Therapeutic Strategies for Targeting CDKN2A Loss in Melanoma

Inger Z.M. Kreuger1,2, Roderick C. Slieter2,3, Tim van Groningen1,2 and Remco van Doorn1,2

Loss of the tumor suppressor gene CDKN2A, encoding p16 and p14, is a frequent event driving melanoma progression. Therefore, therapeutic strategies aimed at CDKN2A loss hold great potential to improve melanoma treatment. Pharmacological inhibition of the p16 targets CDK4/6 is a prime example of such a strategy. Other approaches exploit cell cycle deregulation, target metabolic rewiring, epigenetically restore expression, act on dependencies resulting from co-deleted genes, or are directed at the effects of CDKN2A loss on immune responses. This review explores these therapeutic strategies targeting CDKN2A loss, which potentially open up new avenues for precision medicine in melanoma.


INTRODUCTION

The survival of patients with metastatic melanoma has considerably improved after the introduction of targeted therapy with BRAF/MEK inhibitors and immunotherapy with anti–PD-1 and anti–CTLA-4 antibodies. However, not all patients respond to these treatments, and therapy resistance is often acquired. The 5-year survival rate of patients with metastatic melanoma is currently approximately 30% (Siegel et al., 2022). Hence, the development of more refined therapeutic strategies for melanoma remains a priority. Melanoma development and progression are driven by genetic and epigenetic alterations affecting the BRAF, NRAS, CDKN2A, TERT, NF1, PTEN, ARID2, and other genes (Cancer Genome Atlas Network, 2015; Hayward et al., 2017; Shain et al., 2015). The identification of the molecular drivers of melanoma has enabled precision medicine, which has thus far primarily involved the targeting of oncogenes among the affected genes. In recent years, cancer treatments have been developed directed at non-oncogenic dependencies, for instance, arising as a consequence of tumor suppressor gene loss (Hahn et al., 2021; Liu et al., 2015). Targeting tumor suppressor gene inactivation could advance melanoma treatment.

The CDKN2A gene is the most commonly inactivated tumor suppressor gene in melanoma (Cancer Genome Atlas Network, 2015; Curtin et al., 2005; Hayward et al., 2017; Hodis et al., 2012). This critical gene is located adjacent to the CDKN2B gene on chromosome 9p21 and encodes two tumor suppressor proteins, p16 (also known as p16INK4a) and p14 (also known as p14ARF). The proteins are transcribed from different first exons, exon 1α for p16 and exon 1β for p14, leading to distinct reading frames in the shared exons 2 and 3 (Figure 1a) (Sherr, 2001). An analysis of melanoma data in The Cancer Genome Atlas shows p16 and p14 inactivation in 67.5% and 27.4% of cases, respectively (Figure 1b). Other studies have also reported frequent CDKN2A inactivation, ranging from 40 to 70% of melanoma cases (Cancer Genome Atlas Network, 2015; Ming et al., 2020). Inactivation may be the result of somatic mutations, deletions, or epigenetic alterations, such as promoter hypermethylation. In addition, in 1994, it was discovered that CDKN2A can be inactivated by inherited gene variants (Hussussian et al., 1994; Kamb et al., 1994). This discovery constitutes a crucial milestone in melanoma genetics and established CDKN2A as the first predisposing gene, which is now estimated to explain 10–40% of hereditary melanoma cases (Goldstein et al., 2007; Holland et al., 2021; Pissa et al., 2021; Potjér et al., 2018).

The canonical function of the p16 protein is binding and subsequent inhibition of CDK4 and CDK6, thereby hampering the phosphorylation of Rb, which leads to G1/S cell cycle arrest and senescence (Serrano et al., 1997, 1993). Recent studies have identified additional non-canonical functions of p16, including in cell metabolism (reviewed by Buj and Aird [2019]). In melanoma, loss of p16 promotes proliferation, delays senescence, and initiates invasion, emphasizing the significance of p16 loss in melanoma progression (Haferkamp et al., 2008; Monahan et al., 2010; Sharpless and Chin, 2003; Straume et al., 2000; Sviderskaya et al., 2003, 2002; Zeng et al., 2018).

