RESEARCH ARTICLE

REVISED CDK4/6 inhibitors display a class effect in inducing differentiation of neuroblastoma cells

[version 2; peer review: 1 approved, 2 approved with reservations]

Kirsty M. Ferguson[1], Fiona M. Y. Abou Grealy[1], Anna Philpott[1,2]

[1]Jeffrey Cheah Biomedical Centre, Cambridge Stem Cell Institute, Cambridge, CB2 0AW, UK
[2]University of Cambridge Department of Oncology, Cambridge, CB2 0XZ, UK

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Abstract

Background

Neuroblastoma is the most common extracranial solid tumour in infants and children, accounting for approximately 15% of paediatric cancer mortality. These tumours are unique in that a subset, namely stage MS, frequently undergo spontaneous regression or differentiation. Differentiation therapy, where cancer cells are re-routed back down their correct developmental pathway, is therefore a promising therapeutic avenue. We have previously shown that the CDK4/6 inhibitor palbociclib induces both decreased proliferation and enhanced neuronal differentiation of neuroblastoma cells *in vitro*. When combined with retinoic acid, already used clinically for maintenance therapy, this differentiation is enhanced.

Methods

Here, we investigate two additional CDK4/6 inhibitors, abemaciclib and ribociclib, to induce differentiation of the relapsed, high-risk MYCN-amplified neuroblastoma cell line SK-N-BE(2)C, with and without retinoic acid. We culture SK-N-BE(2)C cells in both adherent and three-dimensional culture and monitor proliferation and differentiation using readouts including live-imaging, immunocytochemistry, qRT-PCR and EdU incorporation.

Results

We find the CDK4/6 inhibitors palbociclib, abemaciclib and ribociclib all enhance retinoic acid-induced differentiation in both adherent SK-N-BE(2)C cells and 3D spheroids.

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1. **Feng-Hou Gao**, Shanghai Jiao Tong University School of Medicine, Shanghai, China
2. **Yang Li**, Sun Yat-Sen University, Guangdong, China
3. **Marie Arsenian-Henriksson**, Karolinska Institutet, Stockholm, Sweden
Mohammad Alzrigat, Karolinska Institutet Institutionen for neurovetenskap, Stockholm, Sweden

Any reports and responses or comments on the article can be found at the end of the article.

Conclusions

CDK4/6 inhibitors display a class effect in inducing neuronal differentiation together with retinoic acid, both in adherent neuroblastoma cell lines and three-dimensional tumour spheroids. This is an important consideration for potentially developing CDK inhibitor-induced differentiation as a therapy in the clinic.

Plain language summary

Neuroblastoma is the most common solid tumour outside of the brain in children and infants. These tumours happen when developing cells in the sympathetic nervous system fail to specialise properly and begin dividing uncontrollably. Rerouting neuroblastoma cells back down their correct pathway is a promising therapeutic strategy that may present fewer long-lasting side-effects than therapies that directly kill cells.

We have previously found that a drug called palbociclib, which interferes with cell division and is used to treat some types of breast cancer, triggers neuroblastoma cells to specialise into neurons. Combining this with retinoic acid, a derivative of vitamin A already used to prevent neuroblastoma tumours regrowing, enhances this effect even more.

In this research article, we wanted to see if this effect is specific to the drug palbociclib. To do this, we test two other drugs which interfere with cell division in the same way as palbociclib. We find that all three drugs, together with retinoic acid, trigger aggressive neuroblastoma cells to specialise into neurons. These drugs therefore have a ‘class effect’. This is important knowledge for developing this therapy from the bench to the clinic, where the drug used must be balanced with factors such as efficacy, availability and cost.

Keywords

neuroblastoma, CDK4/6, retinoic acid, differentiation, palbociclib, abemaciclib, ribociclib

Corresponding author: Anna Philpott (ap113@cam.ac.uk)

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Competing interests: We have filed an application seeking patent protection on the use of the combination CDK4/6 inhibitors and retinoic acid in neuroblastoma.

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REVISED **Amendments from Version 1**

In response to reviewer feedback, we have made several changes to improve our manuscript. In the Methods, we have expanded the text relating to the Edu incorporation assay. In the Results, we have added to the text to clarify that retinoic acid is viewed as a positive standard for differentiation induction. In the Discussion, we have significantly expanded our discussion to include interesting areas of future research suggested by the reviewers, including assessing the functionality of cells following CDK4/6 inhibitor + RA treatment and comparing the efficacy of CDK4/6 inhibitors to one another. We have also expanded our discussion on literature relating to the effect of the cell cycle on cell fate determination. Additional references have been added as appropriate.

Any further responses from the reviewers can be found at the end of the article

Introduction

Neuroblastoma is the most common extracranial solid tumour in infants and children, accounting for approximately 15% of paediatric cancer mortality. Novel, kinder therapies are desperately needed to treat affected children while minimising long-term side-effects. Neuroblastoma is a disease of development gone awry, where developing sympathoadrenal cells go down the wrong path. A subset of tumours, namely International Neuroblastoma Risk Group (INRG) stage MS, frequently undergo spontaneous regression into benign ganglioneuroma[1]. Re-routing neuroblastoma cells back down their correct developmental path is therefore a promising therapeutic strategy. Previously we found that the CDK4/6 inhibitor, palbociclib (PB, Ibrance, Pfizer; PD-0332991) both decreases proliferation and induces neuronal differentiation of adrenergic (ADRN) neuroblastoma cells. When combined with retinoic acid (RA), already used clinically in high-risk neuroblastoma as maintenance therapy for minimal residual disease, the oncogenic core regulatory circuitry was reset, proliferation was further reduced, and differentiation further enhanced, compared to PB or RA treatment alone[2].

Dysregulation of the Cyclin D-CDK4/6-INK4-Rb pathway and subsequent unchecked cell proliferation, is a common feature of human cancers; CDK4/6 activity is therefore a key target to attenuate tumour growth. Palbociclib (PD-0332991, Pfizer) was the first CDK4/6-specific inhibitor to be discovered and to show clinical efficacy[3–7]. Ribociclib (LEE011, Novatis and Astex Pharmaceuticals) and abemaciclib (LY2835219, Eli Lilly and Company) later followed. All three are now FDA-approved for combinatorial treatment of HR-positive and HER2-negative breast cancers with endocrine therapy[8], each displaying unique *in vitro* specificity, pharmacokinetics and clinical toxicity profiles[3]. For example, while abemaciclib is structurally different from palbociclib and ribociclib, abemaciclib and ribociclib have shown a higher potency to CDK4 than CDK6, while palbociclib shows no difference in potency between the two CDKs[9]. While all drugs are orally available, palbociclib and ribociclib are dosed twice daily, with a dose interruption due to grade 3–4 neutropenia observed in 60% of patients[10,11]; in contrast, abemaciclib is dosed twice daily, continuously, and while only 21% of patients experience grade 3–4 neutropenia, 10% develop a different side-effect of grade 3 diarrhoea[3,10,11].

Several clinical trials are now ongoing investigating CDK4/6 inhibitors in paediatric cancers. For example, a phase 1/2 study is underway to evaluate palbociclib in combination with irinotecan and temozolomide or in combination with topotecan and cyclophosphamide in paediatric patients with recurrent or refractory solid tumours (Clinical trials GovID: NCT03709680). The efficacy and safety of ribociclib in combination with topotean and temozolamide (TOTEM) in paediatric patients with relapsed or refractory neuroblastoma and other solid tumours is also being investigated (Clinical trials GovID: NCT05429502), as is abemaciclib in combination with other treatments in children with solid tumours such as neuroblastoma (ClinicalTrials.gov ID NCT04238819). In this study, we set out to investigate the ability of these three CDK4/6 inhibitors: palbociclib (PB), abemaciclib (ABE) and ribociclib (RIBO), to induce differentiation of the relapsed, high-risk MYCN-amplified neuroblastoma cell line SK-N-BE(2)C, with and without retinoic acid (RA). We find that CDK4/6 inhibitors display a class effect in inducing neuronal differentiation together with retinoic acid, both in two-dimensional and three-dimensional *in vitro* neuroblastoma cell cultures.

