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Original Research

Combined targeting of the p53 and pRb pathway in neuroblastoma does not lead to synergistic responses

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MDM2 inhibitor;
Idasanutlin;
CDK4/6 inhibitor;
Abemaciclib

Abstract ***Background:*** Despite intensive treatment protocols and recent advances, neuroblastomas still account for approximately 15% of all childhood cancer deaths. In contrast with adult cancers, p53 pathway inactivation in neuroblastomas is rarely caused by p53 mutation but rather by altered MDM2 or p14ARF expression. Moreover, neuroblastomas are characterised by high proliferation rates, frequently triggered by pRb pathway dysfunction due to aberrant expression of cyclin D1, CDK4 or p16INK4a. Simultaneous disturbance of these pathways can occur via co-amplification of *MDM2* and *CDK4* or homozygous deletion of *CDKN2A*, which encodes both p14ARF and p16INK4a.

Methods and results: We examined whether both single and combined inhibition of MDM2 and CDK4/6 is effective in reducing neuroblastoma cell viability. In our panel of ten cell lines with a spectrum of aberrations in the p53 and pRb pathway, idasanutlin and abemaciclib were the most potent MDM2 and CDK4/6 inhibitors, respectively. No correlation was observed between the genetic background and response to the single inhibitors. We confirmed this lack of correlation in isogenic systems overexpressing MDM2 and/or CDK4. In addition, combined inhibition did not result in synergistic effects. Instead, abemaciclib diminished the pro-apoptotic effect of idasanutlin, leading to slightly antagonistic effects. *In vivo* treatment with idasanutlin and abemaciclib led to reduced tumour growth compared with single drug treatment, but no synergistic response was observed.

Conclusion: We conclude that p53 and pRb pathway aberrations cannot be used as predictive biomarkers for neuroblastoma sensitivity to MDM2 and/or CDK4/6 inhibitors. Moreover, we

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advise to be cautious with combining these inhibitors in neuroblastomas.
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1. Introduction

Neuroblastomas account for approximately 15% of all childhood cancer deaths [1]. Using an intensive treatment protocol, which includes high-dose multi-agent chemotherapy, complete remission is nowadays achieved in many high-risk patients. Nevertheless, up to 60% of them will eventually relapse and succumb to the disease due to the treatment-resistant nature of these relapse tumours [2]. Similar to many other tumours, neuroblastomas are characterised by high proliferation rates, making the cell cycle and its regulation an attractive therapeutic target. The pRb and p53 pathways are essential in this process by regulating cell cycle progression and cell cycle arrest or apoptosis, respectively.

Unlike many adult tumours, neuroblastomas are known to have low frequencies of inactivating mutations in *TP53* [3]. However, p53 functionality can be disturbed by aberrant expression of upstream proteins, most importantly the E3 ubiquitin-protein ligase MDM2. Overexpression of MDM2 has been described in up to 53% of the patients [4], whereas gains and amplifications occur at lower frequencies (19% and 13%, respectively) [5,6]. MDM2 inactivates p53 by blocking its transcriptional activity, stimulating proteasomal degradation and inhibiting its translation [7]. MDM2 and p53 form a negative feedback loop, as MDM2 is one of p53's transcriptional targets.

The pRb pathway, on the other hand, is disturbed by high cyclin D1 mRNA levels in 67% of the patients [8]. Overexpression, amplification or gain of CDK4 also occur in up to 5% of neuroblastomas [9,10]. Cyclin D1 forms an activating complex with CDK4, enabling phosphorylation of Rb protein. This phosphorylated form is no longer able to bind and thereby inactivate the transcription factor E2F, which then stimulates the expression of G1/S phase-promoting genes.

Interestingly, *MDM2* (12q15) and *CDK4* (12q14.1) are located in close proximity to each other. Although rare, co-amplification of these genes has been reported as a recurrent event in neuroblastomas, with higher frequencies in relapse tumours [11–13]. Other aberrations that affect both pathways are those in *CDKN2A*, encoding both p14ARF (which blocks the binding of MDM2 to p53) and p16INK4a (which inhibits the CDK4-cyclin D1 complex). Homozygous deletions of *CDKN2A* are infrequent in primary neuroblastomas but occur in up to 11% of relapse tumours [14].

