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Single-Vehicular Delivery of Antagomir and Small Molecules to Inhibit miR-122 Function in Hepatocellular Carcinoma Cells by using "Smart" Mesoporous Silica Nanoparticles**

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Abstract: *MicroRNAs (miRNAs) regulate a variety of biological processes. The liver-specific, highly abundant miR-122 is implicated in many human diseases including cancer. Its inhibition has been found to result in a dramatic loss in the ability of Hepatitis C virus (HCV) to infect host cells. Both antisense technology and small molecules have been used to independently inhibit endogenous miR-122 function, but not in combination. Intracellular stability, efficient delivery, hydrophobicity, and controlled release are some of the current challenges associated with these novel therapeutic methods. Reported herein is the first single-vehicular system, based on mesoporous silica nanoparticles (MSNs), for simultaneous cellular delivery of miR-122 antagomir and small molecule inhibitors. The controlled release of both types of inhibitors depends on the expression levels of endogenous miR-122, thus enabling these drug-loaded MSNs to achieve combination inhibition of its targeted mRNAs in Huh7 cells.*

MicroRNAs (miRNAs) are single-stranded, small regulatory noncoding RNA endogenously expressed in plants and animals, which control around 30% of human genes and a variety of biological processes by binding to the 3′-untranslated regions of specific targeted mRNAs.[1] The regulation of many endogenous miRNAs is tightly associated with diseases including cancer, cardiovascular diseases, neurological disorders, and viral infection.[1,2] As the most abundant liver-specific microRNA, miR-122 is found to regulate cholesterol, fatty acid, and lipid metabolism.[3] In addition, it is known to interact with the genome of Hepatitis C virus (HCV) and facilitate viral replication in host cells. With an estimated 150–200 million infected people worldwide, HCV is a major cause of chronic liver diseases. Inhibition of endogenous miR-122 in hepatocellular carcinoma cells (HCCs) such as Huh7 has led to dramatic decreases in HCV RNA levels, indicating down-regulation of miR-122 could be highly effective in conferring antiviral effects.[4] Therefore, miR-122 has been pursued as a novel therapeutic target for HCV infection.

To suppress endogenous miR-122 expression, different strategies have been explored.[5–7] With antisense technology,[5,6] chemically modified antisense oligonucleotides (ASOs) were artificially introduced into host cells, in which these molecules effectively bound to intracellular miR-122 (through sequence complementarity) and inhibited its function. Amongst various ASOs, the antagomirs (sometimes also referred to as anti-miRs), which are nucleic acids containing phosphorothioates in place of the normal phosphodiester linkage in the oligonucleotide backbone with periodic insertion of 2′-methoxy nucleotides (i.e. Ant122 in Scheme 1), are highly effective in silencing miR-122.[5a] These chemical modifications were shown to render the antagomir highly resistant toward intracellular nuclease degradation, and at the same time significantly improve its binding affinity toward target miRNAs.[5b,c,6] In many cases, addition of a cholesterol moiety at the terminus of the antagomir could further improve its cellular activities.[5] In contrast, administration of unmodified ASOs (i.e. antisense-miR-122 in Scheme 1) into host cells showed no effect on the level of endogenous miR-122,[5a] presumably due to rapid degradation by endogenous exo- and endonucleases. Notwithstanding, intracellular delivery of ASOs remains a formidable challenge in antisense gene therapy.[5b,c] Recently, small-molecule inhibitors of miR-122 have been reported.[7] By taking advantage of a cell-based screening assay with a dual luciferase reporter system, Deiters and co-workers discovered a novel compound (sm122 in Scheme 1), which was able to inhibit endogenous miR-122 function by effectively blocking the synthesis of pri-miRNA.[7] Such chemical genetic approaches are highly desirable for gene regulation, owing to improved chemical stability and cell permeability of small molecules. One major shortcoming, however, is the poor water solubility of small molecules because of their hydrophobic nature under physiological conditions, which in many cases make them difficult to deliver to host cells without the help of organic solvents.[8] Another problem is that, upon cell internalization, the controlled release of small molecules could not be readily modulated, leading to undesirable cellular effects.[9]

We envisioned a single-vehicular system, capable of simultaneous delivery of antagomir/small molecule into cells and subsequent "controlled release" of both, upon dose-dependent stimulation of cellular targets (e.g. miR-122). Such a system should solve some of the long-standing problems associated with existing miR-122-based therapeutics. Herein,

