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Targeting the Production of Oncogenic MicroRNAs with Multimodal Synthetic Small Molecules

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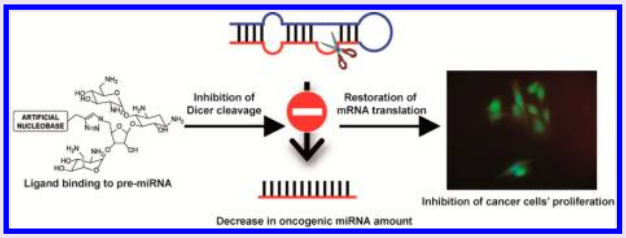
Supporting Information

ABSTRACT: MicroRNAs (miRNAs) are a recently discovered category of small RNA molecules that regulate gene expression at the post-transcriptional level. Accumulating evidence indicates that miRNAs are aberrantly expressed in a variety of human cancers and revealed to be oncogenic and to play a pivotal role in initiation and progression of these pathologies. It is now clear that the inhibition of oncogenic miRNAs, defined as blocking their biosynthesis or their function, could find an application in the therapy of different types of cancer in which these miRNAs are implicated. Here we report the design, synthesis, and biological evaluation of new small-molecule RNA ligands targeting the production of oncogenic microRNAs. In this work we focused our attention on miR-372 and miR-373 that are implicated in the tumorigenesis of different types of cancer such as gastric cancer. These two oncogenic miRNAs are overexpressed in gastric cancer cells starting from their precursors pre-miR-372 and pre-miR-373, two stem-loop structured RNAs that lead to mature miRNAs after cleavage by the enzyme Dicer. The small molecules described herein consist of the conjugation of two RNA binding motives, i.e., the aminoglycoside neomycin and different natural and artificial nucleobases, in order to obtain RNA ligands with increased affinity and selectivity compared to that of parent compounds. After the synthesis of this new series of RNA ligands, we demonstrated that they are able to inhibit the production of the oncogenic miRNA-372 and -373 by binding their pre-miRNAs and inhibiting the processing by Dicer. Moreover, we proved that some of these compounds bear anti-proliferative activity toward gastric cancer cells and that this activity is likely linked to a decrease in the production of targeted miRNAs. To date, only few examples of small molecules targeting oncogenic miRNAs have been reported, and such inhibitors could be extremely useful for the development of new anticancer therapeutic strategies as well as useful biochemical tools for the study of miRNAs' pathways and mechanisms. Furthermore, this is the first time that a design based on current knowledge about RNA targeting is proposed in order to target miRNAs' production with small molecules.



MicroRNAs (miRNAs or miRs) are single-stranded noncoding RNAs of 18−25 nucleotides that act as post-transcriptional regulators of gene expression upon binding to the 3′ untranslated regions (3′-UTR) of specific target messenger RNAs (mRNAs). The recognition of the mRNA target usually leads to gene silencing by repression of mRNA translation and acceleration of mRNA degradation.[1] In some cases, activation of translation has also been observed.[2] The miRNAs' biogenesis process starts with the transcription of a long primary RNA of several kilobases (pri-miRNA), which is processed in the nucleus by the enzyme Drosha into a shorter (ca. 70 nucleotides) stem-loop-structured precursor (pre-miRNA). This latter is exported to the cytoplasm, where it becomes a substrate for the Dicer enzyme. Processing by Dicer produces the mature miRNA, which is loaded onto the RNA-induced silencing complex (RISC) and subsequently targets complementary sequences on mRNA.[3]

A wide number of studies have demonstrated that the dysregulation of miRNAs plays a pivotal role in a large range of human diseases, including cancer, immune disorders, and cardiovascular pathologies.[4] With respect to human cancer specifically, abnormalities in miRNAs' expression have been linked to initiation, progression, and metastases by the overexpression of oncogenic miRNAs or by the decrease of tumor suppressor miRNAs' amount, these variations being a hallmark of malignant phenotype.[5,6] On the basis of these findings, it is now clear that miRNAs represent a particularly interesting target for the discovery of new therapeutic strategies against cancer.[7] To date, only few examples of small-molecule drugs have been reported to modulate miRNA expression by targeting their transcription and/or their processing.[8,9] These have been discovered after the screening of large chemical libraries[10−14] or after the study of established RNA ligands.[15,16] It is now clear that the discovery of small molecule inhibitors of oncogenic miRNAs would be a very promising approach for the development of targeted cancer therapy.[17]

Here we report the first example of multimodal RNA ligands aimed to inhibit the biogenesis of oncogenic miRNAs. In

