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Chemical Perturbation of Mcl-1 Pre-mRNA Splicing to Induce Apoptosis in Cancer Cells

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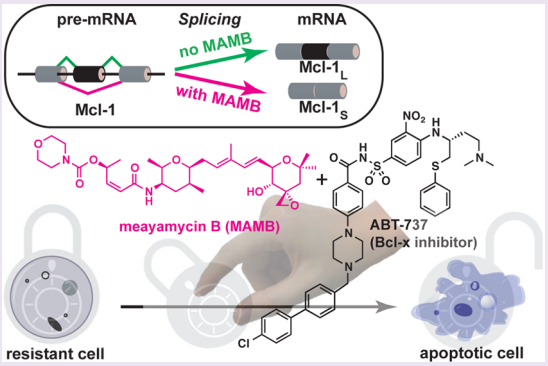
Supporting Information

ABSTRACT: The myeloid cell leukemia-1 (*MCL1*) gene encodes antiapoptotic Mcl-1_L and proapoptotic Mcl-1_S proteins. In cancer, the Mcl-1_L/Mcl-1_S ratio is very high, accounting for the antiapoptotic nature of cancer cells. As such, reducing this ratio can render the cancer cells prone to apoptosis. The Mcl-1_L/Mcl-1_S ratio is determined in the alternative pre-mRNA splicing step that is regulated by splicing factor 3B1 (SF3B1). Here, we report that meayamycin B, a potent inhibitor of SF3B1, reversed the dominant isoform from Mcl-1_L to Mcl-1_S at the mRNA and protein levels. The resulting proapoptotic cellular environment was further exploited; when meayamycin B was combined with Bcl-x_L inhibitor ABT-737, the combination treatment triggered apoptosis in nonsmall cell lung cancer A549 and H1299 cells that were otherwise resistant to ABT-737. These results demonstrate that perturbation of the *MCL1* splicing with small molecule inhibitors of SF3B1 provides a means to sensitize cancer cells toward Bcl-x_L inhibitors.



Many B-cell lymphoma-2 (Bcl-2) family genes undergo alternative pre-mRNA splicing and produce both proapoptotic and antiapoptotic protein isoforms. For example, *BCL2L1* and myeloid cell leukemia-1 (*MCL1*) genes are alternatively spliced into proapoptotic Bcl-x_S, Mcl-1_S, and Mcl-1_{ES}, antiapoptotic Bcl-x_L and Mcl-1_L, respectively.[1−3] In cancer, antiapoptotic isoforms are predominant.[4] Although other antiapoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-w are present in cancer cells, inhibition of both Bcl-x_L and Mcl-1_L is necessary and sufficient to trigger massive cell death.[5] In support of this notion, ABT-737, a small molecule that selectively binds to and antagonizes Bcl-2, Bcl-x_L, and Bcl-w, but not Mcl-1_L,[6,7] encountered resistance in cancer cells that overexpressed Mcl-1_L.[8−12] The inhibition of Mcl-1 expression by biological or pharmacological means restored the anticancer activity of ABT-737.[8,10,13,14] However, the only endeavor successfully sequestering Mcl-1_L by perturbing the alternative splicing of Mcl-1 pre-mRNA was antisense morpholino oligonucleotides.[15] So far, there is no small molecule reported to have such activity.

The expression of Mcl-1_S and Bcl-x_S mRNAs was upregulated when splicing factor 3B 1 (SF3B1; a.k.a. SAP155) was knocked down, indicating that SF3B1 is involved in the alternative splicing of these apoptosis-related genes.[16] SF3B1, an essential subunit of U2 snRNP, is critical for the faithful selection of the 3′ splice site in homeostatic cells.[17] SF3B1 has also been identified as a *trans*-acting splicing factor that enhances the production of Bcl-x_L in A549 cells.[18] Meanwhile, natural product FR901464 has been found to be a low nanomolar inhibitor of SF3B1.[19] Subsequently, we have developed a low to middle picomolar analogue of FR901464, meayamycin B (Figure 1).[20−22] Hence, we hypothesized that meayamycin B could switch the alternative splicing of Bcl-x and/or Mcl-1 pre-mRNAs toward the overexpression of Bcl-x_S and Mcl-1_S.