Recurrent findings of (co-occurring) p53 and pRb pathway disturbances in neuroblastomas let us to hypothesise that these patients might benefit from drugs targeting those pathways. We used the MDM2 inhibitors idasanutlin, SAR405838 and HDM-201, which occupy the p53 binding pocket of MDM2, thereby preventing p53 degradation [15]. To target the pRb pathway, we used the three different CDK4/6 inhibitors (palbociclib, ribociclib and abemaciclib) that are currently approved for adult malignancies and tested in paediatric patients. These inhibitors bind to the ATP binding pocket of CDK4 and CDK6 [16], thereby inhibiting the ability of the CDK4-cyclin D1 complex to phosphorylate pRb. Since *MDM2* and *CDK4* are co-amplified in a subset of patients, we hypothesised that targeting both pathways might be of added value in the treatment of high-stage neuroblastoma.

Here, we tested MDM2 and CDK4/6 inhibitors in a panel of neuroblastoma cell lines representing the clinically observed p53 and pRb pathway aberrations, as well as in isogenic MDM2 and/or CDK4 overexpression systems, to examine whether p53 and pRb pathway disturbances act as predictive biomarkers for drug sensitivity. Given the fact that combination therapies are often more effective and can hamper the development of resistance mechanisms [17,18], we also studied simultaneous inhibition of these pathways, both *in vitro* and *in vivo*.

2. Material and methods

2.1. Cell culture

Cell lines were obtained from the American Type Culture Collection (SKNSH, SKNAS and SKNBE) or from historic collaborations, and their identity was validated by short tandem repeat analysis. Cells (Fig. 1a) were cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, #41965), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were grown at 37 °C and 5% CO_2 and tested for mycoplasma infection every six weeks.

2.2. Cell viability assay

For single compound testing, cells were seeded in triplicates in a 96-well plate at a density of

2000–20,000 cells per well, depending on the cell line. Cells were given 24 h to attach and subsequently treated with the targeted compound using a five-fold concentration range from 0.64 nM to 10 μM. For combination testing, 400–8000 cells were seeded in duplicates in a 384-well plate (for Shep2, 2000 cells were seeded in a 96-well plate). Inhibitors (Supplementary Table 1) were added in a matrix of five-fold concentration ranges from 0.128 nM to 50 μM using the D300e Digital Dispenser (TECAN). After 72 h, cell viability was measured using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium (MTT) assay [19].

2.3. Xenograft experiments

Ethical approval was obtained under project number AVD3990020173068, study protocol PMC.63.3068.1901. NGP neuroblastoma cells (5×10^6) were xenografted into both flanks of three Naval Medical Research Institute (NMRI) nu/nu mice at 5–7 weeks of age (obtained from Charles River). Tumour size was monitored twice a week by calliper measurements and determined using the formula $(\pi/6)^*d3$. Once tumours reached 1000 mm^3 in size, tumour pieces were xenotransplanted into recipient mice. Daily oral treatment started once tumours reached a size of 250 mm^3. Treatment groups (n = 6 or 7 mice per group) were idasanutlin (25 mg/kg) + vehicle abemaciclib, abemaciclib (50 mg/kg) + vehicle idasanutlin, a combination of abemaciclib and idasanutlin or the appropriate vehicles (Supplementary Table 2). Animals were sacrificed after 28 days of treatment (n = 3) or when tumour volumes exceeded 2000 mm^3.

Plasmid generation, virus production and generation of overexpression cell lines, Western blot analysis and cell cycle analysis are described in the supplementary material and methods.

3. Results

In vitro efficacies of MDM2 inhibitors idasanutlin (RG7388) and SAR405838 were studied in a panel of ten neuroblastoma cell lines with different genetic backgrounds (Fig. 1a). Lowest IC_{50} values were found for idasanutlin with a range from 13 nM to 309 nM for wildtype p53 cell lines (Supplementary Fig. 1a). IC_{50} values exceeded 10 μM for p53 mutant cell lines SKNAS and SKNBE, confirming that MDM2 inhibitors are dependent on functional p53 [15]. No correlations were observed between aberrations that should lead to higher MDM2 activity (i.e. *MDM2* amplification and homozygous *CDKN2A* deletion) and compound sensitivity (Fig. 1b and c and Supplementary Fig. 1a).

