Inhibition of miR-17 and miR-20a by Oridonin Triggers Apoptosis and Reverses Chemoresistance by Derepressing BIM-S

Hengyou Weng, Huilin Huang, Bowen Dong, Panpan Zhao, Hui Zhou, and Lianghu Qu

Abstract

Cancer cell chemoresistance arises in part through the acquisition of apoptotic resistance. Leukemia cells resistant to chemotherapy-induced apoptosis have been found to be sensitive to oridonin, a natural agent with potent anticancer activity. To investigate its mechanisms of action in reversing chemoresistance, we compared the response of human leukemia cells with oridonin and the antileukemia drugs Ara-C and VP-16. Compared with HL60 cells, K562 and K562/ADR cells displayed resistance to apoptosis stimulated by Ara-C and VP-16 but sensitivity to oridonin. Mechanistic investigations revealed that oridonin upregulated BIM-S by diminishing the expression of miR-17 and miR-20a, leading to mitochondria-dependent apoptosis. In contrast, neither Ara-C nor VP-16 could reduce miR-17 and miR-20a expression or could trigger BIM-S-mediated apoptosis. Notably, silencing miR-17 or miR-20a expression by treatment with microRNA (miRNA; miR) inhibitors or oridonin restored sensitivity of K562 cells to VP-16. Synergistic effects of oridonin and VP-16 were documented in cultured cells as well as mouse tumor xenograft assays. Inhibiting miR-17 or miR-20a also augmented the proapoptotic activity of oridonin. Taken together, our results identify a miRNA-dependent mechanism underlying the anticancer effect of oridonin and provide a rationale for its combination with chemotherapy drugs in addressing chemoresistant leukemia cells. Cancer Res; 74(16); 4409–19. ©2014 AACR.

Introduction

Apoptosis, the most common form of programmed cell death (PCD), is critical in maintaining tissue homeostasis in multicellular organisms. Defects in apoptotic signaling pathways may result in the unlimited proliferation of cells and might ultimately lead to the development of cancer. As one of the hallmarks of cancer (1), the ability to evade apoptosis helps cancer cells escape from the host immune system and, more severely, makes them resistant to therapy, such as chemotherapy (2). For example, the K562 chronic myeloid leukemia (CML) cell line was found to be resistant to apoptosis induced by a variety of traditional chemotherapeutic agents, including cytarabine (Ara-C), etoposide (VP-16), diphtheria toxin, campothecin, and paclitaxel (3–7). Therefore, restoring the sensitivity of cancer cells to apoptosis by manipulating apoptotic signaling pathways may have great significance in improving the efficacy of cancer therapy.

Proteins of the BCL-2 family play crucial regulatory roles in mitochondria-dependent apoptosis (8–10). In this family, all members contain at least one of four BCL-2 homology (BH) domains, designated BH1 to BH4 (11). Members of the BH3-only subfamily, including BIM, BIK, BID, BAD, PUMA, NOXA, BMF, and HRK, contain only the BH3 domain and act as proapoptotic proteins (12). BIM (BCL-2 interacting mediator of cell death) was first identified in an expression screen for proteins that bind to BCL-2 (13), and since then, many isoforms resulting from alternative splicing have been found. Of these, BIM-EL (variant 1), BIM-L (variant 6), and BIM-S (variant 11) are the three main isoforms that have been studied intensively.

Recognized as one of the two BH3-only activator proteins, BIM can activate the apoptotic executor proteins BAX and BAK, leading to the release of cytochrome c from mitochondria and the subsequent initiation of apoptosis (14, 15). Increasing evidence indicates that BIM acts as a tumor suppressor, and loss of BIM function can lead to tumorigenesis and the acquisition of resistance to chemotherapy (16). Multiple levels of regulation have been found to modulate the expression and the activity of BIM in cells. For example, phosphorylation of BIM-EL and BIM-L by proteins of the MAPK family (including ERK, JNK, and MAPK14) can either enhance their apoptotic activities or promote their degradation (17–20). A class of small noncoding RNAs, called microRNAs (miRNA; miR), has also been shown to regulate the expression of BIM-EL and BIM-L.

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posttranscriptionally (21–25). Although BIM-S has been reported to be the most potent isoform in inducing apoptosis (13, 16), research on the regulation of BIM-S remains in its infancy.

Oridonin is a natural diterpenoid isolated from genus *Isodon* plants in the 1970s that has attracted increasing attention in recent years for its potential as a cancer treatment (26). In our previous mechanistic study, we found that oridonin altered the expression of MYC-regulated miRNAs in K562 cells, including miR17-92 and the let-7 family of miRNAs, and induced potent apoptosis in K562 cells (27). However, it remains unclear why oridonin can trigger apoptosis in K562 cells that are resistant to chemotherapy drugs.

