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Small Molecule Modifiers of MicroRNA miR-122 Function for the Treatment of Hepatitis C Virus Infection and Hepatocellular Carcinoma

Douglas D. Young,‡ Colleen M. Connelly,‡ Christoph Grohmann, and Alexander Deiters*

Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695

Received December 4, 2009; E-mail: alex_deiters@ncsu.edu

Abstract: MicroRNAs are a recently discovered new class of important endogenous regulators of gene function. Aberrant regulation of microRNAs has been linked to various human diseases, most importantly cancer. Small molecule intervention of microRNA misregulation has the potential to provide new therapeutic approaches to such diseases. Here, we report the first small molecule inhibitors and activators of the liver-specific microRNA miR-122. This microRNA is the most abundant microRNA in the liver and is involved in hepatocellular carcinoma development and hepatitis C virus (HCV) infection. Our small molecule inhibitors reduce viral replication in liver cells and represent a new approach to the treatment of HCV infections. Moreover, small molecule activation of miR-122 in liver cancer cells selectively induced apoptosis through caspase activation, thus having implications in cancer chemotherapy. In addition to providing a new approach for the development of therapeutics, small molecule modifiers of miR-122 function are unique tools for exploring miR-122 biogenesis.

Introduction

MicroRNAs (miRNAs) are single-stranded noncoding RNAs of 21−23 nucleotides. They are a recently discovered class of gene regulators that function by binding the 3′ untranslated regions of specific target mRNAs leading to gene inactivation by repression of mRNA transcription or induction of mRNA degradation.[1−3] MicroRNAs are transcribed from the genome and undergo several post-transcriptional processing steps via a dedicated microRNA pathway. It is estimated that 1000 miRNAs exist in humans, controlling approximately 30% of all genes, and thus are involved in almost every genetic pathway and many human pathologies, e.g. cancer, heart disease, and viral infection.[4]

MicroRNA miR-122 is a liver specific miRNA and the most abundant miRNA in the liver.[5] It was discovered that miR-122 is greatly down-regulated in hepatocellular carcinoma (HCC). Identified targets of miR-122 in primary liver carcinomas and the HCC cell lines Hep3B and HepG2 are cyclin G1 (CCNG1) and Bcl-w, an antiapoptotic Bcl-2 family member.[6,7] HCC is a primary cancer of the liver, and it is the third largest cause of cancer-related death behind only lung and colon cancers.[8] Treatment options of HCC and prognosis are usually poor (with a median survival time of 3 to 6 months), as only 10−20% of hepatocellular carcinomas can be removed completely using surgery.[8] Transfection of miR-122 into cancer cells induced apoptosis and led to reduced cell viability.[9,10] Consequentially, the induction of apoptosis in malignant hepatocytes through the activation of miR-122 expression represents a potential treatment for HCC.

The liver-specific miR-122 is also necessary for hepatitis C virus (HCV) replication and infectious virus production through interaction with the viral genome.[11−14] HCV infection is one of the major causes of chronic liver disease, including cirrhosis and liver cancer and is therefore the most common indication for liver transplantation. The role of miR-122 in HCV replication suggests that it could be an excellent target for antiviral therapy, since knock-down of miR-122 results in a dramatic decrease

‡ These authors contributed equally to this work.

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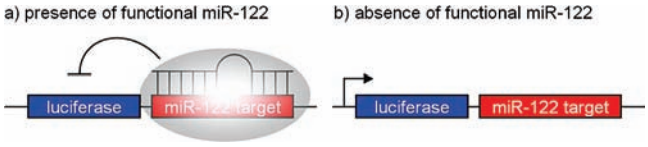

Figure 1. MicroRNA miR-122 assay. (a) The developed luciferase reporter can detect the presence of a functional mature miR-122 through repression of the luciferase signal. (b) Small molecules are assayed for their ability to alleviate the suppression by inhibition of miR-122, thus inducing luciferase expression.

of HCV RNA in human liver cells,[13,14] without any toxic effects in mice and primates.[11,15,16]

The discovery of small molecule modifiers of miR-122 will validate this miRNA as a new therapeutic target. Recently, we reported the first small molecule inhibitors of miRNA function, specifically miR-21 function.[17] These compounds displayed specificity for miR-21 and induced a reduction of both mature-miR-21 and primary-miR-21 levels. Additionally, the small molecule enoxacin has been demonstrated to be a general activator of both the siRNA and miRNA pathways,[18] presumably through promoting the processing and loading of siRNAs/miRNAs into RISCs by facilitating the interaction between TAR RNA-binding protein and RNAs.

