Multiple effects of CDK4/6 inhibition in cancer: From cell cycle arrest to immunomodulation

Commentary

Multiple effects of CDK4/6 inhibition in cancer: From cell cycle arrest to immunomodulation

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Abstract

Dysregulation of the cell cycle is a hallmark of cancer that leads to aberrant cellular proliferation. CDK4/6 are cyclin-dependent kinases activated in response to proliferative signaling, which induce RB hyper-phosphorylation and hence activation of E2F transcription factors, thus promoting cell cycle progression through the S phase.Pharmacologic inhibition of CDK4/6 by palbociclib, ribociclib, or abemaciclib has been showing promising activity in multiple cancers with the best results achieved in combination with other agents. Indeed, CDK4/6 inhibitors are currently approved in combination with endocrine therapy for the treatment of estrogen receptor-positive, human epidermal growth factor receptor 2-negative advanced or metastatic breast cancer. Moreover, a number of clinical trials are currently underway to test the efficacy of combining CDK4/6 inhibitors with different drugs not only in breast but also in other types of cancer.Beyond the inhibition of cell proliferation, CDK4/6 inhibitors have recently revealed new effects on cancer cells and on tumor microenvironment. In particular, it has been reported that these agents induce a senescent-like phenotype, impact on cell metabolism and exert both immunomodulatory and immunogenic effects. Here we describe recent data on the anti-tumor effects of CDK4/6 inhibitors as single agents or in combined therapies, focusing in particular on their metabolic and immunomodulatory activities.

Abbreviations: AMPK, AMP-activated protein kinase; BC, breast cancer; CCND, cyclin D; CCNE, cyclin E; CDKs, Cyclin-Dependent Kinases; DNMT1, type 1 DNA methyl-tranferase; ER, estrogen receptor; FA, fatty acids; FDA, Food and Drug Administration; GLN, glutamine; GLUT, glucose transporter; HCC, hepatocellular carcinoma; HNSCC, Head and Neck Squamous Cell Carcinoma; HR, hormone receptor; HER2, human epidermal growth factor receptor 2; IFN, interferon; MHC, major histocompatibility complex; NET, neutrophil extracellular traps; NSCLC, non-small cell lung cancer; OXPHOS, oxidative phosphorylation; PDA, pancreatic ductal adenocarcinoma; PDK1, pyruvate dehydrogenase kinase; PD-1, programmed cell death protein 1; PD-L1, programmed cell death protein 1 ligand; PPP, pentose phosphate pathway; RB, Retinoblastoma protein; SASP, senescence-associated secretory phenotype; SPOP, Speckle-type POZ protein; T-ALL, T-cell acute lymphoblastic leukemia; TCA, tricarboxylic acid; TNBC, triple negative breast cancer

Keywords: CDK4/6 inhibitors; Cell cycle; Senescence; Metabolism; Immune system

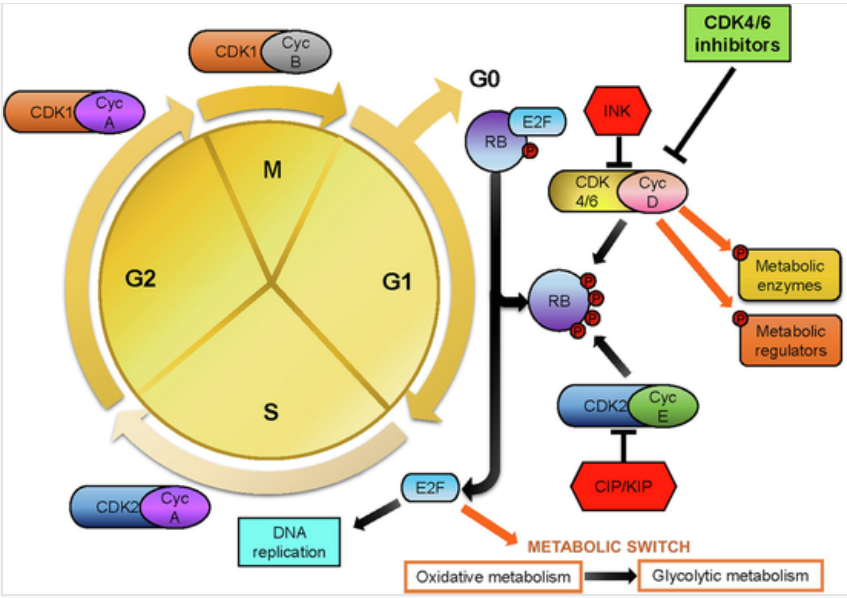
1 Introduction

Many human cancers harbor alterations in the CDK4/6-cyclin D-RB-E2F pathway and targeting cyclin-dependent kinases (CDK)/cyclin complexes has emerged as an effective strategy for cancer therapy. Palbociclib (PD0332991), ribociclib (LEE011), and abemaciclib (LY2835219) are selective CDK4/6 inhibitors that block tumor suppressor retinoblastoma protein (RB) phosphorylation with induction of cell cycle arrest. Although CDK4/6 inhibitors have been shown to induce apoptosis in some types of tumors [1,2], their anti-tumor activity is generally associated with the inhibition of cell cycle and proliferation. However, beyond their cytostatic effect, these drugs have recently revealed new non-canonical functions, inducing a senescent-like phenotype, altering cell metabolism, and influencing the immune system and the tumor microenvironment. Here, we mainly focus on their metabolic and immunomodulatory activities.

1.1 Cell cycle control

Cell cycle progression is sustained by a variety of molecules such as growth factors and hormones and involves a series of tightly regulated molecular events that culminate in cell division. In particular, the cyclin proteins, binding to and activating their kinase partners, the CDKs, drive the progression through the cell cycle [3] ~~(Fig. 1).~~(Figure 1).

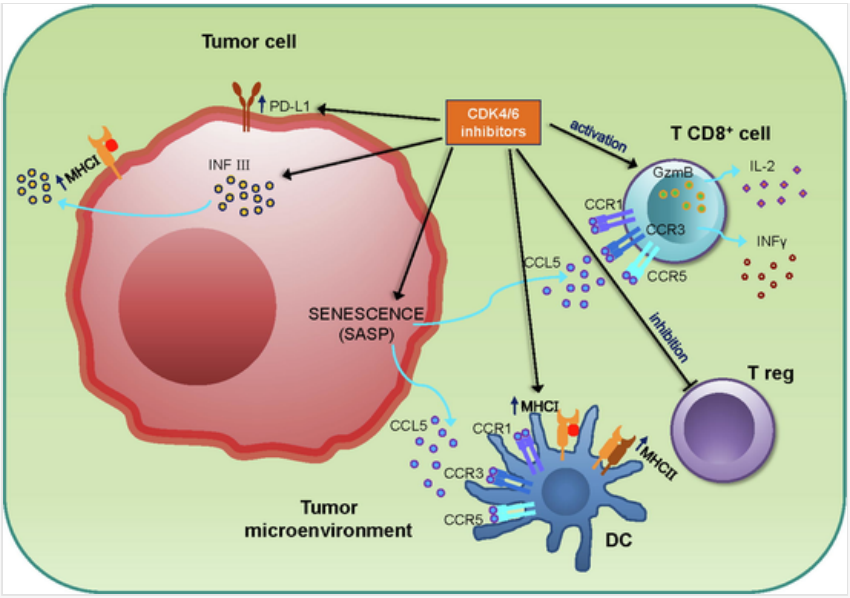
Fig. 1



Interplay between cell cycle regulation and metabolism. CDK4/6-Cyclin D complex promotes the progression of the cell cycle towards the S phase, through the release of E2F. Beside the regulation of cell cycle, CDK4/6-Cyclin D complex and E2F exert effects on cell metabolism, modulating the expression/activity of metabolic enzymes and of metabolic regulators. CDK4/6 inhibitors impact on both cell cycle progression and metabolism.

