Combination Therapy using CDK4/6 Inhibitors for Cancer Treatment

Abstract

Neuroblastoma is an extracranial solid tumor located in the sympathetic nervous system and covers 5-7% of the pediatric cancers. Clinical outcome differs greatly, as patients with low-risk disease have very high chances of survival, but patients with high-risk disease often have a poor prognosis. Multiple therapeutic agents are available for neuroblastoma, but partially due to limitations of pediatric cancer treatment, no optimal cure is found yet. Possible future therapeutics for pediatric high-risk neuroblastoma are Proteolysis Targeting Chimeras (PROTACs); molecular compounds designed to selectively degrade target proteins. Possible targets of these PROTACs are cell cycle regulators CDK4/6. Targeting CDK4/6 will lead to the cell cycle arrest, which result in the suppression of tumor growth. Currently, there are multiple clinically improved small molecule inhibitors for CDK4/6, which can be used in PROTACs design. A lot of research has been conducted in combining CDK4/6 inhibitors with other therapeutics, to obtain an synergistic effect and improve the clinical outcome for various cancers. In this review, therapeutics are discussed to provide an overview of very promising combinations that can be used in the future with CDK4/6 targeting PROTACs, for the treatment of high-risk pediatric neuroblastoma.

1. Introduction

1.1. Pediatric Cancer

Cancer is the dominant cause of death for children and adults worldwide, although pediatric cancer is rare compared to adult cancer (1). Adult cancer is a genetic disease that is often caused by an accumulation of unrepaired mutations that arise throughout life. These mutations can be caused by environmental factors or internal factors. There is a big overlap in mechanisms involved in cancer and ageing, including genomic instability, epigenetic changes and telomere attrition (1, 2). There are various tumor suppressor genes and oncogenes that can result in cancer when mutated. About 66% of the mutations that cause cancer are a result of environmental factors, which are therefore not likely to arise in children. However, of other mutations that are not associated with environmental factors, development of similar tumors and locations would be expected in children and adults, although that is not the case. Requirements for treatment also differs between children and adults. First, long term effects of treatments have a stronger impact on children than on adults (1). Secondly, there is a lack identifiable targets. Pediatric cancer is more often a disease of altered protein expression than of mutated proteins, meaning that that patients express a limited number of tumor-specific targets (1) (3). This can be an obstacle in using precision or personalized medicine that target mutationally malfunctioning pathways, an approach that is promising for cancer treatment (1). A lot of research is needed to understand the mechanisms of pediatric cancer in order to develop and improve treatment strategies (1).

1.2. Neuroblastoma

Neuroblastoma is an extracranial solid tumor, predominantly located in the adrenal medulla or elsewhere in the sympathetic nervous system (4). Worldwide, the occurrence of neuroblastoma at birth is one per 10.000, covering 5-7% of pediatric cancer types (4, 5). Around 30% of neuroblastoma cases arise during the first six months after birth, being the predominant cancer type for patients younger than one (4, 6). The location and stage of the tumor determines symptoms and clinical phenotypes (4, 7). Early symptoms are mostly nonspecific such as weight loss, fever and possibly acute pain caused by spontaneous bleedings of the tumor (4). Severe symptoms arise when the tumor gained

a critical size or has metastasized (4). The clinical outcome for stage 1 and 2 tumors shows a positive prognosis such as spontaneous regression, whereas high-stage tumors often result in a fatal outcome (8). Hallmarks of low-stage neuroblastoma tumors are multiple changes of chromosomal copy numbers, while high-stage tumors contain structural chromosomal defects (8). Gene mutations are infrequent in patients with neuroblastoma, making it hard to find efficient druggable targets (9). There is a wide genetic variation in neuroblastoma patients (10). In high risk neuroblastoma, recurrent somatic mutations in genes like *ATRX*, *ALK* and *PTPN11,* are found in less than 25% of high risk neuroblastoma patients. *ALK* is the oncogene that is mutated most frequently in pediatric neuroblastoma (11). Chromosomal alterations like *17q* gain, *11q* loss and *MYCN* amplification are found in around 90% of high risk neuroblastoma patients (10). *MYCN* is the most frequently amplified oncogene and a very important driver of neuroblastoma (12). In primary neuroblastomas*, MYCN* is amplified in 20% of the patients, and has a strong correlation to advanced staged neuroblastoma, a fast disease progression and a fatal outcome (4, 12).

1.3. The Role of CDK4/6 in the Cell Cycle

Neuroblastoma arises from sympathoadrenal lineage of neural crest cells, most likely due to malfunctioning of regulators in the G1-S transition within the cell cycle (13, 14, 15). Cyclin-dependent kinases (CDKs) are serine/threonine kinases which can be divided into a cell-cycle-related subfamily and a transcriptional subfamily (16). CDKs tightly regulate cell division via their essential role in the progression of the cell cycle into the G1, S, G2 and M phases (17). Cell cycle regulators that could be involved in neuroblastoma are CDK4, a member of the cell-cycle related subfamily, and CDK6, a member of both subfamilies (17, 13). CDK4/6 are key components in driving the cell cycle from the G1 phase (prophase of DNA synthesis) into the S phase (DNA synthesis), although they play no dominant role in the rest of the cell cycle (18, 19). Pro-mitosis signaling through receptors, such as human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGFR), can activate multiple pathways such as the protein kinase signaling pathway PI3/AKT/mTOR and the RAS/MAPK pathway to increase expression of cyclin D (17, 20). Amplification or deregulated transcription of the *CCND1* gene, can additionally cause deregulated cyclin D–CDK4/6 activity, since it encodes a cyclin D1 isoform (21). Additionally, when the locus *CDKN2A* is deactivated, there is no expression of $p16^{INK4A}$, which inhibits the kinase activity of cyclin D–CDK4/6 (21). After an increase in expression of cyclin D, CDK4/6 and cyclin D form a complex that regulates phosphorylation of retinoblastoma protein (Rb) (17, 20). Phosphorylated Rb induces dissociation of transcription factor E2F, which in turn binds to DNA to promote gene expression of cell cycle transcription genes and to regulate DNA replication and cell division in the S phase (17, 20). When Rb remains unphosphorylated, cell cycle progression from G1 to S phase is arrested (22). There are multiple endogenous factors that can regulate cell proliferation by inhibiting components in this pathway (17). However, gain-of-function mutations in CDK4/6 or overexpression of CDK4/6 or cyclin D can lead to an uncontrolled cell cycle, possibly leading to the tumorigenesis in multiple cancers such as neuroblastoma (17).

1.4. Small Molecule Inhibitors of CDK4/6

Small molecule inhibitors (SMIs) are therapeutic agents of ≤500 Da that are often orally administered and used for cancer treatment. SMIs are used as targeted therapies to bind specific molecular targets intracellularly, extracellularly or on cell surfaces (17). Multiple SMIs targeting CDKs are developed and approved by the FDA, such as the selective CDK4/6 inhibitors ribociclib, abemaciclib and palbociclib; ATP-competitive inhibitors that form hydrogen bonds with the adenosine triphosphate (ATP)-binding sites of CDK4 and -6, since these sites are almost identical (17). Using CDK4/6 inhibitors, cell cycle progression can be arrested and lead to subsequent tumor growth suppression (Figure 1) (17).

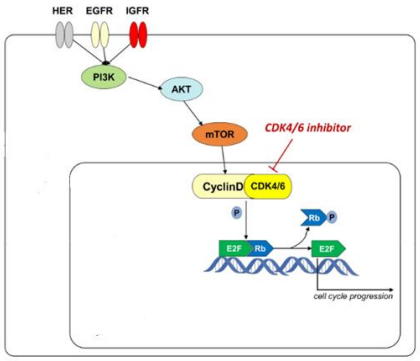

Figure 1: Schematic illustration of signaling processes involved in G1-to-S-phase transition via the cyclin D – CDK4/6 complex *. The protein kinase signaling pathway PI3/AKT/mTOR gets activated by growth factors through HER2, EGFR and IGFR and induces binding of cyclin D to CDK4/6 to form a complex, which will lead to cell cycle progression via the activation of E2F transcription factor. CDK4/6 inhibitors arrest the cell cycle by inhibiting the CDK4/6 kinase function, leaving Rb unphosphorylated and E2F inactivated to induce gene transcription, leading to cell cycle arrest. Adjusted from (17).*

Ribociclib, abemaciclib and palbociclib have different dosing schedules, potencies, toxicities and pharmacokinetics (23). Although the three CDK4/6 inhibitors are widely used, the development of therapeutics with higher selectivity for CDK4/6, less side effects, improved efficacy and to overcome resistance continues (17, 24).

1.5. PROTACs

Proteolysis Targeting Chimeras (PROTACs) are molecular compounds designed to selectively degrade target proteins involved in various cancers, immune disorders, viral infections and neurodegenerative diseases (25, 24). PROTACs are a new field in small molecule therapy, introduced in 2001 by Sakamoto et al. (26). PROTACs are heterobifunctional molecules consisting of a ligand for target protein binding connected via a linker to a ligand, which recruits an E3 ubiquitin (E3) ligase (25). After ternary complex formation the E3 ligase induces ubiquitination of the target protein, which subsequently is degraded by the 26S proteasome; a component of the ubiquitin-proteasome system (UPS) (25, 24). Via a chemical knockdown strategy, PROTACs can regulate protein levels in living cells (Figure 2) (24).

