

Spotlight on Molecular Profiling

MicroRNAs modulate the chemosensitivity of tumor cells

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Abstract

MicroRNAs are strongly implicated in such processes as development, carcinogenesis, cell survival, and apoptosis. It is likely, therefore, that they can also modulate sensitivity and resistance to anticancer drugs in substantial ways. To test this hypothesis, we studied the pharmacologic roles of three microRNAs previously implicated in cancer biology (*let-7i*, *mir-16*, and *mir-21*) and also used *in silico* methods to test pharmacologic microRNA effects more broadly. In the experimental system, we increased the expression of individual microRNAs by transfecting their precursors (which are active) or suppressed the expression by transfection of antisense oligomers. In three NCI-60 human cancer cell lines, a panel of 60 lines used for anticancer drug discovery, we assessed the growth-inhibitory potencies of 14 structurally diverse compounds with known anticancer activities. Changing the cellular levels of *let-7i*, *mir-16*, and *mir-21* affected the potencies

of a number of the anticancer agents by up to 4-fold. The effect was most prominent with *mir-21*, with 10 of 28 cell-compound pairs showing significant shifts in growth-inhibitory activity. Varying *mir-21* levels changed potencies in opposite directions depending on compound class; indicating that different mechanisms determine toxic and protective effects. *In silico* comparison of drug potencies with microRNA expression profiles across the entire NCI-60 panel revealed that ~30 microRNAs, including *mir-21*, show highly significant correlations with numerous anticancer agents. Ten of those microRNAs have already been implicated in cancer biology. Our results support a substantial role for microRNAs in anticancer drug response, suggesting novel potential approaches to the improvement of chemotherapy. [Mol Cancer Ther 2008;7(1):1–9]

Introduction

The molecular genetic basis of sensitivity and resistance to cancer therapeutics is complex, involving multiple processes such as drug transport, drug metabolism, DNA repair, and apoptosis. The targets and modulators of therapy most in focus have traditionally been DNA, mRNA, and proteins. Therefore, mutations, copy number changes, and epigenetic variables at the DNA level and expression changes at the mRNA and protein levels have been widely studied to probe mechanisms that determine the pharmacologic response (1–7). Because of the ease of detection on a large scale, expression profiling has been most extensive at the mRNA level, but levels of mRNA and the encoded proteins are often not proportional. That lack of proportionality could have a number of causes, among them, the regulatory influences of microRNAs.

microRNAs (8–10) are noncoding regulatory RNAs of 21 to 25 nucleotides that are generated from larger RNA precursors. One strand of the mature double-stranded microRNA is incorporated into the RNA-induced silencing complex, which down-regulates target mRNAs by degrading them or, perhaps more commonly, by inhibiting their translation (9). microRNAs play important roles in the regulation of normal gene expression for developmental timing, cell proliferation, and apoptosis. In addition, aberrant microRNA expression is strongly implicated in cancer genesis and progression (11–30). Because many of the same biological processes are relevant to cancer chemosensitivity and chemoresistance, we hypothesized that microRNAs could broadly affect the response to anticancer drugs.

To assess the potential roles of microRNAs in cancer chemotherapy, we (31) have measured the expression levels of most known microRNAs in 60 human cancer cell lines

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(the NCI-60) used by the Developmental Therapeutics Program of the National Cancer Institute to screen >100,000 compounds as potential anticancer drug agents (1, 32). Analogous molecular profiling of mRNA had previously enabled us to identify the genes involved in drug response by assessing the correlations between mRNA expression levels and the potencies of the tested compounds (for example, see refs. 5, 33). More recently, we did a similar but preliminary assessment of the correlations between microRNA expression and compound potencies, revealing potentially relevant drug-microRNA pairs (31). To follow up on those pilot findings, we selected three microRNAs previously implicated in cancer, mir-21, let-7i, and mir-16, and tested the effect of their expression on the potencies of a number of compounds with anticancer activity. All microRNAs are thought to target multiple mRNAs that largely remain to be discovered, but those three each have at least one known target relevant to cancer biology (34); let-7i targets RAS, (16), mir-16 targets BCL2, (9) and mir-21 targets PTEN (27). However, new targets continue to be discovered (for example, TPM1 for mir-21; ref. 35), indicating that those microRNAs may have multiple mechanisms of action. mir-21, in particular, is highly expressed in various cancer types (22, 30).

microRNA functions can be cell context- and tissue-dependent. For example, inhibition of mir-21 using antisense oligonucleotides increased the growth of HeLa cells (derived from cervical cancer) but did not significantly affect the growth of A549 cells (non-small cell lung cancer; ref. 15). Chan et al. (14) showed that inhibition of mir-21 resulted in increased apoptosis in human glioblastoma cells. Previous studies have also indicated that mir-21 could play a role in drug response. For example, suppression of mir-21 expression in a cholangiocarcinoma cell line increased sensitivity to gemcitabine (27). Similarly, growth inhibition of MCF7 (breast cancer) cells by topotecan (a clinical camptothecin analogue) was increased ~40% by transfection with mir-21 antisense oligonucleotides.

To manipulate microRNA levels in selected cell lines, we transfected the cells with microRNA precursors or antisense inhibitors (15, 27, 29). We then challenged the cells with up to 14 structurally diverse chemotherapeutic agents that have various putative mechanisms of action to assess the roles of microRNAs in drug sensitivity and resistance (Fig. 1). The compounds tested included standard anticancer agents and compounds that we have tested in previous pharmacologic studies (4–7). Because different phenotypic contexts might lead to different results, we selected three cell lines, A549 (non-small cell lung), SNB-19 (central nervous system; glioma), and OVCAR-3 (ovarian) that show intermediate, high, and low mir-21 expression, respectively (31). The result was that the microRNAs each affected the potencies of various compounds, revealing a pervasive role in drug response.

To gain a broader perspective on the potential role of microRNAs in drug response, we also analyzed correlations in the NCI-60 cell panel between microRNA expression patterns and potency patterns for a large set of

compounds, applying stringent statistical criteria to control for false-positive results. Ten of 31 microRNAs that showed disproportionately large numbers of statistically significant correlations with compound activity had previously been reported as up-regulated in cancers or implicated in the biology of various cancer types.

