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Sulfuretin-induced miR-30C selectively downregulates cyclin D1 and D2 and triggers cell death in human cancer cell lines

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ABSTRACT

Sulfuretin (3′,4′,6′-trihydroxyaurone), one of the key flavonoids isolated from *Rhus verniciflua*, is known to suppress inflammation and oxidative stress. However, the anti-cancer properties of sulfuretin as well as its mechanism of action remain poorly understood. Here, we show that the expression of miR-30C is markedly enhanced in sulfuretin-stimulated cells, consequently promoting apoptosis and cell cycle arrest in human cancer cell lines. The transient transfection of pre-miR-30C resulted in greater than 70% growth inhibition in PC-3 cells and provided strong evidence that miR-30C selectively suppresses the expression of cyclin D1 and D2, but not cyclin D3. Target validation analysis revealed that 3′-UTR of cyclin D2 is a direct target of miR-30C, whereas suppression by miR-30C of cyclin D1 may occur through indirect mRNA regulation. In addition, silencing miR-30C expression partially reversed sulfuretin-induced cell death. Taken together, our data suggest that miR-30C, a tumor suppressor miRNA, contributes to anti-cancer properties of sulfuretin by negatively regulating cyclin D1 and D2, providing important implications of sulfuretin and miR-30C for the therapeutic intervention of human cancers.

1. Introduction

Rhus verciniflura Stokes (Anacardiaceae) used in traditional herbal medicine, has been known to control many biological responses including inflammation and oxidative stress. Recently studies also showed that the standardized extract of *R. verniciflua* stabilizes cancer progression, prolonging overall survival either alone or in combination with chemotherapy for gastric, colorectal, and lung cancer patients [1,2]. Moreover, such anti-cancer properties of extract of *R. verniciflua* is known to be closely associated with a flavonoid fraction mainly consisting of protocatechuic acid, fustin, fisetin, butein, and sulfuretin [3].

Sulfuretin, one of the major flavonoids isolated from *R. verniciflura*, has been reported to be anti-diabetic [4] and have therapeutic potential to treat allergic airway inflammation *in vitro* and *in vivo* [5]. Few studies have shown anti-tumor effects of *R. verniciflura* extract, and the direct pharmacological relevance of sulfuretin in tumor suppression remains unclear.

In recent years, many insights have been made into the mechanisms by which chemotherapeutics interrupt the proliferation of cancer cells. MicroRNAs (miRNAs) are a class of small, single-stranded, non-coding RNAs consisting of 20–25 ribonucleotides that regulate a variety of genes by binding to the 3′-untranslated region (UTR) of their target messenger RNAs (mRNAs), leading to mRNA degradation or translational repression during both normal development and pathological responses [6]. miRNA research is an extremely fast growing field of cancer research, and microRNA-based therapeutics holds great potential for the development of therapeutic targets as well as pharmacological markers for diagnosis, prognosis and therapeutic effectiveness. The aberrant expression of miRNAs such as miR-17, miR-372, miR-10b and miR-203 is reported to correlate with tumorigenesis and cancer progression [7,8], and high levels of miR-21and miR-155 are known to be associated with resistance to anti-cancer therapeutics by targeting Bcl2, PTEN, Fas ligand [9,10], and FOXO3a [11]. Therefore, a better understanding about the biological functions and molecular mechanisms of miRNA-mediated processes may contribute to the development of new anti-cancer therapeutics.

In this study, we report the anti-cancer properties of sulfuretin and the possible mechanism of its action, providing evidence that sulfuretin induces apoptosis and cell cycle arrest in human cancer cell lines by downregulating cyclin D1 and D2 through the induction of miR-30C. Our study also suggests cyclin D1 and D2 as potential targets of miR-30C.

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2. Materials and methods

2.1. Materials

Sources were as follows: anti-cyclin D3 (#2936), anti-cyclin D1 (#2926) and anti-PARP (#9542) antibodies (Cell Signaling Technology, Boston, MA, USA); anti-cyclin D2 (sc-593) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-β-actin (A-5441) antibody (Sigma Chemical Co., St. Louis, MO, USA); characterized fetal bovine serum (FBS) (HyClone, Logan, UT, USA). Sulfuretin was provided by Dr. Hyunju Jung at Wonkwang University.

