Alterations of MicroRNA Expression Patterns in Human Cervical Carcinoma Cells (Ca Ski) toward 1'S-1'-Acetoxychavicol Acetate and Cisplatin

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Abstract

The aims of this study were to investigate the combined effects of a natural compound 1'S-1'-acetoxychavicol acetate (ACA) with cisplatin (CDDP) on HPV-positive human cervical carcinoma cell lines (Ca Ski—low cisplatin sensitivity and HeLa—high cisplatin sensitivity), and to identify microRNAs (miRNAs) modulated in response toward ACA and/or CDDP. It was revealed that both ACA and CDDP induced dose- and time-dependent cytotoxicity when used as a stand-alone agent, while synergistic effects were observed when used in combination with a combination index (CI) value of 0.74 ± 0.01 and 0.85 ± 0.01 in Ca Ski and HeLa cells, respectively. A total of 25 miRNAs were found to be significantly differentially expressed in response to ACA and/or CDDP. These include hsa-miR-138, hsa-miR-210, and hsa-miR-744 with predicted gene targets involved in signaling pathways regulating apoptosis and cell cycle progression. In conclusion, ACA acts as a chemosensitizer which synergistically potentiates the cytotoxic effect of CDDP in cervical cancer cells. The altered miRNA expression upon administration of ACA and/or CDDP suggests that miRNAs play an important role in anticancer drug responses, which can be manipulated for therapeutic purposes.

Keywords

1'-acetoxychavicol acetate, microRNA, cisplatin, cervical cancer, combination chemotherapy

Introduction

Despite cervical cancer having a good prognosis through early detection, it remains the second most common cancer and cancer-related mortality in women worldwide with an estimated 529 409 new cases and 274 883 deaths in 2008.¹ Incidence and mortality rates in developed countries were lower due to effective cervical screening and ongoing active health education programs as compared to developing countries, which accounts for 86% of cervical cancer cases worldwide.² In Malaysia, a total of 1074 cases of cervical cancer were registered by National Cancer Registry (NCR) in 2006, making it the third most common cancer in women after breast and colorectal cancer.³

Natural bioactive compounds have been extensively used in the treatment of cancer for many years either as stand-alone or as adjuvants to improve therapeutic efficacy by reducing drug-induced toxicity and drug resistance.^{4,5} One example is the naturally occurring phenylpropanoid, 1'S-1'-acetoxychavicol acetate (ACA) isolated from the wild ginger, *Alpinia conchigera*. 1'S-1'-Acetoxychavicol acetate has been shown to exhibit various biological activities such as inhibition of tumor promoter-induced Epstein-Barr virus activation⁶ and induction of apoptosis in Ehrlich ascites tumor cells through polyamine metabolism and caspase 3 activation.⁷ Furthermore, ACA was

shown to inhibit the cellular growth of myeloid leukemic cells in vitro and in vivo through induction of apoptosis via mitochondrial- and Fas-mediated dual mechanism.⁸ More recently, we have also reported that ACA induced comparable levels of dose- and time-dependent cytotoxicity on a variety of other tumor cell lines to current commercial anticancer drugs, without any adverse effects on normal cells.⁹ Despite numerous studies reporting its efficacy on cancer cells over the past years, its combined effects with other anticancer drugs as well as its effect on microRNAs (miRNAs) expression have not been reported.

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Cisplatin (CDDP) is a platinum-based anticancer drug widely used in treatment of various cancers, such as ovarian, testicular, lung, cervical as well as head and neck carcinomas.¹⁰ Even with extensive development of cisplatin analogues, only very few have entered clinical trials, with the resulting approved drugs such as carboplatin and oxaliplatin having limited advantages over cisplatin.¹¹ Although CDDP treatment results in high initial responsiveness, treatment failures due to toxicity and acquired resistance remains a major clinical problem. Hence, combination therapies with multiple drugs have been introduced over the years in the treatment of cancers to improve therapeutic efficacies through synergistic drug interaction.¹²

MiRNAs are a class of evolutionary conserved small noncoding RNAs that regulate target genes posttranscriptionally.¹³ It is postulated that a single miRNA is capable of regulating up to 100 different target mRNAs on average,^{14,15} and that each of these miRNAs play critical roles in various biological processes such as human tumorigenesis,¹⁶ cell proliferation and metabolism,¹⁷ and programmed cell death.¹⁸ As many of these biological processes are pertinent to chemosensitivity and chemoresistance, it is hypothesized that miRNAs could play an important role in modulating response toward anticancer drugs.

