A Small-Molecule Modulator of the Tumor-Suppressor miR34a Inhibits the Growth of Hepatocellular Carcinoma

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Abstract

Small molecules that restore the expression of growth-inhibitory microRNAs (miRNA) downregulated in tumors may have potential as anticancer agents. miR34a functions as a tumor suppressor and is downregulated or silenced commonly in a variety of human cancers, including hepatocellular carcinoma (HCC). In this study, we used an HCC cell-based miR34a luciferase reporter system to screen for miR34a modulators that could exert anticancer activity. One compound identified as a lead candidate, termed Rubone, was identified through its ability to specifically upregulate miR34a in HCC cells. Rubone activated miR34a expression in HCC cells with wild-type or mutated p53 but not in cells with p53 deletions. Notably, Rubone lacked growth-inhibitory effects on nontumorigenic human hepatocytes. In a mouse xenograft model of HCC, Rubone dramatically inhibited tumor growth, exhibiting stronger anti-HCC activity than sorafenib both in vitro and in vivo. Mechanistic investigations showed that Rubone decreased expression of cyclin D1, Bcl-2, and other miR34a target genes and that it enhanced the occupancy of p53 on the miR34a promoter. Taken together, our results offer a preclinical proof of concept for Rubone as a lead candidate for further investigation as a new class of HCC therapeutic based on restoration of miR34a tumor-suppressor function. Cancer Res; 74(21): 6236–47. © 2014 AACR.

Introduction

Hepatocellular carcinoma (HCC) remains to be one of the most common malignancies and is a leading cause of cancer-related deaths due to the high mortality and unsatisfactory treatment options available (1). It is of urgent significance to develop more effective therapeutics for HCC. microRNAs (miRNA) are a class of single-stranded noncoding RNAs (2), and it is estimated that 25% to 70% of human genes are regulated by miRNAs (3, 4). The patterns of miRNAs expression were proved to be correlated with cancer types, stages, and other clinical variables (5). Therefore, the expression of miRNAs could be applied as a tool for cancer diagnosis and prognosis. In cancer cells, miRNAs could function as oncogenes or tumor suppressors. Their roles are manifested in almost all aspects of cancer biology, such as proliferation, apoptosis, invasion/metastasis, and angiogenesis (6). Recently, a miR122 inhibitor has been evaluated in phase Ia clinical trial for treating hepatitis C virus (HCV) infection. This builds a bright prospect for developing modulators of miRNAs for disease treatment (7).

miR34 family constitutes three members: miR34a, miR34b, and miR34c. All miR34 family members are direct targets of p53, miR34b and miR34c share the same primary transcript while miR34a resides in another one (8). miR34a has been well documented as a tumor suppressor in previous studies (9–13). Upregulation of miR34a could decrease the expression of a series of targets such as B-cell lymphoma 2 (Bcl-2), cyclin D1, cyclin-dependent kinase 6 (CDK6), Forkhead box protein P1 (FOXP1), Notch 1, and Sirtuin 1 (SIRT1). Thereby, miR34a could induce cell apoptosis, senescence, and inhibit cellular differentiation and proliferation (14–16). miR34a expression was dramatically downregulated or silenced in various cancers including human HCC. Ectopic expression of miR34a inhibited HCC cell migration and invasion (17). Thus, restoration of miR34a might represent a potential therapeutic for HCC.

Few studies have focused on small-molecule modulators of miRNAs so far. Previously, the small-molecule modulators of miR21 and miR122 have been identified with potent biologic activities (18, 19). However, the small-molecule modulators of miR34a have not been identified yet. In this study, we developed a luciferase reporter system for the screening of small-molecule modulators of miR34a from a natural product library. After library screening, one hit compound named Rubone was found to activate miR34a expression in HCC cells. We further

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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investigated the anticancer activity of miR34a modulator in both cell culture and animal models.