2.2. Cell culture

Four human cancer cell lines, PC-3, MDA-MB-231, MiaPaCa and H460 cells, were maintained in DMEM supplemented with 10% FBS, grown at 37 °C in a 95% air/5% CO_2 environment, and passaged every 3–4 days. All experiments were performed in low-serum conditions (DMEM containing 1% FBS and 15 mM HEPES).

2.3. Western blot analysis

Samples were analyzed by immunoblotting as described previously [12,13]. Briefly, cells were plated at a density of 2×10^5 cells/2 ml/well in 6-well plates in DMEM + 1% FBS + 15 mM HEPES and treated with sulfuretin at various doses (0–40 μM) for the indicated times. Thirty to fifty μg of protein was electrophoresed through 8% or 10% Tris–glycine gels, and electrophoretically transferred to nitrocellulose membranes. Membranes were probed with the indicated primary antibodies and the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoReseach Laboratories, West Grove, PA, USA). The Super Signal Chemiluminescence Substrate System (Pierce) was used to visualize protein bands.

2.4. Pre-miR-30C-mediated up-regulation and miRNA inhibitor-mediated down-regulation of miR-30C

PC-3 cells were transfected with 50 nM pre-miR-30C (UGUAAACAUCCUACACUCUCAGC, Ambion, Grand Island, NY, USA) or control miRNA using the Lipofectamine Plus reagent (Invitrogen, Grand Island, NY, USA) as described previously [14] to upregulate its expression or were infected overnight with pLL3.7_hsa-miR-30c lentivirus to knock down its expression. Pre-miR-negative and pLL3.7 lentivirus were used as negative controls.

2.5. miRNA quantification and real-time quantitative RT-PCR

The expression level of miR-30C was quantified using the TaqMan microRNA assay (Applied Biosynthesis, Grand Island, NY, USA) according to the manufacturer's instructions and normalized relative to RNU43 small nuclear RNA (endogenous control).

2.6. Reporter vector and DNA constructs

The 3′-untranslated regions (UTR) of cyclin Ds were PCR-amplified and cloned downstream of the CMV-driven firefly luciferase cassette in the pMIR-Repoter vector (Ambion) as described previously [15]. A reporter vector containing a direct matched miRNA-binding site oligonucleotide for miRNA-30C was used as a positive control. In order to validate targets for miRNA-30C, cells were transfected with 50 ng of each firefly luciferase reporter construct, 150 ng of empty pCDNA3 vector, 200 ng of pCDNA3 harboring the renilla luciferase gene, and 30 pmol of pre-miR-30C or pre-miR-neg (Ambion). After 24 h-transfection, lysates were subjected to dual luciferase assay following manufacturer's guideline (Promega). The firefly luciferase activity was normalized by renilla control luciferase activity.

2.7. Crystal violet nuclei staining and cell viability assay

Cells were plated at a density of 1×10^5 cells/1 ml/well in 12-well plates or 2×10^5 cells/2 ml/well in 6-well plates in DMEM + 1% FBS + 15 mM HEPES, and cultured for the indicated times in the presence of Sulfuretin or control vehicle. Cells were then fixed with 2% formalin-PBS and incubated with 0.2 mg/ml crystal violet solution for nuclei staining. After washing twice with PBS, the dye was eluted by adding 1% Triton X-100 in PBS, followed by spectrophotometry at 550 nm.

2.8. Flow cytometry

Detached cells (1.5×10^6) were washed once in PBS, fixed in 90% methanol, treated with 0.1 mg/ml RNaseA followed by 50 μg/ml propidium iodide, and analyzed with a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). Sub-G1 cells, which have less than 2*n* DNA content, were considered apoptotic.

2.9. Statistical analysis

Statistical significance was calculated with an ANOVA and student's unpaired *t*-test, and accepted at the level of $P < 0.05$.