Materials and Methods

The NCI-60 Cancer Cell Lines

Cell stocks were obtained from the National Cancer Institute Developmental Therapeutics Program and cultured under the same conditions as that used for other molecular studies of the NCI-60 at the National Cancer Institute. Cells were grown in tissue culture flasks at 37°C in 5% CO₂ in RPMI 1640 with L-glutamine and 10% fetal bovine serum. For microarray studies, cell culture and harvests were done under the standard protocol of the Genomics and Bioinformatics Group at the National Cancer Institute (36).⁷ The cells were re-fed with medium 1 day before harvest. Just prior to harvest, each flask was examined under the microscope to rule out infection or other anomalies, and the time from the incubator to stabilization of the preparation was kept to <1 min. Total RNA was extracted at ~80% confluence using TriZol (Invitrogen) according to the manufacturer's instructions. To avoid loss of low-molecular weight RNAs, the procedure used no column separation.

microRNA Transfections

All microRNA transfections and compound dose-response studies were done in triplicate. Chemically modified RNA-based anti-miR microRNA inhibitors, pre-miR microRNA precursors, and control oligomers were purchased from Ambion.⁸ Anti-miR inhibitors are chemically modified, single-stranded nucleic acids designed to bind specifically to, and inhibit, endogenous microRNA molecules (37). Pre-miR precursors are small, chemically modified double-stranded RNA molecules designed to mimic endogenous mature miRNA molecules (37). As a control oligo, we used anti-miR negative control no. 1, which is a random sequence anti-miR molecule that has been validated to produce no identifiable effects on microRNA function (37). To transfect cells with microRNA inhibitor, precursor, or control, we diluted 12.5 μL of 2 mmol/L oligomer to 100 μL with Opti-MEM (Invitrogen Corp.), diluted 10 μL of LipofectAMINE 2000 (Invitrogen) to 100 μL with Opti-MEM, mixed the transfection components, added 2 μL of diluted cell suspension containing 20,000 cells, and incubated the plate at 37°C. After 24 h, the medium was removed, and the cells were trypsinized. For the growth inhibition assay, the expression levels of the target microRNA were assessed by reverse transcription-PCR (RT-PCR) 48 h after transfection.

SDS-PAGE, Western Blotting, and Antibodies

Lysates for protein analysis of transfected A549 cells were prepared according to the manufacturer's recommendation,

⁷ <http://discover.nci.nih.gov>

⁸ <http://www.ambion.com>

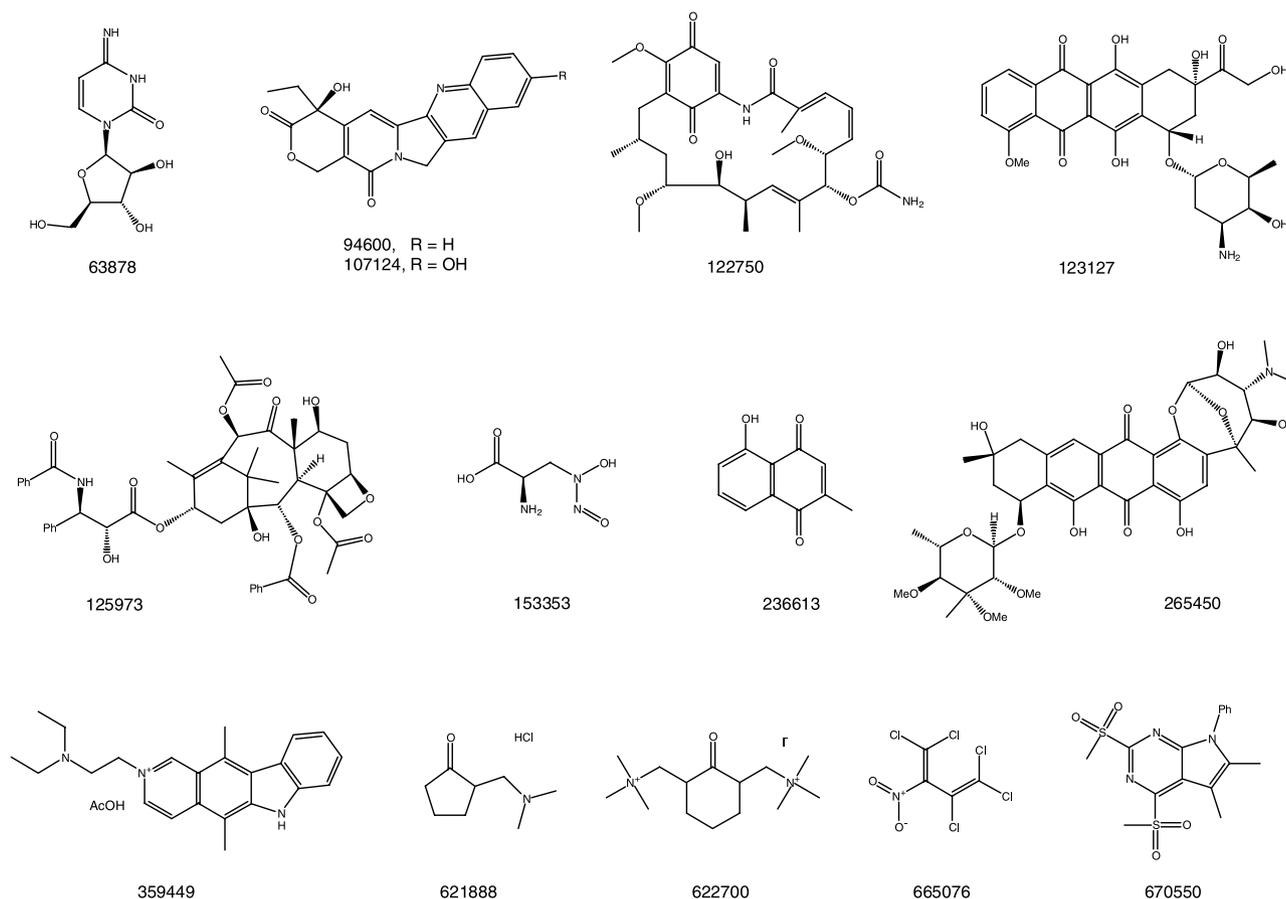


Figure 1. Structures of tested compounds. Current clinical drugs tested included cytarabine (NSC 63878), a DNA synthesis inhibitor; doxorubicin (NSC 123127), a DNA intercalator and topoisomerase II inhibitor; paclitaxel (NSC 125973), a tubulin-binding antimetabolic agent; camptothecin (NSC 94600) and 10-hydroxycamptothecin (NSC 107124), both topoisomerase I inhibitors and close analogues of the clinical agents topotecan and irinotecan; and geldanamycin (NSC 122750), an HSP-90 inhibitor and close analogue of 17-AAG, which is in clinical trials.

