miR-181a and miR-630 Regulate Cisplatin-Induced Cancer Cell Death

Lorenzo Galluzzi, Eugenia Morselli, Ilio Vitale, Oliver Kepp, Nicolas Servant, Caroline Paccard, Philippe Hupel, Thomas Robert, Hugues Ripoche, Emmanuel Barillot, and Guido Kroemer

Abstract

MicroRNAs (miRNA) are noncoding RNAs that regulate multiple cellular processes, including proliferation and apoptosis. We used microarray technology to identify miRNAs that were upregulated by non–small cell lung cancer (NSCLC) A549 cells in response to cisplatin (CDDP). The corresponding synthetic miRNA precursors (pre-miRNAs) per se were not lethal when transfected into A549 cells yet affected cell death induction by CDDP, C2-ceramide, cadmium, etoposide, and mitoxantrone in an inducer-specific fashion. Whereas synthetic miRNA inhibitors (anti-miRNAs) targeting miR-181a and miR-630 failed to modulate the response of A549 to CDDP, pre-miR-181a and pre-miR-630 enhanced and reduced CDDP-triggered cell death, respectively. Pre-miR-181a and pre-miR-630 consistently modulated mitochondrial/postmitochondrial steps of the intrinsic pathway of apoptosis, including Bax oligomerization, mitochondrial transmembrane potential dissipation, and the proteolytic maturation of caspase-9 and caspase-3. In addition, pre-miR-630 blocked early manifestations of the DNA damage response, including the phosphorylation of the ataxia-telangiectasia mutated (ATM) kinase and of two ATM substrates, histone H2AX and p53. Pharmacologic and genetic inhibition of p53 corroborated the hypothesis that pre-miR-630 (but not pre-miR-181a) blocks the upstream signaling pathways that are ignited by DNA damage and converge on p53 activation. Pre-miR-630 arrested A549 cells in the G0-G1 phase of the cell cycle, correlating with increased levels of the cell cycle inhibitor p27Kip1 as well as with reduced proliferation rates and resulting in greatly diminished sensitivity of A549 cells to the late S-G2-M cell cycle arrest mediated by CDDP. Altogether, these results identify miR-181a and miR-630 as novel modulators of the CDDP response in NSCLC. Cancer Res; 70(5); OF1–11. ©2010 AACR.

Introduction

Cisplatin (CDDP) is one of the principal chemotherapeutic agents used for the treatment of non–small cell lung cancer (NSCLC), a frequent and aggressive form of lung cancer that is responsible for more than 1 million deaths worldwide annually (1, 2). Usually, an initial success associated with partial responses or disease stabilization is followed by the selection of chemotherapy-resistant tumor cells, leading to chemotherapeutic failure (3, 4). Thus, the elucidation of the mechanisms of chemoresistance to CDDP may have important therapeutic implications for the management of NSCLC.

MicroRNAs (miRNA) are small noncoding RNA molecules that inhibit gene expression at the posttranscriptional level. Thus, miRNA binding to the 3′ untranslated region of target mRNAs can result in mRNA degradation or translation inhibition, depending on the degree of complementary base pairing (5). Since the discovery of lin-4 in Caenorhabditis elegans (6), more than 4,500 miRNAs have emerged as epigenetic regulators in organisms as diverse as vertebrates, flies, worms, plants, and viruses (5, 7, 8). This number is destined to grow in the future with the discovery of other miRNA-encoding loci. Recent computational estimations suggest that each miRNA regulates ~200 target mRNAs, implying that more than one third of protein-coding genes are controlled by miRNAs. It is therefore no surprise that miRNAs regulate multiple processes, including metabolism, proliferation, differentiation, development, and cell death (5). Aberrant miRNA expression has been associated with oncogenesis, and some miRNAs act as bona fide tumor suppressors, whereas others as oncogenes (9). Moreover, miRNA expression profiling may have both diagnostic and prognostic value (10).
Driven by these observations, we decided to investigate whether miRNAs play a role in the apoptotic response triggered by CDDP in human NSCLC A549 cells. Microarray analysis allowed us to identify miRNAs that are specifically upregulated by CDDP, as well as miRNAs whose levels also change in response to other proapoptotic agents such as C2-ceramide (C2-CER) and cadmium dichloride (CdCl2). Here, we describe the molecular pathways of CDDP-induced apoptosis that are regulated by miR-181a, which has previously been shown to exert tumor-suppressive functions in glioma cells (11), and by miR-630, which to the best of our knowledge has not been studied in detail before.

