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Development and validation of an RNAi screen for ABT-737 sensitizers

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Abstract: The chemical compound ABT-737 is a nanomolar inhibitor of several antiapoptotic Bcl-2 family members with potential therapeutic efficacy for a variety of cancers. Herein, we describe the development of a complementation-based RNAi assay that can be used to identify mechanisms of ABT-737-resistance. HeLa cells, which were resistant to ABT-737, were optimized for reverse-transfection efficiency and tested for siRNA-mediated silencing. The developed assay utilized HeLa cell reverse-transfection with 10 nM siRNA, followed by 48 h incubation, ABT-737 or DMSO treatment for 24 h, and cell viability measurement using ATPlite (which measures ATP levels as an indicator of cell viability). As a validation, the kinase subset of the Ambion Silencer Human Druggable Genome siRNA Library V2, which consisted of 865 genes (three siRNA sequences per gene), was screened. Several assay-positive siRNAs were tested and confirmed to sensitize cells to ABT-737. Transfection of cells with siRNAs targeting Bcl-2 family member Mcl-1 also potently sensitized HeLa cells to ABT-737. The current assay thus represents a screen that can be utilized to identify ABT-737-sensitizing siRNAs and correspondingly, potential new targets for drug discovery.

Keywords: ABT-737, Bcl-2, apoptosis, RNAi screen, siRNA

Introduction

Cell death includes such processes as apoptosis, autophagy, and necrosis, with apoptosis being common under nonpathological circumstances.[1–4] The pathological inhibition of apoptosis is a causative and/or contributing factor in autoimmune disease, and is considered a hallmark of cancer.[1,5,6] Particularly common in cancer is the dysregulation of the Bcl-2-family of proteins, which function to either inhibit (eg, Bcl-2 (BCL2), Bcl-X_L (BCL2L1), Mcl-1 (MCL1), Bcl-B (BCL2L10), Bfl-1 (BCL2A1), and Bcl-W (BCL2L2) in humans) or promote (eg, Bax (BAX), Bak (BAK1), Bim (BCL2L11), and Puma (BBC3)) apoptosis at mitochondria.[7–9]

Bcl-2 was initially cloned in the 1980s from follicular lymphomas, wherein its involvement in a reciprocal translocation results in its overexpression and the inhibition of apoptosis.[10–13] Changes in gene structure, gene copy number, microRNAs, epigenetics, and post-translational modifications have all since been shown to affect the Bcl-2 family proteins in cancer.[7,14–17] The over-expression of antiapoptotic Bcl-2-family proteins and under-expression of proapoptotic Bcl-2 family proteins not only contributes to cancer pathogenesis, but also allows malignant cells to resist chemotherapeutic interventions.[4,18] Accordingly, much attention has focused on inhibiting antiapoptotic Bcl-2 family proteins or mimicking proapoptotic Bcl-2 family members.[19–24]

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Synthetic compounds such as ABT-737 (and ABT-263), GX15-070, and HA-14-1, as well as natural products such as (-)-Gossypol and (-)-epigallocatechin gallate (EGCG), bind to a hydrophobic cleft on the surface of antiapoptotic Bcl-2-family proteins, inhibiting their cytoprotective activity.[4,18,25–28] Perhaps the most advanced antiapoptotic Bcl-2 family-targeted compound is ABT-737, a nanomolar inhibitor of Bcl-xL, Bcl-2, and Bcl-w that has progressed to clinical trials in the form of ABT-263.[26,29] ABT-737 shows efficacy in various cancer cell lines, primary patient-derived cells, and animal models.[26,30–33] However, several ABT-737-resistant tumor cell lines have been identified, such as A549, NCI-H82, NCI-H196, and OCI-AML3, most likely related to the compound's inability to effectively inhibit the antiapoptotic Bcl-2 family proteins Bcl-B, Mcl-1, or Bfl-1 (A1).[26,30,34]

