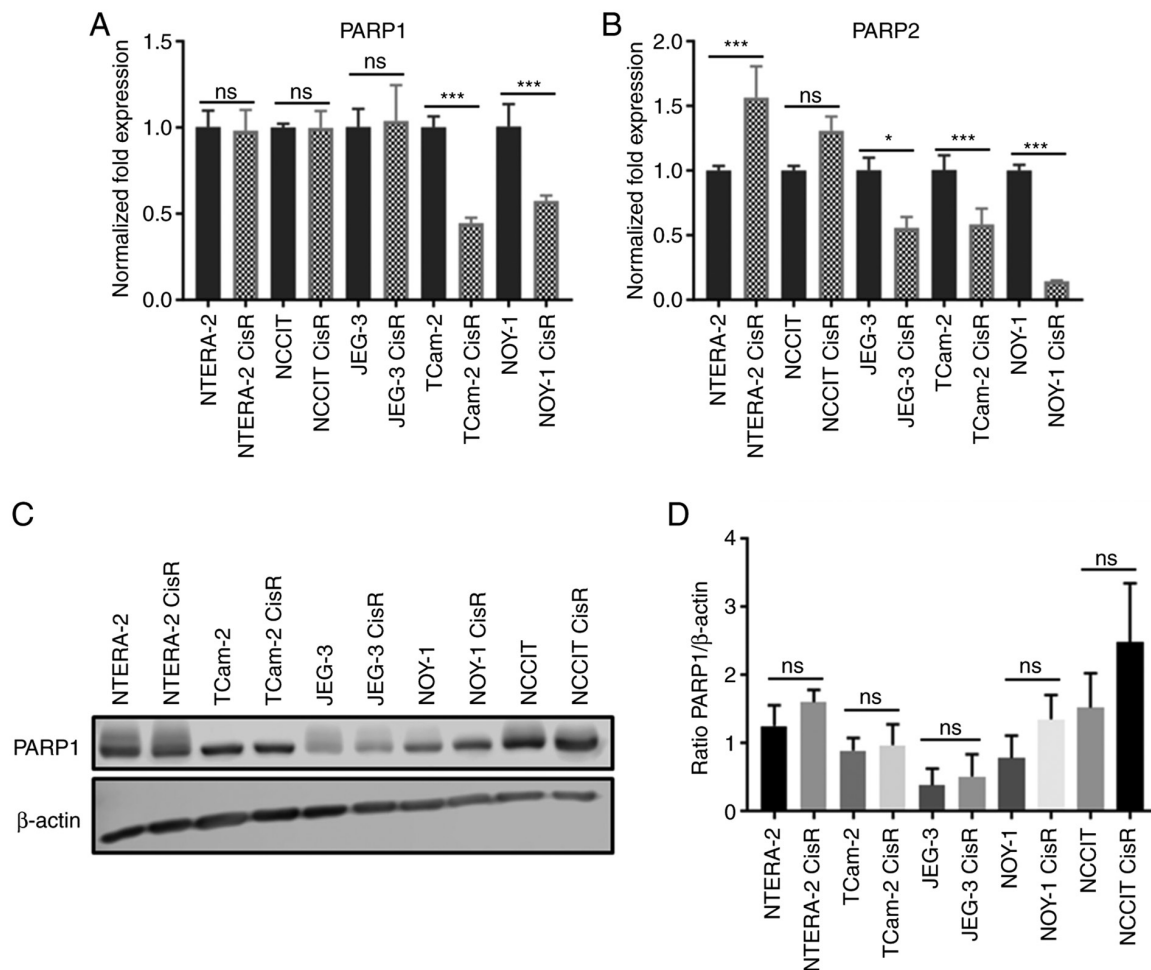


Figure 1. Analysis of PARP expression at the mRNA and protein levels in different germ cell tumor cell lines. (A) Relative *PARP1* gene expression determined by RT-qPCR. (B) Relative *PARP2* expression determined by RT-qPCR (C) Western blot analysis of PARP1 revealed the presence of this protein in all tested cell lines. (D) Densitometric analysis revealed no significant differences between parental and cisplatin-resistant cell line pairs. Unpaired Student's t-test, * $P < 0.05$, *** $P < 0.001$, ns-not significant. PARP, poly (ADP-ribose) polymerase; RT-qPCR, reverse transcription-quantitative PCR; CisR, cisplatin-resistant variant of parental GCT cell line.

Results

Model GCT cell line selected for the study. Five GCT cell lines and their cisplatin-resistant variants, which have been previously characterized (33-35), were used in this study. NTERA-2 and NCCIT represent pluripotent embryonal carcinoma (EC) cell lines, JEG-3 is a choriocarcinoma (ChC) cell line, and NOY-1 was derived from ovarian yolk sac tumor (YST). The only available seminoma (SE) model is the TCam-2 cell line, which was also included in our analyses.

Expression of PARP in different GCT cell lines. PARP1 and PARP2 gene expression was analyzed in all five GCT cell lines and their cisplatin-resistant variants using quantitative RT-PCR. We observed significant downregulation of PARP1 in TCam-2 CisR and NOY-1 CisR cells compared to parental cell lines (Fig. 1A). Similarly, a significant decrease in PARP2 expression was detected in these two cell lines. PARP2 downregulation was also observed in the JEG-3 CisR cell line. Only cisplatin-resistant NTERA-2 CisR cells showed significant PARP2 overexpression compared to sensitive cells (Fig. 1B). Next, we investigated PARP1 protein levels using Western blot

and densitometric analyses. Western blot analysis revealed the highest levels of PARP1 in the EC cell lines NTERA-2 and NCCIT and their resistant variants. Only low levels of PARP1 protein were observed in the ChC cell lines JEG-3 and JEG-3 CisR (Fig. 1C). However, no significant changes between parental and resistant cell lines were detected by densitometric analysis (Fig. 1D).

