

Navitoclax (ABT-263) Accelerates Apoptosis during Drug-Induced Mitotic Arrest by Antagonizing Bcl-xL

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Abstract

Combining microtubule-targeting antimetabolic drugs with targeted apoptosis potentiators is a promising new chemotherapeutic strategy to treat cancer. In this study, we investigate the cellular mechanism by which navitoclax (previously called ABT-263), a Bcl-2 family inhibitor, potentiates apoptosis triggered by paclitaxel and an inhibitor of kinesin-5 (K5I, also called a KSP inhibitor), across a panel of epithelial cancer lines. By using time-lapse microscopy, we showed that navitoclax has little effect on cell death during interphase, but strongly accelerates apoptosis during mitotic arrest, and greatly increases the fraction of apoptosis-resistant cells that die. By systematically knocking down individual Bcl-2 proteins, we determined that Mcl-1 and Bcl-xL are the primary negative regulators of apoptosis during prolonged mitotic arrest. Mcl-1 levels decrease during mitotic arrest because of an imbalance between synthesis and turnover, and turnover depends in part on the MULE/HUWE1 E3 ligase. The combination of Mcl-1 loss with inhibition of Bcl-xL by navitoclax causes rapid apoptosis in all lines tested. Variation in expression levels of Mcl-1 and Bcl-xL largely determines variation in response to antimetotics alone, and antimetotics combined with navitoclax, across our panel. We concluded that Bcl-xL is a critical target of Bcl-2 family inhibitors for enhancing the lethality of antimetotic drugs in epithelial cancers, and combination treatment with navitoclax and a spindle specific antimetotic, such as a K5I, might be more effective than paclitaxel alone. *Cancer Res*; 71(13); 4518–26. ©2011 AACR.

Introduction

Antimetotic drugs, such as paclitaxel, are important for treatment of a variety of solid tumors. They induce prolonged mitotic arrest through activation of the spindle assembly checkpoint (1). Drug treated cells then either die in mitotic arrest, or slip out of mitotic arrest into an abnormal G₁ state in which they may die, arrest in a tetraploid G₁ state, or continue to proliferate (2–6). Although antimetotics at sufficiently high concentration can cause mitotic arrest in all proliferating cells, sensitivity to induction of cell death during or after this arrest is highly variable across different cancer cell lines in syngeneic mouse tumors (7) and cultured human cell lines (8). This poorly understood variation in cell death sensitivity may contribute to large variation in patient responses.

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-10-4336

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Although nonapoptotic death has been reported (9, 10), most of the available data, including our own, show that antimetotics induce cell death during mitotic arrest, or after mitotic slippage, by the intrinsic apoptosis pathway, in which mitochondrial outer membrane permeabilization (MOMP) is the defining event (11–13). Thus, one way to potentially enhance antimetotics-induced cell death, and reduce variation in death sensitivity across cancers, is to combine antimetotics with drugs that directly stimulate MOMP. ABT-737 and its orally active analog, navitoclax (formerly ABT-263), are among the most promising candidates for such drug combinations (14–16). ABT-737 is a potent inhibitor ($K_i < 10$ nmol/L) of several Bcl-2 homologs that inhibit MOMP, including Bcl-2, Bcl-xL, and Bcl-w (14). These drugs have already been shown to potentiate the cytotoxic effect of paclitaxel in cell culture, animal models, and early-stage clinical trials (17, 18). However, the cellular mechanism by which ABT-737 and navitoclax enhance paclitaxel-mediated apoptosis, and how this mechanism varies across different cell types, are still poorly understood. For example, it is unclear whether the taxane + navitoclax combination kills cells in interphase, during mitotic arrest, or after slippage from arrest. It is also unclear which of the various targets of navitoclax are relevant for promoting cancer cell killing by taxanes, which precludes development of more selective drugs. Answering these questions might also provide clues to the still unsolved problem of how antimetotics trigger MOMP at the molecular level.

Here, we investigate the cellular mechanism by which navitoclax enhances cancer cell killing by 2 antimetotic drugs,

the microtubule-stabilizing drug, paclitaxel, and an allosteric inhibitor of motor protein, kinesin-5 (K5I, also called a KSP inhibitor), both of which promote prolonged mitotic arrest, but by different mechanisms. We test which Bcl-2 family members are important for inhibiting and enhancing cell death during mitotic arrest caused by these drugs in epithelial cancer cells and thus identify relevant targets of navitoclax when it is combined with an antimetabolic. Our primary technique for measuring apoptosis was time-lapse microscopy, which allows conceptual synchronization of cell cycle without the need for additional drugs, and, naturally, accounts for the very large variation between individual cells in death sensitivity and kinetics (4, 6). Our findings have important implications for how the navitoclax + taxane combination may work or fail in the clinic. Moreover, as neither K5Is nor navitoclax are neurotoxic, our results also suggest that navitoclax, or a comparable Bcl-xL inhibitor, would strongly potentiate the clinical response to K5Is, without causing neurotoxicity that is characteristic of microtubule-targeting antimetabolites.

Materials and Methods

Cell culture

All cell lines were purchased from American Type Culture Collection and cultured under 37°C and 5% CO₂ in appropriate medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 µg/mL streptomycin. HeLa was maintained in Dulbecco's modified Eagle's medium, U2OS was maintained in McCoy's, OVCAR-5 was maintained in RPMI, and A549 was maintained in F-12K.