Here, we report that meayamycin B upregulates the proapoptotic Mcl-1_S and downregulates Mcl-1_L at the pre-mRNA splicing level. The resulting dominance of Mcl-1_S at the

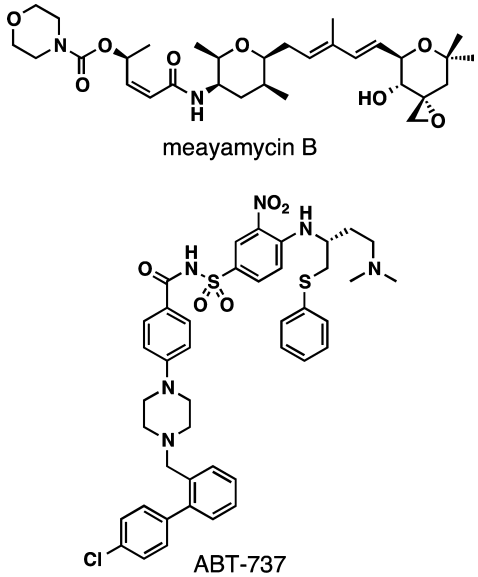


Figure 1. Structures of meayamycin B and ABT-737.

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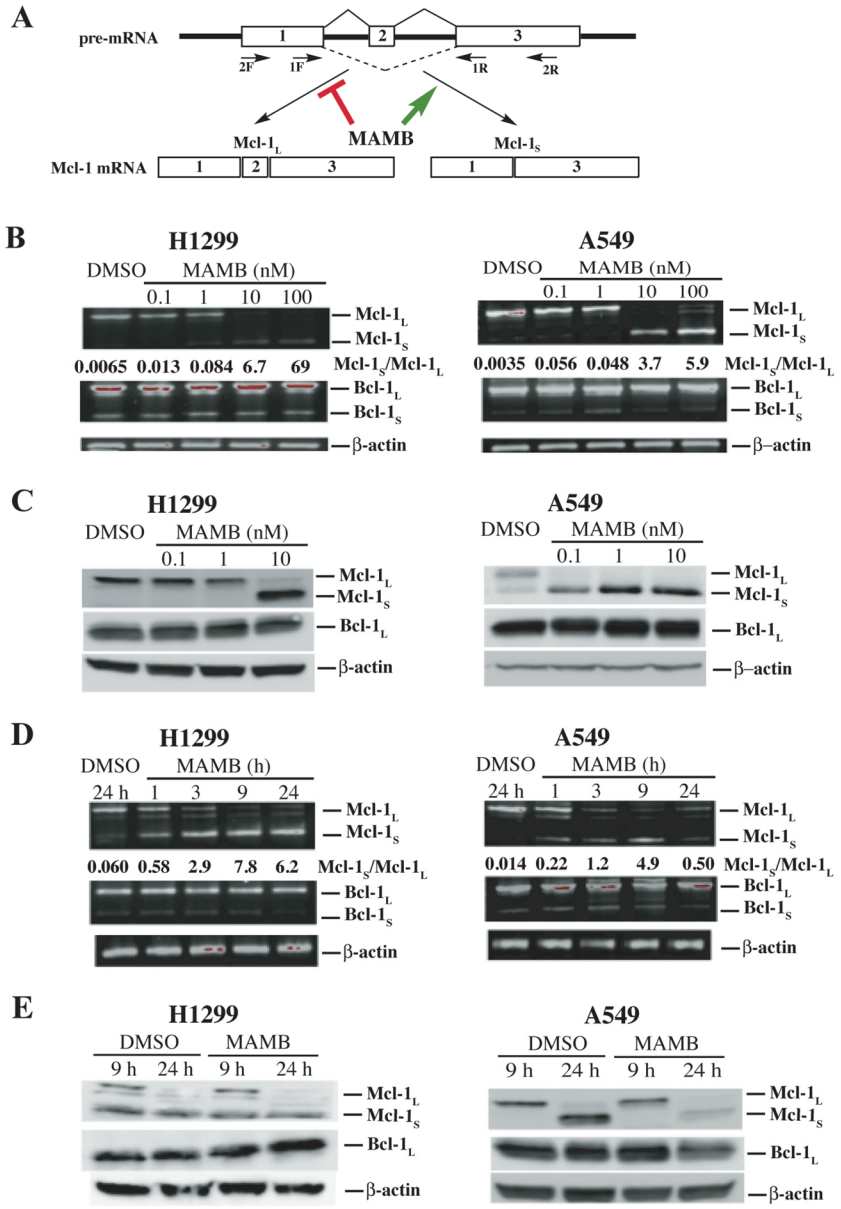