Materials and Methods

Chemicals, cell cultures, and transfections. Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich and cell media were from Life Technologies-Invitrogen. NSCLC A549, H1650, H1975, and HCC827 cells [American Type Culture Collection (ATCC)]; colon carcinoma HCT116 cells (kindly provided by Bert Vogelstein, Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD, routinely tested for p53 status by immunoblotting; see ref. 12); and cervical carcinoma HeLa cells (ATCC) were cultured in standard conditions (13, 14). A549 cells were transfected with a green fluorescent protein (GFP)-Bax–coding plasmid (kindly provided by Dr. Shigemi Matsuyama, Case Western Reserve University, Cleveland, OH; ref. 15), as described (16). On selection with 1 mg/mL G418, individual clones were isolated on a FACSVantage cell sorter (BD Biosciences). miRNA precursors (pre-miR) and inhibitors (anti-miR) were purchased from Ambion; control (siUNR; ref. 14) and p53-targeting (sip53) small interfering RNAs (siRNA; ref. 17) were from Sigma-Pro-ligo. A549 and HeLa cells were transfected with Oligofectamine (Invitrogen), whereas HCT116 cells were transfected with HiPerFect (Qiagen).

MicroRNAome analysis, cytofluorometry, and cell proliferation tests. Total RNA was extracted and processed as previously described (14) and then hybridized to G4470A 15K Human miRNA Microarrays (Agilent Technologies). miRNA microarrays underwent standard posthybridization processing and image acquisition (18). Apoptosis-related parameters and cell cycle distribution were assessed by means of a FACSCalibur cell sorter (BD Biosciences), miRNA precursors (pre-miR) and inhibitors (anti-miR) were purchased from Ambion; control (siUNR; ref. 14) and p53-targeting (sip53) small interfering RNAs (siRNA; ref. 17) were from Sigma-Pro-ligo. A549 and HeLa cells were transfected with Oligofectamine (Invitrogen), whereas HCT116 cells were transfected with HiPerFect (Qiagen).

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Immunoblotting and (automated) (immuno)fluorescence microscopy. A549 cells were lysed in "semireducing" (1% CHAPS, 10 mmol/L HEPES, 150 mmol/L NaCl + protease inhibitor tablets from Roche) or "fully reducing" (1% NP40, 20 mmol/L HEPES, 10 mmol/L KCl, 1 mmol/L EDTA, 10% glycerol, 1 mmol/L orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT + protease inhibitor tablets) conditions followed by standard immunoblotting (24, 25) with antibodies (from Cell Signaling Technology, Inc., unless otherwise indicated) specific for Bax (Upstate), caspase-3, caspase-9, glyceraldehyde-3-phosphate dehydrogenase (Chemicon International), p27\textsuperscript{kip1} (Oncogene Research Products), p27\textsuperscript{kip1}, phospho-p53(Ser15), phospho-p53(Ser19), p53, and Puma. Immunofluorescence microscopy was performed as previously described (18, 26), with antibodies recognizing phospho–ataxia-telangiectasia mutated (ATM; Ser\textsuperscript{1981}, Upstate) and phospho-H2AX(Ser\textsuperscript{1399}) [histone H2AX (γ-H2AX, from Trevigen). For automated fluorescence microscopy, a BD Pathway 855 High-Content Biomager (BD Biosciences) was used, followed by image processing with BD AttoVision (BD Biosciences) software. The region of interest (ROI) was defined by a 10-pixel ring mask around the nucleus. GFP-Bax granularity was evaluated by comparing the SD of the mean fluorescence intensity of groups of adjacent pixels within a ROI with the mean fluorescence intensity of the entire ROI and normalized to the granularity of mock-transfected cells. The percentage of phospho-ATM and γ-H2AX positivity was assessed by scoring cells that exhibited more than five very bright nuclear spots (DNA damage foci).

