Up-regulation of Pro-apoptotic Protein Bim and Down-regulation of Anti-apoptotic Protein Mcl-1 Cooperatively Mediate Enhanced Tumor Cell Death Induced by the Combination of ERK Kinase (MEK) Inhibitor and Microtubule Inhibitor

Background: MEK inhibitors enhance apoptosis induction by microtubule inhibitors.
Results: MEK and microtubule inhibitors together induced up-regulation of Bim and down-regulation of Mcl-1 in association with prolongation of mitosis.
Conclusion: The drug combination tips the balance between pro- and anti-apoptotic signaling toward induction of cell death.
Significance: The combination of MEK inhibitors with agents that down-regulate or inactivate anti-apoptotic proteins is a promising anticancer strategy.

Blockade of the ERK signaling pathway by ERK kinase (MEK) inhibitors selectively enhances the induction of apoptosis by microtubule inhibitors in tumor cells in which this pathway is constitutively activated. We examined the mechanism by which such drug combinations induce enhanced cell death by applying time-lapse microscopy to track the fate of individual cells. MEK inhibitors did not affect the first mitosis after drug exposure, but most cells remained arrested in interphase without entering a second mitosis. Low concentrations of microtubule inhibitors induced prolonged mitotic arrest followed by exit of cells from mitosis without division, with most cells remaining viable. However, the combination of a MEK inhibitor and a microtubule inhibitor induced massive cell death during prolonged mitosis. Impairment of spindle assembly checkpoint function by RNAi-mediated depletion of Mad2 or BubR1 markedly suppressed such prolonged mitotic arrest and cell death. The cell death was accompanied by up-regulation of the pro-apoptotic protein Bim (to which MEK inhibitors contributed) and by down-regulation of the anti-apoptotic protein Mcl-1 (to which microtubule and MEK inhibitors contributed synergistically). Whereas RNAi-mediated knockdown of Bim suppressed cell death, stabilization of Mcl-1 by RNAi-mediated depletion of Mule slowed its onset. Depletion of Mcl-1 sensitized tumor cells to MEK inhibitor-induced cell death, an effect that was antagonized by knockdown of Bim. The combination of MEK and microtubule inhibitors thus targets Bim and Mcl-1 in a cooperative manner to induce massive cell death in tumor cells with aberrant ERK pathway activation.

Aberrant activation of the ERK signaling pathway contributes to the pathogenesis of many types of human cancer (1, 2). In particular, activating mutations of the epidermal growth factor receptor (3), Ras (4), and Raf (5), all of which culminate in the activation of ERK kinase (MEK) isoforms 1 and 2 (MEK1/2) and ERK1/2 (6), have been associated with various human cancers. The ERK pathway thus represents a promising target for the development of anticancer drugs, and highly selective small-molecule inhibitors of MEK1/2, including PD184352, PD0325901, and AZD6244, have been developed (7, 8).

We have previously shown that specific blockade of the ERK pathway by MEK inhibitors results in marked suppression not only of the proliferation (9) but also of the invasiveness (10) of tumor cells in which the pathway is constitutively activated. However, blockade of the ERK pathway by itself is largely cytostatic rather than cytotoxic, resulting in only a moderate induction of apoptosis in such tumor cells (9). Thus, although PD184352 or AZD6244 totally suppressed the proliferation of T24 cells in culture (11) or the growth of HT-29 or BxPC3 tumor xenografts in vivo (12), respectively, these tumor cells remained viable and resumed proliferation after removal of the inhibitor or cessation of drug administration. Consistent with these observations, recent clinical studies of MEK inhibitors in individuals with advanced cancers have shown that, although PD184352 or AZD6244 achieved target inhibition at well tolerated doses, these drugs alone exhibited insufficient antitumor activity (13, 14). Strategies to improve the anticancer activity of MEK inhibitors might therefore prove to be therapeutically beneficial for cancer patients.