A series of checkpoints block the cells from progressing into the next phase of the cell cycle ~~(Fig. 1)~~. The first checkpoint, also known as restriction point, occurs at G1-S transition and after that, the completion of the cell cycle is independent of mitogen stimulation. During G1 phase, in response to mitogenic stimuli, cells synthetize Cyclin D proteins (Cyclin D1, D2 and D3) that promote the CDK4 and CDK6 activation. The CDK4/6-Cyclin D complexes catalyse the mono-phosphorylation of RB, a protein that in the hypo-phosphorylated state binds and inhibits E2F family transcription factors. The consequent production of Cyclin E promotes the formation of the CDK2-Cyclin E complex, which in turn hyper-phosphorylates RB at all the 14 sites. At this point, E2F dissociates from RB leading to the transcription of genes required for progression into S phase, including the Cyclin E gene (*CCNE*) and c-*myc* [4]. The CDK2-Cyclin A and the CDK1-Cyclin A complexes are required for S-G2 transition, while the second checkpoint (G2-M) is controlled by the CDK1-Cyclin B complex ~~(Fig. 2)~~.

Fig. 2



Cross talk between CDK4/6 inhibition and immune system. CDK4/6 inhibitors induce favorable conditions for immune-surveillance and effective immunotherapy through multiple mechanisms: induction of antigen processing and PD-L1 expression on tumor cells, recruitment and activation of T lymphocytes, depletion of T regulatory cells, and enhancement of antigen presentation capability by dendritic cells (DCs).

CDKs activity is also negatively regulated by the INK4 and CIP/KIP families of proteins. The former includes $p16^{INK4A}$, $p15^{INK4C}$, $p18^{INK4C}$, and $p19^{INK4D}$, which inhibit the ability of CDK4/6-Cyclin D complexes to phosphorylate RB leading to G1 cell cycle arrest. The CIP/KIP family includes $p21^{CIP1}$ and $p27^{KIP1}$. $p21^{CIP1}$ interferes with CDK2-Cyclin E activity and is an important transcriptional target of p53 mediating the DNA-damage-induced cell cycle arrest in G1 and G2; $p27^{KIP1}$ inhibits the activity of CDK4-Cyclin D and CDK2-Cyclin E complexes at the G1 phase [5].

1.2 Dysregulation of CDK4/6-cyclin D-RB-E2F pathway in cancer

The CDK4/6-Cyclin D-RB-E2F pathway is one of the most frequently dysregulated in cancer, indeed about 40% of human tumors display alterations in cyclins, CDKs, or in CDK modulators.

Cyclin D1 gene (*CCND1*) amplification and protein overexpression have been observed in more than 50% of esophageal carcinomas and, less frequently, in head and neck, bladder, hepatocellular and endometrial carcinomas, cholangiocarcinomas, melanomas, breast and lung cancers. *CDK4* gene amplification has been found in numerous types of cancer including sarcoma, malignant glioma, and melanoma, whereas amplification of *CDK6* has been found in esophageal, gastric, head and neck, and pancreatic carcinomas [6,7].

In addition, a large number of cancers show genetic alterations leading to a constitutive activation of mitogenic signaling pathways, such as the RAS/RAF/MEK/ERK and the PI3K/AKT/mTOR pathways, which can induce an increase in Cyclin D level with subsequent CDK4/6 activation.

Inactivation of $p16^{INK4A}$ can occur through *CDKN2A* deletion, methylation, or mutation, and is a frequent event in many tumors. Indeed, *CDKN2A* alterations have been found in more than 30% of cases of esophageal carcinoma, pleural mesothelioma, glioblastoma, head and neck carcinoma, pancreatic adenocarcinoma, lung

squamous cell and bladder carcinoma, melanoma and large B-Cell lymphoma. Aberrations in the *RB1* tumor suppressor gene are documented in about 25% of sarcomas and bladder carcinomas and in about 10% of endometria, hepatocellular, esophageal, cervical squamous cell carcinomas, prostate adenocarcinomas, ovarian tumors, and triple negative breast cancers (TNBCs) [6–8]. Commonly, the *RB1* mutations/loss are mutually exclusive with other cell cycle gene aberrations, such as *CDKN2A* and *CDK4/6* alterations [9].

1.3 CDK4/6 inhibitors

Since cell cycle gene abnormalities are common in solid tumors, in recent years, small molecule inhibitors of CDK4/6 pathway have been developed. The three highly selective reversible inhibitors palbociclib, ribociclib, and amebaciclib bind to CDK4 and CDK6 ATP-binding pocket, leading to the inactivation of CDK4/6-Cyclin D complexes with subsequent inhibition of RB phosphorylation and induction of G1 phase arrest.

These drugs are orally administered, once daily for palbociclib and ribociclib, and twice daily for abemaciclib, and undergo hepatic metabolism. Palbociclib and ribociclib were approved by the Food and Drug Administration (FDA) in February 2015 and March 2017, respectively, in combination with the aromatase inhibitor letrozole for the treatment of hormone receptor-positive (HR^+), human epidermal growth factor receptor 2-negative ($HER2^-$) advanced or metastatic breast cancer (BC) as first-line treatment in postmenopausal women. The approval was based on data from two international, randomized, double-blind, placebo-controlled, clinical trials: PALOMA-2 (palbociclib plus letrozole vs placebo plus letrozole) and MONALEESA-2 (ribociclib plus letrozole vs placebo plus letrozole) [10,11].

As single agents they are fundamentally cytostatic, and limited clinical benefit has been reported in clinical trials when tested as monotherapy in different cancer types. Abemaciclib, being more potent than the two other compounds with higher selectivity for CDK4 and CDK9 [12], has shown activity also as a single agent. Based on the data from MONARCH-1 [13] and MONARCH-2 [14] trials, in September 2017 abemaciclib received FDA approval as monotherapy or in combination with fulvestrant for the treatment of HR^+, $HER2^-$ metastatic BC with disease progression following endocrine therapy. In February 2018, following the positive results of the MONARCH-3 trial [15], abemaciclib was also approved in combination with an aromatase inhibitor as initial endocrine-based therapy for postmenopausal women with HR^+, $HER2^-$ advanced or metastatic BC.

The cross talk between the CDK4/6 pathway and different mitogenic signaling pathways has provided a rationale for therapeutic strategies based on the combination of CDK4/6 inhibitors with other targeted therapies, as recently reviewed [16].

