Cytokinetically quiescent (G₀/G₁) human multiple myeloma cells are susceptible to simultaneous inhibition of Chk1 and MEK1/2
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Effects of Chk1 and MEK1/2 inhibition were investigated in cytokinetically quiescent multiple myeloma (MM) and primary CD138⁺ cells. Coexpression to the Chk1 and MEK1/2 inhibitors AZD7762 and selumetinib (AZD6244) robustly induced apoptosis in various MM cells and CD138⁺ primary samples, but spared normal CD138⁻ and CD34⁺ cells. Furthermore, Chk1/MEK1/2 inhibitor treatment of asynchronized cells induced G₀/G₁ arrest and increased apoptosis in all cell-cycle phases, including G₀/G₁. To determine whether this regimen is active against quiescent G₀/G₁ MM cells, cells were cultured in low-serum medium to enrich the G₀/G₁ population. G₀/G₁–enriched cells exhibited diminished sensitivity to conventional agents (eg, Taxol and VP-16) but significantly increased susceptibility to Chk1 ± MEK1/2 inhibitors or Chk1 shRNA knock-down. These events were associated with increased γH2A.X expression/foci formation and Bim up-regulation, whereas Bim shRNA knock-down markedly attenuated lethality. Immuno-fluorescent analysis of G₀/G₁–enriched or primary MM cells demonstrated colocalization of activated caspase-3 and the quiescent (G₀) marker statin, a nuclear envelope protein. Finally, Chk1/MEK1/2 inhibition increased cell death in the Hoechst-positive (Hst⁺), low pyronin Y (PY)–staining (2N Hst⁺/PY⁻) G₀ population and in sorted small side-population (SSP) MM cells. These findings provide evidence that cytokinetically quiescent MM cells are highly susceptible to simultaneous Chk1 and MEK1/2 inhibition.

Introduction

Multiple myeloma (MM) is an accumulative disorder of mature plasma cells that is almost universally fatal. MM treatment has been revolutionized by novel agents such as immunomodulatory drugs (eg, lenalidomide) and proteasome inhibitors (eg, bortezomib). One barrier to successful MM treatment is that it is a low-growth-fraction disease before the late phase supervenes and that MM cells can rest in a quiescent, nonproliferative state with < 5% of cells actively cycling. Moreover, low proliferation of tumor cells, including MM cells, may contribute to resistance to conventional or novel targeted agents.

Cellular defenses against DNA damage are mediated by multiple checkpoints that permit cell-cycle arrest, DNA repair, or, if damage is too extensive, apoptosis. Checkpoint kinases (Chk1 and Chk2) play key roles in this DNA-damage response network. In contrast to Chk2, which is inactive in the absence of DNA-damaging stimuli, Chk1 is active in unperturbed cells and is further activated by DNA damage or replicative stress. Chk1 activation occurs even in nonproliferating cells. Given its critical role in the DNA-damage response, Chk1 represents an attractive target for therapeutic intervention. Previous studies have shown that pharmacologic Chk1 inhibitors abrogate cell-cycle arrest in transformed cells exposed to DNA-damaging agents, triggering inappropriate G₀/M progression and death through mitotic catastrophe.

Dysregulation of the Ras/Raf/MEK/ERK cascade in transformed cells, including MM cells, has prompted interest in the development of small-molecule inhibitors. Multiple agents target the dual specificity kinases MEK1/2, which sequentially phosphorylate ERK1/2, leading to activation. The MEK1/2 inhibitor PD184352 (CI-1040) has been supplanted by other MEK1/2 inhibitors with superior PK/PD profiles, such as selumetinib (AZD6244/ARRY142886). AZD6244 has shown significant in vivo activity in a MM xenograft model system, and trials of AZD6244 in MM are under way.

Previously, we reported that interruption of the Ras/MEK1/2 cascade by PD184352 dramatically increased the lethality of the multit kinase and Chk1 inhibitor UCN-01. It is important to extend these studies to more specific Chk1 and MEK1/2 inhibitors currently in clinical trials, such as AZD7762 and AZD6244. Moreover, the possibility exists that Chk1-inhibitor strategies abrogating DNA-damage checkpoints might be ineffective in cytokinetically quiescent MM cells, as is the case for more conventional therapies. The results reported herein demonstrate that regimens using AZD7762 and AZD6244 potently induce MM-cell apoptosis in all phases of the cell cycle, including G₀/G₁. Furthermore, this strategy selectively targets primary MM cells while sparing their normal counterparts. Our findings indicate that, in addition to cycling cells, cytokinetically quiescent (G₀/G₁) MM cells are highly susceptible to concomitant Chk1/MEK1/2 inhibition.

Methods

Cells and reagents

The human MM cell lines NCI-H929 and U266 were purchased from ATCC. RPMI8226 cells were provided by Dr Alan Lichtenstein (University of California, Los Angeles). The IL-6–dependent MM cell lines ANBL-6 and KAS-6/1 were provided by Dr Robert Orlowski (The M. D. Anderson Cancer Center, Houston, TX). BM samples were obtained with informed consent. The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 USC section 1734.

