REVIEW

Overcoming CDK4/6 inhibitor resistance in ER-positive breast cancer

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Abstract

Three inhibitors of CDK4/6 kinases were recently FDA approved for use in combination with endocrine therapy, and they significantly increase the progression-free survival of patients with advanced estrogen receptor-positive (ER+) breast cancer in the first-line treatment setting. As the new standard of care in some countries, there is the clinical emergence of patients with breast cancer that is both CDK4/6 inhibitor and endocrine therapy resistant. The strategies to combat these cancers with resistance to multiple treatments are not yet defined and represent the next major clinical challenge in ER+ breast cancer. In this review, we discuss how the molecular landscape of endocrine therapy resistance may affect the response to CDK4/6 inhibitors, and how this intersects with biomarkers of intrinsic insensitivity. We identify the handful of pre-clinical models of acquired resistance to CDK4/6 inhibitors and discuss whether the molecular changes in these models are likely to be relevant or modified in the context of endocrine therapy resistance. Finally, we consider the crucial question of how some of these changes are potentially amenable to therapy.

Overview of the cell cycle

Dysregulation of the complex regulatory network that controls cell cycle progression is a hallmark of cancer (Hanahan & Weinberg 2011). A major axis of dysregulation is the gateway to cell cycle entry, which is controlled by the retinoblastoma (Rb) protein. Rb restricts progression from G1 phase into S phase by binding and suppressing E2F transcription factors. This is overcome by cyclin-dependent kinase 4/6 (CDK4/6) phosphorylation of the Rb protein, which leads to E2F release, and a transcriptional program for proliferation is activated, committing the cell to G1 exit. Subsequently, there is a cascade of downstream signalling events that ultimately promotes the activity of cyclin E/CDK2 complexes, the phosphorylation of further target proteins and progression into S phase and DNA replication (Kato et al. 1993, Burkhart & Sage 2008).

Regulation of CDK4/6 activity is key to the deactivation of the Rb protein. CDK4 and CDK6 become active when CDK4/6 form heterodimers with D-type cyclins, which are upregulated and post-translationally modified in response to mitogenic signalling by the RAS/MAPK and PI3K/ AKT/mTOR signal transduction cascades. Entry into the cell cycle is suppressed by two families of CDK inhibitors, INK4 (p16INK4A, p15INK4B, p18INK4C and p19INK4D) and CIP/KIP (p21Waf1/Cip1, p27Kip1, p57Kip2) (Sherr 1996). INK4 proteins selectively inhibit the cyclin D–CDK4/6 complex (Sherr & Roberts 1999, Sheppard & McArthur 2013) to induce cell cycle arrest and senescence.
(Kim & Sharpless 2006). CIP/KIP proteins inhibit both CDK4/6 and CDK2 complexes but have the additional effect of stabilising these complexes and preventing cyclin degradation (Prall et al. 1997). In the presence of stable p16INK4a-cyclin D-CDK4/6, the binding of p21Waf1/Cip1 and p27Kip1 to CDK2 complexes will reinforce cell cycle arrest (Sherr & Roberts 1999).

Dysregulation of the cyclin-CDK-Rb axis by upregulation of cyclin–CDK activity and/or abrogation of suppressors are features of many tumour types. It is therefore unsurprising that this axis is recognised as a key target for therapeutic intervention (Musgrove et al. 2011). Research has focussed on small-molecule inhibition of CDK function and such CDK inhibitors have been designed, developed and trialled in the clinic with increasing success over the last few years.

The development of CDK inhibitors

CDK4 and CDK6 are part of the CDK family of serine/threonine kinases (Peyressatre et al. 2015). The initial discovery of cyclin-dependent kinases was in the context of the cell cycle where ‘cyclins’ were cyclically degraded and includes CDK1, CDK2, CDK4 and CDK6. Since then, further CDK functions have been identified for the transcriptional machinery (CDK7, CDK8, CDK9, CDK12), DNA damage response (CDK12) and in tissue specific functions (CDK5) (Reviewed by Malumbres 2014, Lenjisa et al. 2017, Philip et al. 2018). Despite these diverse functions, the CDKs are structurally very similar, due to the fact that context-specific cyclins are activated to control each function.

CDK inhibitors are a class of pharmacological agents used to target dysregulated CDK activity in malignant cells. The mechanisms of several first-generation CDK inhibitors have been studied in a variety of cancer types, but few have successfully transitioned to a clinical setting (Asghar et al. 2015). A major barrier to the clinical development of first-generation inhibitors was lack of selectivity due to structural similarity between the CDKs (Shapiro 2006, Michaud et al. 2010). Compounds such as flavopiridol (CDK1, CDK2, CDK4, CDK6, CDK7 and CDK9, trialled in patients with a range of haematological malignancies and solid tumours) and roscovitine (CDK2, CDK7 and CDK9, trialled in patients with non-small-cell lung cancer (NSCLC) (NCT00372073), triple-negative breast cancer (NCT01333423) and other advanced solid tumours (NCT00999401)) failed clinical trials, demonstrating limited efficacy and considerable toxicity (Lapenna & Giordano 2009). Second-generation pan-CDK inhibitors had greater selectivity across a smaller number of CDKs, and a reduced toxicity profile, but they showed a lesser degree of CDK4/6 inhibitory activity. For example, the pan-CDK inhibitors dinaciclib and SNS-032 target CDK1, CDK2, CDK5, CDK9 and CDK12, and CDK2, CDK7 and CDK9 respectively (Asghar et al. 2015). Dinaciclib is currently being trialled on patients with multiple myeloma, NSCLC, melanoma, advanced hematologic, breast and pancreatic malignancies and other solid tumours and has reached phase II clinical trials for some applications. More recently, dinaciclib was shown to be a potent inhibitor of CDK12 with implications for its use in homologous repair-deficient tumour types in combination with PARP inhibitors (Johnson et al. 2016), a finding that is now being investigated in patients (NCT01434316).

