Evasion of apoptosis is a known feature of cancer cells. One mechanism of deregulating the apoptotic pathway is through overexpression of antiapoptotic BCL2 family members. ABT-263 (navitoclax) is a first-in-class BCL2 family inhibitor that restores the ability of cancer cells to undergo apoptosis. However, many cancer cells are resistant to ABT-263 due to high levels of a BCL2 family member, MCL1, which is not targeted by the drug. MCL1 expression is regulated transcriptionally, translationally, and through proteasome-mediated degradation. Recently, MCL1 expression was shown to be affected by microRNAs (miRNA). To identify miRNAs that modulate the sensitivity of cancer cells to ABT-263, we screened a library of 810 human miRNA mimics in HCT-116 cells in the presence of ABT-263. The screen revealed 19 miRNAs that sensitize HCT-116 cells to ABT-263. Fifteen of these miRNAs were also shown to sensitize CHL1 melanoma cells to the same agent. We further evaluated 12 of the strongest sensitizers in these cell lines. We found that these sensitizers induced apoptosis only in the presence of ABT-263. In addition, whereas all 12 of these miRNAs reduced MCL1 protein expression, only 10 of them targeted MCL1 through direct binding to the 3′-untranslated region of the gene, raising the possibility that other resistance regulators of MCL1 expression may be identified using our method. Finally, because sensitizing miRNA expression is lower in tumors compared with normal tissues, our data can facilitate the design of miRNA replacement therapies to increase sensitivity to BCL2 antagonists.

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(e) miRNA expression versus target gene expression data (19), and (f) secondary structure of the target that is conducive to miRNA binding (20). Unfortunately, the current target prediction tools are unreliable, resulting in a large number of false positives and false negatives (13). Recently, proteomics has also been used to facilitate the identification of miRNA targets (21, 22).

In this study, we describe a synthetic lethal screen for miRNAs that sensitizes cancer cells to ABT-263. A library of 810 synthetic miRNAs was screened in the presence of ABT-263 in a colorectal cancer cell line, HCT-116. The functional screen yielded 19 miRNAs that kill HCT-116 cells in combination with ABT-263. Most of these miRNAs were shown to downregulate MCL1 expression by directly targeting the 3′-UTR of the MCL1 gene. Our results suggest that miRNAs play a role in the regulation of MCL1 expression in the cell. The data generated can be used to design miRNA replacement therapies to increase sensitivity of tumors to BCL2 family inhibitors.

Materials and Methods

Reagents

ABT-263 was synthesized at Abbott Laboratories. All small interfering RNAs (siRNA) and miRNAs were purchased from Dharmacon.

Cell culture, transfection, and cell-based assays

HCT-116 and CHL1 cells were purchased from the American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen Corp.). All cell lines were maintained in a humidified chamber at 37°C containing 5% CO2. These cells were authenticated by morphologic, cell proliferation, and Mycoplasma tests recommended in the ATCC Technical Bulletin No. 8 (2007). siRNAs or miRNAs were introduced into the cells by reverse transfection using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). Briefly, siRNAs or miRNAs were first mixed with Lipofectamine 2000 in Opti-MEM (Invitrogen). Cells were added at 1.5 × 10⁴ to 2.5 × 10⁴/100 μL in 96-well tissue culture plates after 15 minutes. For MCL1 and BCL-xl siRNA transfections, a final concentration of 12.5 nmol/L siRNA was used. For miRNA transfections, a final concentration of 50 nmol/L miRNA was used. The cells were then grown in medium without antibiotic for 2 days before harvesting. For reporter experiments, 100 ng of pcDNA3–MCL1–3′-UTR–luciferase reporter construct (23) were included in the liposome complex mix. Forty-eight hours after transfection, cells were assayed for viability using CellTiter-Glo luminescent cell viability assay according to the manufacturer’s protocol (Promega). Caspase-3/7 activity was measured using the Caspase-Glo 3/7 assay (Promega). Luciferase activity was measured using the Steady-Glo reagent according to the manufacturer’s protocol (Promega).

miRNA mimic library screen and data analysis

HCT-116 cells were reverse transfected as described above. Three replica plates were treated with 1 μmol/L ABT-263 in media. After 48 hours, the cell viability was assessed using the CellTiter-Glo assay. To determine the dynamic range of our screen, we calculated the Z score. In brief, \[ Z = \frac{x - \mu}{\sigma}, \] where \( x \) is the raw data point to be standardized, \( \sigma \) is the SD of the miRNA population, and \( \mu \) is the mean of the miRNA population.