Next, similar experiments were performed using CDK4/6 inhibitors palbociclib, ribociclib (LEE011) and abemaciclib (LY2835219). In accordance with previous reports [16], we found that abemaciclib was the most potent inhibitor, with IC_{50} values ranging from 4 nM to 8913 nM (Supplementary Fig. 1b). We did not see a correlation between cells with expected higher activity of CDK4, caused by its amplification or *CDKN2A* loss,

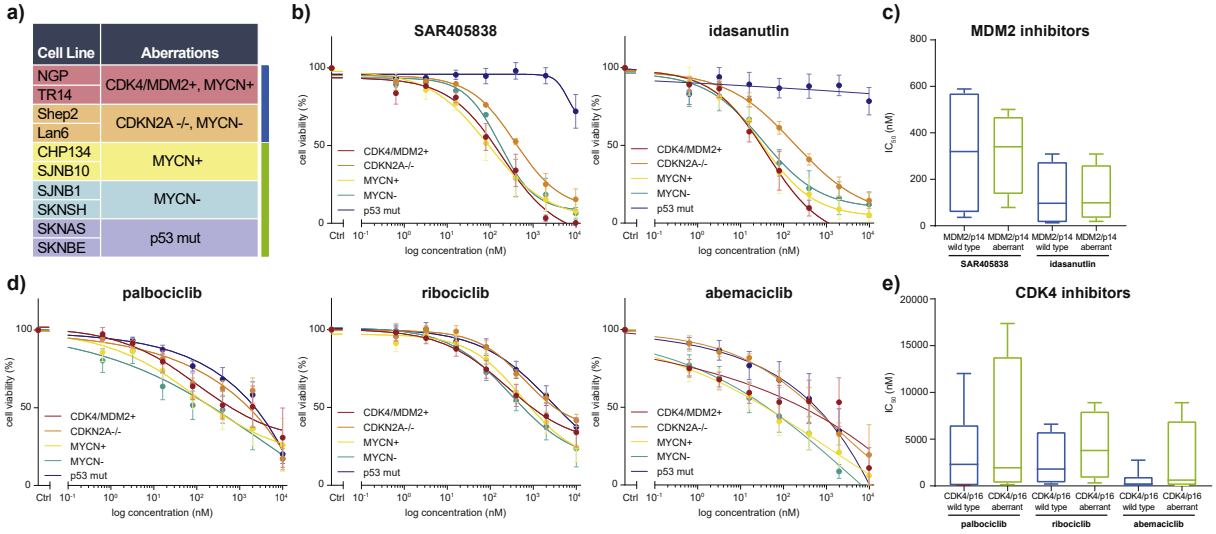

Fig. 1. MDM2 and CDK4/6 inhibitors reduce cell viability, but response does not correlate with genetic background. (a) Classical neuroblastoma cell lines used in this study and their relevant genetic background. Dose-response curves after 72-h treatment with (b) MDM2 inhibitors and (d) CDK4/6 inhibitors. The two cell lines with the same genetic background are merged here, curves represent the average of two replicates and error bars indicate the standard error of the mean (SEM). Box plots of IC_{50} values after (c) MDM2 and (e) CDK4/6 inhibitor treatment. Cell lines were grouped depending on the presence (blue; NGP, TR14, Shep2 and Lan6) or absence (green) of p53 and Rb pathway aberrations. No statistic significance was reached ($p < 0.01$).