Chemicals

Paclitaxel was purchased from Sigma. The potent K5I (EMD534085) was provided by Merck-Serono. Responses to EMD534085 have been shown to be similar to *s*-trityl-cysteine, a commercially available K5I (4). Navitoclax (ABT-263) was purchased from Selleck.

Time-lapse microscopy

Cells were plated in 35-mm imaging dish (µ-dish, ibidi, Germany) and cultured in phenol red-free CO₂-independent medium (Invitrogen) supplemented with 10% FCS, 100 U/mL penicillin, and 100 µL streptomycin. Cell images were acquired with the Nikon TE2000-PFS inverted microscope enclosed in a humidified chamber maintained at 37°C. Cells were imaged every 10 minutes by using a motorized stage and a 20× objective (NA = 0.95). Images were viewed and analyzed by using the MetaMorph software (Molecular Dynamics).

Gene knockdown by RNA interference

siRNAs for knocking down individual Bcl-2 family proteins were obtained from the following sources: Bcl-2 (J-003307-16), Bcl-w (J-004384-07), Bcl-xL (J-003458-11), Bak (J-003305-07), and Bax (J-003308-12) from Dharmacon; Mcl-1 (s120644) and Cdc20 (4392420) from Ambion; Bim (#6461) from Cell Signaling. The above siRNAs were used at final concentration of 20 nmol/L for RNA interference (RNAi) in all 4 cell lines. siRNA against MULE/HUWE1 (5'-GAGUUUGGAGUUUGUGAAGtt-

3') was synthesized by Dharmacon and used at final concentration of 50 nmol/L. Dharmacon On-Target plus siControl (D-001810-01) was used as nontargeting siRNA control. All siRNA transfections were done by using either DharmaFect 1 (Dharmacon) or HiPerFect (Qiagen) according to manufacturers' instructions. Experiments were conducted after 48 hours of gene silencing.

Gene expression by transient transfection

To express Mcl-1-GFP or Bak-EGFP, U2OS cells were seeded in 6-well plate and transiently transfected with the desired plasmids by using Fugene6 (Roche) according to manufacturer's instruction. Drug treatment experiments of U2OS cells with Mcl-GFP overexpression as well as time-lapse imaging experiment of U2OS cells expressing Bak-EGFP were conducted 48 hours after transient transfection. Both the Mcl-1-GFP and Bak-EGFP constructs were generous gift from Dr. Peter Sorger's lab at the Department of Systems Biology, Harvard Medical School.

Western blot analysis

Cell lysates were obtained by using LDS sample buffer (NuPAGE; Invitrogen). Proteins were resolved on 10% or 12% Tris-glycine gels and transferred onto polyvinylidene difluoride membranes. Blots were probed with commercial primary antibodies and chemiluminescent detection by using ECL-plus (Amersham). Antibodies: PARP1 (#9542), Bcl-w (#2724), Bcl-xL (#2762), Bim (#2933), Bak (#3814), and Cdc20 (#4823) were purchased from Cell Signaling; Bax (#sc-493) and Mcl-1 (#sc-819) from Santa Cruz; Bcl-2 (#M088901) from Dako. Anti-actin (#A5316) from Sigma was used as a loading control.

For Western blot analysis of synchronized mitotic cells, we grew large volume of cells in 25-cm dishes to 90% confluency, then treated the cells with 150 nmol/L paclitaxel or 1 µmol/L K5I to induce mitotic arrest. After 3 hours of drug treatment, the mitotic fraction of cells was collected by gently shaking and washing the dish to detach the mitotic cells from the bottom.

Results

Bcl-2 family expression does not obviously predict apoptosis sensitivity

We previously profiled the sensitivity of a panel of cancer-derived, and one noncancer telomerase-immortalized (RPE), cell lines for sensitivity to apoptosis in response to antimetabolic drugs (8). At drug concentrations that were saturating for mitotic arrest, the extent and speed of apoptosis varied greatly across the cell line panel, but correlated strongly for 3 different antimetabolic drugs, suggesting they trigger apoptosis by similar mechanisms. In every case subsequently studied, cell death occurred during or after slippage from mitotic arrest, by MOMP followed by caspase activation (ref. 6 and Supplementary Fig. S1). To probe the mechanistic basis of differential MOMP sensitivity across the panel, we measured levels of known MOMP regulators by immunoblotting (Fig. 1). Basal expression of Bax, Bak, Mcl-1, and Bcl-w were relatively similar

