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Cisplatin sensitivity mediated by WEE1 and CHK1 is mediated by miR-155 and the miR-15 family

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Abstract

Resistance to platinum-based therapies arises by multiple mechanisms, including by alterations to cell-cycle kinases that mediate G2/M phase arrest. In this study, we conducted parallel high-throughput screens for microRNAs (miRNAs) that could restore sensitivity to cisplatin-resistant cells, and we screened for kinases targeted by miRNAs that mediated cisplatin resistance. Overexpression of the cell cycle kinases WEE1 and CHK1 occurred commonly in cisplatin-resistant cells. miRNAs in the miR-15/16/195/424/497 family were found to sensitize cisplatin-resistant cells to apoptosis by targeting WEE1 and CHK1. Loss-of-function and gain-of function studies demonstrated that miR-15 family members controlled the expression of WEE1 and CHK1. Supporting these results, we found that in the presence of cisplatin altering expression of miR-16 or related genes altered cell cycle distribution. Our findings reveal critical regulation of miRNAs and their cell cycle-associated kinase targets in mediating resistance to cisplatin.

Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II)] and its analogs carboplatin and oxaliplatin are used to treat a range of malignancies including testicular, ovarian, head and neck, bladder, esophageal, and small cell lung cancer (1–4). Due to acquired or intrinsic resistance, tumors are not eliminated by treatment with the notable exception of testicular cancer (3–5). Cisplatin resistance studied in cultured cancer cells often results from a cellular defense mechanism that results in a highly complex pleiotropic phenotype that confers resistance by reducing apoptosis, up-regulating DNA damage repair mechanisms, altering cell cycle checkpoints, and disrupting assembly of the cytoskeleton (4). These alterations to the cytoskeleton disrupt cellular protein trafficking, and redirect transporters away from the cell surface. This results in cells that are not only permanently resistant to cisplatin but also show

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Disclosure of Potential Conflicts of Interest

We declare that we have no conflicts of interest related to the publication of this manuscript.

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cross-resistance to other compounds that usually enter into cells via uptake transporters (such as methotrexate and toxic metals). The pleiotropic mechanisms underlying cisplatin resistance are well described, but poorly understood in their entirety and in terms of clinical significance (4, 6).

In cisplatin resistant (CP-r) cancer cells, cell cycle mechanisms allow the cell to compensate for drug toxicity (3, 6–8). When cancer cells are treated with a Pt-based chemotherapeutic, the drug forms Pt-DNA adducts that are generally believed to be responsible for cell death (4, 9). The lesion formed is recognized, activating DNA repair or apoptotic pathways (2, 7, 9). The classical cellular response to cisplatin is G₂ arrest, allowing the cell to repair critical DNA damage before moving forward with mitosis. Cells that attempt to progress through G₂ without sufficient repair undergo apoptosis (2, 9, 10). The length of time spent in G₂ is therefore dependent on the extent of DNA platination (11). Entrance into G₂ and the G₂/M checkpoint is mediated by kinase-controlled signaling pathways (12). Multiple kinases have been shown to have an effect on sensitivity to chemotherapy, in part by regulating cell cycle checkpoints (13–18).

MicroRNAs (miRNAs) are small non-coding regulatory factors 18 to 25 nucleotides in length. miRNAs affect gene expression by complementary pairing with the 3'-untranslated region of mRNAs leading to translational repression, by either degradation or sequestration of mRNA. miRNAs have crucial roles in diverse biological processes such as cell differentiation and phenotypic stabilization, and have also been shown to be involved in tumor growth and response to chemotherapy (19–21). The extent of miRNA involvement in cisplatin resistance is not understood, but studies have begun to identify miRNA-mRNA targets involved in sensitizing or causing resistance, providing potential new targets and mechanisms as treatment options (21–26).

We hypothesized that miRNAs play a role in mediating cisplatin resistance through multiple pathways, including regulation of kinases critical to cell cycle control. In order to address this hypothesis, we designed a high-throughput screen with two purposes: 1) to identify miRNAs that confer sensitivity to cisplatin in resistant cells by mimicking miRNA, and 2) to identify kinases that increase the sensitivity of cisplatin-resistant cells when silenced. Kinase hits were validated by siRNA experiments, examined for expression in cell lines, and tested for involvement in CP-r with small molecule kinase inhibitors. miRNA hits from the screen that were predicted to target kinases identified by the screen were validated using loss-of-function/gain-of-function cell survival curves and Western blots to demonstrate a connection between miRNA activity and kinase regulation. We also tested our screen by selecting a miRNA that was not identified in our screen but was predicted to target the top kinase hit.

Materials and Methods

Cell lines and cell culture

The parental human epidermoid carcinoma cell line KB-3-1, and two cisplatin-resistant sub-lines, KB-CP.5 and KB-CP20 were employed for this study. KB-CP.5 cells were originally selected in a single step in 0.5 µg cisplatin/mL (1.6 µM) in our laboratory, as described previously (27, 28). KB-CP20 cells were selected by stepwise increases to 20 µg of cisplatin/mL of medium (66.7 µM), as described previously (29, 30). The CP-r cells were maintained in the presence of cisplatin, but it was removed from growth medium three days prior to all experiments. All cell lines were grown as monolayer cultures at 37°C in 5% CO₂, using Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L glucose (Invitrogen, Carlsbad, CA), supplemented with L-glutamine, penicillin, streptomycin and 10% fetal bovine serum (BioWhittaker, Walkersville, MD). Cisplatin was purchased from Sigma (St.

Louis, MO). Cross-resistance of cisplatin-resistant cells to cisplatin is confirmed on a regular (at least monthly) basis, using cell viability assays as described herein.

High-throughput screen

KB-CP.5 (resistant) cells were screened with both human kinome siRNA (Ambion SilencerSelect) and human miRNA mimic (Qiagen) libraries. The kinome library targets 704 genes with 3 siRNAs per gene arrayed in individual wells of 384 well plates (see Supplemental Table 2 for a full list of genes targeted). The miRNA mimic library is based on Sanger miRBase 13.0 and contains 875 mimics (Supplemental Table 3). For transfection, siRNA or miRNA mimic (0.8 pmol) was spotted to 384 well plates (Corning 3570) and 0.1 μ L of Lipofectamine RNAiMax (Invitrogen) was then added in 20 μ L of serum free media. This mixture was incubated at ambient temperature for 30 minutes prior to adding cells (1,200) in 20 μ L of DMEM containing 20% FBS, yielding final transfection mixtures with 20 nM siRNA or miRNA mimic. Transfected cells were incubated for 24 h at 37 °C prior to addition of cisplatin or vehicle only (PBS). Cells were incubated with compound for 72 h prior to assaying cell growth (CellTiter Glo, Promega).

