*Original Research*

# Potential New Therapeutic Approaches for Cisplatin-Resistant Testicular Germ Cell Tumors

André van Helvoort Lengert[1],*, Leticia do Nascimento Braga Pereira[1],
Eduardo Ramos Martins Cabral[1], Izabela Natalia Faria Gomes[1], Lais Machado de Jesus[1],
Maria Fernanda Santiago Gonçalves[1], Aline Oliveira da Rocha[1], Tiago Alexandre Tassinari[1],
Luciane Sussuchi da Silva[1], Ana Carolina Laus[1], Daniel Onofre Vidal[2],
Mariana Tomazini Pinto[1], Rui Manuel Reis[1,3,4], Luiz Fernando Lopes[1,2,5],*

[1]Molecular Oncology Research Center, Barretos Cancer Hospital, 14784400 Barretos, Sao Paulo, Brazil
[2]Brazilian Childhood Germ Cell Tumor Study Group, from the Brazilian Pediatric Oncology Society (SOBOPE), 14784400 Barretos, São Paulo, Brazil
[3]Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, 4710057 Braga, Portugal
[4]ICVS/3B's - PT Government Associate Laboratory, 4806909 Braga/Guimaraes, Portugal
[5]Barretos Children's Cancer Hospital from Hospital de Amor, 14784400 Barretos, São Paulo, Brazil
*Correspondence: ahlengert@gmail.com (André van Helvoort Lengert); lf.lopes@yahoo.com (Luiz Fernando Lopes)
Academic Editors: Simona Daniele and Rebecca Piccarducci
Submitted: 19 April 2022   Revised: 9 June 2022   Accepted: 13 June 2022   Published: 16 August 2022

## Abstract

**Background**: Testicular germ cell tumors (TGCTs), a group of heterogeneous neoplasms, are the most frequent tumors of teenagers and young men, with the incidence rising worldwide. High cure rates can be achieved through cisplatin (CDDP)-based treatment, but approximately 10% of patients present refractory disease and virtually no treatment alternatives. Here, we explored new strategies to treat CDDP-resistant. **Methods**: *In vitro* TGCT CDDP-resistance model was established and differential mRNA expression profiles were evaluated using NanoString technology. Then, TGCT cell lines were treated with four potential drugs (PCNA-I1, ML323, T2AA, and MG-132) to overcome CDDP-resistance. **Results**: We found several differentially expressed genes related to DNA repair and cell cycle regulation on CDDP-resistant cell line (NTERA-2R) compared to parental cell line (NTERA-2P), and the proteasome inhibitor MG-132 demonstrated cytotoxic activity in all cell lines evaluated, even at a nanomolar range. MG-132 also enhanced cell lines' sensitivity to CDDP, increasing apoptosis in both NTERA-2P and NTERA-2R. **Conclusions**: MG-132 emerges as a potential new drug to treat CDDP-resistant TGCT. Targeted therapy based on molecular mechanism insights may contribute to overcome acquired chemotherapy CDDP-resistance.

**Keywords**: testicular germ-cell tumor; cisplatin resistance; MG-132

## 1. Introduction

Testicular germ cell tumors (TGCTs) are a group of heterogeneous neoplasms resulting from a defective primordial germ cell development and are currently considered the most common tumors of teenagers and young men (15–40 years), representing 0.4% of new cases from all sites, with increasing incidence rates worldwide [1–4].

TGCTs are classified into two main histological subtypes, including seminoma and non-seminoma germ cell tumors (NSGCT), representing 60% and 40% of the cases, respectively [5,6]. NSGCTs are subdivided into several histologies, such as embryonal carcinoma, yolk sac tumors (YST), teratoma, choriocarcinoma, and mixed NSGCT [5,6]. Thus, TGCTs are frequently heterogeneous tumors identified by their specific histology.

Platinum-based treatment is frequently used as first-line therapy for pediatric and adult TGCTs, and international guidelines have established a consensus for its application [7,8]. Indeed, TGCTs are highly curable, exhibit-ing one of the highest sensitivity levels to platinum-derived compounds, and present an overall disease-free survival rate of approximately 80% for metastatic disease [9]. Even patients with advanced metastatic disease can achieve complete remission through systemic treatment and secondary resection of residual masses [10]. Its unique sensitivity is likely multi-factorial and associated with the germ cell origins of these tumors. Additional factors are known that may help explain this feature: TGCTs present a hypersensitive apoptotic response to DNA damage agents; show a decrease in the repair capacity of platinum-induced damage; only around 1% of TGCTs have *TP53* mutations [11,12]. Notably, however, about 10–20% of TGCT patients with advanced disease are refractory to platinum-based chemotherapy and have a less favorable prognosis with relapses, and treatment options for this group are extremely limited [13]. Studies have shown that late relapses occur more frequently in non-seminomas (3.2%) compared to seminomas (1.4%), in which the most often histological components are ter-

atomas (60%) and YST (47%) [13–15]. Recent encouraging advances have been published in this area [16–18], but still, approximately 3–5% of all TGCT patients will eventually die of their disease [19–21].

Several reports have described molecular mechanisms related to cisplatin (CDDP) resistance of TGCTs, including *TP53* and *MDM2* alterations [22], induction of differentiation [23], global and specific DNA methylation alterations [24], deregulation of the PDGFR$\beta$/AKT pathway [25], inactivation of *REV7* [26]. However, none of these was determinant, so far, to develop new approaches to overcome the acquired resistance or to stratify the patient's risk. An alternative pathway of CDDP-resistance is based on the cell's tolerance to damage caused by CDDP, merely replicating the damaged DNA through a mechanism known as translesion DNA synthesis (TLS) [27]. This mechanism has been connected to CDDP-resistance in several tumors, although it has still been little examined in TGCTs [28–31].

TGCT is a complex disease with various histological and clinical characteristics, so identifying specific molecular features critical for CDDP response will likely be necessary to effectively use this drug and/or find new treatment strategies [19].

Here, we established a TGCT model of resistance to CDDP, created after CDDP long-term exposure of parental NTERA-2 cell line, to identify mechanisms that are central for acquired CDDP-resistance in TGCTs. We performed an extensive phenotypic and molecular characterization of NTERA-2R and found that several genes related to DNA repair and cell cycle regulation are differentially expressed on resistant cells in response to CDDP. Moreover, the proteasome inhibitor MG-132 demonstrated cytotoxic activity in all TGCT cell lines evaluated, even at a low nanomolar range. MG-132 also enhanced cell lines' sensitivity to CDDP in both, NTERA-2P and NTERA-2R, indicating that it could be a potential new strategy to overcome TGCTs treatment failure.

## 2. Materials and Methods

### 2.1 Cell Culture and Drugs

Two TGCT cell lines obtained from the European Collection of Authenticated Cell Cultures (ECACC) were used. NTERA-2 clone D1 (ECACC Cat# 01071221, RRID:CVCL_3407) is a cell line derived from a human testicular embryonal carcinoma and was first described in 1984 [32]. 577MF (ECACC Cat# 06011802, RRID:CVCL_2290) is a cell line derived from a human testicular teratocarcinoma and was first described in 1980 [33]. Both were cultured following ECACC recommended conditions and incubated in a humidified atmosphere with 5% $CO_2$ at 37 °C. CDDP-resistance model was developed by growing the sensitive cell line NTERA-2 in increasing sublethal concentrations of CDDP in the growth medium. The starting dose was approximately the $IC_{25}$ (inhibitory concentration, 25%) of the cell line for 72 hours. The medium

was then replaced to let the cells recover for a further 72 hours. This development phase was conducted for approximately 8 months, after which the $IC_{50}$ concentrations were re-assessed. The resistant cell line obtained (NTERA-2R) was cultivated in the same conditions of the parental cell line. Cell lines were negative for mycoplasm contamination (MycoAlert Mycoplasma Detection Kit, Lonza; tested monthly) and were authenticated by short tandem-repeat analysis at the Barretos Cancer Hospital facilities as reported [34].

Cisplatin (CDDP) (PHR1624; Sigma-Aldrich) was prepared at 5 mM in 0.9% NaCl. PCNA-I1 (SML0730; Sigma-Aldrich), ML323 (SML1177; Sigma-Aldrich), T2AA (SML0794; Sigma-Aldrich) and MG-132 (M7449; Sigma-Aldrich) were prepared at 10 mM in DMSO (Sigma-Aldrich). All drugs were aliquoted and stored at –20 °C until use.

### 2.2 Measurement of Cell Viability

Exponentially growing cells were seeded at a density of $5 \times 10^3$ cells per well in 96-well plates. After leaving cells to adhere overnight, the drugs were added at zero (vehicle only) and seven increasing concentrations (0.0625, 0.125, 0.5, 1.0, 5.0, 15.0, 30.0, 50.0 $\mu$M). Cells were then incubated for a further 72 hours before measuring viability using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega). The optical density at 570 nm (OD570) was measured with a Varioskan Flash plate reader (Thermo Fisher Scientific) and expressed as a percentage of the value obtained from control cells. The assays were performed in both technical and biological triplicate.

$IC_{50}$ concentrations were obtained in GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA), through the equation: log(inhibitor) *vs.* normalized response, using a Variable slope model.

The NTERA-2R $IC_{50}$ was also verified after four months straightly in CDDP-free media culture to confirm the resistance phenotype stability.

