Abbreviations:

BEP: bleomicin – etoposide – cisplatin

CI: combination index

DMF: dimethylformamide

FA: fraction affected

HER2: epidermal grow factor 2

HR: hormone receptor

NT2/D1: Ntera-2/cl. D1

P-pRb: phospho-protein of retinoblastoma

pRb: protein of retinoblastoma

PTU: phenylthiourea

STR: short tandem repeats

TGCT: testicular germ cell tumors

VIP: etoposide – ifosfamide – cisplatin

CISPLATIN CYTOTOXICITY IN HUMAN TESTICULAR GERM CELL TUMOR CELL LINES IS ENHANCED BY THE CDK4/6 INHIBITOR PALBOCICLIB

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HIGHLIGHTS

- Cytotoxic drugs as well as the target therapy induced cancer cell resistance
- The combination therapy gives advantage to cure cancers, included TGTC
- Translational research may help to find the better combination
- Combined cisplatin/palbociclib decreased *in vitro* and *in vivo* TGCT cell viability
- The advantage was confirmed with a lower cell recover in drug withdrawal experiments

ABSTRACT

Background: Cisplatin-based chemotherapy is the mainstay of pharmacological treatment of Testicular Germ Cell Tumors (TGCTs) that, together with the early diagnosis and surgery and/or radiotherapy, has dramatically improved the prognosis. However, under the pressure of the pharmacological therapy, both the classical cytotoxic drugs as well as the target therapy drugs, cancer cells may develop resistance. Thus, the combination therapy that may include cytotoxic drugs and target therapy could give advantage to cure cancers. Here, we investigated the *in vitro* and *in vivo* antitumor activity of cisplatin, as a single-agent or in combination with palbociclib.

Materials and Methods: Cell viability of Ntera-2/cl.D1 (NT2/D1) and 833K after exposure to palbociclib and/or cisplatin was evaluated by MTT dye reduction assay and/or by ATP-Lite Luminescence Assay. Gene and protein expression was evaluated by q-RT-PCR and by western blot. Flow cytometric cell cycle analysis was performed as well. The *in vivo* experiments were conducted on NT2/D1 xenografts in AB Zebrafish embryos exposed to the drugs.

Results: palbociclib and cisplatin decreased TGCT cell viability both *in vitro* and *in vivo*. This effect is additive/synergic when cells were exposed to the drug combination. In the NT2/D1 cell lines, the drug combination exerted a positive effect also on delaying cell recovery after the toxic insult. In the combination experiments, cisplatin-induced cell accumulation in G2/M was predominant versus palbociclib effect.

Conclusions: These results could give the rationale to develop further studies to improve the pharmacological treatment of the TGCTs, that however needs to be demonstrated in a dedicated clinical trial.

Keywords: cisplatin**,** palbociclib, combined treatment, NT2/D1 - 833K cell line

MICROABSTRACT

- Cisplatin-based chemotherapy is the choice for Testicular Germ Cell Tumor (TGCTs) treatment. Here, at preclinical level we studied whether the cisplatin combination with the Cdk4/6 inhibitor palbociclib could give advantage in TGCT cell models.
- Palbociclib and cisplatin decreased TGCT cell viability *in vitro* and *in vivo*.
- We suggest a rationale to design a clinical study to evaluate this combination approach.

1. INTRODUCTION

Testicular cancer is a relatively rare cancer accounting for 1% to 2% of all neoplasms in male patients [1], but is the most common malignancy in young adult men aged 15-40 years [2]. The testicular cancers derived from germ cells (Testicular Germ Cell Tumors, TGCT) are the most common, usually divided into the morphologically homogeneous seminomas, roughly 60% of cases with a peak incidence at 35 years, and the heterogeneous non-seminoma germ cell tumors, with a peak incidence at earlier age [3]. In Europe, relative survival at 5 years after diagnosis is about 97% [4], mainly due to the early diagnosis and treatment efficacy [5]. Standard treatment is radical orchiectomy and/or combination with chemotherapy and radiotherapy or, less frequently, retroperitoneal lymph node dissection [6]. After surgery, low-risk patients usually undergo to active surveliance (AS), while high-risk non-seminoma patients are addressed to adjuvant chemotherapy with bleomycin, etoposide and cisplatin (BEP) (for an extensive review please see [7]).

The systemic treatment for metastatic germ cell tumors is stratified according to the International Germ Cell Cancer Cooperative Group (IGCCCG), based to different clinical and pathophysiological parameters. It indicates different cycles of BEP or VIP, where bleomycin is replaced by ifosfamide [7,8,9]. Local treatment of residual masses after cisplatin-based chemotherapy depends on different approaches, included access to medical care and patient's comorbidities [7,9] or more aggressive chemotherapy protocols [10].

However, under the pressure of the pharmacological therapy, both the classical cytotoxic drugs as well as the target therapy drugs, cancer cells may develop resistance, with a vast array of mechanisms, that may involve the cell metabolism and proliferation and invasion capability at different levels [11]. Thus, the combination therapy that may include cytotoxic drugs and target therapy is essential to cure cancers, as demonstrated in clinical trials, where a potent synergy between targeted molecules and traditional chemotherapy does occur [reviewed in 12]. Accordingly, cyclin –dependent kinases are the key enzymes involved in the control of cell cycle progression and in particular Cdk4 and Cdk6 are the target of cell-cycle checkpoint inhibitors, such as palbociclib, that become a full part of the

combination therapy for the treatment of hormone receptor positive (HR+) and epidermal growth factor 2 negative (HER2-) advanced or metastatic breast cancer [(IBM- Micromedex)]. Interestingly, both *in vitro* and *in vivo* preclinical evidences show its effectiveness also in others type of tumors such as melanoma [13], glioblastoma multiforme [14, 15] and our group demonstrated that also cell lines and primary cell cultures derived from adrenocortical carcinoma are sensitive to the cytotoxic effect of palbociclib [16]. More recently, it has been shown that Cdk4/6 inhibitors induce cell cycle arrest and apoptosis in different types of TGCT, suggesting that they could present a therapeutic potential [17]. Accordingly, a small non-randomized phase 2 trial on 30 patients with refractory, pRB-expressing GCTs treated with palbociclib indicate a favorable 24-week progression-free survival rate, with an acceptable toxicity profile, mainly with hematological toxic effects [18]; however, contrasting results have been as well reported (reviewed in [7]).

Based on this finding and on the well-known therapeutical advantage of a combined pharmacological approach, the aim of this study was to explore whether the combination of cisplatin, the gold standard chemotherapy for TGCT, with palbociclib could enhance the *in vitro* and *in vivo* cytotoxicity in TGCT experimental cell models, namely Ntera-2/cl.D1 (NT2/D1) and 833K cell lines.

2. Material and Methods

2.1 Cell culture

NT2/D1 (ATCC® CRL-1973™) cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as suggested by the manufacturer. 833K cell line [19] was kindly given by Dr. B. Köberle (Karlsruhe Institute of Technology, Karlsruhe, Germany) and Prof. J. Masters (University College London, London, UK). Media and supplements were supplied by Merck (Merck, Darmstadt, Germany). Cell line was tested for mycoplasma and NT2/D1 cells were authenticated by GenePrint® 10 System (Promega Italia, Milan, Italy), according to the protocol suggested by the manufacturer. Using the same approach, the STR expression of 833K cell line was investigated and reported in Supplementary data: table S1.

2.2 Cell treatment

Cells were plated (20.000 cells/well for NT2/D1 cell line; 15.000 cells/well for 833K cell line) in 24-well plates in complete medium and treated with increasing concentration of cisplatin, range: 0.01 - 10 µM; or palbociclib, range: 0.5 – 15 µM in NT2/D1 cell line and 0.5 – 20 µM in 833K cell line for 48 hours, according to the calculated doubling time. Palbociclib and cisplatin were solubilized in water and DMF, respectively. Drugs were purchased from Selleckchem Chemicals (DBA Italia, Milan, Italy). Preliminary experiments were conducted to set up the optimal cell number and the range of drug concentrations in both cell lines.

2.3 Drug combination experiments

Palbociclib and cisplatin combination experiments were performed according to the Chou and Talalay method [20]. Cells were treated for 48 hours with palbociclib (0.29 – 18.4 µM for NT2/D1; 0.479 – 30 µM for 833K) and cisplatin (0.038 – 2.4 µM for NT2/D1; 0.0625 – 4 µM for 833K) alone or in combination at fixed ratio (palbociclib : cisplatin = 7.67 : 1) as recommended for the most efficient data analysis [21]. Cells were analyzed for cell viability using MTT assay. Data were then converted to Fraction affected (Fa, range from 0 to 1 where Fa=0 indicating 100% of cell viability and Fa=1 indicating 0% of cell viability) and analyzed using the CompuSyn software (ComboSyn inc. Paramus,

NJ, USA) to calculate the combination index (CI), being the CI value < 0.9 an indication of synergism, a CI = 0.9-1.1 an indication of addictive effect and CI > 1.1 an indication of antagonism.