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particular, we chose to focus our attention on the targeting of miR-372 and miR-373, implicated for instance in some gastric cancers, with the aim of binding their precursors (pre-miR-372 and -373, Figure 1A) thus inhibiting Dicer cleavage. Gastric

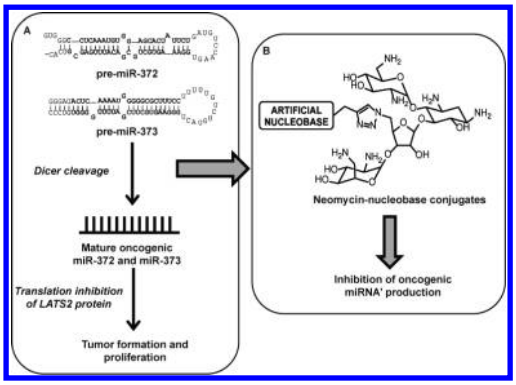

Figure 1. (A) Simplified representation of overexpression of oncogenic miRNA-372 and miR-373 in cancer cells: the overexpression of their precursors (pre-miRNAs) leads, after Dicer cleavage, to the overexpression of mature oncogenic miRNAs able to interfere with the correct mRNA translation, thus inducing tumor formation and proliferation. In pre-miRNAs' sequences bold characters indicate mature miRNAs' sequences. LATS2, Large Tumor Suppressor 2. (B) New neomycin-nucleobase conjugates able to inhibit the production of oncogenic miRNAs at the level of Dicer cleavage.

cancer is the fourth most common and second leading cause of cancer death in the world. Recent studies on miRNA expression profile in gastric cancer found a significant number of deregulated miRNAs, both oncogenic (upregulated) and tumor suppressors (downregulated). miR-372 has been found to act as an oncogene in gastric cancer[18] as well as testicular germ cell tumors,[19] esophageal tumors,[20] and thyroid adenomas.[21] This miRNA belongs to a unique cluster in the genome and shares the same seed sequence (the sequence that recognizes the target mRNA) with miR-373. Both miR-372 and miR-373 are expressed in gastric tumors, while they are undetectable in normal gastric epithelial cells. They target and post-transcriptionally repress the Large Tumor Suppressor homologue 2 (LATS2), which is a serine-threonine kinase involved in cell cycle regulation.[22]

The multimodal ligands described herein are composed of two different RNA binding motives (Figure 1B): (i) an artificial nucleobase designed in order to specifically recognize a RNA base pair of the double-stranded region of the pre-miRNA and (ii) an aminoglycoside (neomycin) known to strongly interact with stem-loop RNAs with high affinity. With regard to the first motif, the artificial nucleobases used in this study have been previously designed and applied in the antigene strategy, i.e., the formation of triple helices of DNA using triplex-forming oligonucleotides.[23] These structures are able to form two or three specific hydrogen bonds (the so-called Hoogsteen interactions) with a T·A or C·G base pairs in physiological conditions and give rise to a DNA triplet.[24] Noteworthy, this approach can also be applied to RNA base pairs, and we recently reported that the conjugation of artificial nucleobases and basic amino acids leads to RNA ligands with high affinity and selectivity for the stem-loop structured HIV-1 TAR RNA.[25] With regard to the second motif, aminoglycosides antibiotics are a class of RNA-binding molecules known to bind to stem-loop structured RNAs and commonly used in the clinic since they bind to prokaryotic rRNA in the decoding A-site and reduce fidelity of protein translation.[26,27] Aminoglycosides can also bind with comparable affinity to other RNA targets, including HIV-1 dimerization RNA element (DIS),[28] transactivating response element (TAR), REV response element (RRE), and several other stem-loop structured RNAs.[29−31] In general, aminoglycosides bind in the major groove of the RNA duplex, where the regular α-helical geometry is distorted by internal loops, bulged out nucleotides, or non-regular base pairs.[32−34]

The conjugation of nucleobases to aminoglycosides allowed us to obtain ligands that bind with high affinity to stem-loop structured pre-miR-372 and pre-miR-373. On the basis of the experiments reported below, we suggest that their binding is linked to the inhibition of oncogenic miRNAs' processing by Dicer enzyme. Some of these compounds also bear a cytostatic effect that is selective for gastric cancer cells overexpressing targeted miRNAs. The experiments reported below strongly suggest that the biological activity is likely correlated to the inhibition of the production of oncogenic miR-372 and miR-373 thus leading to the restoration of normal mRNA translation in LATS-2 protein.