Evaluation of combination treatment was performed using CDDP $IC_{50}$ and a range of MG-132 concentrations from 0 to 5 $\mu$M.

### 2.3 Colony-Forming Assay

Cells were seeded in triplicate in a 12-well plate at a density of $5 \times 10^2$ cells/well. After 14 days, cells were fixed and stained with 1 mL of staining solution (0.5% crystal violet; 20% methanol) for 20 minutes. The plate was washed four times with distilled water, inverted on filter paper to remove the remaining water, and then air-dried for 24 hours at room temperature. The colonies were unstained using 1 mL of 100% methanol for 20 minutes, and the plates were read with a Varioskan Flash plate reader (Thermo Fisher Scientific) at 570 nm (OD570). The means and standard deviation of three independent experiments were analyzed.

## 2.4 Cell Migration Assay

To evaluate cell migration capacity, the monolayer wound-healing assay was performed. Briefly, a confluent monolayer of cells was seeded in a 6-well plate, and a "scratch" with a p1000 pipet tip was made through the cell layer. After washing several times with PBS, a medium containing 10% FBS was added to each well. Two fields of each wound were photographed in regular periods, from 0 to 64 hours. The wound areas were measured using TScratch software [35]. The experiments were performed in biological and technical triplicate.

## 2.5 Flow Cytometry (Apoptosis and Cell Cycle Analysis)

Flow cytometry analysis of apoptosis and cell cycle were performed with FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) and BD Cycletest Plus DNA Kit (BD Biosciences), respectively. Cells were seeded in T25 flasks and treated 24 hours later. After 72 hours, the culture supernatant and the cells were collected, washed twice with 1X PBS, and the specific protocols were followed as recommended by the manufacturer. Cell data was collected using BD Accuri Cytometer. Unstained and single-stained controls were used for color compensation, and at least 10,000 events were collected for each sample. The analysis was performed after two independent experiments, using FCS Express 7 software (De Novo Software, Pasadena, CA, USA).

## 2.6 Expression Profile of CDDP-Resistance in Vitro Model

Expression profile of NTERA-2R and NTERA-2P was performed using nCounter Vantage 3D DNA Damage and Repair panel (Nanostring, USA) as previously reported [36], to evaluate features related to CDDP-resistance. This panel includes 180 genes involved in major DNA damage repair pathways, including base excision repair, nucleotide excision repair, mismatch repair, TLS, and other repair processes (available at: http://nanostring.com).

Total RNA from NTERA-2P and NTERA-2R was isolated in biological triplicate, using TRIzol reagent, according to the manufacturer's protocol. Probe pools, hybridization buffer, TagSet, and 100 ng total RNA (quantified by Qubit 2.0 Fluorometer) were hybridized for 21 hours at 67 °C, followed by purification and RNA/probe complexes immobilization in nCounter PrepStation (Nanostring, USA) and cartridge scanning in Digital Analyzer (Nanostring, USA), according to the manufacturer's protocol.

The nSolver Analysis Software version 4.0 (NanoString Technologies, Seattle, WA, USA) was used for quality control assessment, and further steps were carried out in the R statistical environment, version 3.6.3. Gene expression levels and sample distribution were evaluated with quantro package, version 1.18.0 [37]. Data normalization and differential expression were performed in the NanoStringNorm package, version 1.2.1.1 [38]. Data were quantile normalized, and log2 transformed. Differentially expressed genes were defined by the thresholds of fold change $\geq 1.5$ and $p \leq 0.05$. Heatmaps of differentially expressed genes were built with the ComplexHeatmap package, version 2.0.0 [39].

## 2.7 Online Analysis Tools

Venn diagram was created utilizing Venny 2.1 (RRID:SCR_016561, available at: https://bioinfogp.cnb.csic.es/tools/venny/). The STRING database was used to predict interaction networks from gene expression analysis. Clustering was performed using the K-means clustering method (RRID:SCR_005223, available at: https://string-db.org) [40]. Functional annotation analysis was performed using DAVID (RRID:SCR_001881, available at: https://david.ncifcrf.gov/home.jsp) [41–43].

## 2.8 Western Blot Analysis

Total proteins were extracted after 24 hours of treatment using RIPA buffer, including 10% protease and phosphatase inhibitors (Sigma-Aldrich). After 15 min on ice, samples were centrifuged at 13,000 g for 30 min at 4 °C, and the supernatant was collected. Protein concentration was determined using the Bio-Rad Protein assay, based on Bradford method (Bio-Rad) according to the manufacturer's instructions. Twenty micrograms of protein were denatured at 95 °C for 5 min in 4X Laemmli buffer (Thermo Fisher Scientific), separated on NUPAGE 10 or 15% bis–tris gels at 90 V and transferred to 0.2 $\mu$m polyvinylidene difluoride (PVDF) membranes (GE Biosciences). Ponceau S solution (Sigma-Aldrich; 0.5% in 5% acetic acid) staining confirmed a correct membrane transfer. Membranes were blocked in 5% non-fat milk in TBS with 0.1% Tween (TBS-T, pH = 7.6) for 1 h and then incubated with primary antibodies overnight at 4 °C. Secondary horseradish peroxidase (HRP)-conjugated antibodies were incubated for 1 h at room temperature (RT). Bands were visualized using the ECL detection system (Cell Signaling), and the ImageQuant LAS 4000 (GE Biosciences) was used for imaging. Primary antibody probing was performed with PARP (Cell Signaling Technology Cat# 5625, RRID:AB_10699459/1:1000), and p-$\gamma$H2A.X (Cell Signaling Technology Cat# 2577, RRID:AB_2118010/1:1000). Anti-$\beta$-actin was used as a loading control (Cell Signaling Technology Cat# 8457, RRID:AB_10950489). Quantification was performed using band densitometry analysis from ImageJ software (version 1.6.1), by comparing the band intensity with the loading control $\beta$-actin. Experiments were performed in biological duplicate.

## 2.9 Statistical Analysis

Data are presented as means $\pm$ the standard deviation of the means. Statistical analysis was performed using the GraphPad Prism 5.0 software. Unpaired Student's $t$-test and non-parametric (Mann–Whitney) two-tailed tests were used
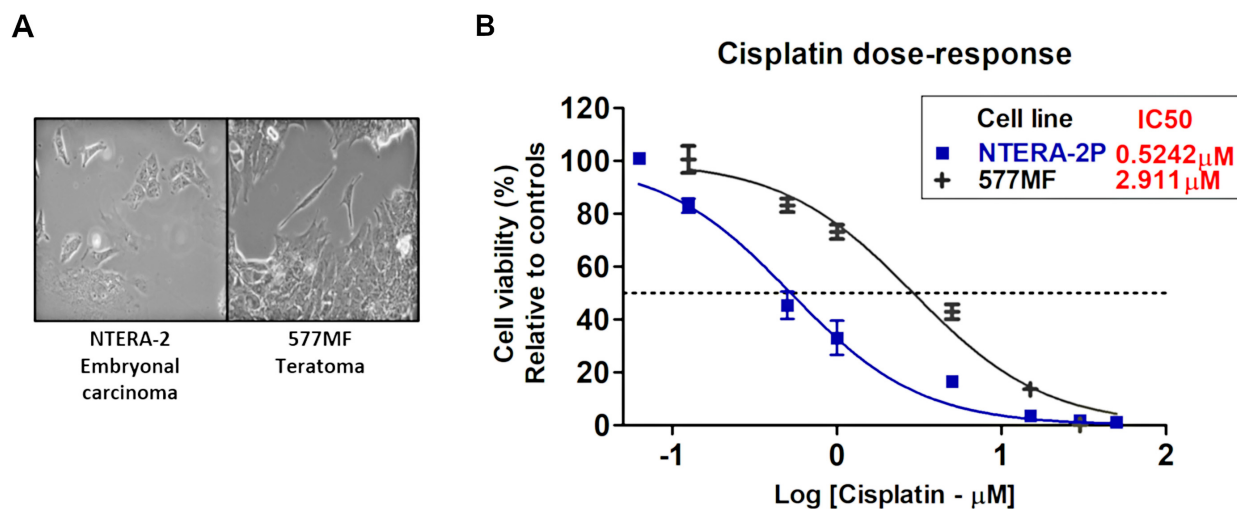
**Fig. 1. CDDP viability effect on NTERA-2 and 577MF TGCT cell lines.** (A) Representative images of the cell lines (Objective: 20x). (B) CDDP at increasing concentrations was used to treat cells for 72 hours, and cell viability was evaluated. $IC_{50}$ values are indicated on the graph.

among all assays, as necessary. A $p$-value $\leq 0.05$ was considered statistically significant.

## 3. Results

### 3.1 The Effect of CDDP in TGCT Cell Lines

We first performed a cell viability assay to determine the half-maximal inhibitory concentration ($IC_{50}$) value of CDDP in two TGCT cell lines. Fig. 1 presents the two cell lines evaluated and their respective cell viability after treatment with increasing CDDP concentrations, ranging from 0.0625 $\mu$M to 50 $\mu$M. $IC_{50}$ for NTERA-2 was 0.524 $\mu$M and for 577MF was 2.911 $\mu$M.