3. Results

3.1. Sulfuretin inhibits cell growth in a variety of human cancer cell lines

To determine whether sulfuretin has anti-tumor properties, we first examined the effect of sulfuretin on the cell growth of a variety of human cell lines including PC-3 (prostate carcinoma cells), MDA-MB-231 (metastatic breast cancer cells), H460 (lung carcinoma cells) and MiaPaCa (pancreatic carcinoma cells). Cells were stimulated with the indicated doses of sulfuretin and maintained for 4 days before cell viability assay using crystal violet. As shown in Fig. 1, treatment with sulfuretin significantly inhibited the growth of human cancer cells in a dose-dependent manner. The growth of PC-3 cells was suppressed by 40% at a dose as low as 2.5 μM and 80% at 20 μM sulfuretin (Fig. 1A). The viabilities of MDA-MB-231 (Fig. 1B), MiaPaCa-2 (Fig. 1C) and H460 cells (Fig. 1D) were also reduced to 16.3%, 39% and 33.7%, respectively, after incubation with 20 μM sulfuretin.

Because we observed significant cell death in sulfuretin-treated cells, we performed flow cytometric analysis after PI staining to measure apoptotic cell death. PC-3 cells were incubated in the absence or presence of 40 μM sulfuretin for 24 h, followed by analysis of the sub-G0/G1 population. As shown in Fig. 1E, 2.98% of cells were apoptotic in non-treated cells, while the percentage of cells in sub-G0/G1 increased up to 18.85% following sulfuretin treatment. These data suggest that sulfuretin suppresses the growth of diverse human cancer cells and induces apoptosis, implying its chemotherapeutic potential against human cancers.

3.2. PC-3 and MDA-MB-231 cells undergo apoptosis following sulfuretin treatment

Caspases, a family of cysteine proteases, act in concert in a cascade triggered by apoptosis signaling and cleave nuclear protein like PARP (poly(ADP-ribose) polymerase) when activated. Since such proteolytic cleavage of PARP and caspase activation have been