Results and Discussion

CDDP-specific changes in the microRNAome of A549 cells. NSCLC A549 cells were exposed for 24/48 hours to CDDP or two other chemicals that induce mitochondrial cell death (27), C2-CER (28) and CdCl2 (29), which we used as controls for chemotherapy-unrelated stress responses. All these agents induced classic manifestations of apoptosis, including dissipation of the mitochondrial transmembrane potential [Δψ\textsubscript{m}; detected with DiOC\textsubscript{6}(3)] and plasma membrane breakdown [monitored with propidium iodide (PI); ref. 19], with comparable dose-response curves (at 48 hours) yet slightly different kinetics (Fig. 1A, see data at 24 hours). To determine the effect of CDDP, C2-CER, and CdCl\textsubscript{2} on mRNA expression levels (by microarray technology), A549 cells were exposed for 12 hours to the LD\textsubscript{50} of these agents as determined at 24 hours (or for 24 hours to the LD\textsubscript{50} of these compounds as determined for 48 hours). This choice was driven by the consideration that exposure to the LD\textsubscript{50} of an agent for half of the time required to achieve half-maximal killing would have minor proapoptotic effects, thereby allowing for the study of microRNAome modifications linked to proapoptotic signaling pathways rather than to unspecific degradative phenomena that operate at end-stage cell death (Supplementary Table S1). Accordingly, hierarchical clustering of the most significant changes in mRNA expression levels (as determined by means of G4470A 15K Human miRNA Microarrays, which include probes for 470 human miRNAs and one-way ANOVA test) resulted in substance-specific rather than time-related profiles (Fig. 1B). Importantly,
whereas the abundance of multiple miRNAs was modulated in a similar fashion by C2-CER and CdCl2, CDDP induced a more specific pattern of modifications (Fig. 1B). In contrast to C2-CER and CdCl2, which triggered a rather generalized reduction in miRNA abundance, CDDP tended to increase the expression levels of multiple miRNAs (Fig. 1C). In line with this observation and with the results from the hierarchical clustering (Fig. 1B), C2-CER–induced and CdCl2-induced microRNAome changes presented a consistent overlap that was not shared with CDDP, which rather exhibited a relevant inducer-specific miRNA signature (Fig. 1C; Supplementary Tables S2 and S3). hsa-miR-181a and hsa-miR-630 (which we will refer to as miR-181a and miR-630) were the miRNAs that were most robustly upregulated by CDDP (Table 1; Supplementary Table S1). Whereas only CDDP induced the upregulation of miR-181a, the expression levels of...
Modulation of CDDP-induced cell death by selected miRNAs. A549 cells were transfected for 48 hours with synthetic precursors of the miRNAs (pre-miRNA) whose expression changed most intensely on CDDP administration, and then monitored for cell cycle progression and apoptosis-related parameters (Fig. 2). At this time point, some miRNA precursors slightly distorted the cell cycle distribution, although none of them induced a G2-M arrest that would be comparable with the one triggered by a sublethal dose (10 μmol/L) of CDDP (Fig. 2A). Thus, whereas pre-miR-210 and pre-miR-765 provoked a minor G2-M blockage, pre-miR-7, pre-miR-654, and, most notably, pre-miR-181a and pre-miR-630 induced a slight but significant (5–15%) increase in the percentage of A549 cells with a G0-G1 DNA content (Fig. 2B). Such cell cycle–modulatory effects were weaker in cervical cancer HeLa cells and more pronounced in colorectal carcinoma HCT116 cells, irrespective of the presence or absence of p53 (Supplementary Fig. S1). Although the administration of CDDP for 48 hours killed ∼50% of A549 cells, at the same point none of the miRNA precursors did induce significant levels of cell death (Fig. 2C and D). In contrast, in HeLa and HCT116 cells, some and most miRNAs, respectively, induced considerable cell death, although in p53−/− HCT116 cells the lethal effects of all miRNA precursors were largely reduced (Supplementary Fig. S2). Altogether, these results point to the existence of cell type–specific and p53-dependent signaling pathways that are modulated by miRNAs.