In this study, the combined effects of ACA/CDDP on a low CDDP-sensitive cell line (Ca Ski)¹⁹ and a CDDP-sensitive cell line (HeLa)²⁰ were investigated, and the responsive miRNAs toward these drugs were elucidated for the first time. In silico determination of gene targets corresponding to selected candidate miRNAs was also identified and a hypothetical pathway model involving miRNAs interaction with their gene targets was proposed. To date, this study describes for the first time, the linkage between miRNA expression profiles in response to both stand-alones and combination chemotherapy.

Materials and Methods

Reagents

Dulbecco modified Eagle medium (DMEM) supplemented with 4.5 g glucose/L and 300 mg/L L-glutamine was purchased from Hyclone Laboratories Inc, Logan, Utah. Fetal bovine serum, penicillin, and streptomycin were purchased from Lonza Inc, Basel, Switzerland. Cisplatin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) reagents were acquired from EMD Chemicals Inc. Keratinocyte serum-free medium (KSFM) and TRIzol Reagent were obtained from Invitrogen, Grand Island, New York. 1'S-1'-Acetoxychavicol acetate was provided by the Centre for Natural Product Research and Drug Discovery (CENAR), Department of Chemistry, University of Malaya, Malaysia.

Cell Lines and Culture Conditions

Human cervical carcinoma cells Ca Ski (HPV-16 positive) and HeLa (HPV-18 positive) were obtained from University Malaya Medical Centre (UMMC, Malaysia) and Institute of

Biological Science, University of Malaya, respectively. Immortalized nasopharyngeal epithelial cells (NP69), used as a normal cell control, were obtained from Cancer Research Initiatives Foundation (CARIF, Malaysia). Ca Ski and HeLa cells were cultured in DMEM, supplemented with 10.0% (v/v) fetal bovine serum, 100.0 U/mL penicillin, and 100.0 µg/mL streptomycin while NP69 cells were cultured in KSFM, supplemented with 5.0% (v/v) fetal bovine serum. All cultures were maintained at 37°C in 5.0% CO₂ and 95.0% relative humidity.

The MTT Cell Viability Assay

The cytotoxic effects of ACA and CDDP on Ca Ski and HeLa cells were determined using the MTT assay. Cells were plated in at 1.0×10^4 cells/well, incubated overnight, and treated with either stand-alone ACA, stand-alone CDDP, or ACA in combination with CDDP at various concentrations and time. Following incubation, 20.0 µL MTT reagent (5.0 mg/mL) was added into each well and incubated in the dark at 37°C for 2 hours. Media containing excess MTT reagent was aspirated and DMSO was added to dissolve the purple formazan precipitates. Results were obtained using microtiter plate reader (Tecan Sunrise, Switzerland), which detects absorbance wavelength at 570 nm with a reference wavelength at 650 nm. The combined effects of ACA and CDDP were evaluated using isobologram and combination index (CI) values to determine the interactions between ACA and CDDP. CI > 1.0 indicates an antagonistic interaction, CI = 1.0 indicates an additive interaction, and CI < 1.0 indicates a synergistic interaction between 2 drugs.²¹

MicroRNA Microarray

Total RNA from cells treated with ACA and/or CDDP for 120 minutes was isolated using TRIzol reagent and analyzed using Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California) according to the manufacturer's protocol. The miRNA microarray was performed using the FlashTag Biotin RNA Labeling Kit for Affymetrix GeneChip miRNA array (Genisphere, Hatfield, Pennsylvania) and GeneChip miRNA array (Affymetrix, Santa Clara, California) according to the manufacturer's protocol. In brief, approximately 1.0 µg of total RNA underwent a tailing reaction followed by ligation of the biotinylated signal molecule to the target RNA sample. The labeled RNA was subsequently used for hybridization on chips containing 46 228 probes, representing over 6703 miRNA sequences (71 organisms) from the Sanger miRNA database v11. Statistical and gene expression analyses of triplicate arrays were performed using Partek Genomics Suite 6.5 (Partek Inc, St Louis, Missouri).