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Members of the Bcl-2 family of proteins possess pro-apoptotic or anti-apoptotic activities and play key roles in the regulation of apoptosis, tumorigenesis, and the cellular response to anticancer therapy (15). The balance between pro-apoptotic and anti-apoptotic signals determines cell fate. In this regard, ERK1/2-mediated phosphorylation of Bim$_{EL}$, a pro-apoptotic protein of the Bcl-2 family, promotes its proteasome-dependent degradation (16), whereas ERK1/2-mediated phosphorylation of Mcl-1, an anti-apoptotic Bcl-2 family protein (15), slows its turnover (17), suggesting that the ERK pathway promotes cell survival. Specific interruption of the cytoprotective function of the ERK pathway by MEK inhibitors has thus been expected to enhance the lethal actions of various cytotoxic anticancer agents by tipping the balance between pro-apoptotic and anti-apoptotic signaling toward cell death. However, MEK inhibitors selectively enhance the induction of apoptosis by microtubule inhibitors in various tumor cell lines with constitutive ERK pathway activation, without affecting the cytotoxicity of many other anticancer drugs, including cytarabine, etoposide, cisplatin, and doxorubicin (11, 18). Enhancement of the therapeutic efficacy of microtubule-stabilizing agents (such as paclitaxel or docetaxel) or microtubule-destabilizing agents (such as TZT-1027 or vinorelbine) by MEK inhibitors has thus been demonstrated for several human tumor xenografts in nude mice (19, 20). The molecular mechanism of this specific interaction between MEK inhibitors and microtubule inhibitors has remained unknown, however. Microtubule inhibitors activate the spindle assembly checkpoint (SAC) and thereby induce mitotic arrest (21). Although the ERK pathway plays an essential role in the G$_0$-G$_1$ transition of the cell cycle, it also contributes to the G$_2$-M transition (22). The combination of a MEK inhibitor and a microtubule inhibitor might thus be expected to act synergistically to induce mitotic catastrophe in tumor cells.

We have examined the molecular mechanism underlying the enhanced antitumor efficacy of the combination of a MEK inhibitor and a microtubule inhibitor, with a focus on the role of Bcl-2 family proteins. We applied time-lapse microscopy to the systematic analysis of >100 individual cells under various drug treatment conditions. The drug combination induced prolonged mitotic arrest in tumor cells with constitutive ERK pathway activation. Down-regulation of anti-apoptotic Mcl-1 and up-regulation of pro-apoptotic Bim$_{EL}$ were apparent in the arrested cells, resulting in the cooperative induction of massive cell death.

EXPERIMENTAL PROCEDURES

**Materials**—Antibodies to ERK1/2, Mcl-1, cyclin B$_1$, poly-(ADP-ribose) polymerase, and Bcl-x$_L$ were obtained from Santa Cruz Biotechnology; those to cleaved caspase-3 (Asp175), survivin, Puma, and Bad were from Cell Signaling Technology; those to BubR1, Mad2, and Bcl-2 were from BD Biosciences; those to diphosphorylated ERK1/2, XIAP, and $\beta$-actin were from Sigma-Aldrich; those to phosphorylated histone H3 (Ser10), Bak, and Bad were from Upstate Biotechnology; and those to Bim were from Calbiochem. Vincristine, paclitaxel, monastrol, and PD0325901 were obtained from Sigma-Aldrich; and vinorelbine diatrate (Navelbine) was from Kyowa Hakko Kirin Co., Ltd. PD98059 and PD184352 were synthesized as described previously (9, 10).

**Cell Culture**—The human tumor cell lines HT-1080 (fibrosarcoma; Health Science Research Resources Bank), HT-29 (colon adenocarcinoma; American Type Culture Collection), and MDA-MB-231 (breast carcinoma; American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. The cultures were maintained for 8 weeks after recovery from frozen stocks (2, 9, 20). In some experiments, HT-1080 cells were synchronized at the G$_1$-S boundary by a double thymidine block, which involved incubation with 2 mM thymidine for 16 h and release into normal medium for 8 h, followed by exposure to 2 mM thymidine for an additional 16 h.

**RNAi**—The sequences 5’-CGGCTCATCGTTCGATTCATCACT-3’ (Mad2 siRNA-1), 5’-GAGTTTTCTCATCCGTGCATACA-3’ (Mad2 siRNA-2), 5’-CACAGATTCGACAAGCTCTGAA-3’ (BubR1 siRNA-1), 5’-CAGCACGTGTTGGCACATACTCA-3’ (BubR1 siRNA-2), 5’-CAGCATTGTTCTTATCTTACGCTT-3’ (Bim siRNA-1), 5’-CAGAGATGATGAGCAGTTGAACTCA-3’ (Bim siRNA-2), 5’-GAAATGATCACAGCGTCTTCTGAA-3’ (Mcl-1 siRNA-1), 5’-CTATTGGTCTACGCACTTGGTT-3’ (Mcl-1 siRNA-2), 5’-CCGGGCAGCTGTCGACGTTCTACA-3’ (Mule) were designed to generate siRNA duplexes specific for human Mad2, BubR1, Bim, Mcl-1, and Mule mRNAs. Stealth RNAi negative control duplexes (low, medium, or high GC) were obtained from Invitrogen. Subconfluent cell cultures were transfected for the indicated times with 50 nM siRNA duplexes with the use of Lipofectamine RNAiMAX (Invitrogen).

