Cell cycle phase-specific drug resistance as an escape mechanism of melanoma cells

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Abbreviations: 2D/3D, two/three-dimensional; BTZ, bortezomib; DAPI, 4’,6-diamidino-2-phenylindole; FUCCI, Fluorescent ubiquitination-based cell cycle indicator; G₁/S/G₂/M-phase, Gap 1/Synthesis/Gap2/Mitosis phases of the cell cycle; MAPK, Mitogen-activated protein kinase; MAPKi, MAPK pathway inhibitors; TMZ, temozolomide
ABSTRACT

The tumor microenvironment is characterized by cancer cell subpopulations with heterogeneous cell cycle profiles. For example, hypoxic tumor zones contain clusters of cancer cells that arrest in G1-phase. It is conceivable that neoplastic cells exhibit differential drug sensitivity based on their residence in specific cell cycle phases. Here, we have used two-dimensional and organotypic melanoma culture models in combination with fluorescent cell cycle indicators to investigate the effects of cell cycle phases on clinically used drugs. We demonstrate that G1-arrested melanoma cells, irrespective of the underlying cause mediating G1-arrest, are resistant to apoptosis induced by the proteasome inhibitor bortezomib or the alkylating agent temozolomide. In contrast, G1-arrested cells were more sensitive to MAPK pathway inhibitor-induced cell death. Of clinical relevance, pre-treatment of melanoma cells with a MAPK pathway inhibitor, which induced G1-arrest, resulted in resistance to temozolomide or bortezomib. On the other hand, pre-treatment with temozolomide, which induced G2-arrest, did not result in resistance to MAPK pathway inhibitors. In summary, we have established a model to study the effects of the cell cycle on drug sensitivity. Cell cycle phase-specific drug resistance is an escape mechanism of melanoma cells that has implications on the choice and timing of drug combination therapies.

INTRODUCTION

Small molecule inhibitors that selectively target mutant BRAF or its downstream effector MEK have provided unprecedented responses in a subset of BRAF mutant melanoma patients (McArthur, 2015). However, development of resistance is common (Homet and Ribas, 2014). Therefore, an improved understanding of the underlying biological mechanisms mediating drug
efficacy and the emergence of resistance is required to develop more effective therapeutic strategies for patients with metastatic melanoma.

MAPK pathway inhibitors (referred to subsequently as MAPKi) effectively induce G1-phase cell cycle-arrest (referred to subsequently as G1-arrest) and apoptosis (Haass et al., 2008; Haferkamp et al., 2013; Lee et al., 2010; Tsai et al., 2008). The 26S proteasome inhibitor, bortezomib, which is approved for treating multiple myeloma and mantel cell lymphoma (Hill et al., 2014; Orlowski and Kuhn, 2008), induces apoptosis of melanoma cells through a mechanism involving induction of G2/M-arrest (Ling et al., 2003; Selimovic et al., 2013; Tamura et al., 2010), whilst having minimal adverse effect on melanocytes (Fernandez et al., 2005). However, while bortezomib induces robust cytotoxicity of proliferating melanoma cells in vitro, the response of melanoma cells to bortezomib is greatly reduced in vivo (Hill et al., 2009). The DNA-alkylating agent temozolomide, a derivative of dacarbazine, induces DNA damage and G2/M-arrest leading to apoptosis in melanoma cell lines in vitro (Eich et al., 2013; Roos et al., 2014), but has limited efficacy in metastatic melanoma patients (Teimouri et al., 2013).

Reduced access to oxygen and nutrients in the tumor center or in areas distant from the vasculature causes G1-arrest (Haass et al., 2014), which can influence the response to chemotherapies that target actively dividing cells (Mitchison, 2012). Hypoxia induces phenotype switching (Haass et al., 2014; O'Connell et al., 2013) and a stress response within melanoma cells that confers a drug-tolerant state (Ravindran Menon et al., 2015). Studies on human lymphocytes revealed that clinically relevant doses of temozolomide only induced apoptosis in proliferating cells (Roos et al., 2004). These findings highlight that environmental factors within
a tumor and/or cell cycle status may alter the response of melanoma cells to therapies (Haass, 2015).

To determine the effect of the tumor microenvironment (Brandner and Haass, 2013) and cell cycle status on melanoma response to bortezomib, temozolomide, MAPKi or combinations of these drugs, we utilized the fluorescent ubiquitination-based cell cycle indicator (FUCCI) (Sakaue-Sawano et al., 2008) in three melanoma cell lines to track the cell cycle in 2D-cultured cells or within 3D collagen-embedded spheroids (Haass et al., 2014). This model allowed us to study the effect of the cell cycle on drug sensitivity in real-time. We found that both pharmacologically and environmentally G₁-arrested melanoma cells are resistant to bortezomib and temozolomide-induced cytotoxicity, but are sensitized to MAPK inhibition – a finding that has implications on the choice and timing of drug combination therapies.