Materials and Methods

Cell lines and drugs
The nontumorigenic human hepatocyte cell line MIHA (20) was obtained from Dr. J.R. Chowdhury’s laboratory at Albert Einstein College of Medicine (New York, NY). The human HCC cell line Huh7 (kindly provided by Dr. H. Nakabayashi, Hokkaido University School of Medicine, Sapporo, Japan; ref. 21) and Bel-7404 (Cell Bank of the Chinese Academy of Sciences; ref. 22) were authenticated with short-tandem repeat profiling by the vendors. The human HCC cell lines HepG2, PLC/PRF/5 (PLC), and Hep3B (American Type Culture Collection) were verified by short-tandem repeat profiling at the GENEWIZ, Inc. within 6 months of use. All cell lines were cultured under the condition as previously described (23). Carboxymethylcellulose was provided by Unitech Chemicals. Sorafenib was purchased from Bayer Healthcare. Dimethyl sulfoxide (DMSO), Cisplatin (CDDP), 5-Fluorouracil (5-FU), and doxorubicin were purchased from Sigma.

Constructs
The pmir-R-REPORT luciferase plasmid (Ambion) was sequentially digested with restriction enzyme SacI and HindIII (10 units each in 50 μl reaction, NEB) and was purified after gel electrophoresis. DNA oligos containing the miR34a-binding site ([5'-CTG GCA GTG TCT TAG CTT GGA A-3'] and 5'-AGC TTA CAA CCA GCT AGG ACA CTG CCA GAG CT-3'), and scrambled miR34a-binding site ([5'-CTG TTT CGT TGG GCG TTT CGA AGA-3'] and 5'-AGC TTC TTC TCC GAA ACG CCC AAC GAA ACA GAG CT-3') were ordered from TechDragon. The oligos were annealed at 95°C and then cooled to 4°C. The annealed DNA fragments were then ligated into pmir-R-REPORT luciferase vector with T4 DNA ligase (200 U in 10-μl reaction, NEB). The generated constructs containing miR34a-binding site or scrambled binding site were named as miR34a reporter or miR34a scr. The luciferase promoter constructs containing miR34a promoter or miR34a mutant promoter were kindly provided by Prof. J.T. Mendell from Johns Hopkins University (Baltimore, MD; ref. 16).

Transfection
miR34a mimics duplex, single-stranded miR34a inhibitors and siRNAs targeting p53 were purchased from Shanghai GenePharma Co. The sip53 sequence was as following: sip53a: 5'-CUA CUU CCA GCC AAG AAC G GdTdT-3'; sip53b: GCA UGA ACC GGG GCC CCA U dTdT. The miR34a mimics, inhibitors, and siRNAs were transfected into HCC cells using DharmaFECT siRNA transfection reagent (Thermo Scientific) according to the manufacturer's protocol. The plasmids mentioned in previous section were transfected into HCC cells using Lipofectamin 2000 (Invitrogen) according to the manufacturer’s protocol. The plasmids mentioned in previous section were transfected into HCC cells according to the manufacturer's protocol. The pmiR-REPORT luciferase plasmid (Ambion) was sequentially digested with restriction enzyme SacI and HindIII (10 units each in 50 μl reaction, NEB) and was purified after gel electrophoresis. DNA oligos containing the miR34a-binding site ([5'-CTG GCA GTG TCT TAG CTT GGA A-3'] and 5'-AGC TTA CAA CCA GCT AGG ACA CTG CCA GAG CT-3'), and scrambled miR34a-binding site ([5'-CTG TTT CGT TGG GCG TTT CGA AGA-3'] and 5'-AGC TTC TTC TCC GAA ACG CCC AAC GAA ACA GAG CT-3') were ordered from TechDragon. The oligos were annealed at 95°C and then cooled to 4°C. The annealed DNA fragments were then ligated into pmir-R-REPORT luciferase vector with T4 DNA ligase (200 U in 10-μl reaction, NEB). The generated constructs containing miR34a-binding site or scrambled binding site were named as miR34a reporter or miR34a scr. The luciferase promoter constructs containing miR34a promoter or miR34a mutant promoter were kindly provided by Prof. J.T. Mendell from Johns Hopkins University (Baltimore, MD; ref. 16).

Library screening with miR34a reporter
Huh7 cells were cultured in complete Dulbecco’s Modified Eagle Medium (DMEM). 5 × 10^5 Huh7 cells were seeded into each well of 96-well plates and incubated for 12 hours at 37°C with 5% CO2. Then, Huh7 cells were cotransfected with miR34a reporter and Renilla luciferase pRL-SV40. The transfection medium was replaced with complete DMEM after 4 hours incubation. Compounds from natural products library (NPL; NPL contained 640 compounds including natural products and their derivatives; TimTec) were added to the cells at a final concentration of 10 μmol/L (0.1% DMSO at final concentration). The complete DMEM containing 0.1% DMSO was used as a control. After 48 hours of incubation, the luciferase activity was measured.