The relative failure of these first- and second-generation inhibitors led to the search for inhibitors highly selective to individual CDKs and their associated cellular functions. Pharmacological (Tadesse et al. 2018, Yin et al. 2018) and computer-aided (Kalra et al. 2017) approaches are now being employed to design the next generation of CDK inhibitors with higher potency and, crucially, higher specificity – a key attribute for the successful deployment of CDK inhibitors. There has been some progress made in targeting non-cell cycle CDKs such as CDK9 and CDK12 (Johnson et al. 2016, Li et al. 2017), but the inhibitors that have progressed furthest and have now entered clinical use are those targeted at CDK4/6.

CDK4/6 inhibitors

All CDK4/6 inhibitor compounds are designed by targeting the ATP-binding domains of the proteins (Asghar et al. 2015). The pharmaceutical lead compounds that have been translated into clinical use are palbociclib (ibrance; Pfizer) (Fry et al. 2004), ribociclib (Kisqali; Novartis) (Infante et al. 2014) and abemaciclib (Verzenio; Eli Lilly) (Patnaik et al. 2016). These compounds are highly selective for CDK4/6 over other members of CDK family compared to their historical counterparts. Both palbociclib and ribociclib have >100-fold higher affinity for CDK4 and CDK6 relative to other cell cycle CDKs and CDK9 (Tadesse et al. 2017). In contrast, abemaciclib is less selective, with only ~six-fold higher affinity for CDK4/6 than it has for CDK9 and has some activity towards CDK1, CDK2 and CDK5 at higher doses (Gelbert et al. 2014, Tripathy et al. 2017) (Table 1). Despite being less selective, in a direct comparison, abemaciclib was found to more efficiently pass through the blood–brain barrier than palbociclib (Raub et al. 2015), which widens
its potential application to brain cancers and secondary brain metastases (NCT02308020). Recent studies using chemoproteomics (Sumi et al. 2015), thermal proteome profiling (Miettinen et al. 2018) and a mass spectrometry-based competition assay have shown that palbociclib, ribociclib and abemaciclib can inhibit a spectrum of other kinases and that the inhibition profiles of palbociclib and abemaciclib were not similar with the exception of CDK4/6 (Cousins et al. 2018). Notably, it was found that in cell line models abemaciclib, but not palbociclib or ribociclib, activated Wnt signalling via inhibition of glycogen synthase kinase (GSK) 3β and subsequent stabilisation of β-catenin. GSK3β activity also plays an important role in the regulation of cyclin D family proteins at both the transcriptional and proteomic level such that inhibition of GSK3β is expected to increase the levels of cyclin D (Takahashi-Yanaga & Sasaguri 2008).

The clinical relevance of the off-target kinase inhibitory activity for each of these drugs has yet to be fully assessed (Chen et al. 2016). The increased potency of abemaciclib toward CDK9 (reported in some, but not all studies) (Gong et al. 2017) correlates with a gastrointestinal toxicity profile specifically in those patients (Shohdy et al. 2017). Conversely, abemaciclib allows for continuous dosing, whereas palbociclib and ribociclib require a break in treatment of 1 week in four in order to allow for neutrophil recovery. All three inhibitors are associated with toxicities resulting in loss of haematocytes (Kassem et al. 2018), consistent with a role for CDK6 in the activation of haematopoietic stem cells (Scheicher et al. 2015). Toxicities associated with all three drugs are, however, considered to be manageable and reversible (Spring et al. 2017).

This family of compounds have found a natural home in the treatment of advanced ER+ breast cancer (Lapenna & Giordano 2009, Musgrove et al. 2011). ER+ breast cancer is by far the most common subtype of breast cancer, representing approximately 70% of breast cancers in women. Estrogen is mitogenic and drives cell proliferation, partly through the increase in levels of cyclin D1 and CDK4/6 activity (Filmus et al. 1994).

**Clinical development of CDK4/6 inhibitors in endocrine-resistant breast cancer**

Endocrine therapy is the bedrock of systemic therapy for ER+ breast cancer. Adjuvant therapy comprises 5–10 years or more of ER-directed endocrine therapy such as tamoxifen and aromatase inhibitors to inhibit ER-driven activation of cell cycle progression. Approximately 50% of patients with early stage ER+ breast cancer obtain benefit from adjuvant endocrine therapy, resulting in a reduction in breast cancer mortality by approximately 40% (Early Breast Cancer Trialists’ Collaborative Group 2015). However, resistance to endocrine therapy leading to early stage ER+ breast cancer recurrence is not uncommon and inevitable in the setting of advanced disease. Importantly, the majority of these cancers have a perturbed but essentially intact Rb axis downstream of the mechanism of endocrine resistance, making them highly suitable for CDK4/6 inhibitor treatment (Ertel et al. 2010) (Fig. 1).

Early preclinical breast cancer studies demonstrated that palbociclib preferentially inhibited the proliferation of ER+ in contrast to ER− breast cancer models in vitro.

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**Table 1** CDK4/6 inhibitor specificities.

<table>
<thead>
<tr>
<th>IC50 (nmol/L)</th>
<th>Palbociclib (Fry et al. 2004)</th>
<th>Ribociclib (Tripathy et al. 2017)</th>
<th>Abemaciclib (Gelbert et al. 2014)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK4-cyclin D1/D3</td>
<td>9–11</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>CDK6-cyclin D1/D2/D3</td>
<td>15</td>
<td>39</td>
<td>10</td>
</tr>
<tr>
<td>CDK1-cyclin B</td>
<td>&gt;10,000</td>
<td>&gt;100,000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>CDK2-cyclin A/E</td>
<td>&gt;10,000</td>
<td>&gt;50,000</td>
<td>&gt;500</td>
</tr>
<tr>
<td>CDK5-p25</td>
<td>&gt;10,000</td>
<td>&gt;40,000</td>
<td>&gt;300</td>
</tr>
<tr>
<td>CDK9-cyclin T</td>
<td>Not determined</td>
<td>Not determined</td>
<td>57</td>
</tr>
</tbody>
</table>

IC50, half maximal inhibitory concentration; in vitro kinase assay.

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**Figure 1**

Regulation of cell cycle in ER+ breast cancer. The key pathways in promoting entry into the cell cycle in ER+ breast cancer and the nodes to which current therapies are targeted.