RESULTS
Bortezomib induces dose-dependent G₂-arrest of melanoma cells.
To track the cell cycle status in melanoma cell lines, we utilized FUCCI in which red fluorescence indicates G₁, yellow early S, and green S/G₂/M-phases, with a short loss of fluorescence just after division (Haass et al., 2014; Sakaue-Sawano et al., 2008).

Bortezomib induces G₂-arrest of cancer cells, including melanoma (Bavi et al., 2011; Hill et al., 2009; Hong et al., 2012; Ling et al., 2003; Yin et al., 2005). Consistently, flow cytometry and image analysis of DAPI-stained FUCCI-C8161, -WM164 and -1205Lu cells demonstrated dose-dependent G₂-phase accumulation after 24h bortezomib treatment in 2D culture (Figure 1a,b,
Supplementary Figure S1a,b). G2-arrested cells appeared yellow (rather than green), which is likely due to inhibition of proteasomal degradation of the fluorescent reporters by bortezomib (Wohlschlegel et al., 2000). There was a significant dose-dependent inhibition of cell viability/proliferation after 48h bortezomib treatment (Figure 1c and Supplementary Figure S1c). These data confirm that bortezomib causes dose-dependent G2-arrest of melanoma cells.

**Bortezomib induces G2- and G1-arrest, but preferential apoptosis of G2-phase cells**

The increase in G2-phase cells following bortezomib treatment for 24h was replicated in 3D spheroids, where FUCCI-C8161 and -WM164 cells accumulated as a yellow/green population (Figure 2, Supplementary Figure S2). However, the G1-arrested spheroid core, a result of hypoxia and nutrient deprivation (Haass et al., 2014), persisted (Figure 2a). Consistently, flow cytometry quantification showed an increase in G2-phase cells after 24h bortezomib treatment in both 2D- and 3D-cultured cells; this increase was more substantial in 2D culture (Figure 2c, Supplementary Figure 2d,e).

Spheroid culture in the presence of 10 nM bortezomib beyond 24h resulted in a gradual reduction in the yellow/green population and an emergence of a primarily red population by 72h, indicating a loss of S/G2/M phase cells and a relative increase in G0/G1-phase cells (Figure 2, Supplementary Figure S2). Flow cytometry cell cycle analysis of DAPI-stained cells after 72h treatment with 10 nM bortezomib confirmed that most of the remaining live cells were red and resided in G0/G1-phase (Figure 2b, Supplementary Figure S2c,d). A similar increase in FUCCI-red G1-cell percentage after 48 and 72h bortezomib treatment was observed in 2D culture (Figure 2c, Supplementary Figure S2e). To exclude the possibility that degradation of bortezomib resulted in synchronization of the cell cycle and re-entry into G1-phase, cell cycle progression of
C8161 spheroids was tracked following replenishment of bortezomib treatment every 24h. Similar to previous experiments where the drug was only added once, there was complete loss of S/G2/M-phase cells by 72h (data not shown), indicating the remaining G0/G1-phase cells have not come from S/G2/M-phase cells re-entering the cell cycle due to degradation of the drug. After 11 days bortezomib treatment of C8161 and WM164-spheroids (fresh drug added every three days), most cells were dead, and the remaining live cells were FUCCI-red (data not shown). This suggests that bortezomib induces both G2- and G1-arrest in 2D and 3D culture, and that G1-arrested cells survive longer than G2-arrested cells.

Flow cytometry of live/dead-stained C8161, WM164 and 1205Lu melanoma cells confirmed that bortezomib induced time-dependent cytotoxicity in 2D culture (Figure 3a, Supplementary Figure S3a). Bortezomib also induced time-dependent cytotoxicity in C8161 in 3D spheroids (Figure 3a), albeit less potently compared to 2D culture, consistent with the observation that spheroids were more resistant to bortezomib-induced G2-arrest.

Time-lapse microscopy of C8161 and 1205Lu FUCCI-expressing melanoma cells in 2D culture confirmed that bortezomib treatment induced primarily S/G2/M-arrest (FUCCI-yellow/green) within 24h (Supplementary Figure S3b), with very few cells completing mitosis after addition of the drug (mean of 18% for C8161, 3.5% for 1205Lu, compared to 100% in the controls). Of the bortezomib-treated cells that stayed alive, 13% (C8161) or 84% (1205Lu) remained in G1 (FUCCI-red) for the entire observation period (40h), compared to 0.5% (C8161) and 3% (1205Lu) in the controls, confirming that a subset of cells undergo G0/G1-arrest in response to bortezomib. Single cell tracking showed that while cells in both G1- and G2-phase died, more
green/yellow S/G\textsubscript{2}/M-phase cells died than red G\textsubscript{1} cells (Figure 3b) and that S/G\textsubscript{2}/M-phase cells died significantly earlier than G\textsubscript{0}/G\textsubscript{1}-phase cells (Figure 3c).