Results and Discussion

Assay Development. We developed an miRNA small molecule modifier screen for miR-122 based on the psiCHECK-2 (Promega) reporter plasmid. This construct expresses both *Renilla* luciferase and firefly luciferase, allowing for the normalization of the signal to account for differential cellular viability (an advantage over our previous reporter constructs[17]). The miR-122 target sequence was inserted downstream of the *Renilla* luciferase gene, between the PmeI and SgfI restriction sites (Supporting Information). Thus, the presence of mature miR-122 will lead to a decrease in the *Renilla* luciferase signal (Figure 1a). The ability to detect endogenous miR-122 was validated by transfecting the generated psiCHECK-miR122 construct into Huh7 and HeLa cells. After a 24 h incubation, the cells were assayed using a Dual Luciferase Assay Kit (Promega). Huh7 cells have previously been demonstrated to express high levels of miRNA-122,[15] whereas miR-122 is not expressed in HeLa cells.[19] The psiCHECK-miR122 reporter verified these results by displaying a >15-fold reduced luciferase signal in Huh7 cells, in contrast to HeLa cells (Supporting Figure S1) and, thus, is a cellular sensor for miR-122 expression. The luciferase signal is readily restored upon the cotransfection with a miR-122 antagomir, suggesting that the reporter can be employed in the discovery of miR-122 inhibitors (Figure 1b). Moreover, a psiCHECK-control reporter (containing an empty multicloning site) is not affected. The signal-to-background ratio is 9.0 and the statistical parameter Z′ is 0.66, demonstrating a robust assay.[20] The variation between plates and from day-to-day is ~10% and, therefore, fairly small.

Discovery of miR-122 Inhibitors and Activators. The psiCHECK-miR122 vector was then employed in a small molecule screen in Huh7 cells to discover modifiers of miR-122 function. Specifically, an increase in the relative *Renilla* luciferase signal indicates a miR-122 inhibitor, while a reduction in the luciferase signal indicates a miR-122 activator. The Diversity Set II (1364 compounds) from the NCI Developmental Therapeutics Program was screened in a 96-well format using Huh7 cells containing the psiCHECK-miR122 reporter. Cells were exposed to the small molecules (10 μM), and the relative luciferase signal was measured after 48 h using a Dual Luciferase Assay Kit (Promega). Gratifyingly, we discovered the compounds **1** (NSC 158959) and **2** (NSC 5476), which induce a 773 ± 38% and 1251 ± 125% increase in the relative luciferase signal (Figures 2 and 3).

The identity and purity (>95%) of **1** and **2** was confirmed by NMR and mass spectrometry. Both compounds were re-assayed in triplicate with both the psiCHECK-miR122 vector and the psiCHECK-control vector (no miRNA target sequence), confirming their activity as miR-122 inhibitors and validating that they do not increase the luciferase signal in a non-miRNA specific fashion (Supporting Figure S2). Both small molecules were also assayed with our previously described miRNA-21 reporter in HeLa cells (Supporting Figure S3),[17] and no activity was found, suggesting that they are not general inhibitors of the miRNA pathway but display a degree of specificity for miR-122.

To validate the activity of **1**, a structure−activity relationship study was undertaken by synthesizing various analogues (Figure 2). Replacement of the halogens with an acetyl (**1a**) or a nitro group (**1b**) at the 4-position of **1** or their removal (**1d**), resulted in small activity changes between 5.7 and 6.7 RLU. However, replacement of the halogens with methoxy groups at the 3- and 4-position (**1c**) led to a complete loss of activity. Modification of the naphtyl group was much less tolerated, since replacement with a larger anthracenyl (**1e**) or a methylene naphtyl group (**1f**) led to virtually complete loss of miR-122 inhibitory activity. The same is true for the smaller phenyl (**1g**), *p*-aminophenyl (**1h**), or *p*-iodophenyl (**1i**) substituents. Similar changes of the naphtyl ring in the miR-122 inhibitor **1a** also led to complete loss of activity (**1j**−**1l**). Methylation of the amide NH was also not tolerated (**1m**).