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Enrichment of G0/G1 cells

MM cells enriched in the G0/G1 phase were obtained by incubating H929, 8226, and U266 in low-serum medium (0.1%, 0.2%, and 0.05% FBS, respectively) for 48 hours after treatment with AZD6244/AZD7762 or PD184352/CEP3891. The purity of CD138 and CD34 cells was > 90% and viability > 95%. Normal BM CD34+ cells (M-101B) were purchased from Lonza. The purity of CD34+ cells was > 95% and viability > 80% when thawed. The MEK1/2 inhibitor BOC-D-fmk was purchased from Enzyme System Products. Reagents were dissolved in sterile DMSO (final concentration < 0.1%) and stored at −80°C.

Assessment of apoptosis and cell death

For a discussion of our assessment of apoptosis and cell death, please see supplemental Methods (available on the Blood Web site; see the Supplemental Materials link at the top of the online article).

Clonogenic assays

Colony-forming ability was evaluated using a soft agar cloning assay described previously.19

RNA interference and stable transfection

For a discussion of RNA interference and stable transfection, please see supplemental Methods.

Western blot analysis

Samples were prepared from whole-cell pellets. Proteins (20 μg) were separated on precast SDS-PAGE gels (Invitrogen) and electrotransferred onto nitrocellulose membranes. Blots were probed with primary Abs: anti-Bim (Calbiochem); anti–phospho-p44/42 (Thr202/Tyr204), anti–MAPK (ERK1/2) and anti-p44/42 MAPK (Cell Signaling Technology); anti–poly adenosine diphosphate-ribose polymerase (anti-PARP, Biomol); anti–caspase-3 (BD Biosciences); anti-cleaved (activated) caspase-3 (Cell Signaling Technology); and anti–statin Ab S-44 (provided by Dr E. Wang, University of Louisville, Louisville, KY). Cytosolic and mitochondrial fractions were prepared using digitonin lysis buffer as described previously.20 Anti–cytochrome c (BD Pharmingen), anti-AIF (Santa Cruz Biotechnology), anti–smac/DIABLO (Upstate Biotechnology), and anti–Bax (Santa Cruz Biotechnology) Abs were used to monitor the release of mitochondrial pro-apoptotic factors and Bax redistribution. Blots were reprobed with anti-actin (Sigma-Aldrich) or anti-tubulin Abs (Oncogene) to ensure equal loading and protein transfer.

Comet assay

Single-cell gel electrophoresis assays were performed to assess single- and double-stranded DNA breaks using a Comet Assay Kit (Trevigen) as per the manufacturer’s instructions.21 Images were captured using fluorescence microscopy at 20×/0.5.

Cell-cycle analysis

Cell-cycle analysis of DNA content after drug treatment by propidium iodide (PI) staining was performed by flow cytometry (BD Biosciences) using Modfit Version LT2.0 software as described previously.25

Distribution of apoptotic cells in cell-cycle phases

Caspase-3 activation/DNA content analysis were performed by dual-parameter flow cytometry to determine apoptotic cells within specific cell-cycle phases.26 Cells were stained with a 1:100 dilution of Alexa Fluor 488 (AF 488)–conjugated anti-cleaved (activated) caspase-3 for 1 hour at 4°C. DNA was stained with PI as described in “Cell-cycle analysis.” In these studies, subdiploid (late apoptotic) cells, which cannot be related to a specific cell-cycle compartment, were gated out and not included in this analysis.

Co-localization of statin and activated caspase-3

Dual statin and activated caspase-3 expression was quantified by flow cytometry to determine apoptotic cells within statin-positive populations (G0/G1 phase).27 After being fixed in 70% cold ethanol, blocked with 5% normal goat serum, and permeabilized with 0.5% Tween 20, cells were incubated with an anti-statin mAb (S-44) or control IgG, followed by secondary AF 594–conjugated goat anti–mouse IgG (Invitrogen). Cells were then stained with anti-cleaved (activated) caspase-3 Ab and analyzed by flow cytometry.

Immunofluorescent staining

In situ coexpression of statin and activated caspase-3 or γH2A.X foci formation was performed by immunofluorescent staining.23,27 Cells fixed on slides were rehydrated, blocked with 5% normal goat serum, and permeabilized with 0.5% Tween 20. Cells were incubated with an anti-statin mAb or control IgG, followed by secondary AF 594–conjugated goat anti–mouse IgG. After washing, slides were stained with either AF 488–conjugated anti-cleaved (activated) caspase-3 Ab or AF 488–conjugated anti–γH2A.X Ab (Cell Signaling Technology), respectively. Images were captured with an Olympus BX40 fluorescence microscope and analyzed with RS Image Version 1.7.3 software (Roper Scientific Photometrics).