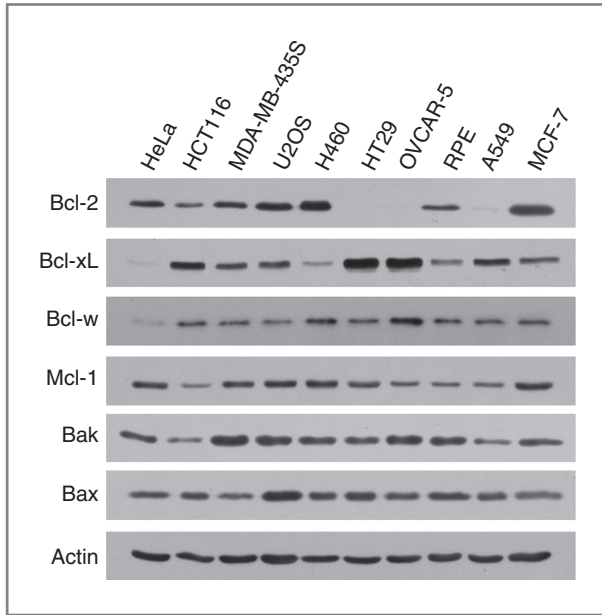


Figure 1. Basal expression of selected Bcl-2 family proteins in a panel of human cell lines we profiled before (8). Cell lines were ordered from left to right in descending sensitivity to antimetotics-induced cell death.

across cell lines, whereas expression of Bcl-2 and Bcl-xL were quite variable. By visual inspection, there was no obvious correlation between overall expression levels of pro- and

antiapoptotic proteins and MOMP sensitivity in response to antimetotics.

Navitoclax accelerates apoptosis during mitotic arrest

All the cell lines expressed antiapoptotic Bcl-2 family members. As a first test of their roles in cell death during or after mitotic arrest, we measured the effect of navitoclax, a potent small molecule inhibitor of Bcl-2, Bcl-w, and Bcl-xL, but not Mcl-1, using time-lapse microscopy. We chose HeLa, U2OS, OVCAR-5, and A549 to cover the spectrum from most to least death sensitive, and also variation in expression levels of Bcl-2 and Bcl-xL. Navitoclax was used at 1 $\mu\text{mol/L}$, a clinically relevant concentration that is probably saturating for Bcl-2 family binding (14). In navitoclax alone proliferation continued normally in HeLa, OVCAR-5, and A549 cells, and we observed less than 5% cell death in these 3 cell lines after 60 hours of drug treatment (gray lines in the top panel of Fig. 2). In U2OS cells, navitoclax alone was cytotoxic, leading to approximately 35% cell death after 60 hours of drug treatment. When navitoclax was combined with an antimetotic drug (paclitaxel or K5I, used at concentrations that are saturating for mitotic arrest in all lines), we observed strong enhancement of cell death for all 4 cell lines. MOMP was observed to precede death in more than 95% of single cell death events, confirming that antimetotics + navitoclax trigger cell death mainly through the mitochondria-dependent apoptosis pathway. Moreover, in antimetotic alone, or antimetotic + navitoclax, no apoptosis occurred before cells entered mitotic arrest.