Figure 2. Dose- and time-dependent regulation of Mcl-1 splicing by meayamycin B (MAMB) in H1299 and A549 cells. (A) The alternative splicing of Mcl-1 regulated by meayamycin B. Horizontal arrows depict the locations of the primers for semiquantitative RT-PCR. Meayamycin B treatment increased Mcl-1_S and decreased Mcl-1_L splice variants. (B,C) A549 and H1299 cells were exposed to DMSO or various concentrations of meayamycin B for 9 h before total RNA and protein were analyzed by semiquantitative RT-PCR (B) and immunoblotting (C) for the alternative splicing of Mcl-1. The same samples were sequentially analyzed by semiquantitative RT-PCR using primer pair 1F and 1R shown in panel A. (D,E) Cells were incubated with 10 nM meayamycin B for indicated periods of time, and the total RNA and protein were extracted and analyzed by semiquantitative RT-PCR (D) and immunoblotting (E) for the alternative splicing of Mcl-1. Data represent results from at least three separate experiments.

protein level overcame the ABT-737 resistance and induced cell death in nonsmall cell lung cancer A549 and H1299 cell lines when both of these compounds were present. Meayamycin B did not perturb the apparent alternative splicing of Bcl-x within 24 h of treatments in our settings.

Mcl-1_L is generally overexpressed in nonsmall cell lung cancer cells including A549 and H1299 cells.[23,24] To test the aforementioned hypothesis about meayamycin B, A549 and H1299 cell lines were exposed to meayamycin B at various concentrations. The exposure time was only 9 h, which was sufficient to generate clear-cut data because the half-life of Mcl-1 mRNA is approximately 40 min.[25,26] The short assay also excludes the downstream effects of meayamycin B. After the 9 h exposure, the relative mRNA levels of Mcl-1 splicing variants including Mcl-1_L and Mcl-1_S were evaluated by means of semiquantitative RT-PCR (Figure 2A). For the RT-PCR experiment, we used primers (see Table S1 in the Supporting Information) that were designed to amplify multiple sites of the gene. Meayamycin B dose-dependently downregulated and upregulated Mcl-1_L and Mcl-1_S, respectively (NCBI GenBank accession number NM_021960 and NM_182763) (Figure 2B).[1] For example, the ratio of Mcl-1_S/Mcl-1_L increased from 0.0065 (H1299 cells) and 0.035 (A549 cells) with DMSO to 69 (H1299 cells) and 5.9 (A549 cells) with 100 nM meayamycin B. In accordance with the change at the mRNA level, immunoblotting showed that meayamycin B abrogated the

