A small molecule modulator of the tumor suppressor miRNA-34a inhibits the growth of hepatocellular carcinoma

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List of Abbreviations

Bcl-2: B-cell lymphoma 2; CDDP: Cisplatin; CDK6: Cyclin-dependent kinase 6; ChIP: Chromatin immunoprecipitation; DMSO: Dimethyl sulfoxide; Dox: Doxorubicin; 5-FU: 5-Fluorouracil; FOXP1: Forkhead box protein P1; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HUVECs: Human umbilical vein endothelial cells; miR-34a: microRNA-34a; MTT: 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Notch1: Notch homolog 1; SIRT1: Sirtuin 1; qRT-PCR: Quantitative real time PCR; DMEM: Dulbecco modified eagle medium; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.
Abstract

Small molecules that restore the expression of growth inhibitory microRNAs downregulated in tumors may have potential as anticancer agents. miR-34a 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 miR-34a luciferase reporter system to screen for miR-34a modulators that could exert anticancer activity. One compound identified as a lead candidate, termed Rubone, was identified through its ability to specifically upregulate miR-34a in HCC cells. Rubone activated miR-34a expression in HCC cells with wild type or mutated p53 but not in cells with p53 deletions. Notably, Rubone lacked growth inhibitory effects on non-tumorigenic 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 miR-34a target genes and that it enhanced the occupancy of p53 on the miR-34a 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 miR-34a tumor suppressor function.
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 are a class of single-stranded non-coding RNAs (2), and it is estimated that 25%-70% of human genes are regulated by microRNAs (3, 4). The patterns of microRNAs expression were proved to be correlated with cancer types, stages, and other clinical variables (5). Therefore, the expression of microRNAs could be applied as a tool for cancer diagnosis and prognosis. In cancer cells, microRNAs 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 miR-122 inhibitor has been evaluated in phase 2a clinical trial for treating hepatitis C virus (HCV) infection. This builds a bright prospect for developing modulators of miRNAs for disease treatment (7).

MiR-34 family constitutes three members: miR-34a, miR-34b and miR-34c. All miR-34 family members are direct targets of p53. MiR-34b and miR-34c share the same primary transcript while miR-34a resides in another one (8). MiR-34a has been well documented as a tumor suppressor in previous studies (9-13). Up-regulation of miR-34a 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), Notch1 and Sirtuin 1 (SIRT1). Thereby, miR-34a could induce cell apoptosis, senescence and inhibit cellular differentiation and proliferation (14-16). MiR-34a expression was dramatically down-regulated or silenced in various cancers including human HCC. Ectopic expression of miR-34a inhibited HCC cell migration and invasion (17). Thus, restoration of miR-34a might represent a potential therapeutic for HCC.

Few studies have focused on small molecule modulators of microRNAs so far. Previously, the small molecule modulators of miR-21 and miR-122 have been identified with potent biological activities (18, 19). However, the small molecule modulators of miR-34a have not been identified yet. In this study, we developed a luciferase reporter system for the screening of small-molecule modulators of miR-34a from a natural product library. After library screening, one hit compound named Rubone was found to activate miR-34a expression in HCC cells. We further investigated the anticancer activity of miR-34a modulator in both cell culture and animal models.

**Materials and Methods:**

**Cell lines and drugs**

The non-tumorigenic human hepatocyte cell line MIHA (20) was obtained from Dr J.R. Chowdhury’s laboratory at Albert Einstein College of Medicine, New York. The human HCC cell line Huh7 (21) (kindly provided by Dr H. Nakabayashi,
Hokkaido University School of Medicine (Japan) and Bel-7404 (22) (Cell Bank of the Chinese Academy of Sciences) 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. Beijing, China within 6 months of use. All cell lines were cultured under the condition as previously described (23). Carboxymethylcellulose was provided by Unitech Chemicals (China). Sorafenib was purchased from Bayer Healthcare (Germany). Dimethyl sulfoxide (DMSO), Cisplatin (CDDP), 5-Fluorouracil (5-FU) and Doxorubicin (Dox) were purchased from Sigma (USA).