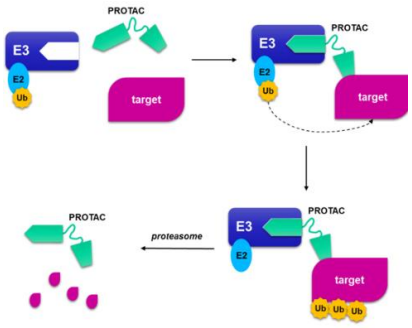

Figure 2: Schematic illustration of PROTACs mode of action*. PROTACs consists of two ligands connected by a linker to bind to a target protein and an E3 ubiquitin ligase. After target protein binding, the E3 ligase induces ubiquitination of the target protein through an E2 enzyme, after which the target protein is fully degraded by the 26S proteasome. After target protein degradation, the PROTAC remains intact and can induce ubiquitination of the next target protein. Adjusted from (17).*

There are numerous advantages of PROTACs compared to SMIs (27). SMIs only inhibit protein function, whereas PROTACs degrade complete target proteins, affecting both the enzymatic as the nonenzymatic functions (24). PROTACs often have an increased selectivity compared to frequently used SMIs and are re-usable after target protein degradation (24). PROTACs possess a catalyzing mode of action for protein degradation and low doses could possibly have a higher efficacy after entering the cell compared to SMIs (25). Treatment with lower doses reduces toxicity and decreases chances of adverse side effects (25). Complete degradation of target proteins prevents the continuous increase of target protein concentration as a resistance mechanism of drug resistant targets (25, 24, 28). The high sensitivity of PROTACs for drug resistant targets can provide a solution for the resistance to chemotherapeutics, which remains the predominant treatment for cancer (24). Currently, phase 1 and 2 clinical trials are conducted for multiple PROTACs (29). Although PROTACs are a promising novel class of therapeutics for human diseases, some limitations need to be resolved (30). Using different linker lengths between a certain target ligand and E3 ligase ligand effects the degradation efficiency, a principle that needs further elucidation (30). Secondly, the selection of the E3 ligase ligand determines the target selectivity and degradation of target proteins. More research should be conducted to determine the suitable E3 ligase ligands per particular disease system (30). Also, the molecular weight of a PROTAC is relatively high, leading to inefficiencies with penetration into the cell (30). Finally, the tissue distribution, toxicity, bioavailability and pharmacokinetics should be further investigated (30).

1.6. PROTACs targeting CDK4/6

Various PROTACs have been designed targeting CDK4/6, resulting in cell cycle arrest and suppression of tumor growth (17, 22). These PROTACs are constructed using palbociclib ribociclib, abemaciclib or palbociclib as the ligand for targeted protein binding, of which the nitrogen atom of piperazine is used to link to an E3 ligase ligand via various linkers (Figure 3) (17). An E3 ligase ligand that has been used is pomalidomide, that binds to E3 ligase cereblon (22, 31). The spatial orientation of the E3 ligase and the target protein and the rigidity and length of the linker can influence the binding affinity of the PROTAC to CDK4/6 (32). Most studies have been conducted on combining pomalidomine and palbociclib with various linkers, although designs with ribociclib and abemaciclib have also been reported (17). The studies indicate that selective degradation of either CDK4 or CDK6 is possible, depending on the type of linker and inhibitor, whilst the individual SMIs do not possess this selectivity and would inhibit both kinases (17).

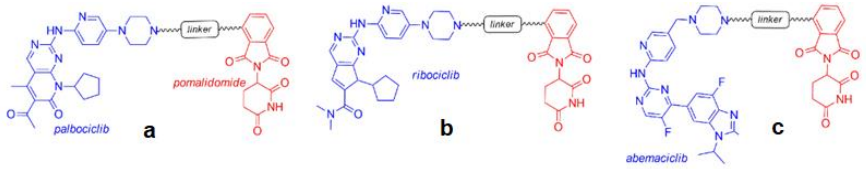

Figure 3: PROTACs targeting CDK4/6, based on different CDK4/6 inhibitors *palbociclib (a), ribociclib (b) and abemaciclib (c) are linked to E3 ligase ligand pomalidomine. Adjusted from (17).*

In this review, literature research has been performed to provide an overview of the current combination therapies using CDK4/6 inhibitors in cancer, particularly focusing on pediatric cancer and neuroblastoma. The aim for the future is to find novel synergistic combinations for neuroblastoma treatment in which CDK4/6 targeting PROTACs are combined with other therapies to improve the clinical outcome. In this overview combination therapies of CDK4/6 inhibitors with various therapeutics, such as inhibitors affecting endocrine systems, PI3K/AKT/mTOR pathway inhibitors, RAS/RAF/MEK/ERK pathway inhibitors, immunotherapeutics, chemotherapeutics and radiotherapeutics, will be discussed in several cancer models. The combinations described are summarized in **Table 1**.

2. Combination therapies with CDK4/6 inhibitors

2.1. CDK4/6 inhibitors combined with endocrine therapy

2.1.1 CDK 4/6 targeting combined with aromatase targeting

A phase 2 study of Finn *et al.* showed that combination therapy using palbociclib with endocrine therapeutic letrozole resulted in a longer progression-free survival compared to letrozole treatment alone (33). Letrozole is an inhibitor of the enzyme aromatase, an enzyme that catalyzes the ultimate step in estrogen biosynthesis (34). Therefore, inhibition of aromatase leads to a decreased estrogen production (35, 34). Palbociclib and letrozole combination therapy exhibited a synergistic effect in women with advanced breast cancer, who are human epidermal growth factor receptor 2 (HER2)–negative and estrogen receptor (ER)-positive (33). The phase 2 study was a double-blind study with progression-free survival as primary end point. Progression-free survival of patients treated with palbociclib and letrozole was 24.8 months (95% confidence interval (CI), compared to 14.5 months 95% (CI) for patients treated with a placebo and letrozole. However, higher myelotoxic effects were observed for patients who received palbociclib and letrozole compared to placebo and letrozole (33).

2.1.2 CDK 4/6 targeting combined with HER2 or ER targeting

A preclinical study of Finn *et al*. examined palbociclib in combination with trastuzumab or tamoxifen in HER2-positive and in ER-positive breast cancer cells, respectively (36). Trastuzumab is a monoclonal antibody that targets HER2, whereas tamoxifen is an antiestrogenic therapeutic that antagonizes the ER (37, 38). Since CDK4/6 function is downstream of ER and HER2, the inhibition of CDK4/6 could be an interesting therapeutic approach (39). *In vitro* sensitivity to palbociclib was measured using 47 human immortalized breast and breast cancer cell lines (36). Measuring dose-response curves, both trastuzumab and tamoxifen showed synergistic effects (with mean CI<1) combined with palbociclib in HER2-positive and ER-positive cells, respectively. Additionally, sensitivity to tamoxifen was enhanced by palbociclib in the tamoxifen resistant cell line MCF7. Conclusively, further clinical development of palbociclib combined with anti-HER2 or anti-estrogen therapy could bring a promising treatment strategy for ER- and HER2-positive breast cancer (36).

2.1.3 CDK 4/6 targeting combined with HER2 targeting

A preclinical study of Witkiewicz *et al*. investigated the effects of palbociclib in combination with the antibody drug conjugate trastuzumab emtansine (T-DM1) (39). T-DM1 consists of humanized antibody trastuzumab, which targets HER2, and is conjugated to DM1, a potent microtubule-poison (40). In cell lines with resistance to HER2 antagonists, cyclin D1 was overactivated and CDK4/6 inhibition showed an effective arrest of cell proliferation by targeting the common pathway (39). Hereafter, the combination of palbociclib and T-DM1 was tested in HER2-positive breast cancer xenografts. Since T-DM1 shows high cytotoxicity, T-DM1 is clinically administered once per 21 days. This could give remaining tumor cells that survived T-DM1 treatment the opportunity to keep proliferating. Simultaneous inhibition of the cell cycle via a CDK4/6 inhibitor might overcome this problem. Witkiewicz *et al*. define mechanisms of action between drugs by immunoblotting and flow-cytometry and a long-term viability assessment. They show that T-DM1 induces suppression of the cell cycle in the S-phase, while palbociclib induces an arrest in the G1 phase. Combination of the two agents showed a hybrid profile of action, although inhibition by palbociclib was dominant compared to the inhibition by T-DM1. Immunoblot analysis showed that T-DM1 suppressed ERK phosphorylation, but it had no significant effect on E2F-regulated genes, indicating that palbociclib and T-DM1 have very different mechanisms of action and can therefore have additive effects on disease control. Cell viability studies showed that addition of palbociclib had no significant effect on the cytotoxicity mediated by T-DM1. Conclusively, this study showed that treatment with palbociclib after initial T-DM1 treatment is successful in preventing tumor outgrowth (39).

2.1.4 CDK 4/6 targeting combined with ER and AKT targeting

In a preclinical study of Alves *et al*., ER-positive breast cancer cells and xenografts were used to examine triple combination therapy, consisting of CDK4/6 inhibitor palbociclib, ER antagonist fulvestrant and pan-AKT kinase inhibitor capivasertib (AZD5363) (41). Ribociclib, abemaciclib and palbociclib are approved by the FDA to combine with endocrine therapeutic fulvestrant (41, 42). When palbociclib, fulvestrant and capivasertib are combined, cell lines show a decrease in tumor cell growth, and in xenografts decreased tumor progression and metastasis is observed (41). When resistance against palbociclib and fulvestrant therapy occurred, a switch to fulvestrant and capivasertib therapy did not prevent further tumor progression. However, when capivasertib was added to the palbociclib and fulvestrant therapy, tumor growth was significantly decreased. This study shows that when CDK4/6 and AKT are targeted, the PI3K/AKT/mTOR and cyclin D/CDK4-6/Rb pathways are efficiently inhibited. Therefore, this study suggests that the triple combination of palbociclib, fulvestrant and capivasertib is a possible treatment for cancers that relapse on therapies that use fulvestrant with or without CDK4/6 inhibitors (41).