Luciferase assay
miR34a reporter, miR34a promoter, and miR34a-mutant promoter were transfected into HCC cells 12 hours before compound treatment. The Renilla luciferase vector pRL-SV40 was cotransfected as an internal control. HCC cells were then treated with compound for 48 to 72 hours. The luciferase activity was measured by the Dual-Luciferase Reporter Assay (Promega) with a Wallac VICTOR^X luminometer. The ratio of firefly luciferase to Renilla expression was calculated for each of the triplicates.

Total RNA and protein extraction
Total RNA from cell cultures and HCC xenograft tumors were extracted using TRIzol (Invitrogen) according to the manufacturer’s protocols. Total RNAs were dissolved into nuclease-free ddH2O. For protein extraction, HCC cells and xenograft tumors were lysed in 1× RIPA buffer with 1 mmol/L phenylmethylsulfonylfluoride (PMSF) and 1× complete protease inhibitor cocktail (Roche). Both RNA and protein were stored at −80°C.

Quantitative real-time PCR analysis
Total RNA was reversely transcribed into cDNAs using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green PCR Master Mix (TaKaRa). GAPDH was used as an internal control. miR34a level was measured by qRT-PCR using SYBR Green PCR Master Mixture (Invitrogen). U6 was measured as an internal control. Fold change was calculated by relative quantification (2^(-ΔΔCt)). The sequences of the primers were listed in Supplementary Table S1.

Western blotting
Protein concentration was measured with BCA protein assay kit (Thermo Fisher Scientific). Of note, 25 to 50 μg protein was separated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and transferred to poly(vinylidene fluoride) (PVDF) membranes (Bio-Rad). Primary antibodies used in this study included anti-cyclin D1 (1:1,000 dilution; cat. no., 2922; Cell Signaling Technology), anti-Bcl-2 (1:1,000 dilution; cat. no., AM2290, ABZOOM), anti-β-actin (1:5,000; cat. no., 4967; Cell Signaling Technology), and anti-p53 (1:1,000; cat. no., Sc-126; Santa Cruz Biotechnology). Membranes were incubated with primary antibodies at 4°C overnight, and then washed three times with TBST (Tris-buffered saline plus Tween-20). The membranes were then
incubated with 1:3,000 peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 hour at room temperature, followed by three washings in TBST. Protein expressions were detected by autoradiography film using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare).

**Cell viability assay**

Cell viability was measured by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (24). Briefly, 1 × 10^4 cells were seeded in 96-well plates in triplicate and treated with compounds at indicated concentrations for indicated time points. At each time point, a set of cells was incubated with 0.5 mg/mL MTT diluted in 1× PBS. After 2 to 4 hours of incubation, the MTT solution was removed. The insoluble MTT was dissolved in DMSO. Absorbance at 570 nm was measured using a Benchmark Plus microplate reader (Bio-Rad).

**In vivo HCC xenograft mouse model**

A total of 5 × 10^3 HepG2 cells were suspended in 100 μL serum-free medium and subcutaneously (s.c.) inoculated into the dorsal flanks of nude mice. When tumors reached 5 to 10 mm in diameter, mice were randomly divided into four groups (n = 5). Rubone and sorafenib were suspended in a carboxymethylcellulose vehicle formulation, which contained 0.4% carboxymethylcellulose sodium and 0.9% NaCl. Tumor-bearing mice were treated with 20 or 50 mg/kg Vehicle alone and sorafenib were used as negative and positive control, respectively. The starting date of treatment was defined as day 0. Tumor volumes and body weight were measured once every 2 days. Tumor volume was calculated as [(length × width × height)/2]. Treatment was continued for 24 days. Finally, mice were sacrificed and tumors were excised. Tumor weight was recorded. Tumor tissues were collected for subsequent RNA extraction or tissue section. All animal experiments were approved by the Animal Experimental Ethics Committee of the Chinese University of Hong Kong (Shatin, Hong Kong).