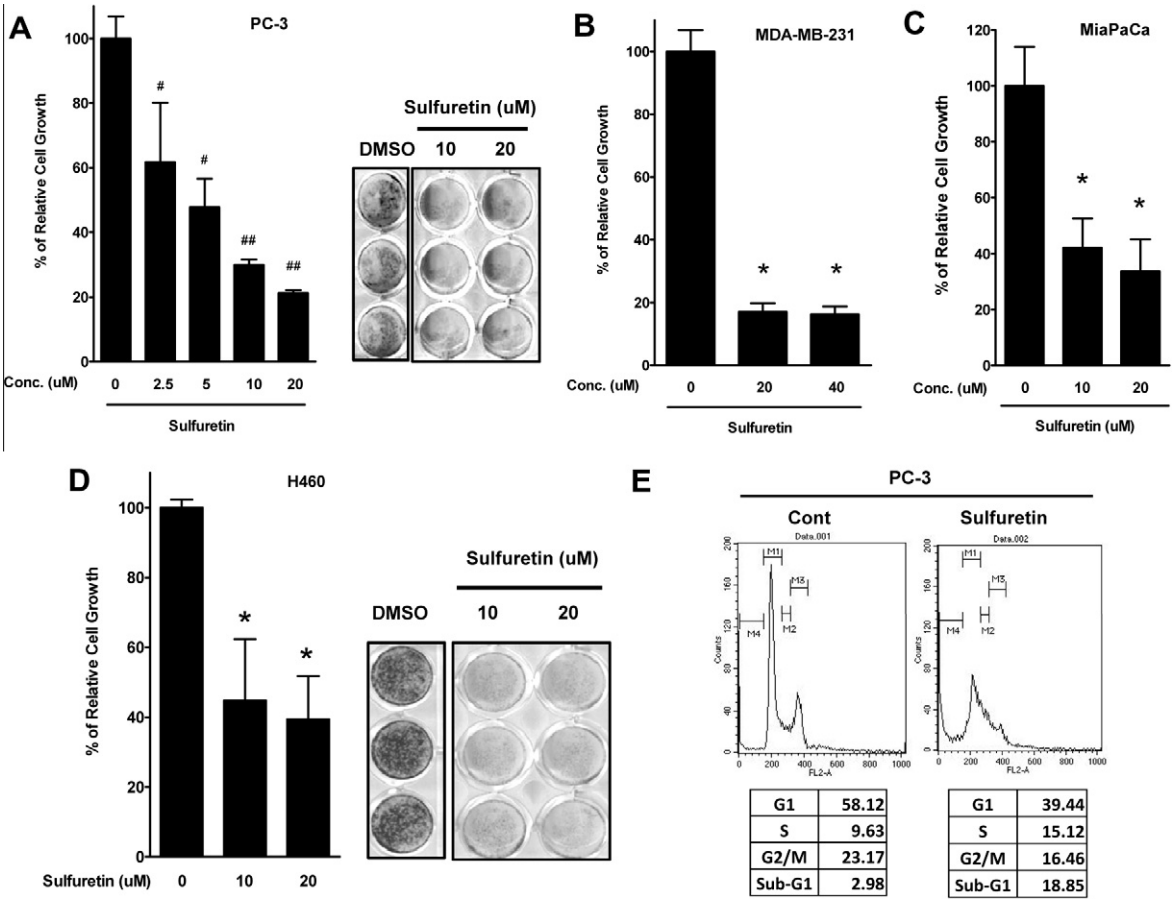

Fig. 1. Sulfuretin inhibits cell growth in a variety of human cancer cell lines. (A–D) Cells were treated with sulfuretin at the indicated concentrations for up to 4 days, and subjected to crystal violet staining to evaluate cell viability by spectrophotometry at 550 nm. The cell lines tested include PC-3 (A), MDA-MB-231 (B), MiaPaCa (C), H460 (D) and (E) PC-3 cells were cultured in the presence or absence of 40 μM sulfuretin, and analyzed for DNA content by propidium iodide staining and fluorescence-activated cell sorting (FACS). Data shown are the average of triplicate determinations ± SD and are representative of two or three independent experiments (A–E). ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{*}P < 0.0001$ vs. control).

considered as a hallmark of apoptosis, we next examined the ability of sulfuretin to induce apoptosis by measuring the cleavage of caspase-3 and PARP. As shown in Fig. 2A, sulfuretin treatment for 24 h activated caspase-3 along with PARP cleavage in PC-3 cells. Similarly, the cleaved form of caspase-3 was detected in MDA-MB-231 cells stimulated at two different doses of sulfuretin, 10 μM and 20 μM (Fig. 2B). We also found that Bcl-xL, an anti-apoptotic member of the Bcl-2 family, was gradually downregulated by sulfuretin in a time-dependent manner with maximum suppression at 24 h of treatment (Fig. 2C).

3.3. Sulfuretin downregulates the expression of cyclin D proteins

Our findings in Figs. 1E and 2 prompted us to test whether sulfuretin regulates the expression of cell cycle-related proteins, such as the cyclin Ds. To do so, cells were treated with sulfuretin at various doses (0–40 μM) for 24–48 h, and cyclin Ds' expression was determined by western blot analysis. As expected, sulfuretin strikingly suppressed cyclin D1 protein level, and similar effects were observed on the protein level of cyclin D2 as well as D3 in both cell lines (Fig. 3A–D). Additionally, such suppression of cyclin Ds by sulfuretin occurred in a dose- and time-dependent manner (Fig. 3C–D). MDA-MB-231 cells appeared more sensitive to sulfuretin, showing similar responses to sulfuretin at doses four times lower than those administered to PC-3 cells, as shown in the right panels of Fig. 3D. Taken together, our data suggest that sulfuretin enhances apoptotic cell death by downregulating cyclin D proteins in both human prostate and breast cancer cell lines.

3.4. miR-30C is upregulated by sulfuretin and selectively targets cyclin D1 and D2, but not cyclin D3, mediating sulfuretin-induced cell death

miRNAs control gene expression at the posttranscriptional level through simultaneous targeting of multiple genes [16], and have been reported to play an important role in tumorigenesis [17,18]. Recent studies showed that miR-30C may play a role in the inhibition of metastasis in cancer [19], but the target genes and functions of miR-30C in human disease are still poorly understood. To identify the mechanism of sulfuretin-induced cell death, we examined whether sulfuretin modulates miR-30C expression in human cancer cell lines. Total RNA from cells stimulated with 5 or 40 μM sulfuretin was extracted and subjected to quantitative real-time PCR. Our results revealed that sulfuretin enhanced the expression of miR-30C up to 160-fold and 5-fold in PC-3 cells and MDA-MB-231 cells, respectively, compared to control (Fig. 4A). These data suggest that miR-30C may function as a tumor suppressor, contributing to cell death mediated by sulfuretin in human prostate and breast cancer cell lines.