**Immunoblot Analysis**—Cell lysates were prepared and subjected to immunoblot analysis as described (23, 24). Immune complexes were visualized with enhanced chemiluminescence reagents (GE Healthcare).

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**FIGURE 1. Combination of MEK and microtubule inhibitors induces pronounced cell death during prolonged mitotic arrest in tumor cells.** A, exponentially growing HT-1080 cells were treated with the indicated agents and observed by time-lapse microscopy, with images being acquired every 5 min for 48 h. Fate profiles of 100 representative cells are shown for each condition, with each horizontal line representing one cell, the length of the line denoting the duration of a given behavior, and the color of the line representing the behavior (upper). Box-and-whisker plots present the time that cells spent arrested in mitosis as a function of subsequent cell fate; cells that either died in mitosis (red) or exited mitosis and returned to interphase (blue) are shown (lower left). The lower boundary of each box indicates the 25th percentile, the line within the box marks the median, and the upper boundary of the box denotes the 75th percentile. Whiskers above and below each box indicate the 90th and 10th percentiles, respectively. HT-1080 cells incubated in the absence (control) or presence of 1 nM vincristine (VCR) for 24 or 48 h were also analyzed for DNA content by flow cytometry (lower right). B and C, fate profiles and box-and-whisker plots for HT-29 (B) and MDA-MB-231 (C) cells exposed to the indicated agents. *, p < 0.05; **, p < 0.01 for the indicated comparisons (n = 100 cells). VNR, vinorelbine; PTX, paclitaxel.
RT-PCR Analysis—Total RNA (1 μg) extracted from HT-1080 cells using Sepasol-RNA I (Nacalai Tesque) was subjected to reverse transcription with a PrimeScript first-strand cDNA synthesis kit (Takara Bio Inc.). The resulting cDNA (0.5 μl) was then subjected to PCR with primers (sense and antisense, respectively) specific for human Mule (5′-GACTGCGAGACCTTAATAG-3′ and 5′-CTCTTTGTCAGATCCCGAC-3′) or GAPDH (5′-CCACCCATGGCAAATTCCATGGAC-3′ and 5′-TCTAGACGGCAGGTCCGGCACC-3′). PCR was performed at 94 °C for 2 min, followed by 25 cycles at 94 °C for 30 s, 58 °C for 42 s, and 72 °C for 1 min. The PCR products were fractionated by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Flow Cytometry—Cells exposed to various agents were harvested by exposure to trypsin, fixed with 70% ethanol, treated with DNase-free RNase A (100 μg/mL; Sigma-Aldrich), stained with propidium iodide (20 μg/mL), and analyzed for DNA content using a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences) (9).

Time-lapse Microscopy and Data Acquisition—Images of cells in 6-well plates were acquired using a Cell Observer system and an Axiovert 200M microscope equipped with an AxioCam MRm camera, a motorized X/Y stage, and an XL incubator (Carl Zeiss). The cells were maintained under a humidified atmosphere of 5% CO2 at 37 °C during the experiments. Time-lapse imaging of cells was performed with the use of AxioVision software (Carl Zeiss), with phase-contrast images being collected every 5 min for 48 h. Image sequences were analyzed by tracking >100 individual cells per experimental condition to determine their behavior. Breakdown of the nuclear envelope was judged as the point at which prophase chromatin lost its smooth linear periphery. Data were collected from at least three non-overlapping fields.

Statistical Analysis—Unless indicated otherwise, quantitative data are presented as means ± S.D. from three separate experiments, each performed in duplicate, and were analyzed with Student’s two-tailed t test. A p value of <0.05 was considered statistically significant. Qualitative data are representative of at least three independent experiments.