With these problems in mind, we examined the different effects of oridonin and two traditional antileukemia drugs, Ara-C and VP-16, on multiple leukemia cell lines. We found that the K562 cell line and its multidrug-resistant subline K562/ADR displayed resistance to Ara-C and VP-16–induced apoptosis but were sensitive to oridonin, whereas HL60 cells were sensitive to all the three drugs. By down-regulating miR-17 and miR-20a miRNAs, oridonin upregulated BIM-S and ultimately led to mitochondria-dependent apoptosis. Furthermore, we demonstrated that inhibition of miR-17 or miR-20a restored sensitivity of K562 cells to VP-16–induced apoptosis and promoted the proapoptotic activity of oridonin.

**Materials and Methods**

**Reagents**

Oridonin was purchased from JiShi Pharmaceutical, while cytosine β-β-arabinofuranoside (Ara-C) and etoposide (VP-16) were purchased from Sigma. The three agents were dissolved in DMSO (Sigma) at a concentration of 40, 100, and 100 mmol/L, respectively, and stored at −20°C before use in cell culture experiments. The final concentration of DMSO was kept below 0.1% in all cell cultures. When used in mice, oridonin and VP-16 were dissolved in DMSO at a stock concentration of 100 mg/mL and freshly diluted with 0.09% NaCl.

**Cell culture and transfection**

K562 and HL60 cell lines were kindly provided by Dr. Shimei Zhuang (Sun Yat-sen University, Guangzhou, China) and cultured in Iscove’s Modified Dulbecco’s Medium (IMDM), while K562/ADR cell line was purchased from Bogu Biotechnology and cultured in RPMI-1640 media. The three cell lines were authenticated using short-tandem repeat (STR) profiling within the last 6 months. HL60/ADR, NB4, and Jurkat cell lines were kindly provided by Dr. Yueqin Chen (Sun Yat-sen University) and passaged for less than 3 months with RPMI-1640 media. All media were supplemented with 10% FBS, 1% glutamine, and 1% penicillin/streptomycin. For K562/ADR and HL60/ADR cells, 1 or 0.5 µg/mL of adriamycin (ADR; Sigma) was added in daily culture, respectively, and removed before experiments. Transient transfections of plasmids and RNA oligonucleotides were performed using the Neon Transfection System (Life Technologies) according to the manufacturer’s instruction.

**Expression plasmids and RNA oligonucleotides**

The coding region sequences of BIM-EL (variant 1), BIM-L (variant 6), and BIM-S (variant 11) were simultaneously amplified from K562 cDNA by PCR using the Bim-Forward (TTGGATCCGCCACATGGCAAACTTCTGATG) and Bim-Reverse (TTGAATTCTGATCAGTCTTCACCCAGG) primers. Then, the corresponding products were cloned into the pcDNA3 vector using the BamHI and EcoRI restriction sites, and the sequences were confirmed by sequencing.

siRNAs against BIM were synthesized by GenePharma. The si-Bim-1 (GCGUGUACUUGUAGAAUA) and si-Bim-2 (GCGGAGAAUCAGGUUUA) siRNAs target all isoforms of BIM, the si-Bim-EL/L (UGACACACGACCAAAACC) siRNA targets the common region of the BIM-EL and BIM-L isoforms, and the si-Bim-S (CUAGCAAGGAGGGUAAUU) siRNA targets the BIM-S isoform only.

miRNA mimics of miR-17 and miR-20a and miRNA inhibitors against all members of the miR17-19b cluster, together with their corresponding negative control RNAs, were purchased from Dharmacon.

**Cell viability and apoptosis assays**

The cells were cultured in 96-well cell culture plates and treated the next day as indicated. The effect of drugs on the number of viable cells was evaluated using MTT assays (Promega) according to the manufacturer’s instructions. Absorbance (A) was determined at 570 nm with a reference wavelength of 630 nm. The relative percentage of viable cells was calculated as follows: viable cells = (A_treatment/A_DMSO) × 100%. Inhibition rate was calculated as follows: inhibition rate = [(A_DMSO − A_VP-16)/A_DMSO] × 100%, which represents the enhanced effect of VP-16 when applied together with miRNA inhibitors or oridonin.

Apoptosis was determined by flow cytometry using the Annexin V–FITC Apoptosis Detection Kit (BioVision). The loss of mitochondrial membrane potential was examined by Rh123 staining and flow cytometry. The cells were analyzed with a FACScalibur flow cytometer (BD Biosciences) immediately after staining. The data were analyzed using FlowJo software (TreeStar).