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https://doi.org/10.1530/ERC-18-0317 © 2019 Society for Endocrinology Published by Bioscientifica Ltd. Printed in Great Britain
(Finn et al. 2009). Phase III clinical trials have shown that the combination therapy of CDK4/6 inhibitors and endocrine therapy for advanced ER+ breast cancer, improves progression free survival (PFS) when compared to endocrine therapy alone; overall survival data are yet to be published. Therefore, the combination of CDK4/6 inhibitor and endocrine therapy is now standard of care as first-line therapy for advanced ER+ breast carcinoma in many countries.

Phase III trials of all three CDK4/6 inhibitors in the first-line setting in combination with non-steroidal aromatase inhibitors and in the second-line setting in combination with fulvestrant has been completed. Palbociclib was the first CDK4/6 inhibitor approved by the FDA in February 2015. It was granted accelerated approval in combination with letrozole for the first-line treatment of advanced ER+ HER2– breast cancer due to the phase II PALOMA-1 trial (Finn et al. 2015). The follow-up phase III PALOMA-2 trial demonstrated that palbociclib and letrozole improved PFS from 14.5 to 24.8 months, when compared to letrozole alone (hazard ratio 0.58; 95% CI 0.46–0.72, \( P < 0.001 \)) (Finn et al. 2016). It also received FDA approval in February 2016 for a second indication, the treatment of advanced ER+ HER2– breast cancer in combination with fulvestrant after progression following endocrine therapy from the phase III PALOMA-3 trial (Cristofanilli et al. 2016) (Table 2).

Also approved for clinical use are ribociclib and abemaciclib. The phase III MONALEESA-2 trial with ribociclib and letrozole as first-line treatment for advanced ER+ HER2– breast cancer showed improved PFS, leading to FDA approval in March 2017 (Hortobagyi et al. 2016, 2018). More recently, the phase III MONALEESA-3 trial with ribociclib and fulvestrant as first- and second-line treatment for advanced ER+ HER2– breast cancer demonstrated an improved PFS (Slamon et al. 2018). Abemaciclib was FDA approved in September 2017 in combination with fulvestrant as a second-line treatment after the phase II MONARCH-2 trial (Sledge et al. 2017) and as monotherapy after progression on endocrine therapy and chemotherapy from the phase II MONARCH-1 trial (Dickler et al. 2017). It was later approved in February 2018 in combination with letrozole as first-line treatment based on results from the phase III MONARCH-3 trial (Goetz et al. 2017).

CDK4/6 inhibitors are given as oral tablets and are generally well tolerated. Common toxicities include nausea, diarrhoea, fatigue, neutropenia (however, febrile neutropenia is uncommon), leukopenia, anaemia and thrombocytopenia (Shah et al. 2018). Patients require regular full blood counts, liver function tests and ECGs.

Current clinical questions include which CDK4/6 inhibitor to choose, how to best sequence therapy and whether to add to the same endocrine therapy regime. The recent TREND study (Malorni et al. 2018) shows that adding palbociclib to the previously administered endocrine therapy led to a PFS advantage in patients who received prior endocrine therapy for >6 months (HR 0.53; 95% CI 0.3–0.9, exploratory \( P \)-value=0.02), but not in those who had received less than 6 months of endocrine therapy.

Resistance to CDK4/6 inhibitors is now the major emerging consideration in pre-clinical and clinical drug development. Clinical areas of interest to address resistance include identifying predictive biomarkers for CDK4/6 inhibitors and novel treatment combinations.

**Does endocrine therapy resistance affect CDK4/6 inhibitor sensitivity?**

Endocrine resistance occurs when tumours bypass the cell cycle inhibition of endocrine therapy and return to a proliferative phenotype. Many mechanisms of acquired endocrine resistance have been described, including the upregulation of ER coactivators (e.g. FOXA1), cyclins (particularly D and E class), CDK proteins (CDK2 and CDK6) and mitogen signalling pathways (PI3K and RAS pathways) and/or downregulation of CDK inhibitor proteins (p16\(^{\text{INK4A}}\), p21\(^{\text{Waf1/Cip1}}\), p27\(^{\text{Kip1}}\)) (Musgrove & Sutherland 2009). Genomic and epigenetic mechanisms of endocrine resistance have also been identified, including activating \( ESR1 \) mutations, which can occur in up to 40% of patients with metastatic disease (Jeselsohn et al. 2014, Schiavon et al. 2015) and hypermethylation of estrogen-responsive enhancers, which is associated with reduced ER binding and decreased gene expression of key regulators of ER activity (Stone et al. 2015). Importantly, in most cases functional Rb protein is retained during the development of endocrine resistance (Musgrove et al. 2011), rendering these tumours amenable to CDK4/6 inhibition (Fig. 1).

CDK4/6 inhibition acts directly downstream of endocrine therapy, and it is therefore inevitable that some mechanisms of resistance will be common to both types of treatments. Endocrine resistance associated with dysregulation of the Rb axis could, in theory, reduce sensitivity to CDK4/6 inhibition as many of these mechanisms could also impinge on the effectiveness of CDK4/6 inhibitors. Despite this, the success of CDK4/6...
Table 2  Seminal phase II/III trials of CDK4/6 inhibitors that led to FDA approval for the treatment of advanced ER+ breast cancer.