2.4 Cell viability assays

Cell viability was determined by 3-(4,5-Dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye reduction assay as previous described [22] and by ATP-Lite Luminescence Assay according to the manufacturer's protocol (PerkinElmer Italia, Milan, Italy). Luminescence was detected by a EnSight Multimode Plate Reader (PerkinElmer Italia).

2.5 Quantitative RT-PCR (qRT-PCR)

RNA was extracted from 1 x 10^6 cells, using RNeasy Mini Kit (QIAGEN, Milan, Italy). Total RNA was eluted in RNAse free water and quantified with NanoDrop instrument (VWR International, Pennsylvania, USA). 1 µg of total RNA was retro-transcribed in cDNA using M-MLV-RT as enzyme (Promega Italia, Milan, Italy). Gene expression was evaluated by qRT-PCR (ViiA7 Real Time PCR System, Applied Biosystems, Milan) using the SYBR Green as fluorochrome (Applied Biosystems). Primer sequences are shown in Supplementary data: table S2. Differences in the threshold cycle Ct values between the β-actin housekeeping gene and the studied genes (ΔCt) were then calculated as an indicator of the amount of mRNA expressed.

2.6 Western Blot

Total NT2/D1 cell lysate was obtained adding to the pellet ice-cold Ripa Buffer containing proteases and phosphatases inhibitor (Roche, Milan, Italy). Total protein quantification was performed with Bradford method and equally amounts of protein were separated by electrophoreses on a polyacrilamide gel NuPage 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA). After the separation, proteins were electroblotted to a nitrocellulose membrane (GE Healthcare Life Sciences, Milan, Italy). The following primary antibodies were used: anti-total Retinoblastoma (pRb) protein (final concentration: 0.656 µg/ml; Cell Signaling Technology, Milan, Italy), anti-phosphorylated pRb protein (final concentration: 0.150 µg/ml; Cell Signaling Technology, Milan, Italy), anti-Cdk4 and anti-Cdk6 (final concentration: 1 µg/ml; Santa Cruz Biotechnology, Dallas, Texas, USA). A mouse

monoclonal antibody direct against α-tubulin (final concentration: 0.01 μg/ml, Sigma Aldrich, Milan, Italy) was used to normalized the values after palbociclib treatment. A mouse monoclonal antibody against GAPDH (final concentration: 0.4 μg/ml, Millipore, Massachusetts, US) was used to normalized the values after cisplatin treatment, since the drug alters the tubulin assembly [23]. The following secondary antibodies were used: goat α-Rabbit 926-32211 (IRDye 800CW, LI-COR), goat α-Mouse 926-68070 (IRDye 680RD, LI-COR). The specific signal was visualized with Odissey (LI-COR Biosciences, Nebraska, USA) and proteins quantified using Image Studio 4.0 Software (LI-COR Biosciences).

2.7 Cell cycle analysis

Flow cytometric cell cycle analysis was performed as previous described, with minor modifications [24]. Briefly, treated and untreated NT2/D1 cells were stained with violet LIVE/DEAD Fixable Dead Cell Stain Kit (Life Technologies, Milan, Italy) fixed, treated with RNase A (12.5 μg/mL), stained with propidium iodide (40 μg/mL) (Life Technologies, Milan, Italy) and analyzed by flow cytometry using an MACS Quant Analyzer (Miltenyi Biotec, GmbH, Germany) for cell cycle status. Data were analyzed using FlowJo (TreeStar Inc, Ashland, Oregon, USA).

2.8 Fish and Embryos Maintenance

Wild-type zebrafish AB strain obtained from Karlsruhe Institute of Technology (KIT, Germany) were used according to the EU Directive 2010/63/EU for animal experiments. The adult zebrafish were maintained by following the protocols approved by the Local Committee for Animal Health (Animal Welfare Organization) and the Italian Ministry of Health (Approved Project 393/2017-PR). They were all kept in tanks containing 3 L of water at 28 °C with 14-h light and 10-h dark cycle. Breeding of adult zebrafish was carried out by natural crosses and embryos were collected; they were staged according to established protocols [25]. The embryos for the experiments were obtained by the breeding of adult male and female zebrafish. After the conclusion of the experiments, the zebrafish embryos were euthanized with 400 mg/L tricaine (ethyl 3-aminobenzoate methane sulfonate salt; Sigma-Aldrich, Milan, Italy).

2.9 Tumor xenograft

Tumor xenograft of NT2/D1 cells was conducted as described in Gianoncelli et al. [26]. Briefly, AB zebrafish embryos at 48 hpf were dechorionated, anesthetized with 0.042 mg/mL tricaine, and microinjected with the labeled tumor cells into the subperidermal space of the yolk sac. Microinjections were performed with the electronic micro- injector FemtoJet coupled with the InjectMan N12 manipulator (Eppendorf Italia, Milan, Italy). Approximately 250 cells in a volume of 4 nL were injected into each embryo (25 embryos for each group), which was then maintained in fish water plus PTU in a 32°C incubator to allow tumor cell survival and growth. A picture of each injected embryo was acquired under a Leica MZ16F fluorescence stereomicroscope 2 hours after treatment (T0). Ten µM cisplatin and/or 10 µM palbociclib or solvent was directly added to the PTU-fish water. After 3 days of treatment (T3), pictures were taken as described previously. The tumor areas of treated and untreated groups at T0 and T3 were measured with Noldus DanioScopeTM software (Noldus Information Technology).

2.10 Statistical analysis

Data analysis was conducted using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Statistical analysis was carried out using one-way ANOVA and Bonferroni's Multiple Comparison test or Student's *t* test. A $P\ value < 0.05$ was considered as statistically significant. Unless otherwise specified, data are expressed as mean ± SD of at least three experiments run in triplicate.

3. Results

3.1 Cytotoxic effect of cisplatin and/or palbociclib in human TGCT cell lines

NT2/D1 and 833K cells were treated with increasing concentrations of cisplatin (0.01–10 μM) for 48 hours and then analyzed by MTT assay. The concentration-response curve showed a decrease of cell viability in both cell lines (Fig. 1A), with the IC_{50} value of, respectively, 0.3 μM (95% IC: 0.25 μM to 0.45 μM) for NT2/D1 cells and 1.02 μM (95% IC: 0.68 μM to 1.53 μM) for 833K cell line.

Cells were then exposed to increasing concentrations of palbociclib (0.5 – 15/20 μM) for 48 hours. Cell viability was reduced in a concentration-dependent manner in palbociclib-exposed cells, reaching about the 16% in NT2/D1 cells and 3% in 833K cells compared to untreated cells (Fig. 1B). Sigmoidal concentration-response function was applied to calculate the IC_{50} value of palbociclib, that was 2.3 μM (95% IC: 1.93 μM to 2.80 μM) for NT2/D1 cell line and 7.7 μM (95% IC: 4.98 μM to 11.81 μM) for 833K cell line. To confirm these data, evaluation of cell viability was performed using also an ATP assay, that resulted in a superimposable trend.

To evaluate whether palbociclib treatment of TGCT cell lines could enhance the cytotoxicity of cisplatin, combination experiments were set up, as described in Methods. Cells were treated with cisplatin alone, palbociclib alone or combined together at fixed molar ratio for 48 hours. Concentration and effect data (Fig. 2A.1) obtained were then converted to Fa values and analyzed with CompuSyn software. The combination index (CI) was calculated and indicated that in NT2/D1 cells, the palbociclib/cisplatin combination exerted mainly an additive/synergic effect within the Fa = 0.13 – 0.60 in NT2/D1 cells (Fig. 2A.2). The graphical representation is reported in figure 2A.3. Results were confirmed using the 833K cell line and are reported in Fig. 2B, showing an additive/synergic effect within the Fa = 0.29 – 0.55 (Fig. 2B.2). These results strongly suggested that the combined palbociclib/cisplatin treatment offered an advantage in inducing TGCT cell cytotoxicity, at low drug concentrations. As expected, when the cytotoxicity of the single drug increased over the 60-70%, the efficacy of combination, measured as Fa value, decreased. The

combination index value for each drug-concentration in each cell line is reported in Supplementary data: table S3.

Finally, in order to evaluate whether the drug(s) treatment exerted a long-lasting effect on cell viability, NT2/D1 cell line were treated for 48 hours with palbociclib and cisplatin, at their respective IC_{50}, as a single drug or in combination, then the drugs were removed and cells were kept in culture in drug-free complete medium, to evaluate the capability of cells to recover after the cytotoxic insult. Results are reported in the Supplementary data: figure S1 and demonstrated that the drug combination slowed down the cell recovery after treatment compared to the single drug.