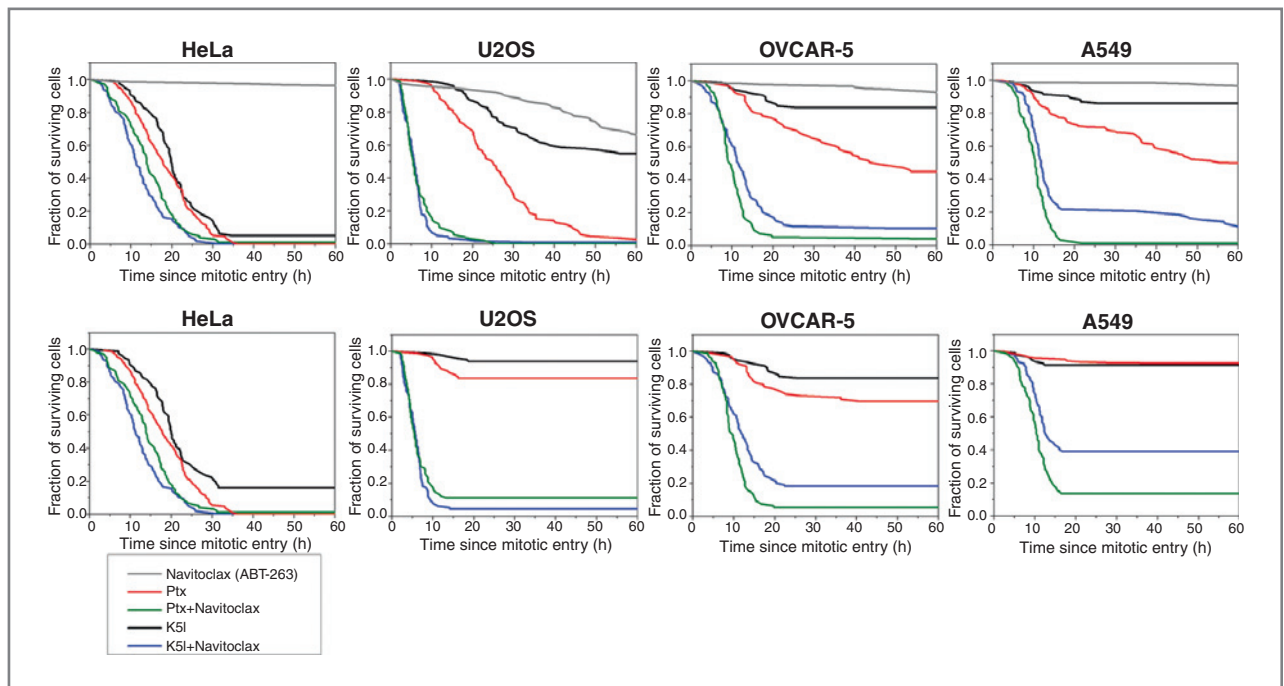


Figure 2. Cumulative survival curves for indicated treatments in 4 cancer cell lines: treatment with 1 $\mu\text{mol/L}$ navitoclax (ABT-263) alone (denoted in gray), 150 nmol/L paclitaxel alone (red), 150 nmol/L paclitaxel + 1 $\mu\text{mol/L}$ navitoclax (green), 1 $\mu\text{mol/L}$ K5I alone (black), and 1 $\mu\text{mol/L}$ K5I + 1 $\mu\text{mol/L}$ navitoclax (blue). Total number of cells analyzed for each curve ranges from 76 to 120 varied between conditions and cell lines. Individual cells were monitored by phase-contrast and fluorescence time-lapse microscopy, and time from mitotic entry to morphologic death was measured and plotted as cumulative survival curves. The top panel quantified kinetics of all cell death and the bottom panel quantified only death during mitotic arrest.

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Evidently, navitoclax enhances the proapoptotic signaling that initiates when cells enter mitotic arrest instead of at time of drug addition (6, 8). We therefore scored death kinetics by measuring the time elapsed from the time of entry into mitotic arrest to the time of morphologic death, that is, cell blebbing and lysing, and plotted cumulative survival curves that provide a metric for the rate of apoptosis induction during and after mitotic arrest (Fig. 2).

The top panel of Figure 2 shows kinetics of all cell death, pooling death during mitotic arrest with death after mitotic slippage. The bottom panel shows only death during mitotic arrest. The black and red lines show death in antimetabolic alone. HeLa is the most sensitive, with most cells dying during mitotic arrest, and A549 the least, with few cells dying. As reported previously (8), paclitaxel (red lines) is significantly more cytotoxic than K51 (black lines) in less death-sensitive lines, even though both drugs cause the same kinetics of mitotic arrest. Addition of navitoclax strongly accelerated death during mitotic arrest in U2OS, OVCAR-5, and A549, and moderately accelerated it in HeLa. This effect was particularly strong for cell line/drug combinations in which relatively little death occurred during mitosis for antimetabolic alone and, in some cases, caused more than 5-fold enhancement of death (e.g., U2OS/K51). Even in the most apoptosis-resistant A549 cells, navitoclax strongly enhanced death during mitotic arrest for both antimetabolics.

Note that we chose to investigate the kinetics of cell response at saturating drug concentrations of navitoclax and antimetabolics, instead of a dose titration study at fixed time point. Dose-dependent effects of antimetabolics are in

general difficult to interpret, as they require consideration of drug efflux pump activity, and single cell behaviors at subsaturating concentrations become very complex. Therefore, we chose to use only one dose and investigate the cellular kinetics in detail so as to seek mechanistic understanding at the level of pathways that mediate mitotic arrest and apoptosis. We picked a saturating concentration to maximize the mechanistic strength of our results and conclusions.

Navitoclax accelerates apoptosis mainly by inhibiting Bcl-xL

To pinpoint the specific Bcl-2 family target(s) of navitoclax during mitotic arrest, we used RNAi to knock down individual antiapoptotic Bcl-2 proteins, including Bcl-2, Bcl-w, Bcl-xL, and Mcl-1, and compared death kinetics to drug treatment. The top panel of Figure 3 and Table 1 compare kinetics of overall cell death following entry into K51-induced mitotic arrest, pooling both death in mitosis and death after slippage, under 7 conditions: control (no siRNA), control siRNA (control for the proapoptotic effect of transfection), Bcl-2 siRNA, Bcl-w siRNA, Bcl-xL siRNA, Mcl-1 siRNA, and navitoclax. The bottom panel of Figure 3 and Table 2 compare kinetics of cell death during mitotic arrest only. Knockdown efficiencies were generally more than 80% and are documented in Supplementary Figure S2. Control siRNA transfection (red lines) slightly sensitized cells to K51-induced apoptosis. Bcl-2 knockdown (blue lines) was similar to control siRNA for all 4 cell lines. Bcl-w knockdown (green lines) also had little effect, except in OVCAR-5 cells, in which it increased the percentage of cell death by approximately 2-fold, but the median time to death, T_d , did not