Quantitative Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Quantitative real-time reverse transcriptase–polymerase chain reaction (qRT-PCR) analysis of miRNA expression

Figure 1. Comparative dose-response curves for stand-alone (A) ACA and (B) CDDP in Ca Ski cells as observed using MTT assays. (C) Combined cytotoxic effect of ACA and CDDP on Ca Ski cells after 24 hours of exposure. Results were expressed as percentage of total viable cells, and data were presented as mean $\pm$ standard deviation of 3 independent replicates. ACA indicates 1'-I'-acetoxychavicol acetate; CDDP, cisplatin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide.

was carried out using TaqMan MicroRNA assay (Applied Biosystems, Carlsbad, California) according to the manufacturer's protocol. The RT reactions contained 5.0 ng of total RNA samples, 2.0 μL stem-loop RT primer, 1.0 μL 10 $\times$ RT buffer, 0.1 μL deoxyribonucleotide triphosphate (dNTP; 100 mmol/L), 0.67 μL MultiScribe Reverse Transcriptase (50 U/ μL), and 0.13 μL RNase inhibitor (20 U/ μL). Polymerase chain reactions contained 0.67 μL RT product, 5.0 μL 2 $\times$ TaqMan Fast Advanced Master Mix, and 0.5 μL TaqMan 20 $\times$ Primer Probe Assay. Reactions were incubated at 50°C for 2 minutes, 95°C for 20 seconds, followed by 95°C for 3 seconds, and 60°C for 20 seconds for 40 cycles using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California) and analyzed using Bio-Rad CFX Manager v1.6 (Bio-Rad Laboratories). U6 small nuclear RNA was used as an internal control to normalize RNA input. Fold changes were calculated using the $2^{-\Delta\Delta C_t}$ method,²² and presented as

fold-expression changes relative to untreated controls after normalization to endogenous controls.

Bioinformatic Analyses of miRNA Gene Targets

The putative gene targets of miRNAs were predicted using TargetScanHuman v5.2.²³ Predicted target genes with total context scores <0 were then selected for gene-annotation enrichment analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7.²⁴

Statistical Analysis

All experiments were carried out in triplicates and presented as mean values $\pm$ standard deviation. Student's *t* test was used to determine the statistical significance of results, where a *P* value of $\leq .05$ was considered significant. Pearson's correlation coefficient (*r*) value was used to determine the association between miRNA microarray and qRT-PCR data.

Figure 2. Cytotoxic effects of both ACA and CDDP on normal cell controls. (A) Comparative dose–response curves in NP69 cells as observed using MTT assays after 24 hours of exposure. (B) Solvent-derived cytotoxicity of DMSO on Ca Ski, HeLa, and NP69 cells after 24 hours of exposure. Results were expressed as percentage of total viable cells, and data were presented as mean $\pm$ SEM of 3 independent replicates. (C) Photomicrograph of Ca Ski and HeLa cells before and after exposure toward respective ACA and CDDP synergistic combination regime for 24 hours ($\times 100$ magnification). Apoptosis-mediated cell death is observed by closed white arrows indicating the occurrence of nuclear condensation. ACA indicates l'-S-l'-acetoxychavicol acetate; CDDP, cisplatin.

Results

Both ACA and CDDP Induced Dose- and Time-Dependent Cytotoxicity

The MTT cell viability assays were first carried out to determine the effects of ACA and CDDP in Ca Ski and HeLa cells when used as a stand-alone agent. Cells were treated with various concentrations of ACA (0–60 $\mu\text{mol/L}$)

or CDDP (0–200 $\mu\text{mol/L}$) for 12 to 48 hours. Results showed that both ACA and CDDP induced dose- and time-dependent cytotoxicity in Ca Ski cells and HeLa cells (data not shown). The IC_{50} values for Ca Ski upon ACA and CDDP exposure for 24 hours were 6.0 ± 0.15 $\mu\text{mol/L}$ and 53.3 ± 0.64 $\mu\text{mol/L}$, respectively (Figure 1A and B), while the IC_{50} values for HeLa were 21.9 ± 0.49 $\mu\text{mol/L}$ and 41.8 ± 0.49 $\mu\text{mol/L}$, respectively. Cell viability in NP69