All screening plates had a full column (16 wells) of both negative (Ambion SilencerSelect Negative Control #2) and positive control (PLK1 siRNA, Ambion, s448, target sequence AACCAUUAACGAGCUGCUUAA) siRNAs. Positive control served to assess transfection efficiency, whereas the median value of each plate's negative control column was used to normalize corresponding sample wells. For vehicle only plates, negative control wells received PBS alone. For compound treated plates, negative control wells received cisplatin (EC₅ or EC₃₀) in PBS. Candidate miRNAs were primarily selected by examining the ratio between the negative control normalized viability of each mimic in the presence and absence of cisplatin. Mimics ranking highest in terms of these ratios at both doses of cisplatin (>2.5 standard deviations from the median ratio value, \approx among the top 3%) were prioritized for follow-up (12 mimics). A similar approach was used to select the top kinases, focusing on those with multiple siRNAs among the top actives at both doses (>2.5 standard deviations from the median, \approx among the top 3%). Genes selected at both doses included ATR, CHEK1, and WEE1. Six additional siRNA sequences (Qiagen) were tested for each of the top genes in the context of a cisplatin dose response (1000 μ M to 0 μ M) (details of siRNAs in Supplemental Materials and Methods). Knock-down by additional siRNAs was confirmed by RT-PCR.

Small molecule kinase inhibitors

Cells were treated with the following kinase inhibitors: SB 218070 against Chk1, MK 1775 against Wee1, and PD 407824 against Wee1 and Chk1 (Tocris Bioscience, Minneapolis, MN).

MTT assay

Cell survival was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at a density of 5,000 cells per well in 96-well plates and incubated at 37°C in humidified 5% CO₂ for 24 h. Serially diluted cisplatin was added to give the intended final concentrations. Cells were then incubated an additional 72 h, and the MTT assay was performed according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Absorbance values were determined at 570 nm on a Spectra Max 250 spectrophotometer (Molecular Devices, Sunnyvale, CA). All MTT assays were performed three times in triplicate. The 50% inhibitory concentration (IC₅₀) values were defined as the drug concentrations required to reduce cellular proliferation to 50% of the untreated control well and are reported here as such.

Flow cytometry for apoptosis and cell cycle analysis

KB-3-1 and KB-CP.5 cells were transfected with siNegative control, miR-16 mimic, or inhibitor. After 24 h, KB-3-1 cells were treated with 5 μ M cisplatin and KB-CP.5 cells with 25 μ M cisplatin. Cells were harvested in PBS 72 h post cisplatin treatment. For evaluation of apoptosis vs. viable populations, cells were washed with PBS and processed with Alexa Flour 488 annexin V/Dead Cell Apoptosis Kit with Alexa Flour 488 annexin V and PI for flow cytometry, as described by the manufacturer (Invitrogen, Eugene, OR). Flow cytometry analysis was performed with FlowJo v.7.6.4. For cell cycle analysis, cells were washed with PBS and fixed overnight in ice-cold 70% ethanol and stored at 4°C. After 24 h, cells were centrifuged and re-suspended in 100 U RNase (Sigma-Aldrich, St. Louis, MO), and incubated at 37°C for 20 min. Propidium iodide (Invitrogen) solution (500 μ L, 50 μ g/mL in DPBS) was added to each tube and incubated in the dark at 4°C overnight. Flow cytometry was performed and analysis was completed with ModFit LT. All data is in triplicate and presented as a percent mean \pm SD.

RNA extraction, cDNA preparation, and quantitative real-time (RT)-PCR

Cisplatin was removed 72 h prior to experiments. RNA was harvested from KB-3-1 and KB-CP.5 cells using the mirVana miRNA Isolation Kit (Ambion, Carlsbad, CA). RNA integrity and concentration were measured using an NP-1000 Spectrometer to measure absorbance (Thermo Scientific, Wilmington, DE). cDNA was prepared from 20ng total RNA following the manufacturer's instructions. RT-PCR was run using Exiqon probes on a 7500 fast real-time PCR system (Applied Biosystems) (details of probes in Supplemental Materials and Methods). The miRNA data was normalized to U6 RNA. Expression level was calculated by the $\Delta\Delta$ Ct method.

Target prediction

miRNAs predicted to target kinases identified by the high throughput screen were identified by using miRanda (<http://www.microrna.org/microrna/home.do>) and TargetScanHuman (<http://www.targetscan.org/>) algorithms.

Transfection of miRNA mimics and inhibitors

Transfection was performed with RNAiMax (Invitrogen, Carlsbad, CA) (2.5 μ L/mL). The pre-designed miRIDIAN miRNA mimics and inhibitors (details of inhibitors in Supplemental Experimental Procedures), and AllStars Negative Control (mock) (Qiagen) were plated with cells on day 1 at 10 nM. The transfection was performed as described by the manufacturer.

Preparation of cell lysates, quantification of protein, and Western blot analysis

Cell samples were obtained by trypsin digestion followed by two washes in PBS. Protein was extracted using Cell Extraction Buffer (Invitrogen) with 50 μ L/mL Protease Inhibitor Cocktail (Sigma). Protein concentrations were determined using Bio-Rad Protein Assay based on the Bradford method as per the manufacturer's instructions. Fifty- μ g protein samples were loaded onto a NuPage 3–8% TA gel (Invitrogen) and run at 125 V. Transfer to nitrocellulose membranes was performed using an iBlot Gel Transfer Device (Invitrogen) at 20 V for 7 min. Membranes were blocked in 5% non-fat milk for 1 h, exposed to primary antibody overnight at 4 °C, and HRP-tagged secondary antibody for 1 h at room temperature (antibodies and conditions listed in Supplemental Experimental Conditions). Blots were incubated with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) and developed on Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK).

Statistical Analysis

We used GraphPad Prism 5 software for graphs and statistics. All data are expressed as mean \pm SD. Comparisons among groups were analyzed using Analysis of Variance (ANOVA). Comparisons between different groups were analyzed using Two-way ANOVA. P values <0.05 were considered significant differences.