1.4 Biomarkers of responsiveness or resistance to CDK4/6 inhibitors

Given that in the absence of RB there is no requirement for CDK4/6 to activate E2F-regulated genes for progression to S phase, a functional RB protein is considered a prerequisite for responsiveness to CDK4/6 inhibitors and lack of RB expression has been generally associated with intrinsic resistance [17].

In contrast with the results derived from preclinical studies [18,19], clinical data demonstrated that *CCND1* amplification and loss of *CDKN2A* were not correlated with sensitivity to palbociclib in BC [10,20,21]. However, a specific group of alternative genomic features, termed “D-Cyclin Activated Features”, including

Cyclin D alterations, Cyclin K loss and F-box protein 31 loss, has been reported to correlate with sensitivity to abemaciclib in different types of cancer [22].

On the other hand, the *CCNE1/RB1* ratio has been recently considered as a possible "pan-cancer" biomarker of CDK4/6 intrinsic resistance to palbociclib [23]. In cell lines derived from different cancer types (pan-cancer dataset), *CCNE1/RB1* significantly correlated, better than *CCNE1* or *RB1* alone, with higher IC_{50} values for palbociclib. It is of interest that the *CCNE1/RB1* ratio was shown to be able to discriminate palbociclib sensitivity versus resistance among patients enrolled in the NeoPalAna trial.

Different mechanisms responsible for acquired resistance after prolonged treatment with CDK4/6 inhibitors have been reported, including acquired *RB1* mutations, loss of *RB1*, loss of function mutations of *FAT-1*, *CCNE1* overexpression, *CDK6* overexpression and *CCNE1/RB1* ratio. The role of RB in the response to CDK4/6 inhibitors and intrinsic/acquired mechanisms of resistance have been extensively reviewed elsewhere [17,24,25].

2 Non-canonical effects of CDK4/6 inhibitors

2.1 Induction of senescence

Palbociclib has been reported to induce cellular quiescence or a senescence-like state in different cell types [26–29] and the transition from quiescence to senescence is known as geroconversion [30]. Senescence is a form of permanent growth arrest characterized by morphological changes, senescence-associated beta-galactosidase activity, presence of senescence associated heterochromatin foci, and production of growth factors, cytokines, proteases, and other proteins and matrix-degrading molecules, collectively known as the senescence-associated secretory phenotype (SASP).

In normal cells, senescence is mainly sustained by the activity of the p53/$p21^{CIP1}$ and $p16^{INK4A}$/RB pathways [31]. Avoiding genomic instability, cellular senescence can be considered as a mechanism of suppression of tumorigenesis and the activation of senescence may be a promising novel approach for cancer treatment [32]. Nevertheless, SASP exerts multiple effects in the tumor microenvironment either inhibiting or stimulating tumorigenesis: SASP can induce the recruitment of immune cells and stimulate paracrine senescence in neighboring cells, or, conversely, it can induce the release of pro-tumorigenic factors and promote invasiveness [33,34].

Differently from classical senescence, senescence induced by CDK4/6 inhibitors may be reversible. Indeed, after a recovery period from treatment, cells from mesothelioma, glioma or hepatocarcinoma were shown to lose the senescent phenotype and re-acquire the ability to proliferate [35–37], suggesting that palbociclib induces a senescent-like quiescence rather than stable senescence. An alternative interpretation is that not all the cells exposed to palbociclib underwent to geroconversion, and, after drug removal, the cells in the quiescent state re-acquired the proliferative capability and progressively substituted the senescent cells. However, it is worth of note that in high-grade serous epithelial ovarian cancer cells a clonogenic assay demonstrated that senescence induced by PARP inhibitors was reverted in the majority of the cell population

and not only in those few cellular clones that escape senescence, indicating that might exist a true reversible senescent status [38].

All the CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib) are trapped into lysosomes and are slowly released extracellularly. The storage and the subsequent release of palbociclib from lysosomes may contribute to induce senescence in a paracrine manner and even cells resistant to palbociclib can release the drug and induce senescence in sensitive cells [39].

The mechanisms by which CDK4/6 inhibitors induce senescence have not been fully clarified. Recently, it has been proposed that proteasome hyper-activation by palbociclib may be involved in the induction of this senescence-like state in BC cells [40]. The authors reported that palbociclib stimulated the dissociation of the proteasomal-inhibitory protein ECM29 from the proteasome, resulting in increased proteolysis and stress induction. Co-treatment with the proteasome inhibitor bortezomib indeed suppressed cell cycle inhibition and senescence, confirming that proteasome activation is required for the maintenance of these phenotypes.

In liposarcoma cell lines, geroconversion in response to CDK4 inhibition was controlled by proteasomal degradation of MDM2 in a p53-independent manner; indeed p53 knock-down or mutant p53 did not affect geroconversion either in liposarcoma cells or in breast, glioma, and lung cancer cell lines [26]. Interestingly, MDM2 down-regulation was associated with positive response to palbociclib in clinic [26]. In addition, we previously demonstrated in mesothelioma cells that palbociclib promoted reversible senescence through a p53-independent, c-myc dependent induction of $p21^{CYP1}$ protein [35]. If senescence induced by CDK4/6 inhibitors is truly reversible, the re-emergence of tumor cells from senescent arrest may represent a hindrance to the efficacy of therapy. Combinatorial strategies with CDK4/6 inhibitors and signal transduction pathway inhibitors may represent a more effective anti-cancer approach. In this context, it is worth of note that mTOR inhibition can cooperate with CDK4/6 inhibition to potentiate senescence, and a prolonged sequential treatment induced an irreversible senescent status in mesothelioma cells [35].

However, considering that SASP may prompt senescent cells to secrete many factors that can modify the microenvironment favoring cancer progression, the elimination of senescent cells could be the best option for eradicating cancer. The selective killing of senescent cells by senolytic agents is known as senotherapy [41].

Recently, a versatile strategy has been proposed to deliver small compounds, including senolytic agents such as the Bcl-2 family inhibitor navitoclax [42], to senescent lesions *in vivo* [43]. In melanoma and lung tumor xenografts treated with palbociclib, senescent tumor cells were specifically targeted with gal-encapsulated navitoclax (GalNP(nav)), improving tumor regression. Importantly, GalNP(nav) had no effect in the absence of palbociclib, demonstrating its efficacy against senescent tumors but not growing tumors.

Collectively, these data suggest that senescence induced by CDK4/6 inhibitors is not a static condition but a dynamic feature that may influence in several ways both tumor and immune cells altering tumor progression.

2.2 Metabolic effects

2.2.1 Control of cell metabolism by cell cycle proteins

In order to divide, the cells must duplicate their cellular content, synthesizing large amounts of DNA, proteins, and lipids. A tight coordination between cell cycle and metabolism is therefore required to allow cell cycle entry and progression only under favorable nutrient conditions, and to ensure that the metabolic needs linked to specific stages of the cell cycle are satisfied. Dysregulation of this cross talk between cell cycle and metabolism has emerged as a relevant feature of cancer.

In cancer cells, some metabolic enzymes exhibit not only altered metabolic activities but also acquired non-canonical functions related to cell cycle progression [44]. Conversely, multiple components of the cell cycle machinery directly and indirectly control metabolic fluxes, likely contributing to the metabolic reprogramming associated with cancer. The CDK4/6-Cyclin D-RB-E2F pathway plays a relevant role in this regard ~~(Fig. 1)~~(. Figure 1).