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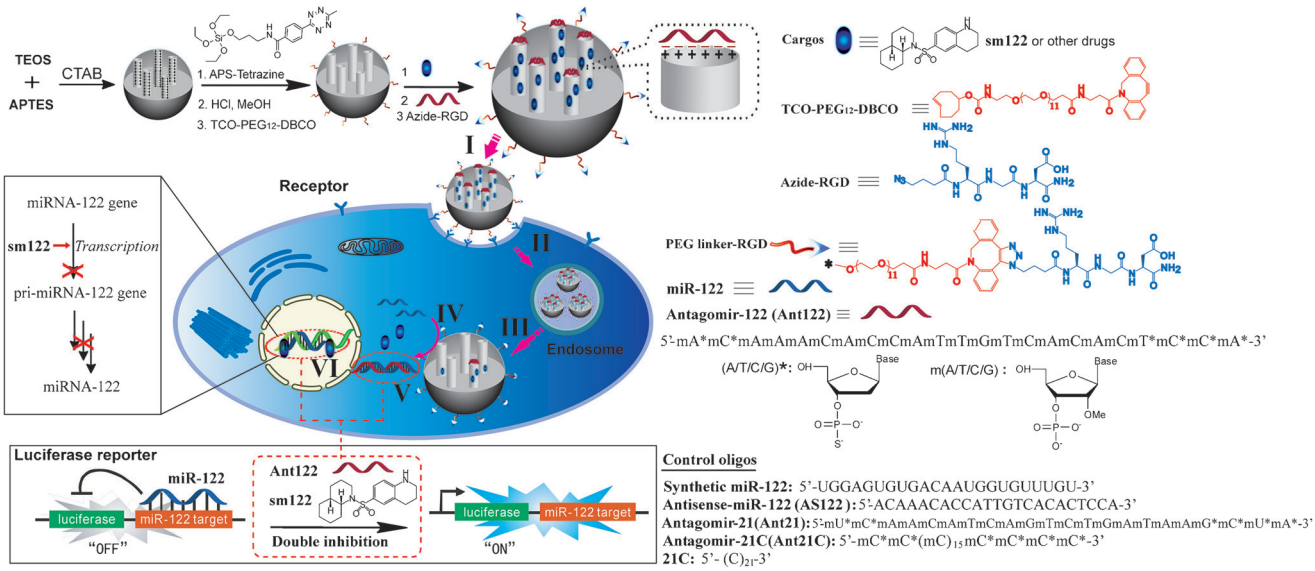

Scheme 1. Overall strategy for the MSN-based, single-vehicle system for simultaneous delivery and controlled release of antagomir and small-molecule inhibitors of miR-122 in Huh7 cells. Step I: RGD-targeted cell recognition; Step II: endocytosis by endosome/lysosome pathways; Step III: endosomal/lysosomal escape; Step IV: controlled release of antagomir/sm122 by Ant122/endogenous miR-122 hybridization; Steps V, VI: combination inhibition of miR-122 function by Ant122 and sm122. Other chemicals used in this study are shown in the Supporting Information.

we report one such system using "smart" mesoporous silica nanoparticles (MSNs; Scheme 1). The system provides the following key features: 1) simultaneous delivery of Ant122 and hydrophobic sm122 to the same Huh7 liver cells, thereby achieving combination inhibition of endogenous miR-122 function with two mechanistically different agents; 2) controlled release of both inhibitors in amounts that depend proportionally upon the expression levels of endogenous miR-122; and 3) the possibility of encapsulating a variety of other small-molecule inhibitors to achieve inhibition of miR-122 function and its downstream targeted enzymes.