The structure−activity relationship of the second, more potent miR-122 inhibitor **2** was also further investigated (Figure 3). Attempts to structurally simplify the *trans*-decahydroquinoline motif by replacing it with piperidine (**2a**) or dihydroquinoline (**2b**) led to a loss of activity. Activity was maintained in the case of aniline (**2c**) but was lost through installation of a *p*-amino group (**2d**). The simple sulfonamide **2e** showed a 50% reduced activity which was then gradually improved almost to the level of the parent compound **2** by installing carbon chains of increasing length, from methyl (**2f**), propyl (**2g**), allyl (**2h**), and propargyl (**2i**), to hexyl (**2j**). The acylation of the NH group of any of the miR-122 inhibitors shown in Figure 3 with $CO_2CH_2CH_3$ led to a complete abrogation of activity (data not shown).

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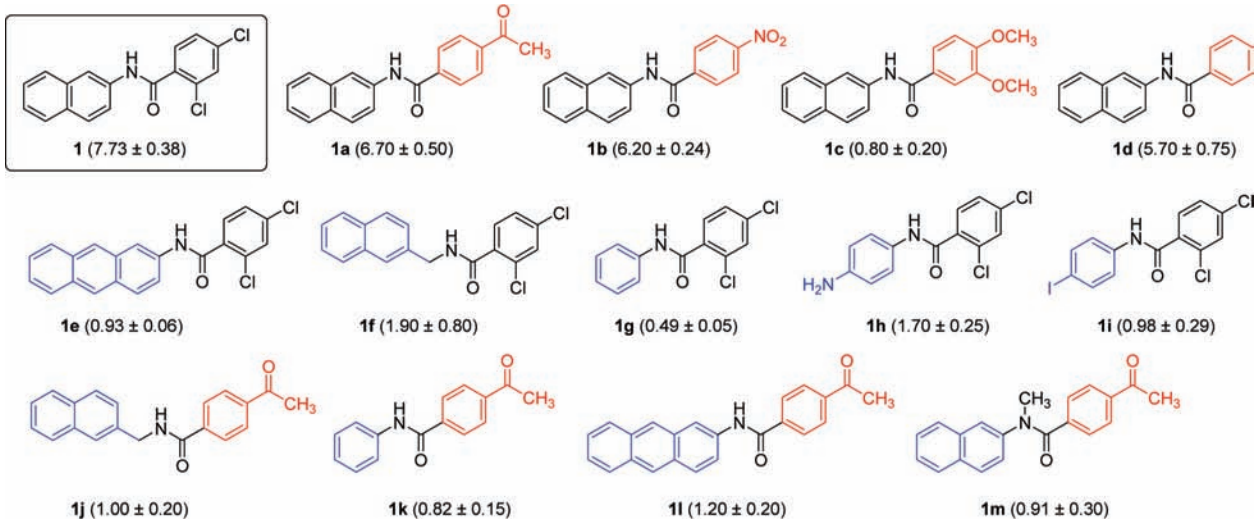

Figure 2. Discovered small molecule inhibitor **1** of miR-122 and preliminary structure−activity investigation. The numbers in parentheses represent relative luciferase units (RLU) normalized to a DMSO control, and the standard deviation is derived from three independent assays.

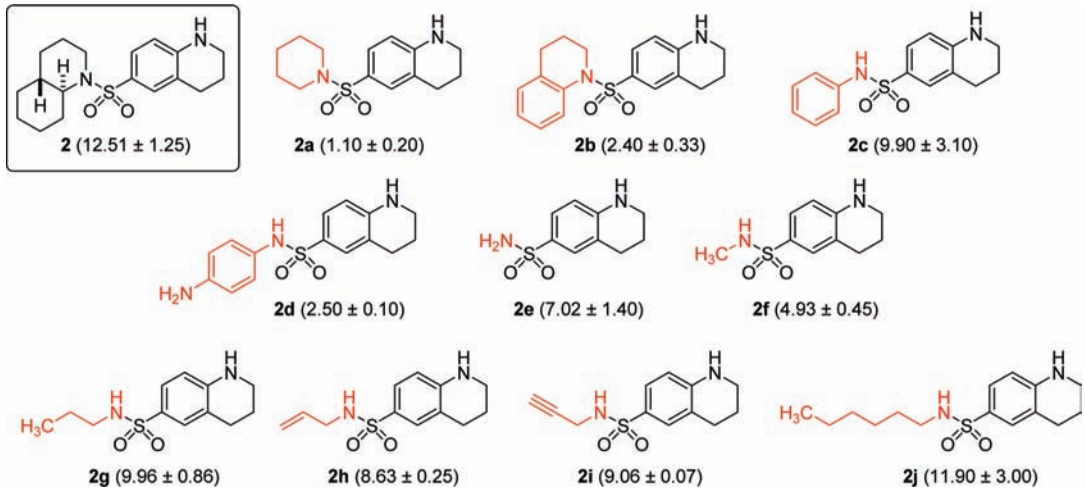

Figure 3. Discovered small molecule inhibitor **2** of miR-122 and preliminary structure−activity investigation. The numbers in parentheses represent relative luciferase units (RLU) normalized to a DMSO control, and the standard deviation is derived from three independent assays.