Multiple lines of evidence indicate that the canonical effector of this pathway, E2F1, promotes a switch from oxidative to glycolytic metabolism through a variety of mechanisms. E2F1 upregulates the expression of the glycolytic phosphofructokinase 2 enzyme [45] and other glycolytic genes, while down-regulating the expression of oxidative genes [46]. Accordingly, in bladder and prostate cancer cell lines, E2F1 was shown to inhibit the expression of Sirtuin 6, a NAD(+)-dependent deacetylase that negatively regulates the transcription of glycolytic genes such as the glucose transporter GLUT1 and pyruvate dehydrogenase kinase 1 (PDK1) [47]. PDK1 is one of 4 kinases that inhibit pyruvate dehydrogenase, reducing the conversion of cytosolic pyruvate to mitochondrial acetyl-CoA and hence its entry into the tricarboxylic acid (TCA) cycle. In another study, PDK1 together with PDK3 isoform have been identified as direct targets of E2F1 in prostate cancer cells [48].

During hepatocellular carcinoma (HCC) progression, aberrant and sustained activation of E2F1 leads to the recruitment of a Pontin/Reptin complex that amplifies the E2F1 transcriptional response with the activation of low binding affinity E2F target genes (*Glut4*, *Pygb*, *Gsk3b*, *Pkm2*, *Pfkl* and *Mct1*) involved in non-cell-cycle functions, such as glycolysis and lactate export, thus contributing to the Warburg effect [49].

Interestingly, the effects of CDK4/6-Cyclin D-RB-E2F pathway on cell metabolism may be ascribed not only to the activity of E2F1, but also to the ability of CDK4 and 6 to directly target a number of proteins involved in metabolism, including metabolic enzymes, metabolic regulators, and molecules regulating the organismal energy homeostasis [50].

CDK4-Cyclin D1 complex has been demonstrated to inhibit the mitochondrial function by directly phosphorylating and repressing the nuclear respiratory factor 1, which controls the expression of nuclear-encoded mitochondrial genes [51]. Depletion of Cyclin D1 in mouse BC cells *in vivo* induced the expression of genes involved in mitochondrial function and biogenesis, increased hexokinase II expression promoting aerobic glycolysis, and activated lipogenesis. Consistently, a reciprocal pattern of gene modulation emerged in Cyclin D1-mediated mammary tumors, confirming that Cyclin D1 inhibits both mitochondrial activity and aerobic glycolysis as well as lipogenesis [52]. A further level of complexity is the evidence that these metabolic alterations do not necessarily require the interaction between Cyclin D1 and CDK4. Indeed, Cyclin D1, independently of CDK4, can compete with hexokinase II to bind to a voltage-dependent anion channel, inhibiting the mitochondrial function [53], and can directly bind to additional mitochondrial proteins and lipogenic enzymes, such as acetyl-CoA carboxylase and fatty acid synthase [54].

In addition to the direct phosphorylation of metabolic enzymes, CDK4 can also modulate cell metabolism indirectly by targeting metabolic regulators such as AMP-activated protein kinase (AMPK) [50]. In particular, CDK4 has been shown to inhibit AMPK activity via direct phosphorylation of the AMPK-α2 subunit, thereby promoting glycolysis and repressing fatty acid oxidation in mouse embryonic fibroblasts [55]. Accordingly, in cell models of human BC the CDK4/6-Cyclin D1 complex inhibited mitochondrial function, induced glycolysis, and restrained autophagy by downregulating AMPK through a mechanism involving phosphorylation of LKB1 [56].

2.2.2 Effects of CDK4/6 inhibitors on cell metabolism

Given the pivotal role of the CDK4/6-Cyclin D-RB-E2F pathway in coordinating the cell cycle with metabolic pathways, the inhibition of its activity by targeting the upstream CDK4/6 modulators may affect tumor growth through a dual inhibition of cell cycle progression and metabolism ~~(Table 1)~~(.Table 1).

Table 1

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Effects of CDK4/6 inhibitors alone or in combination on energy metabolism.

Drugs	Metabolic effects	Cancer types	Refs.
Palbociclib	↓Glycolysis	ER+/HER2-BC	[57]
	↓PPP		
	↓TCA intermediates		[58]
	↑Glycolysis		[59]
	↑GLN metabolism		
	↑OXPHOS		
	↓Glycolysis	TNBC	[64,67]
	↑Glycolysis	~~Pancreatic~~ PDA	[69]
	↑GLN metabolism		
	↑TCA intermediates		
	↑OXPHOS		
	↓Glycolysis	NSCLC	[70]
	↓PPP	T-ALL	[1]
Palbociclib + letrozole	↓Glycolysis	ER+/HER2- BC	[57]

	↓↓PPP		
	↓AA		
	↑Nucleotides		
	↑ FA		
Palbociclib + fulvestrant	↓↓TCA intermediates	ER+/HER2- BC	[58]
	↓AA		
	↓Nucleotides		
	↓Glycolysis		[59]
	↓GLN metabolism		
	↓OXPHOS		
Palbociclib + PI3K/AKT/mTOR inhibitors	↓↓Glycolysis	TNBC	[64]
	↓Glycolysis	Glioblastoma, ~~pancreatic~~ PDA	[68,69]
	↓OXPHOS		
Palbociclib + chemotherapy	↓↓Glycolysis	TNBC	[67]
Abemaciclib	↓TCA intermediates	ER+/HER2- BC	[60]
	↑Glycolysis	~~Pancreatic~~ PDA	[69]
	↑GLN metabolism		
	↑OXPHOS		
	↓Glycolysis	NSCLC	[71]
	↓GLN metabolism		
	↓AA		
Ribociclib	↑Glycolysis	~~Pancreatic~~ PDA	[69]
	↑GLN metabolism		
	↑OXPHOS		
	↓ PPP	Melanoma	[1]

In ER^+ BC, where CDK4/6 inhibitors have found the only approved clinical application, the impact of CDK4/6 inhibition on metabolism has been studied *in vitro* in different therapeutic settings.

A global metabolomics approach was used to investigate the effects of palbociclib alone or in combination with letrozole in $ER^+/HER2^-$ BC cells [57]. Neither palbociclib nor letrozole affected amino acid metabolism when used as single agents, a slight effect was observed on nucleotide metabolism, and only few fatty acids were up-regulated. In contrast, several intermediates of the central carbon metabolism (glycerate 3-phosphate, ribose, 6-phosphogluconic acid, lactic acid) were decreased by palbociclib. When palbocilcib was combined with letrozole, a different scenario emerged: both amino acids and central carbon metabolites were significantly reduced in comparison with individual treatments, whereas fatty acids and nucleic acid precursors were increased. The increased levels of nucleic acid precursors might be caused by treatment-mediated cell cycle arrest, which would prevent their utilization in the S phase for DNA replication.

