Cell cycle-tailored targeting of metastatic melanoma: Challenges and opportunities

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Abstract

The advent of targeted therapies of metastatic melanoma, such as MAPK pathway inhibitors and immune checkpoint antagonists, has turned dermatological oncology from the “bad guy” to the “poster child” in oncology. Current targeted therapies are effective, although here is a clear need to develop combination therapies to delay the onset of resistance. Many antimelanoma drugs impact on the cell cycle but are also dependent on certain cell cycle phases resulting in cell cycle phase-specific drug insensitivity. Here, we raise the question: Have combination trials been abandoned prematurely as ineffective possibly only because drug scheduling was not optimized? Firstly, if both drugs of a combination hit targets in the same melanoma cell, cell cycle-mediated drug insensitivity should be taken into account when planning combination therapies, timing of dosing schedules and choice of drug therapies in solid tumors. Secondly, if the combination is designed to target different tumor cell subpopulations of a heterogeneous tumor, one drug effective in a particular subpopulation should not negatively impact on the other drug targeting another subpopulation. In addition to the role of cell cycle stage and progression on standard chemotherapeutics and targeted drugs, we discuss the utilization of cell cycle checkpoint control defects to enhance chemotherapeutic responses or as targets themselves. We propose that cell cycle-tailored targeting of metastatic melanoma could further improve therapy outcomes and that our real-time cell cycle imaging 3D melanoma spheroid model could be utilized as a tool to measure and design drug scheduling approaches.

KEYWORDS

cell cycle, drug resistance, drug sensitivity, fluorescent ubiquitination-based cell cycle indicator, melanoma therapy, real-time imaging

1 | INTRODUCTION

The development of targeted therapies for metastatic melanoma using either small molecule MAPK pathway inhibitors (MAPKi) or antagonists of the immune checkpoints has revolutionized the field of dermatological oncology. However, MAPKi only work in approximately 40% of cases as a BRAFV600 mutation must be present; moreover, development of resistance is common.[1-3] Immune checkpoint inhibitors (ICI) show response rates of up to 60%, depending on drug or combination, and many of these are durable effects.[4] Multiple resistance mechanisms to MAPKi and ICI have been proposed and confirmed over the years and summarized in recent reviews.[5-9] Additional important contributors to melanoma drug resistance are defective apoptosis,[10,11] tumor heterogeneity and phenotypic plasticity.[12-15] A hallmark of the latter is differential proliferative

Abbreviations: 2D/3D, two/three-dimensional; BTZ, bortezomib; FUCCI, fluorescent ubiquitination-based cell cycle indicator; G1/S/G2/M-phase, Gap 1/Synthesis/Gap2/Mitosis phases of the cell cycle; ICI, immune checkpoint inhibitors; MAPKi, MAPK pathway inhibitors; MAPK, mitogen-activated protein kinase.
behaviour of tumor subpopulations. This dynamic tumor heterogeneity can directly influence drug sensitivity as some drugs only target actively proliferating cells and other drugs may only work in certain cell cycle phases. Reduced access to oxygen and nutrients in the tumor centre or in areas distant from the vasculature causes $G_{\text{s}}$-phase cell cycle arrest, thereby influencing drug responses. Hypoxia induces phenotype switching and a stress response within melanoma cells that confers a drug-tolerant state. While inappropriate cell cycle arrest can reduce drug sensitivity, defective cell cycle checkpoints may offer new therapeutic targeting opportunities. An improved understanding of the contribution of the cell cycle to drug efficacy, and how defective cell cycle controls can be used as targets, is required to develop more effective therapeutic strategies for patients with metastatic melanoma.

2 CELL CYCLE PHASE-SPECIFIC DRUG INSENSITIVITY AS AN ESCAPE MECHANISM OF MELANOMA CELLS

Most anticancer drugs impact on the cell cycle. Alkylating agents and anthracyclines commonly promote $G_{\text{s}}$, $S$, and $G_{\text{2}}$-phase cell cycle arrest, depending on the p53 status of the cell, while mitotic inhibitors arrest in M-phase, and antimetabolites result in S-phase arrest. The arrest or in many cases premature exit from the arrest is the basis of the clinical effect of these agents. In contrast to these "classic genotoxic chemotherapies," kinase inhibitors such as MAPKi induce $G_{\text{1}}$-phase and Aurora kinase inhibitors and the 26S proteasome inhibitor, bortezomib induce $G_{\text{2}}$-phase cell cyclarrest and subsequent apoptosis. Thus, combinations of drugs that block progression into the cell cycle phase essential for the anticancer action of the drugs would be expected to reduce the efficacy of the combination.