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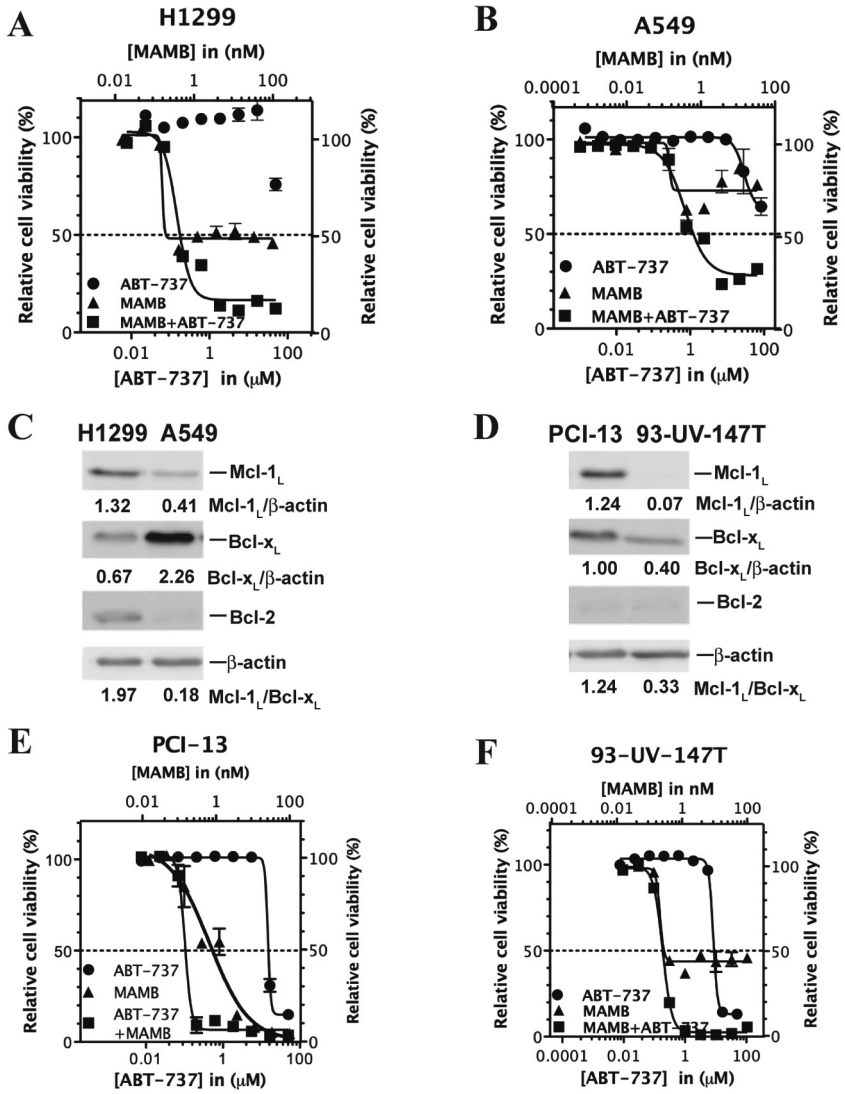

Figure 3. Antiproliferation (viability) assays (72 h) and basal expression of antiapoptotic Bcl-2 family proteins. (A,B) Antiproliferation (viability) assays (72 h) in H1299 and A549 cells. (C,D) Basal antiapoptotic Bcl-2 family protein expression of Mcl-1, Bcl-x, and Bcl-2 evaluated by immunoblotting. (E,F) Antiproliferation (viability) assays (72 h) in PCI-13 and 93-UV-147T cells. Data represent results from at least three separate experiments.

expression of the full length Mcl-1 protein isomer Mcl-1_L (40 kDa) and simultaneously increased Mcl-1_S (35 kDa) (Figure 2C). Mcl-1_L protein was more prevalent in H1299 than in A549, which might explain why 10 nM meayamycin B was required to completely remove Mcl-1_L in H1299 cells. Mcl-1_S protein became the dominant Mcl-1 isoform in A549 cells with only 0.1 nM meayamycin B.