### 3.2 Characterization of TGCT with Acquired Resistance to CDDP

To better understand CDDP-resistance mechanisms, we chose the most sensitive cell line in our previous analysis (NTERA-2) and established a CDDP-resistance model (NTERA-2R). After eight months of CDDP treatment with incremental doses, $IC_{50}$ values were re-evaluated. A significant increase ($p < 0.01$) was observed in the concentration of CDDP required to inhibit 50% of NTERA-2R cells (3.812 $\mu$M), compared to NTERA-2P (0.524 $\mu$M) after 72 hours treatment (Fig. 2A). Subsequently, cells were maintained in culture for four months in CDDP-free media, and the $IC_{50}$ was re-assessed. The $IC_{50}$ fold-increase in NTERA-2R (6.3 fold-increase) was similar to the $IC_{50}$ previously found (7.2 fold-increase), confirming the CDDP-resistance phenotype stability, even without CDDP continuous exposure. We then performed a phenotypical characterization of NTERA-2R and found that it exhibited a higher aggressive phenotype when compared to NTERA-2P, demonstrating a significant increase in cell proliferation capacity (Fig. 2B), increased clonogenic survival

(Fig. 2C,D), and higher migration rates (Fig. 2E,F).

Levels of CDDP-induced apoptosis were assessed after 0.6 $\mu$M CDDP treatment for 72 hours. As expected, a significant increase in the apoptotic response to CDDP was seen in NTERA-2P (Annexin +/7AAD - and Annexin +/7AAD +), while almost no change was observed in NTERA-2R (Fig. 3A). Furthermore, cell cycle changes in response to 0.6 $\mu$M CDDP treatment for 72 hours were more prominent in NTERA-2P, presenting a markedly decrease of cells in G1 and an increase in S-phase and G2 (Fig. 3B). This data suggests the essential role of cell cycle control mechanisms in TGCT CDDP-resistance. Next, we treated both cells with their respective CDDP $IC_{50}$ for 24 hours and performed an immunoblotting assay to verify $\gamma$-H2AX expression levels. We observed no difference in protein levels (Fig. 3C), indicating that the NTERA-2R resistance phenotype was not due to pre-target mechanisms, such as decreased CDDP intracellular accumulation caused by alterations in transporters or increased drug efflux.

To elucidate the molecular changes associated with CDDP-resistance, we performed an analysis of both NTERA-2P and NTERA-2R 72 hours after CDDP ($IC_{50}$) or vehicle (0.9% NaCl) treatment using the nCounter Vantage 3D DNA Damage and Repair panel from NanoString. Gene expression analysis comparing NTERA-2P and NTERA-2R treated with vehicle, revealed five differentially expressed genes (**Supplementary Fig. 1**). Among them, three genes were upregulated in NTERA-2R, two of which were related to DNA repair (*MGMT* and *XPC*), and one was responsible for a small subunit of DNA polymerase delta (*POLD4*). One of the genes downregulated in NTERA-2R encodes the catalytic subunit of DNA polymerase epsilon (*POLE*). Interestingly, high *MGMT* expression levels significantly correlated with a worst disease-free survival in
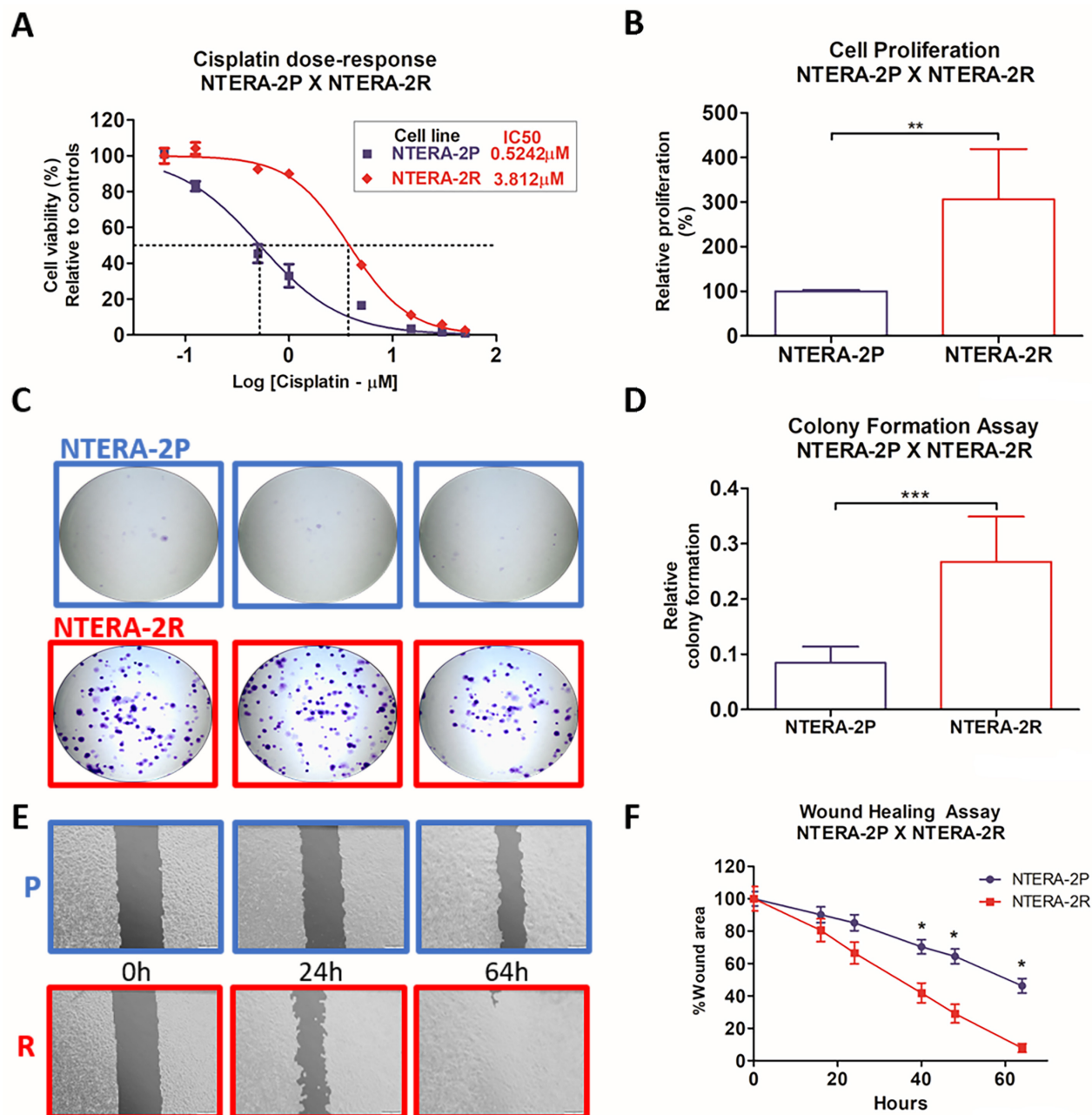
**Fig. 2. Development and phenotypic characterization of TGCT CDDP-resistance model.** (A) Cell viability of NTERA-2P and NTERA-2R after 72 hours of CDDP treatment. $IC_{50}$ values are indicated on the graph. (B) Comparison of cell proliferation capacity, (C,D) cell survival, and (E,F) migration ability in both cell lines. Images are representative of the assays. * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$.

TGCT cohort from the TCGA database ($p = 0.0066$), when considering the 15% patients with higher *MGMT* expression and the 15% patients with lower *MGMT* expression (**Supplementary Fig. 2**). We then performed a differential expression analysis of CDDP induced genes in parental (Fig. 4A) and resistant (Fig. 4B) cell lines, and found three genes that were exclusively altered in NTERA-2P and 21 genes exclusively altered in NTERA-2R. In the following analyses, we considered these 24 exclusively altered genes as the most important to explain our *in vitro* model's resis-

tant phenotype. In addition, we determined which genes were altered in common between NTERA-2P *vs.* NTERA-2PT (NTERA-2P treated with CDDP) and NTERA-2R *vs.* NTERA-2RT (NTERA-2R treated with CDDP) analyzes and nine genes were observed (Fig. 4C), suggesting that the regulation of these genes are independent of cisplatin resistance status.