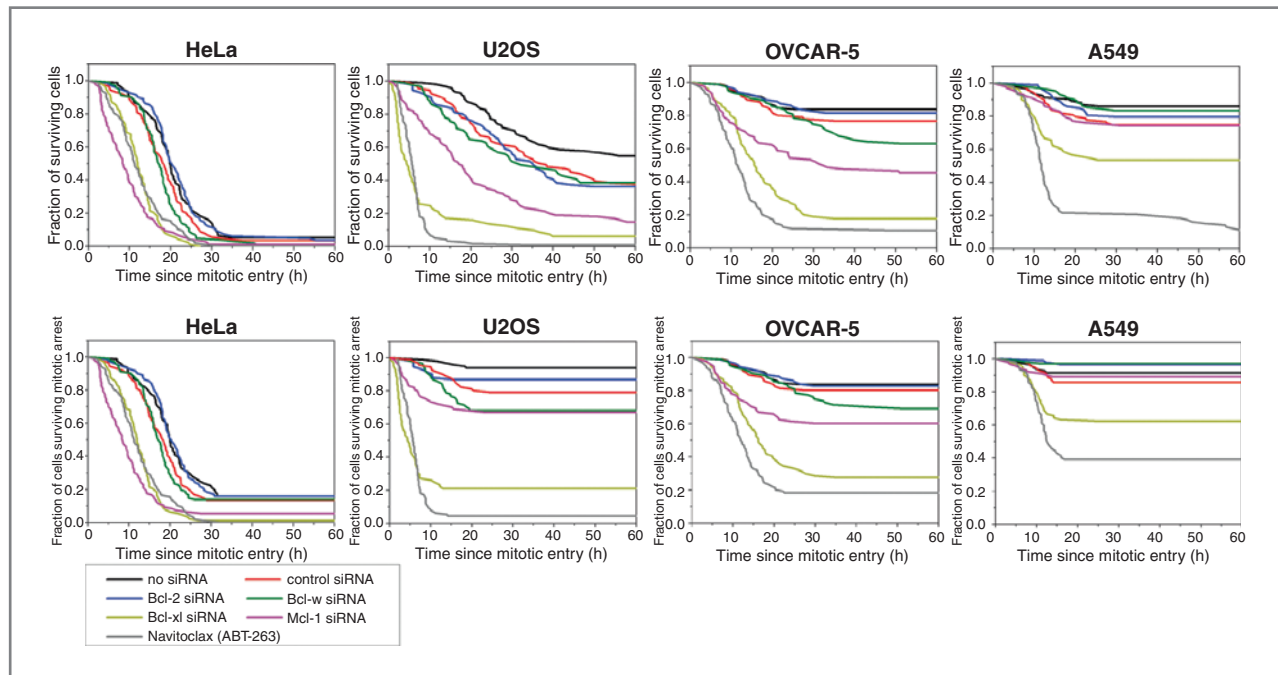


Figure 3. Cumulative survival curves for indicated siRNA treatments or navitoclax in combination with 1 $\mu\text{mol/L}$ K51 in 4 cancer cell lines. The top panel quantified kinetics of all cell death and the bottom panel quantified only death during mitotic arrest. Total number of cells analyzed for each curve ranges from 76 to 144, varied between conditions and cell lines.

Table 1. Percentage of overall cell death and T_d in response to K5I under transfection of different siRNA oligos

K5I+	HeLa		U2OS		OVCAR-5		A549	
	Death (%)	T_d (h)	Death (%)	T_d (h)	Death (%)	T_d (h)	Death (%)	T_d (h)
No siRNA	95	19.5	45	24.6	16	17.5	14	12.0
Scramble siRNA	97	17.8	63	21.5	23	16.8	25	13.5
Bcl-2 siRNA	96	19.7	64	24.7	18	17.2	20	18.0
Bcl-w siRNA	99	16.8	62	18.7	37	23.5	16	18.0
Bcl-xL siRNA	100	12.0	94	3.8	82	13.6	47	10.3
Mcl-1 siRNA	99	8.3	85	15.0	54	11.3	26	12.3
Navitoclax	100	11.3	99	5.7	89	10.8	91	11.3

NOTE: Total number of cells analyzed from the time-lapse movies ranges from 76 to 144, varied between conditions and cell lines. Death denotes the percentage of overall cell death, and T_d is the median time to death since mitotic entry.

change substantially (Table 1). Mcl-1 knockdown (magenta lines) sensitized all lines to apoptosis, especially HeLa, in which it had the strongest effect. This line has high expression of Mcl-1 but little Bcl-xL (Fig. 1). Bcl-xL knockdown (yellow lines) had overall the strongest effect, dramatically accelerating apoptosis in the 3 less apoptosis-sensitive lines. In U2OS and OVCAR-5, the effect of Bcl-xL knockdown was comparable with navitoclax treatment. In A549 it was less effective, but knockdown was noticeably incomplete in this line (Supplementary Fig. S2). As we saw previously with navitoclax treatment, Bcl-xL knockdown and, to a lesser extent, Mcl-1 knockdown had the effect of increasing the fraction of cells that died in the 3 less apoptosis-sensitive lines, as well as shifting death from postslippage to inside mitotic arrest. These data suggested that Bcl-xL is the major target of navitoclax for accelerating MOMP during mitotic arrest, and thus for potentiating cell death caused by antimetabolic drugs.

Mcl-1 is lost by turnover during mitotic arrest

Mcl-1 expression has been shown to negatively correlate with ABT-737 sensitivity in some studies (15). All our cell lines

expressed Mcl-1 at significant levels (Fig. 1), so we next asked why this important antiapoptotic Bcl-2 family member did not protect against MOMP during mitotic arrest when Bcl-xL was inhibited with navitoclax or knockdown by RNAi.