Figure 3. Combined effects of ACA and CDDP on cervical cancer cells as obtained through MTT assays. Isobologram IC₅₀ analysis indicating a synergistic relationship between ACA and CDDP in (A) Ca Ski cells and (B) HeLa cells. (C) Summary of combination index (CI) values representing various ACA and CDDP combination treatment regimes. C1: Simultaneous treatment with ACA at constant concentration for 24 hours; C2: Simultaneous treatment with CDDP at constant concentration for 24 hours; C3: Sequential 12-hour pretreatment with ACA followed by CDDP for 24 hours; C4: Sequential 12-hour pretreatment with CDDP followed by ACA for 24 hours. All data were presented as mean values ± SEM of 3 independent replicates. ACA indicates 1'S-1'-acetoxychavicol acetate; CDDP, cisplatin; SEM, standard error of the mean.

cells was maintained at 75% and 40% following treatment with the highest concentration of ACA and CDDP, respectively (Figure 2A). Solvent-derived cytotoxicity from DMSO did not yield a significant reduction in Ca Ski, HeLa, and NP69 cell viability, where the viability levels were maintained above 80% (Figure 2B). Microscopic

visualization of nuclear condensation in ACA/CDDP-treated cervical cancer cells also served as a very important hallmark, indicating the occurrence of apoptosis-mediated cell death (Figure 2C).

The ACA Potentiates the Effects of CDDP Through Chemosensitization

We next investigated the combined effects of ACA and CDDP in Ca Ski and HeLa cells to determine whether ACA could enhance the efficacy of CDDP. It was found that a 12-hour pretreatment of ACA followed by CDDP exposure further reduced the viability of Ca Ski cells synergistically in comparison to stand-alone exposures (Figure 1C). Both isobologram and CI analyses also showed that significant synergistic relationships were observed when cells were treated with ACA and CDDP simultaneously (Ca Ski and HeLa) over 24 hours, with CI values between 0.74 ± 0.01 and 0.87 ± 0.02 (Figure 3A and B). However, a shift toward an antagonistic relationship in Ca Ski and HeLa cells (CI value = 1.25 ± 0.05 and 1.11 ± 0.03 , respectively) was observed when a sequential 12-hour pretreatment with CDDP was followed by ACA for 24 hours (Figure 3C). Together, these results suggest that ACA acts as a potential chemosensitizer, which sensitizes Ca Ski cells toward apoptosis through undetermined mechanisms prior to CDDP exposure, an observation which was less notable in HeLa cells.

Alterations in miRNA Expression Patterns by ACA and/or CDDP Treatment

To evaluate the effects of ACA and/or CDDP on miRNA expression in the less CDDP-susceptible Ca Ski cell line, miRNA microarray was used to determine the global miRNA expression profiles following administration of ACA and/or CDDP. A total of 25 miRNAs were found to be significantly differentially expressed in response to ACA and/or CDDP. Among these, 15 miRNAs were upregulated, while 10 miRNAs were downregulated relative to untreated controls. Pattern of miRNA expression between different treatment regimes was markedly different, with the exception of hsa-miR-212 and hsa-miR-210 which showed similar increasing expression patterns in both ACA- and CDDP-treated Ca Ski cells, and ACA in combination with CDDP-treated Ca Ski cells, respectively (Table 1). Data validation also indicated a highly positive correlation between miRNA microarray and qRT-PCR data with a correlation coefficient value, r of .842 and r^2 of .709 (Figure 4A and B). When qRT-PCR on 4 selected dysregulated miRNAs in Ca Ski cells was compared to their expression levels in HeLa cells, it was observed that 2 miRNAs showed similar expression pattern changes (hsa-miR-210 and -224), whereas hsa-miR-138 and -744 did not (Figure 4C). This probed us to further evaluate the roles of these miRNAs in the regulation of their gene targets governing apoptosis following drug exposures.

Table 1. List of miRNA Expression Fold Change Alterations Following the Administration of ACA and/or CDDP on Ca Ski Cells Over 2 hours.^a