Utilizing the fluorescent ubiquitination-based cell cycle indicator (Fucci) in 3D melanoma spheroids, we have established a model to study the effect of the cell cycle on drug sensitivity in real time. Our recent proof-of-principle study on cell cycle phase-specific drug resistance demonstrated that bortezomib induces both $G_{\text{s}}$- and $G_{\text{2}}$-arrest and, strikingly, causes apoptosis preferentially of $G_{\text{2}}$-phase cells (Figure 1 and 2A). Similar results were seen using the DNA-alkylating agent temozolomide, which induces $G_{\text{s}}$/M-arrest leading to apoptosis in melanoma cell lines in vitro. Importantly, we found that both pharmacologically (eg by MAPKi) and environmentally (eg by hypoxia and/or nutrient deprivation) $G_{\text{s}}$-arrested melanoma cells were resistant to bortezomib and temozolomide, indicating cell cycle phase-specific drug sensitivity. Similarly, cell cycle-mediated drug sensitivity and resistance has been shown for the taxanes, which induce M-arrest followed by apoptosis. Thus, induction of $G_{\text{s}}$-arrest in gastric and breast cancer cells through pretreatment with flavopiridol resulted in resistance to paclitaxel. Taken together, these findings indicate that cell cycle phase-specific drug insensitivity is a general escape mechanism that can occur in various cancer types and chemotherapy combinations.

**Figure 1** Extended focus confocal microscopy images of a C8161-Fucci 3D spheroid embedded in collagen and treated with 10 nM bortezomib over a course of 64 h (numbers= t in h). There is a random distribution of actively cycling cells (red, yellow and green) at 0 h. Bortezomib initially appears to cause $G_{\text{s}}$-arrest (peak of yellow/green cells at 24-32 h), but only melanoma cells in $G_{\text{2}}$-phase (red) survived prolonged treatment, while cells in $G_{\text{2}}$-phase underwent NOXA-dependent apoptosis.

**Figure 2** Cartoon depicting drug responses in different drug scheduling regimens. (A-E): Random distribution of actively cycling cells (red and green) at 0 h. (A) Treatment with bortezomib (BTZ) initially causes predominantly $G_{\text{s}}$-arrest (green), but only melanoma cells in $G_{\text{s}}$-phase (red) survive prolonged treatment, while cells in $G_{\text{s}}$-phase undergo apoptosis (black cells, green crossbones). (B) Similarly, simultaneous combination of BTZ with a sublethal dose of a $G_{\text{s}}$-arresting agent leads to apoptosis of $G_{\text{s}}$-phase cells (black cells, green crossbones) while $G_{\text{s}}$-phase cells survive (red). (C) Contrarily, pretreatment with a $G_{\text{s}}$-arresting condition (red) cancels BTZ cytotoxicity. (D) The $G_{\text{s}}$-arrested hypoxic core (red) but not the cycling periphery (red and green) of spheroids is resistant to BTZ. (E) Sequential treatment with two $G_{\text{s}}$-inducing drugs (MEKi + BRAFi) results in additive cytotoxicity (black cells, red crossbones).
3 | WOULD OPTIMIZED SEQUENCING OF DRUG COMBINATIONS YIELD BETTER PATIENT OUTCOMES?

We showed that in combination therapies, one drug can sensitize to the other but not necessarily the converse and that reversing the order of the drug combination may impact on treatment efficacy. Indeed, combination and drug scheduling approaches to optimize patient care has become a hot topic in the melanoma field.

In our proof-of-concept study, single-agent bortezomib treatment resulted in preferential NOXA-mediated apoptosis of $G_1$-phase cells, while $G_2$-phase-arrested cells survived (Figure 1 and 2A). Similarly, simultaneous treatment of cells with a conventional $G_1$-phase arrest agent and bortezomib did not affect the preferential apoptosis of $G_2$-phase cells (Figure 2B). In contrast, pretreatment of melanoma cells with agents or conditions that promote $G_1$-phase arrest (MAPKi, hypoxia or nutrient deprivation) resulted in insensitivity to $G_2$-dependent drugs (temozolomide or bortezomib; Figure 2C), whereas the reverse, pretreatment with temozolomide, did not result in resistance to MAPKi. This is supported by another in vitro study of vemurafenib ($G_1$-arrest) in combination with temozolomide (S/G2-arrest) melanoma. Their data showed that cell death was increased when cells were pretreated with temozolomide, and vemurafenib was added after 72 hours, while the cytotoxic effect of temozolomide was reduced when simultaneously combined with vemurafenib. These findings raise the question whether, in a recent clinical trial, the combination of bortezomib with the pan-RAF inhibitor sorafenib was only ineffective because the sequencing of the drugs had not been optimized. Sorafenib may have induced $G_1$-arrest quickly and subsequently neutralized the effect of bortezomib. Could pretreatment with bortezomib prior to commencing sorafenib have improved the outcome?