<table>
<thead>
<tr>
<th>Study</th>
<th>NCT</th>
<th>Phase</th>
<th>No.</th>
<th>Menopausal status</th>
<th>Description</th>
<th>Median PFS; Hazard ratio (95% CI)</th>
<th>ORR (%)</th>
<th>FDA approval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st Line</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PALOMA-1 TRIO-18 (Finn et al. 2015)</td>
<td>00721409</td>
<td>II</td>
<td>165</td>
<td>PMP</td>
<td>Palbociclib + letrozole vs letrozole</td>
<td>20.2 vs 10.2 months; 0.49 (0.32–0.75); P = 0.0004</td>
<td>55.0 vs 39.0</td>
<td>Feb 2015</td>
</tr>
<tr>
<td>PALOMA-2 (Finn et al. 2016)</td>
<td>01740427</td>
<td>III</td>
<td>666</td>
<td>PMP</td>
<td>Palbociclib + letrozole vs letrozole</td>
<td>24.8 vs 14.5 months; 0.58 (0.46–0.72); P &lt; 0.001</td>
<td>55.3 vs 44.4</td>
<td>Feb 2016</td>
</tr>
<tr>
<td>MONALEESA-2 (Hortobagyi et al. 2018)</td>
<td>01958021</td>
<td>III</td>
<td>668</td>
<td>PMP</td>
<td>Ribociclib + letrozole vs letrozole</td>
<td>25.3 vs 16.0 months; 0.57 (0.46–0.70); P &lt; 0.0001</td>
<td>52.7 vs 37.1</td>
<td>Mar 2017</td>
</tr>
<tr>
<td>MONALEESA-7 (Tripathy et al. 2018)</td>
<td>02278120</td>
<td>III</td>
<td>660</td>
<td>Pre-MP</td>
<td>Ribociclib + OFS + tamoxifen/AI vs OFS + tamoxifen/AI</td>
<td>23.8 vs 13.0 months; 0.55 (0.44–0.69); P &lt; 0.0001</td>
<td>51.0 vs 44.0</td>
<td></td>
</tr>
<tr>
<td>MONARCH-3 (Goetz et al. 2017)</td>
<td>02246621</td>
<td>III</td>
<td>493</td>
<td>PMP</td>
<td>Abemaciclib + AI vs AI</td>
<td>NR vs 14.7 months; 0.54 (0.41–0.72); P &lt; 0.0001</td>
<td>59.0 vs 44.0</td>
<td>Feb 2018</td>
</tr>
<tr>
<td>2nd Line</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>PALOMA-3 (Cristofanilli et al. 2016)</td>
<td>01942135</td>
<td>III</td>
<td>521</td>
<td>PMP + Pre-MP</td>
<td>Palbociclib + fulvestrant ± OFS vs fulvestrant ± OFS</td>
<td>9.5 vs 4.6 months; 0.46 (0.36–0.59); P &lt; 0.001</td>
<td>24.6 vs 15.0</td>
<td>Feb 2016</td>
</tr>
<tr>
<td>MONARCH-2 (Sledge et al. 2017)</td>
<td>02107703</td>
<td>III</td>
<td>669</td>
<td>PMP</td>
<td>Abemaciclib + fulvestrant vs fulvestrant</td>
<td>16.4 vs 9.3 months; 0.55 (0.45–0.68); P &lt; 0.001</td>
<td>48.1 vs 21.3</td>
<td>Sep 2017</td>
</tr>
<tr>
<td>3rd line</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MONARCH-1 (Dickler et al. 2017)</td>
<td>02102490</td>
<td>II</td>
<td>132</td>
<td>PMP</td>
<td>Abemaciclib single agent</td>
<td>6.0 months</td>
<td>19.7</td>
<td>Sep 2017</td>
</tr>
</tbody>
</table>

AI, aromatase inhibitor; OFS, ovarian function suppression; ORR, objective response rate; PFS, progression free survival; PMP, post-menopausal; Pre-MP, pre-menopausal.
inhibitors clinically (Table 2) suggests that in general, endocrine-resistant tumours maintain sensitivity to CDK4/6 inhibition, particularly when used in combination with endocrine therapy. This has been retrospectively demonstrated for endocrine-resistant tumours with activating ESR1 mutations. In the PALOMA-3 trial, fulvestrant plus palbociclib improved PFS in patients with ESR1 mutant and ESR1 WT circulating tumour DNA (ctDNA), indicating that CDK4/6 inhibitors are effective irrespective of ESR1 mutation status (Fribbens et al. 2016).

In fact, certain manifestations of endocrine therapy resistance may sensitise breast cancer to CDK4/6 inhibitors. Deficiency of mismatch repair caused by MutL mutation in ER+ breast cancer abrogates CHK2-mediated inhibition of CDK4, leading to endocrine resistance. Consequently, CDK4/6 inhibitors are highly effective in MutL-defective ER+ breast cancer cells, and MutL could prove useful as a biomarker to identify patients suitable for CDK4/6 inhibitors (Haricharan et al. 2017). In a window of opportunity trial in patients with early stage breast cancer, palbociclib has been shown to downregulate an E2F signature associated with letrozole resistance, suggesting that this signature could be used to identify high-risk patients who should receive adjuvant CDK4/6 inhibitors in combination with endocrine therapy (Guerrero-Zotano et al. 2018). Finally, a non-canonical function of palbociclib is proteosomal inhibition via the ECM29 protein. High ECM29 is predictive of poorer relapse-free survival in patients receiving endocrine therapy, and thus, CDK4/6 inhibitor therapy may also prove particularly effective for these patients (Miettinen et al. 2018).

**Biomarkers of response to CDK4/6 inhibitors for ER+ breast cancer**

Patients with advanced ER+ breast cancer are pre-selected for CDK4/6 inhibitor therapy on the basis that these cancers generally have an intact Rb axis, and indeed the incidence of Rb gene deletion/mutation is very rare (3.9%) in ER+ breast cancer (Ciriello et al. 2015). Studies in preclinical models demonstrate a requirement of intact Rb for effective CDK4/6 inhibition, supporting its utility as a biomarker (Konecny et al. 2011, Thangavel et al. 2011). This finding has also been validated clinically (Karacas et al. 2016, Hunt et al. 2017). In an examination of the effects of palbociclib on unselected ex vivo tumour explant breast cancer models (Dean et al. 2012), the 2 of 13 models that were insensitive to palbociclib lacked Rb expression. Studies in glioblastoma cell lines (Michaud et al. 2010) and pancreatic cancer patient-derived xenograft (PDX) models have reached similar conclusions (Chou et al. 2017), with even the low expression of Rb sufficient to confer insensitivity to CDK4/6 inhibitors (Chou et al. 2017). The use of Rb as a biomarker of CDK4/6 inhibitor response in ER+ breast cancer could be further refined in combination with low molecular weight cyclin E1 (LMWE). LMWE is a promiscuous cytoplasmic cyclin E1 fragment (Karacas et al. 2016, Hunt et al. 2017) that when complexed with CDK2 can phosphorylate Rb in the presence of CDK4/6 inhibition (Doostan et al. 2017). Patients with Rb-/- LMWE+ cancers had the shortest PFS in a cohort of 109 patients treated with palbociclib and endocrine therapy (Vijayaraghavan et al. 2017).