RESULTS

Combination of MEK Inhibitor and Microtubule Inhibitor Induces Cell Death during Prolonged Mitosis—Specific blockade of the ERK pathway by MEK inhibitors induces cell cycle arrest in G1 phase (9), whereas microtubule inhibitors induce mitotic arrest (21). To define how tumor cells with constitutive activation of the ERK pathway respond to treatment with the combination of a MEK inhibitor and a microtubule inhibitor, we adapted time-lapse microscopy to allow observation of individual cells. Tumor cells manifest marked intraline variation after prolonged exposure to antimitotic agents (25). Furthermore, population-based approaches such as flow cytometry and immunoblot analysis can generate data that lead to vague and confusing interpretations (26).

HT-1080 cells in the exponential phase of growth were exposed to a MEK inhibitor (PD98059 (50 μM) or PD0325901 (1 μM)), a low concentration (vincristine (1 nM), vinorelbine (10 nM), or paclitaxel (100 nM)) or a high concentration (vincristine (30 nM)) of a microtubule inhibitor, or combinations thereof, and images were acquired every 5 min for 48 h (Fig. 1A). Image sequences were then analyzed by tracking >100 individual cells per condition to determine their behavior, with a focus on the timing of mitotic entry and exit. Fate profiles of 100 representative cells were plotted for each condition (Fig. 1A, upper).

The cells underwent multiple mitoses with intervals of ~12 h in the absence of drugs, with the time spent in mitosis being 26.2 ± 9.2 min (n = 200 cells). Treatment of cells with PD98059 or PD0325901 did not substantially affect the timing of entry into or exit from the first mitosis. After normal division, however, most of the cells (~90%) did not enter a second mitosis but instead remained in interphase for the remainder of the observation period; a small proportion of the cells died in interphase after cell division or without entering mitosis.

Treatment with low concentrations of vincristine, vinorelbine, or paclitaxel induced prolonged mitotic arrest in HT-1080 cells (Fig. 1A, lower left). About 70–80% of the cells subsequently exited mitosis without division (mitotic slippage) (supplemental Figs. 1 and 2) (27), endcycled, and entered a second mitosis; such mitotic slippage generated 4N and 8N cells, which constituted major cell populations after 48 h (Fig. 1A, lower right) and which remained viable. Cells depleted of SAC proteins (Mad2 or BubR1), which do not undergo cytokinesis, have been shown to remain viable through continued cycles of DNA replication up to a DNA content of at least 32N (28). A smaller proportion of the HT-1080 cells (~20–30%) died during a markedly prolonged mitosis or in interphase after exit from mitosis. Treatment of HT-1080 cells with a high concentration (30 nM) of vincristine induced essentially the same behavior as that observed in the presence of 1 nM vincristine, with the exception that most of the cells (~80%) died during prolonged mitosis or in interphase.

Treatment of HT-1080 cells with combinations of a MEK inhibitor and any of the microtubule inhibitors examined resulted in marked enhancement of cell death compared with that observed with the corresponding microtubule inhibitor alone. The effect of MEK inhibitors on death induction by microtubule inhibitors was most pronounced at low concentrations of the latter drugs. The combination of 1 nM vincristine...
and a MEK inhibitor was thus even more effective than 30 nM vincristine alone in inducing cell death. Under such conditions, most cells (80–90%) died during prolonged mitosis. Furthermore, the time to death induction by the drug combinations was markedly shorter than that by the respective microtubule inhibitor alone. Results essentially similar to those obtained with HT-1080 cells were observed when HT-29 cells or MDA-MB-231 cells were treated with PD0325901 or vincristine, alone or in combination (Fig. 1, B and C).

**FIGURE 3.** Combination of PD0325901 and either vincristine or paclitaxel induces up-regulation of Bim and down-regulation of Mcl-1 in HT-1080 cells. A, HT-1080 cells were synchronized at the G1-S boundary by a double thymidine block and then released into the cell cycle. At 1 h after release from the block, the cells were incubated in the absence (control) or presence of 1 μM PD0325901, 1 nM vincristine (VCR), or the combination of these agents. The cells were observed by time-lapse microscopy to determine the timing of mitotic entry (left) or were fixed at the indicated times after release from the block and analyzed for DNA content by flow cytometry (right). B, HT-1080 cells were synchronized at the G1-S boundary, released into the cell cycle, and incubated in the presence of 1 nM vincristine or 100 nM paclitaxel (PTX) alone or in combination with 1 μM PD0325901 as described above. Adherent cells (3 h after release from the block) were collected by scraping, whereas mitotic cells were collected by gentle shaking at the indicated times after release from the block. For reference, synchronized cells were incubated with or without 1 μM PD0325901, and total cells were collected at the indicated times after release from the block. Cell lysates (25 μg of protein) were then subjected to immunoblot analysis with antibodies to the indicated proteins. The brackets indicate BimEL, and open and closed arrowheads indicate the cleaved and intact forms of poly(ADP-ribose) polymerase (PARP), respectively.