The p14 protein antagonizes MDM2-mediated ubiquitination and degradation of p53 (Stott et al., 1998). In murine cells, loss of this function could cause senescence escape and melanomagenesis, which was accelerated by additional loss of the neighboring CDKN2B gene (Kamijo et al., 1997; Krimpenfort et al., 2007; Sharpless and Chin, 2003). However, in human cells, the role of p14 in senescence seems less prominent (Kuilman et al., 2010; Michaloglou et al., 2005; Wei et al., 2001). In addition, a relatively high number of mutations in CDKN2A do not affect the p14 protein but specifically inactivate p16 (Ming et al., 2020). This may relate to the biochemical structure of p14, which is rich in basic...
amino acids and less affected by any specific mutation (Kim and Sharless, 2006). Still, also at the expression level, the p16 protein and not p14 is significantly downregulated in melanomas as compared to nevi (Shain et al., 2018). Therefore, p16 is more likely to be the predominant tumor suppressor in melanoma, with p14 having minor importance.

Accumulating insights into the biological functions of p16 and the development of compounds directed at its downstream targets have opened up novel avenues to target melanomas with CDKN2A inactivation. This review discusses the potential therapeutic strategies directed at melanomas with loss of CDKN2A, in particular inactivation of p16.

RESTORING CDK4/6 INHIBITION

Since the canonical function of p16 is blocking cell cycle entry by CDK4/6 inhibition, CDK4/6 inhibitors can be used to partially restore the functional consequences of p16 loss (Figure 2a and Supplementary Table S1). Currently, three CDK4/6-specific inhibitors are Food and Drug Administration-approved for the treatment of breast cancer, namely palbociclib, ribociclib, and abemaciclib (Sobhani et al., 2019). In vitro and in vivo studies have shown that melanoma cells are also sensitive to these CDK4/6 inhibitors. For instance, ribociclib and abemaciclib impede melanoma growth in preclinical models, and palbociclib inhibits the proliferation of various melanoma cell lines, coinciding with decreased phosphorylation of Rb (Kim et al., 2013; Nassar et al., 2021; Tate et al., 2014; Yadav et al., 2014; Young et al., 2014). Notably, BRAF and MEK inhibitor–resistant cells remain sensitive to palbociclib and abemaciclib, indicating their therapeutic potential for patients who progressed on BRAF/MEK-targeted therapy (Nassar et al., 2021; Yadav et al., 2014; Yoshida et al., 2016).

CDKN2A inactivation further increases sensitivity to CDK4/6 inhibitors in different tumor cell lines, including melanoma (Fennell et al., 2022; Katsumi et al., 2011; Konecny et al., 2011; Li et al., 2019; Ni et al., 2022; Wiedemeyer et al., 2010; Young et al., 2014). A recent example is the knockdown of CDKN2A in BRAF inhibitor–resistant melanoma cells enhances the efficacy of palbociclib (Nassar et al., 2021). However, a few experimental studies in various tumor types found that CDKN2A loss provides no or only intermediate sensitivity to CDK4/6 inhibitors (Franco et al., 2014; Gong et al., 2017; Heilmann et al., 2014). Treatment resistance was suggested to result from increased cyclin E1–CDK2 and PI3K–mTOR signaling in CDKN2A-inactive tumor cells, indicating that additional alterations can diminish CDK4/6 inhibitor efficacy.