As we could not detect any lethal effects of miRNA precursors per se on A549 cells, we next studied their capacity to modulate their apoptotic response to a variety of proapoptotic compounds, including CDDP, C2-CER, CdCl2, etoposide, and mitoxantrone, all of which were administered for 48 hours at their LD50 (defined as the dose that induces plasma membrane breakdown and/or Δψm dissipation in half of the cells). Indeed, these miRNA precursors exerted profound cell death–regulatory effects when cells responded to exogenous agents (Fig. 3). When we ordered the effects of miRNA precursors on CDDP-induced cell death from the most sensitizing (left) to the most cytoprotective (right) ones and maintained the same order for the results related to the other cytotoxic agents (Fig. 3), it became obvious that each compound differs from CDDP with respect to its profile of miRNA-dependent cell death regulation. Thus, CDDP cytotoxicity was globally regulated by individual miRNAs in a fashion that did not correspond to the profiles associated with C2-CER, CdCl2, etoposide, and mitoxantrone (Fig. 3). The profiles of miRNA-mediated cell death sensitization/protection that were most closely related to each other were those of etoposide and mitoxantrone, in line with the notion that these compounds (at least partially) operate as topoisomerase II inhibitors (30, 31). Altogether, these results indicate that miRNAs can affect the apoptotic response of the same cell type to different cell death inducer in a stimulus-dependent fashion.

miRNA-mediated modulation of the caspase cascade. As miR-181a and miR-630 were the miRNAs that were most robustly upregulated by CDDP (Table 1) and both modulated the response of A549 cells to CDDP, we decided to further investigate their cell death–regulatory effects. To rule out the possibility that pre-miR-181a and pre-miR-630 might exert apoptosis-modulatory effects via nonspecific off-target effects, we used the corresponding synthetic miRNA inhibitors (anti-miRNAs) anti-miR-181a and anti-miR-630. Although anti-miRNAs alone failed to modify the response to CDDP of A549 cells, they fully abrogated the sensitizing and protective

### Table 1. miRNA regulation in NSCLC A549 cells

<table>
<thead>
<tr>
<th>Systematic name</th>
<th>CDDP</th>
<th>C2-CER</th>
<th>CdCl2</th>
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<tbody>
<tr>
<td></td>
<td>12 h</td>
<td>24 h</td>
<td>12 h</td>
</tr>
<tr>
<td>hsa-miR-630</td>
<td>+5.04 ± 0.40*</td>
<td>+2.25 ± 0.18*</td>
<td>+2.14 ± 0.17*</td>
</tr>
<tr>
<td>hsa-miR-181a</td>
<td>+2.27 ± 0.28*</td>
<td>+2.46 ± 0.31*</td>
<td>-1.06 ± 0.14</td>
</tr>
<tr>
<td>hsa-miR-194</td>
<td>+2.26 ± 0.25*</td>
<td>+1.86 ± 0.20*</td>
<td>-1.45 ± 0.16</td>
</tr>
<tr>
<td>hsa-miR-191</td>
<td>+2.21 ± 0.37*</td>
<td>+2.39 ± 0.38*</td>
<td>-1.61 ± 0.38</td>
</tr>
<tr>
<td>hsa-miR-50b</td>
<td>+1.18 ± 0.33</td>
<td>+2.11 ± 0.61</td>
<td>-1.42 ± 0.50</td>
</tr>
<tr>
<td>hsa-miR-765</td>
<td>+1.13 ± 0.10</td>
<td>-1.44 ± 0.13*</td>
<td>-1.21 ± 0.11</td>
</tr>
<tr>
<td>hsa-miR-654</td>
<td>+1.10 ± 0.14</td>
<td>-1.32 ± 0.17</td>
<td>+1.07 ± 0.13</td>
</tr>
<tr>
<td>hsa-miR-565</td>
<td>+1.29 ± 0.15</td>
<td>+1.18 ± 0.22*</td>
<td>-1.41 ± 0.17</td>
</tr>
<tr>
<td>hsa-miR-210</td>
<td>-1.38 ± 0.15</td>
<td>-1.04 ± 0.11</td>
<td>1.08 ± 0.11</td>
</tr>
<tr>
<td>hsa-miR-7</td>
<td>-1.44 ± 0.17</td>
<td>+1.05 ± 0.12</td>
<td>-2.76 ± 0.40*</td>
</tr>
<tr>
<td>hsa-miR-27b</td>
<td>-1.44 ± 0.17</td>
<td>-1.01 ± 0.12</td>
<td>-1.55 ± 0.19</td>
</tr>
<tr>
<td>hsa-miR-622</td>
<td>-1.54 ± 0.22</td>
<td>-2.47 ± 0.46*</td>
<td>-2.02 ± 0.32</td>
</tr>
</tbody>
</table>