Methods

Cell line maintenance and drug treatment

Neuroblastoma cell lines SK-N-BE(2)C and SH-EP were cultured in DMEM-F12 with L-glutamine (Sigma, D8437) with 10% FBS (PAN-Biotech, P40-37500) and 1% Penicillin-Streptomycin (Sigma, P0781), with media refreshed every 2–3 days. At least every 3 months cells were confirmed to be *Mycoplasma* negative. Cells were seeded as in 2 to induce aggregation into spheroids. On reaching 200–400 µm in diameter[12], drug treatment of spheroids was commenced. All drugs were dissolved in DMSO (Santa Cruz, sc-358801). Palbociclib (PD-0332991 HCl, SelleckChem, S1116) was used at 1 µM. All-trans retinoic acid (Sigma, R2625) was used at 10 µM. Ribociclib (SelleckChem, S7440) was used at 2 µM. Abemaciclib (SelleckChem S5716) was used at 0.2 µM.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA, ThermoScientific, 15424389) for 10 min at room temperature (RT) then incubated in PBST (PBS with Triton X-100 0.2% (Sigma, X100)) for 10 min at RT. Following incubation in blocking solution (PBS-BSA 3% (Fisher BioReagents, BP9706-100) 0.2% Triton X-100) for 1 hr at RT, cells were incubated overnight with primary antibody at 4 °C in blocking solution (TUBB3, Biolegend 801202, 1:1000). Following washes in PBST, cells were incubated with secondary antibody (Thermo

Fisher Scientific, A11029) in blocking solution (1 hr, RT) and washed again. Nuclear counterstaining was performed using DAPI (Abcam, ab228549) (1:10000 in PBST, 15 min, RT). Imaging was performed on a Leica DMI 6000B Matrix microscope with Leica LAS X software (https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/). Images were processed using FIJI, an open-source platform for biological image analysis (version: 2.14.0/1.544f, https://imagej.net/software/fiji/downloads). 3D spheroid cultures were stained using the same protocol. Spheroids were mounted with ProLong Gold antifade reagent (ThermoFisher, P36930) onto glass slides and imaged on Andor Revolution Nikon spinning disk confocal. Spheroid images were processed in FIJI as maximum intensity projections of Z stacks.

Western blotting

Protein lysates were prepared in RIPA buffer (Sigma, R0278) on ice for 20 min, followed by centrifugation (13,000 rpm, 10 min). Following quantification using the BCA method (ThermoScientific, A55864), 15µg protein was separated on a 4–12% BisTris gel (Invitrogen, NP0301) and transferred to a nitrocellulose membrane (Bio-Rad, 1620115). Membranes were blocked with 5% milk-TBS-T (Serva, 42590) then incubated with primary antibodies diluted at 1:1000 in blocking solution (RB, CST 9309S, pRB, CST 9308S, TBP, Proteintech 22006-1-AP) at 4°C overnight, followed by washing in TBST and incubation with HRP-linked secondary antibodies at 1:10000 in blocking solution (Sigma, NA931 and NA934) for 1 hr at RT. Protein bands were visualised on X-ray films after incubation with ECL chemiluminescent substrate (Thermo Fisher Scientific, 32132). Films were scanned and further processed using FIJI.

EdU incorporation assay

On the 4th day of the 5-day drug treatment, media supplemented with 10 µM EdU was added to the cells for 24 hr. Fixation was performed in 4% PFA (10 min, RT). EdU staining was performed with the Click-iT EdU assay kit (Life Technologies, 15224959), followed by DAPI nuclear counterstaining. After tiled imaging using the Leica DMI 6000B Matrix microscope (~44 fields of view), FIJI image thresholding was used to create binary images and FIJI particle analysis function was used to determine the total cell number from DAPI staining and quantify % EdU positive cells. As standard for fluorescence imaging analysis, the same imaging settings were used for all treatment groups and technical replicates to enable internal comparison.

Confluence analyses and live-imaging

Confluence analysis and live-imaging were performed using the Incucyte® Live-Cell Analysis System (Sartorius). For each treatment, technical triplicates on a 24-well plate were imaged every 6 hours for 5 days. All live imaging was performed three times independently. Confluency was also visualised by crystal violet staining. Cells were fixed in 6 well plates on day 5 with 4% PFA (10 min, RT), stained with 0.5% aqueous crystal violet staining solution (Sigma, V5265 - 30 min, RT) and washed with deionised water.

Quantitative RT-PCR (qRT-PCR)

The RNeasy Mini kit (Qiagen, 74104) and QuantiTect Reverse Transcriptase Kit (Qiagen, 205311) were used for RNA extraction and reverse transcription, respectively. qRT-PCR was performed on an Applied Biosystems StepOne™ Real-Time PCR system using gene-specific primers (see Table S1, Extended data) and SYBR™ Green Master Mix (Applied Biosystems, A25742). Technical replicates were run to ensure pipetting accuracy and data analysed using the ddCt method, normalised to the housekeeping gene, TBP. Data are shown as mean +/- 95% CI Fold change, where fold change = 1 for the calibrator. Before data transformation, statistics were performed and error bars calculated from ddCt values.

Statistical analysis

Statistical analyses were made on GraphPad Prism (version 10.2.2 for macOS, https://www.graphpad.com) from at least three independent experiments unless noted (n numbers in figure legends). R is a free software alternative. GraphPad Prism Viewer is free and allows visualisation of data, analyses and graphs. Different passages of SK-N-BE(2)C plated in independent experiments were taken as biological replicates.

Results

The CDK4/6 inhibitors palbociclib, abemaciclib and ribociclib all reduce proliferation and induce differentiation of adherent SK-N-BE(2)C neuroblastoma cells

We first set out to compare the effects of the three FDA-approved CDK4/6 inhibitors palbociclib (PB), abemaciclib (ABE) and ribociclib (RIBO) on the ADRN-type neuroblastoma cells SK-N-BE(2)C. SK-N-BE(2)C cells are derived from a relapsed tumour that is MYCN-amplified and therefore representative of high-risk disease. Previously, a dosage of 1 µM palbociclib was used, a standard dosage used in cellular studies that is similar to the IC50[2]. We therefore ascertained the IC50 of ribociclib and abemaciclib in SK-N-BE(2)C cells (2 µM and 0.2 µM, respectively) for comparison to palbociclib at 1 µM (Figure S1A, Extended data). An indicator of successful CDK4/6 inhibition is reduced phosphorylation of RB, a tumour suppressor that blocks the G1-S transition[13]. We therefore first confirmed that SK-N-BE(2)C cells treated with PB, ABE or RIBO for 24h resulted in RB hypo-phosphorylation (Figure 1A). A reduction in total RB was also observed, as reported in neuroblastoma cells upon CDK4/6 knock-down[14].

After 5 days of treatments with each CDK4/6 inhibitor, we observed a reduction in proliferation compared to the DMSO control, seen by imaging and crystal violet analysis (Figure 1B,C). Previously, we observed neuronal differentiation co-incident with reduced proliferation upon PB treatment, visible as neurite outgrowth[2]. We also observed this phenomenon upon treatment of SK-N-BE(2)C cells with each of the three