The decrease in amino acid levels led to the inhibition of mTORC1, a master regulator of cell growth and metabolism, resulting in a stronger reduction of cell proliferation as compared to single agent treatments. Interestingly, the dietary xenoestrogens genistein and zearalenone counteracted these inhibitory effects, restoring the amino acid pool with reactivation of mTORC1 signaling, and allowing the recovery of cell proliferative capacity. These findings alert on the potential risk associated with dietary intake of xenoestrogens for hormone-dependent BC patients on treatment with palbociclib and letrozole, and point to the need for further investigation of these aspects *in vivo* in order to define the appropriate targeted nutritional recommendations for these patients.

The same authors have more recently published a study dissecting the metabolic changes associated with the combined treatment of palbociclb and fulvestrant in $ER^+/HER2^-$ BC cells [58]. After 2 days of treatment, palbociclib and fulvestrant synergistically targeted TCA cycle intermediates, while having distinct effects on other pathways; in particular, fulvestrant but not palbociclib mediated the down-regulation of metabolites in both the oxidative and non-oxidative branches of the pentose phosphate pathway (PPP).

After a prolonged combined treatment (7 days), a metabolic signature of oxidative stress and cell death emerged, which was characterized by a dramatic change in TCA metabolism associated with fulvestrant-dependent decrease in amino acids (aspartate, serine, proline, asparagine, tryptophan, tyrosine), NAD^+ and $NADP^+$. In addition, the simultaneous dosing induced a downregulation of genes involved in the biosynthesis of purine nucleotides, an effect opposite to that previously shown for palbociclib-letrozole combination, although this discrepancy may be simply ascribed to the different duration of the treatments. Therefore, palbociclib and fulvestrant, by acting on common or distinct metabolic pathways, produced a combinatorial effect that hampered the macromolecules synthesis and energy production required for cancer cell growth.

Collectively these studies suggest that a more profound effect on BC cell metabolism is achieved by combined therapeutic strategies with palbociclib and endocrine therapy in comparison with monotherapy, possibly contributing to their proven superior efficacy. A different mechanistic explanation to the clinical advantage of such combinations was provided in a previous study [59] showing that fulvestrant counteracts the effects of palbociclib on cell metabolism in $ER^+/HER2^-$ BC cell models. Surprisingly, treatment with palbociclib promoted the activation of genes downstream of mitogenic signaling as well as genes involved in glucose (*G6PD* and *PGM2L1*) and glutamine metabolism. In addition, palbociclib-treated cells displayed an increased number of mitochondria together with higher oxygen consumption rate and ATP levels. By inhibiting these

effects, fulvestrant prevented palbociclib-mediated activation of compensatory growth processes that might contribute to the emergence of resistance to CDK4/6 inhibition.

Differently from palbociclib, abemaciclib alone has been shown to induce extensive metabolic alterations in ER^+ BC cells [60], which may contribute to the effectiveness demonstrated by this drug used as monotherapy in distinct clinical trials [61,62]. In particular, abemaciclib induced a time-dependent reduction in the concentration of the TCA metabolites α-ketoglutarate, fumarate, and malate, together with a minor decrease in succinate concentration, presumably related to alterations in mitochondrial function; α-ketoglutarate was also decreased in a concentration-dependent manner. These metabolic changes were associated with the irreversible arrest of cell cycle and with the induction of senescence and apoptosis. Notably, both senescence and apoptosis were detected earlier and at lower concentrations of abemaciclib in comparison with either palbociclib or ribociclib, confirming the higher potency of the former.

Increasing pre-clinical evidence suggests that not only ER^+ BC, but also TNBC, specifically the RB-proficient subpopulation, may benefit from treatments with CDK4/6 inhibitors used in combinatorial strategies [63]. A recent study from our group demonstrated that the anti-tumor activity of palbociclib in RB-positive TNBC cells is enhanced by the concurrent or sequential treatment with PI3K/mTOR inhibitors, pointing to the inhibition of glucose metabolism as a mechanism contributing to the efficacy of such combination [64]. The rationale for this combination is based on the observation that palbociclib increases the activation of AKT/mTOR signaling [65]. Palbociclib alone down-regulated glucose uptake and consumption as well as GLUT-1 expression under both normoxic and hypoxic conditions; these effects were significantly enhanced by the combination with BYL719, an inhibitor of the p110α catalytic subunit of PI3K. In addition, palbociclib decreased hypoxia-induced HIF-1α accumulation, which might contribute to reduce glucose utilization under hypoxia. The underlying mechanism involved a stronger down-regulation of the E2F1 target c-myc and the inhibition of the AKT/mTOR pathway, both known for their key role in the modulation of cancer energy metabolism [66]. More recently, our group demonstrated that a comparable mechanism, involving the inhibition of both c-myc and AKT signaling, was responsible for the impairment of glucose metabolism induced by the sequential treatment of palbociclib and paclitaxel in TNBC cells [67]. It is worth noting that, while palbociclib alone was cytostatic, its combination with either PI3K/AKT inhibitors or chemotherapy resulted in increased cytotoxicity. In accordance with our findings, the combined treatment of palbociclib with the mTOR inhibitor everolimus in glioblastoma cells elicited anti-proliferative and pro-apoptotic effects associated with the inhibition of both aerobic glycolysis and oxidative mitochondrial function [68]. Combined inhibition of CDK4/6 and mTOR signaling resulted in the suppression of both glycolytic and oxidative metabolism also in cell models of KRAS mutant pancreatic ductal adenocarcinoma (PDA), although the underlying mechanisms were substantially different [69]. Indeed, in contrast with the data obtained by Olmez et al. and our group, palbociclib alone increased glucose consumption and hence glycolytic intermediates, lactate secretion, and extracellular acidification; moreover, palbociclib increased the mitochondrial mass and number with consequent stimulation of oxidative phosphorylation (OXPHOS), and enhanced the glutamine consumption thus augmenting the level of TCA intermediates. Accordingly, a number of genes associated with glycolysis, lysosome, pyruvate metabolism, fatty acid metabolism were induced. This metabolic reprogramming was recapitulated by ribociclib and abemaciclib treatment and was ascribed to the induction of mTOR signaling mediated by CDK4/6 inhibition. Therefore, in this cellular system, the mTOR inhibitors were

combined with palbociclib to counteract these metabolic changes, finally resulting in the induction of cell death.

In cell models from Non-Small Cell Lung Cancer (NSCLC), palbociclib treatment was shown to promote inhibitory effects on both glucose and glutamine metabolism. In A549 cells harboring a *KRAS* mutation as well as *CDKN2A* deletion palbociclib decreased glycolysis and the expression of glycolytic genes, without altering glucose uptake, in contrast with the effects observed in KRAS mutant pancreatic cancer cells [70]. In another study, H460 cells treated with abemaciclib exhibited altered steady-state levels of glycolytic intermediates and amino acids and a reduced uptake of glucose, leucine, and glutamine; these metabolic restrictions, associated with mTOR inhibition, generated nutrient and energy stress conditions that rendered the cells more sensitive to ionizing radiation [71].

A relevant notion emerging from the above-mentioned studies is that the metabolic alterations promoted by combinatorial treatments with CDK4/6 inhibitors influence the final cell fate decision, possibly converting the cytostatic effects of CDK4/6 inhibitors into cytotoxic. In this regard, it is worth to note that treatment with CDK4/6 inhibitors in T-cell acute lymphoblastic leukemia (T-ALL) cells induces apoptosis *per se*, by inhibiting the metabolic functions of the CDK6-Cyclin D3 complex, independently of RB [1].