To identify other genes whose products contribute to ABT-737 resistance, we developed an RNA interference (RNAi) assay using an ABT-737-resistent human tumor cell line. A test screen was performed using the kinase subset (865 genes, three different siRNA sequences per gene) of the Ambion Silencer Human Druggable Genome siRNA Library V2, and several siRNAs were confirmed to sensitize cells to ABT-737. In addition, siRNAs targeting Mcl-1 were highly effective at conferring sensitivity to ABT-737 in this assay, confirming prior results showing that expression of Bcl-2 family members that ABT-737 failed to bind are commonly responsible for resistance to this candidate anticancer drug.[30,34]

Methods and materials

Cell culture

HeLa cells were obtained from ATCC (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA), with 10% FBS (Clontech, Mountain View, CA) and penicillin-streptomycin (diluted according to manufacturer's specifications; Invitrogen) at 37°C, 5% CO_2.

Reverse-transfections were performed using Lipofectamine RNAiMAX (RNAiMAX; Invitrogen) with several modifications to the manufacturer's specifications. Briefly, siRNAs (1 µL/well, 10 nM final) were spotted into white, tissue culture-treated, 384-well plates (Greiner Bio-One, Monroe, NC), followed by 10 µL/well of an Opti-MEM (Invitrogen)-RNAiMAX mixture (100:1 ratio of Opti-MEM:RNAiMAX). The plates were then centrifuged at 1000 rpm for 1 min and incubated at room temperature for 15 min. Cells (40 µL/well, 4000 cells/well) were then dispensed using the ThermoScientific Matrix WellMate bulk dispenser (Thermo Fisher Scientific, Hudson, NH), and the plates incubated at 37°C.

Cell viability relative to untreated cells was measured using the ATPlite Luminescence ATP Detection System (20 µL/well of a 384-well plate; PerkinElmer, Waltham, MA) and an EnVision Multilabel Plate Reader (PerkinElmer) according to the manufacturer's specifications.

Chemicals

ABT-737 was synthesized at the MD Anderson Cancer Center as described previously.[26] ABT-737 was diluted in DMSO and then further diluted in water (0.1% DMSO final). Cisplatin was purchased from Sigma-Aldrich (St Louis, MO).

Immunofluorescence

Cells were reverse-transfected in 384-well polystyrene clear bottom plates (Greiner). After 72 h, the cells were washed with phosphate-buffered saline (PBS), fixed in a 3.7% paraformaldehyde (Sigma-Aldrich)/PBS solution for 15 min, washed with PBS, incubated in a 0.1% Tween-20/PBS solution for 10 min, incubated in a 0.1% Tween-20 (Sigma-Aldrich)/3% skim milk/PBS solution for 2 h, washed with PBS, incubated with mouse anti-GAPDH primary antibody (0.67 µg/mL; Invitrogen) in 0.1% Tween-20/PBS solution at 4°C overnight, washed with PBS, incubated with Rhodamine-labeled anti-mouse secondary antibody (2.67 µg/mL; Invitrogen) in a 0.1% Tween-20/PBS solution at 4°C for 4 h, washed with PBS, and incubated with DAPI (100 ng/mL) for 30 min. Imaging and analyses were performed using the Cell Lab IC 100 (Beckman Coulter, Fullerton, CA) and CytoShop software (Beckman Coulter).

High-throughput screening

HeLa cells (4000 cells/well) were reverse-transfected (using RNAiMAX) with no siRNA (negative control), scrambled control siRNA (siSC; negative control), Mcl-1 siRNA (siMcl-1; positive control), or siRNAs from the kinase subset (865 genes, 3 siRNAs/gene) of the Ambion Silencer Human Druggable Genome siRNA Library V2 (Applied Biosystems, Foster City, CA) (10 nM), and incubated for 48 h. The cells were then treated with 0.1% DMSO or 1 µM ABT-737 (dissolved in 0.1% DMSO) for 24 h, after which cell viability was measured using ATPlite. The ThermoScientific Matrix WellMate bulk dispenser and BioMek FX Laboratory Automation Workstation (Beckman Coulter) were used for liquid dispensing and the EnVision Multilabel Plate Reader was

used for plate reading. The screen was performed twice, with mean results reported. siRNAs were purchased from Ambion, with Ambion identification numbers shown where relevant.