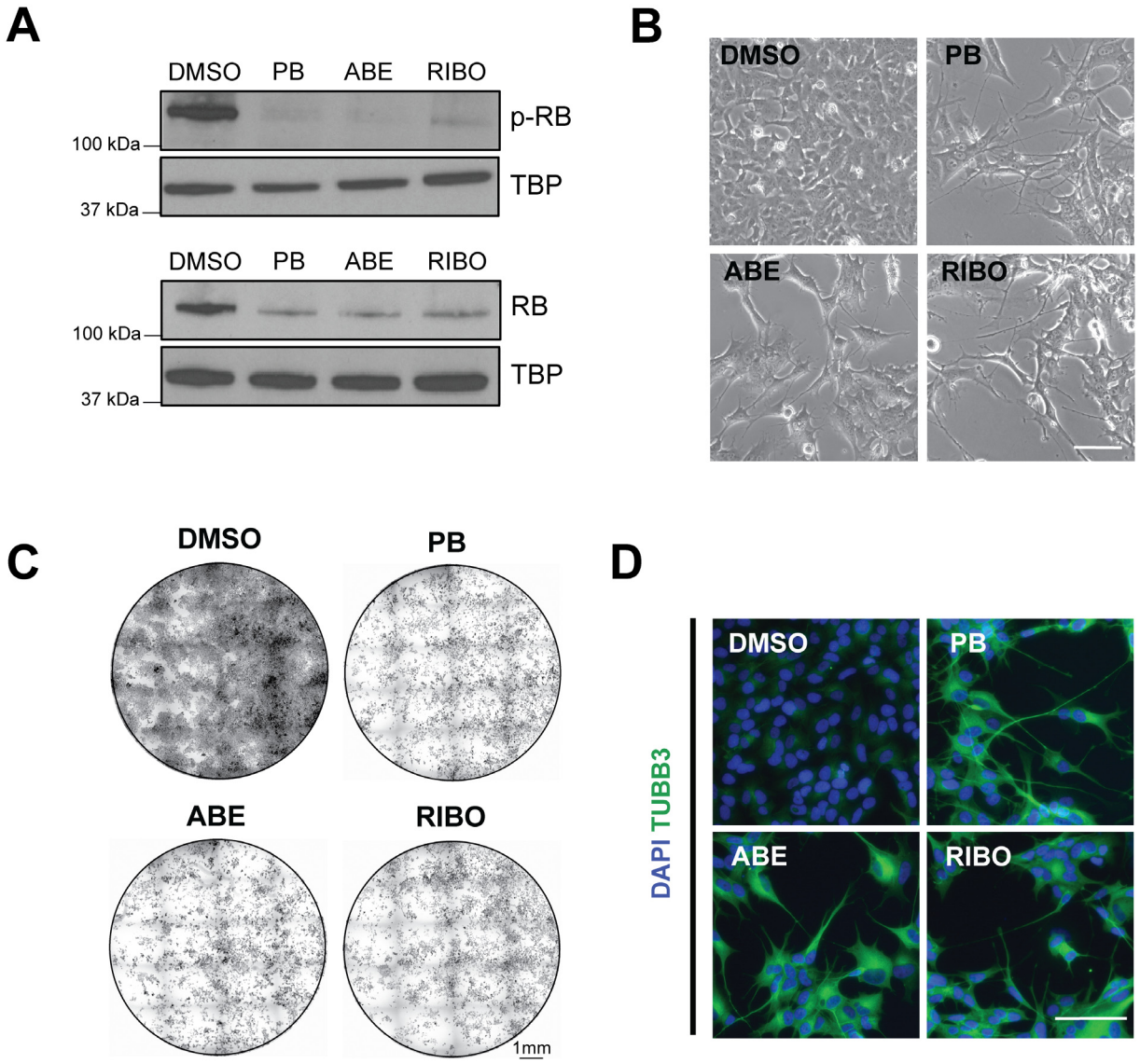

Figure 1. The CDK4/6 inhibitors palbociclib, abemaciclib and ribociclib all reduce proliferation and induce differentiation of adherent SK-N-BE(2)C neuroblastoma cells. (**A**) Western blot analysis of phospho-RB and total RB protein levels in SK-N-BE(2)C cells treated with palbociclib (PB, 1 μM), abemaciclib (ABE, 0.2 μM), ribociclib (RIBO, 2 μM) or DMSO vehicle control for 24 h. TBP = housekeeping loading control. The same concentrations are used throughout the manuscript. (**B**) Representative phase-contrast images of SK-N-BE(2)C cells treated with palbociclib (PB), abemaciclib (ABE), ribociclib (RIBO) or DMSO vehicle control for 5 days. Representative of n = 3 biological replicates. Scale bar: 100 μm. (**C**) Crystal violet staining of SK-N-BE(2)C cells treated with palbociclib (PB), abemaciclib (ABE), ribociclib (RIBO) or DMSO vehicle control for 5 days. Representative of n = 3 biological replicates. Scale bar: 1 mm. (**D**) Immunocytochemistry analysis of neuronal marker βIII-tubulin (TUBB3, green) expression in SK-N-BE(2)C cells following 5 days of palbociclib (PB) , abemaciclib (ABE), ribociclib (RIBO) or DMSO vehicle control treatment. Scale bar: 100 μm. DAPI nuclear counterstain (blue). Representative of n = 3 biological replicates. Note: Figure 1 data forms part of dataset shown in Figure 2.

CDK4/6 inhibitors. Live imaging showed a change of cell morphology during the 5-day treatment, accompanied by negligible cell death (Movie S1, Extended data). The resulting morphology was also visible by immunocytochemistry for the classical neuronal marker βIII-tubulin (TUBB3), whose expression increased upon CDK4/6 inhibition (Figure 1D). By contrast, MES-type neuroblastoma cells, SH-EP, did not show signs of neuronal differentiation with any CDK4/6 inhibitor treatment, in agreement with our previous study (Figure S1B,C, Extended data). Together these data show that the three CDK4/6 inhibitors palbociclib, abemaciclib and ribociclib are capable of reducing proliferation and inducing differentiation of the neuroblastoma cell line SK-N-BE(2)C *in vitro*, without extensive cell death.

The CDK4/6 inhibitors palbociclib, abemaciclib and ribociclib all enhance retinoic acid-induced differentiation in adherent SK-N-BE(2)C cells

Retinoic acid (RA) is a differentiation agent already used as standard of care in maintenance therapy for high-risk neuroblastoma. RA has been found to epigenetically reset the core regulatory circuit of ADRN-type neuroblastoma cells[15] and is viewed as a positive standard for differentiation induction. We found that dual treatment with retinoic acid and palbociclib further suppressed proliferation and enhanced differentiation compared to either drug alone[2]. We therefore next sought to determine if retinoic acid enhances differentiation in combination with palbociclib alone, or withother CDK4/6 inhibitors. For each of the three CDK4/6 inhibitors, CDK4/6 inhibition or dual treatment with retinoic acid showed a greater decrease in proliferation compared to DMSO or RA alone as shown by crystal violet staining, EdU analysis and live-imaging confluency analyses (Figure 2 A,B,D,E, Movie S2, Extended data). The decrease in proliferation by each CDK4/6 inhibitor was consistently enhanced by combinatorial treatment with retinoic acid.

Upon treatment with either CDK4/6 inhibitor alone, or in combination with RA, ICC showed an increase in TUBB3 expressionand neurite extension (compared to DMSO or RA treatment) (Figure 2C). qRT-PCR analysis revealed a greater increase in expression of the differentiation marker *STMN4*, and a greater decrease in expression of the proliferative markers *E2F8, PLK1* and *FOXM1*[16,17], upon treatment of cells with each CDK4/6 inhibitor plus RA, compared to treatment with each CDK4/6 inhibitor alone (Figure 2F).

The CDK4/6 inhibitors palbociclib, abemaciclib and ribociclib all enhance retinoic acid-induced differentiation in 3D SK-N-BE(2)C spheroids

Finally, we wanted to assess the effect of CDK4/6 inhibition on multicellular 3D tumour spheroids. We found a decrease in spheroid growth upon treatment with each CDK4/6 inhibitor compared to DMSO, enhanced by addition of retinoic acid (Figure 3 A,B). Immunostaining of spheroids for the neuronal marker TUBB3 revealed morphological changes to cells and an increase in neurite extension with CDK4/6 inhibitor and CDK4/6i+RA treatment (Figure 3C), compared to DMSO-treated controls. Finally, qRT-PCR analysis revealed a consistent pattern of a greater increase in *STMN4* expression, and a greater decrease in *E2F8, PLK1* and *FOXM1* expression, upon treatment of spheroids with each CDK4/6 inhibitor plus RA, compared to treatment with each CDK4/6 inhibitor alone (Figure 3D). In summary, we find that CDK4/6 inhibitors display a class effect in reducing proliferation and inducing neuronal differentiation together with retinoic acid, both in two-dimensional and three-dimensional *in vitro* neuroblastoma cell cultures.

Discussion

We conclude that treatment of the neuroblastoma cell line SK-N-BE(2)C with any CDK4/6 inhibitor (palbociclib, abemaciclib or ribociclib) at IC50 concentrations results in RB hypo-phosphorylation and both reduces proliferation and induces differentiation, without extensive cell death. Our results demonstrate that combining any of these CDK4/6 inhibitors with retinoic acid (RA) further inhibits cell cycling and enhances differentiation of SK-N-BE(2)C cells in 2D and 3D, compared to treatment with each CDK4/6 inhibitor alone. We therefore conclude this is a class effect; all CDK4/6 inhibitors have a combinatory effect with retinoic acid in inducing differentiation of neuroblastoma cells.

It would be interesting to determine the functionality of the cells following CDK4/6 inhibitor + RA treatment and we invite this for further research. Previously, we observed PB+RA treatment induced ultrastructural features reminiscent of mature neuronal cells, in particular dense-core granules[2]. Additional analyses could include assessment of electrophysiological functions and cell-to-cell signal transduction. Importantly, the use of neuronal cell culture media or more complex *in vivo* systems should be considered, as the microenvironment is likely to influence the ability of differentiated cells to acquire electrophysiological neuronal functions. Such assessments could be usefulfor fields such as Parkinson's disease pathobiology, where differentiation of SH-SY5Y cells with RA alone is used as a model system[18]. While we compared each CDK4/6 inhibitor to the DMSO vehicle control to determine if they exhibit a class-effect, comparing the efficacy of each CDK4/6 inhibitor to one another is also an interesting future researchquestion.