miRNA	miRBase Accession No.	Fold Change ^b	P Value ^c
Treatment: Stand-alone ACA			
hsa-miR-629	MIMAT0004810	2.55 ± 1.12	.010
hsa-miR-212	MIMAT0000269	1.63 ± 0.45	.039
hsa-miR-487a	MIMAT0002178	1.60 ± 0.41	.018
hsa-miR-483-3p	MIMAT0002173	1.56 ± 0.41	.026
hsa-miR-342-3p	MIMAT0000753	1.55 ± 0.22	.004
hsa-miR-376a	MIMAT0000729	1.52 ± 0.13	.015
hsa-miR-1262	MIMAT0005914	-1.53 ± 0.10	.004
hsa-miR-875-3p	MIMAT0004923	-1.60 ± 0.13	.007
hsa-miR-517*	MIMAT0002851	-1.69 ± 0.12	.032
hsa-miR-411	MIMAT0003329	-1.99 ± 0.22	.031
Treatment: Stand-alone CDDP			
hsa-miR-210	MIMAT0000267	2.41 ± 1.32	.047
hsa-miR-1244	MIMAT0005896	2.34 ± 1.32	.024
hsa-miR-663	MIMAT0003326	2.14 ± 0.91	.040
hsa-miR-720	MIMAT0005954	2.07 ± 0.92	.036
hsa-miR-513c	MIMAT0005789	1.77 ± 0.65	.024
hsa-miR-212	MIMAT0000269	1.65 ± 0.52	.032
hsa-miR-134	MIMAT0000447	-1.66 ± 0.14	.047
hsa-miR-337-3p	MIMAT0000754	-1.85 ± 0.11	.031
hsa-miR-130b	MIMAT0000691	-3.40 ± 0.21	.040
Treatment: Combination ACA + CDDP			
hsa-miR-138	MIMAT0000430	2.13 ± 1.09	.049
hsa-miR-744	MIMAT0004945	2.11 ± 1.15	.045
hsa-miR-210	MIMAT0000267	2.02 ± 0.83	.033
hsa-miR-523	MIMAT0002840	1.68 ± 0.44	.044
hsa-miR-922	MIMAT0004972	1.67 ± 0.55	.026
hsa-miR-1271	MIMAT0005796	-1.80 ± 0.06	.002
hsa-miR-224	MIMAT0000281	-1.81 ± 0.20	.048
hsa-miR-21*	MIMAT0004494	-1.87 ± 0.20	.046

Abbreviations: ACA, 1'S-1'-acetoxychavicol acetate; CDDP, cisplatin; miRNA, microRNA; SD, standard deviation.

^a Experiments were performed using Affymetrix GeneChip miRNA arrays followed by data analysis using the Partek Genomics Suite 6.5 Software. Data shown is a mean $\pm$ SD representation of 3 independent biological replicates.

^b Positive values denote upregulation, whereas negative values denote downregulation relative to untreated controls.

^c P values $\leq .05$ were considered significant.

* Denotes miRNA star/minor product.

Predicted Targets of Selected miRNAs Affected by ACA and/or CDDP Treatment

As hsa-miR-138, hsa-miR-210, and hsa-miR-744 exhibited the highest fold change in combination chemotherapy, they were selected for bioinformatic analyses to determine their interaction with their putative gene targets. The putative gene targets of these candidate miRNAs were predicted using TargetScan-Human v5.2, and predicted target genes with total context score <0 were then selected for gene-annotation enrichment analysis using DAVID v6.7 for tumorigenesis and cancer-related pathways. Key signaling pathways involved include the wingless-type MMTV integration site family (WNT) pathway, nuclear factor kappa B (NF- κ B) pathway, both extrinsic and intrinsic pathways, transforming growth factor- β (TGF- β) pathway,

Figure 4. Correlation between miRNA microarray data and qRT-PCR data. (A) Pearson correlation coefficient value, r was .842 with an r^2 of .709, indicating a positive correlation between both sets of data. (B) A total of 5 significantly differentially expressed miRNAs were selected for validation and expressed as normalized fold change expression values. (C) Normalized fold expression comparison of 4 shortlisted miRNAs between Ca Ski and HeLa cells via qRT-PCR upon exposure to ACA and CDDP combinations. All experiments were carried out in triplicates. ACA indicates 1'S-1'-acetoxychavicol acetate; CDDP, cisplatin; miRNA, microRNA; qRT-PCR, quantitative real-time reverse transcriptase-polymerase chain reaction.

hypoxia pathway, and calcium (Ca^{2+}) pathway. Since miRNAs are negative gene regulators, these highlighted gene targets are expected to be downregulated by these upregulated miRNAs. Based on this, a hypothetical network illustrating the interaction between these 3 miRNAs with their specific gene targets was generated, describing the drug-inducing effects of ACA and CDDP on Ca Ski and HeLa cells (Figure 5), with a complete list of miRNA targets summarized in Table 2.

