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Original Research

Target actionability review to evaluate CDK4/6 as a therapeutic target in paediatric solid and brain tumours

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Abstract ***Background:*** Childhood cancer is still a leading cause of death around the world. To improve outcomes, there is an urgent need for tailored treatment. The systematic evaluation of existing preclinical data can provide an overview of what is known and identify gaps in the current knowledge. Here, we applied the target actionability review (TAR) methodology to assess the strength and weaknesses of available scientific literature on CDK4/6 as a therapeutic target in paediatric solid and brain tumours by structured critical appraisal.

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Methods: Using relevant search terms in PubMed, a list of original publications investigating CDK4/6 in paediatric solid tumour types was identified based on relevancy criteria. Each publication was annotated for the tumour type and categorised into separate proof-of-concept (PoC) data modules. Based on rubrics, quality and experimental outcomes were scored independently by two reviewers. A third reviewer evaluated and adjudicated score discrepancies. Scores for each PoC module were averaged for each tumour type and visualised in a heatmap matrix in the publicly available R2 data portal.

Results and conclusions: This CDK4/6 TAR, generated by analysis of 151 data entries from 71 publications, showed frequent genomic aberrations of CDK4/6 in rhabdomyosarcoma, osteosarcoma, high-grade glioma, medulloblastoma, and neuroblastoma. However, a clear correlation between CDK4/6 aberrations and compound efficacy is not coming forth from the literature. Our analysis indicates that several paediatric indications would need (further) preclinical evaluation to allow for better recommendations, especially regarding the dependence of tumours on CDK4/6, predictive biomarkers, resistance mechanisms, and combination strategies. Nevertheless, our TAR heatmap provides support for the relevance of CDK4/6 inhibition in Ewing sarcoma, medulloblastoma, malignant peripheral nerve sheath tumour and to a lesser extent neuroblastoma, rhabdomyosarcoma, rhabdoid tumour and high-grade glioma. The interactive heatmap is accessible through R2 [r2platform.com/TAR/CDK4_6].

1. Introduction

Cancer remains the leading cause of disease-related death in children and adolescents in Western Europe [1]. Despite significant improvements in the overall outcomes of some paediatric cancers over the last decades, the discovery of novel, curative and less toxic therapies is hampered by the rarity and heterogeneity of these diseases (<1% of all cancers) [2]. Small patient numbers and limited economic incentives complicate the development of cancer-specific drugs for children. However, global initiatives and recent changes in the regulation, such as the Research to Accelerate Cures and Equity for Children Act in the US and the obligatory paediatric investigation plan in Europe, now oblige companies to no longer ignore childhood cancers [3]. There certainly have been advances in the targeted treatment of paediatric tumours in recent years [4], though not as big as in adult cancer treatment, compelling paediatric oncologists to turn to off-label use of drugs approved for adults. This off-label use may not only raise key ethical and legal concerns [5], but it also precludes the systematic evaluation of drug efficacy. This argues a strong case for the need to systematically review proof-of-concept (PoC) preclinical data to match paediatric tumour entities to the most promising therapeutic options. To address this, the target actionability review (TAR) methodology [6] was previously established as part of the innovative therapies for children with cancer paediatric preclinical PoC platform (ITCC-P4), an innovative medicines initiative 2-funded public–private partnership between academic research institutions and pharmaceutical companies [7]. In a pilot TAR evaluating the MDM2-*TP53* pathway in primary tumour data and preclinical models of paediatric cancers, we demonstrated that the TAR methodology provided the most comprehensive overview of available preclinical data on targeting of MDM2 in paediatric cancer to date [6]. To extend the TAR series within the ITCC-P4 project, we applied the TAR methodology to systematically review the published literature on CDK4/6 and its inhibitors across a broad panel of 16 paediatric solid and brain tumour types.

CDK4 and its homologue CDK6 are positive regulators of cell cycle progression. Upon binding cyclin D, the complex phosphorylates Rb protein, resulting in the release of E2F transcription factors and the transcription of genes involved in the G1/S transition. Currently, three CDK4/6 inhibitors are approved by the FDA for ER-positive, HER2-negative breast cancer: palbociclib, ribociclib and abemaciclib. In addition, CDK4/6 inhibition seems promising in other solid, as well as haematological, adult cancers [8,9] and gains attention in paediatric oncology. However, the systematic evaluation of preclinical PoC data are currently still lacking for CDK4/6 as a therapeutic target in paediatric tumours.

This TAR provides a comprehensive overview of the available preclinical data on CDK4/6 in paediatric cancers. By summarising and visualising the scores for each tumour type as a heatmap, our review highlights the strengths and gaps in the current preclinical knowledge on CDK4/6 as a paediatric cancer target.