RESULTS AND DISCUSSION

Design of Multimodal Small Molecules as Pre-miRNAs Ligands. The design of the multimodal ligands able to bind selectively and with high affinity to the targeted stem-loop RNAs is based upon the conjugation of an artificial nucleobase to an aminoglycoside, leading to the compounds illustrated in Scheme 1 (**3a−j**). As mentioned above, we decided to introduce artificial nucleobases (**R** in Scheme 1) chosen among the ones conceived for the synthesis of triplex-forming oligonucleotides and able to form specific hydrogen bond interactions (Hoogsteen-type interactions) with DNA and RNA base pairs.[24] **S** nucleobase has been chosen since it is able to form three hydrogen bonds with the T·A base pair,[36,37] while the ureido benzoxazoles **HB-Ar** and **HB**,[38] **D**$_4$,[39] and the benzaminoimidazole-glycyl nucleobase **BIG**[40] are able to form three hydrogen bonds specifically with a C·G. Finally, the 4-(3-benzamidophenyl)imidazole **D**$_3$[41] can form only two hydrogen bonds with T·A and C·G without selectivity. Interestingly, Tor and co-workers previously published a series of conjugates between the aminoglycosides paromomycin and neomycin with natural nucleobases in order to target two different RNA sequences: ribosomal A-site and HIV-1 TAR RNA.[35] In fact, the conjugation of natural nucleobases that are able to base pair with unpaired nucleosides of bulges and loops should also increase the affinity and the selectivity for the target. On the basis of these results, we also conjugated natural nucleobases adenine (A), uracil (U), cytosine (C), and guanine (G) for the synthesis of aminoglycoside-nucleobase conjugates.

In order to identify the best aminoglycoside molecule for the conjugation to nucleobases, we studied seven commercially available aminoglycosides for their ability to inhibit the cleavage by Dicer. Toward this aim, we employed a cell-free assay based on the FRET technique according to a previously published procedure.[42,43] In this assay, targeted pre-miRNAs have been double labeled with a fluorophore (fluorescein or FAM) and a quencher (dabcyl or DAB) at the 3′ and 5′ ends, respectively. In the presence of recombinant Dicer enzyme, this latter cleaves the RNA and the appearance of fluorescence is observed (Figure 2, ∗). If a RNA ligand is able to strongly bind to the structured pre-miRNA and inhibit the cleavage by

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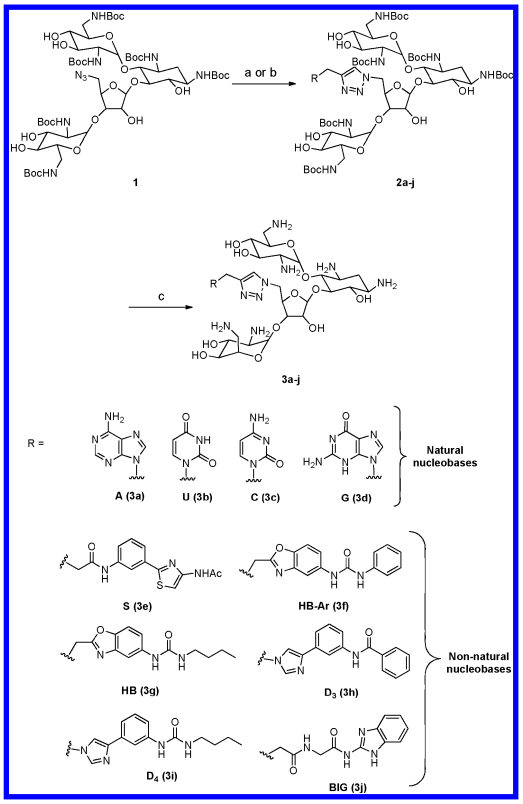
Scheme 1. Synthesis of Neomycin-Nucleobase Conjugates 3a−j[a]



[a]Reagents and conditions: (a) CuI, DIPEA, CH_3CN, RT, overnight; (b) CuI, DIPEA, toluene, Δ, overnight; (c) TFA, CH_2Cl_2, RT, overnight.

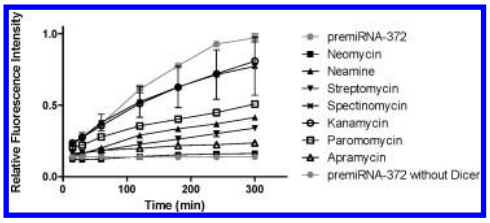


Figure 2. Fluorescence increase upon incubation of 0.25 U recombinant Dicer with 50 nM beacon pre-miRNA372 alone (∗) or in the presence of 250 μM neomycin B (■), neamine (▲), streptomycin (▼), spectinomycin (◆), kanamycin (○), paromomycin (□), and apramycin (△). The fluorescence of premiRNA372 in the absence of Dicer is reported as a negative control (●).