2.2. CDK4/6 targeting combined with PI3K/AKT/mTOR pathway inhibitors

2.2.1 CDK4/6 targeting combined with IGF1R targeting

Guenther *et al*. investigated possible targets and therapies to treat the pediatric solid tumor Ewing sarcoma (43). CDK4/6 inhibitors are possible therapeutics for treating Ewing sarcoma since *CDK4* is an Ewing-selective dependency gene. Activation of insulin-like growth factor 1 receptor (IGF1R) in Ewing sarcoma is a mediator for the resistance against CDK4/6 inhibitors. In this study, combination therapy using ribociclib or palbociclib with IGF1R inhibitor AEW541 showed synergic effects in growth suppression and cell death *in vitro* and *in vivo*. In cells treated with the combination therapy, significant suppression of CDK1 and Cyclin B1 was found. Additionally, levels of phosphorylated Rb were significantly reduced compared to cells that were treated with ribociclib alone. In case of exclusive ribociclib treatment, an increase in cyclin D3 level was observed, which is a catalytic partner in CDK4/6 activation. However, combination therapy of ribociclib or palbociclib with AEW541 showed re-suppression of cyclin D3 levels. Next to alternative expression of cell cycle proteins, increased suppression of the PI3K/AKT/mTOR axis was observed. In comparison to treatment with each inhibitor alone, dual inhibitor treatment had a greater effect on suppressing phosphorylation of 4EBP1 and the S6 ribosomal protein, leading to accumulation of the beta subunit of PI3K and the upstream target p70 S6 kinase. When cells were treated with ribociclib alone, an increase in AKT phosphorylation was observed. However, when cells were treated with AEW541 and ribociclib, the AKT activity was inhibited. Conclusively, the study of Guenther *et al*. suggested that the potential escape mechanism to CDK4/6 inhibition can be prevented by combining treatment with an IGF1R inhibitor (43).

2.2.2 CDK4/6 targeting combined with TEK, CDK1/2/9, mTOR-targeting or demethylation agent

A study of Gopalan *et al*. examined the effect of palbociclib combined with four inhibitors, individually, on tumor cell death, targeting different cellular pathways using non-small cell lung cancer (NSCLC) cell lines (44). Using p16-deficient NSCLS cell lines, MTS viability assays were conducted after treatment with palbociclib combined with and without inhibitors selumetinib (AZD6244), AZD5438, everolimus or decitabine. NSCLC cell lines showed a decrease in phosphorylated Rb after treatment with palbociclib compared to cells without treatment. Cell viability was decreased with 34% using palbociclib only. Selumetinib is a TEK inhibitor, which was administered to test a possible synthetic lethal interaction of CDK4 and K-Ras. MTS viability assays for palbociclib with selumetinib showed 72.0% viability, indicating that this combination did not have a synergistic effect. AZD5438, an CDK1/2/9 inhibitor, was chosen since CDK1 and CDK2 are hypothesized to play a role in resistance to

CDK4/6 inhibitors. MTS viability assays for palbociclib with AZD5438 showed 96.6% viability, which did not confirm the hypothetic role of CDK1 and CDK2. Everolimus is an mTOR inhibitor, which could lead to an arrested upregulation of cyclin D1 and thereby suppress complex formation with CDK4/6. MTS viability assays for palbociclib with everolimus showed 46.4%, viability, with an $p<0.02$ for exclusive palbociclib treatment compared to combined treatment. In NSCLC, CDKN2A is often inactivated through hypermethylation. Therefore, decitabine, a demethylating agent, could indirectly activate CDKN2A, which will lead to an increased expression of the endogenous CDK4/6 inhibitor p16. MTS viability assays for palbociclib with decitabine showed 80.2% viability. Based on these results, inhibitor everolimus shows to be a promising agent to be used in combination with palbociclib for NSCLC treatment (44).

2.2.3 CDK4/6 targeting combined with PI3K/mTOR or MEK1/2 targeting

A study of Franco *et al.* indicated that the combination therapy of palbociclib with PI3K/mTOR inhibitor dactolisib (BEZ235), mTOR inhibitor AZD0855 or PI3K inhibitor apitolisib (GDC0980) has an additive effect in arresting the cell cycle in pancreatic ductal adenocarcinoma (45). Since cyclin D and CDK4/6 activity is regulated by, among others, the PI3K/AKT/mTOR pathway, targeting a component of this pathway may result in increased cell cycle arrest. Using human pancreatic cancer cell line PL5, *Franco et al*. showed that there was resistance to individual treatment with palbociclib or PI3K/mTOR inhibitors. However, combining the two therapies resulted in an absolute arrest of the cell cycle. Decreased expression of cell cycle regulatory proteins cyclin D1, cyclin E1, CDK2 and cyclin A was measured. In treatment with palbociclib exclusively, phosphorylation of Rb was largely suppressed, however residual phosphorylation could be found. In combined treatment with one of the PI3K/mTOR inhibitors, the residual phosphorylation was suppressed, leading to complete cell cycle arrest. Additionally, the effect of mitogen-activated protein kinase kinases 1/2 (MEK1/2) inhibitor AZD8330 combined with palbociclib was measured, showing significant difference in cell cycle arrest between monotherapy and combination therapy. Therefore, it can be suggested that combining CDK4/6 inhibitors with PI3K/mTOR or MEK1/2 inhibitors has an synergisticc effect compared to single compound treatment (45).

2.2.4 CDK4/6 targeting combined with MDM2 targeting

Schubert *et al.* performed a preclinical study which combined abemaciclib and mouse double minute 2 homolog (MDM2) inhibitor idasanutlin as a possible combination treatment for neuroblastoma (46). MDM2 is an E3 ubiquitin-protein ligase, which is overexpressed in 53% of neuroblastoma patients. MDM2 blocks the transcriptional activity of p53 and thereby its pro-apoptotic function. Therefore, overexpression of MDM2 can lead to uncontrolled cell division and tumor growth. Schubert *et al*. examined combination therapy using abemaciclib and idasanutlin looking at cell viability in 10 different cell lines, concluding that there was no synergic effect. In fact, abemaciclib reduced the pro-apoptotic effect of idasanutlin, indicating an antagonistic relationship. It was concluded that the combination of abemaciclib and idasanutlin did not lead to the desired synergistic effects (46).

2.2.5 CDK4/6 targeting combined with ALK targeting

In familiar pediatric neuroblastoma, the predominant mutated oncogene is anaplastic lymphoma kinase (ALK) (11). Wood *et al*. showed that combining ribociclib and ALK inhibitor ceritinib resulted in a synergistic effect on cell cycle arrest, growth inhibition and caspase-independent cell death. In neuroblastoma xenografts possessing *ALK*-F1245C and *ALK*-F1174L *de novo* resistance mutations, the combination therapy overcame ceritinib resistance and resulted in complete regression. When compared to single-agent ceritinib, the combination of ceritinib and ribociclib improved pALK inhibition in the NB-1643 (ALK R1275Q) cell line, and a comparable but less strong effect was shown in the NB-EBc1 and SH-SY5Y (ALK F1174L) cell lines. In a NB-1691 cell line with wild type ALK and amplified

CDK4, an synergistic effect was also measured. Additionally, ribociclib inhibited phosphorylation of Rb in all cell lines, whereas ceritinib alone had no effect on the phosphorylation of Rb in the NB-1691 cell line. However, in the NB-1643 cell line ceritinib decreased the Rb level and thereby phosphorylated Rb. When ceritinib was combined with ribociclib, a reduction of phosphorylated Rb levels occurred at lower doses. Ceritinib monotherapy resulted in a minor G1 arrest in the NB-1643 and SH-SY5Y cell lines, indicating that CDK4 and CDK6 are downstream of active ALK. Exclusive ribociclib treatment generated a more significant dose-dependent cell cycle arrest at the G1 phase, with concomitant reductions in the fraction of cells in the S, G2 and M phases. In all cell lines, combination therapy induced a larger accumulation in the G1 phase than either drug alone, suggesting that cytostasis may contribute to its *in vitro* synergy and efficacy. The plasma concentrations of ribociclib and ceritinib were not affected when administered as combination therapy. In conclusion, the results of Wood *et al*. show that combination therapy of ribociclib and ceritinib provides a synergistic effect on arresting tumor progression and, therefore, could serve as a possible therapy for neuroblastoma in the clinic (11).

2.3. CDK4/6 targeting combined RAS/RAF/MEK/ERK pathway inhibitors

2.3.1 CDK4/6 targeting combined with MEK1/2 targeting

In a study of Hart *et al*., combination therapy was studied for patients with relapsed high risk neuroblastoma, who frequently show mutations that lead to an hyperactivated RAS-MAPK pathway and an increased sensitivity to MEK inhibition therapy (47). In this study, 22 genetically annotated human neuroblastoma cell lines were used to study the effect of combination therapy using ribociclib and binimetinib, a MEK1/2 inhibitor. The study showed that ribociclib and binimetinib have an inversely related sensitivity (r=-0.58, p=-0.009). In sporadic neuroblastoma, about 22% of the tumors possess amplification of *MYCN*, often predicting a bad prognosis (14). Expression and amplification of *MYCN* were related to binimetinib resistance and ribociclib sensitivity, whereas increased MAPK signaling was related to ribociclib resistance and binimetinib sensitivity. Administration of the combination therapy led to synergistic inhibition of cellular growth in all cell lines and tumor growth inhibition in multiple murine xenograft models. Upon termination of the treatment the cell cycle arrest showed to be reversible. The study of Hart *et al*. indicated that the combination of binimetinib and ribociclib can provide an effective therapy in relapsed high-risk neuroblastoma (47).