2. Methods

The TAR method was applied as described previously, with four general steps: (1) extensive literature search for papers on the therapeutic target + paediatric tumours of interest, (2) critical evaluation and scoring of the papers, (3) reviewer adjudication and (4) visualisation of PoC as

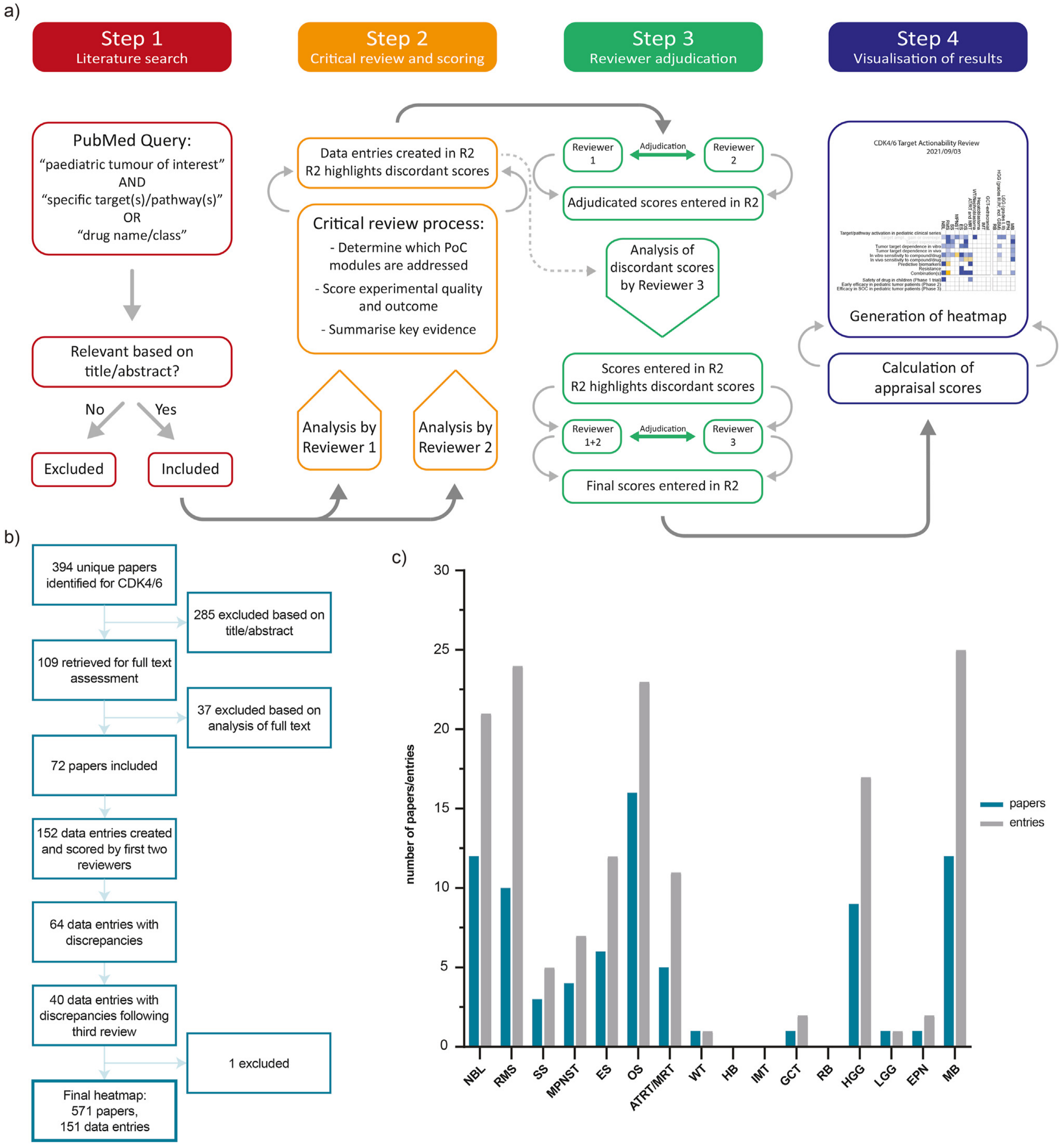

Fig. 1. Overview of the methodology and the studies included in the CDK4/6 TAR. (**a**) Overview of the TAR methodology. Adapted with permission from Schubert *et al.* [6]. (**b**) Study selection process. (**c**) Number of papers and entries per tumour entity. TAR, target actionability review.

a heatmap (Fig. 1a) [6]. Briefly, the first and second reviewers searched PubMed for papers on CDK4/6 and their inhibitors in paediatric solid and brain tumour histologies. After reading the titles and abstracts of the identified papers, the two reviewers agreed on a final list of papers, which included all studies addressing at least one critical appraisal question (CAQ) (Supplementary Table 1). Both reviewers individually performed the full assessment of these papers, i.e. determining the scores for experimental quality and outcome (Tables 1 and 2) and reporting the evidence in the online platform R2. Subsequently, the two reviewers discussed scoring discrepancies and agreed on the final scores. Blinded to these scores, the third reviewer revised the same studies with discordant scores, after which the adjudicated scores of reviewer 1 + 2 and those of reviewer 3 were compared.

Table 1
Rubric for scoring experimental quality.