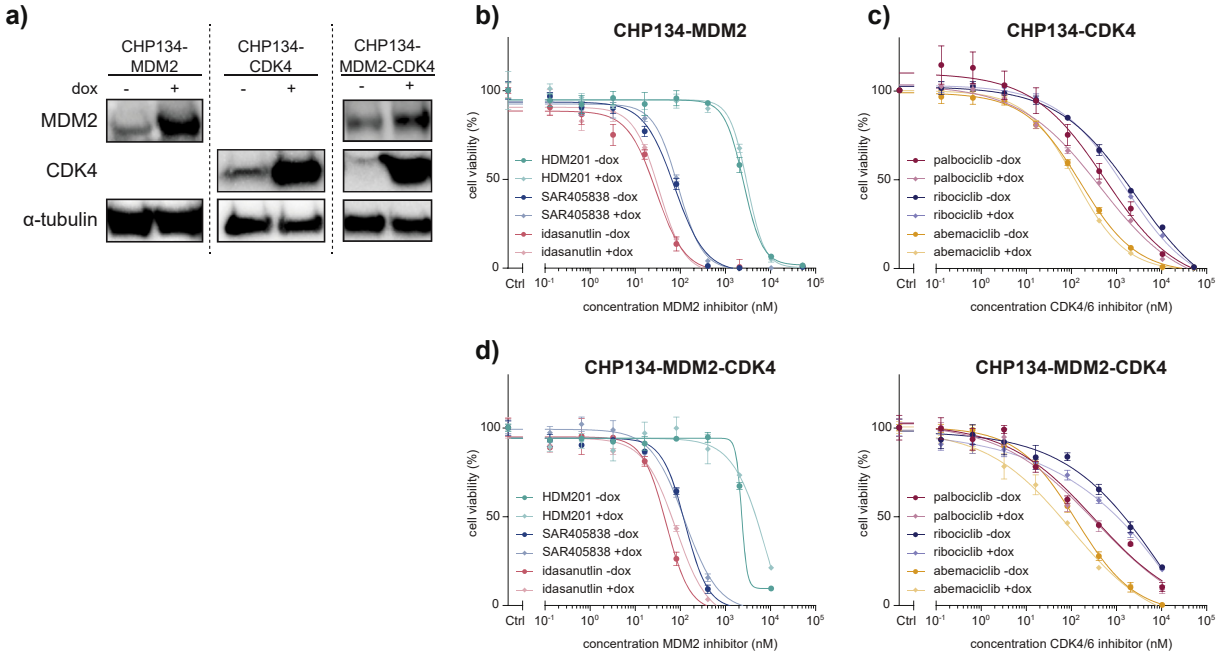

Fig. 2. Overexpression of MDM2 and/or CDK4 does not change response to the inhibitors. (a) Western blot of doxycycline-inducible overexpression of MDM2 and/or CDK4 in CHP134 cells. Dose-response curves after 72-h MDM2 or CDK4/6 inhibitor treatment in (b) CHP134-MDM2, (c) CHP134-CDK4 and (d) CHP134-MDM2-CDK4 (simultaneous overexpression), with and without doxycycline (dox) to induce overexpression. Error bars indicate the SEM.

and compound sensitivity (Fig. 1d and e and Supplementary Fig. 1b).

To further examine this lack of correlation, we established stable doxycycline-inducible overexpression of MDM2 and/or CDK4 in CHP134 (Fig. 2a) and treated these cells with the same inhibitors, including the additional MDM2 inhibitor HDM-201. IC_{50} values were not substantially different when MDM2 and/or CDK4 was overexpressed (Fig. 2b–d), further indicating that these expression levels do not predict sensitivity to MDM2 or CDK4/6 inhibitors.

As *MDM2* and *CDK4* amplifications can occur together (cell lines NGP and TR14) and homozygous deletions of *CDKN2A* affect both pathways (Shep2 and Lan6 cells), we were interested if simultaneous inhibition would be beneficial. We treated our panel of cell lines for 72 h with different concentrations of idasanutlin and abemaciclib (Fig. 3a and Supplementary Fig. 2) and calculated Bliss independence (BI) values as a measure of synergy. BI values were variable between cell lines, despite their genetic backgrounds (Fig. 3b and Supplementary Fig. 3). Nevertheless, BI values indicate an additive to slightly antagonistic effect in most cell lines, with TR14 (*MDM2*/*CDK4*-amplified) and Lan6 (*CDKN2A* loss) showing highest levels of antagonism. To investigate whether first arresting the cell cycle and then stimulating apoptosis would alter the response, we delayed idasanutlin treatment with 24 h. However, this did not result in substantial differences in cell viability or BI values (Supplementary Fig. 4).

To further investigate the adverse effect, we performed cell cycle analyses using propidium iodide staining in combination with flow cytometry in CHP134 and NGP cells treated with idasanutlin and/or abemaciclib. As expected, idasanutlin and abemaciclib both induced G1 arrest (Fig. 4a). In NGP, which contains the co-amplification, this cell cycle arrest seemed to be achieved at lower doses. Both compounds also induced apoptosis, independent of *MDM2* and *CDK4* status, but higher sub-G1 fractions were observed after idasanutlin treatment (Fig. 4b). However, when cells were treated with both compounds simultaneously, the apoptotic fraction was smaller compared with treatment with idasanutlin alone. Unexpectedly, this effect seemed to be more pronounced in NGP cells. Increased protein levels of PARP and cleaved caspase 3 support the induction of apoptosis after idasanutlin treatment and the adverse effect when combined with CDK4/6 inhibition (Fig. 4c). Both single compound treatments led to a reduction of Ser780-phophorylated pRb, while idasanutlin treatment additionally induced p53 and p21 expression, confirming the known mechanism of actions of idasanutlin and abemaciclib. The enhanced expression of p53 upon inhibition with the highest concentration of idasanutlin seemed to be somewhat abolished when combined with abemaciclib. Moreover, MDM2 expression was upregulated and pRb phosphorylation slightly diminished upon combination treatment. These effects were again most prominent in *MDM2*/*CDK4*-amplified NGP cells. Together, these data suggest that

abemaciclib tempers the pro-apoptotic effect of idasanutlin.