Similar to tumors in vivo, 3D spheroids mostly contain a hypoxic centre, which corresponds with areas of $G_1$ arrest. Interestingly in our recent study, the $G_1$-arrested hypoxic core of melanoma spheroids was resistant to bortezomib (Figure 2D). The induction of $G_0$/$G_1$-arrest has been shown to be part of the innate stress response to drug treatment or hypoxic, nutrient-poor conditions within a tumor, and can induce a broad drug tolerance, suggesting that drugs that specifically target the $G_2$-phase may be more commonly and less effective than $G_2$-phase targeting drugs. Highly proliferative cancer cells are more sensitive to bortezomib than slow-cycling cells and conversely, quiescent cells are more resistant to proteasome inhibitors. Similarly, temozolomide requires S-phase progression to exert cell death at clinically relevant concentrations. These data support the importance of microenvironmental factors within a tumor that may alter the response of melanoma cells to therapies.

Sequential treatment with two $G_1$-inducing drugs (MEKi + BRAFi) resulted in additive cytotoxicity (Figure 2E). In contrast to the neutralizing effects of $G_1$-arrest on $G_2$-phase targeting drugs, $G_1$-arrest sensitized melanoma cells to MAPKi, further supporting the rationale for combining BRAF and MEK inhibitors for the treatment of melanoma, which have been shown in numerous clinical trials to improve progression-free survival compared to single-agent treatment.

Furthermore, this indicates that approaches to block the $G_1$-S transition, such as selective CDK4/6 inhibitors currently in clinical trials, may be effective in combination with $G_1$- but not with $G_2$-phase targeting therapies. Interestingly, synchronizing myeloma cells in early S-phase using reversible CDK4/6 inhibition resulted in enhanced bortezomib-induced cytotoxicity compared to synchronization of cells in $G_2$ further demonstrating that strategies that synchronize tumor cells into a drug-sensitive cell cycle phase can improve the antitumor effect of drugs. However, this type of synchronization has been very difficult to achieve in patients. This challenge may be overcome using a novel in vivo reporter model that has been established to quantify the effects of, and resistance to, CDK4/6 inhibitors in a temporal and tumor-selective manner. This will facilitate testing the effects of CDK4/6 inhibitor-based schedules and combinations using the reporter as a direct readout of pathway activity.

The effects discussed above have all been focused on immediate and short-term cell cycle arrest that influences the immediate efficacy of combination treatments. Another, longer term effect is acquired resistance in response to continuous treatment with MAPKi. This suggests that the extended $G_1$-$G_0$-arrest induced by prolonged MAPKi therapy may confer a drug-tolerant phenotype that primes the cell for development of permanent resistance mechanisms, even when re-wiring of the signalling pathways has re-activated proliferative signalling. Therefore, to prevent the progression of melanoma from drug tolerance to resistance, it may be necessary to allow a treatment-free period in the dosing protocol to reduce the general stress response within melanoma cells responsible for development of resistance.

Finally, in our hands, sequential treatment with two $G_2$-inducing drugs (temozolomide and bortezomib) resulted in additive cytotoxicity (Figure 2F). While synergy of bortezomib and temozolomide was shown in a murine xenograft model of human melanoma, a phase I trial with simultaneously combined bortezomib and temozolomide treatment was less promising. Further studies to assess whether sequential rather than a simultaneous treatment using $G_2$-phase targeting drugs may be more effective are warranted.

In summary, these studies indicate that consideration of cell cycle-mediated drug resistance must be taken into account when planning melanoma combination therapies, timing of dosing schedules and choice of drug therapies in solid tumors.

Another approach is to target different tumor cell subpopulations with different drugs. For example, highly proliferative tumor cells are more drug-sensitive than slow growing cells suggesting that distinct therapeutic approaches are needed to target both differentially cycling tumor cell subpopulations. Thus, combinations used should be effective in a particular subpopulation, but not interact with the drugs targeting different subpopulations to reduce their effectiveness. In this sense, each drug in the combination is effective in one subpopulation rather than acting in concert in the same tumor cell. This may explain the clinical effectiveness of drug combinations that appear to go against the principles we have established in our model systems.
4 | COMBINATION OF MAPKi AND ICI

One obvious therapy option is the combination of MAPKi and ICI. The study of the biology behind the impact of this combination on the tumor cells’ cell cycle, the tumor microenvironment and the immune system is complex enough to fill another viewpoint. Marcus Bosenberg’s group has recently generated a series of congenic mouse melanoma cell lines with defined genetic alterations that permit experiments in immune competent systems—the YUMM lines. Such models permit analysis in combination with immune checkpoint inhibitors, which is relevant as inhibitor-induced senescence may alter the tumor immune microenvironment.