** Constructs **

The pmiR-REPORT™ luciferase plasmid (Ambion) was sequentially digested with restriction enzyme Sac I and Hind III (10 units each in 50 μl reaction, NEB) and was purified after gel electrophoresis. DNA oligos containing the miR-34a binding site (5′- CTG GCA GTG TCT TAG CTG GTT GTA-3′ and 5′-AGC TTA CAA CCA GCT AGA ACA CTG CGA GAG CT-3′), and scrambled miR-34a binding site (5′- CTG TTT CGT TGG GCG TTT CGA AGA-3′ and 5′-AGC TTC TTC GAA ACG CCC AAC GAA ACA GAG CT-3′) were ordered from TechDragon (Hong Kong). The oligos were annealed at 95°C and then cooled to 4°C. The annealed DNA fragments were then ligated into pmiR-REPORT™ luciferase vector with T4 DNA ligase (200 units in 10 μl reaction, NEB). The generated constructs containing miR-34a binding site or scrambled binding site were named as miR-34a reporter or...
miR-34a scr. The luciferase promoter constructs containing miR-34a promoter or miR-34a mutant promoter were kindly provided by Prof. Mendell JT from Johns Hopkins University (16).

Transfection

miR-34a mimics duplex, single-stranded miR-34a inhibitors and siRNAs targeting p53 were purchased from Shanghai GenePharma Co (China). The sip53 sequence was as following: sip53a: 5'-CUA CUU CCU GAA AAC AAC G dTdT-3'; sip53b: GCA UGA ACC GGA GGC CCA U dTdT. The miR-34a 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 manufacturer’s protocol.

Library screening with miR-34a reporter

Huh7 cells were cultured in complete Dulbecco Modified Eagle Medium (DMEM). 5×10^3 Huh7 cells were seeded into each well of 96-well plates and incubated for 12 h at 37°C with 5% CO₂. Then, Huh7 cells were co-transfected with miR-34a reporter and renilla luciferase pRL-SV40. The transfection medium was replaced with complete DMEM after 4 h incubation. Compounds from natural products library (NPL) (NPL contained 640 compounds including natural products and their derivatives, TimTec, USA) were added to the cells at a final concentration of
10 μM (0.1% DMSO at final concentration). The complete DMEM containing 0.1% DMSO was used as control. After 48 h of incubation, the luciferase activity was measured.

**Luciferase assay**

miR-34a reporter, miR-34a promoter and miR-34a mutant promoter were transfected into HCC cells 12 h before compound treatment. The renilla luciferase vector pRL-SV40 was co-transfected as an internal control. HCC cells were then treated with compound for 48-72 h. The luciferase activity was measured by Dual Luciferase Reporter Assay (Promega) with a Wallac VICTOR3V 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 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 mM PMSF and 1×complete protease inhibitor cocktail (Roche). Both RNA and protein were stored at -80°C.

**Quantitative real time PCR analysis (qRT-PCR)**
Total RNA was reversely transcribed into cDNAs using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed using SYBR Green PCR master mix (TaKaRa). GAPDH was used as the internal control. MiR-34a level was measured by qRT-PCR using SYBR Green PCR master mixture (Invitrogen). U6 was measured as the internal control. Fold change was calculated by relative quantification \(2^{-\Delta\Delta Ct} \). The sequences of the primers were listed in supplementary table 1.