Protein assays

MCL1 protein expression was measured using an MCL1 assay based on the Luminex technology. In brief, an MCL1 capture antibody (Santa Cruz Biotechnology) was conjugated to Luminex beads (bead region 9) and an MCL1 detection antibody (Santa Cruz Biotechnology) was conjugated to biotin through custom service provided by Millipore. Cells were lysed in MILLIPLEX MAP lysis buffer 1 (Millipore) and diluted with equal volume of MILLIPLEX MAP cell assay buffer 1 (Millipore). MCL1 capture antibody beads were diluted in 25 μL of MILLIPLEX MAP cell assay buffer 1 and added to a Beadlyte filter plate (Millipore). Then, 25 μL of the diluted cell lysate were transferred to each well of the filter plate and incubated overnight at 4°C with shaking. After the overnight incubation, beads were washed twice with cell assay buffer 1, and a biotinylated MCL1 detection antibody diluted in 25 μL of cell assay buffer 1 was added and incubated for 1 hour at room temperature with shaking. After filtering, 25 μL of MILLIPLEX MAP streptavidin-phycocerythrin (Millipore) were added and incubated for 30 minutes at room temperature with shaking. Finally,
cell assay buffer 1 was added after filtering, and the signal was read using a Luminex 200 station.

Expression of miRNAs in tumors and normal tissues
miRNA expression data were downloaded from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) with the series accession number GSE2564 (9). Data were median centered, and the expression of available miRNAs in tumors versus normal tissues was plotted.

Results

Testing of predicted modulators of MCL1
The goal of our study was to identify miRNAs that modulate sensitivity of cells to the BCL2 family inhibitor ABT-263. To accomplish this, we did an miRNA “sensitization” screen using an miRNA mimic library in the presence of ABT-263. The screen was conducted in the colorectal cancer cell line HCT-116 that is intrinsically resistant to ABT-263 due to expression of MCL1. We optimized the screen so that (a) transfection of HCT-116 with the control siRNAs and miRNAs would not be toxic, (b) ABT-263 alone would not affect the cell viability by >25% in the presence of the control siRNA and miRNA, and (c) transfection of HCT-116 cells with MCL1 siRNA would not affect cell viability but significantly sensitize cells to ABT-263 (Supplementary Fig. S1A). In addition, we determined the ability of miRNAs predicted in silico to target MCL1 (miR-29, miR-125, miR-153, miR-193a, and miR-320; ref. 5) to modulate the sensitivity of HCT-116 cells to ABT-263. Synthetic miRNA mimics were introduced into HCT-116 cells by reverse transfection in the presence or absence of ABT-263 (Supplementary Fig. S1A). Transfection of miR-153 and miR-193a sensitized the cells to the BCL2 inhibitor by ~50%, but the effect of miR-29 and miR-320 was much weaker. It is noteworthy that miR-125 did not sensitize HCT-116 cells to ABT-263. These results suggest that miRNA expression may modulate sensitivity of cancer cells to ABT-263. They also pointed at inadequacy of the in silico prediction of miRNA targets.

miRNA sensitization screen
A library of 810 annotated miRNA mimics was introduced into HCT-116 cells in triplicate in the presence of ABT-263 (Fig. 1B). Viability of cells was determined after 2 days. Comparison of the three replicates suggests high reproducibility of the screen (Supplementary Fig. S1B). Both siRNAs and miRNAs targeting MCL1 significantly sensitized the cells to the BCL2 inhibitor (Supplementary Fig. S1C), suggesting high sensitivity of the screen. We used Z scores to select candidate miRNAs for subsequent experiments. Using a Z score cutoff of less than −1.5, we identified 80 miRNAs that either have effects on the cell survival by themselves or sensitize cells to the BCL2 inhibitor. After subtracting miRNAs previously identified to be toxic by themselves (Z < −1.5) and mimic repeats from our hits, we generated a list of 42 miRNAs that modulate the sensitivity to BCL2 inhibitor (with a Z score difference of at least 0.8 between treatment and no treatment). These miRNAs were further evaluated in a secondary screen to assess their effect in HCT-116 cells in the presence or absence of ABT-263. miR-29 and miR-125 were used as control reagents for this screen. Using a cutoff of 25% sensitization to ABT-263 treatment, 19 miRNAs were confirmed to sensitize HCT-116 cells to ABT-263 without significantly reducing the viability of the cells in the absence of the drug (Table 1; Supplementary Fig. S2A).