except that protease inhibitors (protease inhibitor cocktail; Sigma-Aldrich) were added. Protein concentrations were determined using bicinchoninic acid reagents (Sigma-Aldrich), with bovine serum albumin as the standard. SDS-PAGE and Western blotting were done according to procedures recommended for the Bio-Rad Protein III System (Bio-Rad, Inc.). Antibodies against RAS, BCL2, PTEN, actin, and tubulin were purchased from Cell Signaling Technology, and K-RAS-2A antibody was purchased from Santa Cruz Biotechnology, Inc. For Western blotting, protein (20–40 μ g) was fractionated using 8% to 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes using the Trans-Blot Cell system (Bio-Rad). The membranes (S&S, Inc.) were washed four times with 0.1% TBS containing 0.1% Triton X-100 after the first and second antibody reactions. The secondary antibody was anti-rabbit IgG-conjugated to horseradish peroxidase (Amersham Biosciences, Inc.). Membranes were incubated for 1 min facedown in enhanced chemiluminescence substrate (Amersham Biosciences). Fluorescence signals were collected by Hyperfilm XR (Amersham Biosciences) and quantified using ImageQuant software

version 5.1. Equality of the loading of wells was evaluated by Western blotting with actin and/or tubulin as the control.

Real-time Quantitative RT-PCR of mRNA and micro-RNA

Total RNA was prepared using Trizol (Invitrogen), following the manufacturer's protocol. The RNA was quantitated by spectrophotometry. One microgram of total RNA was incubated with DNase I and reverse-transcribed using Superscript II RT-PCR (Life Technologies). One microliter of the reverse transcription product was amplified using primer pairs specific for let-7i, mir-16, mir-21, N-RAS, K-RAS, H-RAS, R-RAS, BCL-2, and PTEN. ACTB (β -actin) and U6 (a small nRNA essential for pre-mRNA splicing; ref. 38) were used as controls for quantitation. Primers for the gene products were designed using Primer Express software (Applied Biosystems). The primers for β -actin were 5'-CCTGGCACCACAGACAAT-3' and 5'-GCCGATCCACACGGAGTACT-3'. The following primers, purchased from Integrated DNA Technologies, Inc., were used for N-RAS, K-RAS 2A, H-RAS, R-RAS, BCL-2, and PTEN: N-RAS, 5'-ATATTTGAGGTTTGAAAATCACTGA-3',

5'-ACTTCCATTGTATTCAAATTTGTG-3'; K-RAS, 5'-GAGTGGCACAGACACAAA-3', 5'-TCCCGGTCCTTAGTAGTGC-3'; H-RAS, 5'-TCAGCAGCCTCCCTTGTG-3', 5'-GGATGTTCAAGACAGTCTGTGC-3'; R-RAS, 5'-ACAGAGTGAGACTCCATCTCAG-3', 5'-CACTTCCCTCAA-CAGGGCGA-3'; BCL2: 5'-ACTTGTGGCTCAGATAGGC-ACCCAG-3', 5'-CGACTTCGAGATGTCCAGCCAG-3'; PTEN, 5'-CAGCCATCAAAGAGATCG-3', 5'-TTGTTCTTGATACGCCTTCAA-3'.

Primers for microRNAs were purchased from Ambion, and microRNA levels (let-7, mir-16, and mir-21) were assayed following the manufacturer's protocol. Relative transcript expression was measured using the GeneAmp 7000 Sequence Detection system (Applied Biosystems).

Growth Inhibition Assays

Drugs and compounds were obtained from Sigma-Aldrich (NSCs 63878, 94600, 107124, 122750, 125973, 236613) or from the National Cancer Institute Developmental Therapeutics Program. Cell growth and drug potency were tested using the sulforhodamine B proliferation assay (39).⁹ In each experiment, 3,000 to 5,000 cells per well were seeded in 96-well plates after transfection with microRNA oligomers and incubated for 24 h. Anticancer drugs were added in a seven-point dilution series, three replicate plate columns per treatment. After 48 h, incubation was terminated by replacing the medium with 100 μ L of 10% trichloroacetic acid (Sigma-Aldrich) in 1 \times PBS and incubating at 4 $^{\circ}$ C for at least 1 h. The plates were then washed with water, air-dried, and stained with 100 μ L of 0.4% sulforhodamine B (Sigma) in 1% acetic acid for 30 min at room temperature. Unbound sulforhodamine B was washed off with 1% acetic acid. After air-drying and resolubilization of the protein-bound dye in 10 mmol/L of Tris-HCl (pH 8.0), absorbance was read in a microplate reader at 570 nm. To determine IC₅₀ values, the absorbance of control cells without drug was set at 1, and dose-response curves were plotted using Prism software (GraphPad Software, Inc.). Each experiment was done independently at least thrice.

Results and Discussion

Manipulation of microRNA Levels

We were able to alter the cellular levels of all three microRNAs (measured by RT-PCR) by transfection of the specific microRNA inhibitor or microRNA precursor (purchased from Ambion). Regulation of selected microRNAs by the inhibitors and precursors was verified by RT-PCR, as shown in Supplementary Table S1. Transfection of let-7 inhibitor decreased the let-7i level by 4.6-fold in A549, and the let-7i precursor increased the level by 6.1-fold. The mir-16 inhibitor was the most efficient, lowering mir-16 levels by 181-fold, and the precursor elevated the level by 7.5-fold. Inhibition of mir-21 decreased mir-21 levels by 6.1-fold in A549, and the precursor elevated the

level by 12-fold. The mir-21 inhibitor/precursor pair was less efficient in OVCAR-3, altering mir-21 levels relative to control by 17-fold and 3-fold, respectively, for an overall difference of 51-fold. The pair was least effective in SNB-19, altering mir-21 levels by only 1.6-fold and 4-fold, respectively, for a 6.5-fold overall difference.