Discussion

To date, topotecan and CDDP combinations are one of the few regimens that demonstrate an improved overall survival, progression-free survival, and response rate compared to single-agent CDDP.^{25,26} Hence, there is an ongoing effort to identify not only new drug combinations but also other therapeutic strategies including the use of miRNAs to ameliorate chemotherapy in cervical cancer. In this study, the cytotoxic effects of ACA and/or

CDDP were first assessed using MTT cell viability assays, and results showed that synergistic effects were observed when cells were treated with ACA and CDDP simultaneously as well as sequentially over 24 hours. Earlier studies on ACA revealed a rapid decrease in intracellular levels of glutathione (GSH),²⁷ which prevents resistance through CDDP inactivation, enhanced DNA repair, and reduced CDDP-induced oxidative stress.²⁸ Furthermore, studies have shown that ACA inhibits activation of NF- κ B and NF- κ B-regulated gene expression such as cyclin D, c-Myc, survivin, inhibitors of apoptosis, B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xL), Bcl-2-related protein A1, and FLICE-like inhibitory proteins.²⁹ Thus, inhibition of these proliferative and antiapoptotic gene products together with a decrease in GSH intracellular levels would theoretically augment CDDP efficacy. The shift toward antagonistic effects in sequential 12-hour pretreatment with CDDP followed by ACA for 24 hours corroborates with our notion, indicating that the chemosensitizing effect of Ca

Figure 5. A hypothetical network of signaling pathways illustrating the interaction of miRNAs and their predicted targets following ACA/CDDP drug exposures in Ca Ski cells. Key signaling pathways involved were predicted to include the TGF- β , Wnt, NF- κ B, hypoxia, calcium, both intrinsic and extrinsic signaling pathways, and cell cycle regulatory elements. Putative mRNA targets of 3 differentially expressed miRNAs were generated using TargetScanHuman v5.2. Predicted target genes with total context scores <0 were then selected for gene-annotation enrichment analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7. Orange colored symbols denote downregulated targets, whereas clear symbols denote targets unaffected by miRNAs. Inhibitory relationships were denoted as flat arrow heads, whereas positive interactions were denoted as open arrow heads. ACA indicates 1'S-I'-acetoxychavicol acetate; CDDP, cisplatin; miRNA, microRNA; mRNA, messenger RNA; TGF- β , transforming growth factor- β ; Wnt, wingless-type MMTV integration site family; NF- κ B, nuclear factor kappa B.

Ski cells by CDDP was absent in comparison to ACA. The inability to obtain significantly synergistic CDDP/ACA relationships in HeLa cells compared to Ca Ski cells was expected since HeLa cells had originally displayed a higher susceptibility toward stand-alone CDDP, making it relatively difficult to identify new synergistic drug partners, which can further improve its existing high CDDP efficacy. Even though HeLa and Ca Ski cells are of similar cervical origin, variations in specific HPV strains present may also contribute toward the alteration of gene expression governing drug sensitivity, resulting in disparities between both cell lines.

Here, we hypothesized that different treatment regimens would exhibit different miRNA expression patterns following different mechanisms of action in ACA and CDDP. Of the 25 miRNAs identified, several have been previously associated with response to chemotherapy. For example, overexpression of miR-212 induced sensitivity toward cetuximab in head and lung squamous cell carcinoma³⁰; whereas downregulation of

miR-342 was associated with tamoxifen resistance in breast cancer cell lines.³¹ It was also reported that silencing of miR-130b promotes multidrug resistance, whereas its overexpression increases sensitivity toward CDDP and taxol in ovarian cancer.³² Dysregulation of other miRNAs such as miR-134 and miR-138 have also been shown to augment the sensitivity of lung tumor cells toward CDDP exposure and subsequent apoptosis.^{33,34} Since our miRNA microarray data showed a directional correspondence to these earlier studies, this indicates that these miRNAs could also play a role in modulating response toward ACA and/or CDDP. Furthermore, the markedly different miRNA expression profile between different treatment regimens showed that specific clusters of miRNAs are, indeed, modulated in response toward ACA and/or CDDP and, thus, can be manipulated for future therapeutic advantages in cervical cancer drug combination treatment options.