**Immunofluorescence and confocal microscopy**

Cells with or without oridonin treatment were collected, resuspended in IMDM medium containing 200 mmol/L Mito Tracker (Life Technologies), and incubated for 15 minutes at 37°C. The next day, cells were fixed in 4% polyformaldehyde for 10 minutes, loaded onto coverslips and dried, and permeabilized with 0.2% Triton X-100 for 10 minutes before they were blocked with 5% BSA for 1 hour. Primary antibody incubations with rabbit anti-BIM (#2819; Cell Signaling Technology) or anti-BAX (#5023; Cell Signaling Technology) were performed at 4°C overnight, followed by incubation with Alexa Fluor 488–conjugated anti-rabbit IgG for 2 hours at room temperature. After incubating cells with DAPI (5 µg/mL) for 10 minutes, coverslips were mounted onto slides in antifade mounting medium (Beyotime). PBS was used for all washing steps. The stained cells were visualized using a TCS SP5 confocal microscope (Leica), and the acquired images were exported using LAS AF (Leica).
Subcellular fractionation

K562 cells with or without oridonin treatment were harvested and washed once in ice-cold PBS buffer. Mitochondria were isolated from the cytoplasm using the Cell Mitochondria Isolation Kit (Beyotime) according to the manufacturer’s instructions. Protein concentrations were determined using the BCA assay (Thermo Fisher).

Quantitative real-time PCR

Total RNA was extracted with TRIzol reagent (Life Technologies). Mature miRNA expression was detected by the StepOnePlus Real-Time PCR Systems (Applied Biosystems) using the miRCURY LNA Universal RT microRNA PCR Kit (Exiqon). U6 was used as an internal control unless otherwise specified. The data were analyzed using StepOne software V2.0 (Applied Biosystems).

Xenograft construction and drug treatment

All procedures for the experiments involving mice were approved by the Animal Care and Use Committee of Sun Yat-sen University.

For the detection of BIM-S in vivo, K562 xenograft-bearing mouse model was constructed using 5-week-old BALB/c nude mice (Sun Yat-sen University) and treated intraperitoneally with oridonin (10 mg/kg) or vehicle (2% DMSO) as previously described (27). All mice were euthanized after a 12-day treatment, and the tumors were collected and subjected to protein extraction using TRIzol reagent before Western blot analysis.

For the evaluation of drug efficacy, K562 xenograft-bearing mouse model was constructed with 7-week-old BALB/c nude mice (Guangdong Medical Laboratory Animal Center, Guangdong, China) as previously described (27). When the tumors reached a volume of 100 to 150 mm³, the mice were randomized into four groups (3 mice/group) and administered intraperitoneally with DMSO vehicle (2%), VP-16 (10 mg/kg), or oridonin (10 mg/kg) every 3 days. Tumor volumes (tumor length × width²/2) were measured with calipers every day and body weights were monitored daily.

Western blot analysis

The cells were lysed in Triton X-100–containing RIPA buffer [150 mmol/L NaCl, 1% Triton X-100, 50 mmol/L Tris–HCl (pH 7.4), 0.5% sodium deoxycholate, 10 mmol/L NaF, and 1 mmol/L Na₃VO₄] supplemented with protease inhibitor cocktail (Roche) or in buffer containing 2% SDS as described previously (27). Equal amounts of protein were loaded and separated by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore), and detected by immunoblotting using the ECL Prime Western Blotting Detection Kit (GE Healthcare). GAPDH was used as a loading control. The primary antibodies for caspase-3 (#9662), caspase-9 (#9502), PARP (#9542), BIM (#2819), BMF (#5889), PUMA (#4976), BAX (#5023), BAK (#6947), BCL-2 (#2870), MCL-1 (#4533), BCL-XL (#2764), COXIV (#4830), caspase-7 (#9492), p-JNK (#4668), JNK (#7080), p-ERK (#9101), ERK (#4695), p-BIM-EL (#4585), and GAPDH (#2118) were all purchased from Cell Signaling Technology.

Statistical analysis

The correlation between oridonin activity and the miRNA expression patterns was evaluated as previously described (27, 28). Briefly, IC₅₀ values of oridonin (NSC 637458) against the 48 NCI-60 cell lines were obtained from the Developmental Therapeutics Program screen (http://dtp.nci.nih.gov) and the drug activity was expressed as −log₁₀ IC₅₀, whereas the miRNA expression patterns were assessed using data from the Israel laboratory (29). The Pearson correlation coefficients (r), Kendall correlation coefficients (τ), and Spearman correlation coefficients (ρ) between the activity of oridonin and the gene expression levels were computed using SPSS software (SPSS Inc.) with the P values provided.

The data throughout the article are presented as the means of three independent experiments (three replicates each). Error bars indicate SD. The significant differences were assessed using Student t test. P < 0.05 was considered statistically significant.