Multiparameter flow cytometric analysis of G0 phase cells

Dual-parametric flow cytometry to monitor DNA and RNA content was used to identify quiescent (G0) cells using the DNA dye Hoechst 33342 (Hst; Molecular Probes) and the RNA-specific dye pyronin Y (PY; Sigma-Aldrich), respectively.24,29 To exclude G1 phase and G0 to G1 transition cells accumulating RNA,28 G0 populations displaying Hst+ and low PY uptake (Hst+/PY−) were gated as R17. To identify cells undergoing apoptosis in the Hst+/PY− (G0) population, 7-amino-actinomycin D (7-AAD) staining (0.5 μg/mL at 37°C for 30 minutes) was adapted for triparametric analysis. Hst+/PY+/7-AAD− cells were gated as R16. Flow cytometry parameter settings were as reported previously.28 Controls for Hst/PY or 7-AAD staining alone and negative controls (without dye) were used as compensation. Fluorescence data were obtained from 30,000 cells per sample. Flow cytometric analysis was performed using Summit Version 4.0 software (Dako Cytomation).

Analysis of SSP cells

Small side-population (SSP) MM cells were isolated as described previously.20,31 In addition, gates were set for both the side population (SP) and low FSC (small size) population (designated SSP) after gating out PI-stained (dead) cells on a flow cytometer (FACS Aria II; BD Biosciences), as described in supplemental Methods. The sorted SSP cells were then exposed to agents as indicated, after which apoptosis was determined by monitoring activated caspase-3 by flow cytometry.

Statistical analysis

For flow cytometric analyses, values represent the means ± SD for at least 3 separate experiments performed in triplicate. Significance of differences
between experimental variables was determined using the Student t test. Median Dose Effect analysis was performed as described previously.21

Results

Novel Chk1 and MEK1/2 inhibitors synergistically promote apoptosis in IL-6–dependent and –independent MM cells

To determine whether the specific, clinically relevant Chk1 inhibitor AZD7762 and the MEK1/2 inhibitor AZD6244 interacted in MM cells, IL–6–independent H929 cells were pretreated with various AZD6244 concentrations for 24 hours, followed by exposure to AZD7762 (200 or 300nM) for another 48 hours, after which time survival was monitored using the MTT assay. Significant reductions in survival occurred with coadministration of 500nM AZD6244 and became more pronounced at higher concentrations (Figure 1A). Parallel results were obtained with annexin V staining (Figure 1B). Pretreatment with minimally toxic concentrations of AZD6244 for 24 hours significantly increased annexin V positivity induced by 48 hours of 100-600nM AZD7762 treatment (Figure 1C). Combined treatment markedly increased cell death, which

Figure 1. The MEK1/2 inhibitor AZD6244 blocks ERK1/2 activation induced by the novel Chk1 inhibitor AZD7762 in human MM cells, leading to synergistic induction of apoptosis and diminished clonogenicity. (A) H929 cells were pretreated (24 hours) with the indicated concentrations of the MEK1/2 inhibitor AZD6244, followed by 200nM or 300nM AZD7762 for another 48 hours, after which time cell survival was monitored with the MTT assay. **P < .01 and ***P < .002 versus values for cells treated with AZD7762 alone. (B-C) H929 cells were sequentially treated as described in panel A with AZD6244 in either the presence or absence of the indicated concentrations of AZD7762 (B), or with the indicated concentrations of AZD7762 in either the presence or absence of 2.5μM AZD6244 (C). After drug treatment, the percentage of apoptotic (annexin V+) cells were determined by flow cytometry. *P < .05, **P < .01, and ***P < .001 versus values for cells treated with AZD6244 or AZD7762 alone. (D) H929 cells were incubated for the indicated intervals with 300nM AZD7762 after preadministration (24 hours) of 2.5μM AZD6244. Cell death was then monitored by 7-AAD staining and flow cytometry. *P < .01 and **P < .001 versus values for cells treated with AZD7762 alone. (E) H929 cells were treated with a range of AZD6244 concentrations for 24 hours and a range of AZD7762 concentrations for another 48 hours, alone or in combination at a fixed ratio (50:1). At the end of this period, 7-AAD+ cells were determined by flow cytometry. Median-dose effect analysis was used to characterize the nature of the interaction. Two additional studies yielded equivalent results. (F) H929 cells were treated with 2.5μM AZD6244 for 24 hours ± 300nM AZD7762 for another 48 hours, and then stained on Cytospin slides by TUNEL. (G) Alternatively, after being treated with the indicated concentrations of AZD6244 for 24 hours and AZD7762 for another 48 hours, cells were washed free of drug and plated in soft agar. After incubation for 14 days, colonies, consisting of groups of >50 cells, were scored, and colony formation for each condition was expressed relative to untreated controls. Results represent the means ± SD for 3 separate experiments performed in triplicate. **P < .005 versus single-drug treatment. (H) In parallel, H929 cells were pretreated for 24 hours with the indicated concentrations of AZD6244 and with 300nM AZD7762 for another 48 hours, after which time Western blot analysis was performed to evaluate ERK1/2 phosphorylation and caspase-3 cleavage. Each lane was loaded with 20 μg of protein; blots were stripped and reprobed with anti-tubulin Ab to ensure equal loading and transfer. Results are representative of 3 separate experiments.