To better understand the significance of sulfuretin-induced miR-30C expression, we studied whether enforced expression of miR-30C could inhibit the growth of PC-3 cells. Because the miR-30C precursor (pre-miR-30C) is processed inside cells to produce

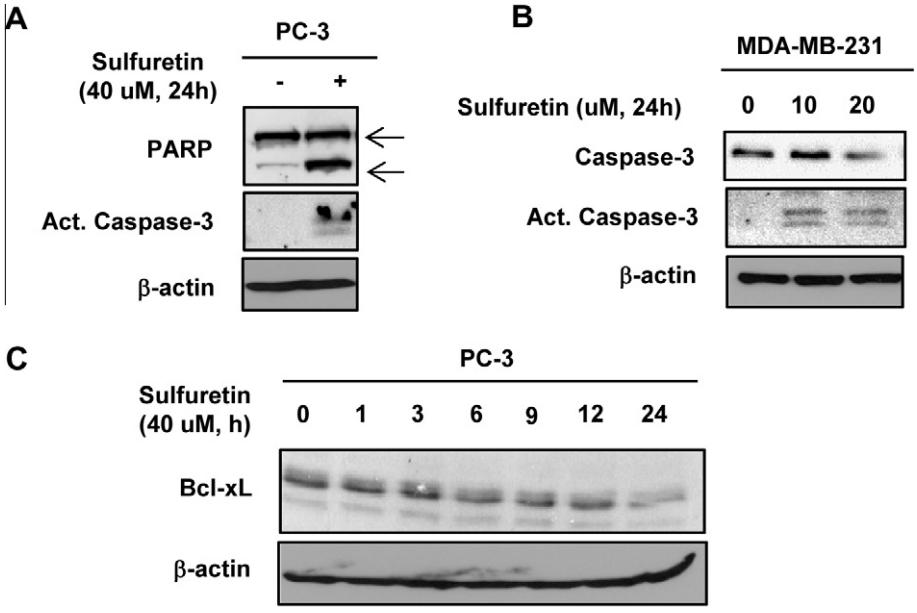

Fig. 2. Sulfuretin regulates diverse genes involved in apoptosis in PC-3 and MDA-MB-231 cells. (A) PC-3, (B) MDA-MB-231 cells were stimulated with 10–40 μM or sulfuretin for 24 h and (C) Separately, sulfuretin-treated PC-3 cells were incubated for the indicated times. Thirty to fifty μg of whole cell extract was subjected to Western blotting to measure the protein levels of PARP, cleaved caspase-3 and Bcl-xL. The results are representative of two to three different experiments (A–C).