Uniquely among characterized Bcl-2 family proteins, Mcl-1 turns over rapidly in interphase cells, with a typical half-life of less than 1 hour (19, 20). It is constitutively ubiquitinated by E3 ligases and then degraded by proteasomes (21). Transcription is silenced during mitotic arrest and translation is inhibited (22–24), so we suspected Mcl-1 levels might decay during arrest by a continuation of the interphase turnover mechanism. We first measured time courses of Mcl-1 levels in cells treated with antimetabolic drugs and found that its levels decrease at the time point where mitotic arrest peaks (~15–30 hours; Fig. 4A). In HeLa, the level subsequently decreases to zero as all cells die but in the more resistant line, it recovers as cells slip out of mitotic arrest. To more accurately measure Mcl-1 levels during mitotic arrest, we synchronized cells by mitotic shake-off in drug and measured Mcl-1 levels by immunoblotting. Mcl-1 levels were found to decay with a half-life of approximately 4 hours in HeLa and

Table 2. Percentage of cell death in mitosis and the median time to mitotic death (T_d^m) in response to K5I under transfection of different siRNA oligos

K5I+	HeLa		U2OS		OVCAR-5		A549	
	D in M (%)	T_d^m (h)	D in M (%)	T_d^m (h)	D in M (%)	T_d^m (h)	D in M (%)	T_d^m (h)
No siRNA	84	19.3	6	13.7	16	17.5	8	8.8
Scramble siRNA	86	16.2	20	12.8	20	13.8	14	10.5
Bcl-2 siRNA	84	19.0	13	8.5	17	17.2	3	12.3
Bcl-w siRNA	86	16.0	32	12.8	31	22.0	3	7.0
Bcl-xL siRNA	99	11.8	79	3.0	73	13.2	38	9.2
Mcl-1 siRNA	95	8.2	33	4.7	40	9.5	11	6.0
Navitoclax	100	11.3	96	5.5	82	10.8	62	10.3

NOTE: D in M denotes the percentage of cell death occurring in mitotic arrest, and T_d^m is the median time to mitotic death since mitotic entry.

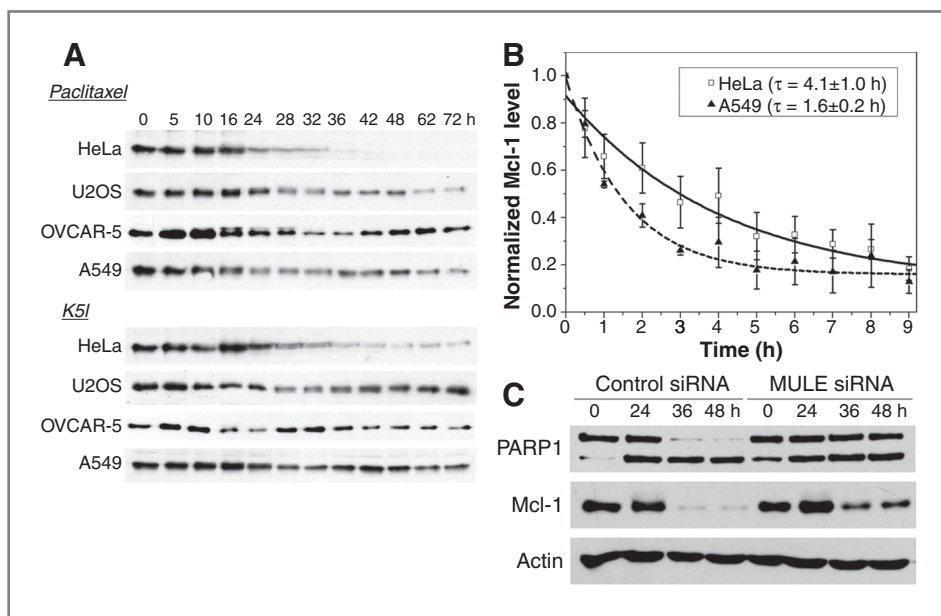


Figure 4. A, Mcl-1 levels at 12 selected time points (unit: hour) in the 4 selected cell lines treated with either 150 nmol/L paclitaxel or 1 μ mol/L K51. Actin, which served as loading control, was not shown, as loading was similar in all samples. B, comparison of Mcl-1 kinetics during mitotic arrest in HeLa (death sensitive) and A549 cells (death resistant). Pure mitotic HeLa and A549 cells were obtained by synchronizing cells in mitosis with 3 hour treatment of 1 μ mol/L K51 and then gently shaking off the mitotic fraction. Mcl-1 level was quantified from Western blots, normalized to initial level at time 0, and fit to an exponential decay to derive the half-life τ . Values of Mcl-1 level were averaged from 3 separate experiments. C, attenuation of Mcl-1 depletion by RNAi of MULE/HUWE1 (50 nmol/L) and the resulting reduction in apoptosis (indicated by PARP1 cleavage) compared with no knockdown of MULE/HUWE1.

approximately 1.6 hours in A549 during mitotic arrest (Fig. 4B). In A549, Mcl-1 plateaus at approximately 20% of the initial level. In HeLa, it may continue to drop, and we suspect complete loss of Mcl-1 may trigger MOMP.