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Selectivity of the AZD7762/AZD6244 regimen was examined. CD138+ MM cells while sparing their normal hematopoietic counterparts. CD34+ cells (Figure 2B). Primary CD138+ MM cells isolated from BM samples of 9 unselected MM patients were exposed for 24 hours to AZD7762 ± AZD6244, after which time cell death was monitored. For each sample, individual drug treatment induced minimal to modest lethality, whereas combined treatment substantially increased cell death (Figure 2C). Combined treatment induced minimal lethality in CD138+ BM cells (Figure 2D). A representative sample stained for fluorescein-labeled activated caspase-3 highlights the pronounced killing of primary CD138+ MM cells while sparing their CD138- counterparts (Figure 2E). Western blot analysis revealed that AZD7762 induced ERK1/2 activation in primary CD138+ MM cells, an effect abrogated by AZD6244 and accompanied by BimEL up-regulation (Figure 2F), as observed in cultured MM cell lines.20 Similar phenomena were also observed with another specific Chk1 inhibitor, CEP389123 (data not shown).

MEK1/2 inhibitors promote DNA damage induced by Chk1 inhibitors in both MM cell lines and primary CD138+ MM cells

Whereas exposure of MM cell lines (eg, H929 or RPMI8226) to AZD7762 or AZD6244 individually had minimal effects, combined treatment robustly increased the expression of γH2A.X (Figure 3A), a double-strand DNA damage marker.21 Coexposure of primary CD138+ MM cells to AZD7762/AZD6244 clearly increased DNA damage, manifested by increased γH2A.X nuclear...
foci (Figure 3B top panels) and a comet assay (Figure 3B bottom panels). Similar results were obtained in primary CD138+ cells exposed to CEP3891 and PD184352 (data not shown). The pan-caspase inhibitor BOC-D-fmk attenuated MM cell apoptosis but not γH2AX expression (Figure 3C-D), arguing against the possibility that DNA damage resulted from apoptosis.

**Cotargeting Chk1 and MEK1/2 results in G0/G1 arrest of MM cells and increased apoptosis in all phases of the cell cycle, including G0/G1.**

To elucidate effects of this regimen on the cell cycle, flow cytometry was performed in H929 cells exposed to AZD6244 for 24 hours, followed by AZD7762 for an additional 24 hours, before the induction of extensive apoptosis (Figure 1D). AZD6244 or AZD7762 alone modestly increased the G0/G1 population (Table 1). However, combined treatment significantly increased the G0/G1 population by > 80% (P < .01). Similar results were obtained in other MM cell lines such as 8226 (Table 1) and U266 (data not shown), indicating that Chk1/MEK1/2 inhibition arrests MM cells in G0/G1.

To examine effects of cell-cycle distribution on responses to Chk1/MEK1 inhibition, dual-parameter flow cytometric analysis of cleaved (activated) caspase-3 and cell cycle (PI staining for DNA content) in 8226 cells was performed to monitor caspase-3 activation specifically in each cell-cycle phase after gating out cells.

**Table 1. Cell-cycle distribution of MM cells after being exposed to AZD7762 ± AZD6244 for 24 hours**

<table>
<thead>
<tr>
<th></th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
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<tr>
<td><strong>H929</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl</td>
<td>56.6 ± 2.6</td>
<td>18.6 ± 1.4</td>
<td>21.2 ± 1.9</td>
</tr>
<tr>
<td>AZD7762 (200nM)</td>
<td>65.0 ± 2.0</td>
<td>13.3 ± 0.6a</td>
<td>18.0 ± 1.4</td>
</tr>
<tr>
<td>AZD7762 (300nM)</td>
<td>66.8 ± 1.7</td>
<td>13.0 ± 0.4a</td>
<td>21.2 ± 1.7</td>
</tr>
<tr>
<td>AZD6244 (1.5μM)</td>
<td>63.4 ± 1.5</td>
<td>19.3 ± 0.7</td>
<td>19.0 ± 0.8</td>
</tr>
<tr>
<td>6244 + 7762 (200nM)</td>
<td>81.3 ± 1.3b</td>
<td>9.1 ± 0.5†</td>
<td>10.1 ± 1.0†</td>
</tr>
<tr>
<td>6244 + 7762 (300nM)</td>
<td>80.5 ± 1.3b</td>
<td>9.5 ± 0.5†</td>
<td>10.9 ± 1.2†</td>
</tr>
<tr>
<td><strong>8226</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ctrl</td>
<td>58.5 ± 2.0</td>
<td>29.1 ± 2.2</td>
<td>12.6 ± 1.0</td>
</tr>
<tr>
<td>AZD6244 (3μM)</td>
<td>71.0 ± 2.7a</td>
<td>17.5 ± 2.0</td>
<td>10.5 ± 1.7</td>
</tr>
<tr>
<td>AZD7762 (300nM)</td>
<td>61.4 ± 0.7</td>
<td>26.7 ± 1.2</td>
<td>12.0 ± 0.5</td>
</tr>
<tr>
<td>6244 + 7762</td>
<td>79.6 ± 1.2†</td>
<td>11.7 ± 1.02a†</td>
<td>7.7 ± 0.8</td>
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Values represent the means ± SD of triplicate determinations.