Compared to healthy liver tissue, miR-122 is reduced by ~85% in the HCC cell line Huh7 and by 99.5% in the HCC cell lines HepG2 and Hep3B.[5,10] Thus, the screening results were also analyzed for a further reduction in the relative *Renilla* luciferase signal, since this could reveal small molecules that would activate miR-122 function in Huh7 cells. Several compounds were identified and reassayed in triplicate with both the psiCHECK-miR122 vector and the psiCHECK-control vector. Compound **3** (NSC 308847; Figure 4) was found to be an activator of miR-122, inducing a 7-fold reduction of the *Renilla* luciferase signal (0.11 ± 0.02 RLU). No effect of **3** on miR-21 controlled luciferase activity[17] was observed in HeLa cells, indicating that **3** is not a general activator of the miRNA pathway, in contrast to the small molecule enoxacin,[18] but shows specificity toward miR-122 activation. The molecule's identity and purity (>95%) were confirmed by both NMR and MS analysis.

Structural modifications of the miR-122 activator **3** (Figure 4) were performed. Removal of the NH_2 group (**3a**) displayed retention of activity; however, replacement with a nitro group (**3b**) completely abrogated miR-122 activation. The removal of both *N*-methyl groups (**3c**) and substitution of the ethylamino group with a proton (**3d**) led to an increasing loss of activity. Other modifications of that group, e.g. with a methylcarboxylate

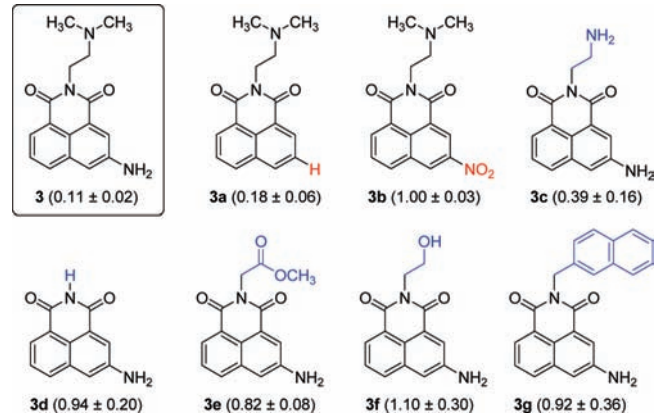

Figure 4. Discovered small molecule activator **3** of miR-122 and preliminary structure−activity investigation. The numbers in parentheses represent relative luciferase units (RLU) normalized to a DMSO control, and the standard deviation is derived from three independent assays.

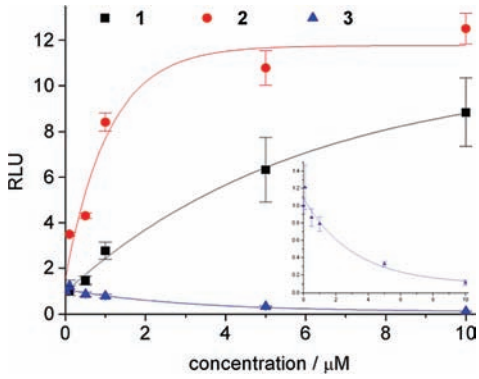

Figure 5. Luciferase assay dose response curves for compounds **1**−**3**. The insert shows only the activator **3**. All assays were conducted in triplicate and normalized to a DMSO control. Error bars represent standard deviations.

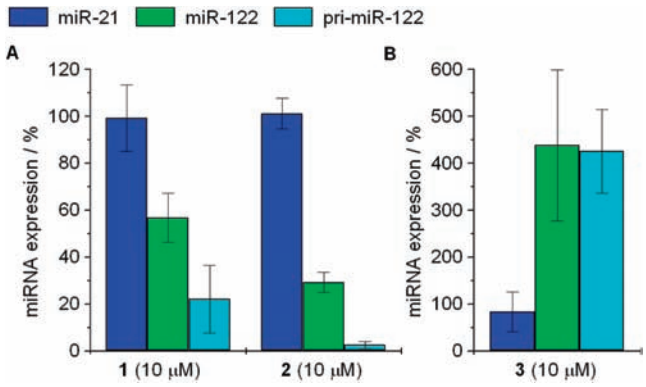

Figure 6. RT PCR quantification of miR-122 and pri-miR-122 in Huh7 cells, and miR-21 in HeLa cells exposed to (A) inhibitors **1** and **2** (10 μM) and (B) activator **3** (10 μM). All experiments were conducted in triplicate, and the data was normalized to a DMSO control.