To test whether loss of Mcl-1 during mitotic arrest contributes to apoptosis induction, we attenuated Mcl-1 depletion by RNAi knockdown of MULE/HUWE1. This is the first E3 ligase that was shown to play a role in Mcl-1 turnover (21), subsequently others have been implicated (see below). We used a custom synthesized siRNA used in previous study (25) and measured the resulting apoptosis response to antimetotics in HeLa cells by PARP1 cleavage (Fig. 4C). MULE/HUWE1 knockdown decreased the extent of Mcl-1 loss and also decreased PARP1 cleavage. These data confirmed that depletion of Mcl-1 is a proapoptotic signal that contributes to apoptosis during mitotic arrest. Because Mcl-1 levels were only partially protected by RNAi of MULE/HUWE1, we suspected that other turnover mechanisms were important; anaphase promoting complex (APC/C; ref. 26), NOXA (27), and another E3 ligase, FBW7 (28, 29) have all been implicated in recent studies. In Supplementary Figure S3, we compared kinetics of Mcl-1 depletion in mitotic shake-off HeLa cells under 3 conditions: control, MULE/HUWE1 siRNA, and Cdc20 siRNA (Cdc20 RNAi was used to inhibit APC/C activity). It is evident that RNAi of both MULE/HUWE1 and Cdc20 delayed and reduced Mcl-1 depletion during mitotic arrest. However, neither completely rescued Mcl-1 depletion, pointing to the existence of multiple degradation factors for Mcl-1 during mitotic arrest.

Bim does not trigger MOMP during mitotic arrest

The activator BH-3 protein Bim was previously implicated as the major trigger of cell death in BMK cells treated with paclitaxel, but where in the cell cycle Bim-dependent apoptosis occurred was not determined (30). To determine whether Bim is required to activate apoptosis during mitotic arrest, we knocked it down by using siRNA, and measured kinetics of cell death in HeLa, which die mainly during mitotic arrest (Fig. 5). Knockdown was largely complete by immunoblotting analysis (Supplementary Fig. S4). Both the extent and kinetics of death in mitosis were very similar under treatments of K51 alone (black line in Fig. 5), K51 plus control siRNA (red line), and K51 plus Bim siRNA (blue line), with 80% to 90% cell death occurring during mitotic arrest and a median time to death of 19 hours. Together with Western blot analysis that showed no attenuation in PARP1 cleavage under Bim knockdown (Supplementary Fig. S4), we concluded that Bim is not required to activate cell death during mitotic arrest in HeLa. Note that Bim is a fast turnover protein with a half-life of approximately 3 hours (31). Therefore, on the Western blot levels of Bim exhibited significant reduction during mitotic arrest because of transcriptional silence (24-hour time point in Supplementary Fig. S4). In contrast, knockdown of Bax (green line) and Bak (yellow line) caused substantial protection as expected for a MOMP-dependent death pathway. Immunoblotting analysis showed no upregulation of any known sensitizer BH-3 only proteins (Supplementary Fig. S5), and we previously found no cleavage of Bid in antimetotic treated cells (8). These data suggested that activator BH3 proteins or upregulation of

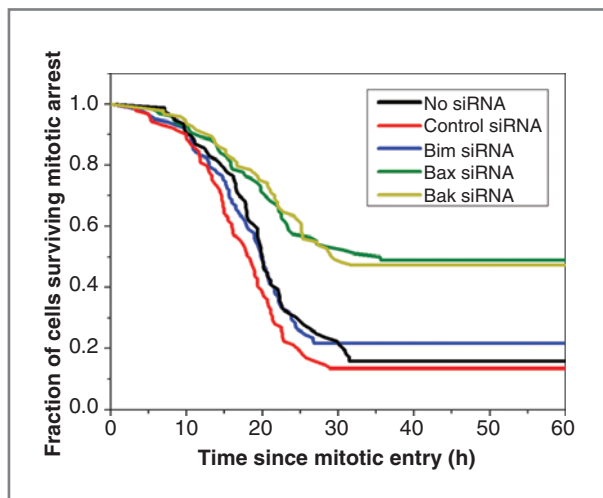


Figure 5. Cumulative survival curves for indicated siRNA treatments in combination with 1 $\mu\text{mol/L}$ K51 in HeLa cells. The survival statistics quantified only death during mitotic arrest.

sensitizer BH-3 only proteins are not required for cell death during mitotic arrest.

Discussion

We observed strong acceleration of apoptosis during mitotic arrest when navitoclax was combined with antimetabolic drugs, as well as recent data from xenograft cancer models (32), pointing to exciting potential of this combination for treatment of common epithelial cancers. Navitoclax has been known to potentiate paclitaxel-mediated cytotoxicity since the first description of ABT-737 (17), but ours is the first study to pinpoint where in the cell cycle this potentiation occurs, and to document its kinetics at the single cell level. The major target of navitoclax in the cells we tested was Bcl-xL, not Bcl-2 or Bcl-w, as evidenced by similar acceleration of apoptosis during mitotic arrest with drug treatment and RNAi knockdown of Bcl-xL alone. The potent lethality of inhibiting Bcl-xL during mitotic arrest is because of the fact that another important and widely expressed antiapoptotic protein, Mcl-1, is naturally depleted during mitotic arrest as a result of imbalance between synthesis and degradation. The pathway by which Mcl-1 is proteolyzed has been the subject of several recent studies. MULE/HUWE1 was implicated early as an E3 ligase (21). Our data (Fig. 4C) suggest it indeed plays a role during mitosis, though it seems not to act alone. APC/C was recently implicated as an alternative E3 ligase (26). Our data are consistent for a role of this factor (Supplementary Fig. S3), though caution is required in interpreting this observation, because APC/C plays a central role in mitotic regulation, and its effects on Mcl-1 might be indirect. We have not yet tested the role of other candidates, including NOXA (27) and FBW7 (28, 29). Whatever the precise mechanism of Mcl-1 proteolysis during mitosis, the important conclusion from our perspective is that its loss is strongly synergistic with inhibition of Bcl-xL by navitoclax, leading to rapid apoptosis during mitotic arrest,

even in lines in which little apoptosis is seen in antimetabolic alone.