To test if this adverse effect would also be seen *in vivo*, we established a xenograft model by injecting NGP cells subcutaneously into NMRI nu/nu mice. Once the tumours reached a size of 250 mm^3, 28-day treatment with either 25 mg/kg idasanutlin, 50 mg/kg abemaciclib or both started. All treatments were well tolerated. Idasanutlin monotherapy resulted in diverse reactions, ranging from complete regression to tumour progression comparable with vehicle-treated tumours (average tumour growth on day 28 was 158% versus 470% in the vehicle group) (Fig. 5 and Supplementary Fig. 5). Abemaciclib slowed down tumour growth compared with vehicle-treated animals, but tumours were still progressing (average tumour growth 220%). Combined treatment led to stable tumour sizes in all six animals, a response comparable to stable disease, but no tumour regression (average tumour growth 14%). Thus, combining idasanutlin and abemaciclib resulted in smaller average tumour sizes than either targeted compound alone. However, average tumour sizes are higher than expected based on an additive BI value for most time points, showing that there is no synergistic effect *in vivo* (Fig. 5a). Instead, the combination shows an additive to slightly antagonistic effect in mice, as was observed earlier in cell lines.

4. Discussion

In the era of precision medicine, one of the main focuses is finding biomarkers for specific targeted drugs. Besides the fact that drug development is still mainly focused on adult conditions, the identification of biomarkers in paediatric cancer is hampered by small patient numbers, with even smaller genetic subpopulations. Multinational initiatives, such as the Innovative Therapies for Children with Cancer (ITCC) and the Neuroblastoma New Drug Development Strategy (NDDS), which focus on accelerating paediatric drug development, are essential in this process [20,21]. MDM2 and CDK4/6 inhibitors were amongst the targeted drugs selected by the NDDS [21,22]. To prevent further delay by (clinical) studies that are unlikely to be effective, it is pivotal to also publish negative findings.

Here, we examined whether *MDM2* and *CDK4* amplification or homozygous *CDKN2A* deletions can be used as predictive biomarkers of response for MDM2 and/or CDK4/6 inhibitors in neuroblastoma.