To assess the intrinsic effects of microRNAs on cell growth, we used the sulforhodamine B assay to compare the growth of A549 cells transfected with microRNA inhibitor or precursor with the growth of cells transfected with control oligonucleotide. The measurements were made 72 h after transfection; the time at which GI₅₀ values (the concentration causing 50% cell growth inhibition) for drugs were determined using the same assay. The data (from triplicate samples) were analyzed for differences by unpaired, two-tailed *t* test. Transfection with let-7i precursor or mir-16 precursor slightly reduced cell growth to ~80% of control (*P* = 0.002 and 0.008, respectively). However, none of the three microRNA inhibitors or the mir-21 precursor significantly affected cell growth (at the *P* = 0.05 level). That observation, which set the stage for

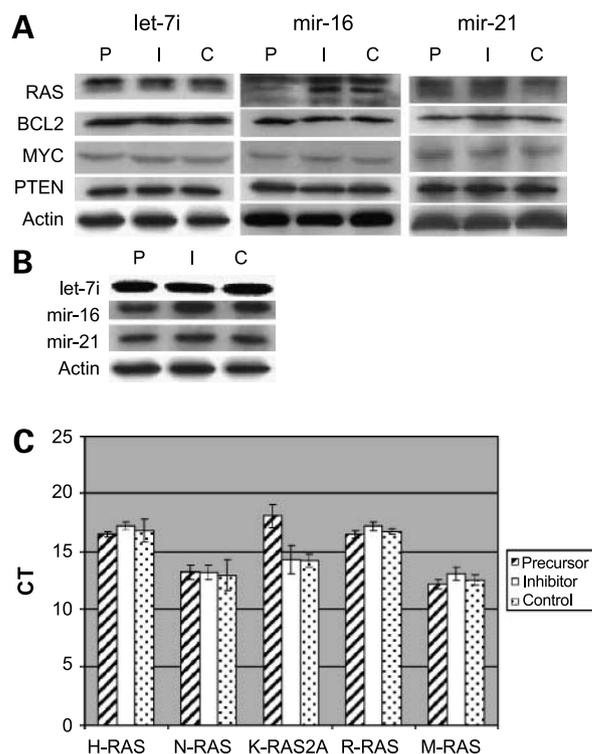


Figure 2. Western blots and quantitative RT-PCR for microRNA targets in A549 cells. **A**, Western blots for known targets of let-7 [RAS, Myc (predicted)], mir-16 (BCL2), and mir-21 (PTEN, indirectly BCL2). Actin served as the control. Gel images from A549 cells transfected with precursor (lane P), inhibitor (lane I), and control (lane C). **B**, Western blots for K-RAS-2A in A549 cells transfected with precursor, inhibitor, and control for let-7i, mir-16, and mir-21 plus actin. **C**, quantitative RT-PCR for RAS family genes in A549 cells transfected with mir-16 precursor, inhibitor, and control. Forty-eight hours after transfection, total protein and mRNA were assessed by immunoblotting and quantitative RT-PCR, respectively.

⁹ <http://www.dtp.nci.nih.gov>

Table 1. Effects on drug potency of silencing and forced expression of microRNAs in A549 cell lines

NSC	let-7i			mir-16			mir-21		
	Inhibitor	Control	Precursor	Inhibitor	Control	Precursor	Inhibitor	Control	Precursor
63878	0.91 ± 0.08	0.88 ± 0.15	0.53 ± 0.07	0.35 ± 0.08	0.49 ± 0.03	0.67 ± 0.11	0.91 ± 0.03	0.86 ± 0.03	0.38 ± 0.01
107124	0.29 ± 0.08	0.29 ± 0.04	0.30 ± 0.03	0.17 ± 0.06	0.26 ± 0.08	0.30 ± 0.03	0.40 ± 0.01*	0.40 ± 0.01*	0.37 ± 0.01*
125973	1.75 ± 0.25	1.47 ± 0.27	1.00 ± 0.06	1.61 ± 0.06	1.57 ± 0.19	1.04 ± 0.10	1.83 ± 0.12	1.59 ± 0.21	1.10 ± 0.10
236613	0.71 ± 0.13	0.86 ± 0.15	1.29 ± 0.02	1.16 ± 0.11	0.55 ± 0.12	0.36 ± 0.03	0.58 ± 0.02	1.38 ± 0.08	1.52 ± 0.12
265450	1.72 ± 0.08	1.47 ± 0.19	0.91 ± 0.06	1.87 ± 0.18	1.47 ± 0.12	1.28 ± 0.13	1.86 ± 0.14*	1.49 ± 0.05*	0.64 ± 0.02*
359449	0.76 ± 0.09	0.89 ± 0.14	1.15 ± 0.07	0.67 ± 0.06	0.65 ± 0.03	0.6 ± 0.09	0.56 ± 0.04	0.61 ± 0.03	0.75 ± 0.04
621888	0.81 ± 0.12	1.10 ± 0.07	1.19 ± 0.13	1.09 ± 0.12	1.02 ± 0.06	0.66 ± 0.06	0.66 ± 0.07	0.99 ± 0.11	1.32 ± 0.06
622700	0.65 ± 0.05	0.94 ± 0.09	1.17 ± 0.07	1.15 ± 0.10	0.81 ± 0.04	0.61 ± 0.04	0.94 ± 0.20	2.16 ± 0.08	2.27 ± 0.08
665076	0.72 ± 0.13	0.68 ± 0.08	1.17 ± 0.04	1.20 ± 0.14	0.64 ± 0.3	0.90 ± 0.07	0.89 ± 0.07	1.01 ± 0.08	1.12 ± 0.12
670550	0.76 ± 0.04	1.67 ± 0.19	1.86 ± 0.15	1.70 ± 0.09	1.30 ± 0.13	0.59 ± 0.04	0.50 ± 0.08	1.35 ± 0.10	1.95 ± 0.10

NOTE: Complete dose-response curves were run for each compound and condition (seven concentrations, each point in triplicate except where indicated). $\log_{10}(GI_{50})$ values (\pm SE) were calculated by four-variable logistic fits to the dose-response curves using Prism (GraphPad Software) and were scaled such that $1 = 0.1 \mu\text{mol/L}$. Potency data in boldface indicate entries for which three criteria were met: more than a 2-fold difference between microRNA inhibitor and precursor configurations, $P < 0.01$ for the t test, and $P < 0.01$ for the slope test. Higher $\log_{10}(GI_{50})$ values mean greater resistance to the compound.

*Replicates $n = 6$.

analysis of the experiments on drug effect, is consistent with the report by Cheng et al. (15) that inhibition of mir-16, mir-21, or members of the let-7 family tested did not significantly affect A549 cell viability.

Western Blot Assessment of Target Protein Levels

To study the effects of microRNA manipulation on known targets for let-7i, mir-16, and mir-21, we used quantitative RT-PCR and Western blots to measure mRNA and protein levels, respectively, in A549 cells. Figure 2 shows Western blots for RAS, a target of let-7 (23); BCL2, a target of mir-16 (16), indirectly regulated by mir-21 (29); PTEN, a target of mir-21 (27); and MYC, a predicted target of let-7 and mir-16. Actin was used as the control. Transfection of A549 with the mir-16 precursor caused varying degrees of reduction in RAS-related bands, including a 10-fold reduction in one of the bands (Fig. 2A). Although RAS mRNAs had not previously been identified as targets of mir-16, it seems that K-RAS 2A has a putative mir-16 target site. Use of an antibody specific for K-RAS-2A (Fig. 2B) revealed a 2-fold reduction in K-RAS-2A protein levels. Taken together, the results shown in Fig. 2 support a statistically significant reduction in RAS proteins, at least in part attributable to K-RAS-2A.