(**3e**), ethylhydroxy (**3f**), or a naphtyl group (**3g**), also induced a complete loss of miR-122 activation.

A dose dependent response was established for all three compounds, revealing EC_{50} values of 3 μM and 0.6 μM for the inhibitors **1** and **2**, respectively. The miR-122 activator **3** displayed an IC_{50} of 3 μM (Figure 5).

The activity of the small molecule modifiers was then analyzed by quantitative RT PCR to measure their direct effects on miR-122 expression levels in Huh7 cells. Cells were incubated with **1**−**3** (10 μM) for 48 h, followed by total RNA isolation (miR Premier miRNA Isolation Kit; Aldrich) and quantification by RT PCR using TaqMan primers (Applied Biosystems). The sulfonamide **2** elicited a 72% knock-down of mature miR-122 levels relative to DMSO treated cells (Figure 6). The amide **1** displayed only a 45% knock-down, which is consistent with the lower activity of **1** observed in the functional assay. The small molecule activator **3** led to a 438% increase in miR-122 expression levels.

Preliminary studies on the mode of action of the small molecules **1**−**3** were conducted by investigating their interaction with the miRNA oligonucleotide. Melting experiments were performed, which demonstrated that the small molecule modifiers do not seem to directly interact with the RNA (see Supporting Information, Figures S5−S8). To further investigate the mode of action of **1**−**3**, the intracellular levels of the primary miRNA were measured by quantitative RT PCR with primers specifically designed to be unique to the pri-miR-122 sequence (Figure 6),[21] indicating a down-regulation to 22% and 3% for **1** and **2**, respectively. Thus, the miR-122 inhibitors **1** and **2** seem to be targeting the transcription of the miRNA gene into primary miRNA, rather than other components of the miRNA pathway. Interestingly, the pri-miR-122 levels are reduced further than the miR-122 levels in the presence of the small molecule inhibitors. One explanation could be that in the presence of the small molecule inhibitors the pri-miRNA processing to mature miRNA is faster than the transcription of the miRNA gene. Up-regulation of pri-miR-122 was observed in the presence of **3**, thus indicating that the repression of miR-122 expression in hepatocellular carcinoma cells may be caused on the transcriptional level. All compounds were additionally assessed by RT PCR for miR-21, and within the error margin, no effect was observed. This confirms the data obtained in the luciferase assays and again suggests a degree of specificity of the small molecule modifiers **1**−**3** for miR-122.

Reduction of Hepatitis C Virus Replication by miR-122 Inhibitors. The inhibition of miR-122 by antisense oligonucleotides (antagomirs) results in a reduction of HCV replication in human liver cells (Huh7).[11−14] A binding site for miR-122 was predicted to reside close to the 5′ end of the viral genome, revealing a genetic interaction between miR-122 and the viral RNA genome. Moreover, it has been shown that interferon β, currently the most common HCV therapeutic (together with interferon α), modulates the expression of several miRNAs which have target sequences in the HCV genome.[22] MicroRNA miR-122 was the only down-regulated miRNA (by ∼80%), and it was demonstrated that this down-regulation plays an important role in the antiviral effects of interferon β against HCV. Due to the efficient down-regulation of miRNA-122 by the small molecule inhibitors **1** and **2**, both compounds were tested for their ability to inhibit HCV replication in Huh7 cells. The pHtat2Neo/QR/KR/FV/SI plasmid (provided by Dr. Stanley Lemon) was used to generate genotype 1a H77c RNA, which was subsequently transfected into Huh7 cells.[23] The cells were then either transfected with a miR-122 antagomir (positive control) or treated with compounds **1** and **2** (10 μM) or DMSO (negative control). After 48 h, total RNA was isolated and HCV RNA levels were measured by quantitative RT PCR (Figure 7). In agreement with previous reports, the antagomir reduced HCV RNA levels to 20%.[13,14] Moreover, the small molecule miR-122 inhibitors **1** and **2** elicited a reduction in viral load to 48% and 47%, respectively. These results provide an opportunity for the development of fundamentally new small molecule drugs for the treatment of HCV infection. Since the compounds **1** and **2** target a critical host component at the miRNA-mRNA host−pathogen interface, the virus will most likely not be able to develop a resistance to those molecules or other miR-122 inhibitors.