Profiles of miRNA expression vary among cancer cell lines and normal tissues, possibly resulting in differences in the effects of the same mimic on different lines. We tested our panel of 42 miRNAs in CHL1 melanoma cells. Seventeen out of 42 miRNAs sensitized CHL1 cells to ABT-263 by >25% (Table 1; Supplementary Fig. S2B). It should be noted that silencing MCL1 in the absence of ABT-263 affected CHL1 cell viability because CHL1 cells partially depend on MCL1 for survival as shown by MCL1 siRNA (Supplementary Fig. S2B). Fifteen of these miRNAs overlapped with the hits from HCT-116 cells (Table 1; Supplementary Fig. S2D), confirming the sensitization effect of these miRNAs.

Recently, we developed a liquid-phase quantitative protein assay based on the Luminex technology that measures the levels of MCL1 in cells. Western blotting was used to validate the assay with MCL1 siRNA and five predicted MCL1-targeting miRNAs (Supplementary Fig. S3). Using this MCL1 quantitative assay, we measured the effect of these miRNAs on the MCL1 protein levels in CHL1 cells. All the sensitizing miRNAs potently silenced MCL1

| Table 1. miRNAs that sensitize HCT-116 and/or CHL1 cells to ABT-263 |
|---------------------------------|--------|--------|
|        | HCT-116 | CHL1   |
| miR-153 | +       | +       |
| miR-892b | +       | +       |
| miR-744* | +       | +       |
| miR-193a | +       | +       |
| miR-582 | +       | +       |
| miR-148b* | +       | +       |
| miR-876-3p | +       | +       |
| miR-29b-2* | +       | +       |
| miR-886-3p | +       | +       |
| miR-29b-1 | +       | +       |
| miR-518a-5p | +       | +       |
| miR-218-1* | +       | +       |
| miR-605 | +       | +       |
| miR-380-5p | +       | +       |
| miR-105 | +       | +       |
| miR-661 | +       | +       |
| miR-101 | +       | +       |
| miR-1233 | +       | +       |
| miR-935 | +       | +       |
The levels of sensitization to ABT-263 correlate with the levels of MCL1 protein knockdown (Supplementary Fig. S2E). Taken together, our results suggest that the sensitizing miRNAs identified in our functional screen represent important modulators of MCL1 expression that determine the sensitivity of different cancer cells to BCL2 family inhibitors.

To determine the potency of these miRNAs, two published and two novel miRNAs (miR-101, miR-153, miR-744*, or miR-886) were transfected into HCT-116 cells, exposed to a range of doses of ABT-263, and assessed for viability after 2 days (Fig. 2A). In the control assay, the MCL1 siRNA was used. MCL1 siRNA and the four miRNAs significantly shifted the dose-response curve compared with control siRNA and the control miRNA. Their EC_{50} was lower by a factor of 4 to 16 than that for the controls. Our data suggest that miR-101, miR-153, miR-744*, and miR-886 are strong sensitizers, similar to MCL1 siRNA. To confirm and extend these results, HCT-116 cells were transfected with the 12 strongest sensitizing miRNAs (in both HCT-116 and CHL1 cells) and assessed for viability. These miRNAs all sensitized HCT-116 cells to ABT-263 (Fig. 2B).

**Sensitizing miRNAs induce caspase-3/7 activity in the presence of ABT-263**

To determine if the sensitizing miRNAs induce apoptosis, HCT-116 cells were transfected with the 12 strongest sensitizing miRNAs and caspase-3/7 activity was measured in the presence or absence of ABT-263. miR-29 and miR-125 were used as positive and negative controls, respectively. As shown in Fig. 3, all the sensitizing miRNAs significantly increased caspase-3/7 activity in the presence of ABT-263. Our results indicate that the sensitizing miRNAs restore the ability of ABT-263–resistant cells to undergo apoptosis.
MCL1 is the direct target of most of the BCL2 inhibitor–sensitizing miRNAs