Using quantitative RT-PCR, we also measured mRNA levels (Fig. 2C) for the RAS family in A549 cells 48 h after transfection with mir-16 precursor, inhibitor, or control oligonucleotide. Cells treated with mir-16 precursor showed ~16-fold reduction in K-RAS-2A mRNA levels compared with the control. None of the other RAS family members assayed (including N-RAS, H-RAS, and R-RAS) showed significant differences as a consequence of transfection with mir-16 inhibitor or precursor.

Similarly, we did not observe any changes in protein levels for the three known microRNA-target pairs (i.e., let-7i/RAS, mir-16/BCL2, or mir-21/PTEN). These results indicate that, in the cell lines tested, the three microRNAs may regulate other target mRNAs. To determine which

mRNAs are regulated by the three microRNAs, we used microarrays to measure mRNA and microRNA profiles after treatment of A549 cells with mir-21 precursor, inhibitor, or control.

Changes in mRNA and microRNA Profiles Caused by Up-regulation or Down-regulation of mir-21

To test whether changes in mir-21 levels affect a single target or cause more pervasive changes, we measured microRNA and mRNA levels in A549 cells that had been treated with mir-21 inhibitor, precursor, or control. The expression levels of a number of microRNAs and mRNAs were systematically affected (Supplementary Tables S2 and S3), but none of the affected mRNAs are known targets of mir-21. As expected on the basis of our protein analysis (Fig. 2A), PTEN, the principal known target of mir-21, was unaffected. We conclude that mir-21 has multiple downstream effects that may combine to alter anticancer drug potencies.

Effects of microRNA Silencing and Forced Expression on Drug Potency

Table 1 documents the effects on drug potency of manipulating levels of let-7i, mir-16, and mir-21 in A549 cells. The differences in expression level between precursor-treated and inhibitor-treated cells were at least 32-fold for all three microRNAs, whereas drug potencies were affected up to 4-fold. Even relatively small 2-fold shifts in drug potency may be relevant to anticancer treatment outcomes as differential cytotoxic effects between normal and tumor cells are critical determinants. Figure 3 shows dose-response curves for NSC 265450 (nogamycin, a DNA intercalator) and NSC 670550 at the three levels of mir-21 in A549 cells (i.e., after transfection with precursor, inhibitor, or control). Transfection with the mir-21 inhibitor enhanced the potency of NSC 670550, and the precursor decreased it. In contrast, the mir-21 precursor enhanced the potency of NSC 265450, but the inhibitor had only a statistically non-significant negative effect on potency.

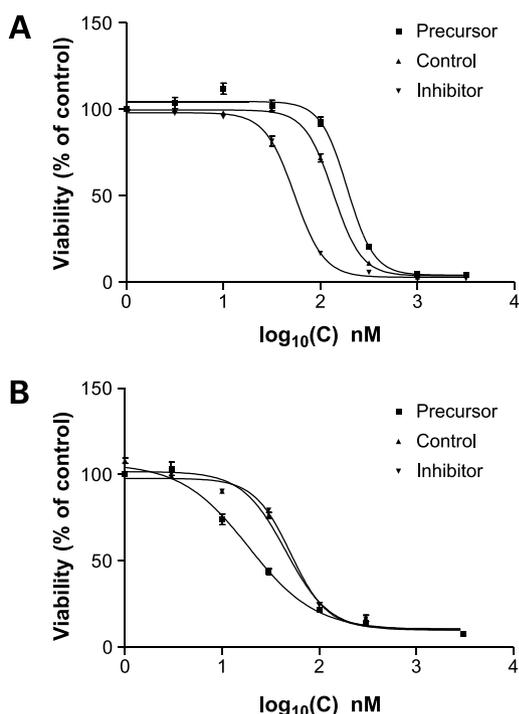


Figure 3. Dose-response curves for (A) NSC 670550 and (B) NSC 265450 in A549 cells transfected with mir-21 precursor, inhibitor, and control.

We applied two statistical tests to the replicate $\log_{10}(\text{GI}_{50})$ data for a compound-microRNA cell combination. First, we used the *t* test (unpaired, two-tailed) to compare the mean $\log_{10}(\text{GI}_{50})$ values for cells transfected with the microRNA precursor with the mean $\log_{10}(\text{GI}_{50})$ value for cells transfected with the inhibitor. *P* values for the null hypothesis of zero difference in the means were adjusted for multiple comparisons by the method of Benjamini and Hochberg (40). Second, we built a linear model with replicate $\log_{10}(\text{GI}_{50})$ values as the dependent variable and ΔC_T values from Supplementary Table S1 for the microRNA inhibitor, control, and precursor as the independent variable, in which ΔC_T is the difference in PCR cycle threshold between the test sample and U6 snRNA (internal standard). We then calculated a two-tailed *P* value for the null hypothesis in which the slope of the regression line was zero (henceforth called the "slope" test). As a third criterion for significance, we calculated the mean ratio $\log_{10}\text{GI}_{50}^{\text{prec}}/\log_{10}\text{GI}_{50}^{\text{inhib}}$ [denoted *P/I*; note the unusual sign convention: a larger positive value of $\log_{10}(\text{GI}_{50})$ means greater resistance to the compound]. We considered that microRNA level had a significant effect on compound potency in a given cell line if *P* < 0.01 for both the *t* test and the slope test, and if there was at least a 2-fold difference in mean ratio (i.e., *P/I* < 0.5 or *P/I* > 2). Figure 4 compares $\log_{10}(\text{GI}_{50})$ values in A549 cells transfected with mir-21 precursor, inhibitor, and control for the 14 compounds in Fig. 1. Using those stringent criteria, compounds for which the mir-21 level seemed to have a significant effect on potency, are indicated.