3.2 In vitro functional effects of cisplatin and/or palbociclib exposure of TGCT experimental cell model NT2/D1.

The expression of the main palbociclib target proteins, namely Cdk4, Cdk6 and pRb was evaluated, firstly by gene expression. Results are reported in the Supplementary data: table S4 and revealed that exposure of NT2/D1 cells to 2.3 µM palbociclib for 48 hours did not modify the mRNA expression level of the above mentioned genes. Translation into their respective proteins was then evaluated by western blot. Results are reported in Fig. 3A and demonstrated that the expression of Cdk4 and Cdk6 were both increased by palbociclib treatment, with an increase of the protein level of 277 % ± 89.7% for Cdk4 and of 163.3% ± 34.6% for Cdk6, while we observed a not significant decrease of pRb. In order to verify the palbociclib mechanism of action, the total pRb and the phosphorylated pRb (P-pRb) protein levels were then analyzed. Cells were left untreated or exposed to 10µM palbociclib for 6, 12 and 24 hours and results reported in Supplementary data: figure S2 demonstrated that P-pRb significantly decreased in a linear time-dependent manner up to 24 hrs in treated compared to untreated cells.

With the hypothesis to investigate the cytotoxic effect of the palbociclib/cisplatin combined treatment, we evaluated whether cisplatin treatment could modify palbociclib target protein expression. NT2/D1 cells were then treated for 48 hours with cisplatin at its IC_{50} and Cdk4, Cdk6 and pRb expression were measured both at the gene and protein level. q-PCR-RT analyses demonstrated

that the mRNA levels of Cdk4 and pRb were not modified by cisplatin treatment, while the mRNA encoding Cdk6 was significantly increased in treated cells compared to untreated control cells (Supplementary data: table S5). Results obtained measuring the protein expression by western blot are in line with the gene expression results and demonstrated that the amount of Cdk4 protein and pRb protein did not vary significantly, while we observed a significant increase of 100.9% ± 38% in Cdk6 expression ($P < 0.01$) (Fig. 3B).

NT2/D1 cells were treated with palbociclib and cisplatin as single drug and then in combination and the cell cycle distribution was analyzed by flow cytometry (Fig. 4). As expected, an increase in the proportion of cells in the G0/G1 phase after palbociclib treatment (untreated cells: 26.97 ± 4.51% vs palbociclib-treated cells: 45.85± 3.06%, $P<0.05$) was observed.

Similarly, due to the mechanism of cisplatin-induced DNA damage preventing cell cycle progression [27, 28], cisplatin treatment led to accumulation of cells in the G2/M (untreated cells: 31.28 ± 0.01% vs cisplatin-treated cells: 71.35 ± 3.29%, $P<0.001$). The addition of palbociclib to cisplatin did not modify the percentage of cells that remained in the G2/M peak: the percentage of cells distribution within the different phases was similar in cisplatin alone compared to combined treatment (Fig. 4A) thus suggesting that Cdk4/6 inhibition may have a scarce impact on the DNA damage response. A representative graph of cell cycle distribution after drugs treatment is reported in Fig. 4B. Finally, exposure of NT2/D1 cells to either a single drug at their respective IC_{50} or to the combination activated apoptosis, as observed with the AO/EtBr staining reported in Supplementary data: table S6 and figure S3.

3.3 NT2/D1 tumor xenograft in zebrafish embryos

Then, we explored whether the cytotoxic effect of palbociclib, cisplatin and drug combination was observed as well in the *in vivo* model of tumor xenograft in zebrafish embryos. Preliminary experiments were conducted to evaluate the drug toxicity in 48 hpf zebrafish embryos maintained up to 120 hpf in PTU-fish water added with increasing palbociclib and cisplatin concentrations. Up to, respectively, 50 µM palbociclib and 50 µM cisplatin, no phenotype alterations nor death were

detected in embryos (not shown). Thus, the concentration of 10 µM of each drug was chosen, that is the highest concentration used in the *in vitro* cytotoxicity experiments. NT2/D1 cells were injected in AB zebrafish embryos and embryos were then divided into different groups as indicated and maintained in PTU- fish water added, respectively, with solvent, 10 µM cisplatin, 10 µM palbociclib, 10 µM cisplatin/ 10 µM palbociclib combination. After 3 days (T3), pictures were taken and the tumor area was calculated both in solvent- and drug-exposed embryos. Results in Fig. 5 indicated that, in solvent-exposed embryos, the injected cell area at T3 displayed a two-fold increase compared to T0: 58793 μm^2 ± 5069 μm^2 T3 area vs 28701 μm^2 ± 8307 μm^2 T0 area. In T3 cisplatin-treated embryos, the drug completely inhibited the NT2/D1 cell growth, as the tumor area (23272 μm^2 ± 6965 μm^2) was similar to T0. As well, in T3 palbociclib-treated embryos, the area (31888 μm^2 ± 7449 μm^2) was significantly reduced compared to T3 solvent-exposed embryo area. When embryos were exposed to the drug combination, the area at T3 was significantly reduced compared to T0: tumor area of combined-drug exposed embryos: 17204 μm^2 ± 4500 μm^2, - 41.76% ± 14.92% vs solvent-exposed embryo tumor area, ($P < 0.01$) (Fig. 5A). A representative image of NT2/D1 xenograft in zebrafish embryos is reported in Fig. 5B.

4. Discussion

Combination drug therapy in cancers aims to increase and prolong the therapeutic benefits of a single therapy, controlling side effects and reducing the development of resistance. The availability of drugs with new mechanisms of action, selectivity for specific molecular targets and different adverse effect profiles permits the use of new drug combinations and regimens, that often included however the classical chemotherapic cytotoxic drugs, still representing a milestone in the cancer therapy [29].

Among classical chemotherapic cytotoxic drugs, cisplatin and, more in general, platinum compounds are among the most efficacious drugs, widely used in the treatment of different cancers. Despite the therapeutic effects of platinum, however, these drugs are endowed with the appearance of severe side effects that are dose-limiting. These side effects included the general cell-damaging effects and platinum-specific nephro- and neuro-toxicity [30]. Cisplatin is frequently used in combination with other classical chemotherapic drugs, with positive results. However, it is mandatory to find a combination therapy in which the clinical effects of cisplatin could be enhanced by the contemporary use of another drug, giving the maximum clinical effect in patients, while producing no additional side effects. Accordingly, preclinical studies demonstrated the combination of cisplatin with an inhibitor of the Cdk4/6 could be of interest, as it was demonstrated, i.e. in the oesophageal squamous cell carcinoma [31], in ovarian [32, 33] and bladder cancer [34].

Recently it has been shown that the up-stream block of this pathway elicited by the Cdk4/6 inhibitors could exert an inhibitory effect in TGCTs [17], in line with the observation that an aberrant pRb pathway is frequently observed in TGCTs [35,36]. Here, we reported that cisplatin induced a concentration dependent cytotoxicity in two cell lines derived from human TGCT. Interestingly, after cisplatin treatment we observed a selective increase of Cdk6 in NT2/D1 cell line, that it has been shown to be a signal of cisplatin resistance in other types of cancers, as observed in the epithelial ovarian cancer [32]. This phenomenon however seemed to be not common in all cancers, as contrasting results have been reported in NSCLC, where a decrease of Cdk6 involving miR-145 activity has been proposed for cisplatin-induced resistance [37]. In line with previous results, we

report that palbociclib exerted a concentration-dependent inhibition of TGCT cell viability, through the reduction of P-pRb level and the induction of apoptosis. However, exposure of NT2/D1 cells to palbociclib induced a rapid increase Cdk4/6 protein levels, that may limit the cytotoxic effect observed and that suggest the capability of this cell line to overcome palbociclib effect, inducing a drug-resistance, as indicated also by the observed rapid recover of this cell line after palbociclib withdrawal. The cytotoxic effects obtained with the single drug in our experimental models, together with the cisplatin-induced increase of Cdk6 allowed us to evaluate whether the cisplatin/palbociclib combination could offer a better approach. Indeed, the combination demonstrated to induce an additive/synergic effect, in particular and most important, at low concentrations. The advantage of the drug combination was confirmed also in the drug withdrawn experiments, showing a longer cell growth recovery when the two drugs are administered together rather than individually. In particular, as already mentioned, the cell recovery after palbociclib withdrawn was quite rapid, in line with the cytostatic effect elicited by this drug [38], while the cytotoxic effect induced by cisplatin caused a damage that was not fully repaired upon withdrawal. The drug combination strongly prolonged the recovery, suggesting that palbociclib and cisplatin cooperated to reduce the cell recovery capacity. One of the mechanism underlying the positive cytotoxic effect of palbociclib/cisplatin could reside in the effect of these drugs on the cell cycle. Indeed, as expected, palbociclib blocked the NT2/D1 cell cycle in G0/G1 phase [38], while cisplatin-exposed cells showed a cell cycle arrest in G2/M phase [39]. The combination of the two drugs revealed a prevalent arrest of cells in the G2/M phase, suggesting that cisplatin may exert a prevalent role in the cytotoxic combined effect. We would like to underline that cells that overcome the G1 block of palbociclib were then block by cisplatin in G2/M phase and this could support the observed additive/synergic effect observed in our models.

The prevalent arrest in G2/M with the combined drugs may be related to the known functions of Cdks in the DNA damage response [40,41], which is essential for response and recovery from cisplatin exposure [42]. Indeed, it has been shown that the Cdk4/6 inhibition sensitizes ovarian cancer cells to cisplatin, due to an impairment to DNA damage response pathways [33]. The increased cytotoxic

effects of the combination may find its rationale in this dysregulation. However this is an hypothesis that needs to be investigated.

