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 in 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...
MicroRNAs Modulate Chemosensitivity

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). 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.

Materials and Methods

MicroRNA Transfections

All microRNA transfections and compound dose-response studies were done in triplicate. Chemically modified RNA-based anti-miR microRNA inhibitors, premiR microRNA precursors, and control oligomers were purchased from Ambion. 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,
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 μg) 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 microRNA**

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′-CCTGGCACCCAGCAAT-3′ and 5′-GCCGATCCACCGGAGTACT-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′-ATATTTGAGGTTTGAATAATCACTGA-3′.
5'-ACTTCCATTGTATTCAATTTGTG-3'; K-RAS, 5'-GACTGAGACTCCATCTCAG-3'; H-RAS, 5'-TCAGCAGCTCTCCCGTTC-3'; R-RAS, 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; R-RAS, 5'-ACAAGTGACACTCCATCTCAG-3'; 5'-CAGCTGGCACAGAGACCAAA-3'; 5'-ACTTCCATTTGTATTCAAATTTGTG-3'; 5'-GGATGTTCAAGACAGTCTGTGC-3'; 5'-ACTTGTGGCTCAGATAGGC-3'; BCL2: 5'-CGACTTCGAGATGTCCAGCCAG-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; H-RAS, 5'-ACTTCCATTTGTATTCAAATTTGTG-3'; 5'-GGATGTTCAAGACAGTCTGTGC-3'; 5'-ACTTGTGGCTCAGATAGGC-3'; BCL2: 5'-CGACTTCGAGATGTCCAGCCAG-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; K-RAS, 5'-ACTTCCATTTGTATTCAAATTTGTG-3'; 5'-GGATGTTCAAGACAGTCTGTGC-3'; 5'-ACTTGTGGCTCAGATAGGC-3'; BCL2: 5'-CGACTTCGAGATGTCCAGCCAG-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; R-RAS, 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; R-RAS, 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CTCCGGTCTCTTAGTGACTGC-3'; 5'-CAAGTGGTCAAACAGTCTGTGC-3'; 5'-CACTTTCCCTTCAA-3'.
Table 1. Effects on drug potency of silencing and forced expression of microRNAs in A549 cell lines

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<th>mir-16</th>
<th>mir-21</th>
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<tr>
<td></td>
<td>Inhibitor</td>
<td>Control</td>
<td>Precursor</td>
<td>Inhibitor</td>
</tr>
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<td>63878</td>
<td>0.91 ± 0.08</td>
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<td>107124</td>
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<td>125973</td>
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<td>1.06 ± 0.00</td>
<td>1.61 ± 0.10</td>
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<td>0.91 ± 0.06</td>
<td>1.87 ± 0.18</td>
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<td>1.15 ± 0.07</td>
<td>0.67 ± 0.06</td>
</tr>
<tr>
<td>621888</td>
<td>0.81 ± 0.12</td>
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<td>1.19 ± 0.13</td>
<td>1.09 ± 0.12</td>
</tr>
<tr>
<td>622700</td>
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<td>0.94 ± 0.09</td>
<td>1.17 ± 0.07</td>
<td>1.15 ± 0.10</td>
</tr>
<tr>
<td>665076</td>
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<td>0.68 ± 0.08</td>
<td>1.17 ± 0.04</td>
<td>1.20 ± 0.14</td>
</tr>
<tr>
<td>670550</td>
<td>0.76 ± 0.04</td>
<td>1.67 ± 0.19</td>
<td>1.86 ± 0.15</td>
<td>1.70 ± 0.09</td>
</tr>
</tbody>
</table>

NOTE: Complete dose-response curves were run for each compound and condition (seven concentrations, each point in triplicate except where indicated). \( \log_{10}(G_{50}) \) values were calculated by four-variable logistic fits to the dose-response curves using GraphPad Software and were scaled such that 1 = 0.1 \( \mu \)M/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 \( \log_{10}(G_{50}) \) values mean greater resistance to the compound.

*Replicates \( n = 6 \).

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-7i (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-7i and mir-16. Actin was used as the control. For Table 2A, the let-7i precursor enhanced the expression of K-RAS and PTEN, whereas the mir-21 precursor had no effect on these proteins. Similarly, the let-7i inhibitor reduced the expression of PTEN, whereas the mir-21 inhibitor had no effect on this protein. The results are consistent with our protein analysis, which showed a 2-fold reduction in K-RAS protein levels after transfection with the mir-21 inhibitor.

Table 2B documents the effects on drug potency of silencing and forced expression of microRNAs in A549 cell lines.

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 microRNAs were known targets of mir-21. As expected on the basis of our protein analysis, PTEN, the principal known target of mir-21, was unaffected. We conclude that mir-21 has multiple downstream effects that may contribute to its anticancer effect.

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 (nogalamycin, 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.
MicroRNAs Modulate Chemosensitivity

We applied two statistical tests to the replicate \( \log_{10}(GI_{50}) \) data for a compound-microRNA cell combination. First, we used the \( t \) test (unpaired, two-tailed) to compare the mean \( \log_{10}(GI_{50}) \) values for cells transfected with the microRNA precursor with the mean \( \log_{10}(GI_{50}) \) 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 \( \log_{10}(GI_{50}) \) values as the dependent variable and \( \Delta C_T \) values from Supplementary Table S1 for the microRNA inhibitor, control, and precursor as the independent variable, in which \( \Delta C_T \) 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 ‘slopes’ test). As a third criterion for significance, we calculated the mean ratio \( \log_{10}(GI_{50})^{\text{prec}} / \log_{10}(GI_{50})^{\text{inhb}} \) [denoted \( P/I \); note the unusual sign convention: a larger positive value of \( \log_{10}(GI_{50}) \) 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 \( \log_{10}(GI_{50}) \) 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 A549 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 different 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 [\( \log_{10}(\text{expression}) = 14.5 \); ref. 31], and OVCAR-3 expresses low levels [\( \log_{10}(\text{expression}) = 8.2 \); 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
MicroRNAs Modulate Chemosensitivity

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

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<th>NSC</th>
<th>A549</th>
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<td>Inhibitor</td>
<td>Control</td>
<td>Precursor</td>
</tr>
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<td>94600</td>
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</tbody>
</table>

NOTE: Complete dose-response curves were run for each compound and condition (seven concentrations, each point in triplicate except where indicated). log10(G0/C00) values (± SE) were calculated by four-variable logistic fits to the dose-response curves using Prism (GraphPad Software) and were such that P < 0.05 for the t test, and P < 0.01 for the slope test. Higher log10(G0/C00) 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 < 10−10, 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 log10(G0/C00) 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


Mol Cancer Ther 2008;7(1). January 2008
MicroRNAs modulate the chemosensitivity of tumor cells


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