Of the three microRNAs tested, mir-21 had a significant influence on potency for the largest number of compounds; 6 of 10 compounds were significantly affected. NSC 621888, NSC 622700, and NSC 670550 showed an increase in potency with decreasing mir-21 levels. That is, mir-21 acted as a chemoresistance factor for those compounds. The compound most affected by altering mir-21 levels was NSC 670550. Inhibition of mir-21 led to a 2.7-fold increase in A549 sensitivity, and forced expression resulted in a 1.4-fold decrease, for an overall 3.9-fold difference between inhibitor and precursor. NSC 621888 and NSC 622700 showed similar trends. Decreasing the mir-21 levels resulted in 2- and 2.4-fold increases in drug sensitivity, respectively, between inhibitor- and precursor-treated cells. Those results are consistent with recent reports that mir-21 inhibition increased sensitivity of malignant cholangiocytes to gemcitabine (27) and increased the sensitivity of MCF7 cells to topotecan (29).

In contrast, the other two compounds, NSC 63878 (cytarabine, a DNA synthesis inhibitor) and NSC 265450 (for which the mir-21 level had a significant effect on compound potency) showed the opposite effect, an increase in sensitivity of A549 cells with increasing mir-21 level. The differences in sensitivity with mir-21 inhibitor and precursor were 2.4-fold for NSC 63878 and 2.9-fold for NSC 265450.

The expression levels of the other two microRNAs tested, let-7i and mir-16, had significant influence on the potencies of only 1 out of 10 and 2 out of 10 compounds tested, respectively. Inhibition of mir-16 decreased the sensitivity of A549 cells to NSC 236613 (plumbagin, an inhibitor of AKT activation; ref. 41), resulting in a 3.2-fold difference in compound potency between inhibitor and precursor. mir-16 inhibition also resulted in the decreased sensitivity of A549 cells to NSC 670550 (2.9-fold). In contrast, let-7i inhibition increased the sensitivity of A549 cells to NSC 670550 (2.5-fold). Thus, altering microRNA levels of let-7i, mir-16, or mir-21 had differing but significant influences on the potency of NSC 670550. Those observations might mean that the three microRNAs target different components of the same biological network mediating drug resistance or else that distinct networks are involved. Because the different microRNAs had disparate effects on drug activity levels, the results are consistent with complex relationships between microRNA levels and chemoresistance, perhaps involving more than one target.

Cell Line Differences in the Pharmacologic Response to Silencing and Forced Expression of mir-21

To determine whether cell lines differ in their pharmacologic responses to silencing and forced expression of microRNAs, we compared mir-21's effects in A549 cells, SNB19 glioblastoma cells, and OVCAR-3 ovarian cancer cells. SNB19 expresses high levels of mir-21 [$\log_2(\text{expression}) = 14.5$; ref. 31], and OVCAR-3 expresses low levels [$\log_2(\text{expression}) = 8.2$; ref. 31]. Table 2 documents the effects on drug potency of manipulating mir-21 levels in the three cell lines. Statistically significant compound-microRNA correlations are highlighted in boldface.

In general, the trends across the three cell lines (Table 2) were consistent. If decreased levels of mir-21 increased the sensitivity of A549 to a compound, they also increased sensitivity in the other cell lines. Doxorubicin (NSC 123127, a DNA intercalator and topoisomerase II inhibitor) was the exception; it showed a significant increase in potency (2-fold) in SNB19 as mir-21 was increased, but a decrease in potency in A549 and OVCAR3 as mir-21 was increased. That observation suggests that the relationship between microRNA levels can be dependent on the cell context, but additional studies would be necessary to test the robustness and breadth of that conclusion.

In the case of camptothecin (NSC 94600, a topoisomerase I inhibitor), mir-21 inhibition by antisense affected potency in OVCAR-3 (2.1-fold compared with control and 2.8-fold compared with precursor). The effects were similar in A549, but there was no effect in SNB19 (which expresses high baseline mir-21). The difference may have resulted from inefficient inhibition of mir-21 levels in SNB-19 (see Supplementary Table S1). Inhibition of mir-21 also increased the potency of topotecan (NSC 609699, a camptothecin analogue), as reported for MCF7 cells in a previous study (29). In contrast, manipulation of mir-21 levels had no effect on the potency of 10-hydroxycamptothecin (NSC 107124, also a topoisomerase I inhibitor).

The results in Tables 1 and 2 suggest a role for microRNAs in chemoresistance and chemosensitivity. However, the shifts in drug potency detected did not exceed 4-fold in terms of differences between cells treated with microRNA

precursor and with inhibitor. These are several possible explanations for these modest shifts in compound potency. MicroRNAs are expected to have multiple cellular targets, and many mRNAs are targeted by multiple microRNAs. Moreover, a microRNA target could be far upstream from the factors that directly affect the potency of a compound. However, even small changes in activity could make the difference between the success and failure of cancer chemotherapy.

We applied stringent criteria for identifying compound-microRNA cell combinations in which the microRNA level had a significant effect on potency. It is noteworthy that 36% (10 of 28) of the compound mir-21 pairs tested showed at least a 2-fold difference in potency between inhibitor and precursor. Even though we have tested only a relatively small number of compounds thus far (14 of them), the high percentage suggests that mir-21 levels could play a broad role in sensitivity to chemotherapeutic agents.

Analysis of Compound-microRNA Correlations

To gain a broader perspective on the potential roles of microRNAs in cancer chemotherapy, we calculated Spearman correlation coefficients across the NCI-60 between expression patterns of the 279 microRNAs and the potency patterns of the 3,089 compounds selected previously (31). In general, the six leukemias were more sensitive to cytotoxic compounds than the other cell lines were. Because a number of microRNAs were overexpressed or underexpressed in the leukemias (in comparison with the other cell types), an extreme correlation coefficient might