Proof-of-concept module (PoC)	Description	Scoring and criteria	
PoC 1: CDK4 or CDK6 activation in paediatric clinical series	Number of paediatric samples Type of analysis	3	$n \geq 20$ paediatric patient samples ≥ 2 different methods OR next-generation sequencing
		2	$20 > n > 10$ paediatric patient samples ≥ 1 reliable method
		1	$n \leq 10$ paediatric patient samples 1 method
PoC 2: tumour target dependence *in vitro*	Methodology Tumour cell viability Biological pathway readout	3	Different methods to alter target expression in ≥ 3 cell lines Phenotypic analysis of knockdown
		2	Single method to alter target expression in <3 cell lines
		1	Questionable alteration of gene expression
PoC 3: tumour target dependence *in vivo*	Model used Tumour formation/growth Biological pathway readout	3	Transgenic mouse model or ≥ 2 different xenografts with appropriate controls and/or different methods of genetic modification *in vivo* (shRNA/CRISPR)
		2	≥ 2 different xenografts without appropriate control
		1	1 xenograft model without appropriate control
PoC 4: *in vitro* sensitivity to compound/drug	Number of cell lines Measurement of PD markers and/or phenotypic response	3	5+ cell lines + ≥ 2 appropriate controls; validation
		2	2-5 cell lines + ≥ 1 appropriate controls; validation
		1	1 cell line and/or lack of control and/or validation
PoC 5: *in vivo* activity of compound/drug	Number and type of models used Measurement of PD markers and/or phenotypic response	3	≥ 2 xenograft models or 1 transgenic mouse model with appropriate control; treatment with clinically relevant dose; validation
		2	1 xenograft model with appropriate control; treatment with clinically relevant dose; validation
		1	1 xenograft model OR use of supra-clinical dose levels; no appropriate control or validation
PoC 6: predictive biomarkers	Confirmation of correlation Patient selection	3	Correlation molecularly confirmed in ≥ 2 models (e.g., silencing, overexpression, etc.); patient selection
		2	Correlation confirmed in one model
		1	Correlation not confirmed
PoC 7: resistance	Mechanism of resistance Molecular analysis Method to overcome resistance	3	Reported resistance and comprehensive analysis and reversing/overcoming resistance
		2	Reported resistance and analysis of molecular changes underlying/due to resistance
		1	Only reporting resistance
PoC 8: combinations	Concentrations tested *In vitro* combination index values *In vivo* combination	3	>4 concentrations of each compound are tested (*in vitro*) and synergy values calculated (*e.g.*, CI); combination evaluated *in vivo*
		2	1-4 concentrations of each compound are tested (*in vitro*) and synergy values calculated (*e.g.*, CI); with or without evaluation of combination *in vivo*
		1	Only 1 concentration of each compound is tested; no evaluation of combination *in vivo*
PoC 9: clinical trials (phase I-III)	Compound tested Patient cohort	3	The drug targets only CDK4/6; patients <18 years with a paediatric tumour
		1	The drug has more targets (*e.g.*, pan-CDK inhibitor); patients ≥ 18 years with a paediatric tumour

CI: combination index; strikethrough and underlined text indicate deviations from the original methodology as described in ref. 6.

The remaining discrepancies were resolved by the three reviewers and the final heatmap was generated in R2 [r2platform.com/TAR/CDK4_6].

For this TAR, we made a few adjustments to the standard methodology as defined in [6]. These changes are underlined in the scoring tables for experimental quality (Table 1) and experimental outcomes (Table 2).

3. Results

In this study, we applied the TAR methodology to evaluate the potential actionability of CDK4/6 in paediatric solid and brain tumours. To obtain a list of papers that was as complete as possible with studies addressing CDK4/6 or their respective inhibitors in paediatric malignancies, we used only minimal keywords as our search terms for PubMed (Table 3).

Using these search terms (search date: 24 November 2021), 394 unique papers were identified (Fig. 1b). Of these, 18 (4.6%) were review papers and 30 (7.6%) were case reports and thus excluded immediately. We further filtered out 38 papers (9.6%) published before 2000, based on our experience with previous TARs that older publications typically used experimental techniques that would score poorly on quality, thus having minimal impact on the final heatmap. After reading the titles and

Table 2
Rubric for scoring experimental outcomes.

Proof-of-concept module (PoC)	Description	Scoring and criteria	
PoC 1a: CDK4 or CDK6 activation in paediatric clinical series	Prevalence of CDK4 or CDK6 amplification, gain or overexpression (OE)	3	More than 10% of the cohort with amplification/gain/OE of either CDK4 or CDK6
		1	Between 2-10% with amplification/gain/OE of either CDK4 or CDK6
		-3	$\leq$2% of the cohort with amplification/gain/OE of either CDK4 or CDK6
PoC 1b: CDK4 or CDK6 activation in paediatric clinical series	Expression of CDK4 or CDK6 (generally, as determined by immunohistochemistry)	3	More than 10% of the cohort was positive for CDK4 or CDK6
		1	Between 2-10% of the cohort was positive for CDK4 or CDK6
		-3	$\leq$2% of the cohort was positive for CDK4 or CDK6
PoC 2: tumour target dependence *in vitro*	Level of dependency and phenotypic recapitulation	3	Full dependency (>75% cell death OR transformation)
		1	Partial dependency (<75% cell death OR altered growth)
		-3	No dependency
PoC 3: tumour target dependence *in vivo*	Level of dependency and phenotypic recapitulation	3	Full dependency (CR) after knockdown/knockout or transformation in GEMM
		1	Partial dependency (<75% response)
		-3	No dependency
PoC 4: i*n vitro* sensitivity to compound/drug	IC_{50} observed after 72hr exposure	3	$IC_{50} < 500$ nM or $\leq$ clinically relevant concentration[a]
		1	$IC_{50} = 500–1500$nM
		-1	$IC_{50} > 1500$ nM
		-3	No activity ($IC_{50} > 10$ μM)
PoC 5: *in vivo* activity of compound/drug	*In vivo* tumour response	3	Response comparable to PR/CR
		1	Response comparable to SD
		-1	Very minor response (between SD and PD, slight TGI)
		-3	No activity or clear PD, growth comparable to control
PoC 6: predictive biomarkers	Correlation of biomarker status with the anti-cancer activity of a targeted drug *in vitro/in vivo*	3	A strong correlation (presence of biomarker results in significantly different drug response)
		1	A moderate correlation (presence of biomarker results in different drug response, not significant)
		-3	No correlation (presence of biomarker does not correlate with drug response)
PoC 7: resistance	Reported resistance with drug exposure	3	Resistance reported at clinically relevant concentration/dose and identification/description of mechanism
		1	Resistance reported with no mechanism
PoC 8: combinations	Synergy in combination testing at clinically relevant dosages in relevant *in vitro* and/or *in vivo* models	3	Strong synergy reported – combination index (CI) <0.5
		1	Moderate synergy/additive effect - CI 0.5–0.9
		-1	Very minor synergy/additive effect observed - CI 0.9–1.1
		-3	No combination benefit
PoC 9: clinical trials	Phase I	3	Toxicity profile was acceptable[b], RP2D identified and early efficacy observed
		1	DLT was observed with still acceptable safety and no efficacy was observed
		-3	Toxicity profile was not acceptable
	Phase II	3	The efficacy observed was greater than historical ORR, DoR, and/or PFS and acceptable toxicity
		1	Limited efficacy observed above the historical ORR, DoR, and/or PFS and acceptable toxicity
		-3	No efficacy observed and/or unacceptable toxicity
	Phase III	3	Added efficacy over SOC in appropriate pivotal trial with acceptable benefit/risk profile.The new drug is now part of SOC.