**Immunohistochemical staining**

Immunohistochemical staining was performed on xenografted tumor tissues with proliferating cell nuclear antigen (PCNA; 1:400 dilution; cat. no., SC-56; Santa Cruz Biotechnology), p16 (1:250 dilution; cat. no., SC-397; Santa Cruz Biotechnology), p21 (1:400 dilution; cat. no., SC-56; Santa Cruz Biotechnology)-specific antibodies. Subcutaneous tumor tissue sections were incubated with antibodies overnight at 4°C. Mean positive cells were calculated by averaging positive cells from three random fields per slide.

**TUNEL assay**

Analysis of apoptotic cells in xenografted tumor tissues was performed by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining. The in situ cell death detection kit was used by following the manufacturer’s protocol (Roche Diagnostics). Images of the sections were taken by a fluorescent microscope. Mean positive cells were calculated by counting TUNEL-positive cells from three random fields per slide.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChiP) assay was performed as described previously (25). Cross-linked chromatin was incubated overnight with anti-H3 (cat. no., 4620; Cell Signaling Technology), IgG (cat. no., 2729; Cell Signaling Technology), p53 (cat. no., sc-126; Santa Cruz Biotechnology). The precipitated DNA were quantitated absolutely by real-time PCR and normalized by respective 2% input.

**Statistical analysis**

GraphPad Prism 5 (GraphPad Software) was used for statistical analysis. Four-parameter logistic model was applied to calculate IC_{50}. The two-tailed Student t test was applied for paired data analysis. All data are expressed as mean ± SD from three separate experiments performed in triplicate. P values less than 0.05 were considered statistically significant.

**Results**

**Identification of small-molecule modulator of miR34a by library screening**

We first established a luciferase reporter by cloning the complementary sequence of miR34a into pmiR-Reporter vector (Fig. 1A). The scrambled miR34a-binding sequence was used as a control. Theoretically, this miR34a reporter could be used for screening miR34a binders by measuring luciferase signals (Fig 1B). The endogenous miR34a level in HepG2 cells was significantly higher (22.29-fold; P = 0.0017) than that of Huh7 cells as measured by RT-PCR (Supplementary Fig. S1A). We transfected miR34a reporter into HepG2 and Huh7 cells respectively and measured the luciferase activities. The luciferase activities in Huh7 cells was significantly higher (15.66-fold, P = 0.044) than that of HepG2 cells (Supplementary Fig. S1B). Cotransfection of miR34a mimics and miR34a reporter decreased the luciferase signal in Huh7 cells (Supplementary Fig. S1C). Conversely, cotransfection of miR34a inhibitor and miR34a reporter increased the luciferase signal in HepG2 cells (Supplementary Fig. S1D). No luciferase signal was changed in control group where miR34a reporter was replaced with miR34a scr. These results indicated that miR34a reporter was responsive to miR34a level.

Using the miR34a reporter system, we screened the NPL for miR34a modulators. The screening process was depicted in Fig 1C. After a primary screening of 640 compounds in Huh7 cells, one hit compound named Rubone was found to be a potential miR34a modifier (The full library screening dataset was listed in Supplementary Table S2). Rubone was re-assayed in triplicate with miR34a reporter system. Figure 1D showed Rubone inhibited the luciferase activity in a dose-dependent manner, with an IC_{50} value at 3.8 μmol/L. Rubone was a chemically synthesized plant chalcone derivative, which was also named 2'-hydroxy-2,4',5,6'-pentamethoxychalcone. Its molecular weight was 374.39 and its chemical structure was shown in Fig. 1D. Rubone has not been previously reported to have any known biologic activities.
We next examined whether Rubone modulated miR34a expression in HCC cells. As shown in Fig 2, both primary and mature miR34a levels significantly increased after treatment with Rubone in Huh7 and HepG2 cells. However, Rubone caused no change in the expression of primary and mature miR34a in Hep3B cells (Fig. 2). To note, mutant p53 and wild-type p53 was expressed in Huh7 and HepG2 cells, respectively, while p53 was deleted in Hep3B cells. These results indicated that Rubone modulated miR34a expression in HCC cells expressing either wild-type or mutant p53 but not in HCC cells with p53 deletion.