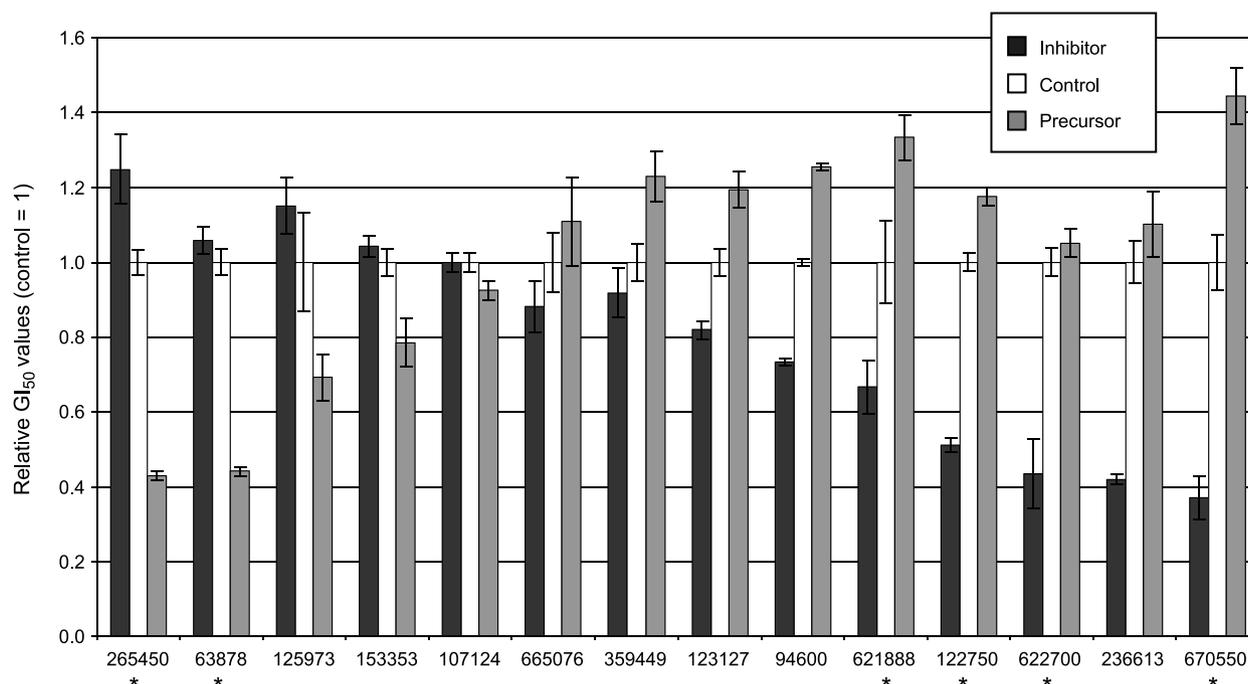


Figure 4. Comparison of drug activities in A549 cells transfected with mir-21 precursor, inhibitor, or control for compounds in Fig. 1. Columns, mean; bars, SE ($n = 3$). Values for each compound were scaled to control = 1 for comparison, and compounds were ordered by the ratio $\log_{10}GI_{50}^{prec}/\log_{10}GI_{50}^{inhib}$. The numerator and denominator are the $\log_{10}(GI_{50})$ values for precursor and inhibitor, respectively. *, compound potencies significantly affected by mir-21 levels.

Table 2. Effects on drug potency of silencing and forced expression of mir-21 in A549, OVCAR-3, and SNB-19 cell lines

NSC	A549			OVCAR-3			SNB-19		
	Inhibitor	Control	Precursor	Inhibitor	Control	Precursor	Inhibitor	Control	Precursor
94600	0.72 ± 0.01*	0.98 ± 0.01*	1.23 ± 0.01*	0.37 ± 0.09	0.78 ± 0.14	1.02 ± 0.14	0.23 ± 0.01*	0.28 ± 0.00*	0.29 ± 0.01*
122750	0.85 ± 0.03	1.66 ± 0.04	1.95 ± 0.04	0.71 ± 0.05	0.83 ± 0.03	1.03 ± 0.09	3.37 ± 0.11	8.12 ± 0.32	9.78 ± 0.64
123127	0.68 ± 0.02	0.83 ± 0.03	0.99 ± 0.04	1.1 ± 0.09*	1.41 ± 0.02*	2.13 ± 0.19*	2.33 ± 0.05*	2.17 ± 0.03*	1.17 ± 0.08*
153353	14.6 ± 0.4	14.0 ± 0.5	11.0 ± 0.9	14.7 ± 1.5	12.9 ± 0.4	12.4 ± 0.2	11.2 ± 1.6	8.63 ± 0.45	6.94 ± 0.36
236613	0.58 ± 0.02	1.38 ± 0.08	1.52 ± 0.12	1.13 ± 0.00	1.77 ± 0.01	2.15 ± 0.04	0.88 ± 0	1.44 ± 0.01	1.66 ± 0
265450	1.86 ± 0.14*	1.49 ± 0.05*	0.64 ± 0.02*	1.75 ± 0.14	1.53 ± 0.09	0.72 ± 0.01	2.07 ± 0.04	1.96 ± 0.24	1.26 ± 0.24
670550	0.50 ± 0.08	1.35 ± 0.10	1.95 ± 0.10	1.22 ± 0.01	1.67 ± 0.02	1.92 ± 0.02	1.02 ± 0	1.77 ± 0.01	2.14 ± 0

NOTE: Complete dose-response curves were run for each compound and condition (seven concentrations, each point in triplicate except where indicated). $\log_{10}(\text{GI}_{50})$ values (\pm SE) were calculated by four-variable logistic fits to the dose-response curves using Prism (GraphPad Software) and were such that $1 = 0.1 \mu\text{mol/L}$. Potency data in boldface indicate entries for which three criteria were met: more than a 2-fold difference between microRNA inhibitor and precursor configurations, $P < 0.01$ for the t test, and $P < 0.01$ for the slope test. Higher $\log_{10}(\text{GI}_{50})$ values mean greater resistance to the compound.

*Replicates $n = 6$.

have resulted from the intrinsic sensitivity of the leukemias, not from selective chemoresistance or chemosensitivity associated with microRNAs. Therefore, to put all of the cell lines on an equal footing, we shifted compound potency values so that all lines had equal means across the compounds prior to calculating compound-microRNA correlation coefficients.

Compound-microRNA correlation coefficients ranged from -0.66 to $+0.72$. There were 721 compound-microRNA correlations with raw $P < 10^{-4}$, corresponding to an approximate false discovery rate of 12%. We chose that value as a significance cutoff because it gave a good balance between the expected numbers of false-positive and false-negative correlations. Of the 721 significant correlations, 567 were positive, ranging in value from $+0.51$ to $+0.72$, and 154 were negative, ranging from -0.66 to -0.51 . None of the compound-microRNA pairs that we tested experimentally with microRNA precursor and inhibitor had statistically significant correlations by those stringent criteria.

Thirty-one microRNAs showed disproportionately large numbers of significant correlations with compound potencies. Supplementary Table S4 lists those that reached an α -level of $P = 0.01$. Importantly, 10 of the microRNAs listed in Supplementary Table S4 have previously been identified (30) as dysregulated in cancer according to the shared signatures of six solid tumor types. Included are mir-17-5p, mir-20a, mir-21, mir-24-1, mir-24-2, mir-25, mir-32, mir-92-2, mir-106a, and mir-146a.