**Western blotting**

Protein concentration was measured with BCA protein assay kit (Thermo Fisher Scientific). 25-50 μg protein was separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to PVDF membranes (Bio-Rad). Primary antibodies used in this study included anti-cyclin D1 (1:1000 dilution, Cat #: 2922, Cell signaling), anti-Bcl-2 (1:1000 dilution, Cat #: AM2209, ABZOOM), anti-β-actin (1:5000, Cat #: 4967, Cell signaling) and anti-p53 (1:1000, Cat #: Sc-126, Santa Cruz). Membranes were incubated with primary antibodies at 4°C overnight, and then washed thrice with TBST (Tris-Buffered Saline Tween-20). The membranes were then incubated with 1:3000 peroxidase conjugated secondary antibody (Santa Cruz) for 1 h at room temperature, followed by three washings in TBST. Protein expressions were detected by autoradiography film using Amersham ECL PlusTM Western Blotting Detection Reagents (GE Healthcare).
Cell viability assay

Cell viability was measured by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described (24). Briefly, 1×10^3 cells were seeded on 96-well plates in triplicate and treated with compounds at indicated concentrations for indicated time points. At each time point, a set of cells were incubated with 0.5 mg/ml MTT diluted in 1×PBS. After 2 to 4 h 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

5×10^6 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 5-10 mm in diameter, mice were randomly divided into 4 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 mg/kg or 50 mg/kg Rubone in 200 µl vehicle by gavage once every two days. 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 two 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.

Immunohistochemical staining

Immunohistochemical staining was performed on xenografted tumor tissues with PCNA (1:400 dilution, Cat #: SC-56, Santa Cruz), p16 (1:200 dilution, Cat #: BM0174, ABZOOM), p21 (1:250 dilution, Cat #: SC-397, Santa Cruz) 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 (ChIP) assay

ChIP assay was performed as described previously (25). Cross-linked chromatin were incubated overnight with anti-H3 (Cat #: 4620, Cell Signaling), IgG (Cat #:
2729, Cell Signaling), p53 (Cat #: sc-126, Santa Cruz). 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 IC50. Two tailed Student’s 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 miR-34a by library screening**

We first established a luciferase reporter by cloning the complementary sequence of miR-34a into pmiR-Reporter vector (Fig. 1A). The scrambled miR-34a binding sequence was used as a control. Theoretically, this miR-34a reporter could be used for screening miR-34a modulators by measuring luciferase signals (Fig 1B). The endogenous miR-34a level in HepG2 cells was significantly higher (22.29-fold, p=0.0017) than that of Huh7 cells as measured by qRT-PCR (Supplementary fig. 1A). We transfected miR-34a 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. 1B). Co-transfection of miR-34a mimics and miR-34a reporter decreased the
luciferase signal in Huh7 cells (Supplementary fig. 1C). Conversely, co-transfection of miR-34a inhibitor and miR-34a reporter increased the luciferase signal in HepG2 cells (Supplementary fig. 1D). No luciferase signal was changed in control group where miR-34a reporter was replaced with miR-34a scr. These results indicated that miR-34a reporter was responsive to miR-34a level.

Using the miR-34a reporter system, we screened the NPL for miR-34a 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 miR-34a modulator (The full library screening dataset was listed in supplementary table 2). Rubone was re-assayed in triplicate with miR-34a reporter system. Fig 1D showed Rubone inhibited the luciferase activity in a dose-dependent manner, with an IC50 value at 3.8 μM. Rubone was a chemically synthesized plant chalcone derivative which was also named 2'-Hydroxy-2, 4, 4', 5, 6'-pentamethoxychalcone. Its molecular weight was 374.39 and its chemical structure was shown in figure 1D. Rubone has not been previously reported to have any known biological activities.

We next examined whether Rubone modulated miR-34a expression in HCC cells. As shown in Fig 2, both primary and mature miR-34a levels significantly increased after treatment with Rubone in Huh7 and HepG2 cells. However, Rubone caused no change in the expression of primary and mature miR-34a 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 miR-34a expression in HCC cells expressing either wild-type or mutant p53 but not in HCC cells with p53 deletion.