miRNAs could modulate sensitivity to ABT-263 through several mechanisms: by directly targeting the MCL1 gene 3′-UTR and repressing protein translation or by targeting other genes involved in regulating MCL1 expression or function. In addition, some miRNAs may modulate ABT-263 sensitivity in an MCL1-independent fashion. To determine the exact mechanism, we measured the effect of two MCL1 siRNAs and the 12 strongest sensitizing miRNAs on the MCL1 protein levels in HCT-116 cells using an MCL1 quantitative assay. As shown in Fig. 4, two siRNAs targeting either the open reading frame (ORF) or 3′-UTR of MCL1 strongly knocked down the expression of MCL1 in HCT-116 cells. We then showed that all the sensitizing miRNAs decreased the expression of MCL1 (Fig. 4), suggesting that the sensitization of cells to ABT-263 was due to the modulation of MCL1 expression.

To determine whether the effect of these strongest sensitizing miRNAs on the MCL1 gene was direct, two approaches were used: (a) in silico prediction of binding to the 3′-UTR of MCL1 and (b) an MCL1 3′-UTR reporter assay. The binding of miRNA to its target is determined by multiple factors, including the seed sequence and favorable thermodynamic hybridization.
Therefore, we identified the seed sequence of miRNA hits binding to the 3′-UTR of MCL1 using two prediction algorithms, TargetScan (18) and miRANDA (24, 25), or by seed sequence analysis. TargetScan and miRANDA predicted that three miRNAs (miR-661, miR-153, and miR-193a) and seven miRNAs (miR-892b, miR-876-3p, miR-101, miR-518a-5p, miR-582-5p, miR-153, and miR-193a) can bind to the 3′-UTR of MCL1, respectively (Supplementary Fig. S4). Only two miRNAs (miR-153 and miR-193a) are predicted by both algorithms. Interestingly, we found binding site of the remaining four miRNAs (miR-886-3p, miR-124*, miR-744*, and miR-605) using seed sequence analysis.

To test if these miRNAs bind to the MCL1 3′-UTR, we used a reporter assay in which ~1 kb of the MCL1 3′-UTR immediately adjacent to the ORF was subcloned downstream of a luciferase reporter into the pcDNA3 vector. Potential binding sites of the test mimics within this 1 kb of the MCL1 3′-UTR are shown in Supplementary Fig. S4. The specificity of the reporter assay was first evaluated by the cotransfection of the MCL1 siRNAs targeting either the ORF or 3′-UTR regions and the reporter construct in HCT-116 cells. Although both siRNAs substantially reduced the protein levels of MCL1, only the siRNA targeting the 3′-UTR strongly reduced the luciferase activity (Fig. 4). Next, the 12 strongest sensitizing miRNA mimics and the MCL1 3′-UTR–luciferase reporter construct were cotransfected into HCT-116 cells. After 2 days, the luciferase activity was measured. All the mimics that potently knocked down MCL1 expression, except miR-605 and miR-744*, substantially reduced the luciferase activity (Fig. 4). Both miR-605 and miR-744* affected the MCL1 protein expression but did not reduce the luciferase activity, suggesting that these miRNAs regulate the expression of a modulator of MCL1 protein or pathway.

Lower expression of sensitizing miRNAs in tumors compared with normal tissues

To evaluate the potential role of the sensitizing miRNAs in cancer, we compared their expression in tumors versus normal tissue using existing miRNA expression database (9). The expression of only four miRNAs (miR-29b, miR-101, miR-153, and miR-193) was available. As shown in Fig. 5, the expression of these miRNAs is much lower in six tumor types than in the normal tissue. Interestingly, almost all solid tumor cell lines are resistant to ABT-737/ABT-263 due to high MCL1 expression (26–28). It is possible that suppressing the expression of these miRNAs may lead to high expression of MCL1 in tumors and thus cause resistance to ABT-263.

Discussion

ABT-263 is an antiapoptotic BCL2 family protein inhibitor that possesses high affinity for BCL-xl, BCL2, and BCL-w, but not for MCL1 or A1 (2). ABT-263 is orally bioavailable and is being evaluated as a single agent for the treatment of relapsed or refractory chronic lymphocytic leukemia, lymphoid malignancies, and advanced small cell lung cancer in a series of phase I/IIa clinical trials. The known mechanism of resistance to ABT-263 is MCL1. In this study, we conducted an miRNA sensitization screen to identify miRNA modulators of sensitivity to ABT-263. Interestingly, all the miRNAs identified in the screen (miR-101, miR-148b*, miR-153, miR-193a, miR-518, miR-582, miR-605, miR-661, miR-744*, miR-876-3p, miR-886-3p, and miR-892b) reduced the expression of MCL1. In addition, a reporter assay revealed that 10 of these 12 miRNAs (except miR-605 and miR-744*) directly regulate transcription of the MCL1 gene.