2.3.2 CDK4/6 targeting combined with B-RAF targeting

In a preclinical study of Yadav *et al*., a combination therapy using abemaciclib and vemurafenib, a V600E B-RAF inhibitor, was tested as a therapeutic strategy for melanoma patients presenting a V600E *B-RAF* mutation (48). RAF is an oncogenic serine-threonine protein kinase that plays a role in the MAPK pathway, which regulates cell growth (49). As a result of the activating V600E *B-RAF* mutation, patients have an overactivation of the MAPK pathway. When the MAPK pathway is malfunctioning, uncontrollable growth arises which may lead to cancer (49). Long term treatment with vemurafenib resulted in resistance after 5-7 months due to MAPK signaling reactivation, leading to an upregulation of cyclin D1 (48). In 38% of melanoma patients, CDKN2A deletions result in insufficient expression of $P16^{INK4a}$, which is a tumor suppressor gene and a natural inhibitor of CDK4, leading to acceleration of the cell cycle. Administration of abemaciclib and vemurafenib showed additive tumor growth inhibition *in vitro* and *in vivo*, showing that combined therapy is effective to overcome resistance (48).

2.4. CDK4/6 targeting combined with immunotherapy

2.4.1 CDK4/6 targeting combined with PD-1 targeting

CDK4/6 inhibitors are capable of inducing an anti-tumor immune response (23). There are multiple possible responses, such as activation of effector T lymphocytes, increased antigen presentation of tumor cells, and an decreased proliferation of immunosuppressive regulatory T cells (50)(51). Rugo *et al*. conducted a phase Ib study for HR-positive and HER2-negative breast cancer combining abemaciclib with the humanized antibody pembrolizumab, which is used for cancer immunotherapy in multiple cancers (52). Pembrolizumab targets programmed cell death protein 1 (PD-1), an inhibitory T-cell surface receptor (53). Activation of PD-1 by its ligands leads to the suppression of T-cell activation (53). When pembrolizumab is administered PD-1 is blocked, meaning that T cell activation cannot be suppressed (53). Rugo *et al*. previously showed that in murine models for HR-positive and HER2-negative breast cancer, abemaciclib and pembrolizumab had a synergistic effect on tumor immunogenicity and antitumor efficacy (52). Hereafter, a phase Ib study that was non-randomized, multicenter, multi-cohort, and open-label was conducted using breast cancer patients to characterize the safety. Prior to the administration of abemaciclib and/or pembrolizumab, patients had received one or two chemotherapy regimens. The study showed that there was a tolerable safety profile when combining the therapeutics. There was a higher rate of transaminase elevations measured for the combined therapy than for the individual therapies. Additionally, slightly higher overall survival, progression-free survival and objective response rates were measured in patients that received combined therapy compared to therapy with abemaciclib exclusively. This suggest that immunotherapy targeting PD-1 can be combined with CDK4/6 inhibitors to improve treatment of breast cancer (52).

2.5. CDK4/6 targeting combined with chemotherapy

2.5.1 CDK4/6 targeting combined with 5-FU or gemcitabine

Franco *et al*. showed in a preclinical study that chemotherapeutic gemcitabine was antagonized when combined with palbociclib in pancreatic ductal adenocarcinoma cells (45). Gemcitabine is a cytotoxic drug approved as treatment for multiple cancers. Treatment with gemcitabine and palbociclib resulted in a reduced cell death compared to treatment with gemcitabine alone. However, when cells were treated with chemotherapeutic 5-fluururacil (5-FU) and palbociclib, no antagonization was detected and in some cell lines cooperative effects were measured. This variation in response could be due to the function of the E2F target genes thymidylate synthase (TS) and deoxycytidine kinase (DCK). When cells are treated with palbociclib, cells obtain lower TS levels. TS is the target of 5-FU, so lower levels of TS lead to increased drug sensitivity. Palbociclib treatment also leads to decreased DCK levels. However, since DCK is required for gemcitabine activation, the antagonistic relationship was observed. Conclusively, the mechanism of action of chemotherapeutics determines whether it is possible to combine it with CDK4/6 inhibitors. Chemotherapeutics that are dependent of cell cycle progression will be antagonized by CDK4/6 inhibitors. Besides chemotherapeutics, this was also indicated for palbociclib combined with anti-mitotic drugs such as PLK1 inhibitors (45).

In another preclinical study, Gelbert *et al*. used gemcitabine combined with abemaciclib as a treatment to inhibit tumor growth (54). In calu-6 lung xenografts, both therapeutics showed tumor growth inhibition when administered separately, however subsequential or combined administration showed a more significant antitumor activity. When abemaciclib was administered to the calu-6 lung xenograft, no inhibition of Rb phosphorylation or cell cycle arrest was detected. However, when abemaciclib was combined with gemcitabine, the expression of Rb/E2F-regulated protein ribonucleotide reductase catalytic subunit M1 (RRM1) was significantly inhibited. RRM1 is a subunit of an enzyme that coverts

ribonucleotides into deoxyribonucleotides, and is a molecular target of gemcitabine (54, 55). RRM1 expression is additionally known to be highly sensitive to phosphorylated Rb regulation (56). Chemosensitivity to gemcitabine, both *in vitro* and *in vivo*, is correlated with RRM1 expression. The study of Gebert *et al.* showed consistent results on the inhibition of RRM1 expression when gemcitabine and abemaciclib are combined (54). Conclusively, Gelbert *et al*. showed that in calu-6 lung xenograft tumors, combined abemaciclib and gemcitabine have an additive antitumor activity and an additive suppression of RRM1, while in absence of cell cycle arrest (54).

2.5.3 CDK4/6 targeting combined with doxorubicin

A preclinical study of Gogolin *et al*. used CDK4 inhibitor RO050124 and chemotherapeutic doxorubicin (doxo) as possible combination therapy for *MYCN*-amplified neuroblastoma (57). Genotoxic chemotherapies such as doxo are often used for the treatment of multiple cancers such as leukemia, lymphoma, carcinoma and neuroblastoma (58, 59, 60). In *MYCN*-amplified neuroblastoma, drug resistance leading to relapse is the predominant cause of death (57). Patients have increased levels of CDK4 as a result of MYCN overexpression, which leads to an increased competition between CDK2 and CDK4 for p21 binding. CDK2 is an important cell cycle regulator for the G1/S and S/G2 transition, and its function can be blocked through p21 binding (61, 62). When doxo is used to induce DNA damage, there is insufficient p21 to inhibit CDK2, resulting in increased CDK4 and CDK2 kinase activity and thereby an increase in cell proliferation. By using CDK4 inhibitor RO050124, arrest at the G1-S cell cycle checkpoint is partly restored, and cell viability is reduced. Twelve neuroblastoma cell lines were treated with doxo and/or RO050124 (57). The combination treatment resulted in increased G_0 phase fractions or S phase fractions for nine and six cell lines, respectively. Compared to cell lines exclusively treated with doxo, G_2/M fraction was reduced in cell lines treated with doxo and RO050124. Combination treatment had an additive effect on cell viability reduction in cell lines that had additional aberrations in the p53 pathway, while doxo treatment alone resulted in little cell death. Gogolin *et al.* indicated that combination treatment resulted in a G_1-S arrest and not in cell death. Conclusively, after chemotherapy-induced DNA damage, the CDK4/cyclin D-phosphorylated Rb axis regulates cell cycle progression as an additive control next to the p53-p21 axis. CDK4 inhibition combined with chemotherapy therefore could be a future therapeutic for *MYCN*-amplified neuroblastoma (57).

A preclinical study of McClendon *et al.* combined doxo with palbociclib as a possible treatment for triple negative breast cancer (TNBC) (58). A predominant determinant is the Rb tumor suppressor pathway in disease progression of TNBC. This study showed that doxo treatment combined with palbociclib exerted an additive cytotoxic effect on Rb-proficient cell lines in the short term. However, in the long term palbociclib antagonized doxo-mediated cytotoxicity. In Rb-deficient cells, palbociclib did not affect the therapeutic response of doxo. This indicated that the efficacy of chemotherapeutics and CDK4/6 inhibitors combined is dependent on the Rb pathway in TNBC (58).