Table 2 (*continued*)

Proof-of-concept module (PoC)	Description	Scoring and criteria	
		1	Added efficacy over SOC but new agent not part of SOC, due to trial design issues and/or benefit/risk assessment
		-3	Insufficient efficacy in a pivotal trial

Amplification: >8 copies, based on next-generation sequencing (NGS) techniques, array CGH, FISH or Southern blotting; gain: 2,5–8 copies, based on NGS techniques, array CGH, FISH or Southern blotting; overexpression: z-score >2 in the related cohort. If definitions are not clearly mentioned in papers, it is assumed that the authors used similar definitions, CR: complete regression, the disappearance of tumour; PR: partial regression, ≥30% decrease of tumour volume; SD: stable disease, neither PR nor PD criteria met; PD: progressive, disease, ≥20% increase of tumour volume; TGI: tumour growth inhibition; criteria based on RECIST criteria [10]; underlined text indicates deviations from the original methodology as, described in ref. 6.

RP2D: recommended phase 2 dose; DLT: dose-limiting toxicity; ORR: overall response rate; DoR: duration of response; PFS: progression-free survival; SOC: standard-of-care, NB: if publications did not address the experimental outcomes according to these criteria, the outcomes were estimated and scored based on this table.

[a] Clinically relevant concentration: the dose that corresponds to the maximum plasma concentrations reached in patients without signs of toxicity.

[b] Toxicity profile is acceptable if adverse events are not life-threatening (no higher than Grade 3 based on the Common Terminology Criteria for Adverse Events) [11].

Table 3
Search terms.

General search terms		"(histology[Title/Abstract]) AND (CDK4[Title/Abstract])" "(histology[Title/Abstract]) AND (CDK6[Title/Abstract])" "(histology[Title/Abstract]) AND (palbociclib[Title/Abstract])" "(histology[Title/Abstract]) AND (ribociclib[Title/Abstract])" "(histology[Title/Abstract]) AND (abemaciclib[Title/Abstract])"	# publications identified
Histologies	neuroblastoma (NBL)	"neuroblastoma"	63
	rhabdomyosarcoma (RMS)	"rhabdomyosarcoma"	38
	synovial sarcoma (SS)	"synovial sarcoma"	8
	malignant peripheral nerve sheath tumour (MPNST)	"MPNST"	15
	Ewing sarcoma	"Ewing"	20
	Osteosarcoma	"osteosarcoma"	141
	atypical rhabdoid tumour (ATRT)/ malignant rhabdoid tumour (MRT)	"rhabdoid"	11
	Wilms tumour (WT)/nephroblastoma	"Wilms" "nephroblastoma"	4
	hepatoblastoma (HB)	"hepatoblastoma"	5
	inflammatory myofibroblastic tumour (IMT)	"inflammatory myofibroblastic tumor"	5
	extracranial germ cell tumour (GCT)	"germ cell tumor"	9
	retinoblastoma (RB)	"retinoblastoma"	5
	high-grade glioma (HGG)/low-grade glioma (LGG)	"glioma"[a]	45
	ependymoma (EPN)	"ependymoma"	6
	medulloblastoma (MB)	"medulloblastoma"	38

[a] "AND (pediatric OR child)" was added to the search terms in an attempt to exclude papers on adult gliomas.

abstracts of the remaining 308 publications, another 199 papers were excluded. Most of these excluded studies did not focus on CDK4/6 (inhibitors) or did not include any paediatric patients. Finally, 109 papers (27.7%) were left for a full assessment. 37 more papers were excluded (reasons included adult-only patient cohorts, none of the CAQs was addressed, the study used a non-targeted compound or miRNA), resulting in 72 papers (152 data entries) that were scored. Of all data entries, 64 (42.1%) were discordant after the assessment by the first two reviewers. Following the third reviewer's assessment, 40 (26.3%) data entries still had discrepant scores. Subsequently, discrepancies were discussed between the three reviewers and a consensus was reached for all data entry scores. One additional paper was excluded because it did not clearly fit one of the PoC modules, resulting in a final heatmap with 151 data entries from 71 papers.