Prolonged Mitotic Arrest Is Essential for Induction of Cell Death by Combination of MEK and Microtubule Inhibitors—Antimitotic agents activate the SAC, resulting in mitotic arrest.
To determine whether the prolonged mitotic arrest elicited in tumor cells by the combination of a MEK inhibitor and a microtubule inhibitor is essential for the induction of cell death, we examined the effect of RNAi-mediated depletion of Mad2 or BubR1, which are essential components of the SAC (21), in HT-1080 cells. Immunoblot analysis revealed that transfection of the cells with siRNAs specific for Mad2 or BubR1 resulted in pronounced and selective depletion of the corresponding proteins (Fig. 2A). When HT-1080 cells depleted of Mad2 or BubR1 were exposed to the combination of PD0325901 and vincristine (1 nM), the cells did not arrest in mitosis but instead exited from mitosis more rapidly than did naïve cells (time in mitosis of 22.4 ± 9.1 and 21.6 ± 9.8 min for Mad2- and BubR1-depleted cells, respectively), suggesting that SAC function was impaired. Under these conditions, cell death attributable to the drug combination was suppressed almost completely (Fig. 2A).

In all of our experiments, tumor cells were treated with a MEK inhibitor and a microtubule inhibitor essentially at the same time. When HT-1080 cells were treated first with PD0325901 to induce G1 arrest (preventing progression through S to G2-M phases of the cell cycle) and then with vincristine 12 or 24 h later, the cell death apparent on simultaneous treatment with these drugs was suppressed to an extent that correlated well with the increase in the number of cells arrested in G1 phase (Fig. 2B). In contrast, treatment of the cells first with vincristine to induce mitotic arrest and then with PD0325901 24 h later induced cell death as effectively as did simultaneous treatment with the drug combination. These results indicate that prolonged mitosis is essential for the enhanced cell death induced by the drug combination.

To further confirm the importance of prolonged mitotic arrest for such enhanced cell death, we examined whether MEK inhibitors might also enhance the induction of cell death by antimitotic agents that target components of the mitotic spindle other than microtubules. Monastrol (which targets the mitotic kinesin-5) and Plk inhibitor III (which targets Plk-1,-2,
and -3) (29, 30) each induced prolonged mitotic arrest in HT-1080 cells, with a small proportion (~20%) of the cells dying during a markedly protracted mitosis (Fig. 2C). Treatment of the cells with the combination of PD0325901 and either monastrol or Plk inhibitor III resulted in massive cell death, similar to that observed with the combination of a MEK inhibitor and any of the microtubule inhibitors examined.

Combination of MEK and Microtubule Inhibitors induces up-regulation of Bim and enhanced down-regulation of Mcl-1—To examine the molecular mechanism by which the combination of a MEK inhibitor and a microtubule inhibitor induces cell death during prolonged mitosis, we synchronized HT-1080 cells at the G1-S boundary with a double thymidine block and then released into the cell cycle. After 1 h, the cells were exposed to the combination of 1 μM PD0325901 (PD) and 1 nM vincristine (VCR), and mitotic cells were collected by gentle shaking at the indicated times after release from the block. Cell lysates (25 μg of protein) were subjected to time-lapse microscopy to determine the timing of mitotic entry (0 min) and the timing of death in mitosis (left). Data are means ± S.D. (n = 100 cells). Box-and-whisker plots show the time that cells spent arrested before death in mitosis (right). **, p < 0.01.

For reference, we examined the expression of Bcl-2 family proteins in total cells collected at 3–15 h after release from the block followed by incubation with or without PD0325901. PD0325901 induced dephosphorylation and subsequent accumulation of the pro-apoptotic protein BimEL (32) and elicited a slight down-regulation of the prosurvival protein Mcl-1 (17) in the cells (Fig. 3B).