Immunohistochemical analysis of PARP1 in GCT cell line xenografts. To analyze the expression of PARP *in vivo*, we performed immunohistochemical staining of PARP1 in xenograft models derived after subcutaneous injection of GCT cell lines into an immunodeficient NSG mouse model. Weak to strong PARP1 positivity was present in all xenografts except the ChC JEG-3 and JEG-3 CisR cell line xenograft models, where positivity was absent or very weak corresponding to the data from protein analysis. Representative pictures are shown in Fig. 2A-J, and the results of immunohistochemistry scoring using the QS method are given in Table SI. Semiquantitative analysis of representative xenografts indicated no major differences in percentages of positive cells and/or their staining intensity in parental vs. resistant NCCIT and TCam-2 resulting in similar QS score

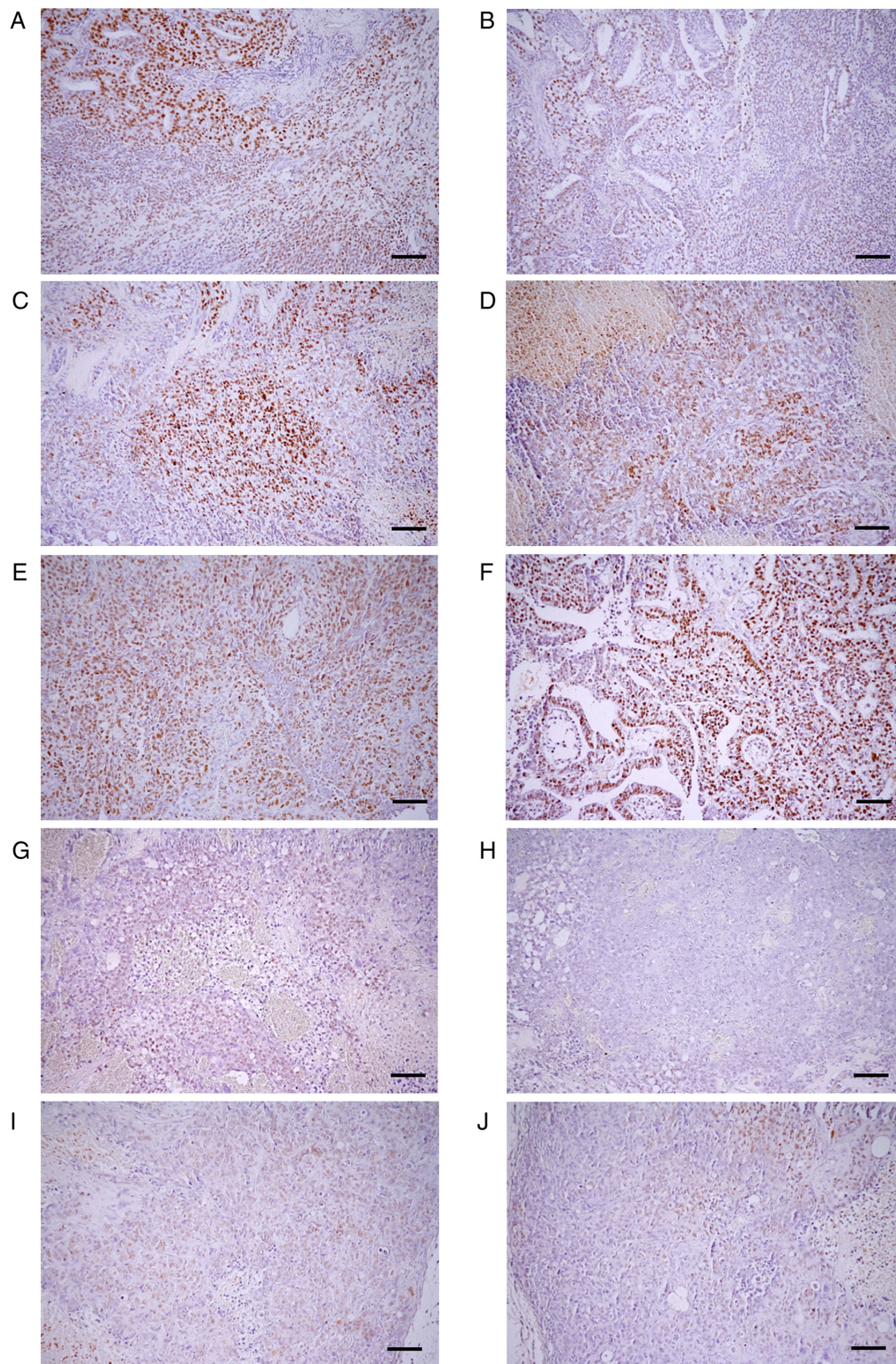


Figure 2. Immunohistochemical analysis of poly (ADP-ribose) polymerase 1 in germ cell tumor cell line xenografts. (A) NTERA-2 xenograft, diffuse strong to weak positivity in tumor cells. (B) NTERA-2 CisR, focal weak to intermediate positivity in tumor cells. (C) NCCIT, diffuse strong to weak positivity in tumor cells. (D) NCCIT CisR, diffuse strong to weak positivity in tumor cells. (E) TCam-2, diffuse intermediate to weak positivity in tumor cells. (F) TCam-2 CisR, diffuse strong to weak positivity in tumor cells. (G) JEG-3, negativity to scattered weak positivity in tumor cells. (H) JEG-3 CisR, negativity in tumor cells. (I) NOY-1, diffuse weak to intermediate positivity in tumor cells. (J) NOY-1 CisR, focal weak to intermediate positivity in tumor cells. Original magnification, x200; scale bar, 100 μ m. Visualization with 3,3'-diaminobenzidine. CisR, cisplatin-resistant variant of parental GCT cell line.

values. NOY-1 CisR cells exhibited lower proportion both in percentages and weak/intermediate intensity of positive cell staining *in vivo*. Interestingly, parental NTERA-2 xenograft exhibited high proportion of PARP1 positive cells (~90%),

which decreased in the resistant variant (~35%) accompanied by the absence of cells with strong staining and low (~5%) proportion of cells with intermediate intensity *in vivo* as reflected in decrease in QS value. We observed, that the NOY-1 CisR and