Results

A high-throughput screen identifies miRNA and kinases that can sensitize cell lines to cisplatin

We developed a high-throughput screen (HTS) to identify miRNAs that when mimicked (increasing abundance) could sensitize CP-r KB-CP.5 cells to cisplatin (Supplementary Fig. S1). KB-CP.5 cells were screened with miRNA mimics (Sanger miRBase 13.0), and then challenged with cisplatin at IC₅ (5 μ M) and IC₃₀ (25 μ M) doses. After 72 h, cell viability was determined using a Promega CellTiter-Glo luminescent assay that reports on cell survival based on cellular ATP levels in each well. Conditions that sensitized the resistant cells (indicated by low cell number compared with cells transfected with negative control siRNA) were examined (Supplementary Table S1, available online). Twelve miRNA mimics were shared among the top hits for both the EC₅ and EC₃₀ doses, inducing > 2 -fold change at the EC₃₀ dose (Table 1, Supplementary Table S3). Five of these mimics belong to the miR-15/16/195/424/497 family. This family shares an identical seed sequence (a seven nucleotide sequence that complements and selects target mRNA for sequestering or degradation). miR-16-1* and miR-424* are part of the family but are matured from the 3' strand of the hairpin loop of the pre-miRNA and therefore are a perfect complement to the family seed sequence. miR-16 transfection conferred a 5-fold increase in cisplatin sensitivity, and its family member miR-15a had a 3.4-fold increase providing two of the top three hits in the primary screen.

As part of the HTS, in parallel we evaluated a kinase library in order to determine which kinases had a significant effect on cisplatin resistance in our cell lines. Here, we screened for kinases that, when silenced, conferred sensitivity to CP-r cells. To accomplish this, the kinome siRNA (Ambion SilencerSelect, 704 genes, 3 siRNAs per gene) library was run under the same conditions as those used for the miRNA mimics (CP.5 cells with two low toxicity cisplatin doses, IC₅ and IC₃₀), with three siRNAs being tested for each kinase. Selected candidates had at least 2 of 3 siRNAs scoring at the top in terms of sensitizing CP-r cells to cisplatin at both doses (Table 2, Supplementary Table S4). The three strongest candidates were WEE1, ATR and CHEK1.

ATR, WEE1, and CHEK1 expression and function confer resistance to cisplatin while interference by RNAi or small molecule inhibitors increases sensitivity

In order to confirm that the top three kinase hits from the screen affect cisplatin resistance, an additional 6 siRNAs for each gene were used in a follow-up dose-response validation (Fig. 1). Four siRNAs against ATR accorded a 3 to 10-fold increase in sensitivity to cisplatin. Six siRNAs against *CHEK1* and six against *WEE1* increased sensitivity 2 to 14-fold, indicating that these kinases play an important role in inhibiting cell killing by cisplatin (Fig. 1A). Knock-down of additional siRNAs was confirmed by RT-PCR (Supplementary Figure S4).

To confirm that ATR, WEE1, and CHK1 are involved in cisplatin resistance, we examined the expression of ATR, WEE1, and CHK1 protein in KB-3-1 (sensitive), KB-CP.5 (resistant), and KB-CP20 (highly resistant) cells (Fig. 1B). All three kinases showed an increase in expression in the CP-r lines compared to parental KB-3-1 cells. In addition, KB-

CP20 cells showed an increase in expression compared to KB-CP.5, indicating that the expression of *ATR*, *WEE1*, and *CHK1* correlate with the level of cisplatin resistance in each cell line, though whether the increased protein levels result in greater activity has not been determined. As *ATR* was not predicted as a target for miRNA identified in our screen, it was not studied further.

We sought to determine whether inhibiting *WEE1* and *CHK1* kinase activity could sensitize resistant lines and modulate resistance. To this end, we evaluated the response of the KB-3-1 and KB-CP.5 lines to the following small molecule kinase inhibitors: MK 1775 (*WEE1*), PD 407824 (*WEE1/CHK1*), and SB 218078 (*CHK1*) (Fig. 1C). All inhibitors demonstrated cytotoxicity towards both the KB-3-1 and KB-CP.5 lines. MK 1775 (*WEE1*) provided the strongest effect, killing 50% of the cell population (IC_{50}) at $0.4 \pm 0.1 \mu\text{M}$ irrespective of cell line (Table 3). KB-CP.5 cells showed cross-resistance to SB 218078 (*CHK1*, KB-3-1 $IC_{50} = 1.7 \pm 0.2 \mu\text{M}$, KB-CP.5 $IC_{50} = 2.8 \pm 0.2 \mu\text{M}$) and PD 407824 (*CHK1/WEE1*, KB-3-1 $IC_{50} = 2.7 \pm 0.5 \mu\text{M}$, KB-CP.5 $IC_{50} = 4.4 \pm 0.6 \mu\text{M}$).

Next, KB-3-1 and resistant KB-CP.5 cells were examined to determine whether inhibiting *WEE1* or *CHK1* sensitized them to cisplatin treatment. This was achieved by treating cells with a sub-toxic dose of each kinase inhibitor; MK 1775 (*Wee1*, 100 nM), PD 407824 (*WEE1/CHK1*, 10 nM), and SB 218078 (*CHK1*, 10 nM). Kinase inhibitors were used at a subtoxic dose to ensure the assay identified cell death dependent on cisplatin and not the inhibitors themselves. Inhibiting the function of kinases produced an increased sensitivity to cisplatin, albeit to a lesser degree than occurred with the siRNA (Fig. 1D), with MK 1775 (*WEE1*) being the most effective. It may be that the kinase inhibitors did not fully inhibit kinases at sub-toxic doses, leading to the lesser effect compared with siRNA. Sensitive KB-3-1 cells showed a greater degree of sensitization than resistant KB-CP.5 cells (Table 3). This may be due to the lower expression of *WEE1* and *CHK1* in KB-3-1 cells, as there is a lower concentration of kinase to inhibit with an identical dose. We cannot rule out, however, that the CP-r phenotype restricts cell entry of the kinase inhibitors. These data indicate that resistance to cisplatin includes increased expression of kinases responsible for G_2/M checkpoint regulation, and inhibiting these same kinases sensitizes CP-r cells. The CP-r phenotype confers cross-resistance to kinase inhibitors against *WEE1* and *CHK1*.