Results

K562 and K562/ADR cells are sensitive to oridonin but not to Ara-C or VP-16

We first compared the efficacy of Ara-C, VP-16, and oridonin on K562 and K562/ADR cell lines, as well as in the drug-sensitive cell line HL60, by MTT assays. After a 24-hour treatment, the percentage of viable HL60 cells was significantly reduced by Ara-C and VP-16 in a concentration-dependent manner (P < 0.01; Fig. 1A). In contrast, K562 and K562/ADR cells were minimally affected by either Ara-C or VP-16, even at a concentration as high as 100 μmol/L. When we treated the three cell lines with oridonin, however, all these cell lines exhibited high sensitivities. In particular, treatment with 20 or 40 μmol/L oridonin resulted in a reduction in viable cells of more than 70% or 65% for the two drug-resistant cell lines, K562 and K562/ADR, respectively (Fig. 1A).

We next examined the effect of the three agents on apoptosis. FITC–Annexin V/PI double-staining showed that all three agents effectively induced apoptosis in HL60 cells, whereas oridonin displayed proapoptotic activity in K562 and K562/ADR cells (Fig. 1B). Moreover, oridonin significantly increased the proportion of Rh123-negative cells (Fig. 1C), which indicates the occurrence of mitochondria-dependent apoptosis. It should be noticed that the concentration of oridonin required for inducing obvious apoptosis in K562/ADR cells is higher than that in K562 cells (Fig. 1B and C). In contrast, both Ara-C and VP-16 failed to induce mitochondria-dependent apoptosis in K562 and K562/ADR cells as expected (Fig. 1C). These results consistently demonstrate that both K562 and K562/ADR cells exhibit different sensitivities to the three agents, being resistant to Ara-C and VP-16 but sensitive to oridonin.

Oridonin, but not Ara-C or VP-16, induces apoptosis through upregulation of BIM-S

Given the important role of BCL-2 family proteins in the process of mitochondria-dependent apoptosis, we looked for change in their expression upon treatment of K562 cells with...
the three agents. As shown in Fig. 2A, oridonin depleted the expression of pro-caspase-3 and pro-caspase-9 and decreased the expression of PARP in K562 cells, demonstrating its pro-apoptotic ability. Of the BCL-2 family proteins detected, the expression levels of the proapoptotic proteins BMF, PUMA, BAX, and BAK, as well as the antiapoptotic proteins BCL-2, MCL-1, and BCL-XL, were not affected by the three agents. However, the expression of the proapoptotic protein BIM was remarkably altered by oridonin, but not by Ara-C or VP-16 (Fig. 2A). Treatment with oridonin resulted in the occurrence of higher molecular weight bands above the BIM-EL and BIM-L isoforms and increased the expression level of the BIM-S isoform by 2.3-fold in K562 cells. The response of BIM in K562/ADR cells to these agents was very similar to that in K562 cells, with retarded bands over BIM-EL and BIM-L and an increase of BIM-S by 1.6-fold in the presence of oridonin but not Ara-C or VP-16 (Fig. 2A). Interestingly, the increase of BIM-S by oridonin was consistently observed in other leukemia cell lines, including HL60/ADR, NB4, and Jurkat (Supplementary Fig. S1). Combining these data, we hypothesized that changes in BIM expression upon oridonin treatment might be related to the apoptosis-inducing ability of oridonin.

In an attempt to understand the response of BIM to oridonin treatment, we performed immunofluorescence

Figure 1. Oridonin displays potent antiproliferative and proapoptotic effects on leukemia cells compared with Ara-C or VP-16. A, MTT assays showing inhibition of viable cells after a 24-hour treatment with different drugs as indicated. B and C, cells were treated with Ara-C (10 μmol/L), VP-16 (10 μmol/L), oridonin (20 μmol/L for K562 and HL60 and 40 μmol/L for K562/ADR), or DMSO for 24 hours, then the percentages of cells undergoing apoptosis were determined by FITC-Annexin V/PI staining and flow cytometry and shown in B, and the percentage of Rh123-negative cells are shown in C.

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staining in K562 cells. Only a fraction of the BIM pool was found to colocalize with mitochondria in K562 cells in the absence of oridonin. However, after treatment with 20 μmol/L oridonin for 24 hours, BIM colocalized very well with the aggregated mitochondria (Fig. 2B). The same localization pattern was also observed for BAX (Fig. 2B), suggesting activation of BAX. In addition, the Western blotting results supported the increase and accumulation of BIM-S at mitochondria after oridonin treatment (Fig. 2C).