Mcl-1 has a very short half-life at the mRNA and protein levels.[25,26] Therefore, we thought it would be possible to observe changes in the expression of *MCL1* soon after the addition of meayamycin B to the aforementioned cells. Thus, H1299 and A549 cells were exposed to 10 nM meayamycin B for 1, 3, 9, and 24 h before relative expression of Mcl-1 splicing variants were determined at the mRNA and protein levels. The semiquantitative RT-PCR analysis revealed that the increase of the Mcl-1_S mRNA was detectable after 1 h of treatment (Figure 2D). In addition, the suppression of the Mcl-1_L mRNA by meayamycin B was complete in 9 h and remained as such for the next 15 h. We also observed larger RT-PCR products that increased over time. These products were partially spliced Mcl-1 pre-mRNA retaining both intron 1 and intron 2 (Figure S1 in the Supporting Information), indicating that meayamycin B acted as both a constitutive splicing inhibitor and an alternative splicing modulator for Mcl-1 pre-mRNA. At the protein level (Figure 2E), Mcl-1_S was the dominant Mcl-1 isoform in both A549 and H1299 cell lines after 9 h of exposure to meayamycin B.

SF3B1 is also a *trans*-acting splicing factor for the Bcl-x gene.[18] In neither dose- nor time-dependent treatments did meayamycin B significantly increase Bcl-x_S (Figure 2C,D). This is in agreement with the results from the Webb group using sudemycins (analogs of FR901464) in pediatric rhabdomyosarcoma cell line Rh18 for 24 h.[27] The different responses between the Mcl-1 system and the Bcl-x system toward meayamycin B might be due to the much longer half-life of Bcl-x mRNA. Therefore, although biological means (siRNA) to inhibit SF3B1 resulted in the perturbation of the alternative splicing of both Bcl-x and Mcl-1 systems, the chemical approach was selective for the Mcl-1 system in a short time

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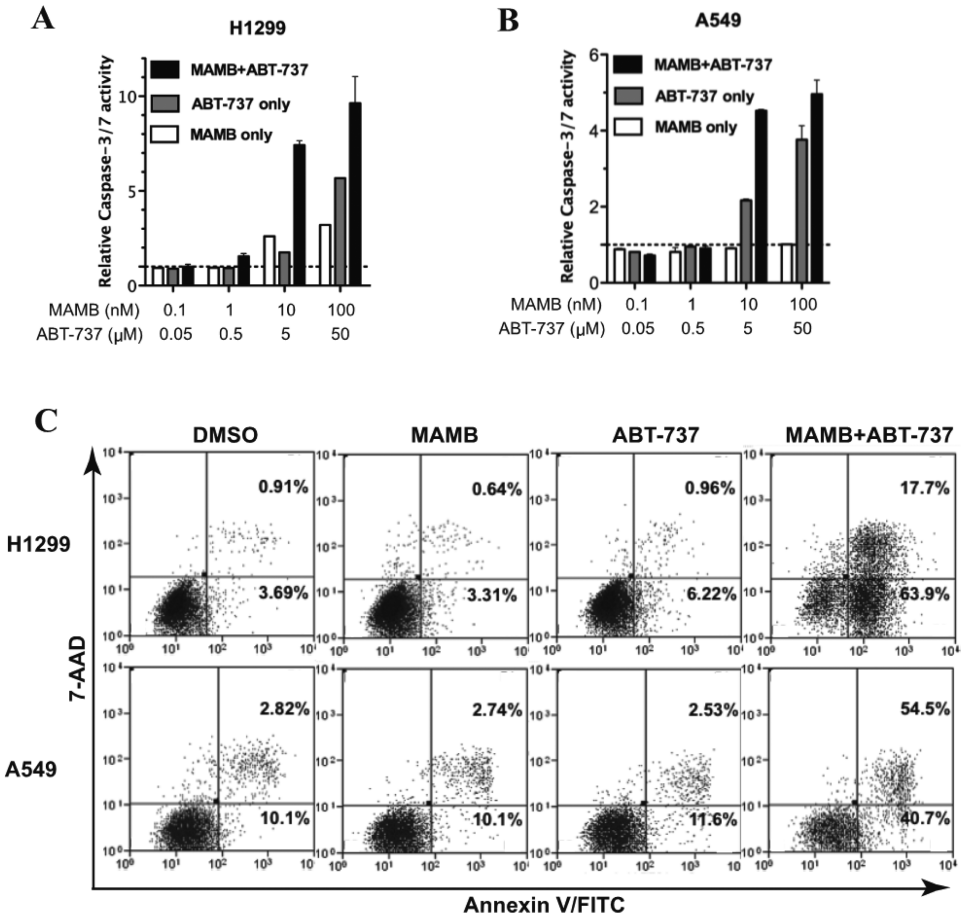