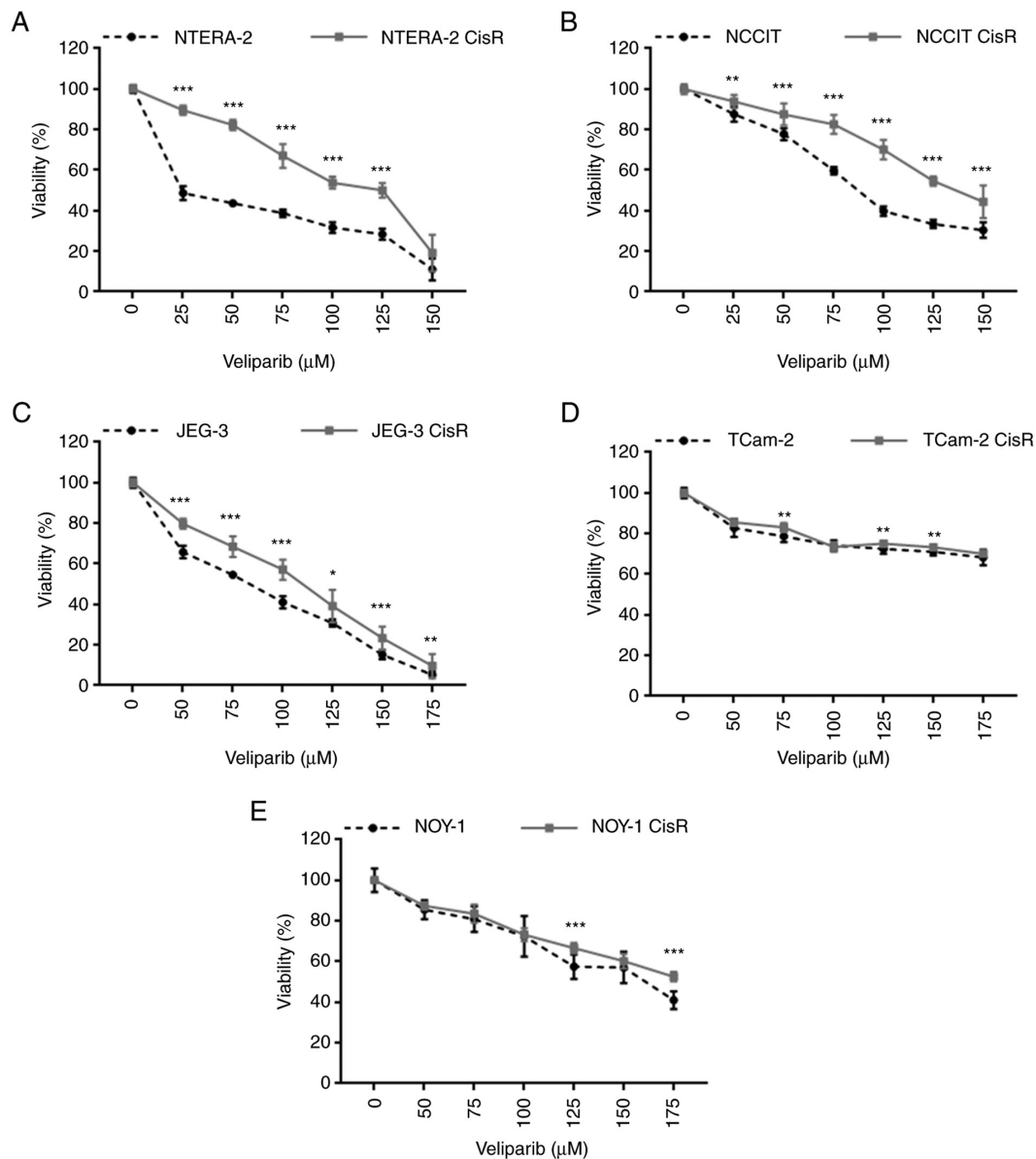


Figure 3. Sensitivity of parental and cisplatin-resistant germ cell tumor cell lines to the poly (ADP-ribose) polymerase inhibitor veliparib. Cisplatin-resistant (A) NTERA-2 CisR, (B) NCCIT CisR and (C) JEG-3 CisR cell lines were significantly more resistant to veliparib than the parental cells. Only some small significant differences in sensitivity to veliparib were observed in the (D) TCam-2 and (E) NOY-1 cell line pairs. The effect of veliparib treatment was determined using a luminescent viability assay on Day 3. Values are presented as the mean \pm SD. Relative viability of the parental cell line was determined at each indicated veliparib concentration. The viability of the respective CisR variant was compared with the respective parental control at each tested concentration and significant differences are indicated. Mann-Whitney U test; * $P<0.05$, ** $P<0.01$, *** $P<0.001$. CisR, cisplatin-resistant variant of parental GCT cell line.

NTERA-2 CisR chemoresistant variants appear to exhibit lower PARP1 expression in the human xenografts grown on immunocompromised animals *in vivo*. Raw measurements for the tumors are given in Table SII.

Sensitivity of parental and cisplatin-resistant GCT cell lines to the PARP inhibitor veliparib. To evaluate the therapeutic potential of PARP inhibition, we tested the sensitivity of parental and chemoresistant GCT cell lines to veliparib, an oral PARP1/2 inhibitor. Dose-dependent cytotoxic effects of veliparib were observed in all cell lines, but they were not prominent in the TCam-2 pair. The cisplatin-resistant EC cell lines NTERA-2 CisR and NCCIT CisR were significantly more resistant to veliparib treatment than the parental cells (Fig. 3A and B). Similarly, the ChC cell line JEG-3 CisR was

significantly more resistant to this treatment (Fig. 3C). The sensitivity of TCam-2 and NOY-1 cell line pairs to veliparib was comparable. Resistance to this inhibitor was observed only at some concentrations (Fig. 3D and E).

Effect of combined treatment with veliparib and platinum-based drugs in NTERA-2 CisR and NCCIT CisR cells. To test whether the PARP inhibitor veliparib could sensitize chemoresistant cells to cisplatin treatment and yield synergy, we treated NTERA-2 CisR and NCCIT CisR cells with this combination. We observed some synergistic effects in NTERA-2 CisR cells, and the most significant changes were detected when the highest tested concentration of veliparib was used. The viability of NTERA-2 CisR cells was decreased by 13% upon treatment with 0.1 μ g/ml cisplatin alone, but the combination of