To exclude the possibility of non-specific modulation on miR-34a expressions 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 miR-34a (Supplementary fig. 1A). We further demonstrated that other chemotherapeutic drugs such as CDDP, 5'-FU, Dox and sorafenib significantly inhibited HCC cell growth (Supplementary fig. 2B). Among these drugs, Dox could induce the expressions of p53 and its downstream targets including p21 and Puma (Supplementary fig. 2C). However, we found these drugs had no effect on pri-miR-34a or miR-34a expression in HCC cells (Supplementary fig. 2D). These results indicated that Rubone preferentially modulated miR-34a expression in HCC cells. Cyclin D1 and Bcl-2 were two of the most well studied miR-34a 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 miR-34a targets including CDK6, FOXP, Notch1 and SIRT as well as p53 targets including p21 and Puma. Similarly, the expression levels of these miR-34a targets were decreased after Rubone
treatment in Huh7 and HepG2 but not in Hep3B cells (Supplementary fig 2E), although there were no change on the expression of p53 targets in these cells (Supplementary fig 2F).

MiR-34a modulator inhibited HCC cells growth in vitro

We next examined the anti-cancer 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 MTT assay. Rubone 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 non-tumorigenic 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 h. Rubone showed little growth inhibition on Hep3B and MIHA cells while 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 miR-34a. HepG2, Bel-7404 and PLC cells were transfected with miR-34a mimics or
inhibitors and then treated with Rubone. Fig 4C showed that miR-34a mimics showed concomitant effect of growth inhibition with Rubone to all HCC cell lines. Conversely, miR-34a inhibitors attenuated the growth inhibitory effect of Rubone. These results indicated that Rubone inhibited HCC cell growth by modulating miR-34a expression.

**miR-34a modulator inhibited hepatocellular tumor growth in vivo**

We next examined the *in vivo* anti-cancer 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 3, 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 mg/kg 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 3). When comparing the anti-cancer activities of Rubone and sorafenib during the whole treatment process, Rubone exhibited higher anti-cancer 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 to the vehicle control. The tumors from Rubone treated group
were smaller and lighter when compared to the sorafenib treated group (Supplementary fig. 3 A, B, C).

The expression levels of pri-miR-34a, miR-34a, Cyclin D1 and Bcl-2 in the xenografted tumors were measured by qRT-PCR. In Rubone treated tumors, both pri-miR-34a and miR-34a levels were up-regulated, while miR-34a targets Cyclin D1 and Bcl-2 were down-regulated (Fig. 5C and supplementary fig. 4A). Sorafenib treatment caused no change in the expression of miR-34a and its targets (Fig. 5C and supplementary fig. 4A). These results could also be validated in Huh7 xenografted nude mice model (Supplementary fig. 3D). Moreover, there was no obvious body weight loss in the treated mice compared with vehicle control (Supplementary table 3). 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. 4D). These results indicated that Rubone was an effective and safe anti-HCC agent in animal.

**miR-34a 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* anti-proliferative 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). Additionally, p21 and p16-staining 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. 6 C, D). Triggering proliferation inhibition and apoptosis
are important hallmarks for an effective anti-cancer 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. 4B). We next found that Rubone caused a
dose-dependent inhibition on tube formation of HUVECs \textit{in vitro} (Supplementary fig.
4C). These results suggested that anti-angiogenesis may be another potential
molecular mechanism by which Rubone exerts its \textit{in vivo} anti-HCC effect.