Figure 2. Oridonin increases the expression and mitochondrial localization of BIM-S, which in turn contributes to the proapoptotic ability of oridonin. A, K562 and K562/ADR cells were treated with Ara-C (10 μmol/L), VP-16 (10 μmol/L), oridonin (ori; 20 μmol/L for K562 and 40 μmol/L for K562/ADR), or DMSO (CT) for 24 hours and lysed for Western blot analysis. Images showing the expression of the same protein were cropped from the same blot. The expression of BIM-S was quantified and normalized to GAPDH. B, K562 cells were treated with DMSO or oridonin (20 μmol/L) for 24 hours and immunostained before analysis by confocal microscopy. Localization of BIM (green), mitochondria (red), nuclei (blue), and BAX (green) are shown. Scale bar, 5 μm. C, mitochondria from K562 cells treated without (+) or with (-) oridonin were isolated and subjected to Western blot analysis along with the soluble cytosolic fractions. COXIV and caspase-7 are markers for mitochondria and cytosolic proteins, respectively. D, knockdown of BIM dramatically protects cells from oridonin-induced apoptosis. K562 cells were transfected with control (NC) or anti-BIM siRNA. Twenty-four hours later, cells were treated with 20 μmol/L oridonin for 24 hours and subjected to FITC–Annexin V/PI staining and flow cytometry (bottom). The specificity of the siRNAs was confirmed by Western blot analysis (top) and the expression of BIM-S was quantified and normalized to GAPDH. E, oridonin upregulates BIM-S in vivo. Top, Western blot analysis of BIM-S expression in K562 xenograft tumor samples from BALB/c nude mice treated with DMSO or oridonin. Bottom, tumor volumes (length × width² ÷ 2) were measured everyday. Results from five mice are shown for each group. **, P < 0.05; ***, P < 0.01.
These data confirm that oridonin upregulates and activates BIM-S, which was shown to possess the strongest proapoptotic activity of the BIM isoforms. When overexpressed in K562 cells, all three main isoforms of BIM (BIM-EL, BIM-L, and BIM-S) promoted apoptosis, with the percentage of apoptotic cells induced by BIM-S overexpression being the highest (Supplementary Fig. S2), consistent with previous reports (13, 16). However, when we knocked down expression of BIM-S by silencing either all BIM isoforms or the BIM-S isoform specifically, K562 cells gained partial resistance to oridonin-induced apoptosis (Fig. 2D). Therefore, expression of BIM-S contributes to the proapoptotic ability of oridonin in cellulo. In K562 xenograft models in nude mice, intraperitoneal injection of oridonin also led to increased expression of BIM-S and the delay of tumor growth (Fig. 2E).

Downregulation of miR-17 and miR-20a induced by oridonin derepresses BIM-S

As shown in Fig. 2A, upon oridonin treatment, we noticed the appearance of slower migrating bands above the BIM-EL and BIM-L isoforms in K562 and K562/ADR cells, which we presumed might be the phosphorylated forms of BIM-EL and BIM-L, respectively. Indeed, the Western blotting results showed that oridonin treatment increased the level of phosphorylated BIM-EL as well as the levels of phosphorylated JNK and ERK (Fig. 3A), which are two kinases that can phosphorylate BIM-EL and BIM-L, as mentioned above. It has been reported that proteins tagged with ubiquitin that have not been degraded are insoluble in buffers containing only nonionic detergents; however, these proteins can be solubilized in SDS-containing buffer (30, 31). As shown in Fig. 3A, the higher molecular weight bands of BIM-EL and BIM-L disappeared, and the levels of BIM-EL decreased in oridonin-treated K562 cells lysed with the nonionic detergent Triton X-100 but not in cells lysed with the ionic detergent SDS. These data suggest that activation of the JNK and ERK kinases in the presence of oridonin may promote the degradation of BIM-EL and BIM-L.

In contrast, BIM-S expression was consistently increased by oridonin, regardless of which type of detergent was used for lysing the cells (Fig. 3A). Given the potent proapoptotic activity of BIM-S and its role in oridonin-induced apoptosis, we proceeded to investigate the mechanism underlying the upregulation of BIM-S by oridonin. During our previous studies, we found that oridonin significantly downregulated the expression of the miR17-19b cluster (a truncated miR17-92 cluster, including miR-17, miR18a, miR19a, miR-20a, and miR19b). Using data from the Developmental Therapeutics Program screen, we found that the expression of these miRNAs in 48 NCI-60 cell lines correlated well with oridonin activity (Table 1). This finding suggested that these miRNAs might be the targets of oridonin and responsible for the activity of oridonin. In addition, members of the miR17-19b cluster were predicted to have potential binding sites within the 3’untranslated region of oridonin.
Taken together, these results demonstrate that knockdown of miR-17 or miR-20a with the corresponding miRNA inhibitors, and oridonin. Oridonin was used at a concentration of 10 μmol/L, which effectively downregulates miR-17 and miR-20a. "seed sequence," are likely to be the bona fide negative regulators of BIM-S because that only the inhibitors of miR-17 and miR-20a could increase BIM-S protein levels (Supplementary Fig. S4), and that miR-17 miRNA mimics could target the 3′-UTR of BIM-S directly in the luciferase reporter assays (Supplementary Fig. S5). More interestingly, we found that the expression levels of miR-17 and miR-20a were much higher in K562 and K562/ADR cells than in HL60 cells, which is inversely correlated with the expression of BIM-S protein in these cell lines (Fig. 3C). Knockdown of miR-17 or miR-20a with the corresponding miRNA inhibitor in K562 and K562/ADR cells significantly increased the expression of BIM-S, whereas ectopic expression of these miRNAs in HL60 cells reduced BIM-S protein levels (Fig. 3D). Taken together, these results demonstrate that oridonin decreases the expression of miR-17 and miR-20a and therefore derepresses BIM-S.