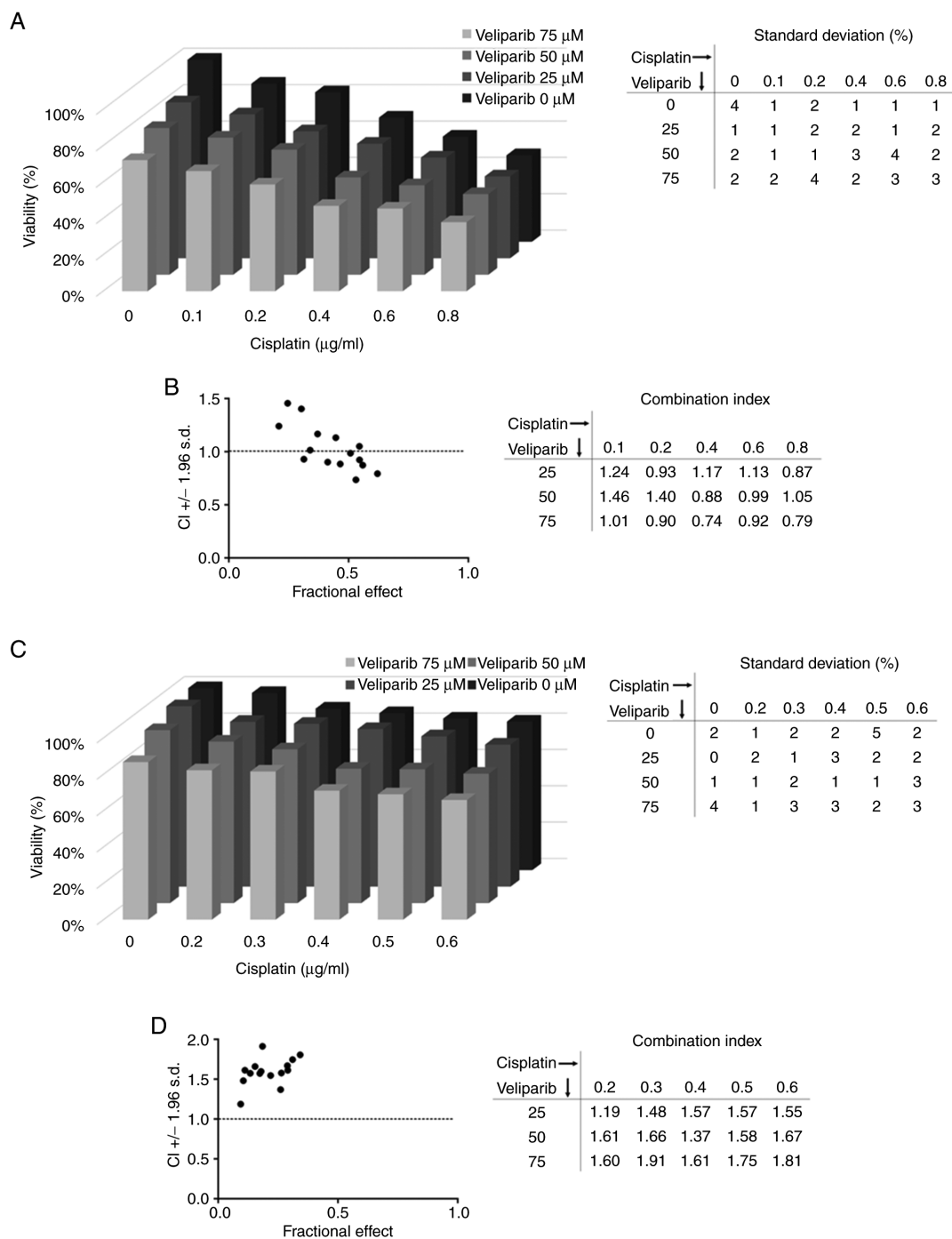


Figure 4. Effect of combined treatment with veliparib and cisplatin in NTERA-2 CisR and NCCIT CisR cells. (A) Veliparib in combination with certain concentrations of cisplatin decreased the viability of NTERA-2 CisR cells. (B) An Fa-CI plot was created according to the data obtained using the luminometric assay and confirmed the synergistic effect of this combination. CI values are indicated in the table. (C) Veliparib did not increase the sensitivity of NCCIT CisR cells to cisplatin. (D) An Fa-CI plot was created and indicated antagonism of this combination over the whole range of concentrations. CI values are indicated in the table. Relative viability was determined using a luminescent viability assay on Day 3. Values are presented as the mean, and SDs are indicated in the table. Data obtained using a luminometric assay were analyzed by Calcsyn software, and Fa-CI plots were created. The plot displays synergism (CI<1), additivity (CI=1) or antagonism (CI>1) for the entire spectrum of effects (38). CI, function of effect level; Fa, fraction affected (Fa=1-% of viable cells/100). CisR, cisplatin-resistant variant of parental GCT cell line.

0.1 µg/ml cisplatin and 75 µM veliparib achieved a 34% reduction in cell viability (Fig. 4A). The combination index (CI) was also below 1, indicating a synergistic effect of veliparib and cisplatin (Fig. 4B). However, veliparib did not sensitize the NCCIT CisR cell line to cisplatin treatment (Fig. 4C), and CI above 1 confirmed antagonism of the veliparib and cisplatin combination in these cells (Fig. 4D).

We also tested another platinum-based chemotherapy drug, carboplatin, and similar results were obtained for both tested cell lines. Carboplatin alone (1 µg/ml) decreased the viability of NTERA-2 CisR cells by 13%, but the combination with 75 µM veliparib induced a 30% reduction in cell viability (Fig. 5A). The combination index was again below 1, indicating synergy of veliparib and carboplatin combination (Fig. 5B).