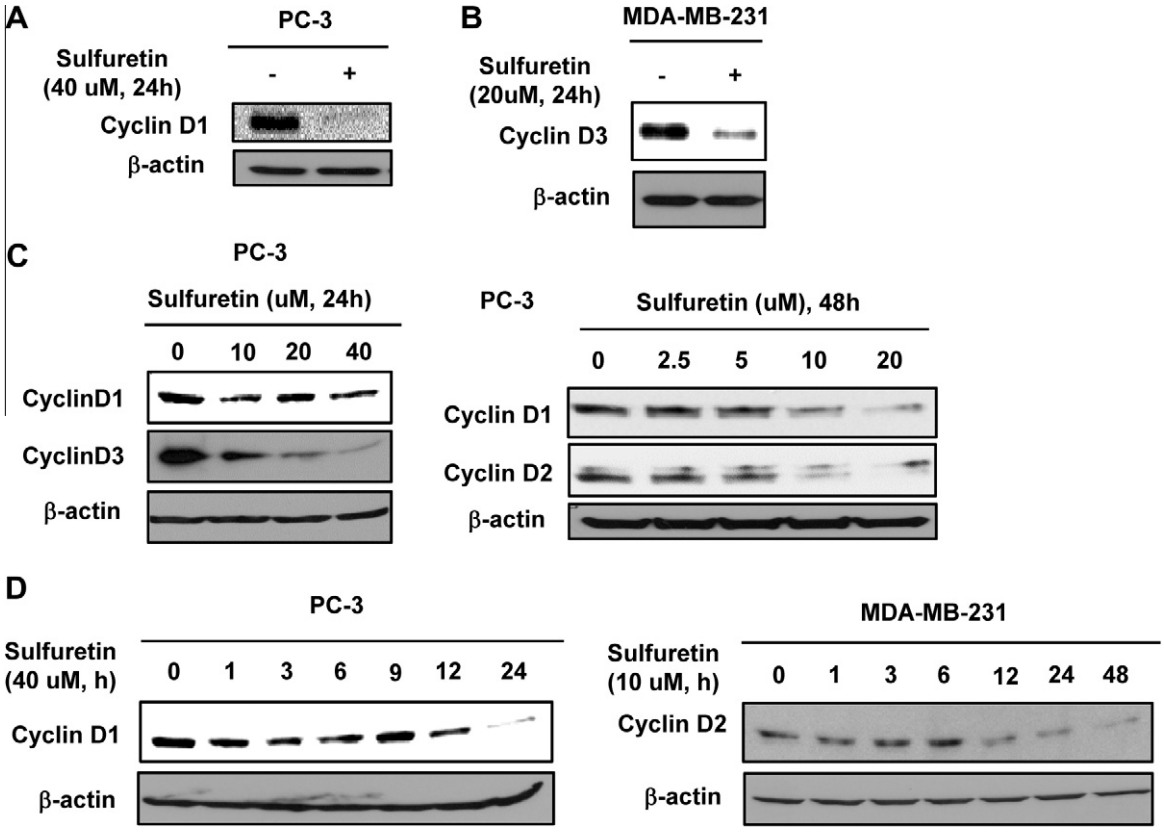

Fig. 3. Cyclin Ds are downregulated by sulfuretin treatment in PC-3 and MDA-MB-231 cells. (A–C) Samples were prepared from cells exposed to sulfuretin at the different doses from 2.5 to 40 μM for either 24 or 48 h. (D) Time course experiments with sulfuretin. Protein samples were subjected to western blot analysis to measure the expression levels of cyclin D1, D2 and D3. The results are representative of two to three different experiments (A–D).

miR-30C, cells were transiently transfected with 50 nM pre-miR-30C or control negative miRNA, and cultured for 24 or 48 h prior to cell viability analysis. As expected, the overexpression of miR-30C substantially suppressed cell growth at 24 (Fig. 4B) and 48 h (data not shown) after transfection, while cells transfected with control negative miRNA remained unaffected. This result was further confirmed using nuclei staining and spectrophotometry (Fig. 4C), showing 75% cell death by the enforced expression of