Figure 4. Meayamycin B (MAMB) and ABT-737 synergistically caused apoptosis. (A,B) Caspase 3/7 activity assays with H1299 and A549 cells. (C) Effects of meayamycin B and ABT-737 on induction of early apoptosis (Annexin V-FITC staining) and late apoptosis (7-AAD staining) in H1299 and A549 cells as measured by flow cytometry. A representative of three separate experiments is shown.

frame. These results warrant further investigation of the role of SF3b (possibly SF3B1) in these alternative splicing systems.

Mcl-1_L expression has been recognized as the culprit for ABT-737 resistance in various cancers, including lung cancer cell monolayer cultures and spheroids.[11,14,28] Therefore, we hypothesized that meayamycin B could overcome the ABT-737 resistance in cancer cells that rely on Mcl-1_L to survive. To test this hypothesis, A549 and H1299 cells were exposed to serial dilutions of meayamycin B and ABT-737 as single agents or in simultaneous combination at a constant ratio (meayamycin B/ABT-737 = 1:500) based on the Chou and Talalay method for 72 h.[29] Post-treatment cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt (MTS) method. ABT-737 did not affect cell viability as a single agent even at a 100 μM concentration that is the upper limit due to its solubility in a cell culture medium (Figure 3A,B). Meayamycin B inhibited cell growth as a single agent with GI_{50} values of 0.14 ± 0.008 and 0.15 ± 0.008 nM (n = 3) in A549 and H1299 cells, respectively. The combination of meayamycin B and ABT-737 induced cell death at doses (≥10 nM and ≥2.5 μM, respectively) that were not cytotoxic with either of the two compounds as single agents. When the treated cells were examined under a microscope, only the combination treatment caused apoptosis-like cell shrinkage (data not shown). Although full cell-killing curves from each compound as a single agent could not be generated due to the poor solubility of ABT-737, preventing us from calculating the combination index values,[29] the remarkable cytotoxic effect from the meayamycin B−ABT-737 combination indicated a strong synergism. Interestingly, under a microscope, H1299 cells displayed more prominent apoptotic morphology than A549 cells upon meayamycin B treatment. This might be related to the different p53 gene status: A549 expresses wild-type p53 protein, while H1299 is p53-deficient.[30] Further studies are warranted since generally the p53-null genotype in H1299 affords them stronger resistance to apoptotic stimuli.[31] Nonetheless, the sensitivity of H1299 cells indicated that the apoptosis triggered by the combination of meayamycin B and ABT-737 does not require the expression of wild-type p53.