Induction of a greater level of cell death by the combination of vincristine and PD0325901 compared with PD0325901 or vincristine alone was confirmed by a corresponding greater accumulation of cells in sub-G1 phase (Fig. 3A, right), enhanced activation of caspase-3, and more pronounced cleavage of poly(ADP-ribose) polymerase (Fig. 3B). Under such conditions, the up-regulation of BimEL was observed, and importantly, the down-regulation of Mcl-1 was markedly enhanced compared with that apparent with either agent alone (Fig. 3B). Essentially similar results were obtained when HT-1080 cells were treated with paclitaxel (100 nM) alone or in combination with PD0325901.

Loss of Bim suppresses and stabilization of Mcl-1 slows onset of enhanced cell death induced by combination of MEK and microtubule inhibitors—To examine the role of the accumulation of Bim and the down-regulation of Mcl-1 in the enhanced cell death induced by the combination of a MEK inhibitor and a
MEK and Microtubule Inhibitors Target Bim and Mcl-1

A

Control siRNA
Mcl-1 siRNA (#1)
Mcl-1 siRNA (#2)
Bim siRNA (#1)
PD0325901 (1 μM)

Mcl-1

BimEL

BimL

Bims

β-Actin

B

Control siRNA/PD0325901 (1 μM)

Mcl-1 siRNA (#1)/PD0325901 (1 μM)

C

PD0325901 (1 μM)

Parent

Control siRNA

Mcl-1 siRNA (#1)

Mcl-1 siRNA (#1) Bim siRNA (#1)

Successful division
Interphase
Death in interphase

D

Cell death (%)

Mock

PD0325901

PD184352

PD98059

Mock

PD0325901

PD184352

PD98059

Mock

PD0325901

PD184352

PD98059

Mock

PD0325901

PD184352

PD98059

Mock

PD0325901

PD184352

PD98059
MEK and Microtubule Inhibitors Target Bim and Mcl-1

microtubule inhibitor, we first transfected HT-1080 cells with siRNAs specific for Bim. Immunoblot analysis revealed that transfection of the cells with Bim siRNA resulted in marked suppression of the up-regulation of Bim induced by PD0325901 (Fig. 4A). Depletion of Bim resulted in slight inhibition of the low level of cell death induced by PD0325901, whereas it markedly suppressed the pronounced cell death induced by the combination of vincristine (1 nM) and PD0325901 to the level induced by vincristine alone (Fig. 4B).

We next attempted to investigate the effect of overexpression of Mcl-1 on cell death induced by the combination of MEK and microtubule inhibitors. However, HT-1080 cells expressing enhanced green fluorescent protein-tagged Mcl-1 did not manifest normal cell cycle progression, rarely entering mitosis (data not shown). We therefore attempted to stabilize Mcl-1 by RNAi-mediated knockdown of Mule, an E3 ubiquitin ligase specific for Mcl-1 (33). Transfection of HT-1080 cells with Mule siRNA resulted in not only marked depletion of Mule mRNA (Fig. 5A) but also substantial stabilization of Mcl-1, without affecting the abundance of other Bcl-2 family members, including Bax, Bcl-2, and Bel-x (Fig. 5B). Although the extent of cell death induced by the combination of vincristine (1 nM) and PD0325901 was not substantially reduced, the onset of such death was delayed by ~120 min in cells depleted of Mule (time to 50% death: control siRNA, 279.3 ± 32.0 min; and Mule siRNA, 396.0 ± 31.6 min) (Fig. 5C). A possible explanation for the observation that knockdown of Mule affected the drug combination-induced cell death only partially is that Mule depletion did not result in complete stabilization of Mcl-1, suggesting that the turnover of Mcl-1 may be regulated in both Mule-dependent and Mule-independent manners. The onset of apoptosis induced by a DNA-damaging agent was previously shown to be delayed by several hours in U2OS cells transfected with Mule siRNA to stabilize Mcl-1 (33).