The *in vitro* cytotoxic effect of cisplatin and/or palbociclib was strengthen by results obtained in the *in vivo* model of TGCT cell line xenograft in zebrafish embryos. This result is of particular interest, as this animal model offers an useful tool for *in vivo* first drug screening, due to the limited number of cells needed, the shortened duration of experiments, with limited costs. We are aware that this animal model cannot completely replace the others already in use; neverthless, we believe that our findings offer a suitable and expeditious model to test drugs potentially useful in testicular cancer.

CONCLUSIONS

Present *in vitro* and *in vivo* results in TGCT experimental cell models strongly indicated that cisplatin when combined with palbociclib could offer a therapeutic advantage, therefore suggesting a potential clinical application to improve the pharmacological treatment of the testicular cancer. These preclinical and translational data are interesting, but the clinical therapeutic advantage of this combination must be demonstrated in a dedicated clinical trial.

Supplementary information

Supplementary data: **Table S1.** STR expression of 833K cell line. **Table S2.** Sequences of oligonucleotide primers for qRT-PCR. **Table S3.** Combination index values derives from combined treatment analysis. **Table S4.** ΔCt values of NT2/D1 after palbociclib treatment. **Table S5.** ΔCt values of NT2/D1 after cisplatin treatment. **Table S6.** Quantification of AO/EtBr staining in NT2/D1 cells after drug treatment.

Figure S1. Effect of drug withdrawal on NT2/D1 cell line. **Figure S2.** Measure of pRb phosphorylation after NT2/D2 palbociclib maximal dose treatment. **Figure S3.** Measure of NT2/D1 apoptotic cells after cisplatin and/or palbociclib treatment.

Declarations

- **Ethics approval and consent to participate:** for human materials not applicable, since we used a cell line. For *in vivo* experiments: wild-type zebrafish AB strain were used according to EU Directive 2010/63/EU for animal experiments. The adult zebrafish were maintained by following the protocols approved by the Local Committee for animal health (Animal Welfare Organization) and the Italian Ministry of Health (Approved Project 393/2017-PR).
- **Consent for publication:** not applicable
- **Availability of data and materials:** the datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.
- **Competing interests:** the authors declare that they have no competing interests
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- **Authors’ contributions***:* ER, AA, SS, DZ participated in research design. ER, VB, AA, VS, GP, RMB conducted experiments. ER, AA, MF, RMB, DZ, VS, DB, GP performed data analysis. ER, DZ, FV, AB, MM, SS, GM wrote or contributed to the writing of the manuscript. All authors have read and approved the manuscript.
- **Acknowledgements:** not applicable

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Figure legends

Figure 1. (A) Effect of cisplatin exposure of NT2/D1 and 833K cell lines. Cells were treated with increasing concentrations of cisplatin for 48 hours and the calculated IC_{50} was 0.3 µM for NT2/D1 and 1.02 µM for 833K cell line. Cell viability was measured by MTT assay. Data are the mean ± SD of three experiments performed in triplicate. * $P < 0.0001$ vs ctrl; $^{\#}$ $P < 0.001$ vs ctrl. **(B) Effect of palbociclib exposure of NT2/D1 and 833K cell line.** The concentrations response curves show the linear cytotoxicity of palbociclib and the calculated IC_{50} was 2.3 µM in NT2/D1 cell line and 7.7 µM in 833K cell line. Cell viability was measured by MTT assay. Data are the mean ± SD of three experiments performed in triplicate. * $P < 0.0001$ vs ctrl.

Figure 2. Palbociclib enhanced NT2/D1 and 833K cytotoxicity induced by cisplatin. (1) Concentrations-response curves. NT2/D1 cells (A1) and 833K cells (B1) were exposed to increasing concentrations of palbociclib and cisplatin alone or in combination for 48 hours, as described. Data are expressed as percent of viable cells vs control (ctrl). Data are the mean ± SD of three experiments performed in triplicate. § $P < 0.05$ vs ctrl; $^{\#}$ $P < 0.001$ vs ctrl; * $P < 0.0001$ vs ctrl. **(2) Logarithmic combination index plot.** Cell viability was converted in Fraction Affected (Fa) values and resulting data were analyzed with CompuSyn software to obtain Combination Index (CI) plot for NT2/D1 cells (A2) and 833K cell line (B2). CI value < 0.9 synergism, CI = 0.9-1.1 additive effect and CI > 1.1 antagonism. **(3) Isobolograms** of NT2/D1 (A3) and 833K (B3) cells.

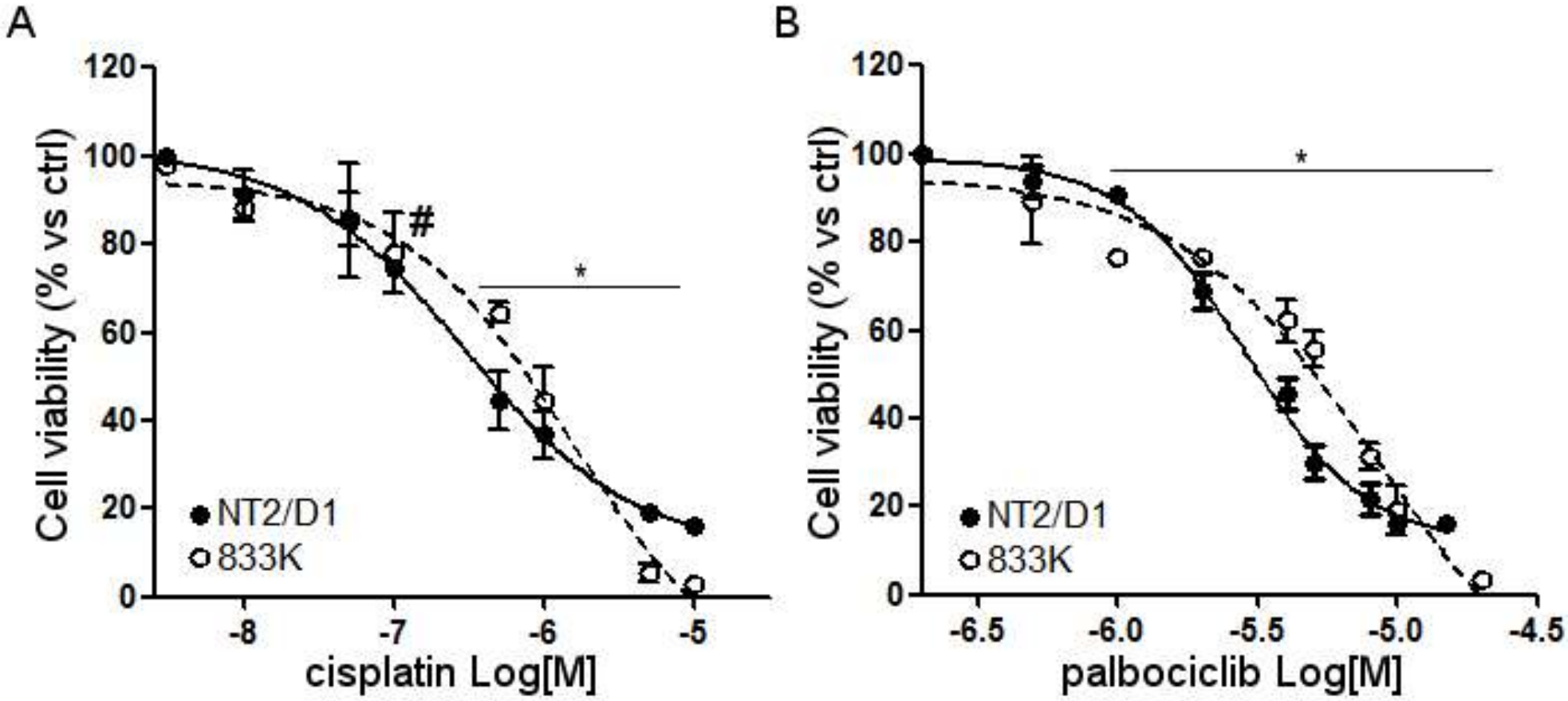
Figure 3. (A) Cdk4/6 and pRb western blot after palbociclib treatment. NT2/D1 cells were treated with palbociclib 2.3 µM for 48 hours, then western blot experiments were conducted as described in methods. The amount of Cdk4 (lane 1), Cdk6 (lane 2) and pRb (lane 3) were visualized with Odissey. The human α – tubulin was used as internal control. A representative wb is shown. **(B) Cdk4/6 and pRb western blot after cisplatin treatment.** NT2/D1 cells were treated with cisplatin 0.3 µM for 48 hours, then western blot experiments were conducted as described in methods. The

amount of Cdk4 (lane 1), Cdk6 (lane 2) and pRb (lane 3) were visualized with Odissey. The human GAPDH was used as internal control. A representative wb is shown.