Annexin V-staining of FUCCI-C8161 and -1205Lu cells after bortezomib treatment indicated the mode of cytotoxicity was primarily apoptosis (Figure 3d, Supplementary Figure S3c). The majority of apoptotic cells were in S/G\textsubscript{2}/M-phase, and the proportion of apoptotic cells increased over time. Apoptotic G\textsubscript{1}-phase cells were also observed, although the proportion was significantly lower and apoptosis was delayed compared to S/G\textsubscript{2}/M-phase cells. These results, together with the imaging data, indicate that bortezomib causes G\textsubscript{2}- and G\textsubscript{1}-arrest, and that melanoma cells in G\textsubscript{1}-phase are less sensitive to bortezomib-induced apoptosis.

As the pro-apoptotic protein NOXA promotes bortezomib-mediated apoptosis in melanoma (Fernandez et al., 2005; Mohana-Kumaran et al., 2014; Podar et al., 2008; Qin et al., 2005; Reuland et al., 2012; Wolter et al., 2007), we chose to investigate if NOXA was also involved in cell-cycle specific bortezomib-mediated apoptosis. FUCCI-1205Lu cells were treated for 24h with 10nM bortezomib and then sorted by cell cycle phases (MoFlo Astrios Cell Sorter, Beckman Coulter, see Supplemental Materials). Immunoblotting of sorted and unsorted samples revealed that NOXA levels increased with bortezomib treatment; this increase was higher in S/G\textsubscript{2}/M-compared to G\textsubscript{0}/G\textsubscript{1}-phase cells (Figure 3e), consistent with the higher level of apoptosis in G\textsubscript{2}-phase. NOXA expression was also lower in un-treated G\textsubscript{0}/G\textsubscript{1}- compared to S/G\textsubscript{2}/M-phase cells. Consistent with the NOXA levels and annexin V-staining, cleaved caspase 3 was detected in bortezomib-treated S/G\textsubscript{2}/M- but not G\textsubscript{1}-phase cells (Figure 3e).
Bortezomib-induced G1-arrest is reversible

As bortezomib is a reversible inhibitor (Hill et al., 2014), we wished to determine if bortezomib-induced G1-arrest is also reversible. To test whether bortezomib-treated G1-arrested cells can re-enter the cell cycle after drug removal, we treated 2D-cultured FUCCI-C8161 and -1205Lu cells for three days, then changed to normal medium. Time-lapse imaging indicated that G1-arrested cells re-entered the cell cycle after bortezomib removal (Supplementary Figure S3d-g, Supplementary Movies 1,2). Re-exposure to bortezomib resulted in an increase in S/G2/M-phase cells and subsequent cell death, indicating retained drug sensitivity (Supplementary Figure S3d-g). While drug-washout after three days led to recovery of the treated cells, continuous exposure to bortezomib (fresh drug replacement after three days) resulted in continuing G1-arrest after day 3 and eventually cell death of most cells at day 6 (Supplementary Figure S3h).

Pharmacologically induced G1-arrest inhibits bortezomib and temozolomide-induced cytotoxicity

Resistance of G1-arrested cells in the spheroid core to bortezomib-induced G2-arrest, as well as the longer survival of G1-arrested bortezomib-treated cells, indicates that G1-arrested cells are less sensitive to bortezomib-induced apoptosis. To investigate this theory further, melanoma cells were pre-treated with G1-arrest inducing drugs before exposure to bortezomib (Figure 4a). We have demonstrated previously that MEK or BRAF inhibition induces G1-arrest (Haass et al., 2014; Haass et al., 2008). Therefore, C8161, WM164 and 1205Lu cells in either 2D or 3D culture were treated for 24h with the MEK inhibitor U0126 or the selective BRAF inhibitor PLX4032 at a concentration that was optimized for each cell line to induce G1-arrest without significant cell death (Supplementary Figure S4a,b). Cells were then treated with bortezomib for further 48h in the continued presence of U0126 or PLX4032. Imaging of FUCCI-C8161, -
WM164 and -1205Lu cells grown in 2D confirmed that U0126 prompted G₁-arrest in all three cells lines after 24h, and PLX4032 in WM164 and 1205Lu cells (Supplementary Figure S4c). C8161 is BRAF<sup>WT</sup> at codon 600 (Davies<sup>et al.</sup>, 2009) and therefore does not undergo G₁-arrest in response to PLX4032 (Lee<sup>et al.</sup>, 2010). Unsurprisingly, PLX4032 did not cause G₁-arrest in C8161 (Supplementary Figure S4c).