Q-PCR

Reverse-transfections were scaled to 12-well plates. At 48 h after reverse-transfection, RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA synthesis was performed using SuperScript III RT (Invitrogen), both according to manufacturer's specifications. SYBR Green quantitative real-time polymerase chain reaction (Q-PCR) (Applied Biosystems) was performed with a Mx3000P (Stratagene, Cedar Creek, TX). The following primers were used:

PPID: GACCCAACACAAATGGTTC, AGTCAG CAATGGTGATCTTC

Mcl-1: AACGGGACTGGCTAGTTAAACA, CCAGCTCCTACTCCAGCAAC

HAK: ATGCCAAGATCTACGCTGCT, TCTCCAATCAGCTCCTCCTC

WNK2: CCCTTGGTGGAGAAGTCAGA, GACCACATGGGAGTCTGAGG

All samples were analyzed in triplicate, with mean fold change in expression was calculated using the $2^{-\Delta\Delta Ct}$ method, where Ct values are normalized to PPID Ct values and compared to untransfected controls.[35]

Statistical analyses

Statistical analyses was performed using GraphPad Prism (La Jolla, CA) as indicated.

Results

Development of an ABT-737-RNAi complementation screen

Our first objective was to develop a screen for detecting siRNAs that sensitize cells to ABT-737. HeLa cells were characterized for this screen due to their robust nature and ease of manipulation for high throughput screening (HTS). Previous studies have shown that ABT-737 decreases cell viability in a variety of different cell types at concentrations well below 1 μM.[26,30] However, HeLa cells were found to be resistant to ABT-737 (Figure 1A).

Reverse-transfection efficiency in 384-well plates was then assessed. Several reverse-transfection conditions were tested (data not shown), with the most effective shown in Figure 1. Cells were reverse-transfected with scrambled control siRNAs (siSC) or GAPDH siRNAs (siGAPDH) in 384-well plates, and after a 72 h incubation, GAPDH protein was immunofluorescently stained (Rhodamine) and imaged using high content screening (HCS) equipment. As shown in Figure 1B, siGAPDH efficiently decreased GAPDH protein expression. GAPDH expression, as determined by Rhodamine fluorescence intensity per cell, was also quantified to further evaluate siRNA-mediated silencing. Mock reverse-transfected HeLa cells and siSC reverse-transfected HeLa cells, whether treated with DMSO (0.1%) or ABT-737 (1 μM, 0.1% DMSO), did not display a decrease in GAPDH protein expression (Figure 1C). In contrast, siGAPDH significantly decreased GAPDH protein expression ($P < 0.05$, ANOVA, n = 3). This decrease was similar whether cells were treated with DMSO (0.1%) or ABT-737 (1 μM, 0.1% DMSO), suggesting that RNAi activity was not affected by ABT-737 and that DMSO would provide for an adequate negative control during HTS.

Finally, to validate positive and negative controls, and screening reproducibility, cells were reverse-transfected with either siSC or Mcl-1 siRNAs (siMcl-1), incubated for 48 h, treated with DMSO (0.1%) or ABT-737 (1 μM, 0.1% DMSO) for 24 h, and assayed for cell viability using ATPlite (which detects ATP levels as an indicator of cell viability) (Figure 1D). As expected, siSC reverse-transfection did not significantly change cell viability. siMcl-1 drastically decreased cell viability in the presence of ABT-737 ($P < 0.05$, ANOVA, n = 12), thus validating it as a positive siRNA control. The Z'-factor between ABT-737-treated siSC and ABT-737-treated siMcl-1 reverse-transfected cells was greater than 0.6, which is considered "excellent".[36]

In summary, the current assay detected siRNAs that sensitize HeLa cells to ABT-737 using DMSO, siSC, and siMcl-1 as controls, and has been validated for siRNA transfection efficiency, siRNA silencing efficiency, and reproducibility (Figure 1E).