To exclude the possibility of nonspecific modulation on miR34a expression by Rubone, we measured the global human miRNAs expression after Rubone treatment. The global human miRNA profiling showed that Rubone preferentially activated the expression of miR34a (Supplementary Fig. S1A). We further demonstrated that other chemotherapeutic drugs such as CDDP, 5'-FU, doxorubicin and sorafenib significantly inhibited HCC cell growth (Supplementary Fig. S2B). Among these drugs, doxorubicin could induce the expressions of p53 and its downstream targets including p21 and Puma (Supplementary Fig. S2C). However, we found these drugs had no effect on pri-miR34a or miR34a expression in HCC cells (Supplementary Fig. S2D). These results indicated that Rubone preferentially modulated miR34a expression in HCC cells. Cyclin D1 and Bcl-2 were two of the most well-studied miR34a targets with important roles in HCC. We examined the expression levels of cyclin D1 and Bcl-2 after Rubone treatment. Rubone significantly reduced the mRNA and protein levels of cyclin D1 and Bcl-2 in Huh7 and HepG2 cells but not in Hep3B cells (Fig. 3). Meanwhile, we measured the expression levels of other miR34a targets including CDK6, FOXP, Notch 1, and SIRT as well as p53 targets including p21 and Puma. Similarly, the expression levels of these miR34a targets were decreased after Rubone treatment in Huh7 and HepG2 but not in Hep3B cells (Supplementary Fig. S2E), although there were no change on the expression of p53 targets in these cells (Supplementary Fig S2F).

miR34a modulator inhibited HCC cells growth in vitro

We next examined the anticancer activity of Rubone in vitro. Five HCC cell lines and MIHA cell line were treated with different concentrations of Rubone at indicated time points. Cell viability was measured by the MTT assay. Rubone

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Identification of miR34a modulator through library screening. A, schematic illustration of miR34a reporter system. The complementary sequences including mature miR34a and the scrambled miR34a sequence were cloned into pMIR-REPORT miRNA Reporter Vector (Ambion) to establish miR34a reporter and miR34a. B, working mechanism of the miR34a reporter. The miR34a reporter could detect the presence of functional mature miR34a as reflected by the rate of luciferase activity repression. C, the process of library screening for the identification of miR34a modulators. D, a small-molecule compound named Rubone significantly inhibited the luciferase activity of miR34a reporter at a dose-dependent manner, Huh7 cells were transfected with miR34a reporter and then treated with indicated concentrations of Rubone for 48 hours. Cell lysates were subjected to luciferase assay. Results are expressed as luciferase activity values. Error bars represent mean ± SD of three independent experiments.
inhibited the growth of HepG2, Bel-7404, Huh7, and PLC in a dose-dependent manner. There was little growth inhibition on MIHA after Rubone treatment, suggesting no cytotoxicity of Rubone to nontumorigenic human hepatocytes (Fig. 4A). Similarly, Rubone caused no growth inhibition on Hep3B cells in which p53 was deleted.

We also compared the growth inhibition effect of Rubone and sorafenib on HCC cells. The results revealed that Rubone exerted stronger growth inhibition on most HCC cell lines including Bel-7404, HepG2, and Huh7, though sorafenib exhibited stronger growth inhibition on PLC cells at 24 hours. Rubone showed little growth inhibition on Hep3B and MIHA cells, whereas sorafenib exhibited significant growth inhibition on these two cell lines at different time points (Fig. 4B).

We asked whether Rubone inhibited HCC cell growth through modulation of miR34a. HepG2, Bel-7404, and PLC cells were transfected with miR34a mimics or inhibitors and then treated with Rubone. Figure 4C showed that miR34a mimics showed concomitant effect of growth inhibition with Rubone to all HCC cell lines. Conversely, miR34a inhibitors attenuated the growth-inhibitory effect of Rubone. These results indicated that Rubone inhibited HCC cell growth by modulating miR34a expression.