A list with all genes differentially expressed (fold change ≥1.5 and $p \leq 0.05$) and their respective fold change is presented in **Supplementary Tables 1,2**.
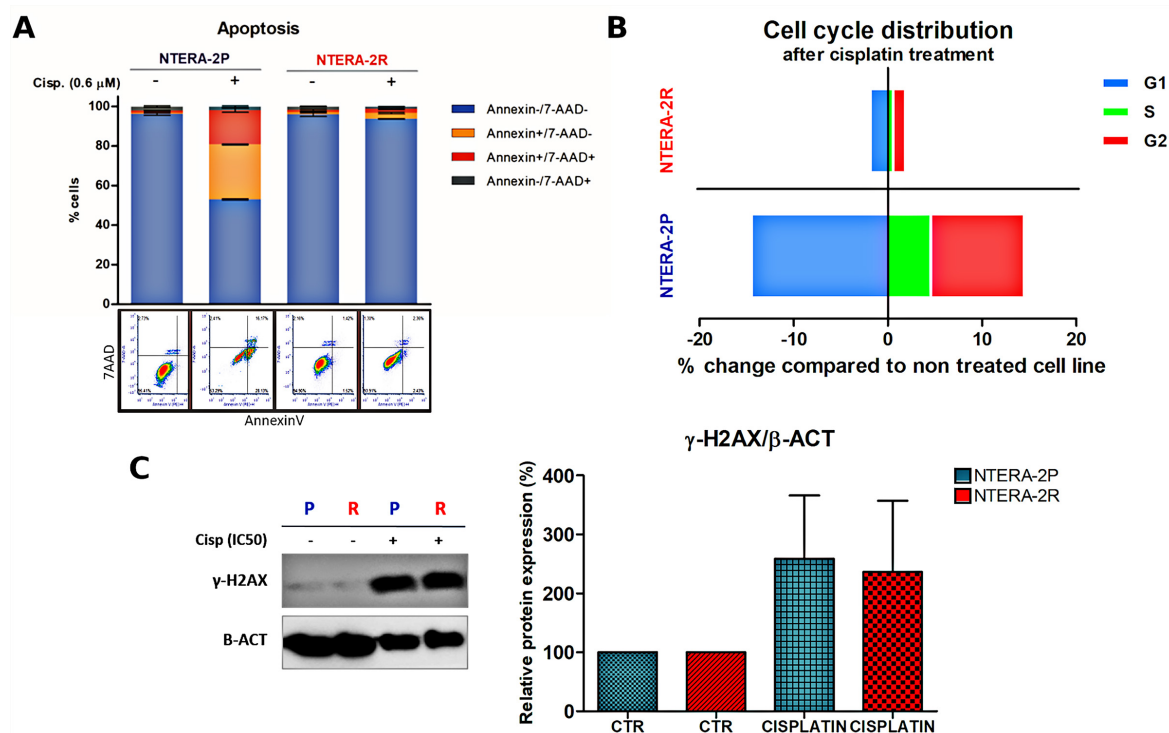
**Fig. 3. Flow-cytometry analysis of CDDP effect on apoptosis and cell cycle distribution.** (A) apoptosis and (B) cell cycle distribution in NTERA-2P and NTERA-2R, after 0.6 $\mu$M treatment for 72 hours. Differences are given in percentage. (C) Measurement of $\gamma$-H2AX protein expression in NTERA-2P and NTERA-2R, after CDDP IC$_{50}$ treatment for 24 hours. The image represents one immunoblotting assay.

We used the STRING database to predict the interactions between the 24 genes. We also used DAVID gene ontology (GO) to find the main regulated processes for these genes. These analyses revealed two clusters mainly related to DNA repair mechanisms and a cluster consisting of genes that regulate cell cycle, apoptosis, and cellular signaling (Fig. 5A). DAVID Functional GO-Analysis identified nine pathways significantly associated with the analyzed genes ($p \leq 0.05$), and DNA repair was the most significant (Fig. 5B). Interestingly, among the genes exclusively altered in NTERA-2P after CDDP treatment, none were related to DNA repair, while in NTERA-2R a set of DNA repair genes was differentially expressed. Another interesting finding was that after CDDP treatment, the antiapoptotic gene *BCL2* was downregulated in NTERA-2P, while the antiapoptotic gene *BCL2L1* was upregulated in NTERA-2R. These results suggest the idea that the main change in NTERA-2R is an increased DNA repair capacity and specific changes in cell cycle control, which may trigger apoptosis evasion and allow cells to proliferate, even in the presence of CDDP adducts.

### 3.3 The Effect of a Panel of New Therapeutic Approaches in TGCT CDDP-Resistance

Considering the central role of cell cycle regulation and DNA repair mechanisms in our CDDP-resistance model, we hypothesized whether a panel of drugs con-

taining three TLS inhibitors and one proteasome inhibitor, which was previously described as targeting TLS, could be an alternative to overcome GCT resistance. We performed a cell viability assay to determine the IC$_{50}$ value of PCNA-I1, MG-132, ML-323 and T2AA, and found that all inhibitors induced some degree of cytotoxicity in both NTERA-2P and NTERA-2R (Fig. 6A). These results indicate that these inhibitors may be part of a new strategy to treat TGCTs, and more importantly, they could overcome CDDP-resistance in TGCTs. MG-132 demonstrated the more significant responses among the four inhibitors, showing strong cytotoxic activity in both NTERA-2P and NTERA-2R (IC$_{50}$: 78.55 nM and 77.52 nM, respectively). Additionally, to confirm this drug's potential, we verified its effect on the 577MF cell line, which is also a TGCT and presents an intermediate sensitivity to CDDP compared to NTERA-2P and NTERA-2R. The 577MF IC$_{50}$ found after the cell viability assay was 88.39 nM (Fig. 6B). These data show that MG-132 effectively reduced cell viability at low nanomolar concentrations in all cell lines analyzed.

### 3.4 MG-132 and CDDP Combination Treatment

In light of these data, we tested the potential of MG-132 and CDDP as a combinatory treatment. We first assessed cell viability treating NTERA-2P and NTERA-2R with a range of MG-132 concentrations combined with
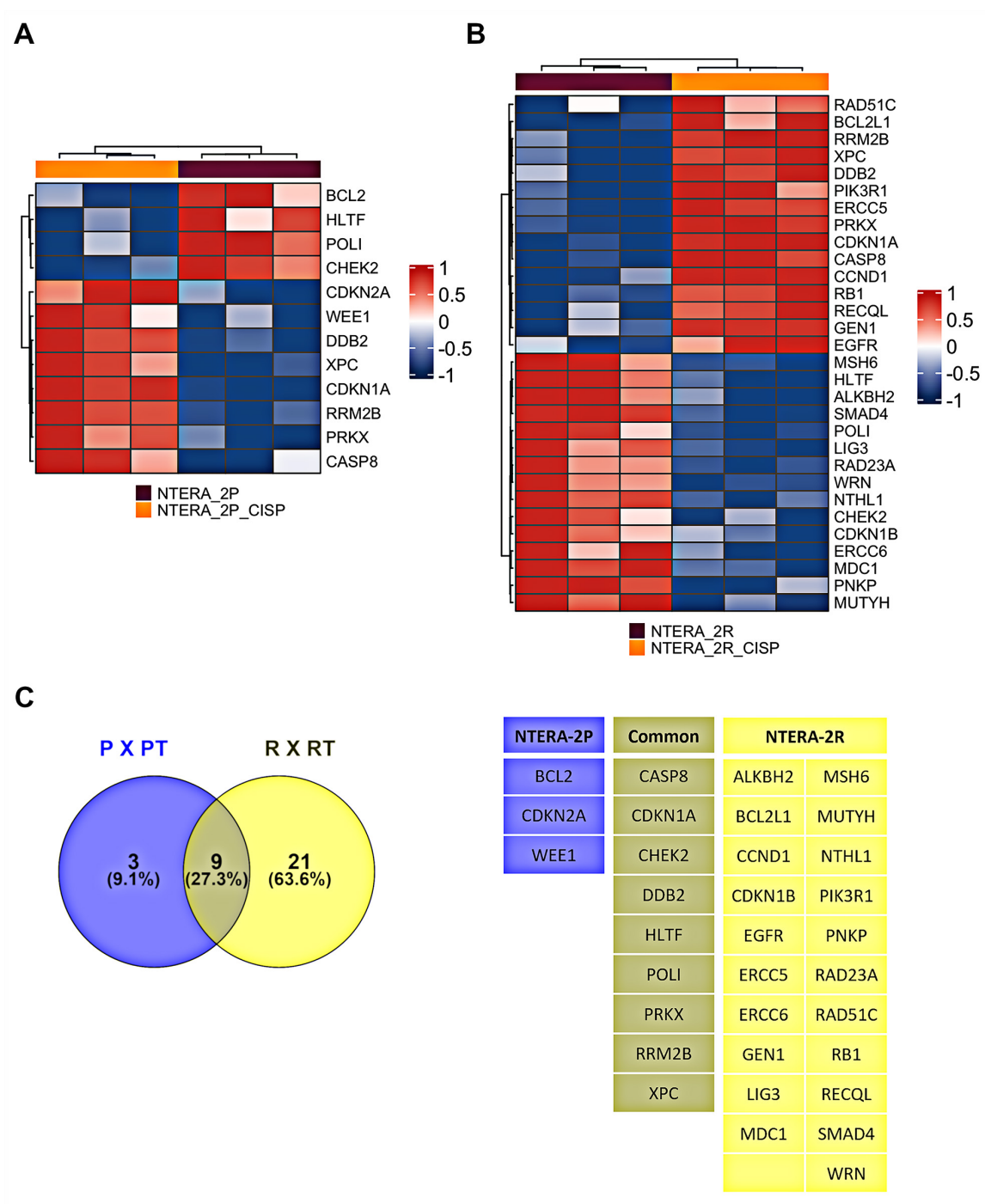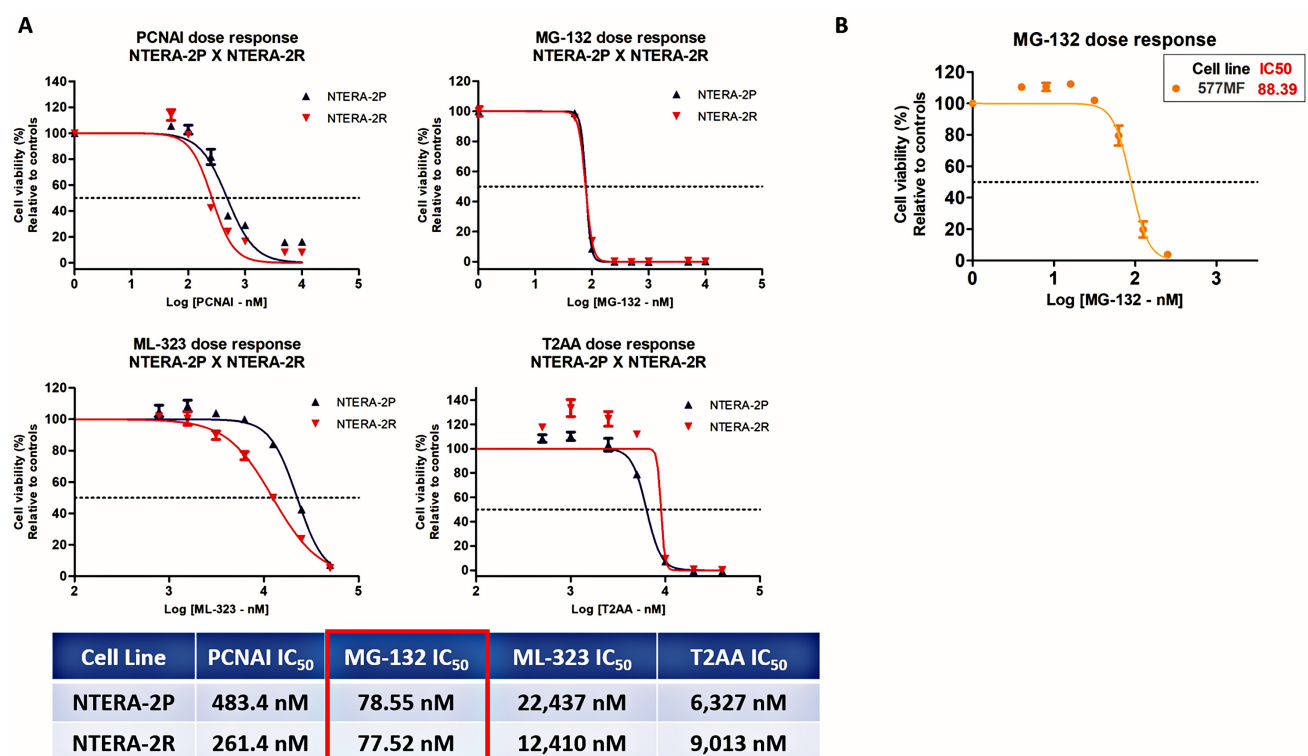
**Fig. 4. Differential expression analysis of CDDP-induced gene expression.** Nanostring DNA Damage and Repair panel revealed genes related to CDDP-resistance in (A) NTERA-2P and (B) NTERA-2R. Only genes with fold change $\geq$1.5 and $p \leq 0.05$ were included. (C) Venn diagram comparing all the genes differentially expressed in the analyses performed in A and B revealed 24 genes exclusively altered in P or R. P, NTERA-2P; R, NTERA-2R; PT, NTERA-2P treated with CDDP; RT, NTERA-2R treated with CDDP.