ABT-737-RNAi complementation screening

The aforementioned assay was used to screen the kinase subset (865 genes, three siRNAs/gene) of the Ambion Silencer Human Druggable Genome siRNA Library V2 twice (to increase accuracy), with mean results reported. As shown in Figure 2, siMcl-1 (positive control) decreased cell viability in the presence of ABT-737 as expected (data points falling below the $y = x$ diagonal line). The 10 genes with the lowest average ABT-737:DMSO cell viability ratio were chosen for further analyses. Twelve additional genes

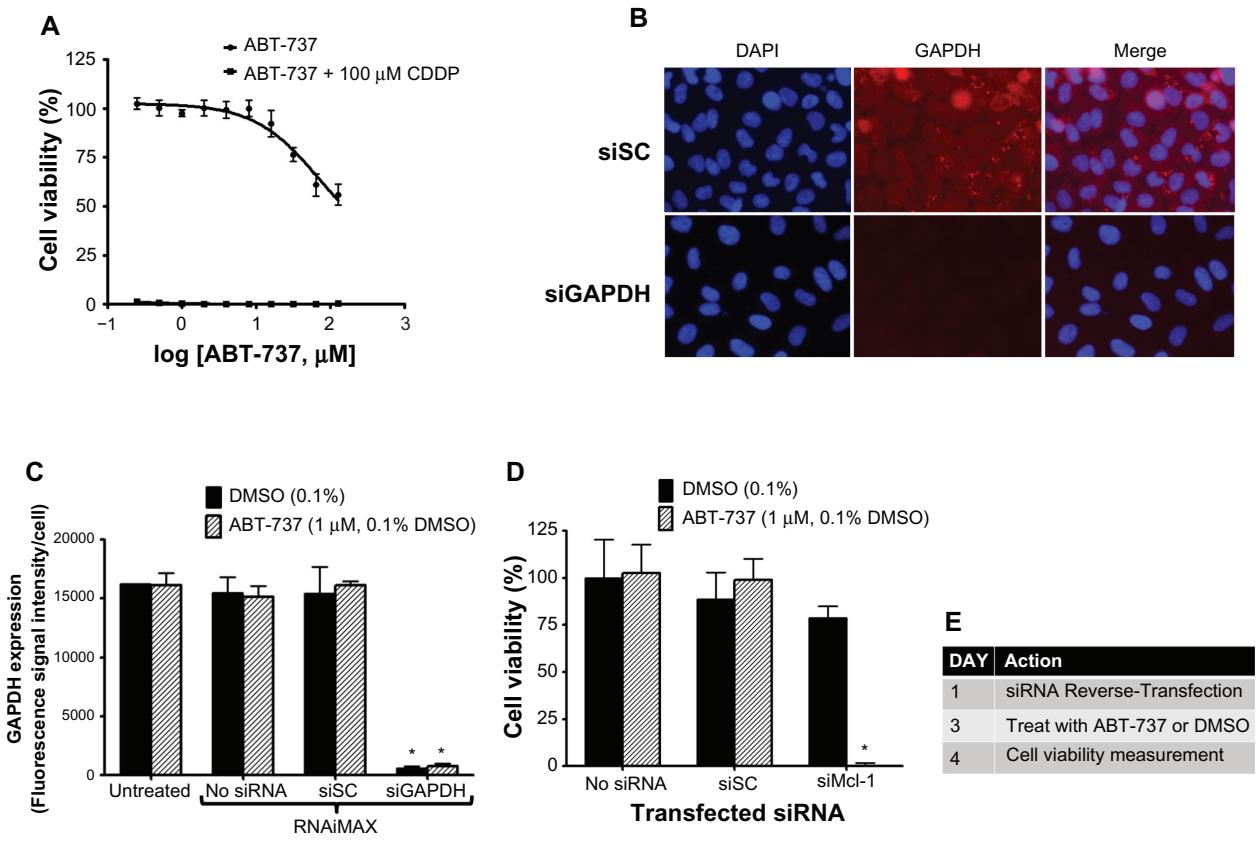

Figure 1 High-throughput ABT-737-RNAi complementation screen development. **A**) HeLa cells are resistant to ABT-737. HeLa cells were seeded in 384-well plates (4000 cells/well), incubated overnight, and treated with ABT-737 or ABT-737 + cisplatin (CDDP; 100 μM; cell death positive control) for 24 h. Cell viability was measured using ATPlite. Mean ± standard deviation (n = 4) are shown. **B**) Efficient siRNA knockdown of GAPDH expression. HeLa cells (4000 cells/well) were reverse-transfected (using RNAiMAX) with scrambled control siRNA (siSC) or GAPDH siRNA (siGAPDH) in 384-well plates. After 72 h, the cells were fixed, immunofluorescently stained (mouse anti-GAPDH primary antibody (0.67 μg/mL), Rhodamine-labeled anti-mouse secondary antibody (2.67 μg/mL), DAPI (100 ng/mL)), and imaged using the Cell Lab IC 100. **C**) Quantification of efficient siRNA transfection and silencing. HeLa cells were reverse-transfected (using RNAiMAX), incubated for 48 h, treated with either 0.1% DMSO or 1 μM ABT-737 (dissolved in 0.1% DMSO) for 24 h, immunofluorescently stained for GAPDH, and imaged (six images/well, three wells/condition). Fluorescence signal intensity per cell was quantified using CytoShop software. Mean ± standard deviation ($n > 200$ cells/condition) are shown, with * indicating $P < 0.05$ (ANOVA). **D**) siMcl-1 