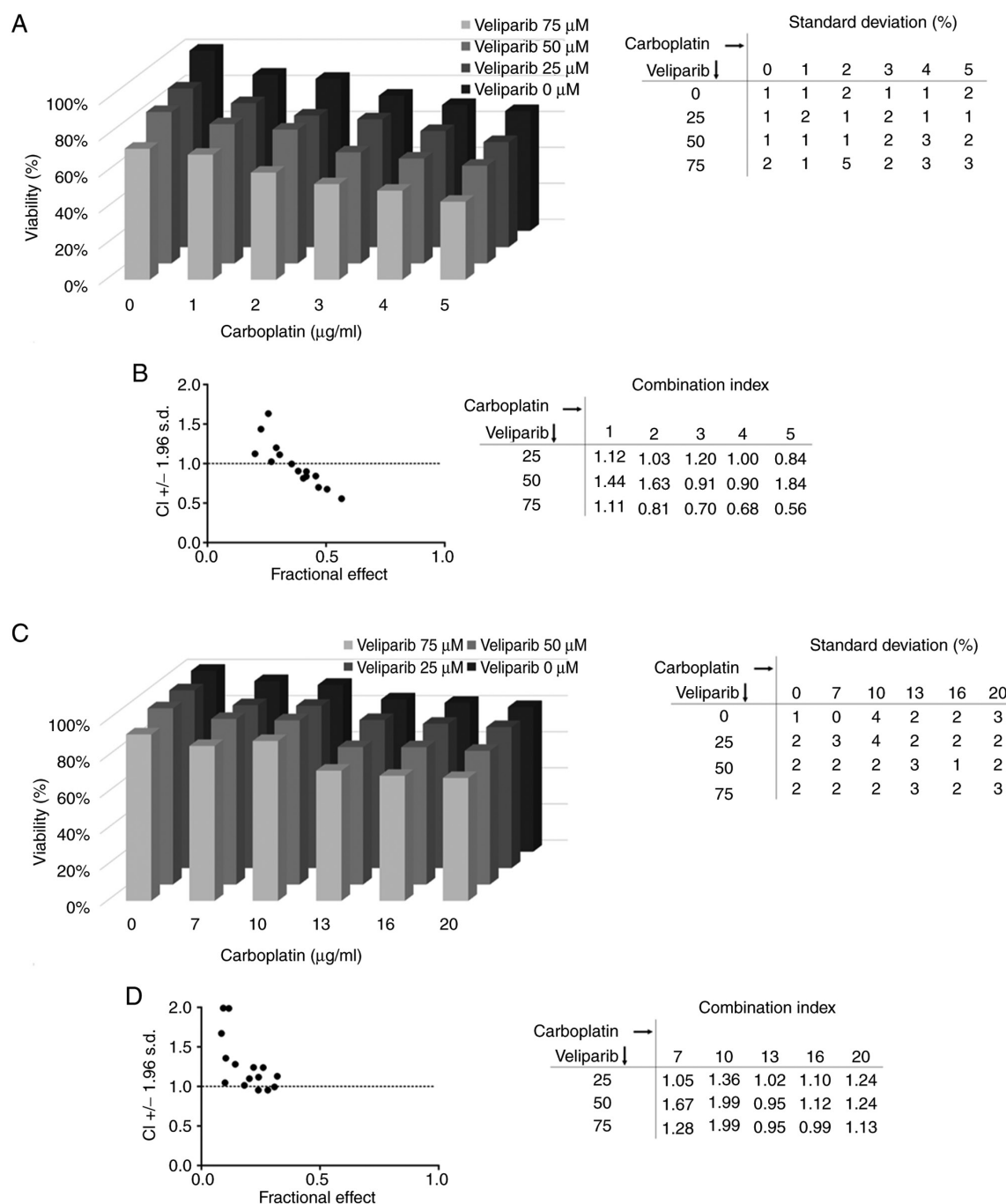


Figure 5. Effect of the veliparib and carboplatin combination on the viability of NTERA-2 CisR and NCCIT CisR cells. (A) Veliparib increased sensitivity to carboplatin in NTERA-2 CisR cells. (B) An Fa-CI plot was created according to the data obtained using the luminometric assay and confirmed the synergistic effect of this combination. CI values are indicated in the table. (C) Combination of veliparib and carboplatin was not more toxic to NCCIT CisR cells. (D) An Fa-CI plot was created and indicated antagonism of this combination. CI values are indicated in the table. Relative viability was determined using a luminescent viability assay on Day 3. Values are presented as the mean, and SDs are indicated in the table. Data obtained using the luminometric assay were analyzed by Calcsyn software and Fa-CI plots were created. The plot displays synergism ($CI < 1$), additivity ($CI = 1$) or antagonism ($CI > 1$) for the entire spectrum of effects (38). CI, function of effect level; Fa, fraction affected ($Fa = 1 - \% \text{ of viable cells}/100$). CisR, cisplatin-resistant variant of parental GCT cell line.

An antagonistic effect of this combination was observed in the NCCIT CisR cell line (Fig. 5C and D).

Effect of veliparib in combination with cisplatin on NTERA-2 CisR xenografts in vivo. To evaluate the effects of PARP inhibition by veliparib on NTERA-2 CisR tumor growth, we performed an *in vivo* experiment using immunodeficient SCID beige mice. NTERA-2 CisR cells were injected s.c. into mouse flanks to produce tumor xenografts and the

animals were divided into 3 treatment groups: i) untreated control/vehicle ($n=4$); ii) cisplatin-3 mg/kg/d ($n=4$); and iii) cisplatin + veliparib-25 mg/kg/d ($n=4$). The treatment started on Day 16 when all xenografts were palpable (Fig. 6A). Tumor growth was not affected by cisplatin or the combination treatment, and statistical analysis did not reveal any significant differences between these three groups (Fig. 6B). Raw measurements for the tumors are given in Table SIII.