CHK1 and WEE1 are predicted to be targets of miR-15a/15b/16/16-1*/424*

Using TargetScanHuman and miRanda, online algorithm programs for the prediction of miRNA targets, we found that the miR-15/16/195/424/497 family identified in the miRNA mimic screen is predicted to target *WEE1* and *CHEK1*, two of the hits provided in the kinome RNAi screen (Table 2). This is consistent with our hypothesis that a miRNA will down-regulate expression of genes associated with cisplatin resistance, leading to loss of phenotype and increased sensitivity to cisplatin (Figure 1). We therefore hypothesize that miR-15a/15b/16/16-1*/424* regulation of *CHEK1* and *WEE1* is causative for cisplatin resistance. miR-155 has been shown previously to target *WEE1* kinase (31), but it was not identified by our screen. In evaluating the connection between the screen hits, miR-155 was incorporated to give us insight into the selectivity of the miRNA screen.

miR-15a/15b/16/16-1*/424* and miR-155 is decreased in cisplatin-resistant cells

RT-PCR was used to determine the endogenous expression of miRNAs in KB-3-1 and KB-CP.5 cell lines (Figure 3A). ΔCT values were obtained using U6 snRNA as an internal control (32). miR-16-1* was the most abundant family member in KB-3-1 cells followed by miR-16 and miR-424*, with all miRNAs being expressed at detectable levels. Expression of all miRNA members showed statistically significant ($p \leq 0.05$) decreased expression in KB-CP.5 cells compared to the parental KB-3-1 cells. miR-16-1* showed the greatest loss in

expression in KB-CP.5 cells, with miR-15a and -15b the smallest change in expression. miR-155 also showed a decrease in expression in KB-CP.5 cells ($p \leq 0.05$) comparable to miR-15a but less than that of family members miR-15b/16/16-1*/424*. This indicates that miR-15a/15b/16/16-1*/424* and miR-155 have a lower expression in KB-3-1 cells consistent with their increased resistance to cisplatin.

miR-15a/15b/16/16-1*/424* target *WEE1* and *CHK1* in KB-3-1 and KB-CP.5 cell lines

To determine whether manipulation of miRNA affects *WEE1* and *CHK1* levels, cells were treated with a miRNA mimic or miRNA inhibitor (siRNA directed against a miRNA precursor) for individual members of the miR-15a/15b/16/16-1*/424* family, and cell lysates were obtained at 48 h. *Chk1* and *Wee1* expression in response to an increase (mimic) or decrease (inhibitor) in miRNA were examined by Western blot (Fig. 2B–F). All miRNA family members elicited a decrease in *WEE1* and *CHK1* levels in response to miRNA mimics in both cell lines (Fig. 2C, E). KB-3-1 cells transfected with miRNA inhibitor showed an increase of *CHK1* in all samples (Fig. 2D,F). miR-15a, miR-15b and miR-16 inhibitors transfected into KB-3-1 also increased expression of *WEE1* (Fig. 2D). KB-CP.5 cells showed increased expression of *WEE1* in response to miR-15a/15b/16/16-1* inhibitors, but did not show a marked response to miR-424* (Fig. 2F). All miRNA inhibitors increased the expression of *CHK1* in KB-CP.5 cells (Fig. 2F).

miRNA inhibitors confer a marked increase in cisplatin resistance

Loss-of-function/gain-of-function experiments were completed by transfecting sensitive and resistant cells with siNegative (control), miRNA mimics, or miRNA inhibitors. After 24 h all samples were challenged with cisplatin (dose gradient from 100 to 0 μM). miRNA loss of function by treatment with inhibitor against each miRNA for the miR-15a/15b/16/16-1*/424* family increased cellular resistance to cisplatin in KB-3-1 cells (Fig. 3, Supplementary Table S2). This was also observed for KB-CP.5 cells, although the low expression of miR-15a/15b/16/16-1*/424* in KB-CP.5 cells (Fig. 2A) means only minimal additional silencing of *WEE1* and *CHK1* is possible. Increasing the expression of miR-15a/15b/16/16-1*/424* decreases the expression of *WEE1* and *CHK1* and results in sensitivity to cisplatin. Cell survival curves demonstrate that all miRNA mimics for the miR-15a/15b/16/16-1*/424* family increase sensitivity to cisplatin for all data points. Transfection of miR-16-1* (Fig. 3D) and miR-424* (Fig. 3E) mimics conferred the greatest degree of sensitization. The effect of miRNA mimics and inhibitors was confirmed by RT-PCR (Supplementary Fig. S2), and no off-target effects were observed for other family members (Supplementary Fig. S3). Generally, miRNA inhibitors (siRNAs) lowered miRNA expression to undetectable levels, whereas the fold-increase in miRNA levels after transfection with miRNA mimic was not as strong. This difference accounts for the lesser effect of mimics compared with inhibitors.

The MTT assay acts as a measure of cellular proliferation, but does not give direct insight into whether cell death or arrest is at play in cells. This is partly because the MTT assay, as well as other analogous assays, provide insight into total cell number. The effect of this is that if cellular proliferation is arrested (which cisplatin is known to do), total cell count would be diminished without necessarily eliciting a high rate of cellular apoptosis. We adopted two experimental approaches to demonstrate that cisplatin's effects on cell growth was related to loss of cell viability (apoptosis) and involved cell cycle arrest, as has been frequently characterized for cisplatin. For both approaches, we examined miR-16 as an exemplar of the family members identified in this report.

First, we assessed cellular apoptosis induced by cisplatin with either miR-16 inhibitor or mimic (with appropriate controls) to demonstrate whether apoptosis occurs. Cell death was

assessed using the Annexin V apoptosis assay, which detects both phosphatidylserine levels on the external leaflet of the cell membrane (apoptotic cells, detected by Annexin V), and cell membrane leakage (permeabilization) indicated by propidium iodide entry (dead cells). We found that, compared with siNeg + cisplatin, both KB-3-1 and KB-CP.5 cell lines were sensitized to cisplatin in the presence of miR-16 mimic (increased apoptosis and death), and apoptosis and death were lowered when the cells were transfected with miR-16 inhibitor (Table 4, Supplementary Figure S5). For example, KB-3-1 cells treated with 5 mM cisplatin (siNeg as control) for 72 h were 81.4 ± 0.3 % viable (8.0 ± 0.5 % apoptotic, 9.9 ± 2.4 % dead). Co-treatment of cells with cisplatin and miR-16 mimic (down-regulation of Wee1/Chk1) resulted in strong sensitization of cells (19.2 ± 4.2 % apoptotic, 60.4 ± 7.3 % dead). Conversely, co-treatment of cells with cisplatin and miR-16 inhibitor (up-regulation of Wee1/Chk1) resulted in inhibition of cell death (0.7 ± 0.5 % apoptotic, 2.8 ± 1.2 % dead). Similar results were observed in KB-CP.5 cells treated with a higher concentration of cisplatin (25 mM, Table 4). The mimic or inhibitor alone did not have an effect on cell growth compared with negative control cells.