complements ABT-737. HeLa cells were reverse-transfected, incubated for 48 h, and treated with either DMSO or ABT-737 for 24 h as previously described. Cell viability was measured using ATPlite. Mean ± standard deviation are shown, with * indicating $P < 0.05$ (ANOVA, n = 12). **E**) Summary of the developed ABT-737-RNAi complementation screening procedure.

selected based on a combination of having one siRNA with an ABT-737:DMSO cell viability ratio lower than 0.66 and literature searches. siRNAs from a total of 22 genes were thus re-assayed (Table 1). Further genes were not evaluated due to resource limitations.

Further validation of ABT-737-RNAi complementation screening "hits"

To further validate our top screening "hits", siRNAs for genes with two siRNAs that possessed confirmed ABT-737:DMSO viability ratios of less than 0.66 were purchased and tested. Dose-response studies confirmed siMcl-1-induced sensitization of cells to ABT-737 (Figure 3A). One out of three HAK siRNAs (siHAK) complemented ABT-737, while all WNK2 siRNAs (siWNK2) showed at least some ABT-737 complementation (Figure 3B, 3C).

The siRNAs were then evaluated for efficient target gene expression knockdown relative to untransfected cells by Q-PCR (Table 2). All siRNAs decreased their target RNAs to at least 0.15-fold levels compared to control. However, siHAK (ID#111041) induced a 0.34-fold change in Mcl-1 and was also the only siHAK to sensitize cells to ABT-737, suggesting that off-target effects were responsible for the observed siHAK complementation. None of the three WNK2 siRNAs markedly decreased Mcl-1 levels, but interestingly, all three decreased HAK levels. At this time it is not clear whether WNK2 affects HAK, or if off-target siRNA effects were observed. In any case, the results indicate that the current RNAi assay can be effectively used for the high throughput identification of siRNAs that sensitize cells to ABT-737.