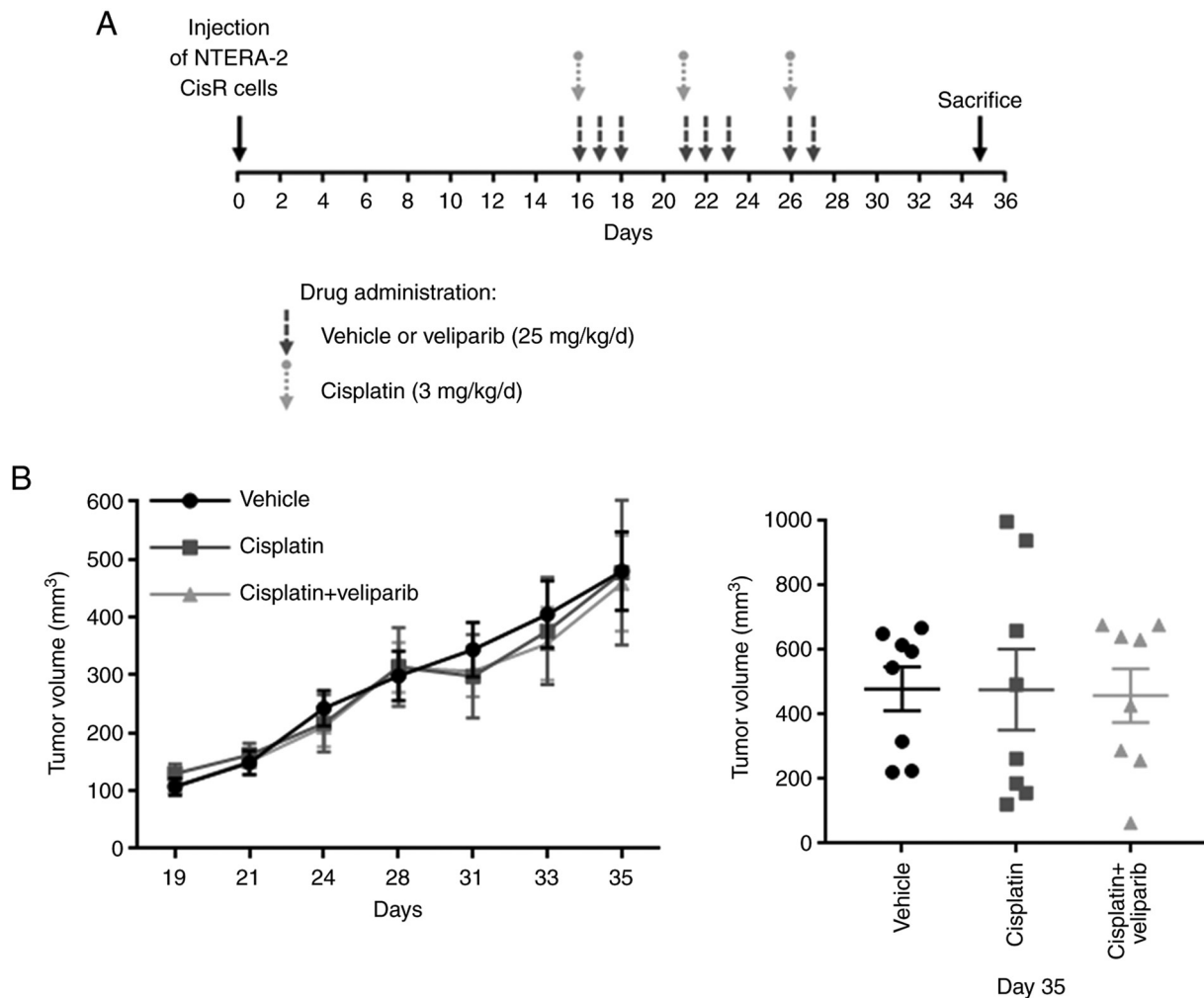


Figure 6. Effect of veliparib in combination with cisplatin on NTERA-2 CisR xenografts *in vivo*. (A) Outline scheme of the treatment. Vehicle, cispt and veliparib were intraperitoneally administered to mice. The timing of drug administration is indicated by arrows. (B) Combination of veliparib with cisplatin did not inhibit the growth of NTERA-2 CisR xenografts. The tumor sizes were similar in all treatment groups, and no significant changes were observed by multivariate analysis one-way ANOVA. CisR, cisplatin-resistant variant of parental GCT cell line.

Effect of combined treatment with veliparib and novobiocin in NTERA-2 CisR and NCCIT CisR cells. The combination of PARPi and polymerase theta (POL θ) inhibitors was recently shown to overcome PARPi resistance and increase the cytotoxic effects of PARPi (41,42). To test whether we could achieve a synergistic effect of this type of combination we used the antibiotic novobiocin, a specific POL θ inhibitor (41), combined with the PARPi veliparib. We treated NTERA-2 CisR cells with this combination and did not observe any synergy (Fig. 7A). The combination index was above 1, indicating an antagonistic effect of veliparib and novobiocin (Fig. 7B). Similar results were observed for other resistant EC cell line, where novobiocin did not sensitize NCCIT CisR cells to veliparib treatment (Fig. 7C and D).

Discussion

The enzymes PARP1 and PARP2 have overlapping functions in the DNA damage response pathway (43,44), but they differ in their substrate preference (45). PARP1- and PARP2-deficient mice display postreplicative genomic instability, whereas doubleknockout mice exhibit lethal genomic instability (46).

Our group previously showed that PARP is overexpressed in TGCTs. Increased PARP expression was present in early precursors of TGCTs-intratubular germ cell neoplasia unclassified- and in less differentiated histological subtypes, such as EC and SE. Its expression decreases with subsequent tumor tissue differentiation toward choriocarcinoma (10). Maximal PARP activity was correlated with PARP1 protein expression in EC cell lines (47). The levels of PARP1 and poly(ADP-ribosyl)ation were heterogeneous among germ cell tumor cell lines (48). Expression analysis confirmed the expression of both *PARP1* and *PARP2* in all GCT parental cell lines; however, only in EC NTERA-2 CisR and NCCIT CisR cell lines the expression on mRNA level remained or increased. Protein levels of PARP1 did not significantly differ between parental and resistant cells, however the lowest level was in ChC cell line pair JEG-3/JEG-3 CisR, which was also confirmed by IHC on the xenografts. The highest PARP1 protein level was observed in EC cell lines NTERA-2 and NCCIT, as also visible in IHC analysis. We detected substantial amount of protein PARP1 in YST cell line pair NOY-1/NOY-1 CisR and seminoma cell line model TCam-2/TCam-2 CisR pair with corresponding positive staining in xenografts. Poor correlations between the