To increase CDK4/6 inhibitor efficacy and combat treatment resistance, combination therapies have been designed. Guo et al. (2020) and Garutti et al. (2021) have previously reviewed the various CDK4/6 inhibitor combination therapies in melanoma. A promising example is the synergistic combination of CDK4/6 inhibitors with BRAF/MEK inhibitors. In clinical trials, more than half of melanoma patients with NRAS or BRAF mutations experienced clinical benefits from this combination treatment (Ascierto et al., 2017; Schuler et al., 2022, 2017; Sosman et al., 2014; Taylor et al., 2014). Of note, one study showed that co-alterations in cell cycle regulators, including in CDKN2A, enhanced responses to combination treatment with a CDK4/6 inhibitor and MEK inhibitor in the difficult-to-treat NRAS-mutant melanoma (Schuler et al., 2022). In a trial that only included melanoma patients with CDKN2A loss, combined CDK4/6 and BRAF inhibition also achieved clinical benefit (Louveau et al., 2019). Currently, clinical trials are ongoing that initially provide standard melanoma therapy but, after progression, assign a follow-up treatment on the basis of the genetic profile (NCT02645149 and NCT02159066). This includes combined BRAF/MEK and CDK4/6 inhibition for patients with CDKN2A deletions. Such trials will provide the evidence necessary to support the clinical use of CDK4/6 inhibitors in the treatment of melanoma patients with CDKN2A loss.

EXPLOITING CELL CYCLE Deregulation

In contrast to restoring cell cycle regulation, one could also exploit the deregulated cell cycle. This is because CDKN2A-deficient cancers may heavily depend on the remaining cell cycle control mechanisms to allow for sufficient DNA damage repair, thereby preventing mitotic catastrophe and apoptosis. Hence, loss of CDKN2A might render cells vulnerable to abrogation of these mechanisms, which is an example of synthetic lethality (Figure 2b).

Various strategies for targeting cell cycle control mechanisms have been devised, distinct from CDK4/6 inhibition
reviewed by Matthews et al. [2022] and Barnaba and LaRocque [2021]. An example hereof is joint inhibition of the cell cycle checkpoint kinases CHK1 and MK2, which normally prevent mitotic entry under genotoxic stress (Reinhardt and Yaffe, 2009). Loss of CDKN2A could significantly predict the synergistic interaction between inhibitors of these kinases in cancer cell lines of differing origins, which was shown to lead to DNA damage and apoptosis (Dietlein et al., 2015).

A second option to exploit cell cycle deregulation is by inhibiting AURKA and AURKB that regulate G2/M and spindle assembly checkpoints (Willems et al., 2018). AURKA/B inhibitors were shown to reduce cell proliferation and increase apoptosis in melanoma cells in vitro, especially in combination with BRAF inhibitors or DNA-damaging agents (Porcelli et al., 2015; Sogutlu et al., 2021; Xie and Meyskens, 2013). Loss of p16 may increase sensitivity to AURKA/B inhibitors because AURKA inhibition is synthetic lethal with Rb loss, being downstream of p16 (Gong et al., 2019; Lyu et al., 2020). In line with this notion, a clinical trial is investigating the effect of the AURKA/B inhibitor ilorasertib in CDKN2A-deficient solid tumors (NCT02478320), which will show the clinical utility of AURKA/B inhibitors.

A third option is to inhibit the cell cycle regulator WEE1, which, potentiated by simultaneous inhibition of CHK1, can induce melanoma cell death (Magnussen et al., 2015, 2012; Margue et al., 2019). In melanoma, WEE1 inhibition sensitivity is associated with deficiency of the G1/S checkpoint due to p53 loss (Bauman and Chung, 2014). This suggests...
that CDKN2A inactivation may also increase sensitivity, although this hypothesis needs experimental validation. Overall, these studies encourage further research into exploiting vulnerabilities caused by aberrant cell cycle regulation in CDKN2A-deficient melanomas.