The TAR revealed that the most studied cancers were osteosarcoma (OS), neuroblastoma (NBL), medulloblastoma (MB) and rhabdomyosarcoma (RMS), whereas no relevant studies on CDK4/6 (inhibitors) were found for hepatoblastoma (HB), inflammatory myofibroblastic tumour (IMT), extracranial germ cell tumour (GCT) and retinoblastoma (RB) (Fig. 1c). Only six studies (8.5%) addressed more than one tumour entity (Supplementary Figure 1a) and 13 studies (18.3%) included one or more tumour subtypes (e.g., different subtypes of MB) (Supplementary Figure 1b). The sensitivity of cell lines to CDK4/6 inhibition (PoC 4) was the most studied module, with a total of 36 out of 151 entries (23.8% - 31 papers), closely followed by *CDK4/6* amplification/gain/overexpression (PoC 1a) with 34 entries (22.5% - 26 papers) (Fig. 2a). The final heatmap is shown in Fig. 2b.

For PoC 1, we grouped target amplification, gain, and overexpression into one module (PoC 1a) and distinguished it from target protein expression (PoC 1b) because studies of DNA/RNA typically show or imply concomitant protein overexpression. OS was the entity most frequently addressed in PoC 1a. However, outcomes were contradictory, which may partly be caused by mixed patient cohorts with both paediatric (≤18 years) and adult cases. In such cases, we lowered the quality scores of PoC 1 by one point to adjust for the fact that adult cases may inflate the actual occurrence of an aberration and consequently the scored outcome [12,13]. *CDK4* copy number variation frequencies of ~10% were reported by three next-generation sequencing studies [14–16]. In RMS, *CDK4* amplification might be more frequent, especially in the alveolar subtype (26.1%) as opposed to the embryonal subtype (7.5%) [17–19]. For NBL, *CDK4* amplification was studied in larger cohorts (ranging from 82 to 628 paediatric patients per study) but seems to be rare (<1.3%) [20–22]. Nonetheless, elevated CDK4 levels were shown to correlate with poor survival in NBL, which is why we increased result scores to +1 [20]. Evidence from this TAR suggests that amplification of *CDK6* is more frequent than *CDK4* in brain tumours, contrary to solid tumours [23]. Overall, overexpression of CDK4/6 seems to be more frequent than gains, which are more frequent than amplification. Moreover, *CDK4* status was studied almost 2.5 times more than *CDK6* status. In summary, there was strongest evidence (average score of ≥3) supporting higher levels of CDK4/6 in RMS, malignant peripheral nerve sheath tumour (MPNST), Wilms tumour (WT), high-grade glioma (HGG) and low-grade glioma (LGG).

12 entries (7.9%) were included for tumour target dependence *in vitro* (PoC 2), compared to only three (2.0%) for tumour target dependence *in vivo* (PoC 3); two studies examined both. Eight papers addressed *CDK4* knockdown/knockout, as opposed to CDK6 knockdown in seven papers. Overall, quality scores for PoC 2 were moderate due to the use of few cell lines, single knockdown methods or the absence of rescue experiments. While either knockdown resulted in decreased cell viability and proliferation, as well as cell cycle arrest and reduced levels of (phosphorylated) pRb, one NBL study found that the effect was lower for *CDK6* knockdown [20]. The biggest effect, i.e. >75% cell death upon *CDK4* knockdown, was seen for ES and OS [24,25].

Tumour target dependence *in vivo* (PoC 3) was only studied in RMS (CDK4) and MB (CDK6). Mice intramuscularly injected with RMS cells with inducible *CDK4* knockdown showed reduced tumour growth compared to control mice [19]. Constitutive overexpression of CDK6 in orthotopic MB xenografts (MB subgroups were one SHH, one former PNET) led to tumour development and shorter survival times [23], whereas another study with transgenic models reported