Complicating the use of Rb as a biomarker is the fact that studies of other cancer types suggest that functional Rb is not an absolute requirement for CDK4/6 inhibitors to demonstrate an effect. In bladder cancer models, palbociclib was found to be as effective in Rb-mutant models as it was in Rb wild-type models, and transcriptome analysis identified FOXM1 as the likely target in the context of Rb mutation (Castellano et al. 2016). Conversely, the presence of Rb does not guarantee a response to CDK4/6 inhibition, as demonstrated by the poor outcomes of a recent phase II study of Rb+ patients with advanced oesophageal or gastric cancer (Karasic et al. 2018).

Whether or not Rb is absolutely required for CDK4/6 inhibitor functionality, it is regarded as the canonical target, and other indicators of intrinsic sensitivity are based on their relationship with Rb. The loss of p16INK4A is postulated to be a marker of sensitivity as this protein inhibits cyclin D1 (Witkiewicz et al. 2011). This is supported by two reported cases with homozygous deletion of the p16INK4A gene CDKN2A, a collecting duct carcinoma and a uterine leiomyosarcoma, where both were exceptional responders to palbociclib treatment (Elvin et al. 2017, Pal et al. 2017). This observation has not been borne out in other studies. Low p16INK4A did not predict response in a phase II study of palbociclib monotherapy in Rb+ breast tumours (DeMichele et al. 2015) or in the PALOMA-1 study of palbociclib with letrozole for ER+ breast cancer. In PALOMA-1, the treatment of patients with tumours harbouring CDKN2A loss had similar PFS compared to unselected patients when assessing combination palbociclib and letrozole treatment versus letrozole monotherapy (Finn et al. 2015). A potential explanation of the difference between the exceptional responders and the patients of the PALOMA-1 trial may be that the PALOMA-1 patients were selected for loss of heterozygosity rather than homozygous deletion.
Conversely, elevated p16INK4A could be associated with reduced CDK4/6 inhibitor sensitivity as p16INK4A is often elevated in the absence of functional Rb (Witkiewicz et al. 2011). For example, Rb-/p16INK4A elevated tumours had reduced sensitivity in the study of explant breast cancers (Dean et al. 2012). The NeoPalAna trial (neoadjuvant anastrozole with or without palbociclib) identified another INK4 protein, p19INK4D, which was elevated in gene-expression analysis across patients with tumours intrinsically resistant to palbociclib (Hunt et al. 2017). One possible explanation may be that p19INK4D is transcribed downstream of E2F activity due to non-functional Rb, and further E2F targets, cyclin D3 and CDKN2D were also identified in the patients with refractory tumours. The same group subsequently identified that another E2F target, thymidine kinase 1, may be useful as a pharmacodynamic biomarker to monitor the initial patient response to CDK4/6 inhibitors (Baggini et al. 2017).

The PALOMA-1 study assessed CCND1 amplification as a biomarker for use of CDK4/6 inhibitors, but patients receiving palbociclib plus letrozole showed the same improvement regardless of CCND1 amplification status (Finn et al. 2015). However, a systematic screen of 560 cancer cells identified cell lines that were highly sensitive to abemaciclib had high cyclin D-CDK4/6 function or ‘D-cyclin-activating features’ (Gong et al. 2017). Notably absent from these was CCND1 amplification, but instead it included CCND2/CCND3 amplification, CCND1 translocation or 3’ UTR loss, and loss of FBX031, which drives the turnover of cyclin D1. While this study confirmed the important role of cyclin D1 in CDK4/6 inhibitor function, it did not identify a suitable single biomarker for CDK4/6 inhibitor sensitivity, including cyclin D1 itself.

With the dearth of suitable single biomarkers for CDK4/6 inhibitor resistance, researchers are now assembling signatures of sensitivity. The ‘D-cyclin-activating features’ signature described above is one such example, and a similar approach is the RBsig signature, a gene expression signature of Rb loss of function derived from E2F1 and E2F2 expression in breast cancers that can predict cell lines with sensitivity to palbociclib (Malorni et al. 2016). An alternative approach is a gene signature for inactive CDK4, which predicted insensitivity to palbociclib across different cell lines (Raspe et al. 2017). This 11 gene expression signature is currently being validated in the NeoRHEA phase II trial (NCT03065621), in biopsies before treatment and following four cycles of neo-adjuvant, pre-operative treatment with palbociclib and endocrine therapy.

The emerging tide of CDK4/6 inhibitor resistance in ER+ breast cancer

The clinical trials on CDK4/6 inhibitors, while highly successful at increasing PFS, have universally yet to demonstrate significant improvement in overall survival. This has slowed the uptake of CDK4/6 inhibitors worldwide, as health economic analyses conclude that the current high costs lead to poor cost-effectiveness ratios for CDK4/6 inhibitor use (Mamiya et al. 2017). Despite this, governments are still being lobbied to support their cost, as their advantages of being orally available with a relatively low toxicity profile means that they provide a tangible improvement in quality of life for patients with advanced ER+ breast cancer. The development of resistance to CDK4/6 inhibitors is inevitable (Xu et al. 2017, Condorelli et al. 2018). In the reported trials that have led to FDA and EMA approval, at least 1/3 of patients recurred on CDK4/6 inhibitors within 2 years, and in the PALOMA-2 trial >70% of patients treated with the palbociclib plus letrozole had progressive disease by 40 months (Finn et al. 2016).

Current knowledge of the molecular mechanisms of CDK4/6 inhibitor resistance is far from complete and is largely based in single agent studies using cell line models. However, several studies have pointed to the acquisition of resistance being a multi-step process: first cells can undergo adaptive changes that may affect durability of response, and this is later followed by long-term acquisition of hard-wired resistance mechanisms (Herrera-Abreu et al. 2016, Martin et al. 2017). An important factor in considering these pre-clinical findings is that CDK4/6 inhibition in ER+ breast cancer will predominantly occur in the context of endocrine therapy treatment and/or resistance (Fig. 2).