Flow cytometry of 2D-cultured cells indicated that U0126- or PLX4032-induced G₁-arrest inhibited bortezomib-induced apoptosis (Figure 4b-d). Furthermore, flow cytometry of 2D-cultured FUCCI-cells and imaging of FUCCI-spheroids, demonstrated that U0126 or PLX4032 prevented bortezomib-induced S/G₂/M-arrest (Figure 4e, Supplementary Figure S4d). As expected, this was not the case for PLX4032/bortezomib combination in C8161 cells (Figure 4d,e, Supplementary Figure S4d). Interestingly, although PLX4032 alone was not cytotoxic for BRAF<sup>WT</sup> cells (C8161, MelRM), PLX4032/bortezomib combination increased cell death and G₂-arrest in BRAF<sup>WT</sup> cells, compared to bortezomib alone (Figure 4d, Supplementary Figure S4d,e). This may be due to off-target effects of PLX4032 or the paradoxical activation of MAPK in BRAF<sup>WT</sup> cells (Hatzivassiliou<sup>et al.</sup>, 2010). Typically, pERK was slightly increased after 48h PLX4032 treatment in C8161 cells (either alone or in combination with bortezomib). However bortezomib alone did not significantly alter pERK levels (data not shown).

To confirm that G₁-arrest inducing pre-treatment was necessary for the rescue effect in BRAF-mutant WM164 cells, we added PLX4032 simultaneously with bortezomib. Indeed there was no rescue from bortezomib-induced apoptosis (Figure 4f). However, as both drugs alone induced some cell death and the combination had no additive effect, there was possibly some protection from bortezomib-mediated apoptosis. Some cells may undergo PLX4032-induced G₁-arrest
before bortezomib becomes effective, which is supported by the reduction of bortezomib-induced 
G2-accumulation when simultaneously combined with PLX4032 (Supplementary Figure S4f).

To determine if pharmacologically induced G1-arrest can rescue MAPKi cytotoxicity, we pre-
treated WM164 cells with low-dose PLX4032 to cause G1-arrest but minimal cytotoxicity, then 
treated with U0126 for further 48h (Figure 4g). Although the amount of cell death was similar 
between single-agent PLX4032 and U0126 treatments, there was a significant increase in cell 
death for the combination. This suggests that rather than rescuing cells from U0126-induced cell 
death, PLX4032-induced G1-arrest sensitizes cells to U0126-induced cell death.

To explore if pharmacologically induced G1-arrest protected from G2-arrest inducing drugs other 
than bortezomib, we tested the effect of U0126 pre-treatment on temozolomide-induced 
cytotoxicity. Temozolomide caused G2-arrest of C8161 after 24h treatment (Figure 4h). U0126 
pre-treatment protected cells from temozolomide-induced G2-arrest and cell death (Figure 4i, 
Supplementary Figure S4g). WM164 and 1205Lu were resistant to temozolomide. At the high 
concentrations required to induce cell death (>100 µM), very little G2-arrest was observed. 
Melanoma cell resistance to temozolomide has been described previously (Mhaidat et al., 2007).

Finally, to determine the effect of G2-arrest on MAPKi cytotoxicity we pre-treated C8161 cells 
with low-dose temozolomide, then added high-dose U0126 for further 48h to induce cell death. 
In this case the cells were not protected from U0126-induced cell death (Figure 4j). U0126 
appeared to be able to overcome the temozolomide-induced G2-arrest and drive cells into G1-
arrest (Supplementary Figure S4h). Cells pre-treated with low-dose temozolomide were also not
protected from bortezomib-induced cell death (Figure 4k). In fact the combination enhanced cell death.

**Environmentally induced G₁-arrest inhibits bortezomib-induced cell death, but enhances MAPK inhibitor cytotoxicity.**

As an environmental approach of inducing G₁-arrest, we serum-starved WM164 cells under hypoxia for 24h (Figure 5a). This environmentally induced G₁-arrest reduced bortezomib-induced cell death (Figure 5c), indicating that multiple methods of arresting cells in G₁ result in protection from bortezomib cytotoxicity. In contrast, environmentally induced G₁-arrest did not protect cells from but rather increased U0126-induced cell death (Figure 5e). This indicates that G₁-arrest is not protective for MAPKi cytotoxicity.

C8161 cells, confluent and serum-starved for 48h, arrested in G₁ (Figure 5b). Also this approach of environmentally induced G₁-arrest resulted in protection from bortezomib- and temozolomide-induced cell death (Figure 5d). Again, environmentally induced G₁-arrest did not protect C8161 cells from MAPKi cytotoxicity (using high-dose U0126), but instead enhanced it (Figure 5f).

To investigate the molecular mechanisms underlying the changes in drug sensitivity of hypoxic/starved G₁-arrested cells, we performed immunoblotting on serum-starved WM164 cells under hypoxia for 24h or 48h (Figure 5g). NOXA levels were decreased under hypoxia, which may contribute to the resistance to bortezomib-induced apoptosis. No changes in pERK levels relative to total ERK were seen under hypoxia (data not shown), indicating that altered MAPK signaling under hypoxia is not responsible for the enhanced sensitivity to MAPKi.
DISCUSSION

We have established a model to study the effect of the cell cycle on drug sensitivity in real-time. Validating this approach we confirmed that bortezomib indeed induces time- and dose-dependent G2-arrest resulting in death of melanoma cells, as previously shown (Hill et al., 2009). Here we demonstrate that bortezomib induces not only G2- but also G1-arrest and, importantly, causes apoptosis preferentially of G2-phase cells, likely via NOXA. Surviving G1-arrested cells re-entered the cell cycle upon bortezomib removal, and regained sensitivity to bortezomib-induced G2-arrest and apoptosis. Both pharmacologically and environmentally G1-arrested melanoma cells are resistant to bortezomib and temozolomide, but are sensitized to MAPKi, indicating cell cycle phase-specific drug sensitivity.