As a result of their high surface areas, large loading capacity, chemically modifiable surfaces, and nontoxicity, MSNs have been used as carriers for effective delivery of a variety of biologically interesting molecules.[10] To date, a variety of stimuli-responsive gatekeepers for controlled release of guest molecules have been developed.[10–12] Among them, nucleic acid-capped MSN delivery systems, in which hybridization/dehybridization of complementary strands of DNA/RNA are used for capping/uncapping MSNs, are worth noting, as they offer a sequence-dependent universal strategy for intracellular drug release.[12] In our system (Scheme 1), the enzyme-resistant, chemically modified antagomir of miR-122 (e.g. Ant122) was used, not only as a gatekeeper of the small-molecule-loaded nanoparticles, but also as an ASO for effective inhibition of endogenous miR-122. Also, the amino-propyl groups on the surface of MSNs were partially modified with PEG-linker-RGD peptide by the bioorthogonal tetrazine-TCO ligation reaction and copper-free DBCO-azide click chemistry.[13] RGD peptide and PEG linker were previously shown to improve the nanoparticles' cellular uptake and prevent their intracellular aggregation.[14] Upon cellular uptake (Steps I, II in Scheme 1) and successful endosomal/lysosomal escape (Step III), the hybridization of the complementary sequences between Ant122 and intracellular miR-122 produced a rigid double helical structure and altered the conformation of Ant122, which would reduce the binding capacity of Ant122 and lead to the uncapping of the MSNs.[12b,15] Subsequently, dose-dependent controlled release of both Ant122 and encapsulated sm122 was triggered in a manner proportional to the endogenous miR-122 expression levels (Step IV). In Huh7 cells, which are hepatocellular carcinoma well-known to express high levels of endogenous miR-122,[16] we obtained unequivocal results showing successful and simultaneous release/inhibition of miR-122 function by both Ant122 and sm122 (Steps V, VI), and leading to combination inhibition of endogenous miR-122 function.

The newly synthesized MSNs were characterized to be highly monodisperse (mean diameter: ca. 120 nm) and possess well-defined pore sizes with high specific areas (Figure S1–S3 in the Supporting Information). We first chose rhodamine B (RhB) as the model drug/cargo to follow the entire process of controlled release both in vitro and mammalian cells (Figures 1,2). The amount of RhB released from loaded MSNs were determined by UV measurement (at 550 nm) in vitro or live-cell bioimaging with confocal laser scanning microscopy (CLSM; Figure 2). We optimized the RhB loading capacity (65.59 $\mu mol\,g^{-1}$) and the amount of Ant122 needed for efficient MSN capping (9.36 $\mu mol\,g^{-1}$) with minimal drug leakage (Figure S4). Minimal nanoparticle aggregation and leakage (< 5%) were observed after 24 h incubation in 10% fetal bovine serum (FBS) (Figure S6). In the presence of synthetic miR-122 (1.0 μM), however, excellent cargo release was observed. Next, more quantitative controlled release of RhB from Ant122-MSN-RhB in vitro was measured, by addition of synthetic miR-122 in a dose-dependent manner.

As shown in Figure 1a, increasing the amount of added synthetic miR-122 resulted in a proportional, time-dependent increase in the released RhB. Similar release profiles were

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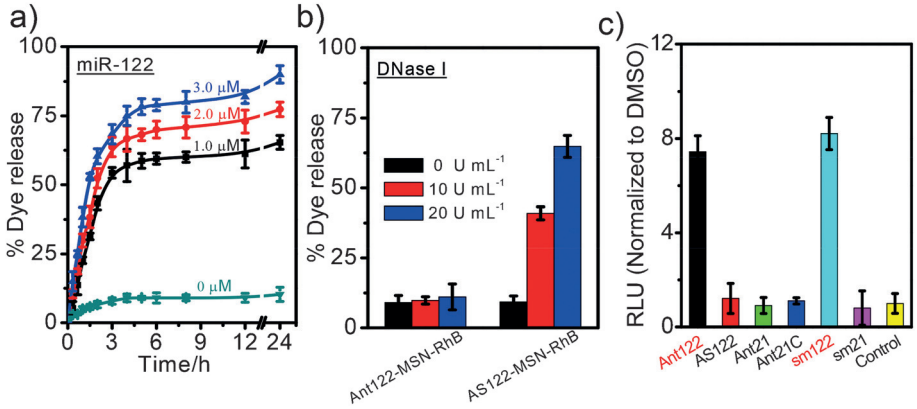

Figure 1. a) In vitro RhB release profiles of Ant122-MSN-RhB (0.1 mg mL^{-1}) with different concentrations of synthetic miR-122 (0 to 3 μM) in PBS buffer (*p*H 7.4). b) In vitro release profiles of RhB from Ant122-MSN-RhB and AS122-MSN-RhB (0.1 mg mL^{-1} each) incubated for 24 h with different amounts of DNaseI (0 to 20 U mL^{-1}) in PBS (pH 7.4). c) Relative luciferase signal (RLU) changes in Huh7 cells after transfection of different ASOs (200 nM each) or addition of small-molecule inhibitors (10 μM with 1% DMSO) for 24 h. All experiments were conducted in triplicate and normalized to DMSO control.