2.5.4 CDK4/6 targeting combined with carboplatin

Carboplatin is a chemotherapeutic agent that can be used as therapy for multiple cancers such as, germ cell tumours, gynaecological cancers and thoracic cancers (63). A study of Roberts *et al*. studied carboplatin-induced myelosuppression in bone marrow in FVB/N wild-type mice after treatment with palbociclib, measuring complete blood cell counts (64). Hereafter, murine models with retinoblastoma-competent and -incompetent breast cancer were used to determine the antitumor activity of palbociclib alone or in combination with chemotherapy using carboplatin. In FVB/N wild-type mice, there was a significant increase of hematocrit, platelet counts, myeloid cells and lymphocytes in mice treated with palbociclib and carboplatin combined, compared to mice that were treated with carboplatin exclusively (64). Murine models with retinoblastoma-competent breast cancer treated with palbociclib alone showed antitumor activity. However, combined therapy with

palbociclib and carboplatin showed a decrease in antitumor activity in retinoblastoma-incompetent models compared to mice exclusively treated with carboplatin. On the contrary, murine models with retinoblastoma-incompetent breast cancer were resistant to palbociclib and combined therapy of palbociclib and carboplatin showed no effect on tumor growth *in vivo,* compared to treatment with carboplatin treatment exclusively. The study of Roberts *et al*. concludes that in cancers that are CDK4/6-dependent, selective CDK4/6 inhibition may reduce the efficacy of chemotherapeutic medicines, of which the mechanism is dependent on cell cycle activity for proliferation. Therefore, not all tumors can be treated with chemotherapy and CDK4/6 inhibitors as a combination (64).

2.6. CDK4/6 targeting combined with radiotherapy

2.6.1 CDK4/6 targeting combined with radiotherapy

Radioresistance is a common cause of radiotherapy failure and a characteristic of cancer stem cells (CSCs) (65). Shimura *et al.* used 82FR-31NR cells; isolated CSCs from human liver cells (HepG2 cells) and human glioblastoma (A172 cells), which show CSC properties and a strong tumorigenic potential, to analyze DNA damage response (65). After radiation-induced DNA damage, 82FR-31NR cells had efficient DNA repair and radioresistance due to activation of the AKT/cyclin D1 survival signaling pathway. In parental cells, radiation-induced DNA damage was persistent and did not activate the AKT/cyclin D1 pathway, which led to apoptosis. Inhibition of CDK4 was shown to be sufficient to overcome radioresistance in CSCs. Cdk4-I (CAS 546102-60-7) is a CDK4 inhibitor that suppresses the activity of the cyclin D1/CDK4 complex. Incidence of cell apoptosis was increased by combining Cdk4-I and radiation in 82FR-31NR cells. Therefore, Cyclin D1, CKD4 and AKT could potentially be used as drug targets to be combined with radiotherapy to overcome radioresistance in CSCs (65).

2.6.2 CDK4/6 targeting with radiotherapy

In a preclinical study of Hagen *et al.* radiotherapy was combined with palbociclib using breast cancer cells (66). During treatment of breast cancer, radioresistance often occurs, which is possibly due to malfunctioning of regulators within the G1 phase machinery. By inhibiting CDK4, phosphorylation of Bad Ser136 is decreased, which is a dominant factor in apoptosis and radioresistance. Treatment with palbociclib increased the sensitivity towards radiation-induced apoptosis, without significant alterations to DNA repair or cell cycle progression. Therefore, it is suggested that certain doses of CDK4/6 inhibitors that do not inhibit the cell cycle progression can be used combined with radiotherapy (66).

Table 1: Clinical and preclinical combination therapies with CDK4/6 SMIs in several cancer types.

CDK4/6 inhibitor	Combined with	Targets	Disease	Phase	Result	Date of publication
Palbociclib	Letrozole	Aromatase	Breast Cancer	Phase 2	Positive	2016-11-17 (33)
Palbociclib	Trastuzumab	HER2	Breast Cancer	Preclinical (*in vitro*)	Positive	2009-11-29 (36)
Palbociclib	Tamoxifen	ER	Breast Cancer	Preclinical (*in vitro*)	Positive	2009-11-29 (36)
Palbociclib	T-DM1	HER2	Breast Cancer	Preclinical (*in vivo*)	Positive	2014-07 (39)
Palbociclib	Fulvestrant, capivasertib	ER, AKT	Breast Cancer	Preclinical (*in vitro and in vivo*)	Positive	2021-08-25 (41)
Ribociclib or Palbociclib	AEW541	IGF1R	Ewing Sarcoma	Preclinical *(in vitro* and *in vivo)*	Positive	2019-02-15 (43)
Palbociclib	Selumetinib (AZD 6244)	MEK1/2	Non-Small Cell Lung Cancer	Preclinical (*in vitro)*	Negative	2013-04-10 (44)
Palbociclib	AZD 5438	CDK1/2/9	Non-Small Cell Lung Cancer	Preclinical (*in vitro)*	Negative	2013-04-10 (44)
Palbociclib	Everolimus	mTOR	Non-Small Cell Lung Cancer	Preclinical (*in vitro)*	Positive	2013-04-10 (44)
Palbociclib	Decitabine	DNA	Non-Small Cell Lung Cancer	Preclinical (*in vitro)*	Negative	2013-04-10 (44)
Abemaciclib	Idasanutilin	MDM2	Neuroblastoma	Preclinical (*in vitro*)	Negative	2020-11-12 (46)
Ribociclib	Certinib	ALK	Neuroblastoma	Preclinical (*in vivo)* Phase I	Positive Unknown	2016-12-16 (11) Started 2016-07
Palbociclib	Dactociclib	mTOR, PI3K	Pancreatic Ductal Adenocarcinoma	Preclinical (*in vitro*)	Positive	2014-07-26 (45)
Palbociclib	AZD0855	mTOR	Pancreatic Ductal Adenocarcinoma	Preclinical (*in vitro*)	Positive	2014-07-26 (45)
Palbociclib	Apitolisib	PI3K	Pancreatic Ductal Adenocarcinoma	Preclinical (*in vitro*)	Positive	2014-07-26 (45)
Palbociclib	AZD8330	MEK1/2	Pancreatic Ductal Adenocarcinoma	Preclinical (*in vitro*)	Positive	2014-07-26 (45)
Ribociclib	Binimetinib	MEK1/2	Neuroblastoma	Preclinical (*in vitro*) Phase Ib/II	Positive Positive	2016-10-11 (47) Started 2013-06
Abemaciclib	Vemurafenib	B-RAF	Melanoma	Preclinical (*in vitro* and *in vivo*)	Positive	2014-08-13 (48)
Abemaciclib	Pembrolizumab	PD-1	Breast Cancer	Phase Ib	Positive	2020-05-20 (52)
Palbociclib	Gemcitabine	DNA	Pancreatic Ductal Adenocarcinoma	Preclinical (*in vitro*)	Negative	2014-07-26 (45)
Palbociclib	5-FU	DNA	Pancreatic Ductal Adenocarcinoma	Preclinical (*in vitro*)	Positive	2014-07-26 (45)

Table 1: (continued).

CDK4/6 inhibitor	Combined with	Targets	Disease	Phase	Result	Date of publication
Abemaciclib	Gemcitabine	DNA	Lung cancer	Preclinical (*in vivo*)	Positive	2014-06-13 (54)
RO050124	Doxorubicin	DNA	Neuroblastoma	Preclinical (*in vitro*)	Positive	2013-04-01 (57)
Palbociclib	Doxorubicin	DNA	Triple-Negative Breast Cancer (Rb-proficient)	Preclinical (*in vitro*)	Negative	2012-07-15 (58)
Palbociclib	Doxorubicin	DNA	Triple-Negative Breast Cancer (Rb-deficient)	Preclinical (*in vitro*)	Neutral	2012-07-15 (58)
Palbociclib	Carboplatin	DNA	Breast Cancer (Rb-proficient)	Preclinical (*in vivo*)	Negative	2012-02-01 (64)
Palbociclib	Carboplatin	DNA	Breast cancer (Rb-deficient)	Preclinical	Neutral	2012-02-01 (64)
Cdk4-I by Calbiochem	Radiotherapy	DNA	Cancer Stem Cells	Preclinical (*in vitro*)	Positive	2012-07-04 (65)
Palbociclib	Radiotherapy	DNA	Breast Cancer	Preclinical (*in vitro*)	Positive	2013-06-25 (66)

3. Discussion

A lot of research has been conducted combining CDK4/6 targeting with other therapies to improve anti-tumor activity in various types of cancer. In order to combine PROTACs with another therapeutic agent as a future treatment strategy for pediatric neuroblastoma patients, various considerations should be made. Fox example, since a substantial number of pediatric neuroblastoma patients achieve long-term survival, radiotherapy is not frequently used, since it induces severe cellular damage (1, 67). This damage can lead to late adverse effects, such as growth abnormalities or diabetes mellitus (67). Secondly, altered protein expression is often the cause of pediatric cancer, whereas adult is often caused by mutated proteins, meaning that pediatric patients only express a small number of tumor-specific targets. In this review, combination therapies have been described that target different pathways and/or systems, besides CDK4/6. Some of the described combinations are more likely to affect tumor progression in neuroblastoma than others as a result of the genomic background of neuroblastoma. Therefore, some might be worthwhile investigating in contrast to others. As previously mentioned, recurrent somatic mutations in genes like *ATRX*, *ALK* and *PTPN11* are frequently found in high risk neuroblastoma patients, of which oncogene *ALK* is most frequently mutated (10, 11). Amplification of the oncogene *MYCN* is found in approximately 20% of neuroblastoma patients, and is strongly correlated to high risk-disease and poor clinical outcome (10, 6). Additionally, there are multiple growth factors, transcription factors and kinases known that play an important role in neuroblastoma.

Most research on endocrine therapies combined with CDK4/6 inhibitors has been conducted focusing on breast cancer (33, 36, 39, 41). Considering that most of these therapies target HER2 or ER, which are not known to play a predominant role in pediatric neuroblastoma, it is not likely that one of the reported endocrine therapy combinations could have an additive effect for neuroblastoma treatment.