**Inhibition of miR-17 or miR-20a by either miRNA inhibitors or oridonin sensitizes leukemia cells to VP-16**

As observed in Figs. 3B and 2A, Ara-C and VP-16 could not reduce the expression of miR-17 and miR-20a in K562 cells, which express high levels of miR-17 and miR-20a, nor could they upregulate BIM-S protein, which might account for the resistance of K562 cells to Ara-C and VP-16. To test this possibility, we knocked down the expression of miR-17 or miR-20a in K562 cells using specific miRNA inhibitors, and evaluated the response of these cells to Ara-C or VP-16 treatment. Transfection with antagomiR-17- or antagomiR-20a did not affect the efficacy of Ara-C in K562 cells (Supplementary Fig. S6). However, treatment with VP-16 in antagomiR17- or antagomiR20a-transfected K562 cells resulted in a remarkable reduction of viable cells (Fig. 4A) and induction of apoptosis (Fig. 4B), compared with cells transfected with the control inhibitor.

Given the reduced expression of miR-17 and miR-20a in K562 cells treated with oridonin, we next examined the possibility of sensitizing K562 cells to Ara-C or VP-16 by cotreatment with oridonin. Oridonin was used at a concentration of 10 μmol/L, which effectively downregulates miR-17 and miR-20a (Fig. 4C) while having only a mild inhibitory effect on K562 cells (Fig. 1A). Similar to the effects of antagomiR17 or antagomiR20a miRNA inhibitor, treatment with oridonin dramatically enhanced the inhibitory effects of VP-16 but not Ara-C in K562 cells (Fig. 4D and E and Supplementary Fig. S7). Noticeably, when analyzed with the TargetScan program (data not shown; ref. 32), and several members of this cluster have been shown to inhibit the translation of BIM-EL and BIM-L, which share a common 3′-UTR with BIM-S (33-35). Combining all of these findings, we hypothesize that the proapoptotic efficacy of oridonin is achieved by downregulating the expression of the mir17-19b cluster and subsequently derepressing BIM-S.

### Table 1. Correlation between oridonin activity and the expression of miRNAs affected by oridonin

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<td>0.523</td>
<td>&lt;0.01</td>
<td>0.219</td>
</tr>
<tr>
<td>miR-126</td>
<td>−1.1</td>
<td>0.254</td>
<td>0.081</td>
<td>−0.04</td>
</tr>
<tr>
<td>miR-223</td>
<td>−2.23</td>
<td>0.232</td>
<td>0.112</td>
<td>0.21</td>
</tr>
</tbody>
</table>
oridonin displayed a synergistic effect with VP-16 both in vitro and in vivo, as shown by the reduced viability (Fig. 4D) and the increased apoptosis (Fig. 4E) in cultured K562 cells, as well as the delay of tumor growth in K562 xenograft-bearing mice (Fig. 4F). Therefore, inhibition of miR-17 or miR-20a by low-dose oridonin (10 μmol/L) was confirmed by qRT-PCR in C. The inhibitory effect of oridonin alone was subtracted from the final results and thus the inhibition rate of cells without VP-16 treatment was set to 0 in D. The final concentrations of oridonin and VP-16 used in E were 10 and 40 μmol/L, respectively. F, the synergistic effect of VP-16 and oridonin in inhibiting tumor growth in K562 xenograft-bearing nude mice. *, P < 0.05; **, P < 0.01; compared with DMSO vehicle-treated control.