Cell cycle-mediated resistance has previously been demonstrated for the taxanes, which stabilize microtubules and induce G2/M-arrest followed by apoptosis (Abal et al., 2003). Indeed paclitaxel cytotoxicity is maximal in cells synchronized in G2/M-phase (Donaldson et al., 1994). Moreover, pre-treatment of gastric and breast cancer cells with flavopiridol, which induces G1-arrest, resulted in resistance to paclitaxel (Motwani et al., 1999). Together with our data, these findings indicate that cell cycle phase-specific drug resistance is a general escape mechanism that occurs in various cancer types and a range of chemotherapies.

We have recently shown that in drug combinations one drug can sensitize to the other but not necessarily the converse (Lucas et al., 2012). Here we demonstrate that reversing the order of the drug combination may impact on treatment efficacy. For example, while pre-treatment of melanoma cells with MAPKi resulted in resistance to temozolomide or bortezomib, pre-treatment with temozolomide did not result in resistance to MAPKi.
Consistent with the synergistic activity of bortezomib with temozolomide against melanoma in mice (Amiri et al., 2004), we show that pre-treatment of melanoma cells with temozolomide, which induces G2-arrest, results in increased bortezomib-induced cytotoxicity, suggesting that sequential combination of bortezomib with other G2-phase-arresting drugs may be an effective therapeutic strategy for patients with metastatic melanoma. Supporting this idea, synchronizing myeloma cells in early S phase using reversible CDK4/CDK6 inhibition resulted in enhanced bortezomib-induced cytotoxicity compared to synchronization of cells in G1 (Huang et al., 2012). In contrast, a phase I trial with combined bortezomib and temozolomide treatment resulted in only one of 19 advanced melanoma patients achieving a partial response (Su et al., 2010). However, drugs in this trial were administered simultaneously. Therefore, sequential rather than a simultaneous treatment with temozolomide or other G2-phase targeting drugs prior to administration of bortezomib may be more effective.

Importantly, we demonstrate that while bortezomib and temozolomide effectively induce death of proliferating melanoma cells they are ineffective against either drug-induced or hypoxia/serum-starve/confluency-induced G1-arrested cells, as well as the G1-arrested core of 3D spheroids. This may explain why the combination of bortezomib and the pan-RAF inhibitor sorafenib in a recent clinical trial was ineffective for the treatment of melanoma (Sullivan et al., 2015), as sorafenib may have quickly induced G1-arrest and hence neutralized the effect of bortezomib. Furthermore, drugs that specifically target cells in G2-phase may be universally less effective than G1-phase targeting drugs in vivo due to the initiation of G0/G1-arrest as part of a general stress response to drug treatment or hypoxic, nutrient-poor conditions within a tumor (Ravindran Menon et al., 2015). However, the level of hypoxia may be important. We used moderate hypoxia (1%),
whereas a previous study using severe hypoxia (<0.2%) showed that hypoxic HeLa cells were more sensitive to bortezomib due to induction of ER stress pathways (Fels et al., 2008). Hypoxia has also been shown to induce drug tolerance though various mechanisms (Shannon et al., 2003), so hypoxia-induced G1-arrest may only increase sensitivity to MAPKi in the short-term. A highly proliferative tumor may also respond better than a slow growing tumor. C8161, which was most sensitive to bortezomib and temozolomide, is the fastest growing cell line and spends the least amount of time in G1-phase (Haass et al., 2014). It has previously been noted that cancer cell lines with a higher proliferation rate are more sensitive to bortezomib (Yerlikaya and Erin, 2008), and that quiescent leukemia cells are more resistant to apoptosis induced by other proteasome inhibitors (Drexler, 1997). Also temozolomide in lymphocytes requires proliferation to exert cell death at clinically relevant concentrations (Roos et al., 2004).

The molecular mechanisms underlying cell cycle phase-specific resistance to bortezomib and temozolomide is not fully elucidated. We demonstrate that NOXA, known to promote bortezomib-induced apoptosis (Fernandez et al., 2005; Mohana-Kumaran et al., 2014; Podar et al., 2008; Qin et al., 2005; Reuland et al., 2012; Wolter et al., 2007), is lower in both cycling G1-phase cells and hypoxia-induced G1-arrested cells. While NOXA is upregulated during bortezomib treatment as expected, we now demonstrate that NOXA is primarily upregulated in S/G2/M- but remains low in G0/G1-phase of bortezomib-treated melanoma cells. Thus it is possible that decreased levels of NOXA have a protective effect on G1-phase cells.