Depletion of Mcl-1 Sensitizes Tumor Cells to MEK Inhibitor-induced Cell Death in Manner Sensitive to Loss of Bim—The MEK inhibitor PD0325901 induced the up-regulation of Bim as well as the down-regulation of Mcl-1 (Fig. 3B), but such inhibitors by themselves failed to induce a substantial level of cell death in tumor cells (Fig. 1A). The extent of MEK inhibitor-induced down-regulation of Mcl-1 was relatively small, however, and might therefore be insufficient to tip the balance between pro-apoptotic and anti-apoptotic signaling toward induction of cell death. To examine this possibility, we determined the effect of Mcl-1 depletion on the susceptibility of HT-1080 cells to death induced by PD0325901 (Fig. 6). Immunoblot analysis revealed that transfection of the cells with Mcl-1 siRNA resulted in effective depletion of Mcl-1 (Fig. 6A). Whereas depletion of Mcl-1 alone was insufficient to induce cell death (Fig. 6D), it conferred sensitivity to death induced by PD0325901, with ~80% of the cells dying in interphase (Fig. 6, B and C). Furthermore, the Mcl-1-depleted cells were again rendered resistant to PD0325901 when up-regulation of Bim by the MEK inhibitor was suppressed by transfection with Bim siRNA (Fig. 6, A, C, and D). Essentially similar results were obtained with other MEK inhibitors and other tumor cell lines (Fig. 6D).

DISCUSSION

We have examined the molecular mechanism by which the combination of a MEK inhibitor and a low concentration of a microtubule inhibitor induces enhanced cell death in tumor cells in which the ERK pathway is constitutively activated. For this analysis, we adapted time-lapse microscopy to monitor the fate of individual cells exposed to such drugs. Individual cells of a given line in the exponential phase of growth exhibited marked variation in their behavior. Whereas some HT-1080 cells entered mitosis immediately after the onset of time-lapse observation, for example, others entered mitosis >18 h later. We found that specific blockade of the ERK pathway by a MEK inhibitor did not affect the entry of most cells into the first mitosis after drug exposure, but it induced arrest in the subsequent interphase, which was accomplished after ~20 h. In this regard, although specific blockade of the ERK pathway by MEK inhibitors induces arrest in G1 phase of the cell cycle in tumor cells in which the ERK pathway is constitutively activated, maximal G1 arrest is apparent ~24 h after the onset of drug treatment, whereas blockade of the ERK pathway is accomplished within 1 h (9). These observations suggest that activation of the ERK pathway is required for the cells to progress through a restriction point early in G1 phase, immediately after cell division.

ERK1 and ERK2 phosphorylate BimEL, a pro-apoptotic Bcl-2 family protein, and thereby trigger its degradation by the proteasome (16). The abundance of Bim is thus low in many tumor cell lines in which the ERK pathway is constitutively activated, including HT-1080, HT-29, and MDA-MB-231 cells. Blockade of the ERK pathway by a MEK inhibitor suppressed BimEL phosphorylation in HT-1080 cells, resulting in the stabilization and accumulation of this protein. Furthermore, ERK1 and ERK2 phosphorylate the anti-apoptotic Bcl-2 family protein Mcl-1 at Thr163, resulting in its stabilization (17). Consistent with this notion, the MEK inhibitor PD0325901 induced the down-regulation of Mcl-1 in HT-1080 cells, although this effect was relatively small. Phosphorylation of Mcl-1 at multiple sites by several kinases contributes to regulation of its stability (34, 35). The inability of MEK inhibitors to induce substantial cell death in tumor cells is likely due to the insufficient down-regulation of Mcl-1, which fails to tip the balance between pro-

FIGURE 6. Depletion of Mcl-1 sensitizes tumor cells to induction of cell death by MEK inhibitors. A, HT-1080 cells were transfected for 48 h with Mcl-1 (#1 or #2), Bim (#1), or control siRNAs and then exposed to 1 μM PD0325901 for 12 h. Cell lysates (25 μg of protein) were subjected to immunoblot analysis with antibodies to the indicated proteins. B, HT-1080 cells transfected with Mcl-1 (#1) or control siRNAs for 48 h were exposed to 1 μM PD0325901 and observed by time-lapse microscopy. Representative time-lapse sequences are shown. Each horizontal line represents one cell, with the length of the line corresponding to the duration of a given behavior (color-coded as described in the legend to Fig. 1). Circled numbers on the line correspond to the position of each image. Arrowheads indicate the same cells in the time-lapse sequences. Scale bar = 20 μm. NEB, nuclear envelope breakdown. C, HT-1080 cells subjected to mock transfection (Parent) or transfected with the indicated siRNAs for 48 h were exposed to 1 μM PD0325901 and observed by time-lapse microscopy for the indicated times. Fate profiles of 100 representative cells are shown for each condition. D, HT-1080, HT-29, or MDA-MB-231 cells transfected with the indicated siRNAs for 48 h were exposed or not (Mock) to PD0325901 (1 μM), PD184352 (10 μM), or PD98059 (50 μM) for 24 h and then analyzed for the proportion of cells in sub-G1 phase (dead cells) by flow cytometry. *, p < 0.05; **, p < 0.01.
apoptotic and anti-apoptotic signaling toward induction of cell death. MEK inhibitors are categorized as cytostatic, not cytotoxic, which limits the therapeutic efficacy of these agents when administered alone (8). It is possible, however, that MEK inhibitors alone would be able to induce substantial cell death in tumor cells in which the abundance of anti-apoptotic Bcl-2 family proteins is low.