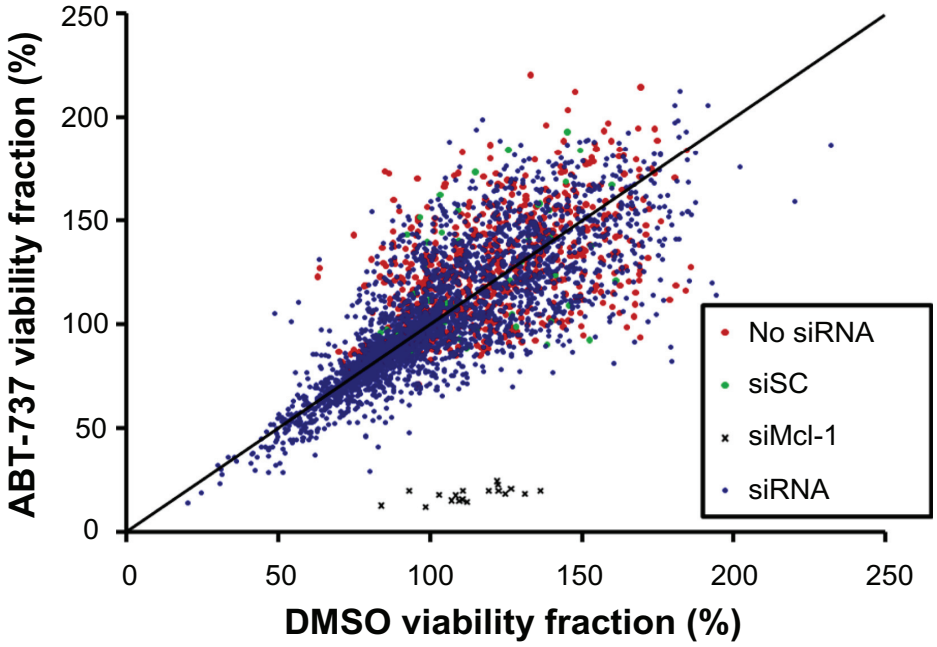

Figure 2 ABT-737-RNAi complementation screening.
HeLa cells (4000 cells/well) were reverse-transfected (using RNAiMAX) with no siRNA (negative control), scrambled control siRNA (siSC; negative control), Mcl-1 siRNA (siMcl-1; positive control), or siRNAs from the kinase subset of the Ambion Silencer Human Druggable Genome siRNA Library V2 (865 genes, three siRNAs/gene) (10 nM), and incubated for 48 h. The cells were then treated with 0.1% DMSO or 1 μM ABT-737 (dissolved in 0.1% DMSO) for 24 h, after which cell viability was measured using ATPlite. The screen was performed twice, with mean results shown. The $y = x$ line is indicated in black.

Table 1 Hit siRNAs re-tested for ABT-737 complementation

Gene	siRNAI	ABT-737/DMSO viability		
		siRMA2	siRNA3	Average
HAK*	0.48	0.75	0.36	0.53
CDC42*	0.91	0.96	0.12	0.66
PRKWNK2*	1.01	0.60	0.41	0.67
MPP6+	0.28	0.86	0.92	0.69
CKMT1+	1.00	0.31	0.77	0.69
FGFR4+	0.59	0.91	0.69	0.73
FGFR3*	1.04	0.97	0.18	0.73
STK23+	0.49	0.80	0.92	0.74
ADORA3+	0.29	0.97	0.97	0.74
STK32A+	0.45	0.89	1.05	0.80
PDPK1+	0.94	0.57	0.89	0.80
FLJ13052+	1.03	0.52	0.93	0.83
TIF1+	0.85	0.68	0.95	0.83
INSRR+	0.74	0.76	1.04	0.85
TXK+	0.75	0.79	1.03	0.86
FLJ32685+	0.85	0.82	0.97	0.88
NTRK1*	0.97	0.92	0.77	0.89
PIK4CB*	0.60	1.02	1.06	0.89
PAK2+	1.05	0.86	0.83	0.91
TEC*	0.96	0.88	0.96	0.93
FER*	1.06	0.87	0.92	0.95
STK22C*	1.06	0.73	1.07	0.95

Notes: *Top ten HTS hits; +additional genes selected based on HTS results and literature searches. HeLa cells were reverse transfected with 10 nM siRNAs, incubated for 48 h, treated with 0.1% DMSO or 1 μM ABT-737 (dissolved in 0.1% DMSO) for 24 h, and assessed for viability using ATPlite. Ratio of ABT-737-treated cell viability to DMSO-treated cell viability is shown.

