Cyclin-Dependent Kinase 4/6 Inhibitors: Is a Noncanonical Substrate the Key Target?

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Cyclin-dependent kinases (CDK), such as CDK4 and CDK6, phosphorylate RB1 to release the transcription factor E2F and drive the transition from G_1 to S-phase of the cell cycle. Inhibitors of these kinases thereby block cell-cycle progression and presumably exert their therapeutic effect. While this mechanism is straight forward, several aspects have seemed problematic, not the least of which is that these drugs seem to have therapeutic effects on a relatively small number of human cancers. Tong and colleagues took an open-ended approach to this mechanistic question, and their results raise the possibility that inhibition of phosphorylation of the transcription factor p73 is a key mechanism of action of these drugs. They show that p73 inhibition and the resultant upregulation of the cell surface receptor DR5 are necessary for the anticancer effects of CDK4/6 inhibitors, including enhancement of immune-mediated cell killing, and that therapeutic benefit relies largely on their use in conjunction with other agents. While many questions remain to be answered, these findings demonstrate the importance of keeping an open mind to mechanistic aspects of therapeutic agents already in clinical use and highlight how rigorous mechanistic studies can answer both basic and translational questions.

See related article by Tong et al., p. 1340

In the late 1980s, research in the fields of viral tumorigenesis, cancer genetics, and cell-cycle regulation converged to lead to the elucidation of how kinases regulated by cyclins, the so-called cyclin-dependent kinases (CDK), could phosphorylate the RB1 protein and regulate cell-cycle entry (1). As with all biological regulatory pathways, there are negative regulators of this process as well. As our understanding of these pathways came in to focus, it became clear that overexpression of cyclins or the CDKs themselves, or loss of expression of RB1 or negative regulators such as CDK inhibitors, could directly lead to cancer pathogenesis (2). Chemists responded with the development of small molecule inhibitors of the CDKs. Currently three such CDK4/6 inhibitors, palbociclib, ribociclib, and abemaciclib, are now approved for clinical use, all for patients with advanced hormone receptor–expressing breast cancers that lack HER2 overexpression. In conjunction with inhibitors of estrogen signaling, these drugs have had a major impact on patients with this disease (3).

However, as with most scientific advances, the success of CDK4/6 inhibitors in breast cancer raised a number of additional questions. If this is such a central pathway in cellular proliferation, why is the clinical activity limited in cancers other than hormone receptor–positive breast cancers? If the only effect of CDK4/6 inhibitors is to block the G_1 to S-phase transition, wouldn't these agents be expected to exert only a cytostatic effect on cells and not the tumor regression commonly seen with their use? In addition, resistance to CDK4/6 inhibitors can occur by mechanisms seemingly unrelated to the regulation of this pathway (4). It is also becoming increasingly apparent to clinical oncologists what chemists and biochemists have long known: Kinase inhibitors developed to one target are likely inhibiting other kinases or are having effects on substrates other than those first proposed. In fact, the pattern of naming kinases based on an identified substrate may lead us down an overly narrow path. Microtubule-associated protein (MAP) kinases, named for their ability to phosphorylate MAP, clearly have other critical targets. Even more notable, casein kinases may never phosphorylate this milk protein in physiologic states. Thus, a natural question is whether CDK4/6 inhibitors may be showing clinical benefit through inhibition of other kinases or inhibiting phosphorylation of substrates other than RB1.

To address this question, Tong and colleagues took an open-ended approach to understand cellular targets and pathways that could be mediators of the therapeutic effect of inhibitors of CDK4/6 (5). Focusing on colorectal cancers, they performed RNA sequencing after exposure of the HCT116 cell line to either palbociclib or siRNA targeting CDK4. This type of approach, combining pharmacologic and genetic inhibition of a target, is a desirable strategy to allow identification of true on-target effects and minimize the chance of following leads due to nonspecific effects. The authors found that death receptor 5 (DR5), a cell surface receptor that binds to TRAIL and can induce apoptosis (6), is upregulated at both the mRNA and protein level by inhibition of CDK4/6. This finding was recapitulated across a range of colorectal cancer cell lines, while nontransformed colorectal epithelial cells did not show this induction of DR5 following treatment with palbociclib or ribociclib.

This observation immediately brings up two questions: What is the mechanism by which CDK4/6 inhibitors lead to increased expression of DR5 and what is the therapeutic implication of the increased DR5 expression? To address the first question, the authors started by examining a number of transcription factors already known to regulate DR5 transcription and found that ablation of p73 abolished the effect of CDK4/6 inhibition on DR5 expression. They then showed that both nuclear localization of p73 and p73 binding to the DR5 promoter increased with CDK4/6 inhibition. They further identified Thr86 as the p73 residue phosphorylated by CDK4/6 and showed that this phosphorylation trapped p73 in the cytoplasm.