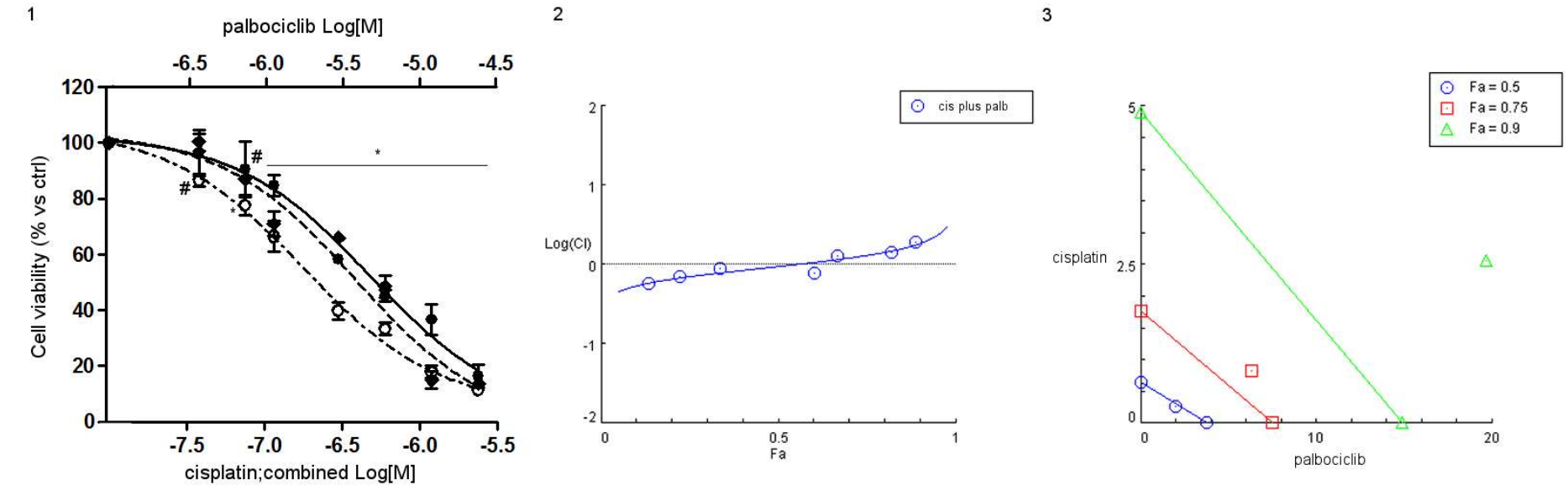
Figure 4. Cell cycle analysis after NT2/D1 drugs treatment. (A) Percentage cell count histograms. Representative bars of one out of three experiments were shown in the figure. **(B) DNA histograms.** Untreated cells (1), 2.3 μM palbociclib (2), 0.3 μM cisplatin (3) and combined (4) for 48 hours treated cells.

Figure 5. NT2/D1 tumor xenograft in AB zebrafish embryos exposed to cisplatin, palbociclib or combined. (A) Tumor area. The tumor area of T0 and T3 palbociclib-, cisplatin- and combination-treated and solvent-treated groups was measured with Noldus DanioScopeTM software (Noldus Information Technology) and analyzed by GraphPad Prism software 6.01 version. The increase of tumor area in solvent-treated vs cisplatin, palbociclib or combination-treated embryos was statistically significant (respectively: ***$P < 0.0001$ vs Ctrl-T3; *$P < 0.05$ vs Ctrl-T3; # $P < 0.01$ vs Ctrl-T0) after one-way ANOVA followed by Dunnett's test analysis. **(B) A representative image of xenografted zebrafish embryos.**

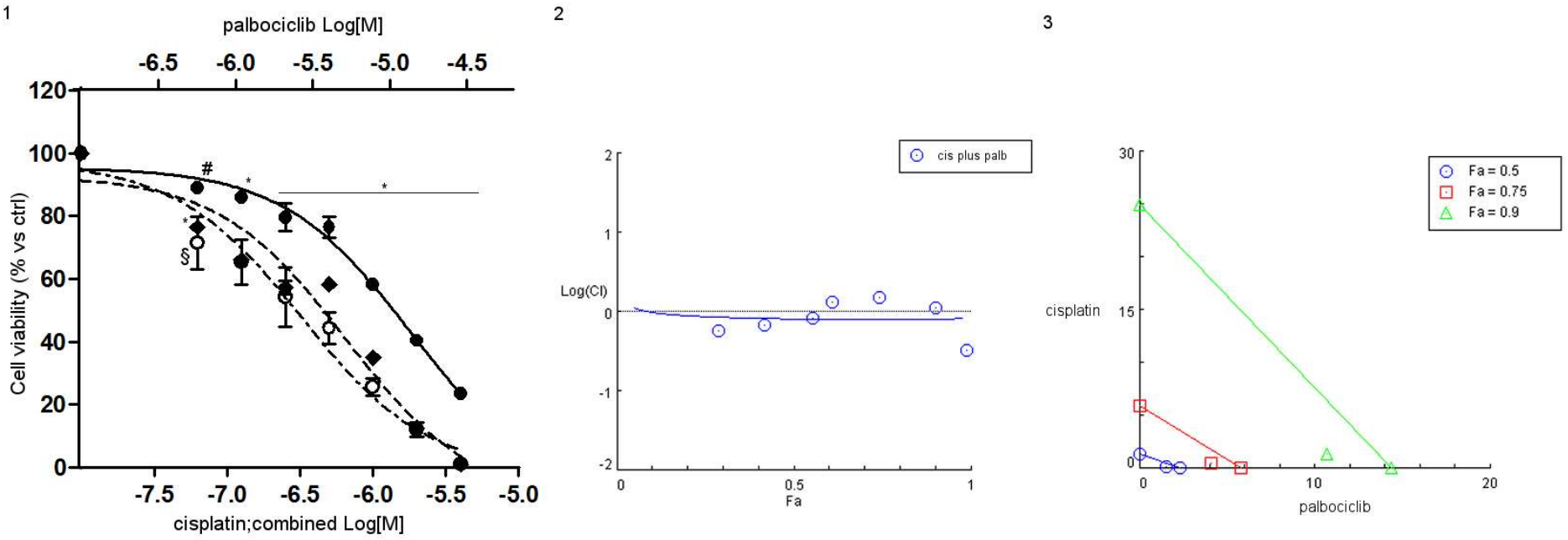
T0-ctrl: time point at injection (control embryos 48hpf); T3-ctrl: time point 3 days later in fish water with solvent alone (untreated embryos 120hpf); T3-cisplatin, palbociclib, combined: time point 3 days later in fish water with single or combined drugs.


A
Cell viability (% vs ctrl)
120
100
80
60
40
20
0
-8
-7
-6
-5
cisplatin Log[M]
NT2/D1
833K
#
*
B
Cell viability (% vs ctrl)
120
100
80
60
40
20
0
-6.5
-6.0
-5.5
-5.0
-4.5
palbociclib Log[M]
NT2/D1
833K
*

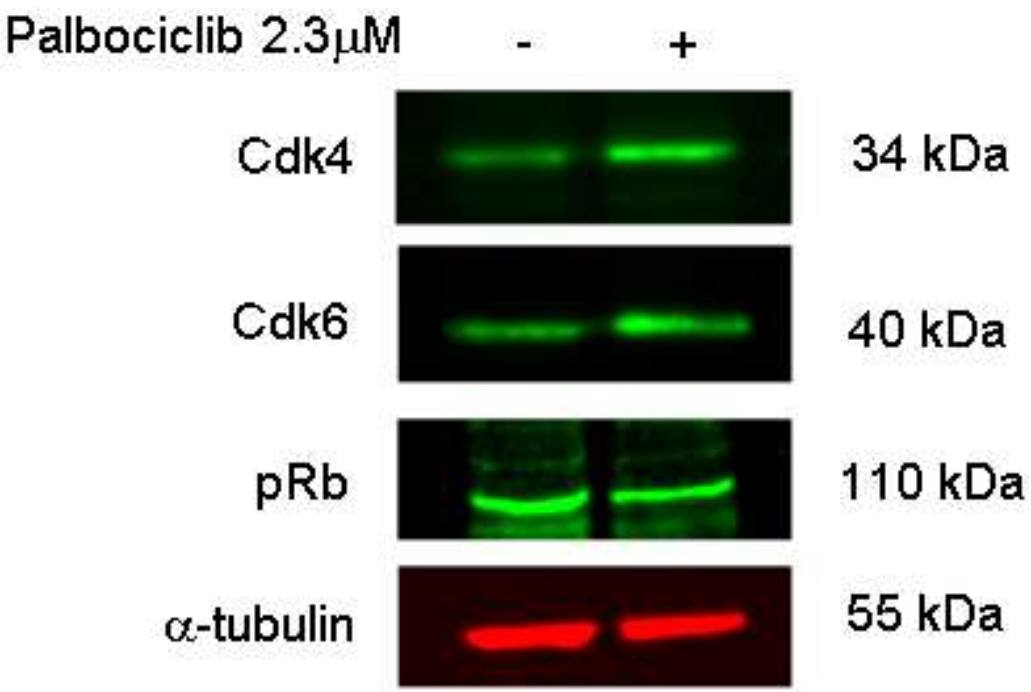
A


1
palbociclib Log[M]
-6.5 -6.0 -5.5 -5.0 -4.5
Cell viability (% vs ctrl)
120 100 80 60 40 20 0
-7.5 -7.0 -6.5 -6.0 -5.5
cisplatin;combined Log[M]
2
cis plus palb
Log(CI)
2 1 0 -1 -2
0 0.5 1
Fa
3
Fa = 0.5
Fa = 0.75
Fa = 0.9
cisplatin
5 2.5 0
0 10 20
palbociclib