CDDP (IC$_{50}$) for 72 hours. A remarkable enhancement of cytotoxicity was observed in the combinatory treatment for both cell lines. Interestingly, NTERA-2R was more responsive than NTERA-2P to the combinatory treatment (Fig. 7A). We then found that the treatment using the combination of CDDP (IC$_{50}$) and MG-132 (25 nM – non-toxic concentration), for 72 hours, increased apoptosis when compared to CDDP alone in both cell lines (Fig. 7B). This result agreed with increased cleaved PARP levels found after the treatment with CDDP and MG-132 combination in both cell lines (Fig. 7C). Finally, we performed flow cytometry to compare the cell cycle distribution after treatment with CDDP (IC$_{50}$) alone and in combination with MG-132 (25 nM) for 72 hours. As presented in Fig. 7D, CDDP alone
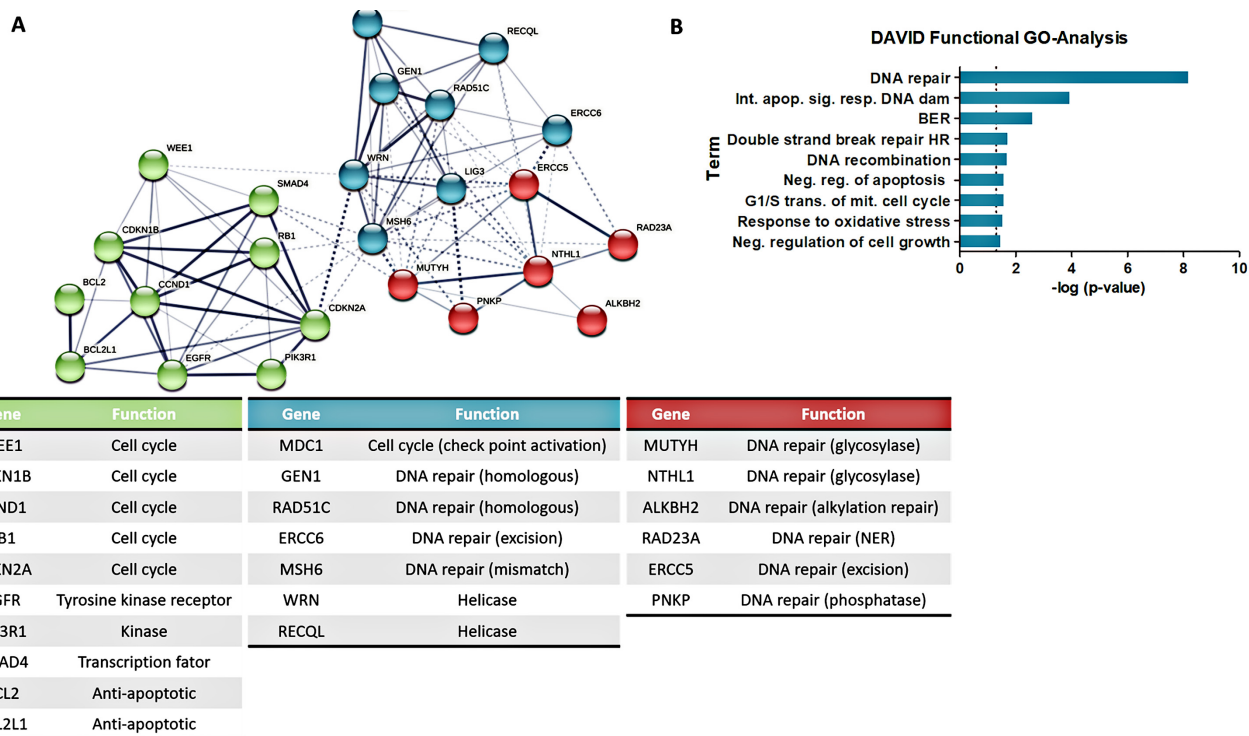
**Fig. 5. Network and functional predictions of CDDP-induced gene expression.** Network and functional predictions indicate the main clusters and pathways of the 24 genes exclusively altered in NTERA-2P and NTERA-2R cells after CDDP treatment. (A) STRING inter-action prediction considering the altered genes. Genes primary function was identified, tabulated, and colors are according to STRING clusters. (B) DAVID Functional GO-Analysis identified nine pathways significantly associated with the analyzed genes ($p \leq 0.05$).



| Cell Line | PCNAI IC$_{50}$ | MG-132 IC$_{50}$ | ML-323 IC$_{50}$ | T2AA IC$_{50}$ |
|-----------|-----------------|-------------------|-------------------|-----------------|
| NTERA-2P | 483.4 nM | 78.55 nM | 22,437 nM | 6,327 nM |
| NTERA-2R | 261.4 nM | 77.52 nM | 12,410 nM | 9,013 nM |