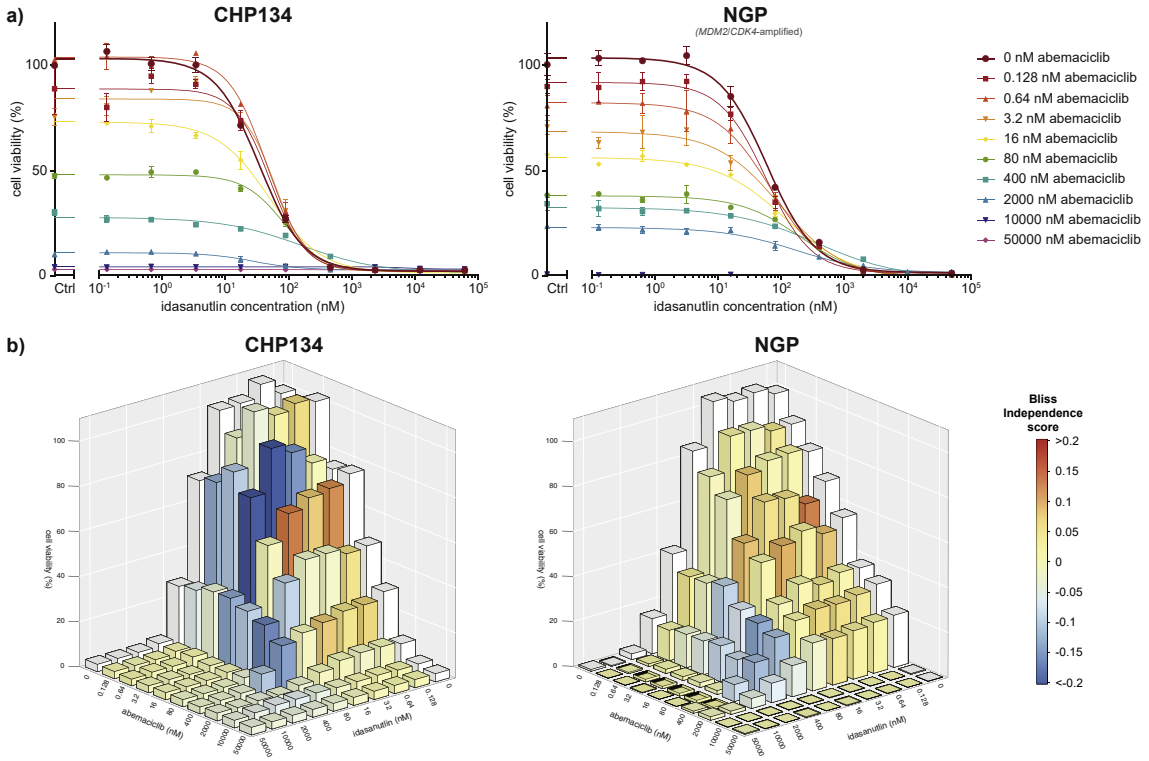

Fig. 3. No consistent synergism is observed after combined inhibition of MDM2 and CDK4/6. (a) Cell viability curves after 72-h treatment with different concentrations of idasanutlin and abemaciclib in CHP134 and NGP cells. Idasanutlin concentration is indicated on the x-axis; abemaciclib concentration is indicated by colour. Error bars indicate the SEM. (b) Plots showing the cell viability after combined inhibition (height of the bars) and the Bliss independence score (colour) in CHP134 and NGP cells. Red indicates synergism, blue indicates antagonism and additive effects are shown in yellow.