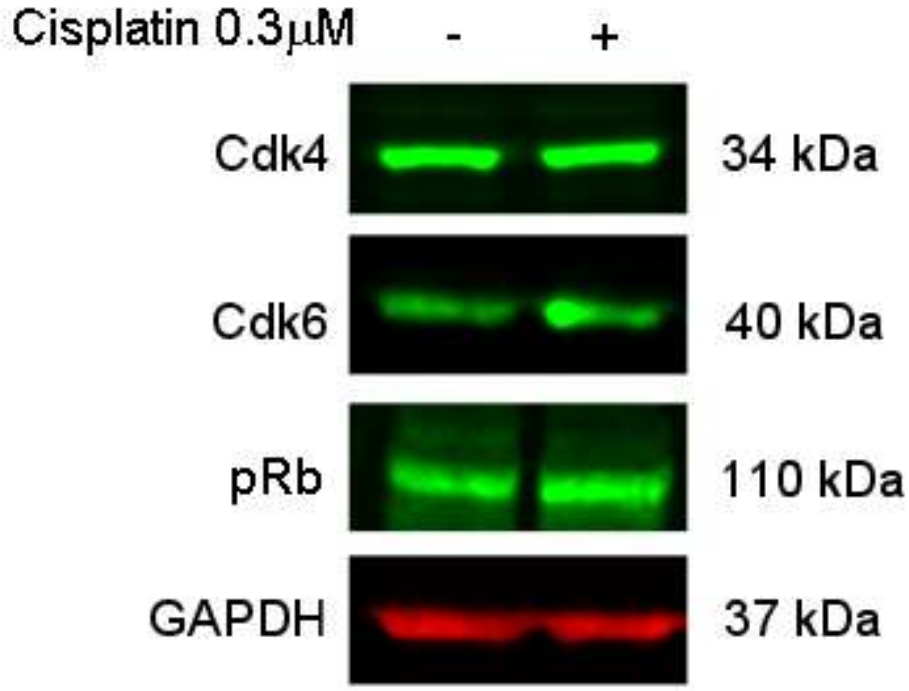
B


1
palbociclib Log[M]
-6.5 -6.0 -5.5 -5.0 -4.5
Cell viability (% vs ctrl)
120 100 80 60 40 20 0
-7.5 -7.0 -6.5 -6.0 -5.5 -5.0
cisplatin;combined Log[M]
2
cis plus palb
Log(CI)
2 1 0 -1 -2
0 0.5 1
Fa
3
Fa = 0.5
Fa = 0.75
Fa = 0.9
cisplatin
30 15 0
0 10 20
palbociclib

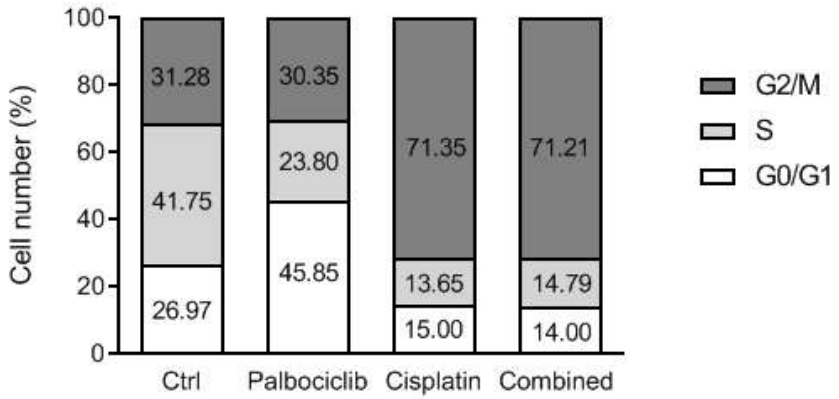
A


Palbociclib 2.3μM
-
+
Cdk4
34 kDa
Cdk6
40 kDa
pRb
110 kDa
α-tubulin
55 kDa

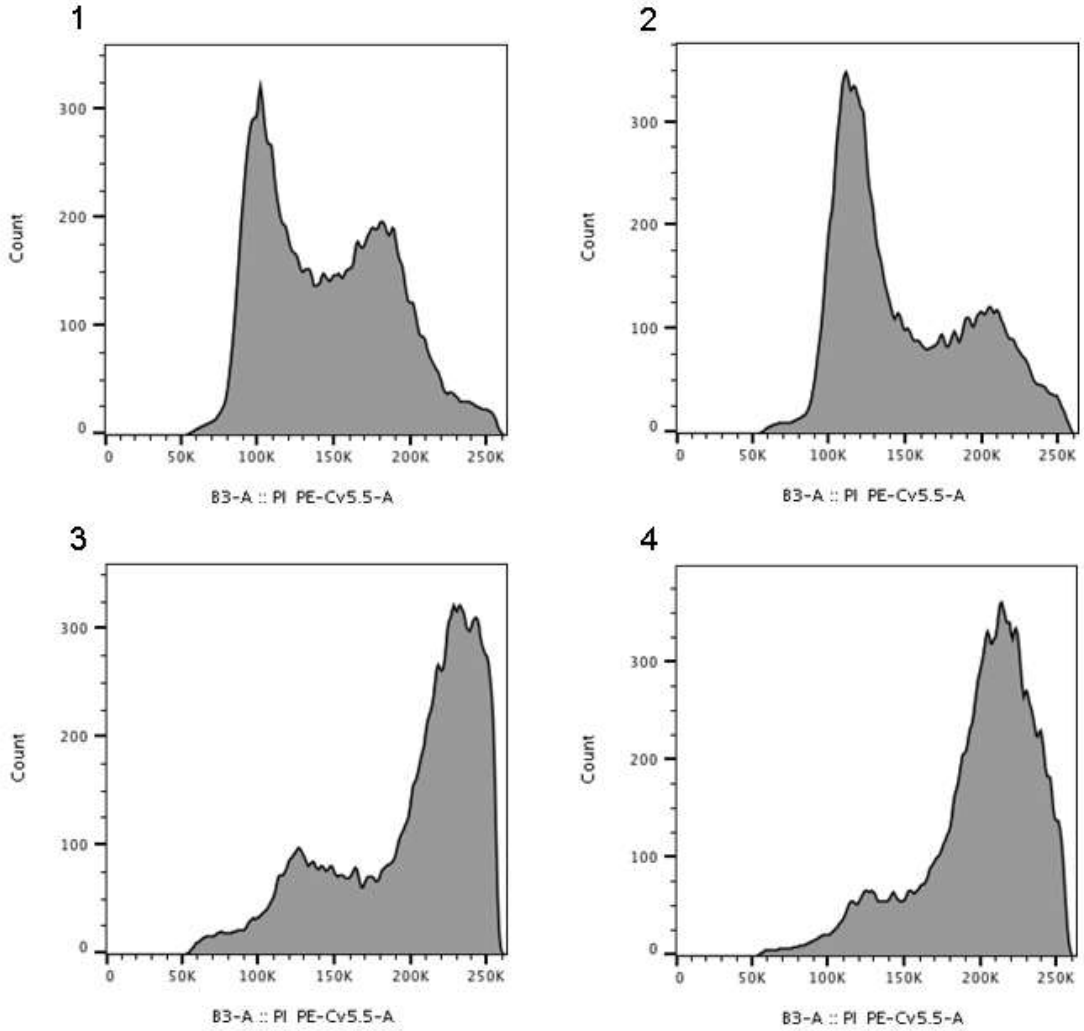
B


Cisplatin 0.3μM
-
+
Cdk4
34 kDa
Cdk6
40 kDa
pRb
110 kDa
GAPDH
37 kDa

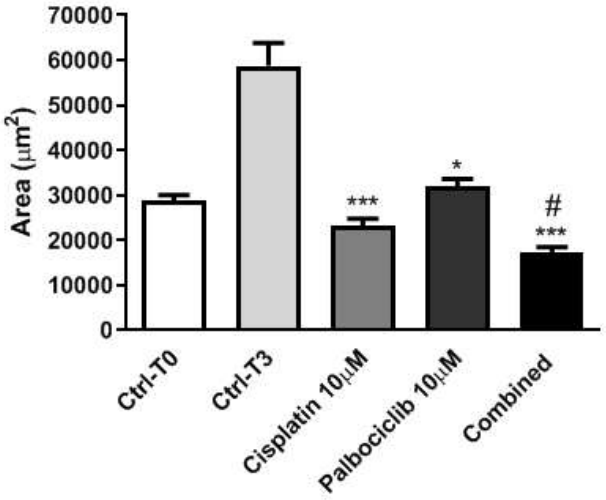
A


Cell number (%)
100
80
60
40
20
0
31.28
41.75
26.97
30.35
23.80
45.85
71.35
13.65
15.00
71.21
14.79
14.00
Ctrl
Palbociclib
Cisplatin
Combined
G2/M
S
G0/G1