NOTE: Values represent expression fold change (mean ± SE). The full lists of miRNAs whose expression in A549 cells is modified by the administration of CDDP, C2-CER, or CdCl2 can be found in Supplementary Table S1.

Abbreviation: hsa, Homo sapiens.

*P < 0.05, Student’s t test with Bonferroni’s adjustment.
effects of the corresponding pre-miRNA (Fig. 4A), in line with the interpretation that pre-miR-181a and pre-miR-630 act in a specific fashion. miR-181a-dependent chemosensitization could be observed in additional NSCLC cell lines (i.e., H1650, H1975, and HCC827) treated with CDDP, as well as in A549 cells challenged with the CDDP-related compounds carboplatin and oxaliplatin (Supplementary Figs. S3 and S4). Conversely, miR-630 exerted cytoprotective effects in A549 cells administered with CDDP (Fig. 4A) and carboplatin yet failed to do so in oxaliplatin-treated A549 cells.
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and in CDDP-treated H1650, H1975, and HCC827 cells (Supplementary Figs. S3 and S4).

To gain further insights into the signaling pathways that are modulated by miR-181a and miR-630, A549 cells that had previously been mock transfected or transfected with pre-miR-181a or pre-miR-630 were challenged with CDDP for 24 hours and then analyzed for hallmarks of the mitochondrial apoptotic pathway. In line with previous reports (18, 23, 26), both cells to per se sublethal doses of CDDP (Supplementary Fig. S5A and B), we found that pre-miR-181a and pre-miR-630 enhanced and reduced, respectively, the proteolytic maturation of caspase-9 and caspase-3 triggered by CDDP. Thus, whereas CDDP induced a minor yet detectable increase in the abundance of the cleaved fragments of both caspase-9 and caspase-3 in mock-transfected cells, pre-miR-181a–transfected cells responded to CDDP by massively activating caspases (which could also be monitored by decreased levels of both procaspase-9 and procaspase-3, Fig. 4B).

**miRNA-mediated modulation of the p53 system.** We then investigated possible effects of miR-181a and miR-630 on the abundance, phosphorylation status, and transcriptional activity of the master regulator of CDDP-induced cell death p53 (32). By immunoblotting with phosphoepitope-specific antibodies, we found that pre-miR-181a barely affected, whereas pre-miR-630 robustly inhibited the phosphorylation of p53 on Ser15 and Ser46 [both of which are involved in the proapoptotic accumulation of p53 (33)] triggered by CDDP in mock-transfected cells (Fig. 4B). At 24 hours, however, this effect was not paralleled by changes in the total levels of p53, nor in its global activity as a transcription factor, as assessed by monitoring the expression of the two p53 targets Puma (a proapoptotic protein from the Bcl-2 family) and p21Waf1 (a cyclin-dependent kinase 1 inhibitor; 18). However, we observed that (on CDDP treatment) pre-miR-181a–transfected and pre-miR-630–transfected cells contained slightly increased and decreased (respectively) levels of another protein that belongs to the early p53 transcriptional network (34), the proapoptotic member of the Bcl-2 family Bax (Fig. 4B). The exact reasons underlying these observations (such as a differential affinity of p53 for the promoters of Puma, Waf1, and Bax) remain elusive. Finally, both pre-miR-181a and pre-miR-630 induced, although to different extents, the upregulation of the cell cycle inhibitor p27Kip1, which in pre-miR-630–transfected (but not in pre-miR-181a–transfected) cells was further increased by CDDP (Fig. 4B).