\textbf{MiR-34a modulator increased miR-34a promoter activities and p53 occupancy
on miR-34a promoter}

Since both primary and mature miR-34a were increased after treatment with
Rubone, we next examined whether Rubone could modulate miR-34a 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 firstly transfected with miR-34a promoter or miR-34a promoter without p53 binding site (miR-34a mutant promoter), and then were treated with Rubone. No luciferase activity change was observed in miR-34a mutant promoter transfected HCC cell lines. MiR-34a 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 miR-34a 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. 5A). Then, we employed two independent siRNAs targeting p53 (sip53a and sip53b) to knock down both wild type and mutant p53 (Supplementary fig. 5B, D), and followed by Rubone treatment in HepG2 and Bel-7404 cells. qRT-PCR results showed that miR-34a level decreased after p53 knockdown (Fig 7B). Moreover, the increased expression level of miR-34a by Rubone was significantly attenuated by p53 knockdown (Fig. 7B). Meanwhile, pri-miR-34a expressions exhibited similar change with that of miR-34a in HepG2 and Bel-7404 cells (Supplementary fig. 5C, E). 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. 5F). These results suggested that p53 played important role in the biological activity of Rubone. We next
employed ChIP assay to examine whether Rubone could modulate p53 activities. The results showed that Rubone treatment significantly increased p53 occupancy on miR-34a promoter in both HepG2 and Bel-7404 cells. The effect of Rubone on p53 was specific to miR-34a promoter, as we showed that Rubone failed to increase p53 occupancy on p21 promoter which was a reported p53 binding region. (Fig. 7D).

Discussion

miR-34a is well known to play important roles in the pathogenesis of human diseases, such as metabolic diseases (26), cardiac diseases (27-29) and malignancy. MiR-34a was dramatically reduced or silenced in a large portion of clinical samples from HCC patients (30-32). Since miR-34a functions as an effective suppressor of malignant properties in cancers (9-13), re-expression of miR-34a in tumors represents a potential approach for HCC treatment. Indeed, such potential has been illustrated in numerous studies. It is showed that systemic miR-34a delivery suppressed tumor growth in xenograft or genetically engineered mouse models (39). Furthermore, overexpression of miR-34a in HCC cells significantly inhibited cell growth and induced apoptosis (17, 33, 34). Despite the need for the development of miR-34a-based therapeutic treatment, few strategies are developed which are limited to direct introduction of miR-34a into human cells such as systemic delivery of miR-34a molecules or viruses. Here, we identified a small molecule Rubone that acted as the modulator of miR-34a level. Rubone induced miR-34a in HCC cells, but not the non-tumor MIHA cells. In contrast, systemic delivery of miR-34a will lead to
uptake of miR-34a by normal cells that induce undesirable miR-34a 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 (35). Sorafenib is a forefront therapeutic agent for HCC treatment, which showed certain efficacy for HCC patients by inhibiting Raf-1 and vascular endothelial growth factor (VEGF) pathways (36). However, sorafenib-treated patients have mild overall survival benefits from the treatment and a significant number of them experiences disease progressions (37). Moreover, sorafenib shows toxicity to normal cells (Fig. 4B) and elicits serious side effect such as hand-foot skin reaction (38) and eruptive melanocytic lesions (39). 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 to sorafenib. Regarding the unspecific toxicity, Rubone did not show any toxicity to non-tumor hepatocytes MIHA cells. In addition, Rubone treatment showed no impairment of the tissues of major organs in BALB/C mice (Supplementary fig.4D). Therefore, Rubone warrants further investigation as a potential effective anti-HCC agent. Besides, miR-34a 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 miR-34a 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 miR-34a expression is p53-dependent. Previous study has revealed p53 directly and positively regulated the expression of miR-34a (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 miR-34a and mutant p53. Our study showed that Rubone required wild type or mutant p53 to effectively reactivate miR-34a expression, suggested for the first time that mutant p53 could also modulate miR-34a 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 miR-34a. Besides, we believed that the multiple molecular role of p53 determined the specificity of Rubone to induce miR-34a 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 wildtype p53 or suboptimal state.
in mutant p53. This function is indispensable for the activation of miR-34a expression. In this respect, it is understandable that Rubone failed to reactivate miR-34a 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 miR-34a expression with wildtype 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.