*P < .05 and †P < .01 = significantly greater or less than values for untreated cells.
subdiploid cells. As shown in Figure 4A, treatment with the microtubule-stabilizing agent Taxol resulted in a 4.7-fold increase over values for untreated cells in caspase 3 activation in G2/M cells, clearly greater than that observed in G0/G1-phase (2.3-fold) and S-phase (2.0-fold) cells (Figure 4A), documenting the reliability of this approach. AZD7762/AZD6244 increased caspase-3 activation within all phases of the cell cycle (eg, 4.0-, 3.3-, and 2.6-fold increases in G0/G1, S, and G2/M phase, respectively; Figure 4A). These findings argue that the Chk1/MEK1/2 inhibitor regimen targets MM cells in each phase of the cell cycle, including G0/G1.

G0/G1–enriched MM cells display increased susceptibility to the AZD7762/AZD6244 regimen

The preceding findings indicated that the Chk1/MEK1/2 inhibitor regimen arrested cultured MM cells in G0/G1, induced apoptosis in the G0/G1 population, and was active toward primary CD138+ MM cells. To validate whether, in addition to cycling MM cells, this regimen might also be active against their cytokinetically quiescent counterparts, H929 cells were cultured in the presence of 10% versus 0.1% FBS, the latter to halt cell proliferation and to enrich the G0/G1 population.11,24,32 Cells maintained in 0.1% FBS medium
exhibited essentially no growth compared with those cultured in 10% FBS medium (supplemental Figure 2A), with 80% of cells accumulating in G0/G1 (Figure 4B), but there were modest differences in cell viability (Figure 4C; P < .05). Interestingly, G0/G1–enriched cells were significantly more sensitive to Chk1 inhibitors (eg, AZD7762 or UCN-01) compared with controls cultured in 10% FBS medium (Figure 4C; P < .01). However, these cells were relatively more resistant to Taxol or VP-16 (Figure 4C; P < .02 in each case), arguing against the possibility that serum-deprived cells were generically more sensitive to cytotoxic agents. Effects on caspase-3 and PARP cleavage were concordant (supplemental Figure 2B). Flow cytometric analysis also demonstrated a clear increase in the subdiploid (sub-G1) fraction in G0/G1–enriched cells after treatment with the Chk1 inhibitors compared with 10% FBS controls (supplemental Figure 2C). Moreover, G0/G1 enrichment also significantly sensitized cells to Chk1 shRNA knock-down (Figure 4D and supplemental Figure 2D), which was accompanied by increased γH2A.X expression (supplemental Figure 2E), arguing for a specific role for Chk1 inhibition in Chk1 inhibitor lethality in quiescent MM cells. G0/G1–enriched cells displayed greater susceptibility to AZD6244/AZD7762 even when these agents were administered for shorter intervals (ie, 24 hours after AZD7762 addition) than those cultured under full-serum conditions (Figure 4E; P < .005 vs 10% FBS controls). AZD6244 exposure resulted in BimEL up-regulation under both conditions, which was associated with inhibition of basal and AZD7762-stimulated ERK1/2 phosphorylation. However, G0/G1–enriched cells exposed to AZD7762/AZD6244 displayed more pronounced γH2A.X expression and caspase-3 cleavage compared with 10% FBS controls (Figure 4F). Similar results were obtained in other MM cell lines (eg, U266 cells) and in cells exposed to an alternative Chk1/MEK inhibitor regimen (eg, CEP3891/PD184352; data not shown).

Bim plays a functional role in the pronounced susceptibility of G0/G1–enriched MM cells to Chk1/MEK1/2 inhibition

As shown in Figure 5, the MEK1/2 inhibitor AZD6244 prevented Bim phosphorylation (particularly the BimEL isoform) in H929 cells exposed to AZD7762, leading to Bim accumulation (Figure 5A), Bax translocation, and the release of mitochondrial apoptotic proteins (ie, cytochrome c, Smac/DIABLO, and AIF; Figure 5B). Similar results were observed in other MM cell lines (eg, RPMI8226 and U266; data not shown). To determine whether
Bim up-regulation also contributed to the susceptibility of cytokinetically quiescent MM cells to the Chk1/MEK1/2 inhibitor regimen, U266 cells stably transfected with Bim shRNA were used. As shown in Figure 5C, coadministration of AZD6244 with AZD7762 markedly increased BimEL expression in G0/G1–enriched U266 cells transfected with negative control shRNA (shCtrl), whereas this event was clearly attenuated in G0/G1–enriched Bim shRNA cells. shRNA knock-down of Bim (2 clones, W12 and W4) significantly diminished the ability of AZD6244 to potentiate AZD7762 lethality compared with shCtrl cells (P < .002 in each case; Figure 5C–D). Similar results were obtained in other MM cell lines (eg, RPMI8226) transfected with Bim shRNA (data not shown). These results indicate that Bim up-regulation plays an important functional role in the susceptibility of G0/G1–enriched MM cells to the Chk1/MEK1/2 inhibitor regimen.