Next, we assessed the impact of miR-16 inhibitor or mimic (with appropriate controls) on arrest of cells in the presence of cisplatin (Table 4, Supplementary Figure S6). We found that in line with the effects on Wee1 and Chk1 and cell viability (described above), miR-16 inhibitor in the presence of cisplatin elevated the proportion of cells in G₂ phase (compared to cisplatin + siNeg). For example, KB-3-1 cells treated with cisplatin showed slow transit S phase (as described by Eastman (2), 82.6 ± 2.9 %) and some accumulation in the G₂ phase (16.7 ± 3.4 %) compared with control cells ($G_2 = 5.5 \pm 1.9$ %). Presence of the miR-16 inhibitor resulted in dramatic arrest in the G₂ phase (93.3 ± 0.3 %), and loss of S phase accumulation. KB-CP.5 cells treated with cisplatin alone showed normal S phase levels, and greater accumulation in G₂ phase, when compared with parental cells, consistent with resistance and elevated checkpoint kinase expression (Fig. 1B). In CP.5 cells, miR-16 inhibitor resulted in greater G₂ accumulation than cisplatin alone. Alternatively, the miR-16 mimic lowered the extent of G₂ arrest to a lesser (but significant) extent (compared to cisplatin + siNeg) (Table 4). The mimic or inhibitor alone did not have an effect on cell cycle distribution.

Interference of miR-155 causes an increase in cisplatin resistance by silencing WEE1

Gain-of-function transfection experiments with miR-155 mimic in KB-3-1 cells did not sensitize cells to cisplatin. In KB-CP.5 cells there was a 20% decrease in percent growth when compared to siNegative treated cells (Fig. 3F). Western blots demonstrate that increasing the level of miR-155 in cell lines with miR-155 mimic silences WEE1 to undetectable protein levels (Fig. 2F). Loss-of-function experiments with miR-155 inhibitors increased resistance significantly (Fig. 3F) and restored expression of WEE1 (Fig. 2B). KB-3-1 cells transfected with miR-155 inhibitor were more resistant to cisplatin. This increased resistance was also seen in KB-CP.5 cells transfected with miR-155 inhibitor. These results indicate that the decreased expression of miR-155 in cisplatin-resistant cells contributes to increasing the amount of WEE1, thereby increasing resistance, but the interference of Wee1 alone by miR-155 is not enough to reverse the CP-r phenotype.

Discussion

We report here the results of two high-throughput screens, with the principal finding that a decrease in the miR-15abc/16/16abc/195/424/497 family mediates resistance to cisplatin in our model, and identified their gene targets, the kinases WEE1 and CHK1. This finding was achieved by developing two complementary high-throughput screens designed to identify kinases whose loss sensitized cells to cisplatin, and miRNA that, when increased by mimicking, achieved the same effect.

875 miRNA were screened for their ability to increase sensitivity to low doses of cisplatin when mimicked in cisplatin-resistant cells (KB-CP.5). Twelve miRNAs were shared amongst the top hits at both doses and 5 were from the same family. The miRNAs selected for further validation were predicted to target the kinase positive hits from the parallel screen, and all miRNAs were confirmed to influence cisplatin resistance by sensitizing cells to cisplatin when mimicked. We also examined miR-155, which was not identified in our screen but has been shown to target WEE1 (31) in our loss-of-function/gain-of-function validation studies. miR-155 did cause an increase in cisplatin resistance when its expression is decreased. However, increasing the abundance of miR-155 does very little to increase sensitivity to cisplatin, accounting for its lack of detection in our screen. An additional family member, miR-497, lay just outside the selection criteria, providing further support for the importance of this miRNA family in cisplatin sensitivity. Furthermore, miR-503, with 6 nt of seed sequence conserved with the 7-nt seed sequence of the miR-15abc/16/16abc/195/424/497/1907 family was also predicted to target CHK1.

The miR-15abc/16/16abc/195/424/497/1907 family are grouped together based on their common seed sequence, and have been identified as tumor suppressor miRNAs with decreased expression, mutations to the coding region, or absence in chronic lymphocytic leukemia, gastric, lung, and prostate cancers (22, 24, 33–36). This family has also been shown to influence apoptotic pathways and response to chemotherapy by targeting BCL2 in gastric cancer (36). However, this is the first report linking the regulation of WEE1 and CHK1 cell cycle kinases by the mi-R15abc/16/16abc/195/424/497 family in response to treatment with cisplatin. This leads us to postulate that increasing the expression of miR-15 family members could reverse resistance to chemotherapy in multiple types of cancers.

While several screens of varying scale have now been performed in the presence of cisplatin, none have examined the sensitization of CP-r cells (37). We believe that potential strategies for resolving cisplatin-based insensitivity is a critical unmet need in the clinic. Arora *et al.* recently reported a siRNA screen against kinases that played a role in the sensitivity of parental (nonresistant) SKOV cells to cisplatin, identifying ATR and CHK1, but not WEE1 (38). ATR, WEE1, and CHK1 are kinases that affect the G₂/M checkpoint in the cell cycle, where cells arrest after exposure to cisplatin (2). Furthermore, CP-r cells develop prolonged G₂ arrest, allowing a greater capacity for nuclear repair (11), supported by our finding that KB CP-r cells show increased expression of ATR, WEE1, and CHK1. While G₂ arrest occurs in response to cisplatin, cell death occurs when the cells undergo an abortive attempt at mitosis after G₂ arrest (2). Therefore, abrogating G₂ by forcing the cell into mitosis before DNA repair has been adequately accomplished increases the efficacy of cisplatin. Decreasing the function or presence of WEE1 and CHK1, for example with small molecule inhibitors, accomplishes this needed abrogation (2, 12, 39). Here we show that this is also true by increasing miR levels with a mimic.