5 | CELL CYCLE CHECKPOINTS AS THERAPEUTIC TARGETS IN MELANOMA

To this point, we have considered the impact of cell cycle stage and progression on standard chemotherapeutics and targeted drugs. An alternative approach is to view cell cycle control and defects in these mechanisms as opportunities for selectively targeting and destroying tumors. Many conventional chemotherapies are genotoxic and necessarily trigger a DNA damage checkpoint response (Figure 3). Intact checkpoint responses can reduce sensitivity to these agents by either triggering repair responses to reduce the damage load or simply slow cell cycle progression to provide for more time to repair or adapt to the damage. This has been the rationale for the development of DNA damage checkpoint inhibitors as chemosensitizers, for example inhibitors ATM/ATR or downstream signalling through CHK1/2. Optimal chemosensitization may require consideration of the cell cycle effects of the chemotherapeutic agent. CHK1 inhibitors very strongly sensitize tumor cells to gemcitabine, and the effect is maximized if the treatments are scheduled with the gemcitabine treatment preceding CHK1 inhibitor treatment by 12 hours to allow the tumor to accumulate in the CHK1 inhibitor-sensitive S-phase checkpoint arrest.

Defects in cell cycle checkpoint controls are common in cancers, and they offer a point of difference between normal tissue and cancers, being defective in only the tumors. An example is the DNA damage response signalling hub, ataxia telangiectasia mutated (ATM). Germline mutation of this gene is responsible for the tumor prone syndrome ataxia telangiectasia, but loss of ATM function is also responsible for increased sensitivity to selective DNA-damaging agents providing a potential avenue to selectively target tumors with somatic acquisition of this specific defect. Thus, it should be possible to stratify patients on the basis of functional status of DNA damage checkpoint signalling in cancers to personalize patients’ chemotherapy regimens. An example is patients with BRCA-mutant breast cancer are more sensitive to cisplatin treatment.

Further opportunities exist in targeting DNA damage checkpoint defects in cancers. Loss of these checkpoints can make tumors more reliant on particular pathways for their viability, and inhibition of these pathways is sufficient to promote selective killing of the checkpoint defective cancers. Inhibiting TSC1/2 in RB-mutant tumors or P38Kα in ATM-dependent G2-phase checkpoint melanomas is synthetically lethal with the checkpoint defect in each case. The ATM defect is a particularly interesting example as it is not the more usual loss-of-function ATM mutation. ATM signalling of DNA damage appears completely intact, but cells are unable to maintain a cell cycle checkpoint arrest, a direct consequence of over-activation of PLK1 driving premature exit from the arrest. This ATM defect is common in melanoma cell lines with a complete loss of ATM-dependent cell cycle arrest in >25% of cell lines tested. Recently, a number of unbiased, systematic screens using either gene targeting (RNAi or gene editing) or small molecules have identified molecular targets/pathways synthetically lethal with defined defects. This is best exemplified in BRCA-mutant breast cancers where loss of BRCA-dependent homologous recombination repair results in cells becoming hypersensitive to inhibition of base excision repair (BER) using PARP inhibitors. This approach has been extended with understanding that BRCA-mutated cancers show defects in the CRISPR-Cas9 genome targeting system, an example of the potential impact of these discoveries on the development of novel therapeutics.

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6 | WHAT HAVE WE MISSED IN THE PAST?

Have drug combination trials that were ineffective and failed been abandoned prematurely, possibly because of the failure to consider
drug cell cycle effects and their impact on the other drugs in the combination? Have we missed the opportunity to develop new effective drug combinations because of a failure to optimize scheduling regimens to adapt the therapies to their impact on cell cycle phases?

7 | FUTURE CLINICAL PERSPECTIVES

Cell cycle-dependent drug insensitivity should be taken into account when planning melanoma combination therapies, timing of dosing schedules and choice of drug therapies. Specifically, if the same cell is targeted by both drugs of a combination, we need to make sure that the drugs do not negatively influence each other, including by accounting for their cell cycle-dependent effects. Further, dynamic tumor heterogeneity needs to be considered. In this case, one drug of a combination is designed to target one tumor subpopulation and another drug targets the other subpopulation, but again these must not negatively impact on each other in tumor cells where the drugs do interact. Additional factors that would contribute to the effectiveness of these combinations include the half-lives of the respective drugs in the patient tumor. We propose the utilization of our real-time cell cycle imaging 3D melanoma spheroid model as a semi-high throughput tool to measure and design drug scheduling approaches, which—knowing the limitations of this model—then need to be validated preclinically in animals. A major goal of this work is to develop FUCCI 3D melanoma spheroid models that replicate the majority of genotypes in melanoma to understand the effects of these genetic backgrounds on drug sensitivity and optimal scheduling of combination treatments. This information can then be used to guide optimized and personalized treatment regimens for patients on the basis of their tumor genotype.

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CONFLICT OF INTEREST

The authors have declared no conflicting interests.

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