Induction of Apoptosis in Hepatocellular Carcinoma Cells by a miR-122 Activator. As discussed above, miR-122 is greatly down-regulated in HCC compared to healthy liver tissue, thus inducing an upregulation of the antiapoptotic miR-122 target Bcl-w, which subsequently leads to a deactivation of caspase-3 and an enhanced viability of cancer cells.[10] Treatment of HepG2

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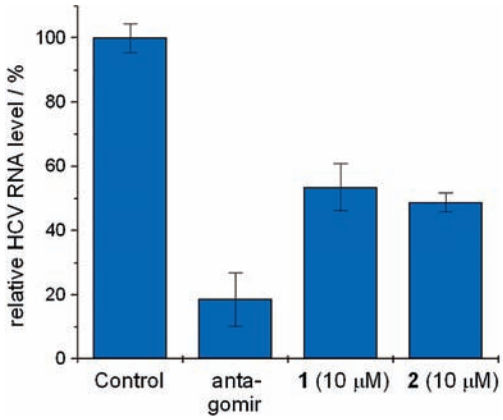

Figure 7. Effects of miR-122 inhibitors **1** and **2** on HCV replication in Huh7 cells. Both compounds led to a substantial reduction of viral RNA in infected cells. Control experiments contain only 1% DMSO and no compound. All experiments were conducted in triplicate.

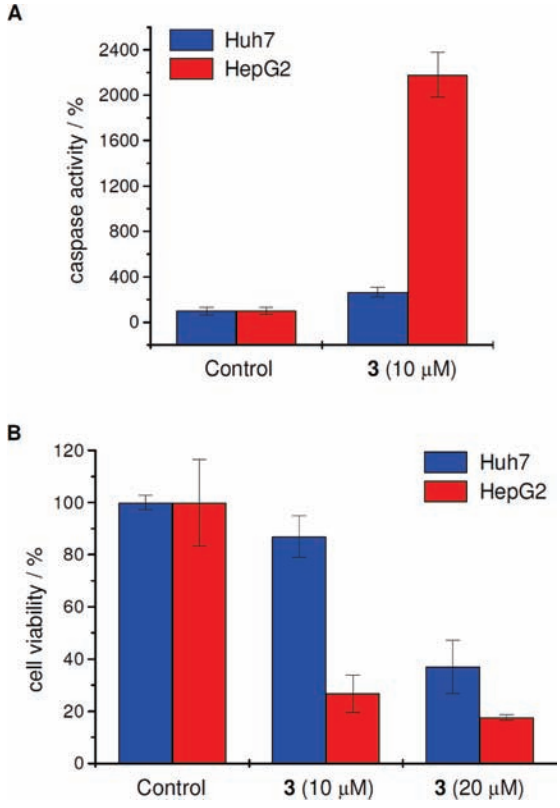

Figure 8. Effects of the miR-122 activator **3** on HepG2 cell viability. (A) An increased caspase 3/7 level is observed in HepG2 cells due to activation of the pro-apoptotic miR-122. This increase is more pronounced in HepG2 than Huh7 cells, due to the different basal miR-122 levels in both cell lines. (B) The increase in caspase-3 activity leads to a loss of cellular viability in HepG2 cells exposed to **3** (10 μM) relative to Huh7 cells. Control experiments contain only 1% DMSO and no compound.

cells with the miR-122 activator **3** (10 μM) led to an approximate 20-fold increase in the activity of caspase-3 and -7 (Caspase-Glo 3/7, Promega), suggesting that the increased levels of miR-122 are capable of inducing apoptosis. This enhanced caspase activity led to a reduced cell viability of ~20% (CellTiter-Glo, Promega) (Figure 8), exceeding previously reported reductions in cell viability due to lentiviral overexpression of miR-122 in HepG2 cells.[10] The cell viability of Huh7 cells, which express 60-fold higher miR-122 levels than HepG2 levels, is only slightly affected by exposure to **3**, suggesting a selective apoptosis inducing effect in cells with pathologically low levels of miR-122. Thus, a small molecule activator of miR-122, like **3**, has therapeutic relevance toward the selective treatment of HCC.