In contrast to the protective effect of G1-arrest on G2-phase targeting drugs, we show that G1-arrest increased sensitivity of melanoma cells to MAPKi, which induce G1-arrest and apoptosis (Lee et al., 2010; Smalley et al., 2006; Wroblewski et al., 2013). This indicates that novel
approaches that aim to block the G1-S transition, such as selective CDK4/6 inhibitors (Yadav et al., 2014) currently in clinical trials (Lee et al., 2015), may be effective in combination with G1- but not with G2-phase targeting therapies. These data also further support 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 (Flaherty et al., 2012; Long et al., 2015). However, the emergence of resistance in response to continuous treatment with these drugs indicates that G0/G1-arrest may confer a drug tolerant phenotype that primes the cell for development of permanent resistance mechanisms and re-activation of proliferative signaling. Exposure to a sub-lethal dose of PLX4032 for 12 days (with cells remaining in G1-arrest) can lead to multi-drug tolerance, where cells become resistant to a subsequent treatment with the MEK inhibitor GSK1120212 or other drugs (Ravindran Menon et al., 2015). The progression of melanoma from drug tolerance to resistance was observed in tumors of patients treated with the BRAF inhibitor dabrafenib, which subsequently became insensitive to treatment with the MEK inhibitor trametinib (Johnson et al., 2014). 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.

This study demonstrates that consideration of cell cycle-mediated resistance to bortezomib, temozolomide and MAPKi must be taken into account when planning melanoma combination therapies, timing of dosing schedules and choice of drug therapies in solid tumors. These results may extend to other drug therapies that cause cell death via cell cycle-arrest, and should be investigated in future studies.
MATERIAL AND METHODS

Cells and cell culture

The human melanoma cell lines C8161, WM164, 1205Lu were genotypically characterized (Davies et al., 2009; Hoek et al., 2006; Smalley et al., 2007a; Smalley et al., 2007b), grown as described (Smalley et al., 2005) (with 4% FBS instead of 2% FBS) and authenticated by STR fingerprinting (QIMR Berghofer Medical Research Institute, Herston, Australia). MelRM were grown in DMEM supplemented with 10% FBS as previously described (Zhang et al., 2001) and authenticated using AmpFISTR profiling (Bowden et al., 2010).

Melanoma 3D-spheroid assays

Melanoma spheroids were prepared as described (Beaumont et al., 2015; Smalley et al., 2008; Spoerri et al., in press). This model mimics in vivo tumor architecture and microenvironment and is used for investigating growth, invasion and viability of melanoma cells (Beaumont et al., 2014; Santiago-Walker et al., 2009). See Supplemental Materials.

Fluorescent ubiquitination-based cell cycle indicator (FUCCI)

To generate stable melanoma cell lines expressing the FUCCI constructs, mKO2-hCdt1 (30-120) and mAG-hGem (1-110) (Sakaue-Sawano et al., 2008) were subcloned into a replication-defective, self-inactivating lentiviral expression vector system as previously described (Smalley et al., 2005). The lentivirus was produced by co-transfection of human embryonic kidney 293T cells, high-titer viral solutions for mKO2-hCdt1 (30/120) and mAG-hGem (1/110) were prepared and used for co-transduction into three biologically and genetically well-characterized melanoma cell lines (see above) and subclones were generated by single cell sorting (Haass et al., 2014; Spoerri et al., in press).
Drugs

Proteasome inhibitor bortezomib (Janssen Cilag; North Ryde, NSW, Australia), MEK1/2 inhibitor U0126 (Sigma-Aldrich, St. Louis, MO), selective BRAF inhibitor PLX4032 (Active Biochem, Maplewood, NJ). For pre-treatment, U0126 and PLX4032 doses were chosen so as to induce G1-arrest without significant cell death (Lee et al., 2010; Smalley et al., 2006).

Drug sensitivity assay

MTS viability assays were conducted as described (McGowan et al., 2011). See Supplemental Materials.

Hypoxia and serum-starvation assay

FUCCI-WM164 cells were plated to 30% confluence in 10-cm dishes. The un-starved/normoxia control was incubated in complete medium at 21% O2 and the serum-starved/hypoxia group in the same medium without FBS and bovine insulin at 1% O2 (CB210, Binder, Germany). 24h later cells were observed for G1-arrest (FUCCI) prior to adding drugs or vehicle. After 48h of treatment live/dead cell analysis was performed.

Confluency and serum-starvation assay

FUCCI-C8161 cells were plated at a density of 2.5x10^4 (low confluency) or 3x10^5 cells/well (high confluency) in 6-well plates in normal medium. Cells were allowed to adhere and the high-confluency wells were changed to serum-free medium. Cells were allowed to grow for 48h, observed for G1-arrest (FUCCI), drugs were added for further 48h before live/dead cell analysis.
Flow cytometry cell cycle, live/dead and annexin V analysis

Flow cytometry cell cycle and live/dead analysis was conducted as described (Beaumont et al., 2015; Haass et al., 2014). Annexin V staining and analysis was performed according to manufacturers instructions. See Supplemental Materials.