Wang et al. demonstrated that the CDK6-Cyclin D3 phosphorylates and inhibits the catalytic activity of two key glycolytic enzymes, i.e. 6-phosphofructokinase and pyruvate kinase M2. This results in the diversion of the glycolytic intermediates into the PPP and serine pathways to promote anabolism and antioxidant metabolism. T-ALL cells depend on Cyclin D3 and CDK6 for their proliferative decision, expressing very low levels of Cyclin D1, Cyclin D2, and CDK4. Therefore, treatment with palbociclib or CDK6 depletion in these cells reduced the flow through PPP and serine pathways, thereby decreasing the antioxidants NADPH and glutathione. As a result, the levels of reactive oxygen species (ROS) increased, triggering apoptosis. Interestingly, these findings were confirmed in melanoma patient-derived xenografts in mice treated with ribociclib, in which long-term regression correlated with high levels of cyclin D3 and CDK6 expression [1].

The comparison between the different studies analyzed leads to the important consideration that the metabolic outcomes correlated with CDK4/6 inhibition are context specific. As reported in Table 1, palbociclib and, even if less characterized, abemaciclib and ribociclib have been shown to influence cell energy metabolism, with effects that are sometimes antithetical depending on the tumor type or even in the same cancer type. These discrepancies may be ascribed to the experimental conditions adopted in different studies (dosage, timing, methodology) or to differences in the genetic background of the tumor cells analyzed. For example, the activation of mTOR signaling frequently observed upon treatment with CDK4/6 inhibitors may result in a metabolic reprogramming towards glycolysis in some cancer models but not in others, depending on the specific oncogenic alterations that determine their metabolic phenotype. Nevertheless, a common conclusion emerging from all these data is that combining CDK4/6 blockade with inhibitors of relevant pathways controlling cell metabolism, such as PI3K/AKT/mTOR inhibitors or antiestrogenic drugs, represents a superior metabolic strategy for halting tumor growth.

2.3 Immunomodulatory effects of CDK4/6 inhibitors

2.3.1 Effects on cancer cells

A number of recent studies have revealed a significant cell cycle-independent activity of CDK4/6 in tumor immunosurveillance and CDK4/6 inhibitors may act as regulators of the immune response against tumor cells.

Firstly, emerging data have shown that CDK4/6 inhibitors may exert a direct immunomodulatory effect on tumor cells (Figure 2). In this regard, Goel and co-workers demonstrated that abemacilib and palbociclib increased the functional capacity of tumor cells to present antigens in BC cell lines and in an *in vivo* model of mammary carcinoma. The underlying mechanism involved the increased production and release of type III interferons (IFN) by tumors after drug treatment, which in turn up-regulated genes encoding for the major histocompatibility complex (MHC) class I molecules (B2m, human HLA-A, HLA-B, HLA-C or mouse H2d1, H2k1) as well as for genes involved in peptide cleavage (Erap1) and transport (Tap1, Tap2). From a mechanistic point of view, the authors demonstrated that CDK4/6 inhibitors, by targeting E2F, reduced the expression of *type 1 DNA methyl-transferase* (*DNMT1*), a E2F target that normally ensures the methylation-dependent transcriptional repression of endogenous retroviral elements. Therefore, abemaciclib treatment resulted in the increased expression of endogenous retroviral elements and intracellular double-strand RNAs, which, in turn, induced the production of type III IFN, mimicking a viral infection [72].

Similar results were obtained in TNBC models following the combined treatment with the PI3Kα inhibitor BYL919 and ribociclib or palbociclib [73]. By performing a gene set enrichment analysis, the authors demonstrated that immune-related pathways were enriched in tumor cells following drug combination treatment. In particular, the up-regulation of genes involved in antigen presentation (HLAs HLA-A, HLA-DMA and TAPBP) and genes encoding for cytokines (IFNα, IFNγ, TNFα) was observed, thus demonstrating the immunomodulatory effect of these treatments.

On the other hand, emerging data demonstrate that CDK4/6 inhibition may promote tumor immune evasion in a variety of tumor models, through the induction of the immunosuppressive programmed cell death protein 1 ligand (PD-L1) expression on tumor cells. The PD-1/PD-L1 immune-chekpoint pathway regulates effector T cell activity in peripheral tissues [74], and PD-1 engagement on T lymphocytes directly inhibits TCR-mediated effector functions, acting as a negative regulator of the immune response. This negative immune-suppressor effect of CDK4/6 inhibitors can be turned to advantage by the recent development of immune-checkpoint inhibitors, whose activity might correlate with PD-L1 expression on tumor cells.

In this regard, Zhang and collaborators [75] documented an inverse correlation between CDK4 activity and PD-L1 expression on tumor cells and demonstrated that the treatment with palbociclib or ribociclib up-regulated PD-L1 expression in a number of BC cell lines and in *in vivo* BC models independently of RB status, thus suggesting that in these models PD-L1 expression was not modulated by the RB/E2F axis. Indeed, the authors demonstrated that PD-L1 protein abundance was regulated by the Cullin 3-based E3 ligase, which interacted with PD-L1 through the adaptor protein specle-type POZ (SPOP). In turn, SPOP stability was controlled by the alternative binding with 14-3-3γ or FZR1 proteins and by CDK4/Cyclin D-mediated phosphorylation. In the presence of CDK4/6 inhibitors, SPOP, no longer phosphorylated, was degraded by FZR1, consequently increasing the expression of PD-L1. In mice tumor models, the combined treatment with palbociclib and an anti-PD-1 antibody markedly retarded tumor progression and significantly improved the

overall survival in comparison with single drug treatments, thus providing the rationale for combining CDK4/6 inhibitors with immune-checkpoint inhibitors as a more effective anti-cancer treatment.

In addition to an increased stability of the protein, the augmented expression of PD-L1 by CDK4/6 inhibitors has been recently related to the activation of the transcription factor NF-kB in RB-proficient cell lines from different tumor types. NF-kB protein p65 is known to regulate PD-L1 expression [76] and its transcriptional activity can be suppressed by the interaction with hyper-phosphorylated RB protein, with the consequent inhibition of *PD-L1* gene expression [77]. Therefore, CDK4/6 inhibitors as well as RB loss, by promoting the release of active p65 protein, induce PD-L1 up-regulation.

The association between CDK4/6 activity and PD-L1 expression has been recently reported also in melanoma patients and combination of CDK4/6 inhibitors with anti-PD-1 antibodies significantly suppressed the tumor growth in mouse models of melanoma [78].

As described in a previous paragraph, CDK4/6 inhibition induces SASP in a variety of cell models. Among the released factors, senescent melanoma cells induced by CDK4/6 inhibitors have been demonstrated to produce CCL5 through the activation of NF-kB [79]. CCL5 is a chemokine that binds to CCR5, CCR3, and CCR1 receptors expressed on a variety of immune cells, such as activated T cells, natural killer, immature dendritic, and others [80]. Using PDX tumors obtained from melanoma patients, the authors demonstrated that cytokines secretion by senescent cells promoted the recruitment of immune cells into the tumor. In particular, they observed a strong positive association between CCL5 release and the expression of molecules associated with T lymphocytes (CD2, CD3, CD8A and B, CXCR3, CCR5) and cytotoxic immune cells (granzymes and FAS ligand).