Summary

We have discovered small molecule modifiers of the important liver-specific microRNA miR-122 and demonstrated for the first time that small molecule inhibitors and activators of miRNA function have therapeutic potential. To achieve this, a reporter system for the activation or inhibition of miR-122 function was established and used for the screening of a small molecule library. The identified miRNA modifiers **1**−**3** are not general inhibitors or activators of the miRNA pathway but induce a down- and up-regulation of miR-122. Further investigation into their mode of action revealed that they act on the transcriptional regulation of miR-122. Most importantly, aberrantly regulated miR-122 is involved in the development of heptocellular carcinoma (HCC), and miR-122 is a cellular component required by the hepatitis C virus (HCV) for viral replication. We demonstrated that our small molecule miR-122 inhibitor **2** inhibits HCV replication in liver cells, thus demonstrating a fundamentally novel approach to the development of small molecule therapeutics for HCV infection. Moreover, the small molecule activator **3** induced an increased expression of the pro-apoptotic miR-122 in the HCC cell line HepG2, leading to increased caspase expression and reduced cell viability. These small molecule miRNA activators and inhibitors represent unique tools for the elucidation of miR-122 biogenesis and regulation in healthy liver tissue and in HCC and have the potential to provide new targets and lead structures for the development of new chemotherapeutics.

Experimental Section

Reporter Plasmid Construction. The psiCHECK-2 plasmid (1 μg; Promega) was sequentially digested with SgfI (10 units, 50 μL reaction; Promega) followed by PmeI (10 units; New England Biolabs) and was gel purified. Insert DNA containing the miR122 binding site was purchased from IDT DNA (5′ CGCAGTAGAGCTCTAGTACAAACACCATTGTCACACTCCAGTTT 3′ and 5′ AAACTGGAGTGTGACAATGGTGTTTGTACTAGAGCTCTACTGCGAT 3′) and hybridized (90 °C, cooled to 4 °C over 5 min, then 4 °C for 60 min) and ligated with T4 ligase (200 units, 10 μL reaction, 1:10 vector/insert ratio; New England Biolabs) into the digested psiCHECK-2 vector. Positive colonies were selected by PCR colony screens, and the construction of the psiCHECK-miR122 vector was confirmed by sequencing (sequencing primer: 5′ GCTAAGAAGTTCCCT 3′; IDT DNA).

Cell Culture. Experiments were performed using Huh7, HeLa, and HeLa-miR-21-Luc cell lines cultured in Dulbecco's Modified Eagle Medium (DMEM; Hyclone) supplemented with 10% Fetal Bovine Serum (FBS; Hyclone) and 2% penicillin/streptomycin (MP Biomedicals) and maintained at 37 °C in a 5% CO_2 atmosphere. Hep2G cells were cultured in Eagle's Minimum Essential Medium (Hyclone) supplemented with 10% FBS (Hyclone) and 0.5 mM sodium pyruvate.

Assessment of psiCHECK-miR122 Reporter System. Huh7 and HeLa cells were transfected at approximately 60% confluency with either the psiCHECK-Control plasmid (the original psiCHECK-2 plasmid containing no known miRNA binding site) or the psiCHECK-miR122 plasmid (0.5 μg) using X-tremGENE transfection reagent (3:2 reagent/DNA ratio; Roche) in Opti-Mem media (Invitrogen). All transfections were performed in triplicate for statistical analysis. The cells were incubated at 37 °C for 4 h followed by the replacement of transfection media with standard DMEM growth media. After 48 h of incubation the media was

removed, and cells were lysed and assayed with a Dual Luciferase Assay Kit (Promega) using a Wallac VICTOR^{3}V luminometer with a measurement time of 1 s and a delay time of 2 s. The ratio of *Renilla* to firefly luciferase expression was calculated for each of the triplicates, the data were averaged, and standard deviations were calculated (Supporting Figure S1).

Assay for Small Molecule Effectors of miR-122. Huh7 cells were transfected with the psiCHECK-miR122 plasmid in 96-well plates as previously described. After 4 h of incubation the transfection media was removed and replaced with DMEM growth media (100 μL) supplemented with 10 μM of the small molecules (NCI Diversity Set II; 1% DMSO final concentration). Cells were incubated for 48 h followed by analysis with a Dual Luciferase Assay Kit (Promega) as previously described.