**Fig. 6. Effect of a panel of drugs in TGCT cell lines viability.** (A) IC$_{50}$ doses of the inhibitors based on MTS data in NTERA-2P and NTERA-2R cells. (B) IC$_{50}$ dose of MG-132 based on MTS data in 577MF cell line.
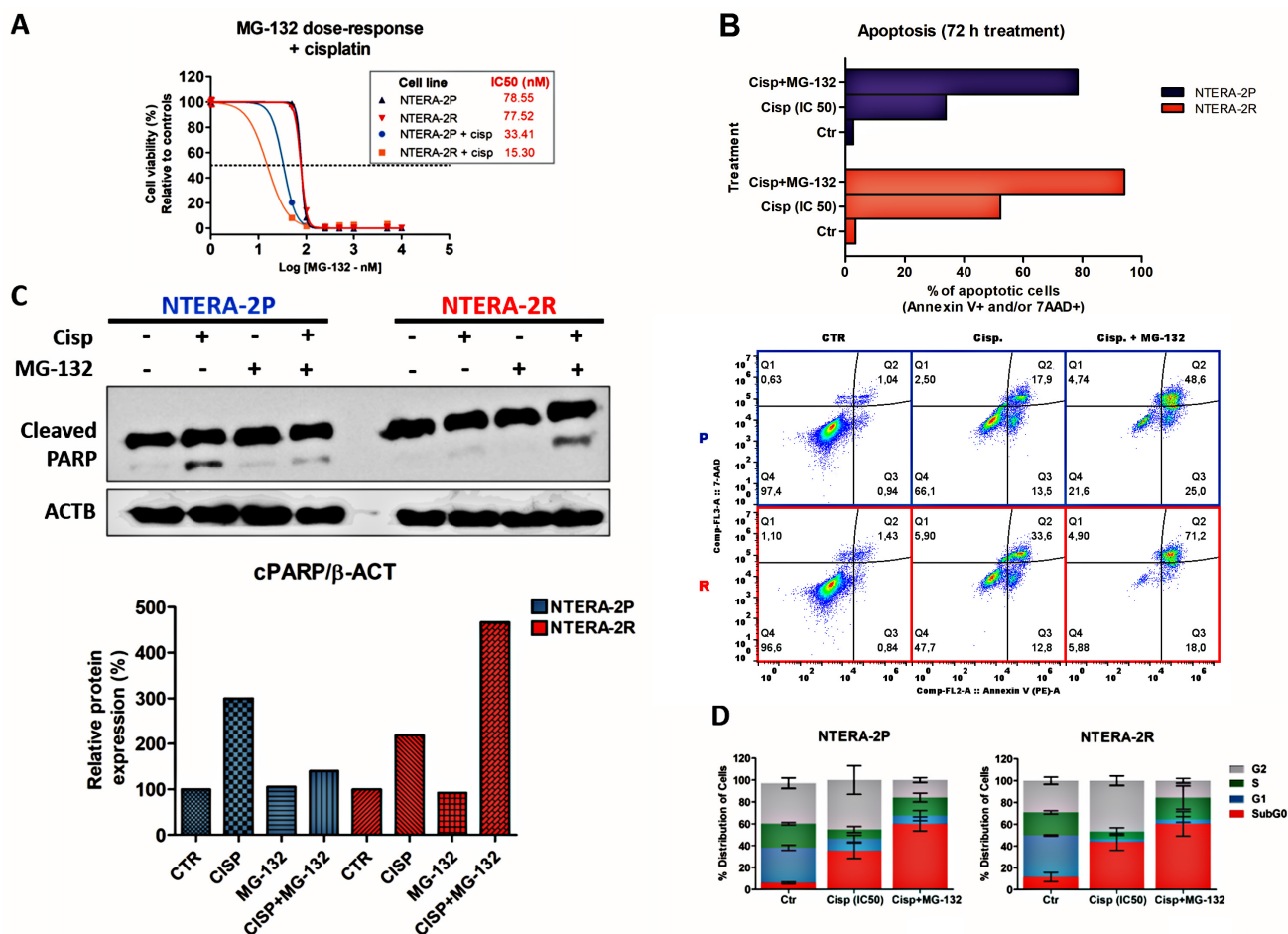
**Fig. 7. MG-132 and CDDP combination indicate great therapeutic potential in both NTERA-2P and NTERA-2R.** (A) Cell viability assay after MG-132 or CDDP ($IC_{50}$) + MG-132 for 72 hours treatment. $IC_{50}$ values are indicated on the graph. (B) Apoptosis assay after CDDP ($IC_{50}$) or CDDP ($IC_{50}$) + MG-132 (25 nM) for 72 hours treatment. (C) Western blot evaluation of PARP protein levels (total and cleaved) in cells treated for 24 hours with CDDP ($IC_{50}$), MG-132 (25 nM), or the combination. $\beta$-Actin was used for data normalization. The image is representative of one immunoblotting assay. (D) Changes in cell cycle distribution after 72 hours of treatment with CDDP ($IC_{50}$) or CDDP ($IC_{50}$) + MG-132 (25 nM) were statiscally significant for both NTERA-2P and NTERA-2R ($p < 0.01$).

increased the G2 population in both cell lines. When combining both drugs, we observed an increase in S population and a remarkable increase in the Sub-G0 population compared to CDDP alone in both cell lines. These results indicate that MG-132 treatment in non-toxic doses restores CDDP sensitivity of the resistant cell line.

## 4. Discussion

CDDP has a significant role in TGCTs treatment, yet a small subset of patients exhibit CDDP-resistance, constituting a critical clinical challenge [44]. Therefore, understanding the molecular mechanisms involved in CDDP-resistance of TGCTs and use this information to identify novel treatment alternatives for these patients is considered a major goal.

To address this objective, we first established and characterized an *in vitro* model of TGCT CDDP-resistance (NTERA-2R). NTERA-2R cell line was established using incremental CDDP doses (NTERA-2P: $IC_{50}$ of 0.524 $\mu$M; NTERA-2R: $IC_{50}$ of 3.812 $\mu$M) and the resistant phenotype's stability was confirmed by cultivating the cell in a CDDP-free media for four months and reassessing its $IC_{50}$. Previous reports show similar results to ours, while the $IC_{50}$ values of NTERA-2P cell lines were 0.45 $\mu$M, the resistant to cisplatin exhibited $IC_{50}$ values of 5.1 $\mu$M [45]. NTERA-2R demonstrated a more aggressive phenotype, with a significant increase in proliferation, clonogenic survival, and migration ability. Differences in response to CDDP were also observed, with NTERA-2R presenting almost no apoptosis when compared to NTERA-2P. Moreover, we explored the changes induced by CDDP in cell cycle phase distribution in both cell lines, since it is well known the relation between DNA damage and cell cycle regulation. Depending on the cell's ability to detect the damage, it can activate the DNA damage response (DDR) and respond by undergoing apoptosis or cell cycle arrest

and DNA damage repair, which may be related to CDDP-resistance. TGCT presents a decreased DNA repair capacity due to low DNA repair protein expression levels, making these tumors typically sensitive to damaging agents as CDDP. Generally, following CDDP exposure, TGCT cells undergo G2 arrest, and apoptosis is induced in this phase of the cell cycle [45]. NTERA-2P exhibited this exact behavior in our study, demonstrating a higher accumulation of cells in S and G2 arrest, and a corresponding decrease of cells in G1, after CDDP treatment. On the other hand, NTERA-2R treated with the same CDDP doses displayed few cell cycle changes, indicating that it probably has an increased and effective capacity to repair DNA damage as a mechanism of CDDP-resistance.

CDDP-resistance mechanisms have been categorized, for organizational purposes, as pre-target, on-target, and post-target, based on the following events after the cell's exposition to the drug [46]. We verified that NTERA-2P and NTERA-2R expressed comparable amounts of the DNA damage sensor γ-H2AX, indicating that CDDP was causing similar DNA damage levels in both. Based on this result, we considered that pre-target mechanisms were not the main ones responsible for NTERA-2R resistance. There is some controversy in the literature about pre-target mechanisms and TGCT CDDP-resistance, but it is conceivable that pre-target mechanisms could contribute to the resistance, but additional studies dedicated to TGCTs are needed [31].

On the other hand, on-target mechanisms have been extensively studied on TGCTs and connected to CDDP-resistance. These mechanisms are related to alterations that implicate DNA adducts formed upon CDDP binding, as DNA repair systems, or alternatively, the bypass of DNA adducts through a system known as translesion DNA synthesis (TLS) [47]. Here, we profiled the gene expression changes in both NTERA-2P and NTERA-2R using the NanoString DNA damage and repair panel. Among the five genes differentially expressed between NTERA-2P and NTERA-2R, we observed an increase in *MGMT* and *XPC* expression in NTERA-2R, which were already described to be involved in CDDP-resistance [48–50]. Moreover, *MGMT* expression levels also significantly correlated with a worst disease-free survival in TGCT cohort from the TCGA database, demonstrating it may be a promising biomarker of CDDP-resistance or even a therapeutic target for TGCT CDDP-resistance. Changes in the expression of *POLD4* and *POLE* genes may indicate the involvement of mechanisms of TLS in our CDDP-resistance model. We also observed that the six genes more upregulated in NTERA-2R after CDDP treatment were also significantly upregulated in NTERA-2P CDDP treated. However, the most downregulated gene in NTERA-2R after CDDP treatment (*ALKBH2*) was exclusively observed in this cell line and may indicate a new mechanism of TGCT CDDP acquired resistance, as previously demonstrated in lung can-

cer cells [51]. In the analysis using STRING database and DAVID gene ontology we observed two clusters associated with DNA repair mechanisms and cell cycle, apoptosis, and cellular signaling. Besides, we found several strongly connected genes related to DNA repair and cell cycle regulation, which led us to hypothesize whether TLS could be related to TGCT CDDP-resistance. TLS has been associated with CDDP-resistance in other tumors with basically a dual role, suggesting that TLS inhibition may sensitize tumors to therapy as well as prevent the emergence of tumor chemoresistance [27,52]. Besides, it has recently been acknowledged that the TLS is an important mechanism that presents a rationale to be further explored in TGCT CDDP-resistance [31].

TLS inhibitors may be an alternative to overcome CDDP-resistance, and the targets can be diverse. The most obvious target though, is the replication sliding clamp Proliferative Cell Nuclear Antigen (PCNA), which is considered the primary regulator of TLS and one of the critical non-oncogenic mediators supporting cancer development [53,54]. In this study, we have chosen three putative TLS inhibitors and one proteasome inhibitor, to evaluate their capacity to inhibit cell proliferation of TGCT cells. (1) PCNA-I1 binds to PCNA and has been shown to reduce the chromatin-associated PCNA in cells, and inhibits the growth of many types of tumors [55]. (2) T2AA is an inhibitor of the PCNA/PIP-box interaction [35] and can inhibit the growth of cancer cells through the induction of early apoptosis [56]. (3) ML-323 is an effective inhibitor of the USP1-UAF1 deubiquitinase complex, responsible for deubiquitinating PCNA [57,58]. ML-323 has been shown to potentiate CDDP cytotoxicity in non-small cell lung cancer and osteosarcoma cells and may be an alternative for overcoming CDDP-resistance [57]. (4) MG-132 is a peptide aldehyde, which effectively blocks the proteolytic activity of the 26S proteasome complex and has been demonstrated to prevent TLS in human cancer cells but not in normal cells [59]. However, the exact mechanism by which MG-132 inhibits TLS remains to be elucidated. Here, we demonstrated that the four TLS inhibitors' present the capacity to reduce TGCT proliferation, even of NTERA-2R cell line. MG-132 exhibited the highest cytotoxic potential, with $IC_{50}$ values for two different embryonal carcinoma cell lines (NTERA-2P and NTERA-2R) and one teratocarcinoma cell line (577MF) in the low nanomolar range.