2.3.2 Effects on tumor microenviroment

In the last few years, it has become clear that the CDK4/6 inhibitors exert their direct activity also on immune cells, thus modifying the tumor microenvironment and affecting the immune response against the tumor, with potential benefit also for patients with RB-deficient tumors ~~.~~(Figure 2).

Indeed, the CDK4/6-Cyclin D-RB-E2F axis plays a critical role in the development of the hematopoietic lineage, as demonstrated in mutant mice lacking both CDK4 and CDK6 or in triple Cyclins D1/2/3 mutant mice: in both cases the mice showed embryonic lethality together with defect in fetal hematopoiesis, whereas the loss of one component impaired the normal development of committed hematopoietic cell lineages [81,82].

In particular, CDK6 has been demonstrated to play a pivotal role in the cell cycle progression of precursors of blood cells [83]. Therefore, it is not surprising that neutropenia has been reported as one of the most frequent adverse effects following palbociclib and ribociclib treatment. Since abemaciclib is a more potent inhibitor of CDK4 than CDK6, neutropenia is less frequent in patients treated with this drug. In addition, some differences in the sensitivity to CDK4/6 inhibitors have been described between the myeloid and the lymphoid lineages [84]. Indeed, the proliferation of naïve lymphocytes in the secondary lymphoid organs is mainly sustained by CDK-cyclin complexes other than CDK4/6-Cyclin D, such as CDK-Cyclin E, and the regular development of T cells has been demonstrated in CDK6-mutant mice [85].

However, specific T cell subsets have shown a more pronounced sensitivity to CDK4/6 inhibitors. In particular, the $FOXP3^+$ regulatory T cell population (Treg) was more sensitive to CDK4/6 inhibitors as compared to other lymphocytes and this behavior has been related to the high expression in these cells of the proteins of the CDK4/6-Cyclin D-RB axis [84,86] or to the reduced activity of DNMT1 [72], although other mechanisms might come into play.

In addition to the reduced Treg accumulation in tumor microenviroment, Deng and collaborators [87] demonstrated that a short-term exposure to palbociclib or trilaciclib (another CDK4/6 inhibitor) significantly enhances T cell activation, thus contributing to anti-tumor effects *in vivo.* This action was related to the release of IL2 by T cells as a consequence of CDK6 inhibition. In particular, the authors demonstrated that CDK6 is an upstream regulator of the NFAT transcription factor; CDK4/6 inhibition decreased the phosphorylation of NFAT, promoting its nuclear translocation and enhancing its transcriptional activity, which resulted in increased IL2 production. Moreover, combining palbociclib with PD-1 blockade enhanced tumor regression both in murine syngeneic models as well as in an *ex vivo* organotypic tumor spheroid culture system.

Also abemaciclib has been shown to increase the activation of human T cells *in vitro* and to modulate the activation status of immune cells within the tumor tissue in an experimental murine BC model [88]. Indeed, abemaciclib treatment significantly up-regulated a number of genes associated with T cell activation and function, such as IFN-γ, granzyme B, co-stimulatory/co-inhibitory receptors and/or ligands, chemokines. However, the increased intra-tumor immune activation and inflammation induced by abemaciclib was related to the enhanced function of immune cells rather than to the increase in their number. Interestingly, the association with an anti-PD-L1 antibody showed a synergistic effect when the drug was administered following a phased schedule of treatment (abemaciclib alone for one week followed by the combined treatment). In this condition, beside the increased activation of T cells, the authors observed an enhanced antigen presentation capability and T cell priming by dendritic cells, indicating that CDK4/6 inhibitors might also modulate innate immunity cells.

In the context of the innate immunity, it has been recently demonstrated that CDK4/6 are involved in the production of neutrophil extracellular traps (NET). Indeed, NET formation is induced by mitogens, and despite neutrophils are non-proliferating and terminally differentiated cells, a functional CDK4/6 activity is required for NET release. Loss of CDK6 impaired NET formation in mice models and rendered the animals more susceptible to fungal infection [89].

In contrast with the previous observations, CDK4/6 inhibitors have been shown to produce negative immunomodulatory effects not only by inducing PD-L1 on tumor cells, as above reported, but also by directly increasing the expression of the negative immune checkpoints PD-1 and CTLA-4 on infiltrating T lymphocytes in a syngeneic TNBC mouse model. However, this effect was observed only when CDK4/6 inhibition was combined with the PI3Kα inhibitor BYL719 and the addition of both anti-PD-1 and anti-CTLA-4 induced stable and durable regression of the tumors [73].

Collectively, these data strongly support the idea that CDK4/6 inhibitors induce tumor immune modulation, and their anti-tumor activity is enhanced by the combination with immune-checkpoint inhibitors.

A summary of clinical trials investigating the safety and efficacy of combining CDK4/6 inhibitors with PD-1/PD-L1 checkpoint inhibitors is presented in ~~Table 2.~~Table 2

Table 2

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Ongoing active and recruiting trials with CDK4/6 inhibitors and PD-1/PD-L1 immune-checkpoint inhibitors (as of September 2019) http://clinicaltrials.gov.

CDK4/6 inhibitors	PD-1/PD-L1 checkpoint inhibitors	In combination with	Disease	Phase	
Palbociclib	Avelumab	Fulvestrant	Pre-treated ER+/HER2- BC	Phase II	NCT03147287*
	Pembrolizumab	Letrozole	Naive ER+/HER2- BC	Phase II	NCT02778685*
	Nivolumab	Anastrozole	ER+/HER2- BC (neoadjuvant)	Phase II	NCT04075604§
Abemaciclib	Nivolumab	–	Pre-treated HNSCC	Phase I/II	NCT03655444*
		–	HCC	Phase II	NCT03781960§
		Anastrozole	ER+/HER2- BC (neoadjuvant)	Phase II	NCT04075604§
	Pembrolizumab	–	Pre-treated or naïve NSCLC	Phase I	NCT02779751**
		±Anastrozole	Pre-treated or naïve ER+/HER2- BC		
		–	Pre-treated or naïve HNSCC	Phase II	NCT03938337§
		–	Pre-treated gastro-esophageal cancer	Phase II	NCT03997448*
	Durvalumab	Aromatase inhibitors	ER+/HER2- BC (neoadjuvant)	Phase I	NCT04088032§
	Lodapolimab		Solid tumors	Phase I	NCT02791334*
Ribociclib	Spartalizumab	±Fulvestrant	ER+/HER2- BC and ovarian cancer	Phase I	NCT03294694*

*:recruiting; **: active, not recruiting; §: not yet recruiting.

Anti-PD-1: Nivolumab, Pembrolizumab, Spartalizumab.
Anti-PD-L1: Atezolizumab, Avelumab, Durvalumab, Lodapolimab.