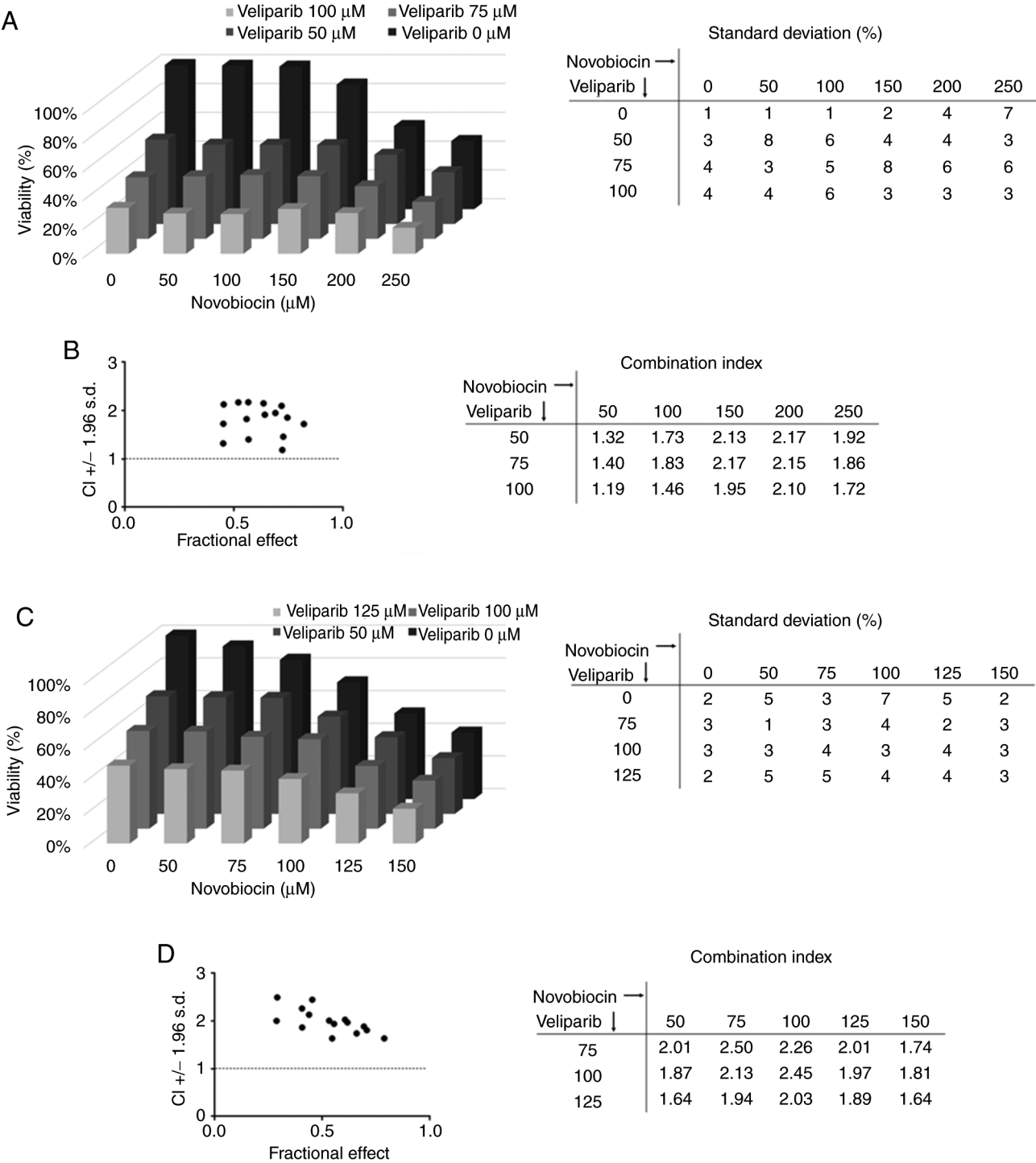


Figure 7. Effect of the combination of veliparib and the DNA polymerase θ inhibitor novobiocin on the viability of NTERA-2 CisR and NCCIT CisR cells. (A) Novobiocin did not increase sensitivity to veliparib in NTERA-2 CisR cells. (B) An Fa-CI plot was created according to the data obtained using the luminometric assay and confirmed the antagonistic effect of this combination. CI values are indicated in the table. (C) Combination of novobiocin with veliparib did not increase the cytotoxic effect of poly (ADP-ribose) polymerase inhibitor in NCCIT CisR cells. (D) An Fa-CI plot was created and indicated antagonism of this combination. CI values are indicated in the table. Relative viability was determined using a luminescent viability assay on Day 3. Values are presented as the mean, and SDs are indicated in the table. Data obtained using the luminometric assay were analyzed by Calcsyn software and Fa-CI plots were created. The plot displays synergism ($CI < 1$), additivity ($CI = 1$) or antagonism ($CI > 1$) for the entire spectrum of effects (38). CI, function of effect level; Fa, fraction affected ($Fa = 1 - \% \text{ of viable cells}/100$). CisR, cisplatin-resistant variant of parental GCT cell line.

level of mRNA and the level of protein were often observed; and may be attributed to many complex post-transcriptional mechanisms involved in protein synthesis; proteins may also differ in their *in vivo* half-lives; and/or there is also technical difference in protein and mRNA experiments. Altogether,

these results are in line with our previously published data from patient samples of GCT demonstrating high PARP1 level in GCT in comparison to healthy tissue (10).

Clinically approved PARPi vary in their effectiveness in trapping PARP onto DNA (from the most to the least

potent): talazoparib >> niraparib > olaparib=rucaparib >> veliparib (49). They are effective in a synthetically lethal interaction against HRR-deficient tumors, such as BRCA1/2-mutated tumors (50). PARPi have been approved for the treatment of breast and metastatic pancreatic cancer and metastatic castration-resistant prostate cancer (51-55). Veliparib was tested as a single agent or in combination with standard chemotherapeutic drugs and markedly improved the therapeutic efficiency in breast, ovarian and lung cancer (56). A phase I study of veliparib with cisplatin and vinorelbine showed increased response rates in advanced triple-negative breast cancer and/or BRCA-mutated breast cancer (57). In a phase III, randomized, placebo-controlled BROCADE3 trial, the addition of veliparib to carboplatin and paclitaxel improved progression-free survival (PFS) in patients with advanced HER2-negative germline BRCA1/2-mutated breast cancer (58). The combination of veliparib plus carboplatin and etoposide demonstrated improved PFS as first-line treatment in patients with extensive-stage small cell lung cancer (59). Promising antitumor activity was also observed in patients with metastatic or advanced non-small cell lung cancer receiving quadruple therapy with veliparib, nivolumab, carboplatin and paclitaxel (60). Moreover, combination therapy with veliparib plus carboplatin and gemcitabine demonstrated promising PFS and response rates in ovarian cancer patients with germline BRCA mutations (30).

In GCT model tumor cell lines, olaparib reduced cell viability in the EC cell lines NCCIT, NTera-2 and 2102Ep, while the SE cell line TCam-2 was the least sensitive. A clonogenic assay further confirmed the differential effect of olaparib in TCam-2 cells compared to that in the tested EC cell lines. Moreover, the least responsive cell lines (NCCIT and TCam-2) exhibited the lowest BRCA1 methylation levels, and high RAD51C and BRCA1 methylation was observed in the two most sensitive cell lines (NTera-2 and 2102Ep). Methylation levels correlated with the expression levels of both of these targets. Altogether, these findings support the evidence that promoter methylation of genes involved in HRR could serve as a predictor of the therapeutic response to PARPi in TGCT patients (61). Similarly, in the present study, we observed dose-dependent cytotoxic effects of the PARPi veliparib in all tested parental and cisplatin-resistant GCT cell lines, but in the TCam-2 cell line pair, these dose-dependent effects were not as profound. This cell line pair was also the least sensitive to veliparib treatment.

Olaparib was able to enhance the toxicity of cisplatin in combination in EC cells, and sensitivity correlated with the levels of PARP activity (47). Combination therapy with olaparib and cisplatin in two cisplatin-resistant EC cell lines, GCT27cis-r and 2102Epcis-r, was efficient (62). Importantly, a phase II clinical trial by De Giorgi *et al* (63) showed that olaparib as a single agent had only marginal activity in heavily cisplatin-pretreated and refractory GCT patients. However, an anecdotic 4-month stable disease was observed in the only patient with a BRCA mutation. The authors also suggested that future studies with olaparib should be conducted in combination or following salvage chemotherapy in less pretreated and more selected GCT patients (63).