Short-term adaptation to CDK4/6 inhibitors

CDK4/6 inhibition results in an immediate and profound G1/G0, cell cycle arrest in Rb+ cells (Fry et al. 2004), but in some cancer models, this wanes within several days. This ‘adaptive response’ is postulated to play a role in the acquisition of resistance or at least in the durability of therapeutic response. In ER+ breast cancer cell lines acutely exposed to palbociclib, cell cycle inhibition was temporary; Rb phosphorylation and markers of S phase entry returned within 72h of first exposure, including increased expression of cyclin D1 (Herrera-Abreu et al. 2016). Non-canonical complexes of cyclin D1 and CDK2 were observed and proposed to be the cause of continued Rb phosphorylation. Increased CDK2 activity was also
observed in acute myeloid leukaemia cells after 96 h of palbociclib treatment, and in this model, the increase in CDK2 activity correlated with a decrease in the p27<sup>kip1</sup> inhibitor protein (Wang et al. 2007).

Interestingly, combination therapies have been shown to inhibit this adaptive response. In breast cancer cells, the addition of a PI3K inhibitor to palbociclib delayed the resumption of S phase entry and abrogated the accumulation of cyclin D1, consistent with the role of the PI3K pathway in promoting cyclin D1 expression (Herrera-Abreu et al. 2016). In an independent study, mTOR pathway inhibition synergised with CDK4/6 inhibition to prevent resumption of proliferation of breast cancer cells, and the combination therapy induced a significant downregulation of E2F target genes (Michaloglou et al. 2018). Finally, endocrine therapy co-treatment with CDK4/6 inhibitors is able to suppress the activation of cell metabolism and cell growth in breast cancer cells (Knudsen & Witkiewicz 2016).

CDK4/6 inhibition mediates cell senescence, as defined by a prolonged proliferation arrest in combination with molecular markers such as β-galactosidase (Bollard et al. 2017). A major CDK4/6 target is FOXM1 (Anders et al. 2011), and when CDK4/6 is inhibited, the hypophosphorylated forms of FOXM1 promote a program of senescence (Sharpless & Sherr 2015). Consequently, the durability of response to CDK4/6 inhibition may affect whether or not cells become senescent with treatment. The program of senescence induced by CDK4/6 inhibitors can be augmented through co-treatment to inhibit other pathways. For example, reduced mTOR signalling can augment entry into senescence induced by CDK4/6 inhibition (Yoshida et al. 2016) and autophagy inhibitors in combination with CDK4/6 inhibitors can augment senescence (Karakas et al. 2016, Hunt et al. 2017, Valenzuela et al. 2017).

Effective induction of senescence by CDK4/6 inhibitors could potentially avert long-term resistance, but equally, it appears that the induction of a G<sub>1</sub> arrest without senescence allows for better synergy with other classes of therapies. For example, palbociclib treatment of melanoma cells for more than 3 days led to the induction of senescence in association with a decreased sensitivity to vemurafenib (Yoshida et al. 2016). Likewise, Rb-expressing sarcoma cell lines with reversible palbociclib-induced cell cycle arrest were sensitive to co-treatment with the WEE1 kinase inhibitor AZD1775 (Francis et al. 2017). Unfortunately, CDK4/6 inhibitor-induced senescence may also result in an undesirable outcome in the stroma through the promotion of a proinflammatory, senescence-associated secretory phenotype (SASP). This SASP phenotype could augment insensitivity to CDK4/6 inhibitors: when palbociclib-induced senescent fibroblasts were co-injected with melanoma cells into an immune-proficient, syngeneic mouse model, it resulted in accelerated tumour growth (Guan et al. 2017).

**Long-term acquisition of CDK4/6 inhibitor resistance**

Prolonged exposure to CDK4/6 inhibitors eventually gives rise to resistant cell populations that undergo hard-wired changes that are distinct to ‘the adaptive response’. A handful of mechanisms have been described in this context, including the loss or mutation of Rb, changes to CDK4/6 and CDK2 signalling and activation of growth signalling pathways. So far, no reports of mutations in either CDK4 or CDK6 that reduce the affinity of CDK4/6 inhibitors have emerged. However, this remains a plausible route for the development of resistance and with the advent of CDK4/6 now being standard of care in the first-line setting, it is likely that previously undescribed mechanisms of CDK4/6i resistance will begin to emerge in the clinic.

**Loss or mutation of Rb**

By far the most frequently observed change in cells resistant to CDK4/6 inhibitors is loss or mutation of the Rb protein. This is observed in multiple cell line models from different tumour types (Taylor-Harding et al. 2015, Herrera-Abreu et al. 2016, Bollard et al. 2017).
Of particular interest is a PDX model exposed to chronic ribociclib treatment that developed partial treatment resistance concurrent with the clonal expansion of a pre-existing Rb-null population (Herrera-Abreu et al. 2016). Interestingly, the parental PDX model was derived from a patient who had been previously treated with endocrine therapy and harboured an activating ESR1 mutation (Y537S), a mutation in TP53 and loss of genes encoding p16INK4a, p15INK4b and p14ARF. Despite these changes, presumably acquired in response to endocrine therapy, the model responded initially to palbociclib treatment. Following on from these pre-clinical results, the first examples of putative attenuation of Rb function through the emergence of potentially deleterious Rb mutations in ctDNA, acquired during the development of resistance to CDK4/6 inhibitors in patients, have begun to emerge (Xu et al. 2017, Condorelli et al. 2018).