Dicer, no fluorescence is detected (Figure 2, ●). Aminoglycosides were screened at 250 μM, and as illustrated in Figure 2, only neomycin is able to completely inhibit Dicer cleavage at this concentration. Apramycin is slightly less active than neomycin, showing a 77% inhibition while the inhibition activity of streptomycin and neamine decreases until 65% and 59%, respectively. Paromomycin, kanamycin, and spectinomycin showed the lowest inhibition activities going from 50% to 20%. Based on this screening, neomycin appeared as the best aminoglycoside for the synthesis of the RNA ligands presented in this study.

Synthesis of Multimodal Ligands. The synthetic route employed in this study is illustrated in Scheme 1. First of all, neomycin was converted to its N_3 derivative **1** using previously reported procedures.[44,45] Concomitantly, natural nucleobases adenine (A, in **3a**), uracil (U in **3b**), cytosine (C, in **3c**), and guanine (G in **3d**) as well as artificial nucleobases **S** (in **3e**), **HB-Ar** (in **3f**), **HB** (in **3g**), D_3 (in **3h**), D_4 (in **3i**), and **BIG** (in **3j**) have been appropriately modified in order to introduce an alkyne side chain (see Supporting Information for experimental details). Thus, compound **1** has been conjugated to alkyne intermediates of each base (compounds **4**, **5**, **7**, **11**, **12**, **20a,b**, **26**, **29**, and **32**, Supporting Information) using CuI and DIPEA in CH_3CN or toluene leading to desired conjugates **2a−j** in 36−87% yields. Finally, a Boc-removal step has been applied to these compounds leading to desired conjugates **3a−j** in 59−95% yields. The synthesis of these compounds is straightforward, and the desired products have been obtained with high purity.

Biological Evaluation of Multimodal Ligands *in Vitro*. In order to screen the inhibition activity of newly synthesized compounds on Dicer cleavage of pre-miR-372 and -373, we applied the cell-free assay described above for the screening of aminoglycosides. Figure 3 shows the results obtained in the presence of compounds **3a−j** at a concentration of 50 μM on pre-miR-372. Regarding compounds **3a−d** containing natural nucleobases, the efficiency in the inhibition of Dicer cleavage was much higher than the one of neomycin alone since we obtained strong inhibition (between 70% and 83%) with compounds **3a**, **3c**, and **3d** (Figure 3A). Compound **3b** showed only 23% inhibition activity, while neomycin alone is completely inactive at this concentration. The low activity of compound **3b**, bearing the uracil base, compared to quite efficient compounds **3a**, **3c**, and **3d**, demonstrates that probably other interactions beside Watson−Crick hydrogen bonds can be formed. In particular, the presence of a $-NH_2$ group in adenine, cytosine, and guanine seems to play an important role in the inhibition activity and thus probably in the interaction with targeted RNAs. More importantly, all conjugates bearing the artificial nucleobases (**3e−3j**) were even more efficient

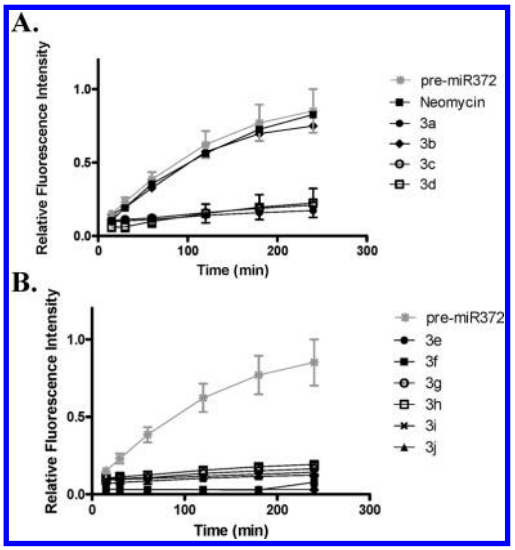


Figure 3. (A) Fluorescence increase upon incubation of 0.25 U recombinant Dicer with 50 nM beacon pre-miRNA372 alone (∗) or in the presence of 50 μM neomycin B (■), **3a** (●), **3b** (◆), **3c** (○), and **3d** (□). (B) Fluorescence increase upon incubation of 0.25 U recombinant Dicer with 50 nM beacon alone (∗) or in the presence of 50 μM **3e** (●), **3f** (■), **3g** (○), **3h** (□) **3i** (×), and **3j** (▲). Conditions: 20 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$, 1 mM DTT in 40 μL in a 384-well plate incubated for 5 h at 37 °C.