Immunoblotting

Immunoblotting was conducted as described (Lucas et al., 2012); see Supplemental Materials. Antibodies used: NOXA (Abcam ab13658), ERK (Cell Signalling 9102S), pERK (Cell Signalling 9101S), cleaved Caspase 3 (Cell Signalling 9664S), α-Tubulin (Sigma T6199), goat anti-mouse IgG-HRP (1:5000, Invitrogen 626520) and goat anti-rabbit IgG-HRP (1:3000, Life Technologies G21234).

Confocal imaging of 3D spheroids

Confocal imaging of 3D spheroids was performed as previously described (Haass et al., 2014). For detail see Supplemental Materials.

Fluorescence microscopy of 2D cultured cells

Imaging of live FUCCI cells before extraction for flow analysis:

Live FUCCI cells cultured in 6-well plates were imaged on a Nikon-300 inverted fluorescence microscope using 10x or 20x objectives, or a Delta Vision Elite microscope using a 20x objective (GE Healthcare Life Sciences, Cleveland, OH).

Imaging of live FUCCI cells for dose response assays:
Cells were cultured in 96-well imaging plates (BD, Franklin Lakes, NJ) and imaged on a Pathway 855 high-content bioimager (BD) using a 10x objective.

**Cell cycle image analysis**

FUCCI-red, -green and -yellow cells in merged spheroid z-stacks or single plane 2D culture images were quantified using automated image analysis (Volocity software, Perkin Elmer, Waltham, MA) as previously described (Beaumont *et al.*, 2015; Haass *et al.*, 2014). Alternatively, images obtained on the BD Pathway 855 were analyzed using BD Attovision software, and red and green cell intensities were exported to FCS files for analysis using FlowJo (TreeStar).

**Live time-lapse imaging and cell tracking**

Live imaging of 2D cultured cells was performed on the Delta Vision Elite microscope with 30-minute intervals for 48-72h. Cells were maintained at 37°C, 5% CO₂. Movies were started approximately 30 minutes after drug addition or medium change. Cell tracking was performed using manual tracking in Volocity software. Individual cells were detected based on emitted fluorescence and cell death was ascertained by cell morphology (blebbing and loss of adherence).

**Statistical analysis**

For group comparisons (normal distribution) one-way ANOVA followed by Tukey’s or Dunnett’s test was used. For comparisons of two samples Student’s t-test (normally distributed) was used. A difference was considered significant if p<0.05. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Experiments were usually repeated at least three times independently.
CONFLICT OF INTEREST

The authors state no conflict of interest.

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FIGURE LEGENDS

Figure 1. Bortezomib induces dose-dependent G2-arrest of melanoma cells.
(a) Flow cytometry cell cycle profile of FUCCI-C8161 cells cultured in 2D treated with vehicle (control) or 10 nM bortezomib for 24h. DNA content histograms are overlaid with the FUCCI distribution. Graphs are representative of n=3 independent experiments.
(b) Fluorescence microscopy image analysis of % G1, early S and S/G2/M FUCCI-C8161 cells cultured in 2D after 24h bortezomib treatment. Representative of n=2 independent experiments.
(c) MTS proliferation assay of C8161 cells treated with bortezomib for 48h at the indicated concentrations; n=3, mean±SEM. All samples were compared to the control sample to determine statistical differences.

Figure 2. Bortezomib induces G2 and G1-arrest.
(a) Confocal extended focus images of collagen-embedded FUCCI-C8161 spheroids treated with 10 nM bortezomib for indicated times. Top slices of the z-stack were removed to reveal the red, G1-arrested spheroid core. Images are representative of n=3 experiments. Grey line = spheroid edge as seen in brightfield. Insets = untreated control. Scale bar = 200 µm.
(b) Flow cytometry cell cycle profiles of live cells of FUCCI-C8161 spheroids treated with vehicle (control) or 10 nM bortezomib for indicated periods. DNA content histogram is overlaid with the FUCCI distribution. Graphs are representative of n=3 independent experiments.
(c) Flow cytometry analysis of live FUCCI-C8161 spheroids (left) or 2D-cultured FUCCI-cells (right) after 24, 48, 72h treatment with 10 nM bortezomib (BTZ); n=2-4 experiments, mean±SEM.
Figure 3. Bortezomib induces apoptosis preferentially of G2-phase cells.

(a) Flow cytometry quantification of % cell death in C8161 cells cultured in 2D or 3D and treated with vehicle (control) or 10 nM bortezomib for the indicated time; n=2-4, mean±SEM.

(b) % cells (2D) in G1 vs. S/G2/M phase at death assessed by time-lapse image analysis. Error bars = mean±SEM, n=2-3. Cell death was determined by cell morphology.

(c) G1 vs. S/G2/M phase at time of bortezomib-induced death (2D) assessed by time-lapse image analysis. Cell death was ascertained by cell morphology. Error bars = mean±SEM, data pooled from n=2-3 experiments.