**miRNA inhibitors of miR-17 or miR-20a enhance oridonin-induced apoptosis**

The results mentioned above indicate a crucial role of miR-17 and miR-20a in the proapoptotic activity of oridonin. Thus, we aimed to determine whether decreasing the levels of miR-17 or miR-20a artificially could enhance the anticancer activity of oridonin. Specific miRNA inhibitors were used to inhibit the expression of miR-17 or miR-20a in K562 cells before a low concentration of oridonin was added. A large decrease in the percentage of viable cells was observed in cells treated with both a miRNA inhibitor and oridonin (Fig. 5A). After treatment with 5 μmol/L oridonin for 48 hours, the relative number of viable cells transfected with antagoniR-17 or antagoniR-20a decreased to 8.3% or 20.7%, respectively, whereas 72.8% of cells transfected with a negative control remained viable (Fig. 5A). In addition, inhibition of miR-17 or miR-20a in K562 cells further increased the percentage of apoptotic cells induced by 5 μmol/L oridonin (Fig. 5B). These results indicate that lowering miR-17 or miR-20a in K562 cells enhanced the efficacy of oridonin, which provides a novel strategy for the clinical use of oridonin at a low concentration.

**Discussion**

The broad-spectrum anticancer activities of oridonin as well as the underlying mechanisms have been studied for many years, in the hope of providing evidence for the use of oridonin in clinical cancer therapy (27, 36–39). Thus far, the mechanistic studies of oridonin focused on proteins involved in many
oncomiR1, was the miR-17 and miR-20a. The miR17-92 cluster, also known as isoform shares the same 3'-UTR as the other two isoforms for the BIM-EL and BIM-L isoforms (34). Because the BIM-S 47). However, this type of regulation was mainly described for the development of B cells and several types of cancer (33, 34, 44). It is shown to be downregulated by the miR17-92 cluster in the miR17/20a-BIM-S pathway, a remarkable finding for the first time that this class of small RNAs participates in the proapoptotic activity of oridonin. By reducing the expression levels of miR-17 and miR-20a, oridonin causes derepression of the proapoptotic BIM-S protein and the subsequent commitment to mitochondria-dependent apoptosis. It seems that downregulation of miR-17 and miR-20a is essential for the induction of apoptosis by chemotherapeutic drugs because Ara-C and VP-16 cannot reduce the expression of miR-17 and miR-20a and are thereby incapable of upregulating BIM-S and triggering apoptosis in K562 cells and K562/ADR cells.

In addition to revealing the proapoptotic mechanisms of oridonin, our results support the posttranscriptional regulation of BIM-S by miRNAs of the miR17-92 cluster, specifically, miR-17 and miR-20a. The miR17-92 cluster, also known as oncomiR1, was the first oncogenic miRNA cluster to be identified in the human genome (41). By targeting different cell cycle-related proteins or tumor-suppressor proteins, miR17-92 participates in several oncogenic signaling pathways crucial for the development of cancer (42, 43). BIM is one of the targets shown to be downregulated by the miR17-92 cluster in the development of B cells and several types of cancer (33, 34, 44–47). However, this type of regulation was mainly described for the BIM-EL and BIM-L isoforms (34). Because the BIM-S isoform shares the same 3'-UTR as the other two isoforms (13, 48), we hypothesized that it was also under the regulation of miR17-92. Indeed, our results showed that miR-17 and miR-20a controlled the expression of BIM-S as well as BIM-EL and BIM-L. Nevertheless, it was worth notice that only the expression of BIM-S was upregulated in the presence of oridonin, whereas the BIM-EL and BIM-L isoforms were most likely phosphorylated by ERK or JNK and degraded. Because BIM-S does not contain the phosphorylation sites found in BIM-EL or BIM-L, it is not surprising that BIM-S has different miRNA-mediated regulation than that of BIM-EL and BIM-L upon oridonin treatment. Considering the potent proapoptotic ability of the BIM-S isoform as shown by us and other groups, it may be sufficient for oridonin to achieve its proapoptotic activity through an upregulation of BIM-S. Downregulation or loss of BIM has been linked to resistance to apoptosis induced by external stimuli (49, 50); thus, our finding that oridonin is able to induce BIM-S expression makes this natural compound a promising agent for BIM-targeted therapy.