Since ACA is able to synergistically potentiate the cytotoxic effects of CDDP when used in combination, pathway analysis

Table 2. Summary of miRNAs Dysregulated in Ca Ski and HeLa Cells Following ACA and CDDP Exposure, and their Putative Targets as Obtained Using TargetScan Human v5.2 and DAVID v6.7 Prediction Software.^a

MiRNA Targets	Description	MiRNAs
ERK signaling		
PDGF	Platelet-derived growth factor	hsa-miR-744
PDGFR	Platelet-derived growth factor receptor	hsa-miR-138, -744, -224
Sos	Son of sevenless homolog	hsa-miR-138, -224
Ras	Rat sarcoma	hsa-miR-224
Raf	Rapidly accelerated fibrosarcoma	hsa-miR-138
MEK	Mitogen-activated protein kinase kinase	hsa-miR-744
ERK	Extracellular signal-regulated kinase	hsa-miR-210, -224
Calcium signaling		
PMCA	Plasma membrane Ca ²⁺ ATPase	hsa-miR-210, -744
NCX	Sodium-calcium exchanger	hsa-miR-210
ROC	Receptor-operated calcium channel	hsa-miR-744
CaV	Voltage-gated calcium channel	hsa-miR-210, -744
TGF- β signaling		
TGF- β 3	Transforming growth factor beta 3	hsa-miR-224
Smad 2/3/4	Mothers against decapentaplegic homolog 2/3/4	hsa-miR-138, -210, -744, -224
p300	E1A-binding protein p300	hsa-miR-138
p15	Cyclin-dependent kinase inhibitor 2B	hsa-miR-138
p21	Cyclin-dependent kinase inhibitor 1A	hsa-miR-224
Cyclin D	Cyclin D	hsa-miR-138, -224
CDK 4/6	Cyclin-dependent kinase 4/6	hsa-miR-138, -744, -224
Cyclin E	Cyclin E	hsa-miR-138
E2F	E2 transcription factor	hsa-miR-138, -210, -224
WNT pathway		
Wnt	Wingless-type MMTV integration site family	hsa-miR-138, -744, -224
Dsh	Disheveled	hsa-miR-138
Fzd	Frizzled receptor	hsa-miR-138, -224
Tcf/Lef	T cell factor/lymphoid enhancer factor	hsa-miR-138, -744
Intrinsic pathway		
Bcl-2	B-cell lymphoma 2	hsa-miR-138, -224
Bad	Bcl-2-agonist death promoter	hsa-miR-744
Bid	BH3 interacting-domain death agonist	hsa-miR-224
Casp 3	Caspase 3	hsa-miR-138, -224
Casp 9	Caspase 9	hsa-miR-224
Survivin	Survivin	hsa-miR-138
NF- κ B pathway		
TRAF2	Tumor necrosis factor receptor-associated factor 2	hsa-miR-138
TRAF4	Tumor necrosis factor receptor-associated factor 4	hsa-miR-224
IKK	Inhibitor of nuclear factor kappa-B kinase	hsa-miR-138, -224
Hypoxia pathway		
HIF- α	Hypoxia-inducible factor, α subunit	hsa-miR-138
HIF- β	Hypoxia-inducible factor, β subunit	hsa-miR-138, -224
p300/CBP	E1A-binding protein p300/CREB-binding protein	hsa-miR-138

Abbreviations: ACA, 1'S-I'-acetoxychavicol acetate; CDDP, cisplatin; miRNA, microRNA; DAVID, Database for Annotation, Visualization and Integrated Discovery.

^a MiRNA targets were categorized according to specific pathways relevant to cancer, apoptosis, and drug resistance/susceptibility.

on 3 primary miRNA candidates (hsa-miR-138, hsa-miR-210, and hsa-miR-744) affected with the highest fold change during combination chemotherapy was evaluated. It was revealed that many members of the extracellular signal-regulated kinase (ERK) signaling cascade were being targeted by these miRNAs, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), fms-related tyrosine kinase 3 ligand (FLT3LG), PDGF receptor (PDGFR), son of sevenless (Sos), and Raf and MAP/ERK kinase (MEK). The ERK is a subfamily of mitogen-activated