A strategy for anti-tumor therapy is targeting CDK4/6 together with the PI3K/AKT/mTOR pathway. Cell survival kinases that play a role in the cell survival pathways of multiple cancers including neuroblastoma are PI3K, FAK, AKT and ALK (68). AKT phosphorylation is more abundant in neuroblastoma cells compared to normal tissue, which correlates with an advanced stage of disease and amplification of the oncogene *MYCN* (45, 47). Cells with upregulated PI3K/AKT show less apoptosis compared to cells with normal PI3K/AKT levels (45). As previously described, the PI3K/AKT/mTOR pathway activates cyclin D to form a complex with CDK4/6 (Figure 1) (45). Targeting components of this pathway in combination with CDK4/6 inhibitors can lead to an enhanced effect, as is shown in a study described in *2.2.3,* where PI3K/AKT/mTOR inhibitors dactociclib, AZD0855 and apitolisib were used in combination with palbociblib for pancreatic ductal adenocarcinoma (45). Targeting this pathway in neuroblastoma could possibly enhance the effect of CDK4/6 inhibitors, as it will suppress the same pathway and thereby encounter cells that could escape CDK4/6 inhibitors (45).

Another interesting strategy is to target CDK4/6 together with *MYCN*, the dominant oncogene in neuroblastoma (69). There are no clinically approved inhibitors of MYCN, so only indirect targeting of MYCN is possible (69). MYCN is an unstable protein, of which its stability is regulated by multiple pathways. One of the main components that regulates MYCN stability is PI3K. PI3K can activate AKT, which can phosphorylate GSK3ß and thereby suppress the kinase activity of GSK3ß. Subsequentially, phosphorylation of MYCN-T58 is decreased, which is essential for targeted proteasome degradation. When PI3K is inhibited, MYCN is destabilized and tumor growth is suppressed (69). Additionally, activation of AKT by PI3K promotes cell growth and survival and is reported as a strong prognostic indicator of a lethal outcome in neuroblastoma patients (70). As described in *2.2.3,* inhibition of PI3K combined with CDK4/6 inhibitors showed additive anti-tumor effects (45). This combination can be a very promising therapeutic strategy in neuroblastoma since the dominant oncogene *MYCN* is targeted indirectly, together with CDK4/6.

Targeting the RAS/RAF/MEK/ERK pathway may also lead to an additive effect when combined with CDK4/6 inhibitors for neuroblastoma. As described in *2.3.1*, B-RAF targeting combined with CDK4/6 inibitors can lead to an additive effect. Somatic mutations in *B-RAF*, although rarely, have been found in neuroblastoma, indicating that combined CDK4/6 and B-RAF targeting could be an interesting strategy (12). Additionally, it is known that when extracellular signal-related kinase 1 (ERK1) is inhibited, tumor growth is arrested in neuroblastoma (71). ERK1 activity can be suppressed by using a MEK1/2 inhibitor (71). Studies described in *2.2.3* and *2.3.1,* combined CDK4/6 inhibitors with MEK1/2 inhibitors AZD8330 or binimetinib for pancreatic ductal adenocarcinoma and neuroblastoma, respectively (45)(47). Both studies have shown that the combination has a synergistic effect in their respective cancer models. A phase Ib/II clinical trial (n=102) has been started in June 2013, using oral ribociclib and binimetinib to treat *NRAS*-mutant melanoma patients (NCT01781572) (72). The maximum tolerated dose was the primary objective of phase Ib, and the evaluation of pharmacodynamics, efficacy and safety were secondary objectives. Results showed that the maximum tolerated dose was 600 mg ribociclib and 45 mg binimetinib for a 21 day cycle and 200 mg ribociclib and 45 mg binimetinib for a 28 day cycle (72). At these doses, treatment had a favorable efficacy and manageable safety profile for *NRAS*-mutant melanoma patients. Since the phase Ib/II clinical trial for *NRAS*-mutant melanoma patients showed promising results, binimetinib with ribociclib could be an effective combination therapy for neuroblastoma patients.

An overarching strategy to affect the multiple pathways mentioned above, is to target anaplastic lymphoma kinase (ALK). ALK regulates the balance between cell differentiation and cell proliferation (14). Signaling of ALK in neuroblastoma goes through the RAS/RAF/MEK/ERK, PI3K/AKT/mTOR and JAK/STAT3 pathways (Figure 6), meaning that targeting ALK will suppress multiple pathways that are

known to play a role in tumorigenesis (9). ALK is expressed in tissues of the developing peripheral and central nervous system, whereas expression on other tissues is limited (9). Overexpression of ALK is often found in neuroblastoma and *ALK* amplification is highly associated with *MYCN* co-amplification (9). Germline mutations in ALK can lead to familial neuroblastomas (<2%), but mutations in ALK also occur in 10-15% of the sporadic neuroblastomas (5)(14). In neuroblastoma, *PHOX2B* was the first gene identified with a predisposition mutation (14). PHOX2B can directly regulate *ALK* gene expression, meaning that mutations in *PHOX2B* can additionally lead to disruption in the same pathways (14). In sporadic neuroblastoma, mutations in *PHOX2B* are relatively rare (14). However, somatic ALK activating mutations (6-10%) or a high level of gene amplifications (3-4%) are found frequently, indicating that ALK is a dominant oncogenic driver in neuroblastoma (14). Ceritinib is a second-generation ALK inhibitor that is also a potent IGFR1 inhibitor, although less strongly (9). The study of Wood *et al*., as described in *2.2.5*, showed that combining ribociclib and ceritinib resulted in an increased cytotoxicity in neuroblastoma cells with mutant ALK compared to wild-type cells (11). In ALK F1245Q and F1174L-mutant cell lines and patient-derived xenografts, complete tumor regression was observed when therapies were combined compared to delayed tumor growth as a result of single drug treatment (11). In July 2016, a phase I clinical trial (n=131) was started for relapsed or refractory neuroblastoma patients, combining ribociclib and ceritinib (NCT02780128) (9). This clinical trial is estimated to be completed in June 2024, no data has been published yet. Based on this information, a CDK4/6 inhibitor combined with an ALK inhibitor, such as ribociclib and ceritinib, could be a promising therapy for neuroblastoma, since multiple pathways are suppressed that contribute to an uncontrolled cell proliferation (9, 11).

Another type of therapy that can be combined with CDK4/6 inhibitors is immunotherapy, which has no direct relation to the previously described PI3K/AKT/mTOR or RAS/RAF/MEK/ERK pathways. Immunotherapy targeting PD-1 with humanized antibody pembrolizumab and CDK4/6 targeting with abemaciclib has been studied by Rugo *et al*. as described in *2.4.1* (52). Combining these inhibitors showed promising results in a phase I clinical trial with breast cancer patients (52). Additionally, research has been conducted into PD-1 targeting in pediatric brain and solid tumors (73). When PD-1 binds to programmed death-ligand 1 (PD-L1), it suppresses T-cell activation. There is growing evidence for PD-L1 expression in pediatric neuroblastoma, possibly associated with a lower survival rate (73). A phase I/II clinical trial (NCT02304458) is currently in progress, where PD-1 inhibitor nivolumab is used in 10 neuroblastoma patients (73). No results are reported yet, as the clinical trial is estimated to be completed in October 2022. Considering that PD-1 targeting with pembrolizumab shows positive results in a phase Ib clinical trials for breast cancer (52), and a phase I/II clinical trial is currently ongoing using nivolumab for neuroblastoma (73), immunotherapy targeting PD-1 can be a promising combination therapy with CDK4/6 inhibitors as parallel treatment for pediatric neuroblastoma.

Additionally, using chemotherapy together with CDK4/6 inhibitors could be an efficient therapeutic strategy for neuroblastoma. Various chemotherapeutics, such as doxo, can be used for neuroblastoma treatment (57). In a study of Gogolin *et al.,* described in *2.5.3*, CDK4/6 inhibitor RO050124 was combined with doxo using neuroblastoma cell lines (57). An additive effect on cell viability reduction was observed when the therapies were combined compared to single drug treatment (57). Although RO050124 is not an approved agent for neuroblastoma, doxo combined with another approved CDK4/6 inhibitor may be promising. This is supported by other studies describing the combination of doxo with CDK4/6 inhibitor palbociclib showing synergy (58). Other described chemotherapeutic agents combined with CDK4/6 inhibitors, such as 5-FU and gemcitabine, are not used for neuroblastoma treatment (45). However, combining chemotherapy with CDK4/6 inhibitors could be a very efficient and promising combination therapy for neuroblastoma (58).

4. Conclusion

Looking at therapeutic combinations with CDK4/6 inhibitors described in this review, multiple options seem highly promising as a strategy for neuroblastoma therapy. A possibility is to use an agent that is in line with the cell cycle pathways. In this case, ALK targeting seems highly promising, since it is at the top of multiple important signaling pathways, leading to an suppressed tumor growth. On top of that, ALK inhibition will suppress MYCN expression, one of the most dominant oncogenes of neuroblastoma. Supported by the fact that there is an ongoing clinical trial (NCT02780128) combining ALK and CDK4/6 inhibition in neuroblastoma patients, targeting ALK combined with a CDK4/6 targeting PROTAC could be very promising. Another promising strategy would be to target PI3K, which indirectly targets MYCN by suppressing its stability. Combining a CDK4/6 targeting PROTAC with PI3K targeting therefore also seems highly effective, since the suppression of the main oncogenic driver MYCN will highly affect the prognosis. Thus, these findings provide an overview of highly promising combinations that can be made with CDK4/6 targeting PROTACs, to improve the treatment and prognosis for pediatric neuroblastoma patients.