CDK inhibitors have been extensively investigated for their ability to arrest proliferation in cancers where cell cycle components are dysregulated. Previously, we hypothesised that by lengthening the G1 phase of the cell cycle, palbociclib may provide a 'phenotype switch' trigger by increasing the available time for cell response to differentiation cues[2,19,20], based on the cell-cycle length model[19]. Indeed, Calegari *et al.*[19,20] provide evidence that G1 lengthening casually induces neuroepithelial differentiation of proliferative neural progenitors. Reduction of serum in cell culture media induces G1 arrest and is often used in combination with retinoic acid to induce neuroblastoma cell differentiation *in vitro*[21]; we therefore surmised that palbociclib was providing a similar, albeit more potent, effect in triggering differentiation. Here, we find that three independent CDK4/6 inhibitors all induce neuroblastoma differentiation, confirming the hypothesis that G1 lengthening is a key mechanistic trigger for this process. This correlation between the cell cycle, in particular G1, and spatial and temporal regulation of cell fate determination has been reviewed across several model systems and stem cell lineages[22–24].

Importantly, all three inhibitors show a combinatorial effect in enhancing differentiation when combined with retinoic acid, already used in maintenance therapy to treat minimal residual disease in high-risk neuroblastoma cases. On potentially developing CDK inhibitor-induced differentiation as a therapy in the clinic, it will therefore be important to consider CDK4/6 inhibitors as a class, balancing their differentiation activity with factors such as availability, cost, pharmacodynamics and toxicity profiles.

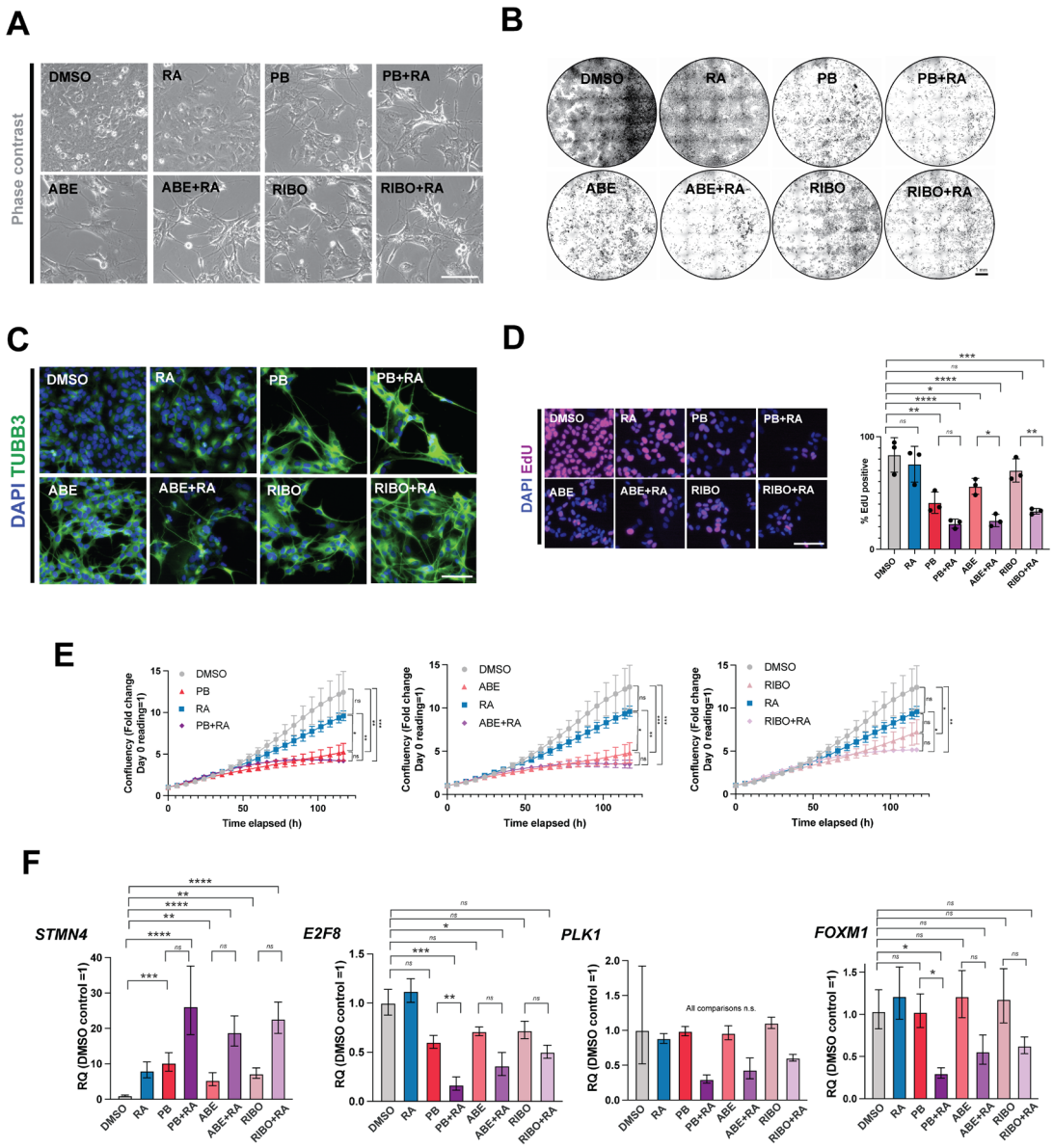

Figure 2. The CDK4/6 inhibitors palbociclib, abemaciclib and ribociclib all enhance retinoic acid-induced differentiation in adherent SK-N-BE(2)C cells. (**A**) Representative phase-contrast images of SK-N-BE(2)C cells treated with palbociclib (PB, 1 μM), abemaciclib (ABE, 0.2 μM), ribociclib (RIBO, 2 μM), retinoic acid (RA, 10 μM) or a combination of PB/ABE/RIBO + RA, or DMSO vehicle control for 5 days. Representative of n = 3 biological replicates. Scale bar: 50 μm. The same concentrations are used throughout the manuscript. (**B**) Crystal violet staining of SK-N-BE(2)C cells treated with palbociclib (PB), abemaciclib (ABE), ribociclib (RIBO), retinoic acid (RA) or a combination of PB/ABE/RIBO + RA, or DMSO vehicle control for 5 days. Representative of n = 3 biological replicates. Scale bar: 1 mm. (**C**) Immunocytochemistry analysis of neuronal marker βIII-tubulin (TUBB3, green) expression in SK-N-BE(2)C cells treated with palbociclib (PB), abemaciclib (ABE), ribociclib (RIBO), retinoic acid (RA) or a combination of PB/ABE/RIBO + RA, or DMSO vehicle control for 5 days. Scale bar: 100 μm. DAPI nuclear counterstain (blue). Representative of n = 3 biological replicates. (**D**) Left: representative fluorescent images of EdU incorporation following a 24-h pulse in untreated SK-N-BE(2)C cells and cells treated with palbociclib (PB), abemaciclib (ABE), ribociclib (RIBO), retinoic acid (RA) or a combination of PB/ABE/RIBO + RA, or DMSO vehicle control for 5 days. Scale bar: 100 μm. Right: analysis of % EdU-positive cells. Mean ± SD. n = 3 biological replicates, each in technical triplicate. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ one-way ANOVA with Tukey's multiple comparison test. Selected comparisons shown for ease of visualisation. (**E**) Confluence analysis SK-N-BE(2)C cells treated with palbociclib (PB), abemaciclib (ABE), ribociclib (RIBO), retinoic acid (RA) or a combination of PB/ABE/RIBO + RA, or DMSO vehicle control for 5 days. Mean ± SD, n=3 biological replicates (each with n=3 technical replicates). Confluency presented as fold change of day 0 reading, where day 0 reading = 1). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ one-way ANOVA with Tukey's multiple comparison test at Day 5 timepoint. (**F**) qRT-PCR analysis of *STMN4, E2F8, PLK1* and *FOXM1* expression levels in SK-N-BE(2)C cells treated with palbociclib (PB), abemaciclib (ABE), ribociclib (RIBO), retinoic acid (RA) or a combination of PB/ABE/RIBO + RA, or DMSO vehicle control for 5 days. n = 3 biological replicates. Mean ± 95% CI. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$; and $^{****}p \leq 0.0001$, one-way ANOVA with Tukey's multiple comparison test. Selected comparisons shown for ease of visualisation.