We further explored the MG-132 potential by testing a combination treatment with CDDP to evaluate its capacity to improve CDDP-based therapy. We found that MG-132 enhanced CDDP activity in TGCT, triggering changes in cell cycle distribution and increased cell death through apoptosis, corroborating studies in ovarian carcinoma and osteosarcoma [60,61].

This is the first pre-clinical study reporting the effectiveness of MG-132 in TGCT treatment, focusing on its potential to overcome CDDP-resistance alone or in com-

bination with CDDP. It is worth mentioning that most of our results were obtained in the NTERA-2 cell line, which is a major limitation in our research. TGCTs are classified into two histological groups, including seminoma and NSGCTs, which includes embryonal carcinoma [5,6]. It has been known that seminomas are highly sensitive to DNA-damaging agents, whereas non-seminomas including embryonal carcinoma and yolk sac tumor, often appear in the metastatic setting resistant to cisplatin, which reflects differences in cell biology [62,63]. Thus, because NTERA-2 cell line is an embryonal carcinoma, the results reported here represent only this histological subtype. Another drawback of our study is that we did not develop other TGCT CDDP-resistant cell lines, which may limit our conclusions of MG-132 effectivity in TGCT CDDP resistance. In order to overcome this limitation, we evaluated the effects of MG-132 on the 577MF TGCT cell line, which was 5.5-fold more resistant to CDDP than NTERA-2P. Besides, 577MF has a different histology (teratocarcinoma), indicating MG-132 potential in histologies other than embryonal carcinoma. Even so, given the complexity and variability of TGCT, the role of MG-132 as a potential new drug to treat CDDP-resistant TGCT should be further evaluated in other cell lines to confirm our findings.

In future studies, we plan to explore in more detail whether and how these drugs may be involved in TGCT CDDP-resistance, assess its potential in TGCT cell lines from different histologies to broadly cover TGCT complexity, and use an *in vivo* TGCT model to evaluate their effectiveness and assess their toxicity profile. These may reveal the most appropriate approach to clinical applications and whether a specific biomarker will be necessary for this new therapy.

## 5. Conclusions

In this study, we demonstrated for the first time the potential of MG-132 used alone or in combination with CDDP to treat CDDP-resistant TGCTs. We developed a CDDP-resistance *in vitro* model and profiled the differentially expressed genes that may be related to the resistance phenotype. We showed the possibility of using new drugs to overcome TGCT CDDP-resistance and found that MG-132 displayed a potent cytotoxic activity against TGCT cells. Moreover, when used in combination with CDDP, MG-132 reduced cell viability and induced apoptosis and changes in cell cycle phases distribution. These results indicate that MG-132 is a potential treatment strategy to overcome TGCT treatment failure.

## Author Contributions

AvHL—Conceptualization, Formal analysis, Methodology, Validation, Writing – original draft. DOV—Conceptualization, Resources, Writing – review & editing. MTP—Conceptualization, Resources, Supervision, Writing – review & editing. RMR—Conceptualization, Resources, Writing – review & editing. LSdS—Formal analysis, Writing – review & editing. LdNBP—Methodology, Writing – review & editing. ERMC—Methodology, Validation, Writing – review & editing. INFG—Methodology, Validation, Writing – review & editing. LMdJ—Methodology, Writing – review & editing. MFSG—Methodology, Writing – review & editing. AOdR—Methodology, Writing – review & editing. TAT—Methodology, Writing – review & editing. ACL—Methodology, Writing – review & editing. LFL—Supervision, Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

## Ethics Approval and Consent to Participate

Not applicable.

## Conflict of Interest

The authors declare no conflict of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2708245.

## References

[1] Ghazarian AA, Trabert B, Devesa SS, McGlynn KA. Recent trends in the incidence of testicular germ cell tumors in the United States. Andrology. 2015; 3: 13–18.

[2] Feldman DR. Medical Treatment of Advanced Testicular Cancer. The Journal of the American Medical Association. 2008; 299: 672.

[3] Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, *et al*. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: A Cancer Journal for Clinicians. 2021; 71: 209–249.

[4] Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2022. CA: A Cancer Journal for Clinicians. 2022; 72: 7–33.

[5] Moch H, Cubilla AL, Humphrey PA, Reuter VE, Ulbright TM.

The 2016 WHO Classification of Tumours of the Urinary System and Male Genital Organs-Part A: Renal, Penile, and Testicular Tumours. European Urology. 2016; 70: 93–105.

[6] Pierce JL, Frazier AL, Amatruda JF. Pediatric Germ Cell Tumors: a Developmental Perspective. Advances in Urology. 2018; 2018: 9059382.

[7] Honecker F, Aparicio J, Berney D, Beyer J, Bokemeyer C, Cathomas R, et al. ESMO Consensus Conference on testicular germ cell cancer: diagnosis, treatment and follow-up. Annals of Oncology. 2018; 29: 1658–1686.

[8] Lopes LF, Macedo CRPD, Aguiar SDS, Barreto JHS, Martins GE, Sonaglio V, et al. Lowered Cisplatin Dose and no Bleomycin in the Treatment of Pediatric Germ Cell Tumors: Results of the GCT-99 Protocol from the Brazilian Germ Cell Pediatric Oncology Cooperative Group. Journal of Clinical Oncology. 2016; 34: 603–610.

[9] Oosterhuis JW, Looijenga LHJ. Human germ cell tumours from a developmental perspective. Nature Reviews Cancer. 2019; 19: 522–537.

[10] Morelli F, Tozzi L, Setola P, Bisceglia M, Barbini VR, Maiello E. Postchemotherapy residual masses in germ cell tumor patients: our experience. Annals of Oncology. 2006; 17: vii132–vii136.

[11] Cavallo F, Feldman DR, Barchi M. Revisiting DNA damage repair, p53-mediated apoptosis and cisplatin sensitivity in germ cell tumors. The International Journal of Developmental Biology. 2013; 57: 273–280.

[12] Sanchez-Vega F, Mina M, Armenia J, Chatila WK, Luna A, La KC, et al. Oncogenic Signaling Pathways in The Cancer Genome Atlas. Cell. 2018; 173: 321–337.e10.

[13] de Vries G, Rosas-Plaza X, van Vugt MATM, Gietema JA, de Jong S. Testicular cancer: Determinants of cisplatin sensitivity and novel therapeutic opportunities. Cancer Treatment Reviews. 2020; 88: 102054.

[14] O'Shaughnessy MJ, Feldman DR, Carver BS, Sheinfeld J. Late Relapse of Testicular Germ Cell Tumors. Urologic Clinics of North America. 2015; 42: 359–368.

[15] Michael H, Lucia J, Foster RS, Ulbright TM. The Pathology of Late Recurrence of Testicular Germ Cell Tumors. The American Journal of Surgical Pathology. 2000; 24: 257–273.

[16] Bakardjieva-Mihaylova V, Skvarova Kramarzova K, Slamova M, Svaton M, Rejlova K, Zaliova M, et al. Molecular Basis of Cisplatin Resistance in Testicular Germ Cell Tumors. Cancers. 2019; 11: 1316.

[17] Oing C, Skowron MA, Bokemeyer C, Nettersheim D. Epigenetic treatment combinations to effectively target cisplatin-resistant germ cell tumors-past, present, and future considerations. Andrology. 2019; 7: 487–497.

[18] Skowron MA, Vermeulen M, Winkelhausen A, Becker TK, Bremmer F, Petzsch P, et al. CDK4/6 inhibition presents as a therapeutic option for paediatric and adult germ cell tumours and induces cell cycle arrest and apoptosis via canonical and non-canonical mechanisms. British Journal of Cancer. 2020; 123: 378–391.

[19] Jacobsen C, Honecker F. Cisplatin resistance in germ cell tumours: models and mechanisms. Andrology. 2015; 3: 111–121.

[20] Zschäbitz S, Distler FA, Krieger B, Wuchter P, Schäfer-Eckart K, Jenzer M, et al. Survival outcomes of patients with germ cell tumors treated with high-dose chemotherapy for refractory or relapsing disease. Oncotarget. 2018; 9: 22537–22545.

[21] Oing C, Alsdorf WH, von Amsberg G, Oechsle K, Bokemeyer C. Platinum-refractory germ cell tumors: an update on current treatment options and developments. World Journal of Urology. 2017; 35: 1167–1175.

[22] Bagrodia A, Lee BH, Lee W, Cha EK, Sfakianos JP, Iyer G, et al. Genetic Determinants of Cisplatin Resistance in Patients with Advanced Germ Cell Tumors. Journal of Clinical Oncol-

ogy. 2016; 34: 4000–4007.