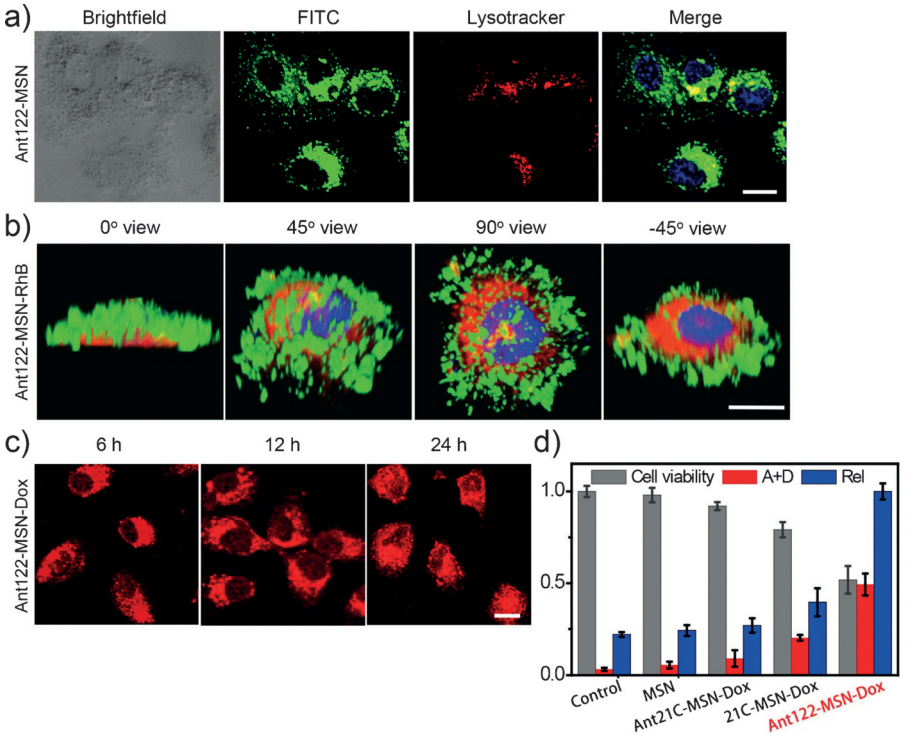

Figure 2. a) CLSM images of Huh7 cells incubated with FITC-labeled Ant122-MSN clicked with Azide-RGD peptide for 6 h. Green: FITC fluorescence channel showing location of MSNs. Red: LysoTracker. The merged channels (green and red) were quantified for co-localization with Image-J software (co-localization = 0.31). b) 3D projections of *z*-stack images at different perspectives (step size, 0.163 μm) of fixed Huh7 cells treated with Ant122-MSN-RhB (12 h). Green: FITC channel showing the location of MSNs. Red: RhB fluorescence. Blue: cell nuclei stained with Hoechst 33342. The global Pearson's R value is 0.41. c) CLSM images of Huh7 cells treated with Ant122-MSN-Dox (10 μg mL^{-1}). d) XTT/FACS/caspase 3 enzymatic activity analysis of the same cells shown in (c) after 24 h MSN treatment. A + D: apoptotic and dead cells determined by FACS using the Annexin-V kit; Rel: relative caspase-3 activity determined by Ac-DEVD-AMC (t = 6 h). See the Supporting Information and Ref. [21] for details. All experiments were conducted in triplicate. Scale bar = 20 μm. Control = Cells not treated with MSNs.

observed in cell culture medium (Figure S7). Addition of DNaseI (up to 20 U mL^{-1}) to the MSNs did not cause any nuclease-triggered release of RhB (Figure 1b). In direct contrast, over 70% release of RhB was observed with the control MSNs (AS122-MSN-RhB), in which unmodified antisense-miR-122 (AS122) was used to cap RhB-loaded MSNs instead, indicating the use of highly cell-environment-stable antagomirs is critical in our current study. Previous attempts to use unmodified ASOs as miRNA-responsive gatekeepers in drug-loaded MSNs which resulted in premature drug leakage and minimal miRNA knockdowns,[12a] were likely a result of nuclease degradation and ineffective inhibition of endogenous miRNAs by such ASOs.[5] The miR-122-triggered release of RhB from Ant122-MSN-RhB was found to be highly sequence-specific under physiological conditions, but tolerate acceptable variations in both pH (5 to 8) and temperature (up to 42 °C); Figure S7). We assessed whether Ant122 and sm122 alone could suppress endogenous miR-122 function in Huh7 cells stably transfected with a dual luciferase reporter (termed psi-CHECK-miR-122 construct).[7] This reporter system could express both firefly and *Renilla* luciferases, allowing normalization of signals to account for differential cellular viability. The miR-122 target sequence was inserted downstream of the *Renilla* luciferase gene. Thus the presence of endogenous miR-122 would lead to a decrease in the *Renilla* luciferase signal (Scheme 1). We first established the dose-response curves in Huh7 cells by transiently transfecting different amounts of Ant122 (negative control: Ant21C), or adding 0–50 μM of sm122 (negative control: sm21—a small molecule inhibitor of miR-21[17]). After 24 h, the cells were assayed using a Dual Luciferase Assay Kit (Figure S8); we observed proportional increases in relative luciferase signals (RLUs) in response to increasing amounts