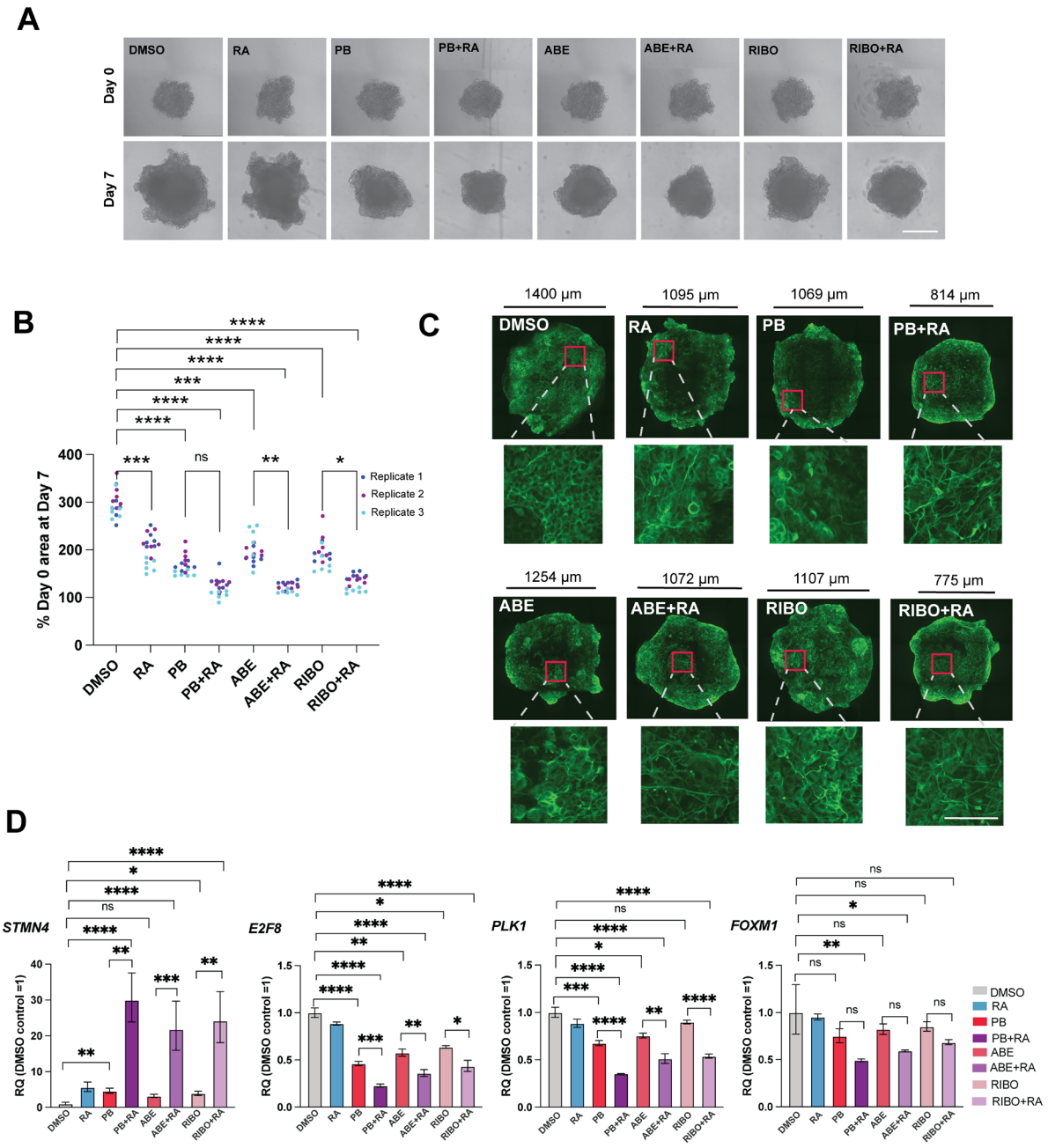

Figure 3. The CDK4/6 inhibitors palbociclib, abemaciclib and ribociclib all enhance retinoic acid-induced differentiation in 3D SK-N-BE(2)C spheroids. (**A**) Representative phase-contrast images of SK-N-BE(2)C spheroids at day 0 and day 7 of treatment with palbociclib (PB, 1 µM), abemaciclib (ABE, 0.2 µM), ribociclib (RIBO, 2 µM), retinoic acid (RA, 10 µM) or a combination of PB/ABE/RIBO + RA, or DMSO vehicle control, at the same concentrations used throughout the manuscript. Scale bar: 500 µm. (**B**) Percentage SK-N-BE(2)C spheroid area at day 7 of treatment compared with day 0. n = 3 biological replicates, with n = 6 spheroids per replicate, each represented by a single data point. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$; and $^{****}p \leq 0.0001$, one-way ANOVA with Tukey's multiple comparison test. Selected comparisons shown for ease of visualisation. (**C**) Immunofluorescence images of tumour spheroids stained for neuronal marker βIII-tubulin (TUBB3, green) at day 7 of treatment. Scale shown for each individual image. Higher magnification images shown with scale bar: 100 µm. Representative of n=3 biological replicates. (**D**) qRT-PCR analysis of *STMN4, E2F8, PLK1* and *FOXM1* expression levels in SK-N-BE(2)C spheroids treated with palbociclib (PB), abemaciclib (ABE), ribociclib (RIBO), retinoic acid (RA) or a combination of PB/ABE/RIBO + RA, or DMSO vehicle control for 7 days (~30 spheroids pooled per replicate, n = 3 biological replicates). Mean ± 95% confidence interval (CI). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$; and $^{****}p \leq 0.0001$, one-way ANOVA with Tukey's multiple comparison test. Selected comparisons shown for ease of visualisation.

Ethics and consent statement

Ethical approval and consent were not required.

Data availability statement

Underlying data

Zenodo: CDK4/6 inhibitors display a class effect in inducing differentiation of neuroblastoma cells. https://doi.org/10.5281/zenodo.13850646[25].

This project contains the following underlying data:

Data for Figure 1A–D, Figure 2A–F and Figure 3A–D.

Figure1A: Western Blot for RB and pRB with TBP loading control.

Figure1B and Figure2A: Representative phase-contrast images.

Figure1C and Figure2B: Representative crystal violet staining images.

Figure1D and Figure 2C: Raw representative immunocytochemistry images, TUBB3 and DAPI staining.

Figure2D: GraphPad Prism file of raw % EdU counts and statistical analysis.

Figure2E: GraphPad Prism file of normalised confluency data from Incucyte® live-imaging and statistical analysis.

Figure2F and Figure3D: GraphPad Prism file of raw qRT-PCR ddCt values, Mean ± 95% CI values and statistical analysis.

Figure3A: Representative phase-contrast images of spheroids at Day 0 and Day 7.

Figure3B: GraphPad Prism file of normalised spheroid areas and statistical analysis.

Figure3C: Raw representative immunocytochemistry images of spheroids, TUBB3 staining.

Extended data

Zenodo: CDK4/6 inhibitors display a class effect in inducing differentiation of neuroblastoma cells. https://doi.org/10.5281/zenodo.13850646[25].

This project contains the following extended data:

Figure S1: related to Figure 1 (IC50 analysis for ABE and RIBO, phase-contrast and crystal violet images of SH-EP treated with PB, ABE, RIBO or DMSO vehicle control for 5 days), legends for Movie S1 and S2, and Table S1: qRT-PCR primer sequences.

FigureS1A: GraphPad Prism file of IC50 value calculation based on % confluency.

FigureS1B: Representative phase-contrast images.

FigureS1C: Representative crystal violet staining images.

Movie S1: Live imaging of SK-N-BE(2)C cells treated with PB, ABE, RIBO or DMSO vehicle control for 5 days. Related to Figure 1. Note: Figure 1 data forms part of dataset shown in Figure 2.

Movie S2: Live imaging of SK-N-BE(2)C cells treated with PB, ABE, RIBO, RA or a combination of PB/ABE/RIBO + RA, or DMSO vehicle control for 5 days, related to Figure 2.

CRediT authorship contribution statement

K.M.F. Conceptualisation, Formal analysis, Investigation, Project administration, Supervision, Validation, Visualisation, Writing – original draft, Writing – reviewing & editing. **F.M.Y.A.G.** Investigation, Formal analysis, Writing - reviewing & editing. **A.P.** Conceptualisation, Project administration, Funding acquisition, Supervision, Writing - reviewing & editing.

Acknowledgments

We would like to thank the Cambridge Stem Cell Institute Tissue Culture and Imaging Core Facilities; the Cancer Research UK Cambridge Institute Research Instrumentation and Cell Services Facility; Dr Aditi Vedi, Prof. Suzanne Turner, Dr Perla Pucci, Prof. Louis Chesler, Dr Evon Poon, and all Philpott lab members for helpful discussions; and Prof. Deborah Tweddle for kindly providing the neuroblastoma cell lines SK-N-BE(2)C and SH-EP.

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http://www.doi.org/10.5281/zenodo.13850646

Open Peer Review

Current Peer Review Status: ? ✓ ?