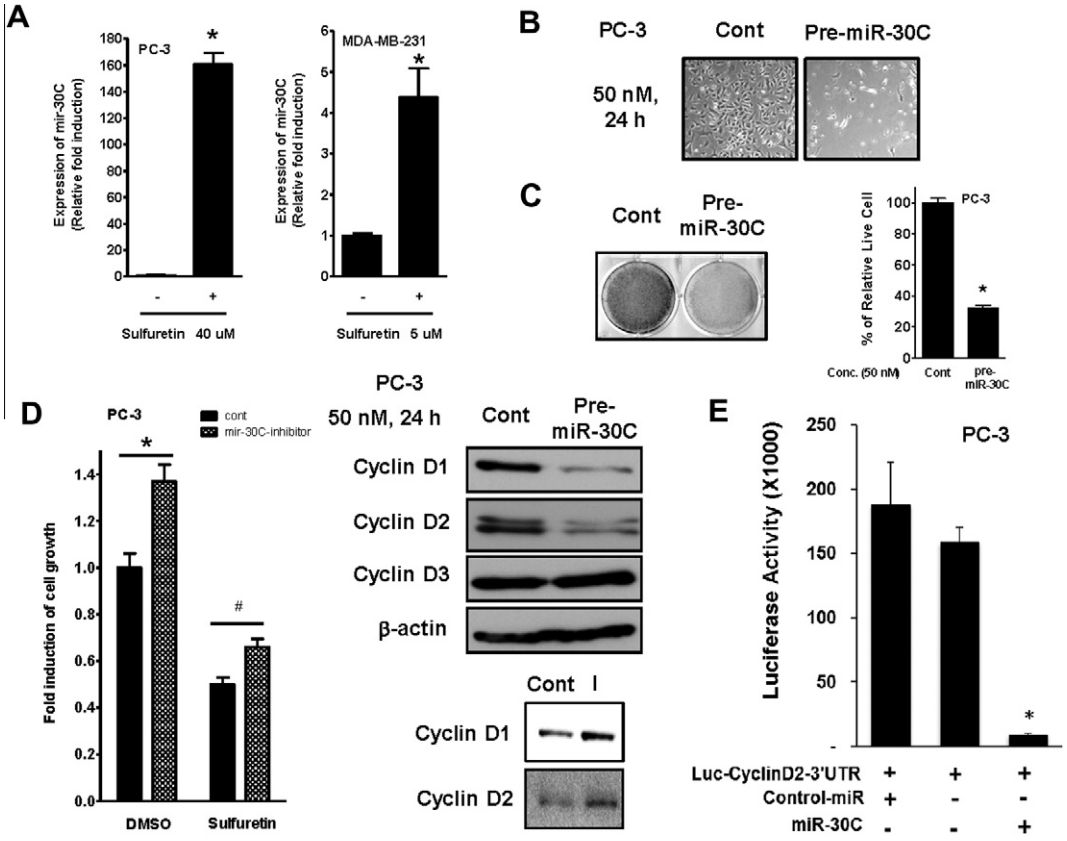

Fig. 4. miR-30C targets cyclin D1 and D2, mediating in part sulfuretin-induced cell death. (A) Total RNA was isolated from cells (*left*, PC-3 and *right*, MDA-MB-231) stimulated for 48 h with 40 or 5 μM sulfuretin, respectively. miR-30C expression was measured using real-time quantitative PCR, (B) PC-3 cells were transiently transfected with either scrambled control sequence (cont) or pre-miR-30C and cultured for 24 h before imaging, (C) Cell viability was measured using crystal violet staining 24 h after transfection with/without pre-miR-30C (*left*), and the eluted dye was subjected to spectrophotometry at 550 nm for quantification (*right*), (D) Protein samples from PC-3 cells transfected with either scrambled control sequence (cont) or pre-miR-30C were subjected to immunoblotting analysis for cyclin Ds. Separately, cells were infected with lentivirus harboring miR-30C inhibitor (I) or control negative miRNA (Cont) to knock down expression of miR-30C and were then cultured for 48 h, followed by sulfuretin treatment and (E) The pMIR-Reporter vector containing the 3′-untranslated regions (UTR) of cyclin D2 were transfected along with pre-miR-30C (miR-30C) or pre-miR-neg (control-miR). After 24hr-transfection, lysates were subjected to dual luciferase assay and the firefly luciferase activity was normalized by renilla control luciferase activity. Data shown are the average of triplicate determinations ± SD and are representative of two or three independent experiments (A–E). ($^{\#}P < 0.05$, $^{*}P < 0.0001$ vs. control).

miR-30C. We next studied whether the inhibition of miR-30C could reverse sulfuretin-induced cell death. To do so, cells were infected with lentiviral cre and lentiviruses containing either cre-regulated miR-30C inhibitor or control vector to block the expression of miR-30C, followed by treatment with 5 or 40 μM sulfuretin for 48 h. Cells were then washed and incubated for 10 days before crystal violet staining. The miR-30C inhibitor slightly induced cell growth and partially reversed sulfuretin-induced cell death in PC-3 cells (Fig. 4D, *left*). We further tested the possibility that miR-30C is involved in the sulfuretin-mediated downregulation of the cyclin D proteins. PC-3 cells were transfected with 50 nM of either control miRNA or pre-miR-30C and expression levels of cyclin Ds were determined by Western blot analysis. As shown in Fig. 4D (*right top*), miR-30C overexpression noticeably reduced the protein levels of cyclin D1 and D2, whereas cyclin D3 level remained unchanged. Separately, miR-30C inhibitor slightly increased protein levels of cyclin D1 and D2 (Fig. 4D, *right bottom*). These results suggest that miR-30C functions as a negative regulator of cell growth, selectively targeting cyclin D1 and D2, but not cyclin D3 (Fig. 4C and D).