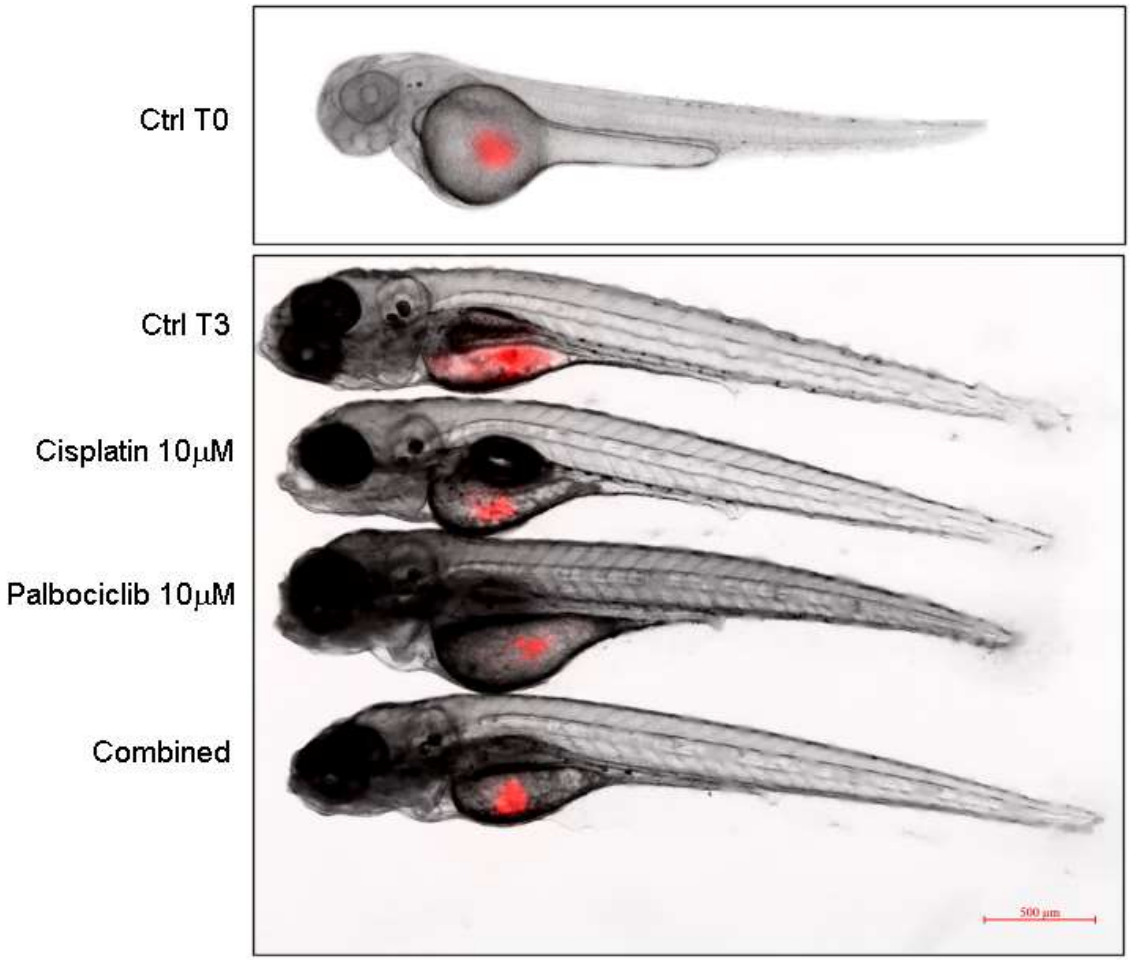
B


1
2
3
4
Count
B3-A :: PI PE-Cy5.5-A
0
50K
100K
150K
200K
250K
100
200
300

A


70000
60000
50000
40000
30000
20000
10000
0
Area (μm²)
***
*
#
***
Ctrl-T0
Ctrl-T3
Cisplatin 10μM
Palbociclib 10μM
Combined

B


Ctrl T0
Ctrl T3
Cisplatin 10μM
Palbociclib 10μM
Combined
500 μm

SUPPLEMENTARY DATA

Supplementary Methods

Drug withdrawal experiments

NT2/D1 cells were plated in 24-well plates and treated, respectively, with the IC_{50} value of cisplatin (0.3 µM), IC_{50} of palbociclib (2.3 µM) or with the combined drugs for 48 hours. After 48 hours treatment, the drug-containing medium was replaced by fresh complete medium without drugs and cell viability was evaluated at different times, up to 12 days. Cells were analyzed for cell viability using MTT assay.

Double staining acridine orange/ethidium bromide

NT2/D1 cells were treated with cisplatin and/or palbociclib at their IC_{50} value for 48 hours. A double staining with acridine orange (AO) and ethidium bromide (EtBr) was performed to visualize and quantify the number of viable, apoptotic and necrotic cells, as previously described [43]. Cells were examined by a Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss AG, Germany). At least 4-6 fields, randomly chosen, were digitalized and scored by using the NIH Image J software.

Supplementary Tables

Supplemental Table 1. STR expression of 833K cell line

Marker	Allele expression
AMEL	X
D3S1358	14 18
D1S1656	15 17
D25441	11.3
D10S1248	14 15
D13S317	11 13
Penta E	5 10
D16S539	9
D18S51	12 16
D2S1338	19 23
CSF1PO	10 11
Penta D	9 10
TH01	6 9
vWA	15 18 19
D21S11	28 31
D7S820	9 11
D5S818	11 12
TPOX	8
DYS391	
D8S1179	13
D12S391	19
D19S433	12 15

FGA	22
D22S1045	16

Supplemental Table 2. Sequences of oligonucleotide primers for qRT-PCR

		Nucleotide Sequences	**Product size (bp)**
Cdk4	F	5’-GCCTCGAGATGTATCCCTGC-3’	118
	R	5’-AGTCAGCATTTCCAGCAGCA-3’	
Cdk6	F	5’-ATCTCTGGAGTGTTGGCTGC-3’	143
	R	5’-GGCAACATCTCTAGGCCAGTC-3’	
pRb	F	5’-CCGTGTGCTCAAAAGAAGTGC-3’	148
	R	5’-AGTCATTTCTGCCAGTTTCTGCT-3’	
β - actin	F	5’-TCTTCCAGCCTTCCTTCCTG-3’	146
	R	5’-CAATGCCAGGGTACATGGTG-3’	

q-RT- PCR was conducted as described in Methods, using SYBR green as fluorochrome and β-actin as housekeeping gene.

Supplemental Table 3. Combination index value

NT2/D1

Palbociclib [μM]	+ Cisplatin [μM]	Fa	CI
0.29	0.0375	0.134	0.58173
0.58	0.075	0.224	0.69991
1.15	0.15	0.336	0.90425
2.3	0.3	0.602	0.78763
4.6	0.6	0.667	1.27901
9.2	1.2	0.821	1.40052
18.4	2.4	0.887	1.90471

833K

Palbociclib [μM]	+ Cisplatin [μM]	Fa	CI
0.479	0.0625	0.28578	0.58593
0.959	0.125	0.41826	0.67589
1.918	0.250	0.553	0.82218
3.835	0.500	0.608	1.33977
7.67	1	0.743	1.53634
15.34	2	0.901	1.13549
30.68	4	0.989	0.33182

Combination of palbociclib with cisplatin against cell viability.

Fa = Fraction affected; CI = Combination Index.

Supplemental Table 4. Palbociclib treatment did not modified target gene expression in NT2/D1 cell line.

Gene of interest	Untreated	Palbociclib treated
Cdk4	4.78 ± 0.37	4.88 ± 0.07
Cdk6	9.33 ± 0.43	9.07 ± 0.35
pRb	9.66 ± 0.26	9.83 ± 0.18

Results are expressed as ΔCt ± S.D. Cells were treated with IC_{50} palbociclib as described for 48 hours, then RNA was extracted, retrotranscribed and the qRT-PCR was conducted as described.