**NON-CANONICAL FUNCTIONS: METABOLIC REWIRING AS A THERAPEUTIC TARGET**

Next to cell cycle regulation, several non-canonical functions of p16 have been discovered (reviewed by Buj and Aird [2019]). The role of p16 in metabolism and consequent metabolic reprogramming upon p16 loss may provide opportunities for therapy (Figure 2c). For instance, knockdown of p16 was linked to an increase in nucleotide metabolism through the mTORC1–RPIA axis (Buj et al., 2019). In the same study, knockdown of RPIA selectively induced senescence in p16-depleted pancreatic, ovarian, and colorectal carcinoma cells. This indicates that increased nucleotide metabolism may be a vulnerability of p16-deficient cancers. This finding has been supported by two recent pancancer synthetic lethality studies. Their computational analyses predicted that CDKN2A loss is synthetic lethal with inactivation of RRM2 (Zhang et al., 2021) and TYMS genes (Benfatto et al., 2021), both of which are involved in nucleotide synthesis (Lane and Fan, 2015). Increased sensitivity to the RRM2 inhibitor cladribine was validated in melanoma cell lines with CDKN2A loss in vitro (Zhang et al., 2021). Nonetheless, whether nucleotide synthesis is a viable therapeutic target in the clinic depends on additional factors, such as TYMP expression levels (Benfatto et al., 2021).

In addition to a role in nucleotide synthesis, loss of p16 is associated with elevated levels of ROS, which occurs through an unknown mechanism independently of the CDK4–Rb pathway (Jenkins et al., 2011; Tyagi et al., 2017). Elevated ROS levels render cells vulnerable to a further increase in ROS, which in turn causes cell death (Perillo et al., 2020). Hence, combining ROS-generating agents with inhibition of antioxidant pathways constitutes a potential therapeutic strategy for p16-deficient melanomas. In pancreatic cancer, CDK4/6 inhibitors elevated ROS levels, which together with the downregulation of antioxidants repressed cell growth (Franco et al., 2016). It is tempting to speculate that such a treatment is especially effective in melanoma cells with p16 loss because it restores CDK4/6 inhibition and exploits the already elevated ROS.

In addition, in a pancreatic cell model, combined inactivation of p16 and mutated KRAS induced NOX4 activity, which enabled the high glycolysis needed for cell growth (Ju et al., 2017). Therefore, in this study, NOX4 was identified as a potential therapeutic target. It will be interesting to determine whether NOX4 can also be a therapeutic target in CDKN2A-deficient melanoma. Taken together, insight into the metabolic functions of p16 has revealed potential new angles for targeting CDKN2A-inactivated melanoma.

**THE CHROMOSOME 9P21 LOCUS: EPIGENETIC REACTIVATION AND COLLATERAL LETHALITY**

In addition to targeting the alterations in cell cycle regulation and metabolism resulting from CDKN2A loss, one could also aim to restore CDKN2A expression, for example, when CDKN2A is inactive because of epigenetic modifications, such as DNA or histone methylation (Figure 2d). This strategy has been successfully tested in various cancer types (reviewed by Zhao et al. [2016]). In melanoma, p16 and p14 expression could be reactivated using the DNA methyltransferase inhibitor 5-aza-2’-deoxycytidine and the histone deacetylase inhibitor suberanilohydroxamic acid (Venza et al., 2015). Another option is targeting the long non-coding RNA transcribed from locus 9p21 called ANRIL, which together with PRC1/2 causes histone H3 lysine 27 trimethylation and stable repression of CDKN2A and CDKN2B, encoding p15, also located on the 9p21 locus (Aquino et al., 2011). Indeed, knockdown of ANRIL or the catalytic subunit of PRC2 was shown to re-express p16 and p15 and hamper melanoma growth (Jin et al., 2020; Xu et al., 2016). However, it should be noted that targeting these epigenetic mechanisms can also affect other tumor-suppressive pathways impacting melanoma growth.

Alternatively, if CDKN2A is lost by deletion of the 9p21 locus, co-deletion of nearby genes may result in vulnerabilities, also known as collateral lethality (Muller et al., 2015). For example, co-deletion of the MTAP gene upstream of CDKN2A results in the accumulation of methylthioadenosine (MTA), which inhibits PRMT5 activity. Consequently, further therapeutic inhibition of PRMT5 and its pathway could impede tumor growth (Figure 2e) (Kryukov et al., 2016; Marjon et al., 2016; Mavrikis et al., 2016). In addition, because of its role in the adenine salvage pathway, MTAP deletion was shown to render cells vulnerable to inhibition of de novo adenine synthesis with antifolate agents, revealing another potential therapeutic vulnerability for patients with co-occurring MTAP/CDKN2A deletions (Alhalabi et al., 2022).