**Chk1/MEK1/2 inhibition induces apoptosis in MM cell lines and primary CD138+ cells expressing the G0 marker statin**

Statin is a 57-kDa nuclear envelope protein primarily expressed in cytokinetically quiescent G0 cells and rapidly down-regulated during progression to the G1 phase.27,33 Western blot analysis revealed that G0/G1–enriched H929 cells exhibited a marked increase in statin expression (supplemental Figure 3A), and immunofluorescent staining demonstrated a marked increase in statin-positive cells after G0/G1 enrichment by culturing in 0.1% FBS (supplemental Figure 3B). Primary CD138+ MM cells clearly expressed statin (supplemental Figure 3C), which is consistent with their quiescent status.1,3 Untreated G0/G1–enriched H929 cells displayed robust red fluorescence (statin) but little green fluorescence (activated caspase-3; Figure 6A). AZD7762/AZD6244-treated cells displayed clear colocalization of statin and activated caspase-3. Parallel studies in primary CD138+ MM cells revealed that AZD7762/AZD6244 exposure resulted in pronounced caspase-3 activation in statin-positive cells compared with untreated cells (Figure 6B). These findings were supported by parallel studies involving alternative Chk1/MEK1/2 inhibitors (eg, CEP3891/PD184352; supplemental Figure 3D–E). Colocalization of statin/activated caspase-3 in G0/G1–enriched H929 cells was further confirmed by dual-parameter flow cytometric analysis, which indicated increased caspase-3 activation in statin-positive cells after brief exposure (12 hours after addition of Chk1 inhibitors) to AZD6244/AZAD7762 and to CEP3891/PD184352 (Figure 6C). Finally, dual-immunofluorescent staining revealed that γH2A.X expression (green fluorescence) colocalized with statin expression (red fluorescence) in AZD6244/AZD7762–treated G0/G1–enriched H929 cells at an early interval (6 hours after AZD7762) (Figure 6D). Parallel results were also obtained with CEP3891/PD184352 (supplemental Figure 4). These findings support the notion that cytokinetically quiescent MM cells are sensitive to DNA damage and apoptosis induced by this regimen.

**Hst’/PY’ G0 MM cells are susceptible to the Chk1/MEK1/2 inhibitor regimen**

To confirm that Chk1/MEK1/2 inhibition kills cytokinetically quiescent (G0) MM cells, a multiparameter flow cytometric analysis was used. This method is specifically designed to identify G0 cells displaying Hoechst positivity (Hst’, corresponding to 2N diploid cells) but low pyronin Y uptake (PY’, because of lack of RNA synthesis) from other populations, particularly G1 (Hst’/PY’).28,29 As shown in Figure 7A panels i and ii, RPMI8226 cells cultured in 0.2% FBS medium displayed an enriched Hst’/PY’ (G0) population (gate R17), compared with 10% FBS controls (19.14% vs 8.18% of the bulk population). 7-AAD staining was then incorporated to monitor cell death (R16) in the Hst’/PY’ (R17 gated-in) population. After 12 hours of treatment with AZD7762/AZD6244, the Hst’/PY’ population exhibited a clear increase in 7-AAD uptake over untreated controls (Figure 7A panels iii and iv; R16, 40.2% vs 26.5%; P < .01). Significant increases in cell death of Hst’/PY’ cells (51.3%) also occurred after CEP3891/PD184352 coexposure (Figure 7A panel v; P < .005 vs. untreated controls), providing further evidence that quiescent (G0), noncycling MM cells are susceptible to Chk1/MEK1/2 inhibitor–mediated lethality.

**Quiescent SSP MM cells are sensitive to Chk1/MEK1/2 inhibitor lethality**

Finally, the effects of Chk1/MEK1/2 inhibition were investigated prospectively in SSP MM cells, representing quiescent cells in which the presumed stem cell compartment resides.30,31 SP cells were sorted from H929 cells cultured in 10% serum (Figure 7B), as described previously.30 SP cells represented a small fraction of cells (0.5% of PI− living cells) excluding Hst (Figure 7B panels i and ii), which was further sorted for SSP cells (Figure 7B panel iii), which have been shown to represent a quiescent population.34 As a control, coincubation of verapamil substantially reduced the SP population (Figure 7B panel iv).31 Approximately 90% of sorted SSP cells resided in G0/G1 (Figure 7B panel v). Fourteen hours of treatment with AZD7762/AZD6244 resulted in a marked increase in caspase-3 activation in the G0/G1 population of the sorted SSP cells compared with untreated controls (Figure 7C). Equivalent results were obtained with the sorted SSP population of RPMI8226 cells (data not shown). These findings provide further evidence that Chk1/MEK1/2 inhibition is active against quiescent MM cells.