Supplemental table 5. Cisplatin treatment selectively increased Cdk6 gene expression in NT2/D1 cell line

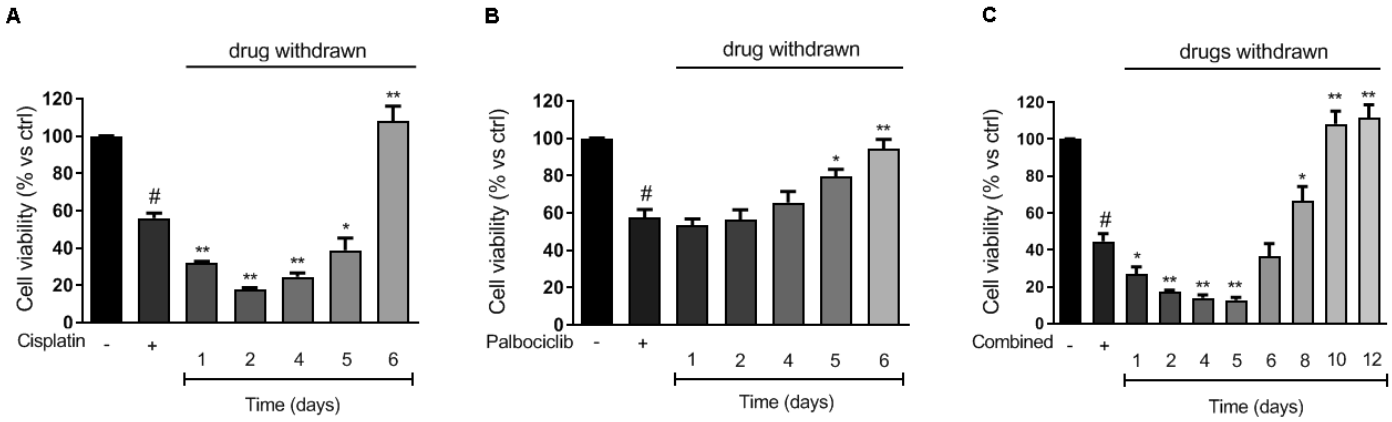
Gene of interest	Untreated	Cisplatin treated
Cdk4	4.77 ± 0.46	5.14 ± 0.20
Cdk6	9.62 ± 0.34	9.06 ± 0.03 ($p < 0.01$)
pRb	9.72 ± 0.14	9.34 ± 0.09

Results are expressed as ΔCt ± S.D. Cells were treated with IC_{50} cisplatin as described for 48 hours, then RNA was extracted, retrotranscribed and the qRT-PCR was conducted as described.

Supplemental Table 6. Quantification of AO/EtBr staining in NT2/D1 cells after drug treatment

Drug treatment	Vital	Apoptotic
Untreated	87 ± 10	13 ± 11
Cisplatin 0.3µM	25 ± 16*	75 ± 16#
Palbociclib 2.3µM	28 ± 13*	72 ± 13#
Combined	12 ± 6.0*	88 ± 6.0#

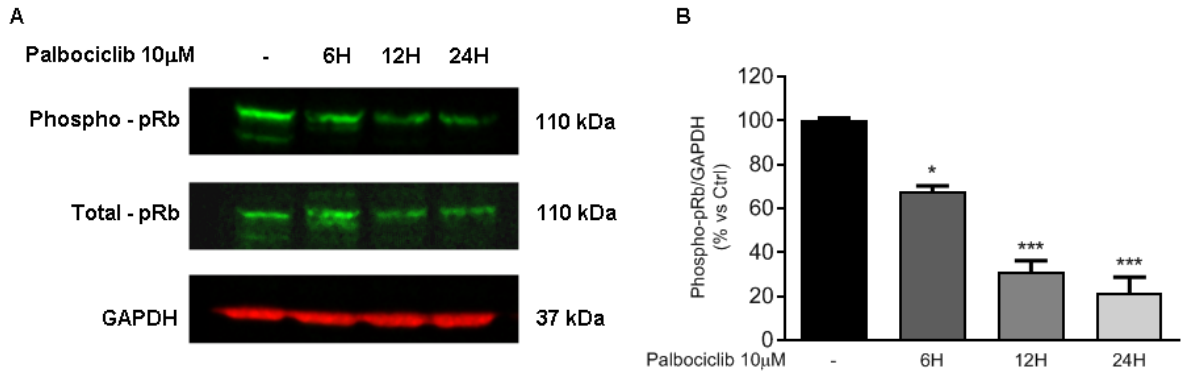
Quantification of AO/EtBr staining in NT2/D1 cells untreated or treated with cisplatin, palbociclib and the drug combination is reported as percentage of viable or apoptotic cells ± SD. * $P < 0.001$ vs ctrl viable cells; #$P < 0.0001$ vs apoptotic ctrl cells.



Supplemental figure 1. Effect of drug withdrawal on NT2/D1 cells. NT2/D1 cells were treated for 48 hours with, respectively, 0.3µM cisplatin (A); 2.3 µM palbociclib (B) or the combination (C) and then transferred in a drug-free medium. Cell viability was measured at the indicated times by the MTT assay. Bars represent the mean ± SD of at least four experiments performed in triplicate. $^{*}P < 0.001$ vs the IC_{50}; $^{**}P < 0.00001$ vs the IC_{50}; $^{\#}P < 0.0001$ vs ctrl.

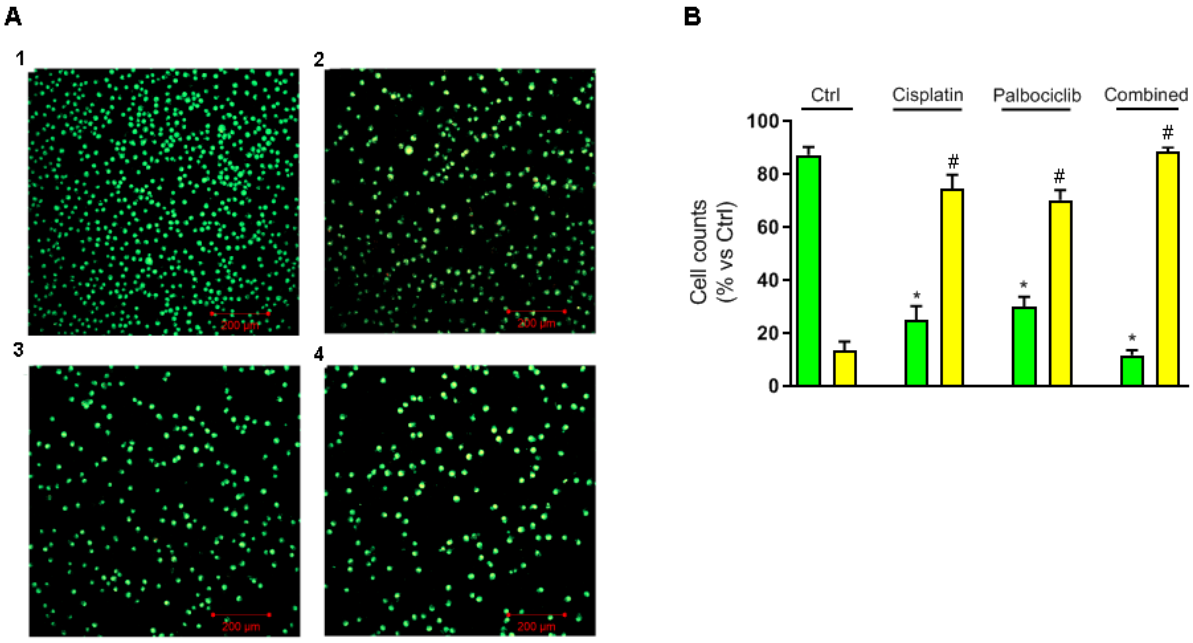
The effect of cisplatin at its IC_{50} concentration lasted up to 4 days withdrawn (% of viability vs ctrl: 24.47 ± 2.09). Cells then recover from the cytotoxic insult and cell viability after 6 days of withdrawn was similar to untreated cells (A). After treatment with palbociclib, cell viability remained low up to 2 days after palbociclib withdrawn (% of viability vs ctrl: 56.38 ± 5.37), then cells recover from drug insult and after 6 days the cell viability was similar to untreated cells (B).

Interestingly, cells viability significantly continued to decrease up to 5 days after drug combination withdrawal, with the cell viability reduced up to 12.85 ± 1.40% compared to control cells. Then cells slowly recovered and 10 days after drug withdrawn, the cell viability was similar to untreated cells (C).



Supplemental Figure 2. Palbociblib maximal dose treatment reduced the phosphorylation of pRb.

(A) NT2/D1 cells were exposed to palbociclib 10 μM for 6, 12 and 24 hours. Phospho-pRb (lane 1) and total pRb (lane 2) expression was investigated using western blot. Human GAPDH was used as internal control. A representative WB is shown. Uncropped blots were shown in Additional file 2: Fig.S5 **(B)** The amount of proteins was visualized with Odissey and proteins were quantified (n=3) using Image Studio Software, as described in materials and methods. Bars represent the mean ± SD. $^{*}P < 0.05$ vs ctrl; $^{***}P < 0.001$ vs ctrl.



Supplemental Figure 3. Cisplatin and/or palbociclib induced apoptosis in NT2/D1 cell line

(A) Cells were left untreated (1) or treated with IC_{50} cisplatin (2) or IC_{50} palbociclib (3) or the combination (4) for 48 hours, then stained with AO/EtBr. Representative images of several acquired fields, with superimposable results.

(B) Viable (green), apoptotic (yellow) cells were scored under a confocal laser-scanning microscope. Bars represent the mean ± SD of three experiments performed in quadruplicate. * $P < 0.001$ vs ctrl viable cells; $^{\#}P < 0.0001$ vs apoptotic ctrl cells.