**BOOSTING ANTI-TUMOR IMMUNE RESPONSES**

Besides targeted therapies, loss of CDKN2A may be relevant for the efficacy of immunotherapy in melanoma. The advent of immune checkpoint inhibitors (ICIs) that augment T-cell-mediated anti-tumor immune responses has revolutionized the treatment of metastatic melanoma. Only a subset of patients responds to ICIs; hence, predictive biomarkers to guide treatment decisions are actively sought. Multiple studies have assessed whether CDKN2A loss can be such a biomarker, with divergent results. A few studies could not significantly associate CDKN2A status with ICI outcome in melanoma (Adib et al., 2021; DeLeon et al., 2020). One study did reveal a trend toward improved melanoma control rate in the CDKN2A-mutant cohort of patients treated with ICI (DeLeon et al., 2020). In addition, in patients with hereditary melanoma due to germline CDKN2A alterations, the response to immunotherapy was found to be superior (Helgadottir et al., 2020). Because melanomas with somatic CDKN2A mutations accumulate an increased number of mutations, CDKN2A loss may lead to the presentation of more neo-antigens, which could explain augmented immune responses (Helgadottir et al., 2020). This would suggest CDKN2A to be a biomarker for ICI sensitivity, supporting the use of ICIs to boost anti-tumor immune responses in patients with inactive CDKN2A.

However, several studies showed a significant association between the loss of CDKN2A and low levels of immune cell...
CONCLUSIONS

Since CDKN2A is inactivated in 40–70% of melanomas, effective therapies directed at CDKN2A loss have great potential for improving melanoma management. The development of CDK4/6 inhibitors has enabled such a therapy, although for the optimal effect these inhibitors should be combined with other targeted therapies that synergize or prevent resistance. An appealing strategy is using CDK4/6 inhibitors to enhance immunotherapy efficacy, which may also benefit from the high mutational load in tumors with CDKN2A loss. However, a concern with combining multiple therapies is the heightened risk of adverse events. Yet, this may be countered by the notion that CDKN2A loss sensitizes cells to treatment, potentially allowing for lower doses and decreased toxicity to healthy cells. In patients with hereditary melanoma due to inactivating CDKN2A variants, therapies may be particularly effective because CDKN2A inactivation is an early cancer driver event in these patients. Currently, clinical trials are underway to explore CDK4/6 inhibitor therapy combinations. It will be especially interesting to examine the effect of these therapies in the subset of patients whose tumors are characterized by CDKN2A inactivation.

While clinical trials are ongoing, it is essential to continue investigating the biological functions of CDKN2A, in particular p16, because this can provide new perspectives on targeting tumor cells with inactive CDKN2A, as exemplified by its role in metabolism and immune responses. In addition, gaining insight into the efficacy and applicability of exploiting cell cycle deregulation, targeting epigenetics, and collateral lethality could provide novel angles for therapy. The discovery of therapeutic approaches directed at CDKN2A loss in melanoma may be further enhanced by next-generation omics and screening techniques specifically applied to melanocytic cells with CDKN2A inactivation. In the future, patients with melanoma will possibly be stratified for targeted treatment on the basis of BRAF mutation as well as CDKN2A status. Thus, therapeutic targeting of CDKN2A loss may ultimately advance precision medicine in melanoma beyond targeting oncogenes, considerably expanding clinical options.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: IZMK, RvD; Supervision: TvG, RvD; Visualization: IZMK, RCS; Writing - Original Draft Preparation: IZMK; Writing - Review and Editing: IZMK, RCS, TvG, RvD

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2022.07.016

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## Supplementary Table S1. Potential Therapeutic Strategies Targeting Loss of CDKN2A, in Particular p16

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<td>Palbociclib, ribociclib, abemaciclib</td>
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<td>RRM2 inhibitors</td>
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Abbreviations: H$_2$O$_2$, hydrogen peroxide.

A selection of references relevant to the therapeutic intervention is provided. References refer to studies in melanoma and/or other (CDKN2A-deficient) tumors.