protein kinases (MAPKs), which regulates cell proliferation and differentiation,³⁵ and whose activation has been associated with cervical cancer.³⁶ It was reported previously that activation of ERK signaling pathway confers survival signals, which counteracts proapoptotic signals activated by c-Jun N-terminal kinases and p38-signaling pathway, the other 2 subfamilies in MAPK.³⁷ Therefore, inactivation of this pathway by miRNAs may lead to increased levels of apoptosis. Despite previous reports indicating that CDDP exposure activates ERK signaling, inhibition of this cascade could in fact

enhance CDDP-based cytotoxicity, suggesting that CDDP-induced ERK activation provided the cells partial protection against CDDP.^{38,39} Our pathway analysis also revealed protein kinase C (PKCs) and calcium channel proteins among the putative targets of these 3 miRNAs, consistent with past reports linking them to CDDP-induced ERK activation.³⁹ Collectively, all these targets have highlighted the ERK-signaling cascade as the most affected pathway following ACA and CDDP exposure through the negative regulation of hsa-miR-138, hsa-miR-210, and hsa-miR-744.

Another pathway found to be affected by these miRNAs is the TGF- β signaling pathway, whereby mutations or disruptions in this pathway have been linked to carcinogenesis.⁴⁰ TGF- β mediates cell cycle arrest by activating Smad2/3/4 complex, which then interacts with p300 to induce the expression of p15 proteins responsible for cyclin D-cdk4/6 complex inactivation and a G₀/G₁ cell cycle arrest.^{41,42} Our in silico pathway analysis not only describes the above proteins as direct putative miRNA targets but also other cell cycle regulatory targets such as cyclin E and E2F. Additionally, we also identified several targets comprising members of the WNT pathway such as Wnt, dishevelled (Dsh), and the Tcf/Lef transcription factor family, which governs the expression of cyclin D⁴³ and c-Myc.⁴⁴ These observations suggest that treatment with ACA in combination with CDDP would lead to cell cycle arrest at G₀/G₁ phase, consistent with previous studies on ACA's cell cycle-arresting effects in oral cancer cell lines.⁹

Targets involved in the mitochondrial/intrinsic pathway of apoptosis were not spared as well, with several miRNA targets including Bcl-2 and Bcl-2-agonist death promoter (Bad) being among those downregulated following ACA and CDDP exposure. Even though Bad is commonly known as a proapoptotic protein, it actually commands a dual role in apoptosis.^{45,46} Caspase 3, a main apoptosis effector protein following both intrinsic and extrinsic pathway inductions, was also found to be a putative target. Although it is contradictory, it is important to note that a downregulation in caspase 3 expression by miRNAs cannot be used to rule out the occurrence of apoptosis as CDDP-mediated cell death can still occur via caspase 3-independent routes as described previously.⁴⁷

We also identified the involvement of several NF- κ B family members such as I κ B kinase (IKK) and tumor necrosis factor receptor-associated factor 2 (TRAF2) among the putative targets. The NF- κ B activation has been associated with CDDP resistance, and NF- κ B inhibitors have been reported to augment CDDP activity in cancer cells.⁴⁸ The occurrence of NF- κ B dysregulation is thought to be mediated by ACA rather than CDDP, as it was previously reported that ACA affects NF- κ B activation through IKK.²⁹ Other putative targets identified and whose expressions have been associated with CDDP resistance in the past are HIF-1 α ,⁴⁹ E-cadherin,⁵⁰ focal adhesion kinase (FAK),⁵¹ c-Jun,⁵² as well as phosphatidylinositol-3-kinase (PI3K) and AKT.⁵³ Thus, downregulation of these targets by miRNAs may increase the sensitivity toward CDDP, leading to increased apoptosis and cell cycle arrest, consistent

with our MTT data explaining the attainment of a synergistic effect when ACA is combined with CDDP.

Conclusion

In summary, this study has demonstrated that ACA synergistically potentiates the cytotoxic effects of CDDP when used in combination through dysregulation of specific miRNAs in Ca Ski and HeLa cervical carcinoma cells. We also showed that the differentially expressed miRNAs affect many important signaling pathways, with the implication of ERK pathway as the main pathway. Therefore, our study provides a platform to methodically study the roles of these miRNAs in modulating response toward anticancer drugs. A better understanding in the interactions between miRNAs with their specific gene targets can help us to delineate the molecular mechanism underlying anticancer drug response and provide potential therapeutic approaches by exploiting the miRNA expression to improve efficacies in combination chemotherapy.

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