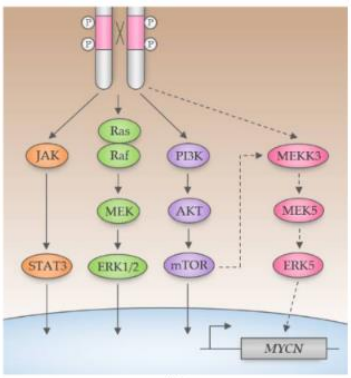

Figure 3: ***Signaling pathways activated by ALK.*** *ALK signals though the JAK/STAT3, RAS/RAF/MEK/ERK, PI3k/AKT/mTOR - pathways. Oncogene MYCN is also activated through ALK signaling. Adjusted from (9).*

References

1. Kattner P, Strobel H, Khoshnevis N, Grunert M, Bartholomae S, Pruss M, et al. Compare and contrast: pediatric cancer versus adult malignancies. Cancer Metastasis Rev. 2019 Dec;38(4):673–82.

2. Aunan JR, Cho WC, Søreide K. The Biology of Aging and Cancer: A Brief Overview of Shared and Divergent Molecular Hallmarks. Aging Dis. 2017;8(5):628.

3. ICGC PedBrain-Seq Project, ICGC MMML-Seq Project, Gröbner SN, Worst BC, Weischenfeldt J, Buchhalter I, et al. The landscape of genomic alterations across childhood cancers. Nature. 2018 Mar 15;555(7696):321–7.

4. Ishola TA, Chung DH. Neuroblastoma. Surg Oncol. 2007 Nov;16(3):149–56.

5. Jones DTW, Banito A, Grünewald TGP, Haber M, Jäger N, Kool M, et al. Molecular characteristics and therapeutic vulnerabilities across paediatric solid tumours. Nat Rev Cancer. 2019 Aug;19(8):420–38.

6. Huang M, Weiss WA. Neuroblastoma and MYCN. Cold Spring Harb Perspect Med. 2013 Oct 1;3(10):a014415.

7. Kildisiute G, Kholosy WM, Young MD, Roberts K, Elmentaite R, van Hooff SR, et al. Tumor to normal single-cell mRNA comparisons reveal a pan-neuroblastoma cancer cell. Sci Adv. 2021 Feb;7(6):eabd3311.

8. Molenaar JJ, Koster J, Zwijnenburg DA, van Sluis P, Valentijn LJ, van der Ploeg I, et al. Sequencing of neuroblastoma identifies chromothripsis and defects in neuritogenesis genes. Nature. 2012 Mar;483(7391):589–93.

9. Trigg RM, Turner SD. ALK in Neuroblastoma: Biological and Therapeutic Implications. Cancers. 2018 Apr 10;10(4):E113.

10. Tsubota S, Kadomatsu K. Origin and initiation mechanisms of neuroblastoma. Cell Tissue Res. 2018 May;372(2):211–21.

11. Wood AC, Krytska K, Ryles HT, Infarinato NR, Sano R, Hansel TD, et al. Dual *ALK* and *CDK4/6* Inhibition Demonstrates Synergy against Neuroblastoma. Clin Cancer Res. 2017 Jun 1;23(11):2856–68.

12. Brady SW, Liu Y, Ma X, Gout AM, Hagiwara K, Zhou X, et al. Pan-neuroblastoma analysis reveals age- and signature-associated driver alterations. Nat Commun. 2020 Dec;11(1):5183.

13. Rihani A, Vandesompele J, Speleman F, Van Maerken T. Inhibition of CDK4/6 as a novel therapeutic option for neuroblastoma. Cancer Cell Int. 2015;15:76.

14. Cheung N-KV, Dyer MA. Neuroblastoma: developmental biology, cancer genomics and immunotherapy. Nat Rev Cancer. 2013 Jun;13(6):397–411.

15. Simões-Costa M, Bronner ME. Insights into neural crest development and evolution from genomic analysis. Genome Res. 2013 Jul;23(7):1069–80.

16. Malumbres M. Cyclin-dependent kinases. Genome Biol. 2014;15(6):122.

Met opmerkingen [SC1]: Niet alle referenties hebben dezelfde stijl. Bijv. ref 25 heeft [internet] erin staat en de link. Zorg ervoor dat alle referenties dezelfde stijl hebben.

17. Ammazzalorso A, Agamennone M, De Filippis B, Fantacuzzi M. Development of CDK4/6 Inhibitors: A Five Years Update. Molecules. 2021 Mar 9;26(5):1488.

18. Gao X, Leone GW, Wang H. Cyclin D-CDK4/6 functions in cancer. In: Advances in Cancer Research. Elsevier; 2020 [cited 2021 Nov 9]. p. 147–69.

19. Du Q, Guo X, Wang M, Li Y, Sun X, Li Q. The application and prospect of CDK4/6 inhibitors in malignant solid tumors. J Hematol OncolJ Hematol Oncol. 2020 Dec;13(1):41.

20. Ribnikar D, Volovat SR, Cardoso F. Targeting CDK4/6 pathways and beyond in breast cancer. The Breast. 2019 Feb;43:8–17.

21. VanArsdale T, Boshoff C, Arndt KT, Abraham RT. Molecular Pathways: Targeting the Cyclin D–CDK4/6 Axis for Cancer Treatment. Clin Cancer Res. 2015 Jul 1;21(13):2905–10.

22. Zhao B, Burgess K. PROTACs suppression of CDK4/6, crucial kinases for cell cycle regulation in cancer. Chem Commun. 2019;55(18):2704–7.

23. Sobhani, D'Angelo, Pittacolo, Roviello, Miccoli, Corona, et al. Updates on the CDK4/6 Inhibitory Strategy and Combinations in Breast Cancer. Cells. 2019 Apr 6;8(4):321.

24. Sun X, Gao H, Yang Y, He M, Wu Y, Song Y, et al. PROTACs: great opportunities for academia and industry. Signal Transduct Target Ther. 2019 Dec;4(1):64.

25. Khan S, He Y, Zhang X, Yuan Y, Pu S, Kong Q, et al. PROteolysis TArgeting Chimeras (PROTACs) as emerging anticancer therapeutics. Oncogene. 2020 Jun;39(26):4909–24.

26. Sakamoto KM, Kim KB, Kumagai A, Mercurio F, Crews CM, Deshaies RJ. Protacs: chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation. Proc Natl Acad Sci U S A. 2001 Jul 17;98(15):8554–9.

27. Burslem GM, Smith BE, Lai AC, Jaime-Figueroa S, McQuaid DC, Bondeson DP, et al. The Advantages of Targeted Protein Degradation Over Inhibition: An RTK Case Study. Cell Chem Biol. 2018 Jan 18;25(1):67-77.e3.

28. Zhao Q, Ren C, Liu L, Chen J, Shao Y, Sun N, et al. Discovery of SIAIS178 as an Effective BCR-ABL Degrader by Recruiting Von Hippel-Lindau (VHL) E3 Ubiquitin Ligase. J Med Chem. 2019 Oct 24;62(20):9281–98.

29. Qi S-M, Dong J, Xu Z-Y, Cheng X-D, Zhang W-D, Qin J-J. PROTAC: An Effective Targeted Protein Degradation Strategy for Cancer Therapy. Front Pharmacol. 2021 May 7;12:692574.

30. Gu S, Cui D, Chen X, Xiong X, Zhao Y. PROTACs: An Emerging Targeting Technique for Protein Degradation in Drug Discovery. BioEssays. 2018 Apr;40(4):1700247.

31. Rana S, Bendjennat M, Kour S, King HM, Kizhake S, Zahid M, et al. Selective degradation of CDK6 by a palbociclib based PROTAC. Bioorg Med Chem Lett. 2019 Jun 1;29(11):1375–9.

32. Su S, Yang Z, Gao H, Yang H, Zhu S, An Z, et al. Potent and Preferential Degradation of CDK6 via Proteolysis Targeting Chimera Degraders. J Med Chem. 2019 Aug 22;62(16):7575–82.

33. Finn RS, Martin M, Rugo HS, Jones S, Im S-A, Gelmon K, et al. Palbociclib and Letrozole in Advanced Breast Cancer. N Engl J Med. 2016 Nov 17;375(20):1925–36.

34. Bhatnagar AS. The discovery and mechanism of action of letrozole. Breast Cancer Res Treat. 2007 Oct;105(S1):7–17.

35. Klein ME, Kovatcheva M, Davis LE, Tap WD, Koff A. CDK4/6 Inhibitors: The Mechanism of Action May Not Be as Simple as Once Thought. Cancer Cell. 2018 Jul;34(1):9–20.

36. Finn RS, Dering J, Conklin D, Kalous O, Cohen DJ, Desai AJ, et al. PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro. Breast Cancer Res. 2009 Oct;11(5):R77.

37. Shagufta, Ahmad I. Tamoxifen a pioneering drug: An update on the therapeutic potential of tamoxifen derivatives. Eur J Med Chem. 2018 Jan;143:515–31.

38. Vu T, Claret FX. Trastuzumab: Updated Mechanisms of Action and Resistance in Breast Cancer. Front Oncol [Internet]. 2012 [cited 2021 Oct 21];2

39. Witkiewicz AK, Cox D, Knudsen ES. CDK4/6 inhibition provides a potent adjunct to Her2-targeted therapies in preclinical breast cancer models. Genes Cancer. 2014 Aug 7;5(7–8):261–72.