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inhibitors of Dicer cleavage (Figure 3B). Compounds **3e** and **3f** were the best inhibitors with almost 100% inhibition. The activity of compounds **3g**−**3j** varies from 80% to 90%. These results show that some of the artificial nucleobases greatly improve the inhibitory effect compared to natural ones. Noteworthy, we also observed that all nucleobases alone are completely inactive, as was the case for neomycin at the tested concentration, thus demonstrating that both neomycin and the nucleobase moiety are necessary for the inhibition activity.

We then pursued the characterization of conjugates, evaluating the IC_{50} for Dicer inhibition of each synthesized compound compared to neomycin. The results for pre-miR-372 are reported in Table 1. Neomycin and compound **3b** showed the lowest inhibition activity, compared to compounds **3a**, **3c**, and **3d**. Accordingly to the preliminary screening, some of the artificial nucleobases greatly improve the inhibitory effect, since compounds **3e**−**j** are very good inhibitors. Compounds **3e** and **3f** showed the best IC_{50} values of 2.42 and 4.17 μM, respectively. Analogous results were obtained in the presence of pre-miR-373 (Table S1 in Supporting Information). Thus, nucleobases **S** and **HB-Ar**, as in compounds **3e** and **3f**, are the most favorable for the inhibition activity. The aliphatic *n*-butyl side chain as in compounds **3g** and **3i** is less favorable for the inhibition activity compared to aromatic side chains of compounds **3f**, **3h**, and **3j**, probably because these latter can form further hydrophobic and stacking interactions. This first set of experiments allowed us to identify compounds **3e** and **3f** as the most efficient in inhibiting Dicer cleavage of both pre-miR-372 and -373. However, even if these results demonstrate that it is possible to inhibit Dicer cleavage, they do not prove unequivocally that the inhibition activity comes from an interaction with the RNA as suggested during the design of these new conjugates.

Table 1. IC_{50} Values and Dissociation Constants (K_d Values) Calculated on pre-miR-372

conjugate	IC_{50} (μM) on pre-miR-372[a]	K_d (nM) on pre-miR-372[b]
neomycin	125 ± 4.2	7400 ± 120
3a	9.75 ± 1.1	485 ± 50
3b	105 ± 3.4	1050 ± 30
3c	40.9 ± 1.1	597 ± 10
3d	11.5 ± 1.2	145 ± 10
3e	2.42 ± 0.06	16 ± 8.8
3f	4.17 ± 1.1	13.9 ± 7.6
3g	16.3 ± 1.3	19.7 ± 7.9
3h	8.30 ± 1.1	18.9 ± 0.9
3i	25.6 ± 1.1	46.1 ± 1.0
3j	10.3 ± 1.0	14.8 ± 6.7

[a]IC_{50} experiments were performed in the presence of 50 nM of pre-miR-372 beacon and 0.5 U of recombinant Dicer in buffer A (20 mM Tris-HCl, pH 7.4, 12 mM NaCl, and 1 mM DTT). [b]Binding affinities were evaluated on 5′-FAM-premiR-372 in buffer A.

In order to support this hypothesis, we studied the affinity of the conjugates for a monolabeled pre-miRNA fragment bearing a fluorescein fluorophore at the 5′-end in the absence of Dicer enzyme. The evaluation of fluorescence variation as a function of the concentration of compounds allows obtaining dissociation constant (K_d) values for all compounds.[46,47] As reported in Table 1, K_d values were in the low micromolar range for natural nucleobases conjugates (**3a**−**d**) and in the nanomolar range (14−46 nM) for artificial nucleobases conjugates (**3e**−**j**). Neomycin is the worst pre-miR-372 ligand with a K_d of 7.4 μM, and this corresponds to the worst IC_{50} value of 125 μM. Overall, compounds bearing the best affinities for target RNAs (**3e**−**3j**) are also the most active and bear the best IC_{50} values, thus suggesting that their activity comes from an interaction with the RNA. The only exception are compounds **3a** and **3d** that bear low affinity toward targeted RNAs but have a good activity that is comparable to those of compounds **3e**−**j**. Compared to other small molecules inhibitors of pre-miRNAs' processing,[10−16] the RNA ligands presented here are among the best inhibitors of Dicer processing with IC_{50} in the low micromolar range.