After examining the potency of meayamycin B in H1299 and A549, we used immunoblotting to assess the basal expression of antiapoptotic Bcl-2 family proteins in these cell lines. It was found that H1299, expressing a higher level of Mcl-1_L, was also more responsive to single-agent meayamycin B. The basal Mcl-1_L level, as measured by the Mcl-1_L/β-actin ratio, was 1.32 in H1299 and 0.41 in A549 (Figure 3C). Meayamycin B reduced the cell viability to approximately 50% in H1299 cells (Figure 3A) and 75% in A549 cells (Figure 3B). The same pattern was also observed in head-and-neck squamous cell carcinoma (HNSCC) cell lines. As an example, the data from PCI-13 and 93-UV-147T showed that the Mcl-1_L/β-actin ratios were 1.24 and 0.07 (Figure 3D). Possibly due to these changes, meayamycin B reduced the cell viability to 0% in PCI-13

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(Figure 3E) but only to 40% in 93-UV-147T (Figure 3F). These data indicate the potential use of meayamycin B for cancer types that overexpress Mcl-1_L.

To determine whether the combination induced apoptosis, we measured the caspase 3/7 activity of the treated H1299 and A549 cells. The cells were exposed to the combination (0.1, 1, 10, and 100 nM meayamycin B; constant meayamycin B/ABT-737 ratio at 1:500) or each agent for 9 h. Although single-agent ABT-737 increased caspase 3/7 activity in a dose-dependent manner, the combination treatment induced a significantly higher level of caspase 3/7 activity in both H1299 cells (Figure 4A) and A549 cells (Figure 4B). Meayamycin B by itself was a weak apoptosis inducer for these two cell lines. The rapid activation of caspase 3/7 observed in the combination-treated cells indicated a strong synergism between meayamycin B and ABT-737 in inducing apoptosis.

To better understand the stages of apoptosis in the cells exposed to the combination of meayamycin B and ABT-737, we stained the cells with fluorescein isothiocyanate (FITC), annexin V, and 7-aminoactinomycin D (7-AAD) and monitored the fluorescence with flow cytometry. Annexin-V stains early apoptotic cells by detecting the externalization of phosphatidylserine,[32] and 7-AAD intercalates double-stranded DNA to detect dead or late-stage apoptotic cells.[33] The cells treated with both meayamycin B and ABT-737 displayed a significantly higher population of annexin $V^+/7\text{-}AAD^-$, indicating that these cells were in an early stage of apoptosis (Figure 4C, lower right quadrants) and annexin $V^+/7\text{-}AAD^+$ (late apoptotic/necrotic; Figure 4C upper right quadrants), indicating synergistic apoptosis stimulation.

In conclusion, meayamycin B switches the alternative splicing of Mcl-1 in a dose- and time-dependent manner in nonsmall cell lung cancer A549 and H1299 cell lines. This is, to the best of our knowledge, the first report of the modulation of Mcl-1 alternative splicing by a single small molecule through spliceosome inhibition. Although both A549 and A1299 cells were resistant to cell death in the presence of either meayamycin B or ABT-737, treatment of these cells with both meayamycin B and ABT-737 induced cell death, presumably through the meayamycin B-mediated modulation of Mcl-1 pre-mRNA splicing. These results support the feasibility of using the combinations of Mcl-1_L and Bcl-x_L inhibitors for both research and therapeutic purposes.

METHODS

Cell Lines. A549 and H1299 cells were obtained from the American Type Culture Collection (ATCC). PCI-13 and 93-UV-147T cell lines are kind gifts from Dr. Robert Ferris (University of Pittsburgh). The A549 and H1299 cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium, PCI-13 were grown in Dulbecco's Modified Eagle's Medium, and 93-VU-147T cell line was grown in DMEM/F12 medium. All media were supplemented with 10% (v/v) fetal bovine serum, 4.5 g L^{-1} D-glucose, 2.0 mM L-glutamine, 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. The cells were cultured at 37 °C in a humidified incubator with 5% CO_2.

A complete methods section can be found in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Complete methods section. Table S1: Primer sequences for RT-PCR. Figure S1: Meayamycin B inhibits the constitutive splicing of Mcl-1 in H1299 and A549 cells. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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