miR34a modulator inhibited hepatocellular tumor growth in vivo

We next examined the in vivo anticancer activities of Rubone in HepG2 xenografted nude mice model. The tumor-bearing mice were gavaged with Rubone. The anti-HCC agent sorafenib was used for efficacy comparison. As shown in Fig 5A and Supplementary Table S3, the tumors in vehicle control showed a fast and stable growth. HepG2 xenografts were sensitive to sorafenib with a tumor growth inhibition rate at 84.22% ($P = 0.0013$ vs. vehicle control) at the dose of 50 mg/kg. Rubone delayed the growth of tumors by 78.27% ($P = 0.0070$ vs. vehicle control) and 89.64% ($P = 0.0010$ vs. vehicle control) at the dose of 20 and 50 mg/kg, respectively. Rubone exhibited a stronger tumor growth inhibition than sorafenib at the same dosage of 50 mg/kg ($P = 0.047$ vs. sorafenib-treated group; Supplementary Table S3). When comparing the anticancer activities of Rubone and sorafenib during the whole treatment process,
Rubone exhibited higher anticancer activities than that of sorafenib (Fig. 5B). Another xenografted nude mice model was established by using Huh7 cells. In this model, we also found that Rubone significantly inhibited tumor growth when compared with the vehicle control. The tumors from Rubone-treated group were smaller and lighter when compared with the sorafenib-treated group (Supplementary Fig. S3A–C).

The expression levels of pri-miR34a, miR34a, cyclin D1, and Bcl-2 in the xenografted tumors were measured by qRT-PCR. In Rubone-treated tumors, both pri-miR34a and miR34a levels were upregulated, while miR34a targets cyclin D1 and Bcl2 were downregulated (Fig. 5C and Supplementary Fig. S4A). Sorafenib treatment caused no change in the expression of miR34a and its targets (Fig. 5C and Supplementary Fig. S4A). These results could also be validated in Huh7-xenografted nude mice model (Supplementary Fig. S3D). Moreover, there was no obvious body weight loss in the treated mice compared with vehicle control (Supplementary Table S3). Meanwhile, we also treated Balb/C mice with Rubone (200 mg/kg) for 24 days. No obvious side effect was observed at the end of the experiment (Supplementary Fig. S4D). These results indicated that Rubone was an effective and safe anti-HCC agent in animal.

miR34a modulator inhibited proliferation and induced apoptosis in HepG2 xenografts

TUNEL staining was performed to assess the in vivo apoptosis induction by Rubone in HepG2 xenografts. The average number of apoptotic cells increased from 13/area in the control group to 109/area (P = 0.0033) in the Rubone-treated group (Fig. 6A). The in vivo antiproliferative effect of Rubone treatment on HCC xenografts was investigated by PCNA immunostaining. Qualitative analysis showed a significant decrease in the average number of PCNA-positive cells after Rubone treatment (P = 0.011 vs. vehicle control; Fig. 6B). In addition, p21 and p16 stainings were performed to assess cell senescence in HCC xenografts. There were no significant change in the number of p21 and p16-stained positive cells between control and Rubone-treated groups (Fig. 6C and D). Triggering proliferation inhibition and apoptosis are important hallmarks for an effective anticancer chemotherapeutic agent. These results indicated Rubone inhibited HCC growth by inhibiting cell proliferation and elevating level of apoptosis but not inducing cell senescence.

We also found that the subcutaneous tumors treated with Rubone were visibly less vascularized compared with the control group. Tumors were then stained with endothelial marker CD31. The new blood vessels were highly vascularized.
in the control group, while tumors treated with Rubone had significantly reduced microvessels (Supplementary Fig. S4B). We next found that Rubone caused a dose-dependent inhibition on tube formation of HUVECs in vitro (Supplementary Fig. S4C). These results suggested that antiangiogenesis may be another potential molecular mechanism by which Rubone exerts its in vivo anti-HCC effect.

miR34a modulator increased miR34a promoter activities and p53 occupancy on miR34a promoter

Because both primary and mature miR34a were increased after treatment with Rubone, we next examined whether Rubone could modulate miR34a promoter activity. HCC cell lines with different p53 status (HepG2: p53 wild-type, Bel-7404: p53 mutant, Hep3B: p53 deletion) were used for promoter activity assay. These three cell lines were first transfected with miR34a promoter or miR34a promoter without p53-binding site (miR34a-mutant promoter), and then were treated with Rubone. No luciferase activity change was observed in miR34a-mutant promoter transfected HCC cell lines. miR34a promoter activities were both increased in HepG2 and Bel-7404 cells but not in Hep3B cells (Fig. 7A). These results suggested a potential role of p53 in Rubone modulation of miR34a promoter activity.