Version 2

Reviewer Report 25 October 2025

https://doi.org/10.21956/wellcomeopenres.27410.r133771

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Marie Arsenian-Henriksson
Karolinska Institutet, Stockholm, Sweden
Mohammad Alzrigat
MTC, Karolinska Institutet Institutionen for neurovetenskap, Stockholm, Stockholm County, Sweden

In this manuscript Ferguson KM, et al. investigated the impact of different CDK4/6 inhibitors on the differentiation of neuroblastoma cells. This study is a follow up of their previously elegant study: "Ferguson KM, et al. Palbociclib releases the latent differentiation capacity of neuroblastoma cells. Dev Cell. 2023 Oct 9;58(19):1967-1982.e8. doi: 10.1016/j.devcel.2023.08.028. Epub 2023 Sep 20" in which they described at the molecular and cellular level how the CDK4/6 inhibitor Palbociclib induces neuronal differentiation in adrenergic neuroblastoma cell lines. Thus, the novelty of this study is somewhat diminished by their previous findings – both the previous article and the current manuscript show that CDK4/6 inhibition induces a reduction in the proliferation and enhance neuronal differentiation of neuroblastoma cells alone or in combination with retinoic acid.

However, since this study as well as the previous work demonstrate for the first time that CDK4/6 inhibition induces neuronal differentiation, this manuscript is suitable for indexing after addressing the following concerns:

Major concerns:

1. Only one cell line of each neuroblastoma subtype was used in this study. The concept of using several cell lines has been shown previously in the Developmental Cell Paper. Still, please include more cell lines in the current study.
2. It is of great importance to the neuroblastoma scientific community to show CDK4 and CDK6 as well as their cell cycle regulated inhibitors at the protein levels in cell lines of MES and ADRN subtypes. Importantly, analysis of Cyclin D1 levels (control versus treated cells) would be of great addition to the results.
3. Considering the impact of cell cycle phases on biological responses to CDKi, the study would benefit from performing differentiation analysis following CDK4/6 inhibition on

synchronized cells. This might shed new insights on the impact of cell cycle on response outcome to CDK4/6 inhibition as well as RA treatment.

4. Withdrawal of CDK4/6i, RA, or combination treatments after 5 or 7-days treatment is of great addition to this current manuscript as well as the previous study. It ids important for its translation into clinical practice to know whether neuronal differentiation is reversed upon stopping treatment. Will all cells differentiate? Will non-differentiated cells take over?

Comments Related to Figure 1:

The authors suggest that the different CDK4/6 inhibitors demonstrated on target effect by reduction of p-RB and total-RB. The on-target effects should only be stated once there is reduction in p-RB without affecting total-RB protein levels. To this end, please analyze earlier time points i.e., 2, 6, 12, and 24 hours to check for on-target effects. Based on the presented data, the reduction in p-RB could be a consequence of reduced levels in total-RB. This also should be done on other ADRN neuroblastoma cell lines like in the previous study.

It is important to state which phosphorylation site has been used to indicate CDK4/6 inhibition. This information is missing in the results, the materials and methods as well as in the discussion.

Treatment of several MES cell lines with their IC50 of CDK4/6is followed by checking on-target effects on p-RB and total-RB is required to uncover if the lack of response in MES cell lines is due lack of effect of the CDKis.

Have the authors treated ADNR and MES for longer time points – longer than 5-days to analyze whether the difference in neuronal differentiation capacity is maintained. Possibly the MES cell lines need longer treatment duration to show response to CDK4/6 inhibition.

Comments Related to Figure 2:

The combination treatment and outcome on differentiation should also be performed on MES cell lines. Maybe under combinatorial settings MES cell lines might undergo neuronal differentiation.

RA has the least impact on confluency despite the high 10 uM concentration used, which is translated by no impact of the expression of E2F8, PLK1 and FOXM1. Images show heterogeneity in BrdU staining. In addition, the authors reflected in prolongation of cell cycle to enhance differentiation. These issues could be addressed possibly by repeating these experiments on synchronized cells which might can help to understand the RA response as well as combinations in ADRN and MES cell lines.

Have the authors analyzed STMN4, E2F8, PLK1 and FOXM1protein levels to make sure that regulation at mRNA levels is translated at the protein level?

Comments Related to Figure 3.

Please add a figure showing the spheroid size/area relative to DMSO at day 0 and day 7. This way readers can appreciate the impact of single verss combination treatments on spheroid size/area.

The black areas in the heart of some of some of the spherids – are they results of resolution, magnification of the image? Or are they areas that did not undergo neuronal differentiation? If so,

is that due to the inefficacy of drug penetration or hypoxia, or cell death?

Other comment related to all Figures:

Quantification of neurite number and length would improve the results and define the impact of each inhibitor alone or in combination on differentiation. RA should be used as positive reference.

References

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Childhood cancer (neuroblastoma, medulloblastoma) and adult cancer (renal and cervical cancer). Our reserach focus on targeting MYC and metabolism as well as studies on neuronal differentiation (neuroblastoma and medulloblöastoma).

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Version 1

Reviewer Report 29 November 2024

https://doi.org/10.21956/wellcomeopenres.25540.r110967

Yang Li
Sun Yat-Sen University, Guangdong, China

This study reveals that CDK4/6 inhibitors induce differentiation in NB cells and, when combined with RA, synergistically enhance differentiation. As CDK4/6 inhibitors are already clinically approved, these findings offer a potential novel therapeutic strategy for differentiation therapy during the maintenance phase in patients with high-risk neuroblastoma, highlighting important clinical implications.

1.Western blot results show that abemaciclib, ribociclib, and palbociclib effectively inhibit CDK4 expression and RB phosphorylation. However, the mechanism underlying the reduction in total RB protein expression remains unclear. Additionally, the lack of quantitative WB band analysis limits the ability to directly compare the inhibitory effects of the three drugs.
2.The absence of functional validation experiments prevents the determination of whether the induced differentiated cells exhibit genuine neuronal functions or if the differentiation state of NB cells is temporary.
3.This study primarily focuses on cellular-level experiments and lacks supporting in vivo data.
4.This study demonstrates that CDK4/6 inhibitors induce differentiation in NB cells; however, the underlying mechanisms have not been thoroughly investigated.
5.The rationale with which the doses of the two drugs were chosen is not clear. A more in-depth study exploring a broad range of concentrations should be done. Furthermore, the assessment of a potential synergistic effect between RA and CDK4/6 inhibitors should be evaluated using appropriate synergy models (i.e. Chou-Talalay model or Bliss Excess).

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Tumor chemotherapy and tumor immunity

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 23 Aug 2025

Anna Philpott

We thank the reviewer for their helpful comments and respond to each of these below.

- **Comment 1:** Western blot results show that abemaciclib, ribociclib, and palbociclib effectively inhibit CDK4 expression and RB phosphorylation. However, the mechanism underlying the reduction in total RB protein expression remains unclear. Additionally, the lack of quantitative WB band analysis limits the ability to directly compare the inhibitory effects of the three drugs.
- **Author response:** To confirm successful CDK4/6 inhibition, we analysed reduced phosphorylation of RB, a tumour suppressor that blocks the G1/S transition. The aim of this research article is to determine if the three CDK4/6 inhibitors, palbociclib, ribociclib and abemaciclib, display a class effect in inducing differentiation together with retinoic acid. For this reason, do not make direct comparisons between the CDK4/6 inhibitors, only comparison of each CDK4/6 inhibitor to its respective control, indeed observing that PB, RIBO or ABE treatment for 24h resulted in RB hypo-phosphorylation. This result is very clear visually and therefore we do not quantify the Western blot bands. It is indeed an interesting future research question to compare the efficacy of each CDK4/6 inhibitor to one another, beyond the aims of this manuscript. We have added this future research question to the Discussion (end of paragraph 2). Regarding the reduction in total RB, this observation has been previously reported in neuroblastoma cells upon CDK4/6 knock-down (PMID: 24045179, Figures 2 and 3); this was noted already of interest in the text and we invite others to investigate the molecular mechanisms for this decrease.

- **Comment 2:** The absence of functional validation experiments prevents the determination of whether the induced differentiated cells exhibit genuine neuronal functions or if the differentiation state of NB cells is temporary.
- **Author response:** We agree with the reviewer that it would be very interesting to investigate the functions of the differentiated cells, such as electrophysiological analyses. In our previous manuscript (PMID: 37734383) we performed extensive transcriptomic and epigenomic studies on PB+RA treated cells and “a mature differentiated state was observed both transcriptionally, by increased expression of neuronal differentiation features, and functionally by the increased presence of dense-core neurosecretory granules as visualized by TEM”. As the aim of our work is to investigate CDK4/6i+RA as a treatment in the paediatric cancer neuroblastoma, our priority, first and foremost, is to assess markers of differentiation and reduction in cell cycle, with the aim of halting tumour growth and enhance the current differentiation therapy of retinoic acid (RA). We invite further research into the functionality of the resulting neurons following CDK4/6i+RA treatment, for biological

and clinical interest. It is possible that use of neuronal cell culture media and/or a more complex *in vivo* microenvironment would influence that ability of the differentiated cells to possess electrophysiological neuronal functions. This could be useful for fields such as Parkinson's disease pathobiology where differentiated SH-SY5Y treated with retinoic acid alone are used as a model system (e.g. PMID: 28118852). We have now highlighted this potential future work in the Discussion text (paragraph 3). Regarding the stability of the differentiation state of the neuroblastoma cells, we began to further explore the differentiation potential of neuroblastoma cell lines *in vitro* in response to palbociclib and retinoic acid treatment in another manuscript (https://wellcomeopenresearch.org/articles/9-671). It would indeed be very interesting to confirm if the differentiation state of the cells is terminally stable, and the treatment regime required to achieve this; such an experiment would be best performed in an *in vivo* system where the neuroblastoma cells are exposed to the tumour microenvironment and we will keep this in mind for future exploration leading to clinical translation.