Discussion

The current study describes a cell viability-based siRNA screen for ABT-737 sensitizers. After testing for siRNA transfection/silencing efficiency and reproducibility, the 865-gene (three different siRNA sequences per gene) kinase subset of the Ambion Silencer Human Druggable Genome siRNA Library V2 was screened to test the assay. After re-assaying siRNAs from 22 genes (10 genes with the lowest average ABT-737:DMSO cell viability ratio and 12 additional genes selected based on having one siRNA with an ABT-737:DMSO cell viability ratio lower than 0.66 and literature searches), siRNAs from two confirmed "hit" genes were further evaluated.

For HAK, only one out of three siRNAs (siHAK ID#111041) sensitized cells to ABT-737. This same siRNA was also the only one of three to decrease Mcl-1 mRNA. siMcl-1 is well-known to sensitize cells to ABT-737 (Figure 1D),[30] thus off-target effects were likely responsible for the sensitization effect of this siHAK.

For WNK2, several siRNAs efficiently decreased WNK2 RNA levels and sensitized cells to ABT-737. These siRNAs did not affect Mcl-1 expression but, interestingly, they decreased HAK expression. Further studies will be required to assess whether WNK2 affects HAK, or if these siWNK2 effects are due to off-target mechanisms.

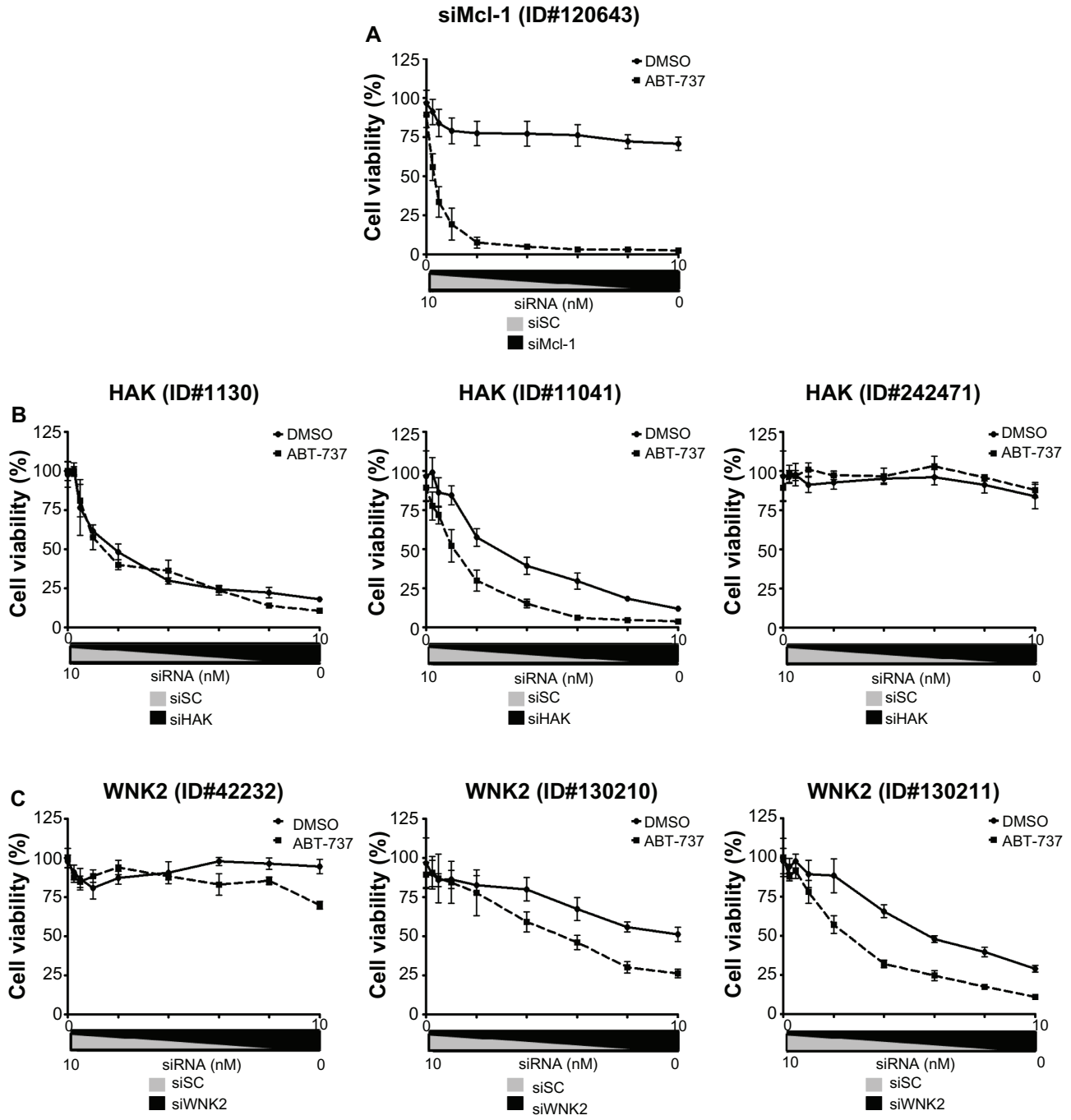

Figure 3 siRNA-mediated ABT-737 sensitization is dose-responsive. HeLa cells (4000 cells/well) were reverse-transfected (using RNAiMAX) with a scrambled control siRNA (siSC, negative control), a specific siRNA, or a mix of the two in varying ratios as indicated (10 nM of total siRNA was transfected in each case). The cells were incubated for 48 h and then treated with 0.1% DMSO or 1 μM ABT-737 (dissolved in 0.1% DMSO) for 24 h, after which cell viability was measured using ATPlite. Mean ± standard deviation ($n \geq 4$) for (**A**) siMcl-1 (positive control), (**B**) siHAK, or (**C**) siWNK2 are shown (Ambion identification numbers are also indicated).