(d) 2D-cultured FUCCI-C8161 cells were treated with 10nM bortezomib for the indicated times. Percentage of annexin-V positive cells in G1- or S/G2/M-phase was quantified by flow cytometry. Error bars = mean±SEM, n=3.

(e) FUCCI-1205Lu cells treated for 24h with 10nM bortezomib were sorted into G1-phase or S/G2/M-phase live cell fractions, or left unsorted prior to immunoblotting. Blots are representative of n=3 experiments. Black line indicates separate blots.

Figure 4. Pharmacologically induced G1-arrest inhibits bortezomib and temozolomide-induced cytotoxicity.

(a) Time-line demonstrating the drug treatment schedule.

Flow cytometry quantification of % cell death in (b) WM164, (c) 1205Lu, (d) C8161 cells cultured in 2D and treated with vehicle (DMSO), 10 nM bortezomib (BTZ), 10 µM U0126 (1µM for WM164), 1µM PLX4032 (0.1 µM for WM164) or the indicated combinations. Bortezomib was added after 24h, for a total of 48h, while the other treatments were applied for the total 72h.
Error bars = mean±SEM, n=3-5. Samples were compared to DMSO+bortezomib to determine statistical differences.

(e) Confocal extended focus images of FUCCI-C8161 spheroids treated with vehicle (DMSO), 15 nM bortezomib (BTZ) alone or combined with 10 µM U0126 or 1 µM PLX4032. Bortezomib was only added after 24h, for a total of 48h, while the other treatments were applied for the total 48h.

(f) Flow cytometry quantification of % cell death in melanoma cells cultured in 2D. Cell line: WM164; treatment: vehicle (DMSO), 10 nM Bortezomib (BTZ), 0.1 µM PLX4032 or PLX + BTZ for 48h. Drugs were added simultaneously. Samples were compared to DMSO+bortezomib to determine statistical differences. Error bars = mean±SEM, n=3.

(g) as (f) with following variables: Cell line: WM164; treatment: 10 µM U0126, 0.1 µM PLX4032 or combination. U0126 was added after 24h, PLX4032 was present for the entire 72h.

(h) Image analysis of the % G1, early S and S/G2/M FUCCI-C8161 cells cultured in 2D after 24h treatment with 10 µM temozolomide (TMZ); n=3, mean±SEM.

(i) as (f) with following variables: Cell line: C8161; treatment: 10 µM U0126, 40 µM temozolomide (TMZ) or combination. temozolomide was added after 24h, U0126 was present for the entire 72h.

(j) as (f) with following variables: Cell line: C8161; treatment: 10 µM temozolomide, 60-80 µM U0126 or combination. U0126 was added after 24h, temozolomide was present for the entire 72h.

(k) as (f) with following variables: Cell line: C8161; treatment: 10 µM temozolomide, 10nM bortezomib (BTZ) or combination. Bortezomib was added after 24h, temozolomide was present for the entire 72h.
Figure 5. Environmentally induced G₁-arrest inhibits bortezomib-induced cell death, but enhances MAPK inhibitor cytotoxicity.

(a) Image analysis of the % G₁, early S and S/G₂/M phase WM164 cells cultured in 2D in normoxia with normal medium, or after 24h hypoxia and serum-starvation. Mean±SEM, n=4.

(b) Image analysis of the % red G₁, early S and S/G₂/M phase C8161 cells cultured in 2D in control, or confluent/starved wells after 48h. Mean±SEM, n=3.

(c) FUCCI-WM164 cells cultured in 2D were pre-treated with either hypoxia and serum-starvation for 24h, or control normoxia and full serum conditions. Flow quantification of the % dead cells was performed after 48h treatment with 15 nM bortezomib or vehicle control. Mean±SEM, n=4.

(d) FUCCI-C8161 cells cultured in 2D were pre-treated with either sub-confluency with normal medium, or confluence with serum-starvation for 48h. Flow quantification of the % dead cells was performed after an additional treatment with 10 nM bortezomib (BTZ), 40 µM temozolomide (TMZ) or vehicle control for 48h. Mean±SEM, n=3-4.

(e) as (c) but treatment with 20 µM U0126. Mean±SEM, n=3.

(f) as (d) but treatment with 60-80 µM U0126. Mean±SEM, n=3.

(g) WM164 cells were cultured in 2D in normoxia with normal medium, or after hypoxia and serum-starvation for 24 or 48h. Western blotting was then performed. Blots are representative of n=3 independent experiments.
A C8161 2D Culture

**C8161 3D Spheroids**

- Control
- Bortezomib

**% Cell Death**

**Time (h)**

24 48 72

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**% Cell cycle phase at death**

- S/G2/M
- G1

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**Time of death (h)**

G1 S/G2/M C8161 1205Lu

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**% Annexin V+ve**

- S/G2/M
- G1

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**Control**

Bortezomib

- No sort
- G1
- S/G2/M

**Western Blot**

- NOXA
- α-Tubulin
- cCaspase 3
- α-Tubulin