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Figure legends

**Fig. 1.** Identification of miR-34a modulator through library screening. (A) Schematic illustration of miR-34a reporter system. The complementary sequences including mature miR-34a and the scrambled miR-34a sequence were cloned into pMIR-REPORT™ miRNA Reporter Vector (Ambion) to establish miR-34a reporter and miR-34a. (B) Working mechanism of the miR-34a reporter. The miR-34a reporter could detect the presence of functional mature miR-34a as reflected by the rate of luciferase activity repression. (C) The process of library screening for the identification of miR-34a modulators. (D) A small molecule compound named Rubone significantly inhibited the luciferase activity of miR-34a reporter at a dose-dependent manner. Huh7 cells were transfected with miR-34a reporter and then treated with indicated concentrations of Rubone for 48 h. Cell lysates were subjected to luciferase assay. Results are expressed as luciferase activity values. Error bars represent means ±SD of three independent experiments.

**Fig. 2.** Rubone increased miR-34a expression levels in HCC cells. Huh7, HepG2 and Hep3B cells were treated with Rubone at the concentration of 10 μM or vehicle control for 48 h. Then, RNA samples were prepared and the fold change of primary and mature miR-34a levels were measured by qRT-PCR. Error bars represent means ±SD of three independent experiments.

**Fig. 3.** Rubone decreased the expression of miR-34a targets. Huh7, HepG2 and
Hep3B cells were treated with Rubone at the concentration of 10 μM or vehicle control for 48 h or 72 h. RNA samples collected at 48 h were applied for measuring miR-34a targets cyclin D1 and Bcl2 by qRT-PCR. The cell lysates collected at 72 h were subjected to western blotting. Specific antibodies against cyclin D1 (1:2500 dilution), Bcl-2 (1:2500 dilution) and β-actin (1:5000 dilution) were used. Error bars represent means ±SD of three independent experiments.

**Fig. 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 μM for 48 h. (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 μM Rubone or sorafenib for 0 h to 72 h. 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 miR-34a expression. HepG2, Bel-7404 and PLC cells were treated with Rubone at the concentration of 10 μM alone or in combination with 2 μM miR-34a mimics or inhibitors for 72 h. All cell viabilities were measured by MTT assay. Error bars represent means ±SD of three independent experiments.

**Fig. 5.** Rubone inhibited HepG2 xenografted tumor growth in nude mice. (A) Rubone
inhibited hepatocellular tumor growth \textit{in vivo} in a dose-dependent manner. Photographs of representative tumors excised from nude mice at Day 24. Scale bars = 1 cm. \textbf{(B)} The comparison between the hepatocellular tumor inhibition effect of Rubone and sorafenib \textit{in vivo}. \textbf{(C)} Modulation of miR-34a and its targets by Rubone \textit{in vivo}. RNA was extracted from the excised tumors. MiR-34a, cyclin D1 and Bcl-2 levels were measured by qRT-PCR. Error bars represent means \pm SD of three independent experiments.

\textbf{Fig. 6.} Rubone inhibited proliferation and induce apoptosis in HepG2 xenografts. \textbf{(A)} Cell apoptosis was detected by TUNEL assay on the HepG2 xenografts after treatment with Rubone. TUNEL-positive cells were stained in green and all nuclei are stained in blue with DAPI. \textbf{(B)} Cell proliferation was examined using immunohistochemistry with PCNA antibody. PCNA positive cells were stained in brown and cell nuclei stained in blue. \textbf{(C, D)} Cell senescence was examined using immunohistochemistry with p16 and p21 antibodies. P16 and p21 positive cells are stained in brown and cell nuclei stained in blue. All scale bars = 50 \(\mu\)m. Error bars represent means \pm SD of three independent experiments.