Although early reports described the exquisite sensitivity of siRNAs in silencing genes, a plethora of both sequence-related and sequence-unrelated off-target effects have been identified.[37–39] Indeed, a common result of many RNAi screens and follow-up studies is not the identification of on-target screening hits, but the discovery of responsible off-target genes and mechanisms.[40–43] Because of the nature of siRNAs and high-throughput studies, larger-scale screens will require more complicated statistical and biological database analyses of screening results, followed by extensive follow-up studies.[44,45] Use of siRNA reagents that apply newer chemistries for reducing off-target effects may also help.[46]

Table 2 siRNA-induced changes in gene expression

siRNA transfected	siRNA (ID#)	Gene expression (fold change normalized to PPID)		
		HAK	WNK2	Mcl-1
Untransfected		1.00	1.00	1.00
Mock Transfected		0.99	1.25	1.10
siSC		1.38	1.30	1.38
siMcl-1	120643	1.50	0.87	0.10
siHAK	1130	0.04	0.94	1.01
siHAK	111041	0.03	0.80	0.34
siHAK	242471	0.10	1.42	1.31
siWNK2	42232	0.45	0.05	1.00
siWNK2	130210	0.67	0.06	0.99
siWNK2	130211	0.23	0.15	1.23

Notes: HeLa cells were reverse transfected with 10 nM of the indicated siRNAs and incubated for 48 h, after which RNA was extracted and analyzed by Q-PCR. (Note: values less than 1 represent a decrease in gene expression, and values greater than 1 represent an increase in gene expression.)

In summation, the current study describes the development and validation of an RNAi screen that identifies ABT-737 sensitizers. Future studies may use this assay for larger-scale screens and applications.

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Disclosure

No conflicts of interest were declared in relation to this paper.

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