We first found there were no significant change in p53 expression level after Rubone treatment in HepG2 and Bel-7404 cells (Supplementary Fig. S5A). Then, we used two independent siRNAs targeting p53 (sip53a and sip53b) to knockdown both wild-type and mutant p53 (Supplementary Figure 4. Rubone inhibited HCC cell growth in vitro. A, Rubone inhibited the growth of HCC cells in a dose-dependant manner. HCC cell lines HepG2, Huh7, Hep3B, Bel-7404, PLC, and MIHA were treated with Rubone at designated concentrations ranging from 1 to 20 μmol/L for 48 hours. B, the comparison between the inhibition effect of Rubone and sorafenib on HCC cell growth at different time points. HepG2, Huh7, Hep3B, Bel-7404, PLC, and MIHA cell lines were treated with 10 μmol/L Rubone or sorafenib for 0 to 72 hours. The differences between Rubone- and sorafenib-treated group was considered as significantly when P < 0.05 (**, P < 0.05; ***, P < 0.01; and ****, P < 0.001 vs. sorafenib-treated group, n = 4)., C, Rubone inhibited HCC cell growth by modulating miR34a expression. HepG2, Bel-7404, and PLC cells were treated with Rubone at the concentration of 10 μmol/L alone or in combination with 2 μmol/L miR34a mimics or inhibitors for 72 hours. All cell viabilities were measured by the MTT assay. Error bars represent mean ± SD of three independent experiments.
Fig. S5B and S5D), and followed by Rubone treatment in HepG2 and Bel-7404 cells. qRT-PCR results showed that miR34a level decreased after p53 knockdown (Fig. 7B). Moreover, the increased expression level of miR34a by Rubone was significantly attenuated by p53 knockdown (Fig. 7B). Meanwhile, pri-miR34a expressions exhibited similar change with that of miR34a in HepG2 and Bel-7404 cells (Supplementary Fig. S5C and S5E). The MTT assay further revealed that the growth-inhibitory effect of Rubone on HCC cells was also significantly reversed by knocking down p53 (Fig. 7C and Supplementary Fig. S5F). These results suggested that p53 played important role in the biologic activity of Rubone. We next used ChIP assay to examine whether Rubone could modulate p53 activities. The results showed that Rubone treatment significantly increased p53 occupancy on miR34a promoter in both HepG2 and Bel-7404 cells. The effect of Rubone on p53 was specific to miR34a promoter, as we showed that Rubone failed to increase p53 occupancy on p21 promoter that was a reported p53-binding region. (Fig. 7D).

Discussion

miR34a is well known to play important roles in the pathogenesis of human diseases, such as metabolic diseases (26), cardiac diseases (27–29), and malignancy. miR34a was dramatically reduced or silenced in a large portion of clinical samples from patients with HCC (30–32). Because miR34a functions as an effective suppressor of malignant properties in cancers (9–13), reexpression of miR34a in tumors represents a potential approach for HCC treatment. Indeed, such potential has been illustrated in numerous studies. It is showed that systemic miR34a delivery suppressed tumor growth in xenograft or genetically engineered mouse models (33). Furthermore, overexpression of miR34a in HCC cells significantly inhibited cell growth and induced apoptosis (17, 34, 35). Despite the need for the development of miR34a-based therapeutic treatment, few strategies are developed that are limited to direct introduction of miR34a into human cells such as systemic delivery of miR34a molecules or viruses. Here, we identified a small molecule Rubone that acted as the modulator of miR34a level. Rubone induced miR34a in HCC cells, but not the nontumor MIHA cells. In contrast, systemic delivery of miR34a will lead to uptake of miR34a by normal cells that induce undesirable miR34a gene silencing.

The discovery of Rubone as the agent against HCC can improve the current therapeutic strategies. The efficacy of available chemotherapeutic drugs for HCC is low (36). Sorafenib is a forefront therapeutic agent for HCC treatment, which...
showed certain efficacy for patients with HCC by inhibiting Raf-1 and vascular endothelial growth factor (VEGF) pathways (37). However, sorafenib-treated patients have mild overall survival benefits from the treatment and a significant number of them experiences disease progressions (38). Moreover, sorafenib shows toxicity to normal cells (Fig. 4B) and elicits serious side effect such as hand-foot skin reaction (39) and eruptive melanocytic lesions (33). Other agents such as sunitinib and brivanib fail to show superiority to sorafenib (40, 41). Therefore, it is still of urgent clinical significance to develop novel therapeutics with better efficacy and less side effect.