Biological Evaluation of Multimodal Ligands in Cancer Cells. The promising results obtained *in vitro* prompted us to study the anti-proliferative activity in the AGS gastric cancer cells where the targeted miR-372 and miR-373 are highly expressed. Toward this aim, we screened all compounds in AGS cultured cells and observed that while conjugates **3a**−**d** were unable to affect cell growth, compounds **3e**−**h** and **3j** were able to inhibit it at a 100 μM concentration (Figure S3 in Supporting Information). The rareness of dead cells floating in the culture supernatant suggests that their effect may be cytostatic rather than cytotoxic. Only compound **3i**, which contains the **HB** nucleobase and a *n*-butyl side chain, presents no activity in cells. This result is in excellent agreement with the extracellular studies where **3i** was the less efficient inhibitor among artificial nucleobases conjugates.

After this first screening neomycin conjugate **3e** has been chosen to study more in detail the inhibition activity of this class of compounds since it appeared as the most efficient inhibitor at the extracellular level as well as in cells. In order to demonstrate that the decrease in the growth rate of AGS cells is linked to an inhibition of miRNAs' production, we performed deeper investigations on the effects of compound **3e**, along with unconjugated neomycin, on miRNA biosynthesis and action. Therefore, AGS cells were treated for 5 days with the compound at concentrations ranging from 5 to 50 μM. Figure 4A shows that AGS cells are sensitive to increasing concentrations of **3e**, which slows down their growth rate in a dose-dependent manner. It is important to note that **3e** led to a growth inhibitory effect that is comparable to that of previously published anti-miR oligonucleotides directed against miRNA-372 and miRNA-373.[22] These antisense oligonucleotides, composed of mixed LNA/DNA sequences, were conceived in order to study the function of targeted miRNAs and to confirm their role in the inhibition of LATS2 translation. It was thus demonstrated that anti-miR oligonucleotides induced at most 50% inhibition of cell growth in the same conditions. Unconjugated neomycin rather exhibits a slight growth promoting effect at these concentrations. Contrarily to AGS cells, MKN74 cells, another gastric epithelial cell line, which does not express miR-372 or miR-373, are not sensitive to the growth inhibitory effects of **3e**, which rather favored cell growth (Figure 4B). These results suggest that **3e** inhibited AGS cell growth because it may be able to impair the biosynthesis of miR-372 and miR-373, on which AGS growth is dependent.[22]

To validate this hypothesis, we measured the residual levels of mature miR-372 and -373 by quantitative RT-PCR after AGS treatment with the neomycin conjugate **3e**. While neomycin does not appear to influence the amount of targeted miRs (Figure 5A), compound **3e** indeed decreases miR-372 and miR-373 levels in a dose-dependent manner (Figure 5B). To make

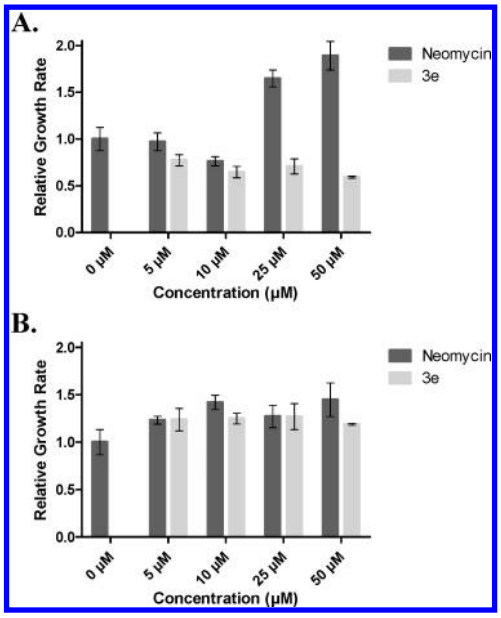

Figure 4. Relative growth rate of AGS cells (A) expressing high miR-372 and miR-373 levels and MKN74 cells (B), which do not express these miRNAs, in the presence of growing concentrations of neomycin (dark gray) and compounds **3e** (light gray). Cell growth was measured using Cell Titer reagent (Promega). Measurements were performed after a 5-day treatment with the compounds at the indicated concentrations. Bars represent the mean ± standard deviation (SD) of cell viability data compared to those of untreated cells.