To check whether p53 is required for the sensitizing and protective effects mediated by miR-181 and miR-630, respectively, we challenged mock-transfected or pre-miRNA–transfected A549 cells with CDDP alone or in combination with the pharmacologic p53 inhibitor cyclic pifithrin-α (PIF-α; 35; Supplementary Fig. S5A and C). Alternatively, cells were cotransfected with pre-miRNAs and either a control or a p53-targeting siRNA (Supplementary Fig. S5B and D). Both pharmacologic and siRNA-mediated inhibition of p53 partially reduced the pre-miR-181a–mediated sensitization of A549 cells to per se sublethal doses of CDDP (Supplementary Fig. S5A and B). In line with previous reports (18, 23, 26), both PIF-α and partial p53 depletion protected A549 cells against...
Figure 4. miR-181a and miR-630 mechanisms of action. A, NSCLC A549 cells were subjected to two 48-h-long rounds of transfection in mock conditions or with pre-miRNAs and anti-miRNAs for miR-181a and miR-630 (in the indicated combinations, for a total period of 96 h) followed by treatment with 50 μmol/L CDDP for 48 h and cytofluorometric quantification of apoptosis-related parameters [Δψm, measured with the cyanine derivative DiOC6(3), and plasma membrane integrity, assessed with PI]. Columns, mean percentage of cells (n = 3) characterized by Δψm dissipation alone [PI−DiOC6(3)low] or in combination with plasma membrane permeabilization [PI+DiOC6(3)low]; bars, SE. *, P < 0.05, compared with mock-transfected cells; #, P < 0.05, compared with mock-transfected CDDP-treated cells. B and C, A549 cells that had previously been mock transfected or transfected with pre-miR-181a or pre-miR-630 for 48 h were left untreated or incubated with 50 μmol/L CDDP for further 24 h followed by protein extraction in semireducing or fully reducing conditions and immunoblotting for the semiquantitative determination of the abundance and/or phosphorylation-dependent activation of the indicated proteins. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was monitored to ensure equal loading of lanes. Act, active caspase; Pro, procaspase. D, A549 cells stably expressing a GFP-Bax fusion protein were transfected as in B and C and then left untreated (Control) or incubated with 50 μmol/L CDDP for 15 h followed by fixation, nuclear counterstaining with Hoechst 33342 and automated fluorescence microscopy for the assessment of Bax aggregation. Representative microphotographs of A549 cells exhibiting diffuse (Control) and aggregated (CDDP) Bax are shown. Scale bar, 10 μm. A full-length movie showing the kinetics of redistribution of the GFP-Bax signal from a diffuse to an aggregated pattern on CDDP treatment can be found in Supplementary Video S1. Columns, mean granularity of the GFP-Bax signal (GFP-Baxgran) normalized to mock-transfected cells (n = 4); bars, SE. *, P < 0.05, compared with mock-transfected cells; #, P < 0.05, compared with mock-transfected CDDP-treated cells.
an overtly proapoptotic dose of CDDP, and an even more pronounced cytoprotection was conferred by pre-miR-630. Importantly, the effects of pre-miR-630 were not amplified by p53 inhibition (Supplementary Fig. S2B and D). Altogether, these results suggest that miR-630 protects A549 cells against CDDP by operating on the same signaling pathway than (and presumably upstream of) p53. Recently, PIF-α has been shown to protect human colon carcinoma HCT116 cells against DNA damage–induced apoptosis downstream of mitochondria and independently of p53 (36). In our experimental setting, however, the effects of PIF-α were consistently mimicked by transfection with a p53-specific siRNA, underscoring the actual contribution of p53 to pre-miR-630–mediated cytoprotection.

miRNA-mediated modulation of Bax activation. As Bax promotes cell death by forming channels in the outer mitochondrial membrane (through which the caspase-9 activator cytochrome c is released; refs. 27, 37), we determined the dimerization/oligomerization of Bax by extracting proteins in semireducing conditions followed by standard immunoblotting. Only in CDDP-treated samples could we detect one band that was compatible with Bax oligomers (i.e., exhibiting a molecular weight multiple of ~21 kDa, which characterizes monomeric Bax). The intensity of this band was significantly enhanced and diminished by pre-miR-181a and pre-miR-630, respectively (Fig. 4C, left). When the same protein extracts were subjected to fully reducing conditions followed by standard immunoblotting. In these conditions, monomeric Bax seemed slightly underrepresented in the sample corresponding to pre-miR-181a–transfected CDDP-treated A549 cells, presumably due to the persistence of higher molecular weight species (Fig. 4C, right, please note the tiny yet discernible band at a molecular weight of ~63 kDa, corresponding to oligomeric Bax). Similar results were obtained by monitoring Bax aggregation in A549 cells engineered for the stable expression of GFP-Bax. CDDP administration progressively increased the granularity of the GFP signal (indicative of Bax aggregation, as assessed by fluorescence microscopy and automated image analysis), and this was enhanced and reduced in cells that had been transfected with pre-miR-181a and pre-miR-630, respectively (Fig. 4D; Supplementary Video S1).