[23] Abada PB, Howell SB. Cisplatin Induces Resistance by Triggering Differentiation of Testicular Embryonal Carcinoma Cells. PLoS ONE. 2014; 9: e87444.

[24] Wermann H, Stoop H, Gillis AJ, Honecker F, van Gurp RJ, Ammerpohl O, et al. Global DNA methylation in fetal human germ cells and germ cell tumours: association with differentiation and cisplatin resistance. The Journal of Pathology. 2010; 221: 433–442.

[25] Juliachs M, Muñoz C, Moutinho CA, Vidal A, Condom E, Esteller M, et al. The PDGFR$\beta$–AKT Pathway Contributes to CDDP-Acquired Resistance in Testicular Germ Cell Tumors. Clinical Cancer Research. 2014; 20: 658–667.

[26] Sakurai Y, Ichinoe M, Yoshida K, Nakazato Y, Saito S, Satoh M, et al. Inactivation of REV7 enhances chemosensitivity and overcomes acquired chemoresistance in testicular germ cell tumors. Cancer Letters. 2020; 489: 100–110.

[27] Yamanaka K, Chatterjee N, Hemann MT, Walker GC. Inhibition of mutagenic translesion synthesis: A possible strategy for improving chemotherapy? PLOS Genetics. 2017; 13: e1006842.

[28] Srivastava AK, Han C, Zhao R, Cui T, Dai Y, Mao C, et al. Enhanced expression of DNA polymerase eta contributes to cisplatin resistance of ovarian cancer stem cells. Proceedings of the National Academy of Sciences. 2015; 112: 4411–4416.

[29] Zhou W, Chen Y, Liu X, Chu P, Loria S, Wang Y, et al. Expression of DNA Translesion Synthesis Polymerase η in Head and Neck Squamous Cell Cancer Predicts Resistance to Gemcitabine and Cisplatin-Based Chemotherapy. PLoS ONE. 2013; 8: e83978.

[30] Yang L, Shi T, Liu F, Ren C, Wang Z, Li Y, et al. REV3L, a Promising Target in Regulating the Chemosensitivity of Cervical Cancer Cells. PLoS ONE. 2015; 10: e0120334.

[31] Lobo J, Jerónimo C, Henrique R. Cisplatin Resistance in Testicular Germ Cell Tumors: Current Challenges from Various Perspectives. Cancers. 2020; 12: 1601.

[32] Andrews PW, Damjanov I, Simon D, Banting GS, Carlin C, Dracopoli NC, et al. Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Differentiation in vivo and in vitro. Laboratory Investigation. 1984; 50: 147–162.

[33] Wang N, Trend B, Bronson DL, Fraley EE. Nonrandom abnormalities in chromosome 1 in human testicular cancers. Cancer Research. 1980; 40: 796–802.

[34] Silva-Oliveira RJ, Silva VAO, Martinho O, Cruvinel-Carloni A, Melendez ME, Rosa MN, et al. Cytotoxicity of allitinib, an irreversible anti-EGFR agent, in a large panel of human cancer-derived cell lines: KRAS mutation status as a predictive biomarker. Cellular Oncology. 2016; 39: 253–263.

[35] Gebäck T, Schulz MMP, Koumoutsakos P, Detmar M. TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. BioTechniques. 2009; 46: 265–274.

[36] Gomes I, Moreno DA, dos Reis MB, da Silva LS, Leal LF, Gonçalves GM, et al. Low MGMT digital expression is associated with a better outcome of IDH1 wildtype glioblastomas treated with temozolomide. Journal of Neuro-Oncology. 2021; 151: 135–144.

[37] Hicks SC, Irizarry RA. Quantro: a data-driven approach to guide the choice of an appropriate normalization method. Genome Biology. 2015; 16: 117.

[38] Waggott D, Chu K, Yin S, Wouters BG, Liu F, Boutros PC. NanoStringNorm: an extensible R package for the pre-processing of NanoString mRNA and miRNA data. Bioinformatics. 2012; 28: 1546–1548.

[39] Gu Z, Eils R, Schlesner M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinfor-

matics. 2016; 32: 2847–2849.

[40] Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, *et al*. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Research. 2019; 47: D607–D613.

[41] Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature Protocols. 2009; 4: 44–57.

[42] Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Research. 2009; 37: 1–13.

[43] Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Research. 2017; 45: W98–W102.

[44] Oing C, Seidel C, Bokemeyer C. Therapeutic approaches for refractory germ cell cancer. Expert Review of Anticancer Therapy. 2018; 18: 389–397.

[45] Oechsle K, Honecker F, Cheng T, Mayer F, Czaykowski P, Winquist E, *et al*. Preclinical and clinical activity of sunitinib in patients with cisplatin-refractory or multiply relapsed germ cell tumors: a Canadian Urologic Oncology Group/German Testicular Cancer Study Group cooperative study. Annals of Oncology. 2011; 22: 2654–2660.

[46] Mueller S, Schittenhelm M, Honecker F, Malenke E, Lauber K, Wesselborg S, *et al*. Cell-cycle progression and response of germ cell tumors to cisplatin in vitro. International Journal of Oncology. 2006; 29: 471–479.

[47] Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O, *et al*. Molecular mechanisms of cisplatin resistance. Oncogene. 2012; 31: 1869–1883.

[48] Singh R, Fazal Z, Freemantle SJ, Spinella MJ. Mechanisms of cisplatin sensitivity and resistance in testicular germ cell tumors. Cancer Drug Resistance. 2019; 2: 580–594.

[49] Chen SH, Kuo CC, Li CF, Cheung CHA, Tsou TC, Chiang HC, *et al*. O(6)-methylguanine DNA methyltransferase repairs platinum-DNA adducts following cisplatin treatment and predicts prognoses of nasopharyngeal carcinoma. International Journal of Cancer. 2015; 137: 1291–1305.

[50] Teng X, Fan X, Li Q, Liu S, Wu D, Wang S, *et al*. XPC inhibition rescues cisplatin resistance via the Akt/mTOR signaling pathway in A549/DDP lung adenocarcinoma cells. Oncology Reports. 2019; 41: 1875–1882.

[51] Chen S, Huang W, Kao W, Hsiao S, Pan H, Fang C, *et al*. O6-methylguanine-DNA methyltransferase modulates cisplatin-induced DNA double-strand breaks by targeting the homologous recombination pathway in nasopharyngeal carcinoma. Journal of Biomedical Science. 2021; 28: 2.

[52] Wu S, Xu W, Liu S, Chen B, Wang X, Wang Y, *et al*. Down-regulation of ALKBH2 increases cisplatin sensitivity in H1299 lung cancer cells. Acta Pharmacologica Sinica. 2011; 32: 393–398.

[53] Xie K, Doles J, Hemann MT, Walker GC. Error-prone translesion synthesis mediates acquired chemoresistance. Proceedings of the National Academy of Sciences. 2010; 107: 20792–20797.

[54] Moldovan G, Pfander B, Jentsch S. PCNA, the Maestro of the Replication Fork. Cell. 2007; 129: 665–679.

[55] Stoimenov I, Helleday T. PCNA on the crossroad of cancer. Biochemical Society Transactions. 2009; 37: 605–613.

[56] Tan Z, Wortman M, Dillehay KL, Seibel WL, Evelyn CR, Smith SJ, *et al*. Small-Molecule Targeting of Proliferating Cell Nuclear Antigen Chromatin Association Inhibits Tumor Cell Growth. Molecular Pharmacology. 2012; 81: 811–819.

[57] Punchihewa C, Inoue A, Hishiki A, Fujikawa Y, Connelly M, Evison B, *et al*. Identification of Small Molecule Proliferating Cell Nuclear Antigen (PCNA) Inhibitor that Disrupts Interactions with PIP-box Proteins and Inhibits DNA Replication. Journal of Biological Chemistry. 2012; 287: 14289–14300.

[58] Liang Q, Dexheimer TS, Zhang P, Rosenthal AS, Villamil MA, You C, *et al*. A selective USP1–UAF1 inhibitor links deubiquitination to DNA damage responses. Nature Chemical Biology. 2014; 10: 298–304.

[59] Huang TT, Nijman SMB, Mirchandani KD, Galardy PJ, Cohn MA, Haas W, *et al*. Regulation of monoubiquitinated PCNA by DUB autocleavage. Nature Cell Biology. 2006; 8: 341–347.

[60] Takezawa J, Ishimi Y, Yamada K. Proteasome inhibitors remarkably prevent translesion replication in cancer cells but not normal cells. Cancer Science. 2008; 99: 863–871.

[61] Guo N, Peng Z, Zhang J. Proteasome Inhibitor MG132 Enhances Sensitivity to Cisplatin on Ovarian Carcinoma Cells in Vitro and in Vivo. International Journal of Gynecologic Cancer. 2016; 26: 839–844.

[62] Sun F, Zhang Y, Xu L, Li S, Chen X, Zhang L, *et al*. Proteasome Inhibitor MG132 Enhances Cisplatin-Induced Apoptosis in Osteosarcoma Cells and Inhibits Tumor Growth. Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics. 2018; 26: 655–664.

[63] Mayer F, Wermann H, Albers P, Stoop H, Gillis AJM, Hartmann JT, *et al*. Histopathological and molecular features of late relapses in non-seminomas. BJU International. 2011; 107: 936–943.