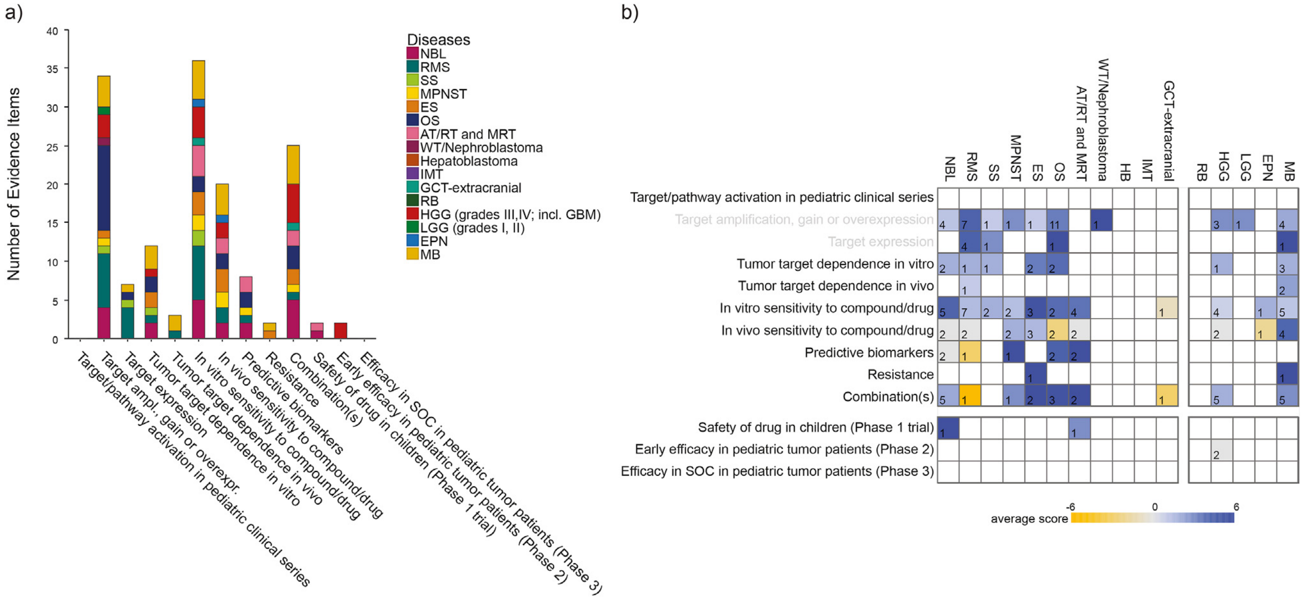

Fig. 2. Overview of the entries included in the CDK4/6 TAR. (**a**) Number of entries included per PoC module and tumour entity. (**b**) Heatmap showing the average scores of all entries made for this CDK4/6 TAR. Numbers indicate the number of included publications. Interactive versions of both figures are accessible through R2 [r2platform.com/TAR/CDK4_6]. PoC, proof-of-concept, TAR, target actionability review.

reduced tumour size and prolonged survival after Cre-mediated homozygous *CDK6* knockout [26]. The positive evidence for *in vitro* target dependence suggests that OS and ES should be further evaluated in an *in vivo* context. Notably, future studies should also aim to evaluate tumour target dependence in other tumour entities.

A total of 31 papers reported testing CDK4/6 inhibitors *in vitro* (PoC 4); 45.2% of these also included *in vivo* studies. Palbociclib was the most studied compound with 23 reports, whereas five studies tested ribociclib and six abemaciclib. Of these, three studies tested more than one CDK4/6 inhibitor. CDK4-specific inhibitors CAS 546102-60-7 and fascaplysin were each used in one study [27,28].

Palbociclib efficacy varied between studies addressing the same entity. There were only three studies that used more than five cell lines, reporting IC_{50} values lower than 500 nM in >10% of NBL and HGG cell lines [29–31]. Atypical rhabdoid tumour/malignant rhabdoid tumour (ATRT/MRT) cell lines were sensitive in three studies that scored lower for quality due to the number of cell lines used [32–34], whereas HGG cell lines seemed rather insensitive [35,36]. For other tumour types, results were mostly conflicting or only based on one study. Ribociclib efficacy *in vitro* was studied in four high-quality studies, showing good responses ($IC_{50} < 500$ nM) in NBL [31,37] and ES [24] cell lines but only moderate efficacy in RMS cells [19]. Abemaciclib treatment was mainly effective in ES [38], NBL [31] and OS [39] cell lines. Two studies (in NBL and EPN) showed superior efficacy of abemaciclib compared with palbociclib or both other CDK4/6 inhibitors [31,40]. Overall, the only entity that scored negatively for PoC 4 is GCT, all other studied entities have average scores between 0.8 (HGG) and 5.7 (ES). Most robust results were seen for NBL, ES and HGG. Studies that scored lower for quality may suggest that CDK4/6 inhibitors are less effective in these tumour entities, but this could be explained by the low number of cell lines included in these studies since we noticed that studies with more cell lines typically also had higher result scores. CDK4/6 inhibition may also be of value in ATRT/MRT, SS, OS, MB and RMS.

The *in vivo* activity of CDK4/6 inhibitors (PoC 5) was assessed by 19 papers, resulting in 20 entries (13.2%). Again, palbociclib was the most studied with 12 papers, followed by abemaciclib (3 studies) and ribociclib (3 studies); and one comparing palbociclib with abemaciclib. Palbociclib treatment (100–150 mg/kg/day orally) resulted in complete remission in MB PDX models (SHH and Group 3) [41]. High-quality studies demonstrated stable disease (SD) in HGG K27M xenografts [30] and MPNST [42]. Interestingly, in the latter study a much lower dose, namely 25 mg/kg/day, was used. In other tumour types, treatment with palbociclib only led to growth inhibition. Ribociclib (75–250 mg/kg/daily) gave the best response (SD) in RMS (ARMS PAX3) [19] and ES [24], while abemaciclib treatment (50 mg/kg/daily) led to SD only in ES [38]. A comparison of palbociclib and abemaciclib treatment in an MB mouse model revealed superior tumour growth-inhibiting potential for palbociclib [26].

Overall, MB, MPNST, ES, RMS, ATRT/MRT and HGG received average scores ≥ 0 for *in vivo* (mouse) studies (all SD with the exception of CR in MB SHH and Group 3). Of these, only MB scored high (>3). Other tumour types received either an average score of 0 due to conflicting results (NBL, RMS, ATRT/MRT and HGG) or a negative score (OS and, based on a single study, EPN).