- **Comment 3:** This study primarily focuses on cellular-level experiments and lacks supporting in vivo data.
- **Author response:** We agree it would be very interesting to test these inhibitors in vivo. Indeed, we are undertaking this as part of a separate study to provide supporting data for clinical application of CDK4/6 inhibitors+RA in treating neuroblastoma, but this work is beyond the scope of this paper.

- **Comment 4:** This study demonstrates that CDK4/6 inhibitors induce differentiation in NB cells; however, the underlying mechanisms have not been thoroughly investigated.
- **Author response:** In our previous manuscript published in Developmental Cell (PMID: 37734383), we performed extensive analyses to show that PB resets the global transcriptional and epigenetic landscapes of ADRNB-type neuroblastoma cells towards a differentiated state. As discussed in the manuscript, "lengthening the cell cycle may be the trigger for this phenotypic switch; for example, G1 lengthening is thought to increase the time available for cells to respond to differentiation cues". As referenced, Calegari et al (PMID: 16014714 and PMID: 14625388) show that G1 lengthening casually induces neuroepithelial differentiation of proliferative neural progenitors. They propose a cell-cycle length model, where "a cell fate determinant may or may not cause a cell fate change, depending on whether the length of time this determinant can act is sufficient" and indeed show that neuroepithelial and radial glial cells in mouse embryos have a longer cell cycle than proliferating progenitors (PMID: 16014714). The effect of cell cycle on lineage commitment has been extensively investigated by Philpott lab and others (PMID: 24859217, PMID: 12593979, PMID: 24791612). We have now expanded on this point in the discussion to address the reviewers' question (Discussion, paragraph 3).

- **Comment 5:** The rationale with which the doses of the two drugs were chosen is not clear. A more in-depth study exploring a broad range of concentrations should be done. Furthermore, the assessment of a potential synergistic effect between RA and CDK4/6 inhibitors should be evaluated using appropriate synergy models (i.e. Chou-

Talalay model or Bliss Excess).
- **Author response:** As in our previous manuscript (PMID: 37734383), cells were treated 1 μM PB; this is a standard dosage used in cellular studies, and is similar to the IC50s that we established for all three lines (Figure S1A of PMID: 37734383). We therefore ascertained and used ribociclib and abemaciclib at their IC50 concentrations in SK-N-BE(2)C cells (2 μM and 0.2 μM, respectively). Cells were treated with all-trans retinoic acid (RA) at a concentration of 10 μM, commonly used in studies to differentiate SH-SY5Y neuroblastoma cells (e.g. PMID: 35205034). Importantly, we confirmed that none of these dosages resulted in cell death. Thank you for the insightful comment on determining drug synergy; this is indeed important for clinical translation to reduce toxic effects and will be determined for *in vivo* assessment of CDK4/6i+RA.

We thank the reviewer for their helpful comments which have guided submission of version 2 of this manuscript. As further experiments cannot be completed at this time as key staff have left the lab, we ask that the reviewer considers this in their responses.

Competing Interests: No competing interests were disclosed.

Reviewer Report 15 November 2024

https://doi.org/10.21956/wellcomeopenres.25540.r110971

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Feng-Hou Gao
Shanghai Jiao Tong University School of Medicine, Shanghai, China

This research focuses on cell differentiation therapy, aiming to redirect cancer cells back to the normal developmental pathway, thus providing new ideas for the treatment of neuroblastoma. In the field of neuroblastoma, previous studies have found that the CDK4/6 inhibitor palbociclib can induce a reduction in the proliferation and an enhancement in the neuronal differentiation of neuroblastoma cells, and the effect is even more significant when combined with retinoic acid. However, the roles of other CDK4/6 inhibitors in the differentiation of neuroblastoma cells remain unclear. This study was carried out under such a background and is of certain innovativeness and exploratory nature.
1. In the Western blot results, only the protein bands were presented, but the quantitative data of phosphorylated RB and total RB protein levels were not clearly provided, making it impossible to accurately judge the differences in the degree of inhibition. For example, although the changing trends between the treatment groups and the control group could be observed, it was difficult to determine whether there were significant quantitative differences in inhibiting RB phosphorylation among abemaciclib, ribociclib and palbociclib, which was insufficient for an in-depth comparison of the effects of different inhibitors.
2. Although the phase contrast microscope images (Figure 1B) could observe the changes in cell

morphology, no quantitative description was made of these changes, such as the measurement and statistical analysis of cell size, neurite length and other characteristics. Merely through image observation, it was difficult to accurately compare the degree and characteristics of cell morphology changes, which might affect the accurate assessment of the differentiation-inducing effects of the inhibitors.
3. The experiment only set DMSO solvent as the control, without setting a positive control (such as other drugs or treatment methods known to be able to effectively induce the differentiation of neuroblastoma cells). This made it difficult to directly compare when evaluating the differentiation-inducing effects of CDK4/6 inhibitors, and it was hard to determine the strength of their effects and their positions in similar studies, which was not conducive to a comprehensive evaluation of the reliability and innovativeness of the research results.
4. In the EdU analysis (Figure 2D), although the analysis results of %EdU positive cells were provided, it was not clearly stated how EdU positive cells were defined, for example, whether a specific fluorescence intensity threshold was set. This made it difficult for other researchers to accurately repeat the experiment and evaluate the reliability of the data. In addition, only some comparisons between treatment groups were presented in the figure, without completely showing all possible pairwise comparison results, which might affect the comprehensive understanding of the differences between different treatment groups.
5. In the immunofluorescence staining images (Figure 3C), although the expression of TUBB3 in tumor spheroid cells was shown, the resolution of some images was not high enough, making it difficult to clearly identify the cell details, such as the fine structures of neurites and the intercellular relationships. Moreover, there was no clear labeling of cell types or regions in the images, making it difficult for readers to accurately judge the specific cell locations and distribution of the enhanced TUBB3 expression, thus affecting the accurate assessment of the cell differentiation state.
6. Although the effects of the inhibitors on tumor spheroid growth and cell differentiation were confirmed by multiple methods, further functional verification experiments were lacking. For example, the functions of the differentiated cells were not detected, such as the electrophysiological functions specific to neurons and cell-to-cell signal transduction. This made the assessment of cell differentiation only stay at the morphological and gene expression levels, and it was impossible to determine whether the differentiated cells had true neuronal functions, which limited the comprehensive understanding of the differentiation-inducing effects of the inhibitors and their evaluation in potential therapeutic applications.
7. The experiment mainly focused on the research at the cell level, lacking in vivo experimental data. The in vivo environment is complex, and factors such as cell-cell interactions and immune responses may affect the effects of the inhibitors. Therefore, it is difficult to directly infer the effectiveness of the inhibitors in clinical treatment solely based on the in vitro experimental results.
8. Although the aspects such as cell proliferation and differentiation were studied, the specific molecular mechanisms by which CDK4/6 inhibitors induce the differentiation of neuroblastoma cells were not thoroughly explored. For example, it remains unclear how the prolongation of the G1 phase triggers the molecular signaling pathways of differentiation, which limits the in-depth understanding and further optimization of this therapy.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Tumor microenvironment and tumor immunity。