A strong correlation between the expression pattern of a microRNA and the growth-inhibitory pattern of a drug may indicate a causal role in drug response. If the relationship is, in fact, causal, it could perhaps be exploited to improve therapy. Because the microRNAs listed above all tend to be up-regulated in cancers (30), a negative correlation with $\log_{10}(\text{GI}_{50})$ might indicate that tumor cells are more sensitive to the drug than the other cell types are. If, to the contrary, the correlation is positive, cotreatment with the microRNA inhibitor might be used to enhance drug potency or reduce toxicity.

mir-21 showed high drug correlations in the analysis, but among the compounds we tested by manipulation of mir-21 levels, only five showed a correlation level with $P < 0.01$. Of the other microRNAs that we tested experimentally, let-7i showed two significant compound correlations (the number expected by chance), whereas mir-16 showed none. Although we observed some effects on drug potency when we altered the levels of those microRNAs experimentally, the effects did not seem to have been strong enough to emerge in the computational correlation analysis. Future work will focus on the microRNAs that displayed the highest correlation values and lowest P values, to assess their role in sensitivity and resistance to anticancer agents.

References

- Weinstein JN. Spotlight on molecular profiling: 'Integromic' analysis of the NCI-60 cancer cell lines. *Mol Cancer Ther* 2006;5:2601–5.
- Dai Z, Barbacioru C, Huang Y, Sadée W. Prediction of anticancer drug potency from expression of genes involved in growth factor signaling. *Pharm Res* 2006;23:336–49.
- Dai Z, Huang Y, Sadée W. Growth factor signaling and resistance to cancer chemotherapy. *Curr Top Med Chem* 2004;4:1347–56.
- Dai Z, Huang Y, Sadée W, Blower PE. Chemoinformatics analysis identifies cytotoxic compounds susceptible to chemoresistance mediated by glutathione and cystine/glutamate transport system xc-. *J Med Chem* 2007;50:1896–906.
- Huang Y, Anderle P, Bussey KJ, et al. Membrane transporters and channels: role of the transportome in cancer chemosensitivity and chemoresistance. *Cancer Res* 2004;64:4294–301.
- Huang Y, Blower PE, Yang C, et al. Correlating gene expression with chemical scaffolds of cytotoxic agents: ellipticines as substrates and inhibitors of MDR1. *Pharmacogenomics J* 2005;5:112–25.
- Huang Y, Dai Z, Barbacioru C, Sadée W. Cystine-glutamate transporter SLC7A11 in cancer chemosensitivity and chemoresistance. *Cancer Res* 2005;65:7446–54.
- Ambros V. The functions of animal microRNAs. *Nature* 2004;431:350–5.
- Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281–97.
- He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* 2004;5:522–31.
- Calin GA, Croce CM. Genomics of chronic lymphocytic leukemia

- microRNAs as new players with clinical significance. *Semin Oncol* 2006; 33:167–73.
12. Calin GA, Croce CM. MicroRNAs and chromosomal abnormalities in cancer cells. *Oncogene* 2006;25:6202–10.
 13. Calin GA, Croce CM. MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res* 2006;66:7390–4.
 14. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res* 2005;65:6029–33.
 15. Cheng AM, Byrom MW, Shelton J, Ford LP. Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res* 2005;33:1290–7.
 16. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A* 2005;102:13944–9.
 17. Croce CM, Calin GA. miRNAs, cancer, and stem cell division. *Cell* 2005;122:6–7.
 18. Engels BM, Hutvagner G. Principles and effects of microRNA-mediated post-transcriptional gene regulation. *Oncogene* 2006;25:6163–9.
 19. Gregory RI, Shiekhattar R. MicroRNA biogenesis and cancer. *Cancer Res* 2005;65:3509–12.
 20. He L, Thomson JM, Hemann MT, et al. A microRNA polycistron as a potential human oncogene. *Nature* 2005;435:828–33.
 21. Hede K. Studies define role of microRNA in cancer. *J Natl Cancer Inst* 2005;97:1114–5.
 22. Iorio MV, Ferracin M, Liu CG, et al. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* 2005;65:7065–70.
 23. Johnson SM, Grosshans H, Shingara J, et al. RAS is regulated by the let-7 microRNA family. *Cell* 2005;120:635–47.
 24. Jovanovic M, Hengartner MO. miRNAs and apoptosis: RNAs to die for. *Oncogene* 2006;25:6176–87.
 25. Kent OA, Mendell JT. A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. *Oncogene* 2006;25:6188–96.
 26. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature* 2005;435:834–8.
 27. Meng F, Henson R, Lang M, et al. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. *Gastroenterology* 2006;130:2113–29.
 28. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 2005;435:839–43.
 29. Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY. miR-21-mediated tumor growth. *Oncogene* 2007;26:2799–803.
 30. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A* 2006;103:2257–61.
 31. Blower PE, Verducci JS, Lin S, et al. MicroRNA expression profiles for the NCI-60 cancer cell panel. *Mol Cancer Ther* 2007;6:1483–91.
 32. Shoemaker RH. The NCI60 human tumour cell line anticancer drug screen. *Nat Rev Cancer* 2006;6:813–23.
 33. Weinstein JN, Myers TG, O'Connor PM, et al. An information-intensive approach to the molecular pharmacology of cancer. *Science* 1997;275:343–9.
 34. Sethupathy P, Corda B, Hatzigeorgiou AG. TarBase: a comprehensive database of experimentally supported animal microRNA targets. *RNA* 2006;12:192–7.
 35. Zhu S, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). *J Biol Chem* 2007;282:14328–36.
 36. Shankavaram U, Reinhold WC, Nishizuka S, et al. Transcript and protein expression profiles of the NCI-60 cancer cell panel: an integrative microarray study. *Mol Cancer Ther* 2007;6:820–32.
 37. Ambion Technote; 11(6), 12(1) and 14(1) Ambion, Inc., Austin TX, <http://www.ambion.com/techlib/>.
 38. Tani T, Takahashi Y, Ohshima Y. Activity of chimeric RNAs of U6 snRNA and (–)sTRSV in the cleavage of a substrate RNA. *Nucleic Acids Res* 1992;20:2991–6.
 39. Boyd MR, Paull KD. Some practical consideration and applications of the National Cancer Institute *In vitro* Anticancer Drug Discovery Screen. *Drug Dev Des* 1995;34:91–109.
 40. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Royal Stat Soc B* 1995;57:289–300.
 41. Kuo PL, Hsu YL, Cho CY. Plumbagin induces G2-M arrest and autophagy by inhibiting the AKT/mammalian target of rapamycin pathway in breast cancer cells. *Mol Cancer Ther* 2006;5:3209–21.