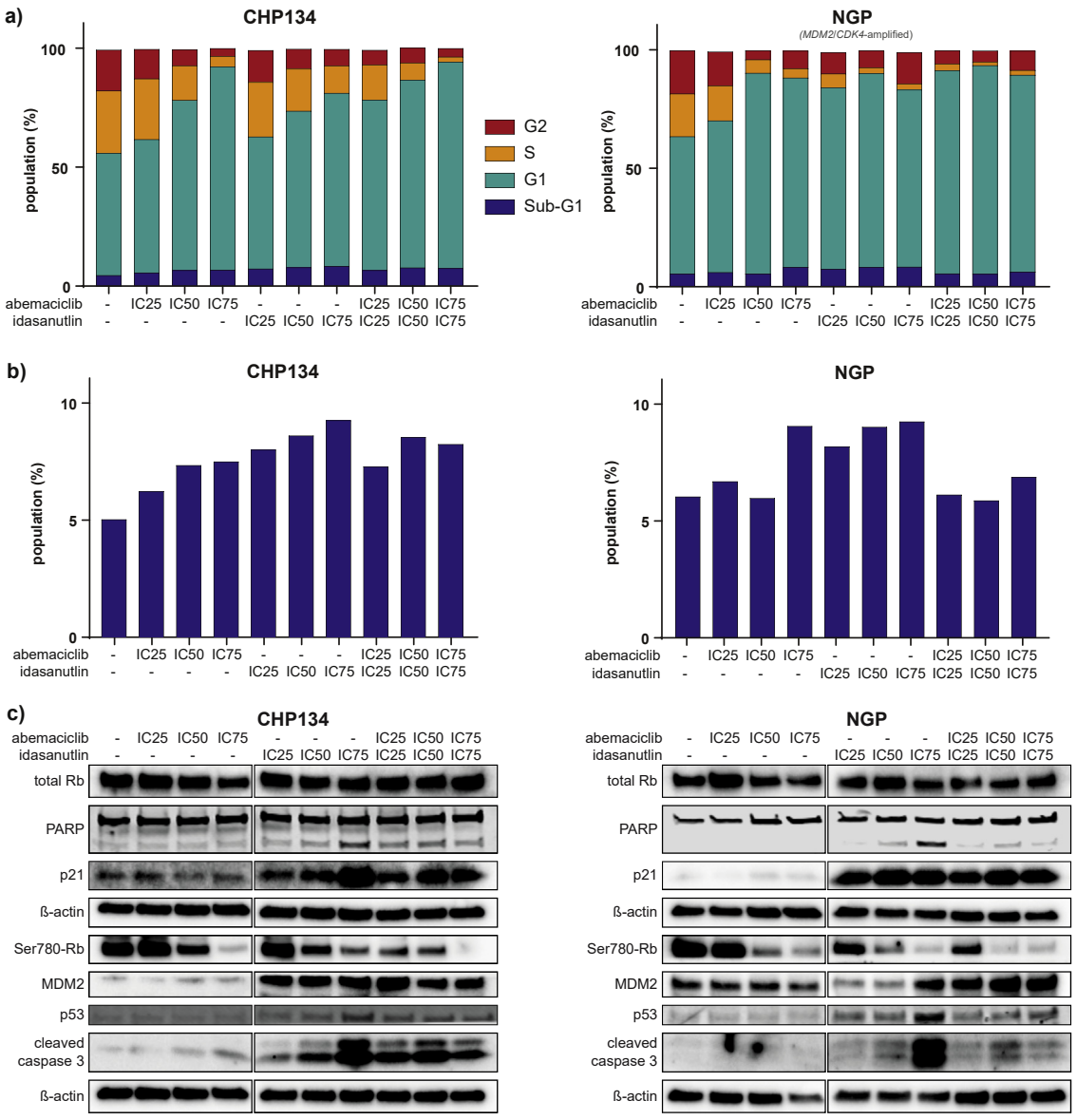

Fig. 4. Cell cycle analyses reveal that abemaciclib reduces the pro-apoptotic effect of idasanutlin. (a) Cell cycle distribution after 24-h treatment with abemaciclib and/or idasanutlin in CHP134 and NGP cells. (b) Apoptotic (sub-G1) fraction highlighted. (c) Protein expression of different cell cycle regulators after treatment in CHP134 and NGP. Concentrations used in CHP134 were 10, 64 and 400 nM for abemaciclib and 15, 33 and 75 nM for idasanutlin; in NGP, the concentrations were 2, 32 and 542 nM for abemaciclib and 26, 65 and 162 nM for idasanutlin, corresponding with the respective IC25, IC50 and IC75 concentrations.

Surprisingly, we did not find a correlation between the genetic background of neuroblastoma cells and their sensitivity to the drugs. The lack of correlation could be confirmed in an isogenic overexpression system. Moreover, we showed that idasanutlin and abemaciclib do not act synergistically. Instead, CDK4/6 inhibitor abemaciclib diminishes the pro-apoptotic effect of MDM2 inhibitor idasanutlin.

Previously, contradicting results were reported regarding a correlation between MDM2 expression levels and MDM2 inhibitor sensitivity in different tumour types [23–25]. Our results are in line with observations by Van Maerken *et al.* [26] and Chen *et al.* [27], showing that this correlation was absent for neuroblastoma cells. Several mechanisms that might influence MDM2 inhibitor sensitivity have been described. First, the relative expression of different splice forms of MDM2 and its homologue MDMX may determine the response [28,29]. *MDM2* amplification might result in expression of MDM2 inhibitor-resistant splice variants [30]. Secondly, other biomarkers, such as MYCN, might determine sensitivity to MDM2 inhibition [27,31]. In our study, we did indeed see a non-significant trend of higher sensitivity in *MYCN*-amplified lines, which might

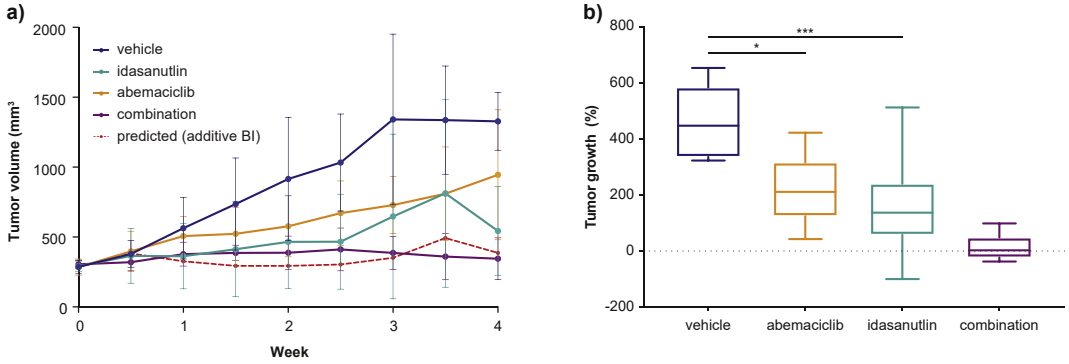

Fig. 5. Abemaciclib and idasanutlin do not act synergistically *in vivo*. (a) Average tumour growth per treatment group (n = 6–7). Error bars indicate the standard deviation (SD). Red dotted line shows the predictive tumour growth if the two drugs would act additively, based on tumour growth after single compound treatments from this experiment. (b) Box plot showing the tumour growth distribution per group after 28 days of treatment. $*p \leq 0.05$; $***p \leq 0.001$.

result from direct transcriptional regulation of p53 by MYCN [32]. Lastly, controversial results about the correlation between *p14ARF* status and MDM2 inhibitor efficacy were reported [26,31]. In our study, cell lines with homozygous *CDKN2A* deletions were among the more resistant ones, but there was no clear correlation with *CDKN2A* status. Despite several undertakings to find a biomarker for MDM2 inhibitor response, up to this date, the only clear marker is p53 [33]. Acquired *TP53* mutations might also explain the variable responses within our group of idasanutlin-treated mice and resistance in one of the tumours [18].