**Discussion**

The preceding findings demonstrate that cytokinetically quiescent (G0/G1) MM cell lines, as well as primary CD138+ myeloma cells expressing quiescent (G0/G1) markers, are fully susceptible to strategies simultaneously targeting Chk1 and MEK1/2. It should be emphasized that G0/G1 MM cells are generally resistant to standard cytotoxic agents, particularly those targeting specific phases (eg, S and M phases) of the cell cycle.1,4,5 The finding that Chk1/MEK1/2–inhibitory regimens are generally resistant to standard cytotoxic agents, particularly those targeting specific phases (eg, S and M phases) of the cell cycle,1,4,5 is generally consistent with the notion that cytokinetically quiescent cells are generally resistant to standard cytotoxic agents, particularly those targeting specific phases (eg, S and M phases) of the cell cycle.1,4,5 The finding that Chk1/MEK1/2–inhibitory regimens (eg, AZD7762/AZD6244 and CEP3891/PD184352) are active against quiescent cells may have particular significance for MM, generally a low-proliferative neoplasm.

Normally, cells respond to genotoxic insults through the coordinated actions of various cell-cycle checkpoints and DNA-repair pathways. The former include biochemical signaling pathways that monitor DNA damage and trigger cell-cycle arrest and DNA repair if the damage is repairable, or induce apoptosis or senescence if it is not.5,7 Transformed cells in general are susceptible to DNA damage and oncogene-mediated DNA-replication stress.35,36 Because checkpoint abnormalities are generally resistant to standard cytotoxic agents, particularly those targeting specific phases (eg, S and M phases) of the cell cycle,1,4,5 the finding that Chk1/MEK1/2–inhibitory regimens (eg, AZD7762/AZD6244 and CEP3891/PD184352) are active against quiescent cells may have particular significance for MM, generally a low-proliferative neoplasm.

Chk1 is a distal signal transducer that is activated by ATM/ATR after DNA damage8 and that has been implicated in the G2/M, intra-S, and mitotic spindle checkpoints.37 More recently, Chk1 has been shown to play a more direct role in cell survival- and DNA repair–related processes.10,18,39 In this context, it has been reported...
that exposure to Chk1 inhibitors (e.g., UCN-01 and CEP3891) or Chk1 shRNA knock-down is sufficient to induce DNA damage, even in the absence of exogenous genotoxic insults. The initial development of Chk1 inhibitors focused on the multikinase inhibitor UCN-01, but because of its unfavorable pharmacokinetic and toxicity profiles, interest has shifted to more specific second-generation Chk1 inhibitors such as AZD7762, CEP3891, XL-844, and PF-477736, among many others. Chk1 inhibitors dramatically enhance genotoxic agent lethality by disrupting the G2/M checkpoint (in the case of DNA-damaging agents such as camptothecin and SN-38) or the intra-S phase checkpoints (in the case of DNA synthesis inhibitors such as gemcitabine). In these settings, cells with DNA damage progress inappropriately through the G2/M or S phase, triggering cell death. Implicit in these mechanistic models is the presumption that cell-cycle progression must occur for such disruptions to be lethal. Consequently, few attempts have been made to apply this approach to disorders involving low-proliferative malignancies, such as MM, or to cytokinetically quiescent (G0/G1) tumor cells, a characteristic of cancer stem (initiating) cells. Studies by our group have shown that interruption of the MEK1/2/ERK1/2 pathway strikingly increases UCN-01 lethality in MM cells. However, the cell-cycle relatedness of this interaction, in contrast to strategies combining Chk1 inhibitors with DNA-damaging agents, has not yet been defined nor have these findings been extended to include newer-generation, more specific Chk1 and MEK1/2 inhibitors currently under clinical development.
evaluation. To minimize—although not completely exclude—the possible contribution of off-target effects, parallel studies were performed using 2 unrelated specific Chk1 inhibitors (AZD7762 and CEP3891) in combination with 2 MEK1/2 inhibitors (AZD6244 and PD184352).

The bulk of evidence indicates that lethality stemming from simultaneous interruption of Chk1 and MEK1/2 occurs independently of cell-cycle progression in MM cells, which, at least at early stages of the disease, display low proliferative indices and largely reside (95%) in G0/G1. This evidence includes the findings that: (1) Chk1/MEK1/2 inhibitor regimens (eg, AZD7762/AZD6244 and CEP3891/PD184352) were highly active against primary CD138+ MM cells, which have a low proliferative capacity1,2; (2) in contrast to regimens incorporating Chk1 inhibitors and genotoxic agents, which lead to inappropriate progression through G2/M,3 Chk1/MEK1/2 inhibition arrested asynchronized MM cells cultured in 10% FBS medium in G0/G1 (notably, the G0/G1 population was as or more sensitive to these regimens than their cycling S or G2/M counterparts); (3) Chk1/MEK1/2 inhibition induced pronounced cell death in G0/G1–enriched MM cells, whereas these cells were relatively less sensitive to VP-16 or Taxol; (4) the Chk1/MEK1/2 inhibitor regimens induced cell death in cultured and primary MM cells characterized by quiescent G0 phase phenotypes29,33; and (5) these regimens were active against the sorted quiescent SSP population of MM cells. These observations suggest that the mechanisms through which combined Chk1/MEK1/2 inhibition induces cell death differ from those responsible for the potentiation of genotoxic agent lethality by Chk1 inhibitors, and that the former are operative in both cycling and quiescent tumor cells. In this context, very recent studies have shown that targeting the SSP population of MM cells represents an important mechanism contributing to the anti-MM activity of lenalidomide.31 It will be interesting to determine whether Chk1/MEK1/2 inhibitor regimens act against this population through similar or different mechanisms.