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 Aug 2025

Anna Philpott

We thank the reviewer for their helpful comments and respond to each of these below.
Comment 1: In the Western blot results, only the protein bands were presented, but the quantitative data of phosphorylated RB and total RB protein levels were not clearly provided, making it impossible to accurately judge the differences in the degree of inhibition. For example, although the changing trends between the treatment groups and the control group could be observed, it was difficult to determine whether there were significant quantitative differences in inhibiting RB phosphorylation among abemaciclib, ribociclib and palbociclib, which was insufficient for an in-depth comparison of the effects of different inhibitors. **Author response**: To confirm successful CDK4/6 inhibition, we analysed reduced phosphorylation of RB, a tumour suppressor that blocks the G1/S transition. The aim of this research article is to determine if the three CDK4/6 inhibitors, palbociclib, ribociclib and abemaciclib, display a class effect in inducing differentiation together with retinoic acid. For this reason, we do not make direct comparisons between the CDK4/6 inhibitors, but compare each CDK4/6 inhibitor to the control. We observe that PB, RIBO or ABE treatment for 24h results in RB hypo-phosphorylation, compared to the DMSO-treated control. This result is very clear visually and therefore we do not quantify the Western blot bands. It is indeed an interesting future research question to compare the efficacy of each CDK4/6 inhibitor to one another, but is beyond the aims of this manuscript. We have added this future research question to the Discussion (end of paragraph 2).
Comment 2: Although the phase contrast microscope images (Figure 1B) could observe the changes in cell morphology, no quantitative description was made of these changes, such as the measurement and statistical analysis of cell size, neurite length and other

characteristics. Merely through image observation, it was difficult to accurately compare the degree and characteristics of cell morphology changes, which might affect the accurate assessment of the differentiation-inducing effects of the inhibitors. **Author response**: In addition to the phase contrast images presented in Figure 1B, we also present crystal violet staining images (Figure 1C) and immunocytochemistry for the neuronal marker TUBB3 (Figure 1D) as supporting data. Figure 1C shows a striking difference in cell proliferation between the DMSO control and PB/ABE/RIBO-treated cells. Figure 1D provides complementary data to Figure 1B, showing a striking upregulation in TUBB3 expression upon PB/ABE/RIBO treatment, with significant changes in cell morphology, notably neurite extensions. We have also provided live imaging of SK-N-BE(2)C cells undergoing these drug treatments in the Extended Data Movie S1; neurite formation can be observed in real-time following drug treatment (with no neurite extension in the DMSO control), supporting the differentiation-inducing effects of the inhibitors.

Comment 3: The experiment only set DMSO solvent as the control, without setting a positive control (such as other drugs or treatment methods known to be able to effectively induce the differentiation of neuroblastoma cells). This made it difficult to directly compare when evaluating the differentiation-inducing effects of CDK4/6 inhibitors, and it was hard to determine the strength of their effects and their positions in similar studies, which was not conducive to a comprehensive evaluation of the reliability and innovativeness of the research results. **Author response:** Thank you for raising this point for discussion. Retinoic acid is used as a differentiation-inducing agent in neuroblastoma cells, and would therefore be considered a positive control. The effect of RA treatment alone can be seen in Figure 2 and Figure 3 of this manuscript, and can be considered a positive control for differentiation. However, as can be seen by our results here and in our previous publication (PMID: 37734383), the differentiation-inducing effects of retinoic acid are substantially enhanced by CDK4/6 inhibition. We have now clarified this in the text (Results section - “The CDK4/6 inhibitors palbociclib, abemaciclib and ribociclib all enhance retinoic acid-induced differentiation in adherent SK-N-BE(2)C cells”).

Comment 4: In the EdU analysis (Figure 2D), although the analysis results of %EdU positive cells were provided, it was not clearly stated how EdU positive cells were defined, for example, whether a specific fluorescence intensity threshold was set. This made it difficult for other researchers to accurately repeat the experiment and evaluate the reliability of the data. In addition, only some comparisons between treatment groups were presented in the figure, without completely showing all possible pairwise comparison results, which might affect the comprehensive understanding of the differences between different treatment groups. **Author response:** For the EdU analysis, FIJI image thresholding and particle analysis functions were used to quantify the % of EdU positive cells, as stated in the methods section using standard analysis methods. As standard for fluorescence imaging analysis, identical settings were used throughout the experiment for all treatment groups and replicates. This has now been further clarified in the Methods section. To aid readability and visualisation, only pairwise comparisons from which we draw conclusions are shown in each plot as stated in the figure legend (‘Selected comparisons shown for ease of visualisation’). However, for those interested in other condition comparisons, all comparisons for the one-way ANOVA with Tukey’s multiple comparison test can be viewed in the Underlying Data ‘Figure2D: GraphPad Prism file of raw % EdU counts and statistical analysis’ using the freely available GraphPad Prism Viewer.

Comment 5: In the immunofluorescence staining images (Figure 3C), although the

expression of TUBB3 in tumor spheroid cells was shown, the resolution of some images was not high enough, making it difficult to clearly identify the cell details, such as the fine structures of neurites and the intercellular relationships. Moreover, there was no clear labeling of cell types or regions in the images, making it difficult for readers to accurately judge the specific cell locations and distribution of the enhanced TUBB3 expression, thus affecting the accurate assessment of the cell differentiation state. **Author response:** The reader can find original high resolution images of the whole spheroids and magnified regions in Underlying data 'Figure3C: Raw representative immunocytochemistry images of spheroids, TUBB3 staining'. In this manuscript we have not determined intercellular relationships or cell locations, as the immunostaining was performed to visually demonstrate morphological changes to cells and an increase in neurite extension with CDK4/6 inhibitor and CDK4/6i+RA treatment. To provide quantitative data, we have performed qRT-PCR analyses of the neuronal differentiation marker STMN4, which shows a striking upregulation in expression in spheroids with CDK4/6i+RA treatment compared to the DMSO control.

Comment 6: Although the effects of the inhibitors on tumor spheroid growth and cell differentiation were confirmed by multiple methods, further functional verification experiments were lacking. For example, the functions of the differentiated cells were not detected, such as the electrophysiological functions specific to neurons and cell-to-cell signal transduction. This made the assessment of cell differentiation only stay at the morphological and gene expression levels, and it was impossible to determine whether the differentiated cells had true neuronal functions, which limited the comprehensive understanding of the differentiation-inducing effects of the inhibitors and their evaluation in potential therapeutic applications. **Author response:** We agree with the reviewer that it would be very interesting to investigate the functions of the differentiated cells, such as electrophysiological analyses. In our previous manuscript (PMID: 37734383) we performed extensive transcriptomic and epigenomic studies on PB+RA treated cells and "a mature differentiated state was observed both transcriptionally, by increased expression of neuronal differentiation features, and functionally by the increased presence of dense-core neurosecretory granules as visualized by TEM". As the aim of our work is to investigate CDK4/6i+RA as a treatment in the paediatric cancer neuroblastoma, our priority, first and foremost, is to assess markers of differentiation and reduction in cell cycle, with the aim of halting tumour growth and enhance the current differentiation therapy of retinoic acid (RA). We invite further research into the functionality of the resulting neurons following CDK4/6i+RA treatment, for biological and clinical interest. It is possible that use of neuronal cell culture media and/or a more complex *in vivo* microenvironment would influence that ability of the differentiated cells to possess electrophysiological neuronal functions. This could be useful for fields such as Parkinson's disease pathobiology where differentiated SH-SY5Y treated with retinoic acid alone are used as a model system (e.g. PMID: 28118852). We have now highlighted this potential future work in the Discussion text (paragraph 3).

Comment 7: The experiment mainly focused on the research at the cell level, lacking in vivo experimental data. The in vivo environment is complex, and factors such as cell-cell interactions and immune responses may affect the effects of the inhibitors. Therefore, it is difficult to directly infer the effectiveness of the inhibitors in clinical treatment solely based on the in vitro experimental results. **Author response:** We agree it would be very interesting to test these inhibitors in vivo. Indeed, we are undertaking this as part of a separate study to provide supporting data for clinical application of CDK4/6 inhibitors+RA in

treating neuroblastoma, but this work is beyond the scope of this study.
Comment 8: Although the aspects such as cell proliferation and differentiation were studied, the specific molecular mechanisms by which CDK4/6 inhibitors induce the differentiation of neuroblastoma cells were not thoroughly explored. For example, it remains unclear how the prolongation of the G1 phase triggers the molecular signaling pathways of differentiation, which limits the in-depth understanding and further optimization of this therapy. **Author response:** Thank you for raising this for discussion. As discussed in the manuscript, "lengthening the cell cycle may be the trigger for this phenotypic switch; for example, G1 lengthening is thought to increase the time available for cells to respond to differentiation cues". As referenced, Calegari et al (PMID: 16014714 and PMID: 14625388) show that G1 lengthening casually induces neuroepithelial differentiation of proliferative neural progenitors. They propose a cell-cycle length model, where "a cell fate determinant may or may not cause a cell fate change, depending on whether the length of time this determinant can act is sufficient" and indeed show that neuroepithelial and radial glial cells in mouse embryos have a longer cell cycle than proliferating progenitors (PMID: 16014714). The effect of cell cycle on lineage commitment has been extensively investigated by Philpott lab and others (PMID: 24859217, PMID: 12593979, PMID: 24791612), and further understanding of the link between G1 lengthening and differentiation is beyond the aims of this study. We have now expanded on this point in the discussion to address the reviewers' question (Discussion, paragraph 3).

We thank the reviewer for their helpful comments which have guided submission of version 2 of this manuscript. As further experiments cannot be completed at this time as key staff have left the lab, we ask that the reviewer considers this in their responses.

Competing Interests: No competing interests were disclosed.