**Activation of CDK4/6 signalling**

Another mechanism of resistance to CDK4/6 inhibition is the upregulation of CDK4 or CDK6 and their cognate cyclins. While CDK6 amplification has been reported (Yang et al. 2017), CDK4 amplification has not been detected in resistance models. Both high and low expression of CDK4 has been noted in resistance models (Bollard et al. 2017, Martin et al. 2017). This may be because while some expression of CDK4 or CDK6 is required for sensitivity to CDK4/6 inhibition, high level amplification of CDK4 on the other hand, as seen in rhabdomyosarcoma, can lead to reduced sensitivity to palbociclib (Olanich et al. 2015). Cyclins D1 and D2 are also upregulated in models of CDK4/6 inhibitor resistance (Taylor-Harding et al. 2015, Jansen et al. 2017, Martin et al. 2017) and high cyclin D3 was observed in patients with ER+ tumours that did not respond to palbociclib (Hunt et al. 2017). Resistance could occur by either non-canonical activation of CDK2 (Herrera-Abreu et al. 2016) or through formation of cyclin D3-CDK4/6 complexes, which appear to phosphorylate Rb even in the presence of synthetic CDK4/6 inhibitors (Paternot et al. 2014).

**Activation of CDK2 signalling**

In normally cycling cells, cyclin E-CDK2 (cyclin E1-CDK2 or cyclin E2-CDK2) complexes phosphorylate Rb subsequent to phosphorylation by cyclin D-CDK4/6, as part of a second wave of signalling. CDK4/6 inhibition has multiple inhibitory effects on CDK2 action. Without the priming of Rb by cyclin D1-CDK4/6 phosphorylation, endogenous levels of cyclin E-CDK2 complexes cannot efficiently phosphorylate Rb to release E2F transcription factors. Cyclin E2 is a transcriptional target of E2F, and hence cyclin E2-CDK2 complexes are also reduced after CDK4/6 inhibition (Caldon et al. 2009). Finally, cyclin E1-CDK2 complexes probably have suppressed activity from the redistribution of p21Waf1/Cip1 and p27Kip1 inhibitors after depletion of cyclin E2-CDK2 complexes (Caldon et al. 2009). In this context, the upregulation of cyclins E1 or E2, or downregulation of their inhibitors can subvert CDK4/6 inhibition.

Cyclin E1, cyclin E2 and CDK2 are upregulated in CDK4/6 inhibitor resistance models (Taylor-Harding et al. 2015, Herrera-Abreu et al. 2016, Bollard et al. 2017, Martin et al. 2017, Yang et al. 2017). Mechanistically this can occur through CCNE1 gene (which encodes cyclin E1) amplification in a single agent CDK4/6 resistant model (Herrera-Abreu et al. 2016), and CCNE2 gene (which encodes cyclin E2) amplification in a combination endocrine therapy/palbociclib resistance model (Martin et al. 2017). Ablation of either cyclin E1 or CDK2 resensitised resistant cells to palbociclib-induced cell cycle arrest (Herrera-Abreu et al. 2016). There are currently no specific CDK2 inhibitors available for clinical use, but newer CDK2 inhibitors currently in development could potentially have a role in CDK4/6 inhibitor-resistant tumours (Caldon et al. 2012).

**Growth factor signalling pathways**

Rb, CDK4/6 and CDK2 are all conduits for growth regulatory signalling pathways to upregulate cell growth and cell cycle progression, and it is not surprising that several signalling pathways can be deregulated to overcome CDK4/6 inhibitors. A kinome-wide siRNA screen identified the PI3K pathway kinase, PDK1, was highly expressed in ribociclib-resistant cells, and sensitised cells to ribociclib (Jansen et al. 2017). While no changes in mTOR signalling components have been documented in long-term resistance models, CDK4/6 inhibitor-resistant cell lines have demonstrated sensitivity to mTORC1/2 inhibition (Michaloglou et al. 2018). Other signalling changes include NRAS amplification or mutation in an NRAS model of melanoma co-treated with MEK1 inhibition and CDK4/6 inhibition (Teh et al. 2018), and alterations in FGF/FGFR signalling in resistance to CDK4/6 inhibitors both alone and in combination with endocrine therapy (Cruz et al. 2018, Formisano et al. 2018, Mao et al. 2018, Shee et al. 2018). Finally, an activating mutation in PIK3CAE545K, was shown to display a resistance phenotype to combination.

https://doi.org/10.1530/ERC-18-0317

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MEK and CDK4/6 inhibitors, and the outgrowth of a clone expressing this mutation caused recurrence in a melanoma patient treated with these inhibitors (Romano et al. 2018).

**Strategies to avert and overcome CDK4/6 inhibitor resistance**

The most frequently detected mechanism of resistance, loss or mutation of Rb, is unfortunately not amenable to targeted therapy. In this situation chemotherapy may be a renewed option, as Rb disruption sensitises tumours to chemotherapy (Zagorski et al. 2007, Witkiewicz et al. 2012), although the efficacy of this is very dependent upon the combination of tumour type and drug mode of action. Also, CDK4/6 inhibitors can be antagonistic when combined with chemotherapy, especially those with an anti-mitotic effect (Franco et al. 2014, Yoshida et al. 2016), and therefore a cautious approach should be taken with such combinations. As more patients present clinically, these therapies can be systematically assessed to determine if they prolong survival effectively.

The clinical efficacy of endocrine therapy doublet combination therapy with mTOR, PI3K, and CDK4/6 inhibitors (Baselga et al. 2012, Finn et al. 2015, Cristofanilli et al. 2016, Finn et al. 2016, Baselga et al. 2017, Goetz et al. 2017, Sledge et al. 2017, Slamon et al. 2018), and the cross talk between these pathways (Fig. 2), have led to the logical development of clinical trials of triplet therapy combinations of CDK4/6 and PI3K pathway inhibitors with an endocrine therapy backbone (Table 3). Further supporting this strategy is data that has demonstrated that CDK4/6 inhibitors can sensitize PIK3CA mutant tumours to PI3K inhibitors (Vora et al. 2014), and the converse, that CDK4/6 resistant cells have been shown to be sensitive to mTORC1/2 inhibition (Michaloglou et al. 2018). Finally, combined targeting of CDK4/6 and PI3K pathways resulted in greater tumour regression compared with PI3K or CDK4/6 inhibition alone; and triplet therapy with CDK4/6 and PI3K inhibitors was more effective than dual therapy with respect to tumour regression (O’Brien et al. 2014, Herrera-Abreu et al. 2016). In two recent studies, cohorts of heavily pre-treated patients who had received everolimus obtained limited benefit from the addition of palbociclib, suggesting that CDK4/6 inhibitors should be used prior to or concurrently with drugs targeting the PI3K pathway (Dhakal et al. 2018, du Rusquec et al. 2018). The multiple types of PI3K inhibitors in clinical development, combined with the three lead CDK4/6 inhibitors, multiple classes of ER-directed therapies, and next generation selective ER degraders, in different lines of therapy, have resulted in a large number of combinations and permutations, creating a challenge when determining the optimal therapeutic strategy for a given patient. Further complicating matters are the potential for overlapping toxicity and financial implications.