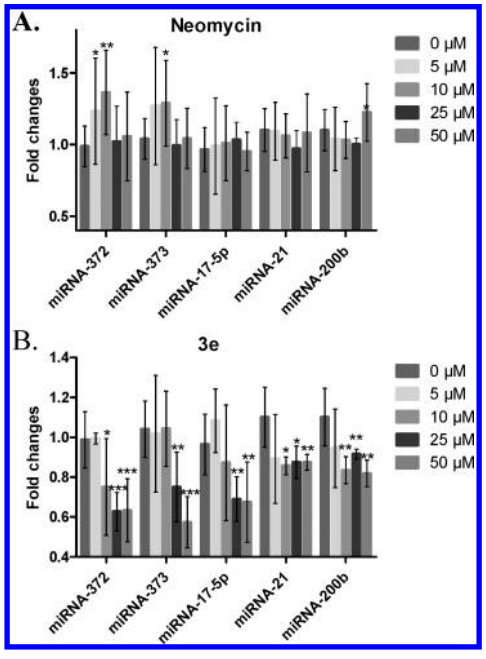

Figure 5. RT-qPCR quantification of miR-372 and miR-373 in comparison with miRNA-17-5p, -21, and−200b after a 5-day treatment of AGS cells in the presence of 0, 5, 10, 25, and 50 μM neomycin (A) or compound **3e** (B). Bars represent the mean ± SD of miRNA expression normalized to the small nucleolar RNA SNORD25 and compared to untreated cells ($n = 4$). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (Student's t test).

sure that these variations are independent of the SNORD25 reference RNA used to normalize the miRNA data, we also expressed the miRNA results using either RNU1A as another individual reference RNA (Figure S5 in Supporting Information) or the six RNU1A, RNU5A, RNA6B, SCARNA17, SNORD25 and SNORA73A reference RNAs (Figure S6 in Supporting Information). All these data are concordant to show the prominent inhibitory effect of the **3e** conjugate at doses over 25 μM on both miR-372 and miR-373 expression. However, they also reveal that this inhibition is not totally specific to these miRNAs, since others are also found down-regulated. MiR-17-5p, another cell cycle-regulating miRNA, is similarly down-regulated in a **3e**-dose-dependent effect. While these three mi-RNAs have been decreased by 35−40% at the highest **3e** dose, the oncogenic miR-21 and the epithelium-specific miR-200b are decreased by only 15−20% independently of the dose. The lack of specificity of compound **3e** for miR-372 and miR-373 could be explained by the structural similarity of the pre-miRNAs. This could actually be an advantage for the eventual therapeutic application of this kind of inhibitors. In fact, cancer cells exhibit a dysregulation of multiple oncogenic microRNAs, and therefore silencing of a single miRNA might not be sufficient for use in cancer where more than one miRNA is overexpressed.[48] More importantly, all compounds showed a very good selectivity for targeted pre-miRNAs with respect to other cellular abundant nucleic acids such as dsDNA and tRNA (Table S2 in Supporting Information).

Noteworthy, the **3e**-dependent down-regulation of miR-372 has also been assessed using a miR-372 sensor, a luciferase reporter vector, in which the 3′-UTR harbors a miR-372 binding site (luc-372PM). As reported in Figure 6, the luc-

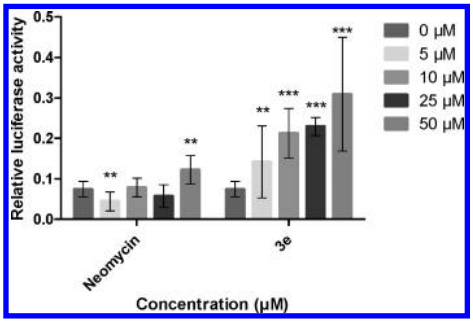

Figure 6. Relative luciferase activity of the miR-372 reporter pGL4-PM372 transfected into AGS cells previously treated for 5 days with either neomycin or **3e** at increasing concentrations. Bars represent the mean ± SD of firefly luciferase activities of pGL4-PM372 normalized to that of pGL4-MM372 ($n = 4$). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (Student's t test).