This beautiful mechanistic study was accompanied by experiments to address the role that this induction of DR5 plays in the therapeutic effects of CDK4/6 inhibitors. First, and most expectedly, CDK4/6 inhibition led to increased sensitivity of colorectal (and breast cancer)

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cells to the apoptotic effects of TRAIL. Of note, CDK4/6 inhibition also led to sensitization of cells to the cytotoxic effects of chemotherapeutic agents. Genetic ablation of DR5 abolished the sensitization of cells to TRAIL as well as to the cytotoxic drug 5-fluorouracil (5FU). This suggests that DR5 upregulation not only leads to the obvious enhanced sensitivity of cells to TRAIL but more broadly enhances the apoptotic sensitivity of these cells, a finding seen with upregulation of other TNF receptor superfamily members.

The authors then tested the effects *in vivo* of the CDK4/6 inhibitor palbociclib alone and in a variety of combinations. Several key points emerged. First, palbociclib alone had only modest effects at slowing the growth of these tumors. Because the phosphorylation of Thr86 of p73 was inhibited by the dose of palbociclib administered, this would suggest that there was inhibition of CDK4/6 kinase activity. This finding alone suggests that a primary effect of these drugs to inhibit the G_1 to S-phase transition is unlikely, as the tumors continued to grow almost to the extent of untreated tumors. However, when palbociclib was combined with either TRAIL or 5FU, near complete inhibition of tumor growth was achieved. Ablation of either DR5 or p73 abrogated this therapeutic effect, supporting the mechanisms uncovered in the *in vitro* experiments.

Finally, the authors showed that CDK4/6 inhibitors induced increased cell surface expression of the damage-associated molecular pattern molecule calreticulin (7) and that this was dependent on the presence of DR5 (and p73). This led to increased phagocytosis of tumor cells by dendritic cells and elicited synergistic effects with immune checkpoint inhibitors.

This would certainly not be the first time that the mechanism of action of a clinically successful anticancer drug has been reappraised. Proteasome inhibitors, such as bortezomib, were first thought to act by preventing activation of the oncogenic transcription factor NF-κB by preventing degradation of the inhibitory IκB subunit. However, several lines of evidence brought this into question, including observations that bortezomib was effective in models of multiple myeloma that lacked activation of NF-κB and that it had limited efficacy in other cancers with constitutive activation of this transcription factor. Ultimately, it was recognized that the protein stress induced in cells that make an overabundance of proteins that are sometimes misfolded, such as in multiple myeloma, was the critical target (8). The so-called IMiD class of drugs, such as thalidomide and lenalidomide, were first developed as putative antiangiogenic agents and then were thought to be immunomodulatory drugs (hence the name "IMiD"). However, elegant biochemical and molecular analyses ultimately revealed the role of these drugs as triggers of targeted degradation of transcription factors of the Ikaros family (9). Thus, the fact that a drug is clinically useful can be used to work backwards to hone and refine our understanding of mechanisms, as in the present study on CDK4/6 inhibitors.

This study raises a number of other important points. First, if the key therapeutic target of CDK4/6 inhibitors is the phosphorylation of p73 on Thr86, then this provides a potentially useful pharmacodynamic biomarker to allow titration of the dose of drug in each patient, allowing truly personalized or precision therapy.

Second, it is likely that many therapies like CDK4/6 inhibitors may alter the apoptotic threshold as single agents but may not trigger cell death by themselves. Perhaps the fact that these drugs have proved effective in the treatment of hormone receptor–expressing breast cancers specifically when used in conjunction with hormonal agents was an important clue to this effect. Thus, it is important not to discard these drugs for other cancer indications in which they lack single agent activity. It may be most important to determine what combinations might achieve the greatest therapeutic effect.

Third, there is a great deal of interest in leveraging signaling inhibitors to enhance the efficacy of an immune-based approach such as immune checkpoint inhibitors. As this study shows, rigorous mechanistic analyses coupled with efficacy studies can provide a huge amount of insight into how these combinations might exert a beneficial effect.

Does this study eliminate the canonical RB1-mediated regulation of G_1 to S-phase transition as the ultimate mediator of the effect of CDK4/6 inhibitors? It is too early to say. However, keeping an open mind to alternative explanations for the effects of drugs, even CDK4/6 inhibitors whose mechanism of action was thought to be well understood, is likely going to remain rewarding.

Authors' Disclosures

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