In response to DNA damage, sensor multiprotein complexes identify DNA lesions, following which ATR and ATM (cell cycle transducer proteins) are activated and signal the downstream checkpoint kinases (12). One of these checkpoint kinases Chk1, a serine/threonine kinase, is a housekeeper present in cells with a normally functioning cell cycle. When signaled by ATR and ATM in response to DNA damage, CHK1 is activated by S345 phosphorylation and prevents CDC2 from activating, thus preventing entry into mitosis (12). CHK1 also phosphorylates p53 and in conjunction with ATM/ATR stabilizes p53 allowing for its activation and accumulation which results in a reversible cell cycle arrest, without requiring *de novo* protein synthesis (40, 41). CHK1 is critical in modulating the G₂/M checkpoint in the cell cycle when cells respond to DNA damaging agents (12) and is therefore one of the most important kinase targets related to the treatment of cancer (12, 42). WEE1 is a tyrosine kinase with a primary function of inactivating CDK1 (encoded by the

CDC2 gene) by phosphorylating it, leading to G₂ checkpoint arrest and therefore preventing entry into mitosis (14, 15). By regulating CDC2 activity, WEE1 determines the length of time spent in G₂/M arrest (43).

Abrogation of the G₂/M checkpoint by targeting CHK1 and WEE1 with kinase inhibitors has been shown to sensitize cancer cells to DNA-damaging drugs (14, 42). Kinase inhibitors against CHK1 and WEE1 are already in clinical trials but cells can develop resistance to the kinase inhibitors. These results highlight a potential strategy for a single therapeutic intervention targeted towards the multiple kinases that mediate cisplatin sensitivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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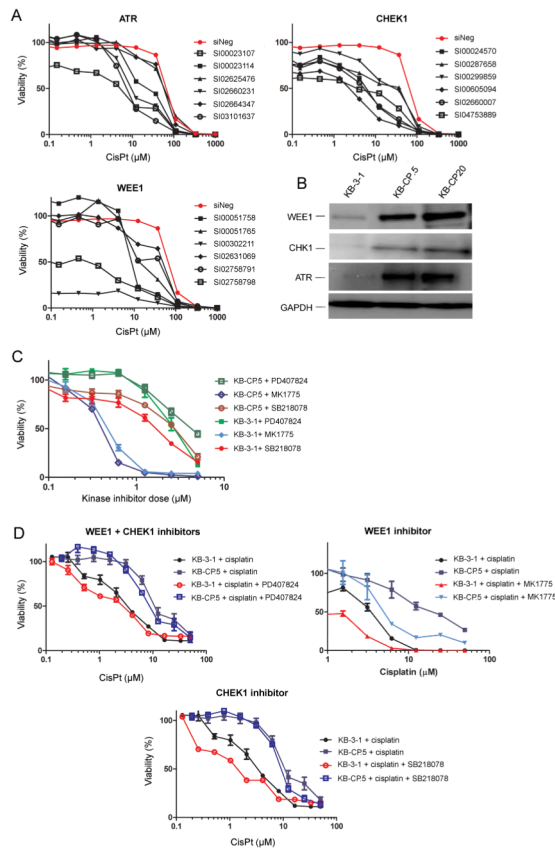


Figure 1. Validation of high-throughput kinase hits

A, Follow-up dose response analyses using additional siRNAs confirms sensitization mediated by the knockdown of *ATR*, *CHK1*, and *WEE1* in KB-CP.5 cells compared to siNegative treated cells. B, Western blots showing normal expression of kinases. *ATR*, *WEE1*, and *CHK1* have increased expression in resistant lines (KB-CP.5 and KB-CP20) compared to sensitive (KB-3-1). The increase in expression is dependent on the level of resistance. C, Small molecule kinase inhibitors PD 407824 (*CHK1* and *WEE1*), MK 1775 (*WEE1*), and SB 218078 (*CHK1*) have concentration-dependent toxic effect on sensitive and resistant cell lines. D, Cells were treated with sub-toxic doses of kinase inhibitors (10nM) and challenged with cisplatin (50 μ L to 0 μ L). KB-3-1 sensitive cells had a 1.2 – 3.9 fold decrease in survival in cisplatin when the kinase function was inhibited with small molecule inhibitors. KB-CP.5 resistant cells showed less of an effect at 1.2 to 1.9 fold increase in sensitivity to cisplatin. CisPt = cisplatin.

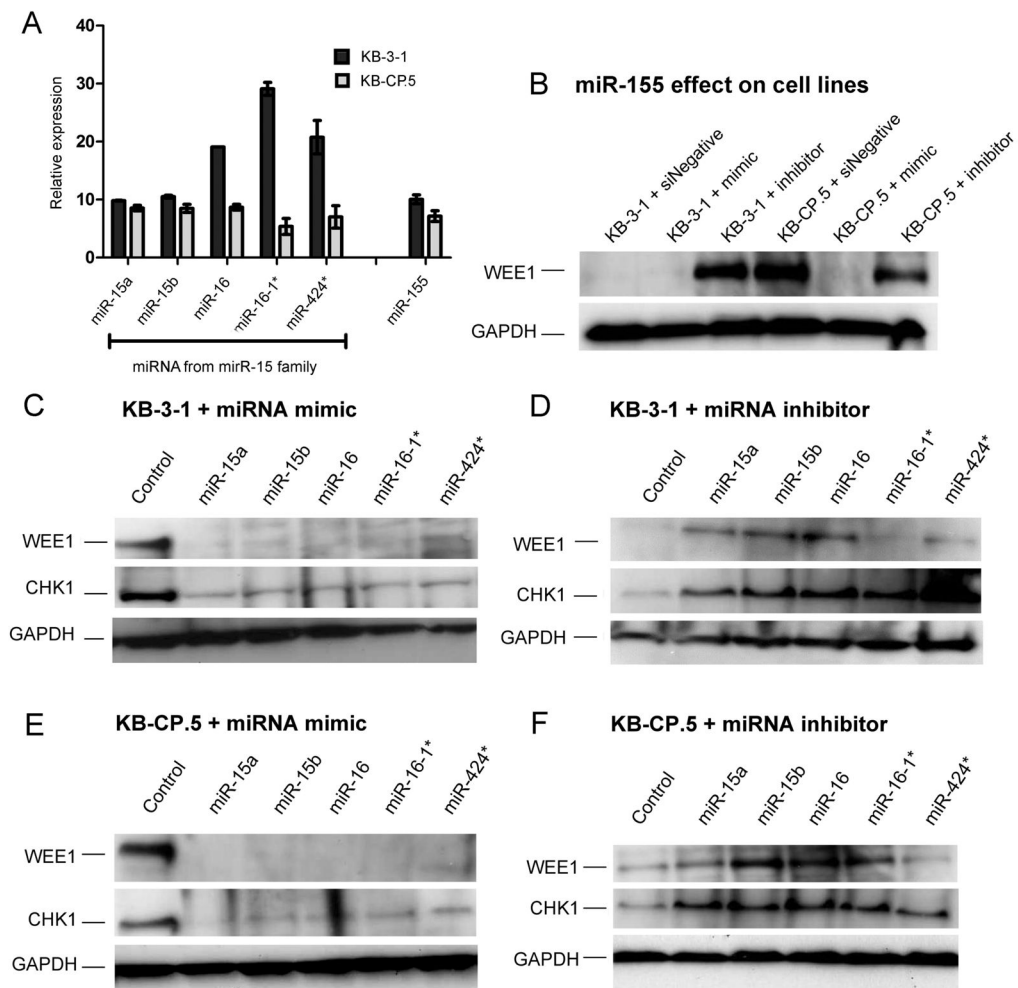


Figure 2. Expression of miR-15 family members and their effect on expression of WEE1 and CHK1 kinases