1 Unpublished data.
Recently, miR-29, miR-101, miR-133b, and miR-153 have been shown to target MCL1 (5–7, 29). In addition, two algorithms most commonly used for identifying miRNA-binding sites, TargetScan (18) and miRanda (24, 25), pointed at 181 and 176 miRNAs that may target MCL1. We found that majority of these predicted miRNAs do not sensitize cells to ABT-263 (data not shown), whereas most of the miRNAs we identified were not predicted by these tools. There could be a few explanations for the discrepancies. First, these in silico tools identify miRNA-miRNA pairs by seed sequence analysis and free energy, but the actual affinities for these pairs are also determined by various factors such as target site accessibility for miRNAs (30) and secondary structures of these binding pairs (20). Thus, many of these miRNAs may either be weak binders or represent false positives. Second, some of the miRNA-binding sites are not conserved among different species and may be excluded from the prediction algorithm. Nevertheless, these miRNAs may play important physiologic roles (12). For example, miR-186-3p is not conserved (data not shown) but could directly target the MCL1 3′-UTR of human (Supplementary Fig. S4).

Third, the expression of some of these miRNAs differs in cell lines, and this may affect the potency of transfected mimics in our cells. In particular, the differences in expression levels of these miRNAs could potentially explain why not all the hits were identical between HCT-116 and CHL1 cells. For instance, miR-133b sensitizes HCT-116 cells to ABT-263 by 24% but only 13% in CHL1 cells and was not evaluated further in our studies. Nevertheless, a recent study verified that miR-133b modulates MCL1 protein expression weakly in lung cancer cell lines (7). Finally, some of the miRNAs identified in our screen, such as miR-148b* and miR-744*, are not in the prediction tools. To summarize, our screen can be regarded as a focused experiment to identify miRNAs that target MCL1.

Although MCL1 is regulated transcriptionally, translationally, and by proteasome-mediated degradation (4), the exact mechanism in different cell types is uncertain. One can envision that our functional screen could facilitate the identification of pathways that regulate MCL1. For example, miR-744* is a strong sensitizer to ABT-263 and it potently knocks down the expression of MCL1 ( Supplementary data). We found that miR-744* does not target MCL1 directly. We suggest that miR-744* may regulate the expression of MCL1 indirectly.

New mechanisms of resistance to ABT-263 independent of MCL1 could be identified using this method. In addition, because miRNAs can target hundreds of transcripts, it is possible that MCL1 is not the sole target that leads to sensitization to ABT-263. Although it will be hard to identify these targets, it is advantageous to identify such miRNAs for designing miRNA replacement therapy for cancer.

Finally, cancer cells are known to have abnormal expression of miRNA. With some exceptions, cancer cells generally suppress the global expression of miRNA (9, 31). We found that at least four ABT-263-sensitizing miRNAs are significantly underexpressed in tumors versus normal tissues (Fig. 5). As almost all solid tumor cell lines are resistant to ABT-737/ABT-263 due to high MCL1 expression (26–28), low expression of these miRNAs may play a role in regulating MCL1 in tumors and response to ABT-263. It should be noted that the effect on MCL1 and resistance to ABT-263 may be due to suppression of multiple miRNAs. Recent studies suggested the potential of miRNA as therapeutic agents against cancer (32, 33). In a recent study, a lentiviral vector was used to deliver miR-26a into hepatocellular carcinoma to show that miRNA replacement therapy is feasible in cancer therapy (32). Because most miRNAs are lost in cancer cells, systemic expression of miRNAs that are expressed at high levels in normal cells would target cancer, but not normal cells. As miRNAs are naturally evolved and they target multiple transcripts, off-target effects would be minimized, thus decreasing the probability of acquired resistance. Although delivery is the major hurdle to use miRNA as therapy for cancer, our study is a first step toward identifying miRNAs that could modulate sensitivity to ABT-263 for cancer treatment.

Disclosure of Potential Conflicts of Interest

All authors are employees of Abbott.

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References


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