At the time of the phase II GCT-SK-004 clinical trial initiation and based on the data available, using veliparib in

the combination with carboplatin and gemcitabine in multiple relapsed/refractory germ cell tumors seemed to be promising strategy (32). At the time of study initiation, other PARPi, such as olaparib or talazoparib, were not available, therefore, we analyzed the effect of veliparib also in our cell line models *in vitro* and *in vivo* to corroborate the results to those from clinical trial. To analyze the effect of combinatorial treatment with veliparib and cisplatin or carboplatin, we selected two cisplatin-resistant EC cell lines, NTera-2 CisR and NCCIT CisR, as they exhibited high levels of PARP1 protein and were sensitive to veliparib treatment in a dose-dependent manner. Synergistic effects of veliparib and cisplatin or carboplatin were observed only in NTera-2 CisR cells. However, this combination failed to enhance the cytotoxic effect of cisplatin *in vivo*, which is in line with the final results of a phase II trial determining the efficacy and toxicity of gemcitabine, carboplatin and veliparib, showing no additive treatment value of veliparib for refractory GCTs (32). Nevertheless, veliparib still remains valuable agent due to its different mechanism of action in comparison to olaparib and talazoparib, and potential synergistic effect with other treatments. Veliparib is a selective PARP1/2 inhibitor with relatively weak affinity, while olaparib and talazoparib have relatively strong affinity. Veliparib mainly selectively inhibits the activity of PARP without holding the PARP protein to DNA damage repair intermediates (64). Meta-data analysis published recently suggest activity of veliparib in combination with platinum agent and chemotherapy in some breast cancer patients with germ line BRCA mutations (65).

The majority of patients develop PARPi resistance despite a good initial response; thus, the identification of potential strategies to overcome these mechanisms could improve the therapeutic outcome of refractory patients (66,67). The most common cause is the restoration of HRR in HRR-deficient tumors, mostly via reversion mutations (68) or epigenetic modifications (69) that induce the re-expression of the BRCA1/2 protein. Another mechanism is stabilization of the replication fork by nucleases followed by inhibition of DNA replication fork degradation in BRCA1/2-deficient cells (70,71). Several other mechanisms, including the upregulation of the drug efflux transporter ABCB1 (P-glycoprotein) (72), inhibition of PARP trapping activity (73) or overexpression of cell cycle regulators (74), have been proposed.

Several clinical trials are currently evaluating possible therapeutic strategies that enhance PARPi sensitivity and overcome or delay PARPi resistance; however, none of them are targeting TGCTs. In solid tumors, PARPi were combined with ionizing radiation (75,76), atezolizumab (77), inhibitors of the G2 checkpoint kinase WEE1 adavosertib (78) and AZD1775 (79), HSP90 inhibitor (80), ATR/CHK1 inhibitors (81,82) or epigenetic drugs (83,84). Importantly, the effects of PARPi on the tumor microenvironment could also pave the way for rational drug combination strategies. PARPi upregulated expression of PD-L1 in breast cancer cell lines and animal models. Consequently, anti-PD-L1 therapy resensitized PARPi-treated cells to T-cell killing, and this combination showed better therapeutic outcomes than either monotherapy in an *in vivo* model (85).

Olaparib induced the differentiation, maturation and anti-tumor activation of macrophages with subsequent activation

of the immune-suppressive signaling pathway. However, the combination of PARPi and macrophage-targeting therapy induced a durable reprogramming of the tumor microenvironment in triple-negative breast cancer (86). Recently, inhibitors of DNA polymerase theta (POL θ) were shown to have synergistic effects with PARPi in the treatment of HRR-deficient tumors. ART558, a selective inhibitor of POL θ , induced DNA damage and synthetic lethality in BRCA1/2-mutated cancer cells and enhanced the effects of olaparib (42). The specific POL θ inhibitor novobiocin killed HRR-deficient breast and ovarian cancer cells *in vivo* and in patient-derived xenografts. Moreover, HRR-deficient tumor cells with acquired PARPi resistance were sensitive to novobiocin *in vitro* and *in vivo* (41). However, in our experiments, novobiocin failed to exert a synergistic effect with veliparib in NTERA-2 CisR and NCCIT CisR cells, suggesting that the synergy will be missing in the subset of tumor cells with increased proficiency in HRR (62).

In summary, we detected the presence of PARP1 protein in all analyzed GCT cell lines, but the levels were low in ChC cell lines, which is in line with our previous observations in clinical samples. GCT cell lines were sensitive to the PARPi veliparib in a dose-dependent manner; only in the Tcam-2 cell line pair was this effect not as prominent. Moreover, the cisplatin-resistant EC cell lines NTERA-2 CisR and NCCIT CisR and the ChC JEG-3 CisR cell line were also more resistant to veliparib treatment than the parental cells. We observed that veliparib synergized with cisplatin or carboplatin in NTERA-2 CisR cells, but this synergy was not confirmed *in vivo*. Neither combination with the POL θ inhibitor novobiocin showed synergy. The limitations of this study can be identified in the lack of direct comparison of other PARPi such as olaparib or talazoparib, however these were not available at the time of study initiation. The lack of high-throughput sequencing of HRR genes in GCT cell lines and absence PARP2 protein detection also represent study limitation. Nevertheless, there is still a rationale to use PARPi in more advanced models including other components of the tumor microenvironment, as GCTs (cell lines, xenografts and also patient tumor tissue (10) also showed PARP1 positivity. Other therapeutic approaches, including combination with anti-PD-L1 therapy or the use of other PARPi, need to be tested to determine the therapeutic efficacy of PARPi combinatorial therapy in GCTs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SS, NU, ZC, SH, KK, MC, LR, MV, LK and MM contributed to the study conception and design. *In vitro* analyses were performed by SS, MC and MV. Expression and western blot analyses were performed by NU and KK. The preparation of xenograft models and *in vivo* experiments were performed by SS, LR and LK. ZC and SH performed immunohistochemical analysis of PARP1 in xenograft models. Material preparation and collection of raw data from measurements were performed by SS and LR, and data analysis and evaluation were performed by LK and MM. The first draft of the manuscript was written by SS, LK and MM, and all authors commented on previous versions of the manuscript. LK and MM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This article does not contain any studies with human participants performed by any of the authors. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Studies involving mice were approved by the Ethic Committee of the Biomedical Research Center, Slovak Academy of Sciences (Dubravska cesta 9, Bratislava, Slovakia) and by the national competence authority (State Veterinary and Food Administration of the Slovak Republic), registration No. Ro 1976/17-221, in compliance with Directive 2010/63/EU of the European Parliament and the European Council and Regulation 377/2012 for the protection of animals used for scientific purposes.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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