Quantitative Real Time PCR Analysis. Huh7 cells were passaged into 6-well plates, grown to 60% confluency, and treated with compounds **1**−**3** (10 μM) or DMSO (1% final DMSO concentration). Each treatment was conducted in triplicate to ensure statistical validity. Cells were then incubated at 37 °C for 48 h (DMEM, 5% CO_2). The media was removed, and cells were washed with PBS buffer (2 × 2 mL, pH 7.4) followed by RNA isolation with the mirPremier microRNA Isolation Kit (Aldrich). The RNA was quantified using a Nanodrop ND-1000 spectrophotometer, and 10 ng of each RNA sample were reverse transcribed using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems) in conjunction with either the miR-122 or miR-21 TaqMan RT primer (16 °C, 30 min; 42 °C, 30 min; 85 °C, 5 min). Quantitative Real Time PCR was conducted with a TaqMan 2× Universal PCR Master Mix and the appropriate TaqMan miRNA assay (Applied Biosystems) on a BioRad MyiQ RT-PCR thermocycler (1.3 μL RT PCR product; 95 °C, 10 min; followed by 40 cycles of 95 °C, 15 s; 60 °C, 60 s). Threshold cycles were used to determine miRNA copy numbers, and the levels of miR-122 and miR-21 were compared via determination of threshold cycle (C_t) and conversion to copy number (copy number = $10[(C_t - 37.4)/-3.3]$).[24] The data were then normalized to the DMSO control. The samples were also analyzed by real time PCR for the presence of pri-miR-122 transcript using the previously described primers (5′ GCTCTTCCCATTGCTCAAGATG 3′ and 5′ GTATGTAACAACAGCATGTG 3′; IDT DNA) and iQ SYBR Green Supermix for the real time PCR (95 °C, 3 min; followed by 40 cycles of 95 °C, 15 s; 60 °C, 60 s).[21]

Effect of Small Molecule miR-122 Inhibitors on HCV Replication. The pHtat2Neo/QR/KR/FV/SI plasmid (provided by Dr. Stanley Lemon)[23] was linearized using *XbaI* (10 units, 50 μL reaction; New England Biolabs), followed by transcription with T7 RNA Polymerase (6 h, 37 °C) and purification on Microcon 10 columns. Huh7 cells were then grown to 60% confluency in a 6-well plate and transfected with 1 μg of RNA using X-tremGENE transfection reagent (Roche Applied Science, 3:2 reagent/RNA ratio) in Opti-Mem media (Invitrogen). After a 4 h incubation at 37 °C, the transfection media was removed and replaced with standard growth media (2 mL) supplemented with 10 μM of **1** or **2** (1% DMSO final concentration). All experiments were conducted in triplicate for statistical validation. Cells were incubated for 48 h at 37 °C, followed by media removal and washing with PBS buffer (2 × 2 mL, pH 7.4), followed by RNA isolation (mirPremier microRNA Isolation Kit, Aldrich). Quantitative RT-PCR was then performed as previously described using the general HCV RT-PCR Primers (5′: CGGGAGAGCCATAGTGGTCTGCG 3′ and 5′ CTCGCAAGCACCCTATCAGGCAGTA 3′) and GADPH primers (5′ TGCACCACCAACTGCTTAGC 3′ and 5′ GGCATGGACTGTGGTCATGAG 3′) as a standard control.

Effects of the miR-122 Activator on Caspase Activity. Both HepG2 and Huh7 cells were passaged into a 96-well plate and grown to 70% confluency. The media was then removed and replaced with standard growth media supplemented with **3** (10 μM) or a DMSO control (1% final DMSO concentration in all wells) and incubated for 48 h at 37 °C. All incubations were conducted in triplicate to ensure statistical validity. The media was removed and cells were assayed with the Caspase-Glo 3/7 (Promega) kit according to the manufacturers directions, and luminescence was measured using a Wallac VICTOR^{3}V luminometer with a measurement time of 1 s and a delay time of 2 s.

Effects of miRNA Small Molecule Modifiers on Cell Viability. HepG2 and Huh7 cells were passaged into a 96-well plate and grown to 60% confluency. The media was then removed and replaced with standard growth media supplemented with increasing concentrations of the miR-122 small molecule modifiers (0−20 μM; 1% final DMSO concentration) and incubated for 48 h at 37 °C. Cellular viability was then assessed using a Cell-Titer Glo Assay (Promega) according to the manufacturers directions, and luminescence was measured using a Wallac VICTOR^{3}V luminometer with a measurement time of 1 s and a delay time of 2 s.

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Supporting Information Available: Additional experimental details and supporting figures, synthetic protocols, and analytical data of organic compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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