40. Burris HA, Tibbitts J, Holden SN, Sliwkowski MX, Lewis Phillips GD. Trastuzumab Emtansine (T-DM1): A Novel Agent for Targeting HER2+ Breast Cancer. Clin Breast Cancer. 2011 Oct;11(5):275–82.

41. Alves CL, Ehmsen S, Terp MG, Portman N, Tuttolomondo M, Gammelgaard OL, et al. Co-targeting CDK4/6 and AKT with endocrine therapy prevents progression in CDK4/6 inhibitor and endocrine therapy-resistant breast cancer. Nat Commun. 2021 Dec;12(1):5112.

42. Van Mater D, Gururangan S, Becher O, Campagne O, Leary S, Phillips JJ, et al. A phase I trial of the CDK 4/6 inhibitor palbociclib in pediatric patients with progressive brain tumors: A Pediatric Brain Tumor Consortium study (PBTC-042). Pediatr Blood Cancer [Internet]. 2021 Apr [cited 2021 Oct 21];68(4).

43. Guenther LM, Dharia NV, Ross L, Conway A, Robichaud AL, Catlett JL, et al. A Combination CDK4/6 and IGF1R Inhibitor Strategy for Ewing Sarcoma. Clin Cancer Res. 2019 Feb 15;25(4):1343–57.

44. Gopalan PK, Gordillo-Villegas A, Zajac-Kaye M, Kaye FJ. Abstract 693: Inhibitory effect of the CDK4/6 inhibitor, PD 0332991, is enhanced by mTOR inhibition in Non-Small Cell Lung Cancer (NSCLC). In: Molecular and Cellular Biology. American Association for Cancer Research; 2013 [cited 2021 Oct 25]. p. 693–693.

45. Franco J, Witkiewicz AK, Knudsen ES. CDK4/6 inhibitors have potent activity in combination with pathway selective therapeutic agents in models of pancreatic cancer. Oncotarget. 2014 Aug 15;5(15):6512–25.

46. Schubert NA, Schild L, van Oirschot S, Keller KM, Alles LK, Vernooij L, et al. Combined targeting of the p53 and pRb pathway in neuroblastoma does not lead to synergistic responses. Eur J Cancer. 2021 Jan;142:1–9.

47. Hart LS, Rader J, Raman P, Batra V, Russell MR, Tsang M, et al. Preclinical Therapeutic Synergy of MEK1/2 and CDK4/6 Inhibition in Neuroblastoma. Clin Cancer Res. 2017 Apr 1;23(7):1785–96.

48. Yadav V, Burke TF, Huber L, Van Horn RD, Zhang Y, Buchanan SG, et al. The CDK4/6 Inhibitor LY2835219 Overcomes Vemurafenib Resistance Resulting from MAPK Reactivation and Cyclin D1 Upregulation. Mol Cancer Ther. 2014 Oct;13(10):2253–63.

49. Patel H, Yacoub N, Mishra R, White A, Long Y, Alanazi S, et al. Current Advances in the Treatment of BRAF-Mutant Melanoma. Cancers. 2020 Feb 19;12(2):E482.

50. Goel S, DeCristo MJ, Watt AC, BrinJones H, Sceneay J, Li BB, et al. CDK4/6 inhibition triggers anti-tumour immunity. Nature. 2017 Aug;548(7668):471–5.

51. Deng J, Wang ES, Jenkins RW, Li S, Dries R, Yates K, et al. CDK4/6 Inhibition Augments Antitumor Immunity by Enhancing T-cell Activation. Cancer Discov. 2018 Feb;8(2):216–33.

52. Rugo HS, Kabos P, Beck JT, Chisamore MJ, Hossain A, Chen Y, et al. A phase Ib study of abemaciclib in combination with pembrolizumab for patients with hormone receptor positive (HR+), human epidermal growth factor receptor 2 negative (HER2-) locally advanced or metastatic breast cancer (MBC) (NCT02779751): Interim results. J Clin Oncol. 2020 May 20;38(15_suppl):1051–1051.

53. Fessas P, Lee H, Ikemizu S, Janowitz T. A molecular and preclinical comparison of the PD-1–targeted T-cell checkpoint inhibitors nivolumab and pembrolizumab. Semin Oncol. 2017 Apr;44(2):136–40.

54. Gelbert LM, Cai S, Lin X, Sanchez-Martinez C, del Prado M, Lallena MJ, et al. Preclinical characterization of the CDK4/6 inhibitor LY2835219: in-vivo cell cycle-dependent/independent anti-tumor activities alone/in combination with gemcitabine. Invest New Drugs. 2014 Oct;32(5):825–37.

55. Bepler G, Kusmartseva I, Sharma S, Gautam A, Cantor A, Sharma A, et al. *RRM1* Modulated In Vitro and In Vivo Efficacy of Gemcitabine and Platinum in Non–Small-Cell Lung Cancer. J Clin Oncol. 2006 Oct 10;24(29):4731–7.

56. Angus SP, Wheeler LJ, Ranmal SA, Zhang X, Markey MP, Mathews CK, et al. Retinoblastoma Tumor Suppressor Targets dNTP Metabolism to Regulate DNA Replication. J Biol Chem. 2002 Nov;277(46):44376–84.

57. Gogolin S, Ehemann V, Becker G, Brueckner LM, Dreidax D, Bannert S, et al. CDK4 inhibition restores G_1-S arrest in MYCN - amplified neuroblastoma cells in the context of doxorubicin-induced DNA damage. Cell Cycle. 2013 Apr;12(7):1091–104.

58. McClendon AK, Dean JL, Rivadeneira DB, Yu JE, Reed CA, Gao E, et al. CDK4/6 inhibition antagonizes the cytotoxic response to anthracycline therapy. Cell Cycle. 2012 Jan 15;11(14):2747–55.

59. Rivankar S. An overview of doxorubicin formulations in cancer therapy. J Cancer Res Ther. 2014;10(4):853.

60. Carvalho C, Santos R, Cardoso S, Correia S, Oliveira P, Santos M, et al. Doxorubicin: The Good, the Bad and the Ugly Effect. Curr Med Chem. 2009 Sep 1;16(25):3267–85.

61. Peng C, Zeng W, Su J, Kuang Y, He Y, Zhao S, et al. Cyclin-dependent kinase 2 (CDK2) is a key mediator for EGF-induced cell transformation mediated through the ELK4/c-Fos signaling pathway. Oncogene. 2016 Mar 3;35(9):1170–9.

62. Brugarolas J, Bronson RT, Jacks T. p21 Is a Critical CDK2 Regulator Essential for Proliferation Control in Rb-deficient Cells. J Cell Biol. 1998 Apr 20;141(2):503–14.

63. Ho GY, Woodward N, Coward JIG. Cisplatin versus carboplatin: comparative review of therapeutic management in solid malignancies. Crit Rev Oncol Hematol. 2016 Jun;102:37–46.

64. Roberts PJ, Bisi JE, Strum JC, Combest AJ, Darr DB, Usary JE, et al. Multiple Roles of Cyclin-Dependent Kinase 4/6 Inhibitors in Cancer Therapy. JNCI J Natl Cancer Inst. 2012 Mar 21;104(6):476–87.

65. Shimura T, Noma N, Oikawa T, Ochiai Y, Kakuda S, Kuwahara Y, et al. Activation of the AKT/cyclin D1/Cdk4 survival signaling pathway in radioresistant cancer stem cells. Oncogenesis. 2012 Jun;1(6):e12–e12.

66. Hagen KR, Zeng X, Lee M-Y, Tucker Kahn S, Harrison Pitner MK, Zaky SS, et al. Silencing CDK4 radiosensitizes breast cancer cells by promoting apoptosis. Cell Div. 2013 Dec;8(1):10.

67. Casey DL, Kushner BH, Cheung N-KV, Modak S, LaQuaglia MP, Wolden SL. Local Control With 21-Gy Radiation Therapy for High-Risk Neuroblastoma. Int J Radiat Oncol Biol Phys. 2016 Oct 1;96(2):393–400.

68. Megison ML, Gillory LA, Beierle EA. Cell survival signaling in neuroblastoma. Anticancer Agents Med Chem. 2013 May;13(4):563–75.

69. Liu Z, Chen SS, Clarke S, Veschi V, Thiele CJ. Targeting MYCN in Pediatric and Adult Cancers. Front Oncol. 2021 Feb 8;10:623679.

70. Petrov I, Suntsova M, Ilnitskaya E, Roumiantsev S, Sorokin M, Garazha A, et al. Gene expression and molecular pathway activation signatures of *MYCN* -amplified neuroblastomas. Oncotarget. 2017 Oct 13;8(48):83768–80.

71. Mayes PA, Degenhardt YY, Wood A, Toporovskya Y, Diskin SJ, Haglund E, et al. Mitogen-activated protein kinase (MEK/ERK) inhibition sensitizes cancer cells to centromere-associated protein E inhibition. Int J Cancer. 2013 Feb 1;132(3):E149–57.

72. Schuler MH, Ascierto PA, De Vos FYFL, Postow MA, Van Herpen CML-, Carlino MS, et al. Phase 1b/2 trial of ribociclib+binimetinib in metastatic *NRAS* -mutant melanoma: Safety, efficacy, and recommended phase 2 dose (RP2D). J Clin Oncol. 2017 May 20;35(15_suppl):9519–9519.

73. Wagner LM, Adams VR. Targeting the PD-1 pathway in pediatric solid tumors and brain tumors. OncoTargets Ther. 2017;10:2097–106.