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of Ant122 or sm122. We next evaluated the effects of other ASOs and small molecules under similar conditions (Figure 1c); as expected, none of the control compounds, including AS122, Ant21 (antagomir of miR-21[18]), Ant21C and sm21, showed any inhibitory effect on miR-122 function, indicating this reporter system could be used to accurately and quantitatively delineate endogenous miR-122 function.

With excellent in vitro profiles of our antagomir-capped nanoparticles being established, we assessed the target release efficiency and specificity of this system in live cells. A good drug-delivery system is characterized by high cellular uptake and endosomal/lysosomal escape, and efficient on-demand drug release with minimal leakage. To investigate these aspects, FITC-labeled Ant122-MSNs loaded with different small molecules were used and cellular-uptake/release experiments were carried out in live Huh7 cells with CLSM (Figure 2). MSNs modified with Azide-RGD peptide showed significantly improved cellular uptake compared to unmodified MSNs (Figure S9), as previously reported.[14] Therefore, for subsequent cell-based experiments, all the MSNs used were modified with RGD peptide, unless otherwise indicated. As shown in Figure 2a, with FITC-labeled Ant122-MSN incubated in Huh7 cells for 6 h, most nanoparticles had been successfully taken up by cells (panel 2), presumably by endocytosis, and more than half of the internalized MSNs had escaped from the endosomes/lysosomes (panel 4) possibly through the mechanism of proton sponge effect as a result of enhanced buffering capacity of the positively charged MSNs upon capping with the negatively charged antagomir.[12a,14,19] A control experiment with FITC-labeled MSN-NH_2 (not capped by an antagomir) was further carried out to confirm this hypothesis (Figure S10a); most of the successfully internalized FITC-labeled MSN-NH_2 remained trapped inside endosomes/lysosomes of Huh7 cells even after 6 h of incubation. To monitor controlled release of drugs, FITC-labeled Ant122-MSN-RhB and Ant122-MSN-Dox (loaded with doxorubicin—an anticancer drug) were used (Figure 2b,c, Figure S11); 3D projections of *z*-stack images of Huh7 cells treated with Ant122-MSN-RhB at different perspectives (Figure 2b; see Supporting Information Video S1) clearly showed the successful release of free RhB and its subsequent subcellular localization to mitochondria (colored in red). The FITC-labeled MSNs (colored in green), on the other hand, were not expected to go into mitochondria owing to their significantly larger size. RhB release was also observed in Huh7 cells treated with Ant21-capping MSNs, as a result of the overexpression of endogenous miR-21 in this cell line (Figure S11 and Video S2).[16c] With control nanoparticles (FITC-labeled Ant21C-MSN-RhB), however, no release of free RhB was observed with nearly complete overlaps (colored in orange/yellow; $R = 0.92$) of RhB/FITC channels (Figure S11 and Video S3). On the other hand, in HeLa cells which are known to express endogenous miR-21 but not miR-122,[16] we observed successful RhB release upon cell treatment with Ant21-MSN-RhB but not Ant122-MSN-RhB (Figure S11 and Videos S4,5, respectively). Huh7 cells treated with Ant122-MSN-Dox also showed successful intracellular drug release (Figure 2c) after 12 h treatment, and nuclear localization of Dox and drug-induced cell apoptosis after 24 h treatment (Figure 2d);[20] XTT cell viability, FACS experiments and cleaved caspase-3 enzymatic assays all indicated successful intracellular delivery and subsequent cell-killing effects of released Dox in Huh7 cells treated with Ant122-MSN-Dox (as well as 21C-MSN-Dox; see below), but not any other nanoparticles. The apparent cell-killing effect of 21C-MSN-Dox (Dox-loaded MSNs capped with 21C) clearly indicated the undesirable drug release profile with unmodified nucleic acids as gatekeepers. Finally, to show different levels of endogenous miR-122 could effectively induce proportional release of both Ant122 and the encapsulated small molecule, Huh7 cells transfected with different amounts of synthetic miR-122 were treated with Ant122-MSN-Dox (Figure S13,S14); an increase in the endogenous miR-122 levels (determined by real-time quantitative PCR, or *q*PCR) led to the corresponding increase in the amount of nuclear-localized Dox and cell death/apoptosis.