Our results showed that Rubone exhibited similar or even higher anti-HCC potency compared with sorafenib. Regarding the unspecific toxicity, Rubone did not show any toxicity to nontumor hepatocytes MIHA cells. In addition, Rubone treatment showed no impairment of the tissues of major organs in BALB/C mice (Supplementary Fig. S4D). Therefore, Rubone warrants further investigation as a potential effective anti-HCC agent. Besides, miR34a was reported to enhance the sensitivity of anti-HCC effect induced by sorafenib (42), which suggested a potential combined treatment of Rubone and sorafenib in HCC. Meanwhile, given previous studies demonstrated increased miR34a in setting of heart disease (27–29), a thorough assessment of Rubone on cardiac function in future studies will be required in the future.

Our study suggested that the action of Rubone to induce miR34a expression is p53-dependent. Previous study has revealed p53 directly and positively regulated the expression of miR34a (8). More than 50% of human tumors contain mutation or deletion of p53 gene (43). However, there is still no report on the relationship between miR34a and mutant p53. Our study showed that Rubone required wild-type or mutant p53 to effectively reactivate miR34a expression, suggested for the first time that mutant p53 could also modulate miR34a expression. Many p53 mutations possess gain-of-function effect, but not inactivation of p53 function. For example, the A220G mutation in huh7 cells lowers the melting temperature and stability of p53 in its DNA-binding domain, causing the denaturation of p53 (44). Study also showed that alterations induced by p53 mutation can be reversible (45). Another HCC
cell line PLC that was responsive to Rubone expressed G249T-mutated p53. It is showed that a designed peptide can rescue the structural distortion of G249T-mutated p53 (46). In these p53-mutant–expressing cells, the function of p53 is diminished but not abolished and evidence suggested that the function of p53 mutant could be modulated. It is possible that Rubone acts as a modulator during the p53-mediated transactivation of miR34a. Besides, we believed that the multiple molecular role of p53 determined the specificity of Rubone to induce miR34a expression. Rubone may restore or enhance a particular molecular function of p53, such as DNA-binding ability or cofactor recruiting ability, which is either in a normal state in wild-type p53 or suboptimal state in mutant p53. This function is indispensable for the activation of miR34a expression. In this respect, it is understandable that Rubone failed to reactivated miR34a in Hep3B cells with p53 deletion. Yet, the effect of Rubone to p53 is still unknown, which requires further investigation. Our studies showed different p53 response induced by Rubone compared with other chemotherapeutic drugs. We speculate that the reason may be that Rubone worked through different mechanism compared with other chemotherapeutic drugs.

Practically, the use of Rubone as therapeutic treatment is feasible in HCC. We showed that Rubone could induce miR34a expression with wild-type or mutant p53, but not in p53-deleted cells. In HCC, the most common type of p53 alteration is point mutation. Over 50% of human aflatoxin-induced HCC harbor G249T mutation (47), while non-aflatoxin- induced, HBV-related and HCV-induced HCC have low mutation rate (48, 49). More importantly, deletion of p53 is rare in human HCC (50). Therefore, Rubone is a suitable agent to treat HCC that retains p53 in cancer cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Chen
Development of methodology: Z. Xiao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Xiao, S.L. Chan, F. Xu, L. Feng, J.J.Y. Sung

Figure 7. Rubone activated miR34a expression by increasing p53 activities. A, Rubone modulated miR34a promoter activity. The arrow above construct P indicates the position of the transcription start site. Filled circles show the position of the p53-binding site. The miR34a promoter reporter constructs were transfected into HepG2, Hep3B, and Bel-7404 cells, respectively. The luciferase activities were measured after 10 μmol/L Rubone treatment. B and C, knockdown of p53 reversed miR34a expression and cell growth inhibition induced by Rubone in HepG2 and Bel-7404 cells. HepG2 and Bel-7404 cells were transfected with 2 μmol/L siRNAs targeting p53 and then treated with 10 μmol/L Rubone. miR34a level was measured by qRT-PCR. Cell viability was measured by the MTT assay. D, Rubone increased p53 occupancy on miR34a promoter. ChIP assay was conducted in HepG2 and Bel-7404 cells treated with 10 μmol/L Rubone. qPCR was used to quantify the p53 occupancy on miR34a and p21 promoter regions. Error bars represent mean ± SD of three independent experiments.
References

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