The mechanism by which mitotic arrest promotes apoptosis is clarified, but not completely solved, by the finding of ours and others (25–29) that Mcl-1 is lost during mitotic arrest because of an imbalance between synthesis and proteolysis. Conceptually, this finding supports a kinetic model in which cells that enter mitotic arrest activate 2 independent and competing pathways, one that leads to MOMP, and the other to slippage by slow proteolysis of cyclin-B (33, 34). Combination of Mcl-1 loss with inhibition of Bcl-xL by navitoclax or siRNA knockdown greatly accelerates the death pathway, so it wins over the slippage pathway in a much larger fraction of cells. Loss of Mcl-1 is clearly an important component of the MOMP activation pathway, less clear is whether this is the only MOMP-activating process during mitotic arrest. It is also not clear to what extent acceleration of apoptosis activation matters clinically, that is, if a cell is going to die eventually, does it matter if it dies within hours following drug addition, as opposed to days or weeks? We suspect it might, that a rapid bolus of apoptosis might alter the tumor environment in ways that a more delayed response does not. The issue of whether apoptosis kinetics really matters for clinical responses will have to be addressed in the tumor context.

Our data have shed new light on cell line variation in antimetabolic drug responses. In cells with low levels of Bcl-xL, such as HeLa, loss of Mcl-1 alone is sufficient to release Bax/Bak from sequestration, which allows their spontaneous oligomerization, and triggers MOMP. In cells with substantial levels of Bcl-xL, such as U2OS, OVCAR-5, and A549, loss of Mcl-1 alone is insufficient, but combining it with Bcl-xL inhibition or knockdown is sufficient to trigger Bax/Bak oligomerization. It is known that Bcl-xL and Mcl-1, but not Bcl-2 or Bcl-w, can directly sequester Bax/Bak, in particular Bak on the mitochondria (35). This might explain why Bcl-2 levels seem less important than Bcl-xL in determining the response to antimetotics, despite wide variation in Bcl-2 expression between cells in our panel. Consistent with a role for Bak, we observed oligomerization of Bak-GFP preceding cell death in response to antimetotics + navitoclax by fluorescence microscopy (Supplementary Fig. S6). We also found that navitoclax remains highly effective in enhancing and accelerating mitotic death in cells overexpressing Mcl-1 (Supplementary Fig. S7). Is loss of Mcl-1 and Bcl-xL activity sufficient to trigger MOMP, or is another signal required? Depletion of Mcl-1 does not greatly accelerate apoptosis in response to antimetotics in any line (Fig. 2), which may indicate the need for an additional signal. Although our data on Bim (Fig. 5) and tBid (8) suggest that neither of the known activator BH3 proteins is required for initiation of apoptosis during mitotic arrest, we cannot rule out that some other, yet to be identified, Bcl-2 family member acts as activator. Further study to determine the molecular model for mitotic death is important for progress on understanding the action of current antimetotics and finding better targets for future drugs.

Antimetabolic drugs that are specific to the mitotic spindle—inhibitors of kinesin-5 (KSP), polo kinase-1, and aurora kinases—have so far shown somewhat disappointing clinical

results (36). K5Is are less proapoptotic than paclitaxel in cell culture (e.g., Fig. 2 and ref. 8), which may contribute to this clinical difference. The reasons are unclear, though the extra death seen with paclitaxel mostly occurs after slippage. One possible cause is greater micronucleation; cells that slip out of paclitaxel-induced arrest always have multiple micronuclei, whereas cells that slip out of K5I-induced arrest typically have one large nucleus. Combination with navitoclax changes this equation, by shifting most death into mitosis, in which the 2 drugs are almost equally proapoptotic. The combination of a K5I and navitoclax is much more proapoptotic than K5I alone or paclitaxel alone, and essentially equal to paclitaxel + navitoclax (Fig. 2). Thus the K5I + navitoclax combination has the promise of efficacy greater than paclitaxel alone, comparable to paclitaxel + navitoclax. Neither K5Is nor navitoclax are neurotoxic, so this combination might realize the promise of an antimitotic treatment that is efficacious but lacks neurotoxicity.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Peter Sorger (Systems Biology, Harvard Medical School) for the IMS-RP retroviral vector, Mcl-1-GFP vector, and Bak-EGFP vector, and the Nikon Imaging Center at Harvard Medical School for conducting part of our imaging experiments. EMD534085 was supplied by Merck Serono.

Grant Support

This work was supported by the National Cancer Institute (CA139980) to T.J. Mitchison and Hong Kong Research Grant Council (#261310) to J. Shi.

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Received December 1, 2010; revised April 5, 2011; accepted April 27, 2011; published OnlineFirst May 5, 2011.

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