A, Expression of miR-15 family members and miR-155 in KB-3-1 and KB-CP.5, with RT-PCR. Samples were normalized to U6. Using ANOVA statistical analysis, columns comparing expression of one miRNA between KB-3-1 to KB-CP.5 have a p-value < 0.05 . Columns comparing different miRNAs to each other in the same cell line have a p-value of 0.0001. Western blots showing expression of WEE1 and CHK1 in response to transfection with miR-15a/15b/16/16-1*/424* miRNA mimics and miRNA. KB-3-1 cells transfected with mimics (B) and inhibitors (C), and KB-CP.5 cells transfected with mimics (D) and inhibitors (E) were collected and protein extracted after 72 h.

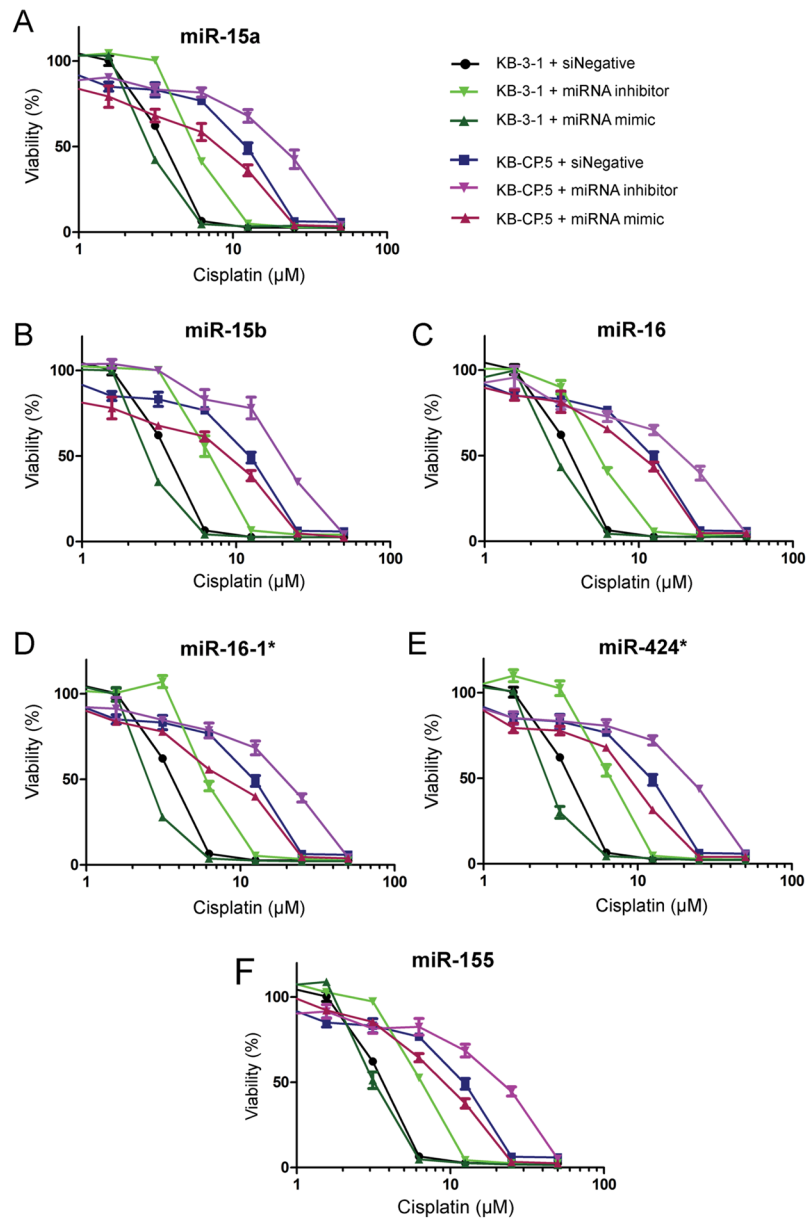


Figure 3. Response of KB cell lines to miRNA mimic/inhibitor data for the miR-15 family
 Cells were treated with a miRNA mimic or an miRNA inhibitor for each family member (miR-15a/15b/16/16-1*/424*) (10nM) and challenged with cisplatin (50 μM to 0 μM). miR-15a/15b/16/16-1*/424* all showed an increase in resistance when the miRNA was inhibited and an increase in sensitivity to cisplatin when the miRNA was increased.

Table 1

miRNA mimics found to sensitize cisplatin-resistant CP.5 cells to cisplatin in the high-throughput screen

	% Proliferation, miRNA mimic	% Proliferation, mimic + IC₃₀ cisplatin	Fold change in % cell proliferation
miR-16	71.3	14	5.1
miR-503	82.9	23.7	3.5
miR-15a	69.5	20.3	3.4
miR-29b-2*	80.3	24.8	3.2
miR-634	100	31.2	3.2
miR-17	98.5	31.6	3.1
miR-424*	79.5	25.6	3.1
miR-1262	67.1	24.5	2.7
miR-16-1*	104.1	43.8	2.4
miR-15b	101.7	43.3	2.4
miR-29b	112.3	51.4	2.2
miR-101	102.6	48.7	2.1

miRNAs were considered to be hits if they were active at both concentrations of cisplatin (IC₃₀ and IC₅ doses). Active miRNA mimics identified are ranked in order of the fold-change sensitization. miR-16, miR-15a, miR-424*, miR-16-1*, and miR-15b all belong to the same family.