2.3.3 Impact of CDK4/6 and immune-checkpoints inhibition on drug resistance

The increase in PD-L1 expression might represent a clinical problem for patients treated with CDK4/6 inhibitors and could be one of the underlying mechanisms accounting for CDK4/6 inhibitor resistance via evasion of immune surveillance checkpoints.

Recent findings demonstrated that immune-checkpoint inhibitors can be a therapeutic strategy to overcome CDK4/6 resistance in BC. In an animal model of palbociclib-resistant $HER2^+$ BC, Wang and collaborators described a population of immunosuppressive immature myeloid cells (IMCs) infiltrating the resistant tumors, together with a reduction of $CD8^+$ T lymphocytes within the tumor. Targeting IMCs with cabozantinib, a tyrosine kinase inhibitor, strongly enhanced the efficacy of immune-checkpoint inhibitors and overcame the resistance to CDK4/6 inhibitors [90].

Conversely, emerging data reported the aberrations in the CDK4/6 pathway as a mechanism of resistance to immune-checkpoint inhibitors. In melanoma patients resistant to immune-checkpoint inhibitors, a single-cell RNA sequencing allowed the identification of a number of mRNAs, indicated as “T cell Exclusion Program”, whose expression was correlated positively or negatively with T cell abundance within the tumors. The mRNAs encoding for CDK4 and its downstream targets, such as E2F and c-myc, were among the induced mRNAs expressed by resistant cells, whereas genes that control antigen presentation (*B2M*, *HLA-A/B/C*, and *TAPBP*), IFN-γ signaling, complement components and immune modulation (CD47 and CD58) were repressed. The same authors demonstrated in melanoma cell lines that the treatment with CDK4/6 inhibitors could shift the malignant cell population to a less immune-resistant state [91]. Also in mouse models *in vivo,* they found that the combination of a CDK4/6 inhibitor with an immune-checkpoint inhibitor repressed the “T cell Exclusion Program”, reducing melanoma tumor growth.

The role of the alterations in the CDK4/6 axis as a mechanism of resistance to immune-checkpoint inhibitors has recently been reported also by Yu and co-workers. In patients with advanced melanoma, mutations in *CDK4* and *CDKN2A* loss, identified by whole-exome sequencing and RNA sequencing profiling, were associated with the lack of clinical benefit to anti-PD-1 immune-checkpoint inhibitors [78]. The CDK4/6-cyclin D-RB-E2F axis has been implicated in the resistance to immune therapy also in cervical cancer cells and in other types of human cancer cells (melanoma, TNBC, colorectal cancer) and high expression of its components negatively correlated with progression-free survival of cervical cancer patients. Recently, the synaptonemal complex protein 3 (SCP3) was shown to drive immune resistance by hyper-activating the CDK4/6/Cyclin D1/E2F1 axis that, in turn, induced the expression of the pluripotent transcription factor NANOG [92]. NANOG mediated multi-aggressive cancer phenotypes, including immune resistance, and NANOG knockdown produced reversal effects on immune modulation and aggressiveness of cancer cells [93,94]. Tumor cells with high levels of SCP3 were more sensitive to palbociclib; in addition, palbociclib inhibited tumor growth in xenografts transplanted with SCP3-high BC cells and the combination of palbociclib with adoptive transfer of CTLs resulted in augmented anti-tumor effects.

These findings add an additional level of complexity to the interplay between CDK4/6 activity and the immune system and suggest that alterations of PD-L1 expression and aberrant CDK4/6 activity could be involved in the resistance mechanisms against CDK4/6 inhibitors or immune-checkpoint inhibitors, respectively. Combination of CDK4/6 inhibition and PD-L1 blockade could result in overcoming such resistances.

3 Conclusions and future directions

CDK4/6 inhibitors were originally developed to inhibit cell cycle progression in an RB-dependent manner, thereby directing their activity to RB-proficient cancers. However, it has become evident that their functions extend beyond G1 arrest, involving other aspects of tumor biology through mechanisms that do not necessarily require RB activation. Induction of senescence, metabolic rearrangements, and immunomodulation are some of the non-canonical effects associated with CDK4/6 inhibitors that are worthy of further exploration to enhance the clinical benefit of these drugs, possibly through more effective combinatorial strategies, and to provide novel therapeutic opportunities even for RB-negative cancers. It is interesting to note that all these non-canonical effects are interconnected and may cooperatively influence the tumor response to CDK4/6 inhibition. For example, SASP, through the release of cytokines and chemokines, may affect the recruitment and activation of lymphocytes and dendritic cells. In addition, the inhibition of glucose or amino acids metabolism induced by CDK4/6 inhibitors, achieved through single or combined treatments depending on the tumor context, may contribute to reinforce the immune surveillance, by reducing the tumor-imposed metabolic restrictions and restoring nutrient availability for immune cells [95]. A deeper understanding of the non-canonical activities of CDK4/6 inhibitors would be important also to uncover the limitations associated with such drug treatments, which cannot be predicted only on the basis of the canonical effects. The induction of senescence mediated by CDK4/6 inhibitors may be considered as a double-edged weapon exerting either anti- or pro-cancer activities. Moreover, the reversibility of the senescent phenotype raises the question of how to ensure a durable clinical response avoiding tumor regrowth after therapy quitting; in this regard, the use of senolytic compounds to selectively kill the senescent cells represents a promising valuable strategy to improve the efficacy of CDK4/6 inhibitors and deserves further *in vitro* and *in vivo* validation.

Finally, a relevant aspect that cannot be neglected is the impact that CDK4/6 inhibitors may have on metabolism at the level of the whole organism, due to the role that the CDK4/6-Cyclin D-RB-E2F pathway has in regulating energy homeostasis. Indeed, it has been demonstrated that multiple cycles of palbociclib administration cause glucose dysregulation associated with pancreatic beta cell degeneration in young rats that have not achieved replicative quiescence, but not in aged rats [96]. These findings suggest that a pediatric population under treatment with CDK4/6 inhibitors may be more susceptible to beta cell failure than adults; this may have direct clinical implications considering that CDK4/6 inhibitors are currently under investigation in pediatric solid tumors [97–99].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank “Associazione Augusto per la Vita”, Novellara (RE) (in memory of Claudio Cavani).

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The corrections made in this section will be reviewed and approved by journal production editor.

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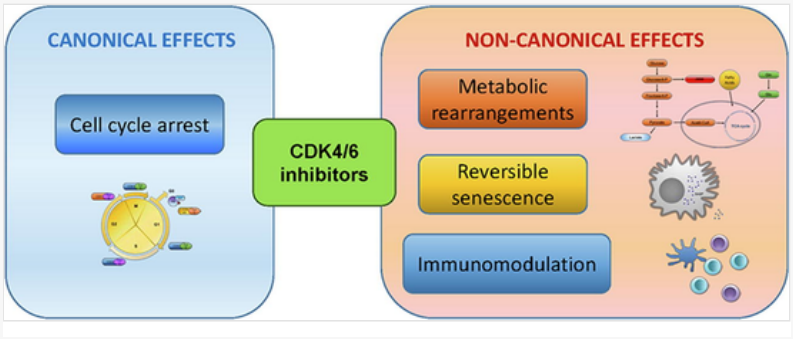
Graphical abstract



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