To confirm that cyclin D1 and 2 are targets of miR-30C, 3′-UTR of cyclin D1 or 2 was cloned into a luciferase reporter construct (Luc-cyclin D2 (or D1)-3′UTR), followed by transiently transfection into PC-3 cells along with pre-miR-30C (miR-30C) or a non-targeting negative control (control-miR). Our results showed that luciferase activity was abolished more than 90% in the cells transfected with the Luc-cyclin D2–3′UTR and miR-30C, while control-miR failed to inhibit it (Fig. 4E). Interestingly, cells transfected with Luc-cyclinD1–3′UTR and miR-30C did not show reduction of luciferase activity (data not shown). These data suggest that cyclin D2 is a direct target for miR-30C, and the suppression of cyclin D1 by mir-30C may occur through indirect mRNA regulation (Fig. 4E).

Taken together, our findings demonstrate that the direct or indirect downregulation of cyclin D1 and D2 by miR-30C, may contribute to the mechanism by which sulfuretin induces cell death in human cancer cell lines.

4. Discussion

A flavonoid fraction from *R. verniciflua* containing sulfuretin has been reported to inhibit proliferation of human cancer cells through blocking Bcl-2 expression, activating caspase-8 and Bax [3,20], and induce cell cycle arrest via accumulation of p27(Kip1) driven by Skp2 reduction [21] and decreased CDK2 activity. Although sulfuretin has been shown to suppress various biological responses including inflammation and oxidative stress *in vitro* and *in vivo* models [5,22], the anti-cancer properties of sulfuretin or its mechanism of action is poorly understood. One publication has been published so far, revealing that proapoptotic activity of sulfuretin is mediated by mitochondrial dysfunction and a Fas/caspase-8 dependent pathway [23]. Our results offered that

sulfuretin not only enhances active form of caspase-3 and PARP cleavage, but also suppresses the protein expression of anti-apoptotic factor, Bcl-xL. Furthermore, we provide evidence for the first time that sulfuretin effectively suppresses protein levels of all three forms of cyclin D (D1, D2, and D3), and miR-30C plays a pivotal role in such downregulation.

Recently, increasing numbers of publications have focused on the anti-cancer properties of miR-30C, showing deregulation of miR-30C expression in a variety of human cancers [17]. Mu et al. reported that single nucleotide polymorphisms (SNPs) A/G in pre-miR-30C might be associated with a high risk of gastric cancer [24], implicating its clinicopathological feasibility as a diagnostic and prognostic marker [25]. However, very little is known about the factors to regulate the expression of and be targets of miR-30C. One study reported epidermal growth factor receptor (EGFR) and MET oncogene suppress as negative regulators of miR-30C expression [17]. The known molecular targets for miR-30C, so far, include metastasis-associated gene-1 (MTA1) [19], KRAS oncogene [26], BCL-9 [27], HMBOX1 transcription factor [28], a cell stress gene REDD1 [29], and the cytoskeleton network genes involving cancer cell invasion such as twinfilin 1 (TWF1) and vimentin (VIM) [30]. Given that miR-30C targets a variety of oncogenes listed above and its expression is enhanced by sulfuretin (Fig. 4A), we investigated our hypothesis that downregulation by sulfuretin of cyclin Ds (Fig. 2) may occur through upregulating expression of tumor suppressive miR-30C, and thus cyclin Ds could be new targets for mir-30C.

Our findings reveal that cyclin D1 and D2, but not cyclin D3, are new possible targets of miR-30C, despite our observation that sulfuretin suppresses all three forms of cyclin D (Figs. 3 and 4). This may explains the partial reversal of sulfuretin-induced cell death by miR-30C inhibition as shown in Fig. 4D. Interestingly, miR-30C seems to differentially regulate the expression between cyclin D1 and D2. Our target validation data demonstrated that only 3′UTR of cyclin D2 is directly targeted by miR-30C, whereas cyclin D1 is likely to be downregulated through indirect mechanism of mRNA modulation where transcriptional factors, RNA-regulatory proteins, or other miRNAs controlled by miR-30C might be involved. The mechanism by which miR-30C regulates cyclin D1 awaits further investigation.

In summary, our data suggest that miR-30C, in part, mediates anti-cancer properties of surfuretin by suppressing cyclin D1 and D2, providing new mechanistic insights into the function of sulfuretin as a potential therapeutic for a variety of cancers.

Acknowledgments

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