Table 2

Kinase siRNAs found to sensitize cisplatin-resistant CP.5 cells to cisplatin

siRNA target	% Proliferation, siRNA	% Proliferation, siRNA + IC ₃₀ cisplatin	Fold change in % survival	Predicted targets of miRNA from screen
WEE1	69.4	13.7	5.1	miR-15a/15b/16/16-1*/424*, miR-17, miR-503
ATR	109.6	28.4	3.9	None
CHEK1	75.6	19.9	3.8	miR-15a/15b/16/16-1*/424*, miR-503

Silenced genes were considered significant if more than 1 siRNA against a given gene significantly sensitized CP.5 cells to cisplatin. Cell growth (proliferation) was determined as the percentage of siRNA-treated surviving cells compared with siNegative cells. The listed values correspond to the highest fold change observed for a given gene. miRNA hits predicted to target kinases using the TargetScanHuman and miRanda programs are listed.

Cytotoxicity (IC₅₀) values for small molecule kinase inhibitors (KIs) against KB-3-1 and KB-CP.5 cells, with and without cisplatin

Table 3

Cell type	Kinase inhibitor (KI)	KI IC ₅₀ (μM)	Subtoxic dose (nM)	Target	Cisplatin IC ₅₀ (μM)	Cisplatin IC ₅₀ (μM) + KI	Sensitization
KB-3-1	PD 407824	2.7 ± 0.5	10	Wee 1, Chek 1	5.1 ± 0.9	4.3 ± 0.6	1.2
KB-CP.5	PD 407824	4.4 ± 0.6	10	Wee 1, Chek 1	12.6 ± 3.6	9.1 ± 0.1	1.4
KB-3-1	MK 1775	0.4 ± 0.1	100	Wee 1	5.1 ± 0.9	0.8 ± 0.3	6.3
KB-CP.5	MK 1775	0.5 ± 0.1	100	Wee 1	12.6 ± 3.6	5.1 ± 0.4	2.5
KB-3-1	SB 218078	1.7 ± 0.2	10	Chek 1	5.1 ± 0.9	1.3 ± 0.01	3.9
KB-CP.5	SB 218078	2.8 ± 0.2	10	Chek 1	12.6 ± 3.6	10.2 ± 0.5	1.2

Cells were treated with KIs alone in order to determine a dose shown to kill 50% of the cell population (IC₅₀), and to identify sub-toxic doses. Cell lines were then treated with cisplatin alone or cisplatin with a subtoxic dose of KIs. Sensitization is calculated as the fold-difference between the cisplatin IC₅₀ divided by KI treated cells. A value > 1 signifies an increase in sensitivity.

Table 4

Cell viability (Annexin V/propidium iodide) and cell cycle analysis of KB-3-1 and KB-CP.5 cells transfected with either siNeg, miR-16 mimic or miR-16 inhibitor alone or in the presence of cisplatin (5 μM for KB-3-1 or 25 μM for KB-CP.5)

	KB-3-1										KB-CP.5									
	Viable (%)	Apoptotic (%)	Necrosis (%)	G ₁ (%)	G ₂ (%)	S (%)	Viable (%)	Apoptotic (%)	Necrosis (%)	G ₁ (%)	G ₂ (%)	S (%)	Viable (%)	Apoptotic (%)	Necrosis (%)	G ₁ (%)	G ₂ (%)	S (%)		
siNeg	99.3 ± 0.3	0.4 ± 0.2	0.18 ± 0.2	65.8 ± 3.4	5.5 ± 1.9	28.7 ± 5.3	99.0 ± 0.8	0.4 ± 0.3	0.1 ± 0.1	54.0 ± 0.8	18.1 ± 0.9	28.0 ± 0.1	99.0 ± 0.8	0.4 ± 0.3	0.1 ± 0.1	54.0 ± 0.8	18.1 ± 0.9	28.0 ± 0.1		
siNeg + cisplatin	81.4 ± 0.3	8.0 ± 0.5	9.9 ± 2.4	0.66 ± 0.5	16.7 ± 3.4	82.6 ± 2.9	82.3 ± 1.5	5.7 ± 1.3	6.8 ± 0.3	1.7 ± 1.2	68.2 ± 3.4	30.1 ± 2.8	82.3 ± 1.5	5.7 ± 1.3	6.8 ± 0.3	1.7 ± 1.2	68.2 ± 3.4	30.1 ± 2.8		
miR-16 mimic	99.2 ± 0.4	0.2 ± 0.1	0.1 ± 0.1	55.15 ± 0.5	7.5 ± 0.4	37.3 ± 0.3	98.6 ± 0.5	0.8 ± 0.6	0.2 ± 0.1	51.0 ± 0.5	17.1 ± 0.4	31.9 ± 0.2	98.6 ± 0.5	0.8 ± 0.6	0.2 ± 0.1	51.0 ± 0.5	17.1 ± 0.4	31.9 ± 0.2		
miR-16 mimic + cisplatin	14.8 ± 3.2	19.2 ± 4.2	60.4 ± 7.3	1.93 ± 0.5	19.4 ± 0.5	78.7 ± 0.5	63.1 ± 2.3	15.6 ± 0.6	16.7 ± 2.2	0.3 ± 0.3	57.01 ± 0.3	42.7 ± 0.3	63.1 ± 2.3	15.6 ± 0.6	16.7 ± 2.2	0.3 ± 0.3	57.01 ± 0.3	42.7 ± 0.3		
miR-16 inhibitor	99.3 ± 0.3	0.4 ± 0.3	0.2 ± 0.2	50.5 ± 0.2	16.7 ± 0.3	32.7 ± 0.3	98.6 ± 0.5	1.2 ± 0.7	0.3 ± 0.1	52.4 ± 0.6	18.5 ± 0.2	29.1 ± 0.7	98.6 ± 0.5	1.2 ± 0.7	0.3 ± 0.1	52.4 ± 0.6	18.5 ± 0.2	29.1 ± 0.7		
miR-16 inhibitor + cisplatin	96.3 ± 0.9	0.7 ± 0.5	2.8 ± 1.2	0.5 ± 0.02	93.3 ± 0.3	6.2 ± 0.2	88.8 ± 1.3	3.2 ± 1.0	5.1 ± 0.7	1.6 ± 0.8	77.8 ± 5.3	20.5 ± 4.7	88.8 ± 1.3	3.2 ± 1.0	5.1 ± 0.7	1.6 ± 0.8	77.8 ± 5.3	20.5 ± 4.7		

KB-3-1 and KB-CP.5 cells were transfected with siNegative control, miR-16 mimic, or miR-16 inhibitor. After 24 hrs cells were treated with cisplatin, and harvested in PBS 72 hrs post cisplatin treatment. Cells were processed for apoptosis or cell cycle analysis as described in Materials & Methods. All data is in triplicate and presented as a percent mean +/- SD