We have shown that prolongation of mitosis is essential for the induction of massive cell death by the combination of a MEK inhibitor and a microtubule inhibitor. Although treatment with low concentrations of microtubule inhibitors did not induce pronounced cell death in tumor cells, it did induce prolonged mitotic arrest. During mitotic arrest, transcription is halted, whereas proteolysis persists, resulting in the depletion of short-lived proteins encoded by short-lived mRNAs; these proteins include several anti-apoptotic molecules such as Mcl-1 and XIAP (27). Furthermore, phosphorylation of Mcl-1 by the CDK1-cyclin B1 complex initiates its Cdc20-dependent degradation during mitotic arrest (35). Consistent with these observations, Mcl-1 underwent substantial down-regulation when HT-1080 cells were treated with a low concentration of vincristine or paclitaxel. The importance of Mcl-1 turnover in the control of cell survival has been described previously (36, 37). However, our results suggest that the down-regulation of Mcl-1 alone is insufficient to induce cell death in tumor cell lines in which the ERK pathway is constitutively activated, including HT-1080, HT-29, and MDA-MB-231 cells. Indeed, although the antiproliferative efficacy of various mitotic inhibitors, including microtubule inhibitors, is established in most tumor cells, cellular responses subsequent to the mitotic arrest vary among cell lines and include apoptosis and mitotic catastrophe but also mitotic slippage and reversal of the mitotic arrest, with many cells remaining viable in the latter two instances (38).

Mcl-1 underwent down-regulation when HT-1080 cells (in interphase) were treated either with PD0325901 or with cycloheximide (to inhibit translation, mimicking mitotic arrest), and this effect was enhanced on exposure of the cells to both of these agents (supplemental Fig. 3). The proteasome inhibitor MG132 prevented the down-regulation of Mcl-1 induced by PD0325901 or cycloheximide, either alone or in combination. Furthermore, RNAi-mediated knockdown of Mule, an E3 ubiquitin ligase specific for Mcl-1, resulted in stabilization of Mcl-1 in HT-1080 cells. These results are consistent with the notion that proteasomal degradation is the major mechanism responsible for the down-regulation of Mcl-1 in tumor cells induced by treatment with anticancer drugs (35, 39). At present, however, the precise mechanism by which microtubule and MEK inhibitors modulate the activity of the proteasomal machinery remains to be elucidated.

We have now shown that the combination of a MEK inhibitor and a low concentration of a microtubule inhibitor induced prolonged mitosis in tumor cells in which the ERK pathway is constitutively activated. The up-regulation of Bim (in which blockade of the ERK pathway by MEK inhibitors plays a role) and down-regulation of Mcl-1 (in which both the prolonged mitotic arrest induced by microtubule inhibitors and blockade of the ERK pathway by MEK inhibitors play a synergistic role) were elicited in such mitotically arrested cells, tipping the balance between pro-apoptotic and anti-apoptotic signaling toward the former and culminating in the induction of massive cell death. Targeting of both the anti-apoptotic protein Mcl-1 and the pro-apoptotic protein Bim by the combination of a MEK inhibitor and a low concentration of a microtubule inhibitor thus represents a promising chemotherapeutic strategy for the treatment of cancer with enhanced efficacy and safety.

Optimal use of molecularly targeted drugs has been suggested to lie in combination therapy, either with classic cytotoxic agents or with other targeted therapies (8, 40). Our data suggest that the combination of a MEK inhibitor with an agent that induces the down-regulation or inactivation of anti-apoptotic proteins such as Mcl-1, Bcl-2, Bcl-xL, or XIAP constitutes a rational strategy for the development of effective anticancer chemotherapies. Such agents include a wide variety of antimitic drugs, not only those that target microtubules but also those that target polo-like kinases or kinesin-5.

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