Despite previous reports that the BCR-ABL oncoprotein contributes to resistance to chemotherapeutic-induced apoptosis in K562 cells (7, 51), the underlying mechanisms remain largely unclear. With our results, we provide a new understanding of the chemotherapeutic resistance of K562 cells as well as K562/ADR cells that involves miRNAs, namely miR-17 and miR-20a. First, the expression levels of miR-17 and miR-20a are much higher in K562 cells and especially in K562/ADR cells than in HL60 cells, and the latter cell type is more sensitive to chemotherapy. The different expression patterns of miRNAs results in relatively low expression of proapoptotic BIM-S in K562 and K562/ADR cells compared with HL60, therefore confers apoptosis-resistance to these two lines. Second, Ara-C and VP-16 could not downregulate miR-17 and miR-20a in K562 and K562/ADR cells and thus were unable to upregulate BIM-S and induce apoptosis. In contrast, oridonin significantly reduced the expression of miR-17 and miR-20a, which led to the release of BIM-S from miRNA-mediated translation inhibition and ultimately triggered mitochondria-dependent apoptosis. Third, inhibition of miR-17 or miR-20a in K562 cells reverses its resistance to VP-16. Interestingly, the miR17-92 cluster has been reported to be the downstream target of BCR-ABL and C-MYC in CML (52). Therefore, our results indicate that the BCR-ABL-MYC-miR-17-20a-BIM-S pathway is the leading cause of chemotherapy resistance of K562 and K562/ADR cells. However, it seems that regulation of the miR-17-20a-BIM-S pathway by oridonin is more generalized regardless of the presence of BCR-ABL, as supported by the data that BCR-ABL-negative cell lines, such as HL60/ADR and Jurkat, show high sensitivity to oridonin that involves regulation of the miR-17-20a-BIM-S pathway as in K562 and K562/ADR cells (Supplementary Figs. S1, S3, and S8).

Emerging evidence suggests the possibility of dealing with chemoresistance by modulating the expression of miRNAs in cancer cells. Meng and colleagues showed that inhibition of

Figure 5. miRNA inhibitors of miR-17 and miR-20a enhance oridonin-induced apoptosis. A, K562 cells were transfected with miR-17 or miR-20a inhibitor and treated with low-dose oridonin (2.5 or 5 μmol/L) 72 hours later. Cell viability was determined by MTT assays after a 24- or 48-hour treatment. B, K562 cells were transfected as described in A and treated with 5 μmol/L oridonin. Apoptosis was determined 24 hours later by FITC–Annexin V/PI staining and flow cytometry. **, P < 0.01.
miR21 and miR200b expression increased the sensitivity of malignant cholangiocytes to gemcitabine (53). In lung cancer cell lines, the expression of miR-17 was upregulated by STAT3 and thus contributed to the resistance to the MEK inhibitor AZD6244. Inhibiting miR-17 expression sensitized the resistant cells to AZD6244 by inducing the expression of BIM (54). Here, we report that inhibition of miR-17 or miR-20a expression results in the upregulation of BIM-S and therefore sensitizes leukemia cells to VP-16, suggestive of a novel approach to overcome resistance to VP-16 by combining VP-16 with miR-17 or miR-20a inhibitors. In addition, miR-17 or miR-20a inhibition by miRNA inhibitors augments the anticancer activity of oridonin, which has great significance for lowering the dose of this natural agent in clinical use to achieve better efficacy and less toxicity. As for Ara-C, inhibition of miR-17 or miR-20a has little effect in reversing the resistance of K562 cells, indicating that effectors other than BIM-S may be required for the anticancer activity of Ara-C.

Because of their ability to modulate miRNA expression (27, 55, 56), natural agents may also be used as a novel strategy in the design of combination therapies to augment traditional approaches (55). The rationale behind designing a combination therapy is to use agents with different and possibly complementary mechanisms of action to achieve a better result in terms of both safety and efficacy (57). Until now, direct evidence for the combination of miRNA-targeting natural agents with traditional chemotherapy was lacking. In this study, we showed that a combination of the natural agent oridonin with the traditional chemotherapeutic drug VP-16 could downregulate miR-17 and miR-20a and could sensitize K562 and K562/ADR cells to VP-16, thus demonstrating a synergistic effect. Furthermore, this synergistic effect can also be observed in K562 xenograft mouse model, in which use of VP-16 alone has little antitumor efficacy. From a clinical view, the use of oridonin is much more applicable than miRNA inhibitors. In addition, oridonin can simultaneously regulate the expression of miR-17 and miR-20a, therefore is more potent than the use of a single miRNA inhibitor alone. Our results provide preclinical evidence for the combined use of oridonin and VP-16 as a better cancer treatment, especially for those cancers with miR-17 and miR-20a overexpression and thus are resistant to standard chemotherapies.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

Conception and design: H. Weng, H. Huang, H. Zhou, L. Qu

Development of methodology: H. Weng, H. Huang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Weng, H. Huang, B. Deng, P. Zhao, H. Zhou, L. Qu

Analysis and interpretation of data (e.g., statistical analysis, biosstatistics, computational analysis): H. Weng, H. Huang, H. Zhou, L. Qu

Writing, review, and/or revision of the manuscript: H. Weng, H. Huang, H. Zhou, L. Qu

Study supervision: H. Zhou, L. Qu

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Inhibition of miR-17 and miR-20a by Oridonin Triggers Apoptosis and Reverses Chemoresistance by Derepressing BIM-S

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