\textbf{Fig. 7.} Rubone activated miR-34a expression by increasing p53 activities. \textbf{(A)} Rubone modulated miR-34a 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. P53 binding site mutation introduced into the miR-34a promoter reporter
construct (Pmut) is shown in red. The miR-34a promoter reporter constructs were transfected into HepG2, Hep3B and Bel-7404 cells respectively. The luciferase activities were measured after 10 μM Rubone treatment. (B, C) Knockdown of p53 reversed miR-34a expression and cell growth inhibition induced by Rubone in HepG2 and Bel-7404 cells. HepG2 and Bel-7404 cells were transfected with 2 μM siRNAs targeting p53, and then treated with 10 μM Rubone. MiR-34a level was measured by qRT-PCR. Cell viability was measured by MTT assay. (D) Rubone increased p53 occupancy on miR-34a promoter. ChIP assay was conducted in HepG2 and Bel-7404 cells treated with 10 μM Rubone. qPCR was employed to quantify the p53 occupancy on miR-34a and p21 promoter regions. Error bars represent means ±SD of three independent experiments.
Fig. 1

A

Luc-miR-34a reporter
miR-34a complementary sequence
Luc-miR-34a scr
miR-34a complementary sequence scramble

B

Small molecule
endogenous miR-34a
miR-34a binding sequence
Luciferase
miR-34a binding sequence
Luciferase

C

Growing Huh7 cells in 96-well dishes

D

Intensity of luciferase signal

Rubone (MW-374.39)
2'-Hydroxy-2,4,4',5,6'-pentamethoxychalcone

Concentration / μM

0 1 2 3 4 5 6 7 8 9 10

Rubone

*p = 0.03
*p = 0.031
*p = 0.019

**p = 0.0068

Cotransfected Luc-miR-34a reporter and Renilla-SV40 plasmids into Huh7 cell, incubating for 4 hours

Adding 640 compounds from NPL into 96-well plates, incubating for 48 hours

Luciferase assay
HepG2: p53 wild type, Huh7: p53 mutant, Hep3B: p53 deficient
Fig. 3

Huh7

Ctrl
Rubone

HepG2

Ctrl
Rubone

Hep3B

Ctrl
Rubone

Actin
Cyclin D1
Bcl-2

Bcl-2 fold change

Cyclin D1 fold change

Ctrl
Rubone

**p=0.0004

Ctrl
Rubone

**p=0.0004

Ctrl
Rubone

**p=0.0004

Ctrl
Rubone

**p=0.0004

Ctrl
Rubone

**p=0.0004
Fig. 4

A

B

C

Legend:
- MIHA
- HepG2
- Huh7
- Bel-7404
- Hep3B
- PLC

**p<0.01
*p<0.05
Fig. 5

A

![Graph showing tumor volume over days for different treatments](image)

B

![Graph showing tumor volume over days for different treatments](image)

C

![Bar graphs showing changes in expression levels](image)

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Fig. 6

A

Tunel

Ctrl

Rubone

**p=0.0033

B

PCNA

Ctrl

Rubone

*p=0.011

C

p21

Ctrl

Rubone

D

p16

Ctrl

Rubone
Fig. 7

(A) Schematic representation of the pGL3-promoter (P), pGL3-promoter mutation (P^{mut}), and pGL3-basic (Empty) constructs. The +578 and -1402 regions are indicated. IRES-Luciferase is shown in the constructs.

(B) Luciferase signal analysis for HepG2, Bel-7404, and Hep3B cells treated with Ctrl or Rubone. The pGL3-promoter mutation (P^{mut}) shows a significant increase in luciferase signal compared to the pGL3-promoter (P).

(C) Cell viability analysis for HepG2 and Bel-7404 cells treated with siN, sip53a, sip53a+Rubone, or siN+Rubone over 1 to 3 days. The pGL3-promoter mutation (P^{mut}) shows enhanced cell viability compared to the pGL3-promoter (P).

(D) p53 ChIP analysis for HepG2 and Bel-7404 cells. The pGL3-promoter mutation (P^{mut}) shows increased p53 ChIP enrichment compared to the pGL3-promoter (P).
A small molecule modulator of the tumor suppressor miRNA-34a inhibits the growth of hepatocellular carcinoma

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