Finally, CDK4/6 inhibitors have also been shown to enhance both tumour antigen presentation, T cell activation and the efficacy of anti-PD-1 immunotherapy,
representing another potential therapeutic combination for this class of drug (Goel et al. 2017, Deng et al. 2018).

**The future of CDK4/6 inhibitor use and resistance in ER+ breast cancer**

The addition of CDK/6 inhibitors into contemporary treatment algorithms for advanced ER+ breast cancer represents a renaissance for the most common subtype of breast cancer and represents the most significant advance in the last decade. While CDK4/6 inhibitors have changed the natural history of ER+ breast cancer by prolonging the PFS, when used in the metastatic context, disease progression and the emergence of resistance is inevitable. As they become standard therapy, combined resistance to endocrine and CDK4/6 inhibitor therapy represents the next wave of clinical challenge to face the breast cancer community. To benefit these patients, we need a detailed mechanistic understanding of CDK4/6 inhibitor resistance in an endocrine sensitive and resistant setting.

The full potential of CDK4/6 inhibitors has yet to be realised, and trials are currently underway to expand its use to other breast cancer subtypes, earlier stages of disease and other cancers. With the success of CDK4/6 inhibitors in advanced ER+ breast cancer, it is now logically being evaluated in early stage ER+ breast cancer. A series of phase II and phase III adjuvant and neoadjuvant trials with CDK4/6 inhibitors are currently underway (NEOLBC (NCT03283384), neoMONARCH (NCT02441946), PALLET (NCT02296801), SAFIA (NCT03447132), monarchE (NCT03155997) and PALLAS (NCT02513394)) (Table 4). The exclusion of HER2+ patients is also being reassessed after the positive results of the abemaciclib monotherapy trial (Patnaik et al. 2016), where 36% (4/11) of ER+ HER2+ patients showed a response compared to 28% (7/25) of ER+ HER2- patients, and several trials such as PATINA (NCT02947685), PATRICIA (NCT03447132), monarchE (NCT03155997) and PALLAS (NCT02513394) are evaluating combinations of CDK4/6 inhibitors, anti-HER2 (trastuzumab, pertuzumab) and endocrine therapy. These emerging treatment scenarios will have different genetic backgrounds and selective drug pressures, potentially giving rise to unique resistance mechanisms in each treatment type.

### Table 4 Ongoing clinical trials of CDK4/6 inhibitors in early stage breast cancer.

<table>
<thead>
<tr>
<th>Study</th>
<th>NCT no.</th>
<th>Estimated enrolment</th>
<th>Breast cancer subtype</th>
<th>Treatment stage</th>
<th>Treatment</th>
<th>Primary outcome measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase III SAFIA</td>
<td>03447132</td>
<td>400</td>
<td>HR+ HER2−</td>
<td>Neo-adjuvant</td>
<td>Palbociclib + fulvestrant vs placebo + fulvestrant</td>
<td>pCR rate</td>
</tr>
<tr>
<td>PALLAS</td>
<td>02513394</td>
<td>5600</td>
<td>HR+ HER2−</td>
<td>Following neo-adjuvant chemotherapy</td>
<td>Palbociclib + ET vs ET</td>
<td>IDFS</td>
</tr>
<tr>
<td>PENELOPE-B</td>
<td>01864746</td>
<td>1250*</td>
<td>HR+</td>
<td>Following neo-adjuvant chemotherapy</td>
<td>Palbociclib + ET vs placebo</td>
<td>IDFS</td>
</tr>
<tr>
<td>EarLEE-1</td>
<td>03078751</td>
<td>52*</td>
<td>HR+ HER2− (high risk)</td>
<td>Adjuvant</td>
<td>Ribociclib + ET vs placebo + ET</td>
<td>IDFS</td>
</tr>
<tr>
<td>monarchE</td>
<td>03155997</td>
<td>3580</td>
<td>HR+ HER2− (high risk)</td>
<td>Adjuvant</td>
<td>Abemaciclib + ET vs ET</td>
<td>IDFS</td>
</tr>
<tr>
<td>Phase II NEOBLC</td>
<td>03283384</td>
<td>100</td>
<td>HR+ HER2−</td>
<td>Neo-adjuvant, stage II or III</td>
<td>AI followed by chemotherapy vs AI + ribociclib</td>
<td>CCCA</td>
</tr>
<tr>
<td>neoMONARCH</td>
<td>02441946</td>
<td>224*</td>
<td>HR+ HER2−</td>
<td>Neo-adjuvant</td>
<td>AI vs abemaciclib vs AI + abemaciclib</td>
<td>Change in Ki67 expression</td>
</tr>
<tr>
<td>PALLET</td>
<td>02296801</td>
<td>306</td>
<td>HR+ HER2−</td>
<td>Neo-adjuvant</td>
<td>AI vs AI then AI + palbociclib vs palbociclib then AI + palbociclib vs AI + palbociclib</td>
<td>Change in Ki67 expression and cCR</td>
</tr>
</tbody>
</table>

AI, aromatase inhibitor; CCCA, complete cell cycle arrest; cCR, clinical complete response; ET, endocrine therapy; HR, hormone receptor; IDFS, invasive disease-free survival; pCR, pathological complete response. * actual enrolment.
**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

**Funding**

E L and N P receive research funding from Novartis Oncology and Bayer. E L and C E C are supported by the National Breast Cancer Foundation. E L is supported by the Love Your Sister.

**Author contribution statement**

N Portman, S Alexandrou, E Lim and C E Caldon contributed equally to this work.

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CDK4/6 inhibitor resistance in breast cancer


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Received in final form 5 September 2018
Accepted 17 September 2018
Accepted Preprint published online 18 September 2018