Having successfully demonstrated the cellular uptake, endosomal/lysosomal escape, controlled release of both the antagomir and small molecules in Huh7 cells, we next focused on combination inhibition of endogenous miR-122 function by our single-vehicular system (Ant122-MSN-sm122). Again, control experiments were carried out with MSNs loaded with other small molecules capped with different antagomirs (Ant122-MSN-sm21 and Ant21C-MSN-sm122). As shown in Figure 3, we observed effective combination inhibition of miR-122 function only with Ant122-MSN-sm122 from both the *q*PCR results (Figure 3 top graph) and the dual luciferase reporter system (bottom graph). Ant122-MSN-sm21 was not as effective, indicating our single vehicle had successfully released not only Ant122 to block endogenous miR-122 by antisense strategy, but also the encapsulated sm122 to cause inhibition of pri-miRNA.[7] Therefore, our "smart" delivery system had achieved simultaneous delivery and controlled release of both antagomir and hydrophobic small molecules to inhibit endogenous miR-122 function, in combination, in a cell-specific manner.

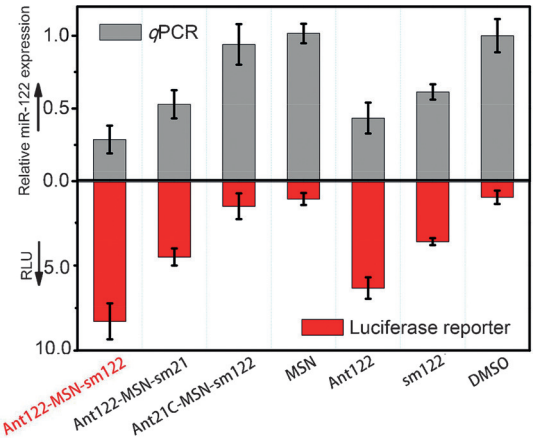

Figure 3. Changes in the endogenous miR-122 expression (determined by *q*PCR; Top) and relative luciferase signal (RLU; Bottom) of Huh7 cells (stably transfected with psiCHECK-miR-122 construct) treated with different MSNs (25 μg mL^{-1}), antagomir (200 nM) and sm122 (2 μM) for 24 h. RLU were normalized to control cells (DMSO-treated). All experiments were conducted in triplicate.

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Inhibition of miR-122 by antagomirs was previously found to cause up/down regulation of many proteins,[3] including a number of important enzymes.[21] To extend our current MSN system into the realm of combination inhibition of miR-122 and its downstream targets, we prepared Ant122-MSN encapsulated with inhibitors of kinases (staurosporine, dasatinib, LY294002[22a]) and Poly(ADP-ribose)polymerase-1 (olaparib[22b]). Both dasatinib and olaparib are FDA-approved cancer drugs. Huh7 cells again were treated with different drug-loaded MSNs and subsequent cellular effects as a result of released Ant122 and the drug were evaluated in a number of cell-based assays (Figure S16–18); expected apoptosis (determined by FACS/WB/caspase enzymatic assay) were observed with MSNs loaded with staurosporine, dasatinib or olaparib, and inhibition of Akt phosphorylation was observed with MSNs loaded with LY294002 (an inhibitor of PI3-kinase). These results are consistent with the successful intracellular drug release of our Ant122-capped MSNs in Huh7 cells.[20,22]

In conclusion, we have developed a single-vehicle system, based on mesoporous silica nanoparticles, for simultaneous cellular delivery of miR-122 antagomir and hydrophobic small-molecule inhibitors. This novel delivery system, by overcoming key challenges in miRNA therapeutics, has good intracellular stability, efficient cellular uptake/endosomal escape and target-triggered release of drugs, and is the first example that successfully demonstrates the combination inhibition of endogenous miR-122 with two mechanistically distinct inhibitors in cell type-specific manner. Preliminary extension indicates the system is equally suitable for delivery of antagomirs and other small molecules that simultaneously target miRNAs and their downstream protein targets.

Keywords: bioimaging · drug delivery · mesoporous silica nanoparticles · microRNA · small-molecule inhibitor

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