The Ras/Raf/MEK1/2/ERK1/2 pathway plays an important role in cell-cycle progression. For example, ERK1/2 activation is
required for progression from the G1 to S phase or through G2/M. Moreover, recent findings suggest a functional role for MEK1/2/ERK1/2 in cell-cycle checkpoints. However, the MEK1/2/ERK1/2 pathway also mediates multiple survival functions, including down-regulation of proapoptotic proteins such as Bad and Bim. The latter protein, implicated in potentiation of Chk1 inhibitor lethality by inhibitors of the Ras/MEK1/2/ERK1/2 pathway in the bulk population of MM cells, also plays a functional role in the lethality of these regimens toward quiescent (G0/G1) MM cells. In this context, the Ras pathway has been linked to checkpoint activation in quiescent cells. Therefore, whereas the lethal consequences of combined exposure to DNA-damaging agents and Chk1 inhibitors have been attributed to inappropriate cell-cycle progression, coadministration of MEK1/2 inhibitors may act coordinately with Chk1 inhibitors to promote genotoxicity in both cycling and quiescent cells. The ability of Chk1 inhibitors (eg, UCN-01 and CEP3891) and Chk1 knockdown by shRNA to induce DNA damage has been well documented. Interestingly, results of one study suggest that nonproliferating ovarian cancer cells are particularly sensitive to the lethal consequences of Chk1 inhibition. These findings raise the possibility that intact Chk1 function is required even in quiescent cells to circumvent the lethal consequences of spontaneously occurring DNA damage. In this context, emerging evidence indicates that, in addition to its classic checkpoint function, Chk1 plays diverse roles, including those related to survival and DNA repair. For example, Chk1 has been implicated in the latter through interactions with the DNA-repair machinery. Conversely, ERK1/2 activation is also involved in DNA repair through regulation of DNA-repair proteins. Interestingly, the type of DNA repair is cell-cycle phase dependent; that is, in quiescent (G0/G1) cells, it primarily proceeds via nonhomologous end-joining, whereas in cycling cells (S and G2/M), homologous recombination predominates. Therefore, it is possible that disruption of Chk1 function, particularly in conjunction with disruption of MEK1/2/ERK1/2–related DNA-repair events, may increase the susceptibility of quiescent (G0/G1) cells to DNA damage. The pronounced induction of DNA damage (eg, γH2AX expression/foci formation) by the Chk1/MEK1/2 inhibitor regimens in G0/G1–enriched cells and in G0/G1–labeled population supports this notion. Although a more direct role for Chk1 in homologous recombination has been recognized recently, evidence specifically relating Chk1 to nonhomologous end-joining or other processes characteristic of G0/G1 cells is not yet available. Further studies will be required to define the mechanism(s), such as disruption of DNA repair, that renders quiescent (G0/G1) cells vulnerable to this strategy.

In summary, the present findings provide evidence that the Chk1/MEK1/2–inhibitory strategy, in addition to killing cycling cells, is also active against cytokinetically quiescent (G0/G1) MM cells while exerting relatively minimal toxicity toward normal cells. Our findings also indicate that the mechanism(s) responsible for inducing cell death in cytokinetically quiescent (noncycling) transformed cells by this regimen may differ fundamentally from those involved in the potentiation of genotoxic agent lethality in cycling cells by DNA-damage checkpoint abrogation. For example, the present results suggest that cytokinetically quiescent transformed cells, which characteristically exhibit resistance to conventional chemotherapeutic agents, may be particularly susceptible to intrinsic DNA damage, possibly reflecting the essential function of Chk1 in the maintenance of genomic stability. In this setting, Chk1 could serve as a death trigger to promote the elimination of cells containing damaged DNA. Dysregulation of the Ras/Raf/MEK/ERK pathway, which is one of the most common aberrations in cancer, including MM, might therefore act to down-regulate Bim, allowing such cells to survive. Conversely, by up-regulating Bim, interruption of MEK/ERK signaling could lower the threshold for apoptosis in transformed cells, including those in a nonproliferative, quiescent state. The finding that Bim up-regulation is required for Chk1/MEK1/2–inhibitor lethality in G0/G1–arrested cells supports this notion. Based on in vitro and in vivo evidence of activity of AZD6244 in MM models, a phase 2 trial of AZD6244 in refractory MM has been initiated. Such a study could provide a foundation for successor trials combining AZD6244 and Chk1 inhibitors such as AZD7762 in MM, which would determine the in vivo relevance of the present findings more definitively.

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Authorship

Contribution: X-Y.P. designed and performed the research, analyzed the data, and wrote the manuscript; L.E.Y., S.C., W.W.B., Y.T., J.F., J.A.A., and L.B.K. performed the research; P.D. helped with the research; and Y.D. and S.G. designed the research, analyzed the data, and wrote the manuscript.

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References


Cytokinetically quiescent ($G_0/G_1$) human multiple myeloma cells are susceptible to simultaneous inhibition of Chk1 and MEK1/2

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