miRNA-mediated modulation of CDDP-mediated cell cycle arrest. Interestingly, as the observation that p27Kip1 was induced by both pre-miR-181a and pre-miR-630 (Fig. 4B), we further studied their possible effects on the cell cycle, knowing that CDDP preferentially kills proliferating cells (14). Cell cycle distributions were determined for A549 cells that had been mock, pre-miR-181a, and pre-miR-630 transfected for 72 hours and incubated during the last 24 hours of transfection with a subapoptotic concentration of CDDP, which at 24 hours induces a consistent late S-G2-M cell cycle blockage that represents the premise of (and can be qualitatively discriminated from) the exquisite G2-M arrest observed at 48 hours (Fig. 2; ref. 38). The slight G2-M blockage induced by pre-miR-181a and pre-miR-630 at 48 hours (Fig. 2) was more pronounced at 72 hours, with an increase in the percentage of cells in the G2-M phases, compared with mock transfection, of ~10% and ~20%, respectively (Supplementary Fig. S6A), which correlated with p27Kip1 levels (Fig. 4B, compare the p27Kip1 band in mock-transfected, pre-miR-181a–transfected, and pre-miR-630–transfected A549 cells not exposed to CDDP). Whereas pre-miR-181–transfected cells remained fully responsive to the late S-G2-M cell cycle blockage induced by CDDP, pre-miR-630 transfection strongly reduced both the G2-M and the S components of the arrest (Supplementary Fig. S6A). Intriguingly, whereas CDDP led to a minor yet detectable decrease in the abundance of p27Kip1 in pre-miR-181a–transfected cells (correlating with the late S-G2-M cell cycle blockage), p27Kip1 was further upregulated by CDDP on pre-miR-630 transfection (which correlated with a less pronounced decrease in the % of cells in G2-M). Thus, whereas CDDP-treated cells fail to enter/complete mitosis due to the activation of the DNA damage checkpoint (and hence accumulate in the S and G2-M phases of the cell cycle), pre-miR-181a and pre-miR-630 induce a qualitatively and quantitatively distinct response, consisting in a slight G2-M cell cycle blockage that is (at least partially) sustained by p27Kip1. We then tested growth rates by colorimetric assays. Pre-miR-630 conferred a proliferative disadvantage to A549 cells, which manifested as early as 24 hours after transfection (Supplementary Fig. S6B). Altogether, these results suggest that pre-miR-630–transfected cells are hindered from progressing through the cell cycle by upregulated p27Kip1, thereby becoming less sensitive to CDDP.

miR-630–mediated inhibition of the DNA damage response. Driven by the observation that miR-630 affects the proapoptotic activation of p53, we monitored hallmarks of the DNA damage response (by immunofluorescence microscopy) during the first 9 hours of CDDP administration, when cells still lacked any morphologic/biochemical signs of apoptosis. By using phosphopeptide-specific antibodies, we assessed the phosphorylation (on Ser139) of γ-H2AX, which forms typical nuclear foci in response to DNA damage (39). Moreover, we determined the activating phosphorylation (on Ser1581) of the DNA damage–sensing kinase ATM, which relocates to DNA damage foci and phosphorylates p53 on Ser15 (Fig. 5A and B; refs. 38, 40). Untreated cells, either mock transfected or transfected for 48 hours with pre-miRNAs, failed to display signs of DNA damage (Fig. 5C and D). In contrast, as early as 3 hours after the administration of a proapoptotic dose of CDDP (50 μmol/L), a sizeable fraction (~40%) of mock-transfected A549 cells displayed γ-H2AX aggregation and ATM activation, and this percentage progressively increased up to ~80% (at 9 hours; Fig. 5C and D). Whereas pre-miR-181a failed to modulate γ-H2AX foci induced by CDDP, pre-miR-630 reduced the fraction of γ-H2AX+ cells to about half of that recorded in mock-transfected cells. Similarly, the activation of ATM triggered by CDDP was not modified by pre-miR-181a yet attenuated by pre-miR-630 (Fig. 5C and D). These data correlate with the results from immunoblotting assessments about the activation of p53 at a later time point (24 hours), altogether...
suggesting that miR-630 inhibits p53 activation by limiting the early DNA damage response.