Papers addressing biomarkers (PoC 6) or resistance mechanisms (PoC 7) were limited, with only eight entries (5.3%) and 2 entries (1.3%), respectively. While *MYCN* amplification had biomarker potential for ribociclib sensitivity in NBL, this was not the case for CDK4 levels, MDM2 levels or *ALK*, *TP53*, *RB1* or *CDKN2A* mutations [31,43]. *CDKN2A* mutations did also not correlate with CDK4/6 inhibitor sensitivity in RMS [44], whereas knockdown of *p16INK4a* (one of the genes encoded by the *CDKN2A* locus) did significantly increase the sensitivity of one MRT cell line to palbociclib [32]. In OS, there is some evidence that pRb function and CDK4 levels correlate with sensitivity [16,39]. In ATRT cell lines, on the other hand, there was no correlation with CDK4 but with cyclin D1 [34]. Additionally, knockdown of RABL6A, a Ras-family oncogene, reduced palbociclib sensitivity in three MPNST cell lines [42]. A genome-scale open reading frame screen in two ES cell lines showed that IGF1R overexpression occurs after prolonged treatment with ribociclib, resulting in increased resistance [45]. A genome-wide CRISPR screen in two MB SHH cell lines identified RPL10 and RPL23A as drivers of resistance upon prolonged treatment with abemaciclib [46].

CDK4/6 inhibitors were combined with different types of treatment: chemotherapy, radiation, other targeted compounds and gene knockdown. In total, 25 entries (16.6%) were made for PoC 8; eight combinations were only tested *in vitro* and six only *in vivo*. Of the chemotherapeutics combined with CDK4/6 inhibitors, only doxorubicin showed some synergistic effects [38,39,47]. The addition of radiotherapy to palbociclib treatment was shown to be synergistic in ATRT, MB and HGG [33,35,48,49]. The CDK4 inhibitor CAS546102-60-7 strongly synergised with DZNep (EZH2), MLN8054 (aurora kinase inhibitor) or bortezomib (protease inhibitor) in rhabdoid tumours [27]. Other synergistic combinations were CDK4/6 inhibitors + ALK inhibitors in NBL and RMS [44,50], palbociclib + temsirolimus (mTOR inhibitor) in HGG [36] and palbociclib + sorafenib (multikinase inhibitor) in OS [51]. In addition, combined inhibition of CDK6 and HSD11β2, an enzyme that produces smoothenedactivation lipids, was synergistic in MB SHH mouse models [46]. Combined targeting of CDK4/6 and MEK in *NF1*-mutant NBL [43], JQ1 in *MYC*-driven MB [52] and CDK1/2/5/9 in MPNST [42] may also be of interest.

Our search identified three clinical studies, up to phase II. The phase I trial for ribociclib included 15 NBL and 15 MRT patients (we excluded the only RMS patient, as this would resemble a case study) and reported a maximum tolerated dose of 470 mg/m^2 and a recommended phase II dose (RP2D) of 350 mg/m^2/d [53]. Stable disease was reached in 7/15 NBL and 2/15 MRT patients. The same dose was used in phase I/II trial with 10 newly diagnosed DIPG (HGG K27M mutant) patients following radiotherapy [54]. Nine patients progressed and one patient discontinued treatment after course 14. Both studies reported manageable adverse events, with neutropenia being the most frequent (up to 90%). The third trial was a phase II study examining palbociclib treatment in 34 patients with grade 3 oligodendroglioma (HGG) >18 years of age [55]. Given the age of the patients, this study received a low quality score. Moreover, the study was discontinued early owing to a lack of efficacy.

Overall, the results of this TAR reveal that extensive preclinical work is still necessary to determine the relevance of targeting CDK4/6 in paediatric cancers. Information on CDK4/6 aberration frequencies is unknown for ATRT/MRT, HB, IMT, GCT, RB and EPN or based on a single publication in SS, MPNST, ES, WT and LGG. The dependency of tumours on these oncogenes is also barely investigated in paediatric cancers. Compound sensitivity should be (further) addressed in all tumour types, especially *in vivo*, and particularly in SS, WT, HB, IMT, GCT, RB, LGG and EPN. Future studies should also focus more on the identification of biomarkers and combinatorial approaches.

4. Discussion

The goal of the ITCC-P4 consortium is to accelerate evidence-driven paediatric cancer drug development by prioritising drugs currently undergoing preclinical investigation (or drugs repurposed from adult malignancies) for clinical development in children suffering from cancer. In this study, we applied the previously established TAR methodology to evaluate the potential actionability of CDK4/6 in paediatric solid and brain tumours. Based on our experience of having a high percentage of discordant scores between the two reviewers, we suggest to adapt the TAR methodology by performing a ‘pilot adjudication’ after the first ten papers that are fully scored (Supplementary Figure 2). This initial comparison will help in identifying pitfalls and different approaches at an early stage, aligning the scoring, and will ultimately result in fewer discordant scores.

Evidence from this TAR suggests that *CDK4/6* aberrations occur in RMS, OS, HGG and MB, and at lower frequencies also in NBL. For most other indications, our search strategy did not capture any or more than one publication(s) reporting aberration frequencies. Overexpression seems to occur more frequently than gain or amplification of *CDK4/6*, suggesting that other mechanisms may contribute to higher levels. It is important to realise that lower incidence rates of certain tumour types could possibly result in smaller sample sizes, thus adding a bias to the quality and the overall score of modules.

There is still a lot of uncertainty regarding the correlation between higher CDK4/6 levels and drug sensitivity. Four studies examined this correlation, but the biomarker status of CDK4 could only be confirmed in OS [16]. Cell lines included in PoC 4 and 5 had all sorts of genetic backgrounds and the relatively low incidence of *CDK4/6* aberrations makes it difficult to draw conclusions. The ambiguous effect of *CDK4/6*, as well as *p16/CDKN2A*, was also reported in adult malignancies [56–59]. As reviewed recently, CDK4/6 overexpression or amplification even correlated with resistance in some adult cancer models [57]. Our included studies suggest only *MYCN* as putative biomarkers for CDK4/6 inhibitor sensitivity, indicating that further research on biomarkers is needed to select the best patient cohort for this intervention. Moreover, not only the dependency on CDK4/6 overexpression but also its exact contribution to the development or proliferation of tumours should be further investigated.