For CDK4/6 inhibitors, varying responses, ranging from a correlation with CDK4 levels to resistance, have been reported across different malignancies [34–37]. *p16INK4a* loss was previously associated with both increased sensitivity and resistance [38,39]. In our study, overexpression of CDK4 did not change sensitivity to CDK4/6 inhibitors. One of the potential mechanisms behind this is the overexpression of cyclin E upon CDK4/6 inhibition [17]. Cyclin E, together with CDK2, is able to phosphorylate pRb, serving as an alternative route into S phase. Alternatively, Guiley *et al* [39]. showed that CDK4/6 inhibitors are unable to bind to CDK4-cyclin D1 complexes that have active p27 bound to it. Instead, they mainly bind to catalytically non-functional CDK4 monomers, and the authors suggest that CDK4/6 inhibition indirectly induces cell cycle arrest by lowering the abundance of cyclin A or increasing the inhibition of CDK2-cyclin E complexes by p21. This indirect effect might be unrelated to the amount of CDK4 and could therefore explain the absence of a correlation between CDK4 levels and compound sensitivity.

To our knowledge, this is the first study using the combination of idasanutlin and abemaciclib, but the approach to simultaneously target MDM2 and CDK4 is not unique. Synergistic effects were obtained in neuroblastoma by combining nutlin-3 (an older generation MDM2 inhibitor) with the pan-CDK inhibitor seliciclib [40], possibly via the inhibition of CDK2 and CDK9, as well as in liposarcoma and melanoma by combining MDM2 inhibition with palbociclib [41,42]. On the contrary, an antagonistic effect was found in sarcoma cells [43]. The two inhibited pathways are interconnected in several ways, which could explain the absence of synergism. Sriraman *et al.* found that the CDK4-cyclin D1 complex can bind to p53, which is essential for transcription of p53 target genes [43]. CDK4/6 inhibitors might block this interaction and counteract the effect of MDM2 inhibition. Moreover, MDM2 inhibition induces expression of cyclin D1 and cyclin E, which could contribute to cells escaping cell cycle arrest despite CDK4/6 inhibition [29]. Another layer of complexness is added by the observation that p53 pathway activation results in upregulation of p27 [44], which can either inhibit CDK2-cyclin E or form the resistant CDK4-cyclin D1-p27 complexes [39]. In these complex and intertwined pathways, protein expression, interactions and turnover levels seem to determine the fate of the cell. As a consequence, it is difficult to pinpoint the exact mechanism responsible for the lack of synergism we observed.

In conclusion, our results contradict the common belief and rationale that inhibition of an overexpressed oncoprotein results in higher compound sensitivity [45]. We showed that *MDM2* and *CDK4* amplifications, as well as homozygous *CDKN2A* deletions, have low potential as predictive biomarkers of response to MDM2 and CDK4/6 inhibitors in neuroblastoma. Further research should focus on finding biomarkers for these inhibitors, as well as on finding better treatment options for patients with p53 and pRb pathway alterations. Based on the lack of synergism observed in our study, we advise to be cautious with the combined inhibition of MDM2 and CDK4 in patients with neuroblastoma.

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Author contributions

Nil A. Schubert: Conceptualisation, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing – Original Draft, Visualisation; **Linda Schild:** Conceptualisation, Methodology, Validation, Investigation; **Stijn van Oirschot:** Investigation; **Kaylee M. Keller:** Investigation; **Lindy K. Alles:** Investigation; **Lindy Vernooij:** Investigation; **Marloes E. Nulle:** Investigation; **M. Emmy M. Dolman:** Conceptualisation, Methodology, Resources; **Marlinde L. van den Boogaard:** Conceptualisation, Methodology, Investigation, Writing – Review & Editing, Supervision, Project administration; **Jan J. Molenaar:** Conceptualisation, Methodology, Writing – Review & Editing, Supervision, Project administration

Conflict of interest statement

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2020.10.009.

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