Concluding remarks. As shown here, a specific panel of miRNAs is upregulated by NSCLC cells in response to CDDP, and some of these miRNAs also strongly modulate this cytotoxic response. Thus, miR-181a sensitized A549 cells to the lethal action of CDDP (as well as to that of carboplatin and oxaliplatin) by stimulating Bax oligomerization and the activation of proapoptotic caspases. Conversely, miR-630 conferred robust cytoprotection against CDDP and carboplatin, (at least partially) resulting from decreased proliferation coupled to upstream inhibition of the signaling cascades that emanate from damaged DNA and converge on p53 activation. A summary of the effects of miR-181a and miR-630 on untreated and CDDP-treated A549 cells can be found in Supplementary Table S4. Apparently at odds with previous reports (11), transfection-enforced upregulation of miR-181a neither did affect the proliferation rates of A549 cells (Supplementary Fig. S6B) nor did it cause obvious apoptosis in A549, HeLa, and HCT116 cells (Fig. 2D; Supplementary Figs. S2). However, pre-miR-181a enhanced the lethality of various chemotherapeutic agents and nonspecific cell death inducers in several NSCLC cell lines (Fig. 3; Supplementary Figs. S3 and S4), lending further support to the hypothesis that miR-181a may indeed represent a bona fide tumor suppressor (11). Recently, miRNA profiling of A549 cells subjected to γ-ray-mediated DNA damage allowed for the identification of 22 miRNAs differentially modulated by low-dose γ-rays.
and high radiation intensities, which, however, included neither miR-181a nor miR-630 (41). Contrarily to miR-181a, which was upregulated by CDDP but not by Cd2-CER and CdCl2, miR-630 levels were increased in a relatively nonspecific fashion. Still, miR-630 failed to protect (and rather tended to sensitize) A549 cells from the cytotoxic effects of Cd2-CER, CdCl2, etoposide, mitoxantrone, and oxaliplatin, thereby behaving in this respect as a rather specific cell death modulator. Thus, our results suggest that miR-630 inhibits p53-regulated proapoptotic signaling pathways that are specifically ignited by CDDP and carboplatin (but not by oxaliplatin). CDDP and oxaliplatin are indeed known to elicit distinct cellular damage responses (42). The observation that anti-miR-181a and anti-miR-630 per se failed to modulate CDDP-induced cell death (Fig. 4A) suggests that these miRNAs are not strictly required for CDDP-elicited signaling pathways, although, as shown above, they do indeed regulate these responses. The miRanda algorithm13 (43) predicts 3,650 and 2,418 targets for miR-181a and miR-630, respectively, strongly suggesting that their functional effects on CDDP-induced cell death are mediated by more than one single (or a few) targets. For instance, miR-181a is predicted to downregulate several proteins whose depletion may sensitize to CDDP-induced cell death, including the essential autophagic modulator ATG5 and several members of the mitogen-associated protein kinase family. As in mammals miRNA-dependent gene regulation mostly occurs at the translational level (5), only large-scale proteomic studies will be able to clarify the contribution of each target of miR-181a and miR-630 to their cell death modulatory role. In the future, it will be interesting to determine whether miR-181a and miR-630 are upregulated in vivo and in NSCLC patients treated with CDDP, as well as whether miR-181a and miR-630 expression levels correlate with (and possibly predict) the chemotherapeutic response.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Lorenzo Galluzzi, Eugenia Morselli, Ilio Vitale, et al.

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