Frequently, tumour entities which scored positively in PoC 4 (*in vitro* sensitivity) scored negatively or at least much lower in PoC 5 (*in vivo* sensitivity), as was the case for NBL, RMS, ES, OS, ATRT/MRT, HGG and EPN. These findings suggest that *in vitro* studies alone are not always predictive of drug efficacy *in vivo*, highlighting the necessity of *in vivo* studies. Based on the included studies, CDK4/6 inhibition may be most promising in MPNST, ES and MB (especially SHH and Group 3). Clinical data showed that CDK4/6 inhibitors are tolerated at relatively high doses, with a maximum tolerated dose of 470 mg/m^2 for ribociclib and an RP2D of 350 mg/m^2, which are comparable to those in adults [53]. Moreover, ribociclib shows good central nervous system penetration [60]. Therefore, entities that scored lower for these modules (mainly NBL, RMS, ATRT/MRT and HGG) may also still benefit from CDK4/6 inhibition, although secondary target inhibition should be examined and prevented. While two studies reported superior *in vitro* efficacy of abemaciclib, assumptions on the most efficient CDK4/6 inhibitor *in vivo* are not possible based on the results of this TAR. However, the MAST study (https://braid.stjude.org/masttour/), which was not found using our search terms, shows that ribociclib has superior efficacy over palbociclib in paediatric solid cancers [61].

That hardly any *in vivo* drug sensitivity studies or the clinical trials were able to achieve a response better than stable disease shows that combination therapies may be necessary to achieve objective responses. Based on current preclinical evidence, CDK4/6 inhibitors in combination with ALK inhibitors in *ALK*-driven tumours, radiation therapy or chemotherapy (mainly doxorubicin) should be prioritised for clinical development. The combination of CDK4/6 inhibitors with chemotherapy indeed shows clinical promise [62]. A key finding reveals the mechanism by which CDK4/6 inhibitors impair recovery from DNA damage induced by chemotherapies that require cycling cells for their activity, suggesting that CDK4/6 inhibitors should be applied after and not before cytotoxic chemotherapy [63]. Additionally, the search for other tumour-specific genetic dependencies that are synergistic with CDK4/6 inhibition should continue.

All three CDK4/6 inhibitors are currently tested in several paediatric clinical trials and included in different precision medicine programs for children with *CDK4/6* amplification or a homozygous loss of *CDKN2A*. Several of these studies or programs use abemaciclib, even though our results show that published preclinical evidence for this drug is still sparse. This disproportion may indicate that results with palbociclib/ribociclib are sometimes extrapolated. Future (pre)clinical studies will have to show whether extrapolation is appropriate, especially given the broader target spectrum of abemaciclib [64].

For clinical trials to succeed, optimal target group selection, taking the molecular status into account, is pivotal. Unfortunately, the data from this TAR shows that preclinical evidence for a positive biomarker status of *CDK4/6* aberrations and *CDKN2A* loss is still scarce and contradictory. Their exact influence on CDK4/6 inhibitor sensitivity will need to be further addressed in future studies. Given the complexity of cell cycle regulation, future studies may also want to look at predictive gene signatures instead of single gene biomarkers.

In conclusion, the heatmap generated from the CDK4/6 TAR reveals that preclinical data are still lacking for many paediatric tumour entities. Indicated by ≤ 2 publications, intensive work across all PoC data modules is necessary for WT, HB, IMT, GCT, RB, LGG and EPN, while for most other tumour types, research should mainly focus on unravelling the dependence of a tumour on CDK4/6 and the identification of biomarkers, resistance mechanisms and combination therapies. Researchers should also be encouraged to differentiate between tumour subtypes where this is applicable. Our data suggest that CDK4/6 inhibition might be most relevant for MPNST, ES and MB (SHH and Group 3) patients, but patients with NBL, RMS, ATRT/MRT and HGG may benefit from this targeted treatment as well. Whether this is indeed the case, will have to be addressed in future clinical studies. The full TAR data is summarised in one publicly accessible online application [r2platform.com/TAR/CDK4_6], where all data can be interactively explored and evaluated.

Author contributions

Nil A. Schubert: methodology, investigation, writing – original draft, visualization Celine Y. Chen: investigation, writing – original draft Ana Rodríguez: methodology, investigation, writing – review & editing, project administration Jan Koster: software, data curation, writing – review & editing, visualization Michele Dowless: investigation Stefan M. Pfister: supervision, writing – review & editing David J. Shield: supervision Louis F. Stancato: supervision, writing – review & editing Gilles Vassal: supervision Hubert N. Caron: methodology, supervision Marlinde L. van den Boogaard: supervision, writing – review & editing Anton G. Henssen: supervision, writing – review & editing Jan J. Molenaar: methodology, supervision, writing – review & editing.

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Conflict of interest statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: MD and LFS are full-time employees of Eli Lilly and Company. AR and HNC are full-time employees of Hoffmann-La Roche. DJS is a full-time employee of Pfizer. All remaining authors have declared no conflicts of interest

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2022.04.028.

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