372PM vector displays only a 7% luciferase activity relative to a control reporter harboring a mismatched binding site (luc-MM), to which miR-372 is unable to pair. This low luciferase activity is due to the efficient post-transcriptional repression exerted by the high endogenous miR-372 levels. Upon treatment with the neomycin conjugate **3e**, the relative luc-372PM activity rises in a dose-dependent manner, in parallel with the decreased mature miR-372 levels. This de-repression is absent with neomycin. Importantly, Figure S7 in Supporting Information shows that **3e**-mediated increase of the relative luc-PM372 activity is exclusively due to the increased luciferase activity of the reporter bearing the perfect match for miR-372 (luc-PM372) and not to that bearing the mismatched site (luc-MM372). On the basis of these data we propose that the inhibition of cancer cells proliferation by compound **3e** is correlated with the decrease in the amount of targeted miR-372

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and -373 that are directly responsible for the growth of AGS cells. These results suggest that the decreased miR-372 levels upon **3e** treatment could de-repress some of the miRNA targets, among which is the cell cycle regulator Large Tumor Suppressor homologue 2 (LATS2).

In order to confirm this hypothesis, we performed immunolabeling experiments on LATS2 protein in AGS cells employing a specific anti-LATS2 antibody revealed by green fluorescence. Figure 7 illustrates the obtained results and shows

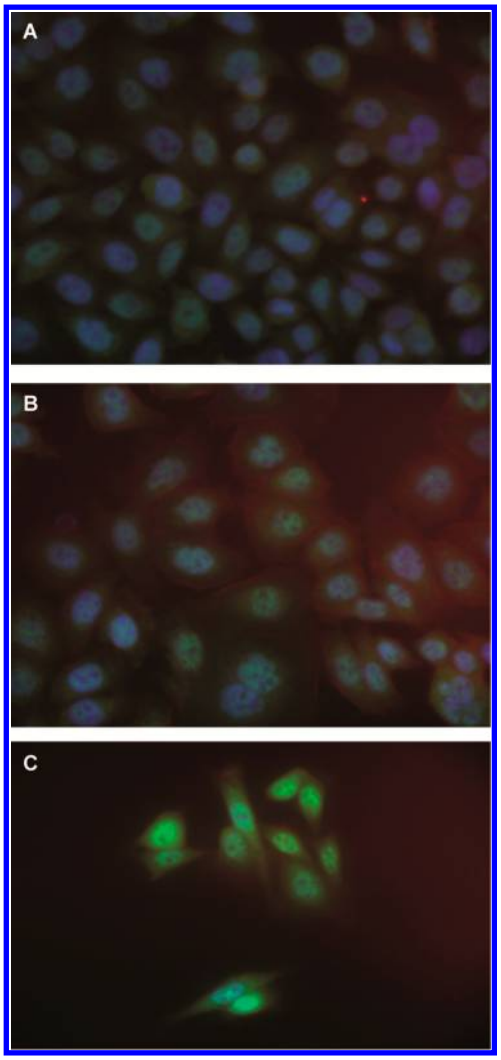

Figure 7. Subcellular localization of LATS2 protein in AGS cells using immunofluorecence microscopy: (A) untreated cells, (B) cells treated with 50 μM neomycin for 5 days, and (C) cells treated with 50 μM compound **3e** for 5 days. Nuclei are revealed by Hoechst staining (blue); LATS2 was labeled using a rabbit anti-human LATS2 antibody followed by an Alexa 488-labeled anti-rabbit antibody and appears in green.

the miR-372 targeted LATS2 protein labeled in green and cell nuclei stained in blue by a DNA labeling dye. Indeed, LATS2 clearly accumulates in the nuclei and cytoplasm of **3e**-treated AGS cells, which then appeared labeled in green. Neomycin-treated cells and untreated cells mostly show blue-stained nuclei and faintly express LATS2 protein. It has to be noted that the nuclei of **3e**-treated cells did not exhibit condensed, fragmented chromatin, which is a hallmark of apoptosis. This further confirms the previous observation in Figure S3 in Supporting Information that the compound may be cytostatic rather than cytotoxic. Furthermore, we also measured the levels of the primary miR-372 and -373 transcript (pri-miR-371-372-373) as well as that of LATS2 mRNA using RTqPCR[22] and found no significant changes in cells treated either by neomycin or by compound **3e** as compared to untreated cells (Tables S2 and S3 in Supporting Information). This indicates on the one hand that the decreased miR-372 and -373 levels in **3e**-treated cells did not result from a decreased transcription of their common primary transcript, and on the other hand that the enhanced LATS2 expression in **3e**-treated cells is mediated by a post-transcriptional event. Therefore, the nuclear LATS2 accumulation mediated by miR-372 down-regulation is likely responsible for the growth inhibition in **3e**-treated AGS cells. The effect of compound **3e** is similar to those of oligonucleotides antisense of miR-372 and miR-373,[22] confirming its activity as miR-372/3 inhibitor.

Conclusion. The work reported here describes the design of new RNA binding agents targeting the production of oncogenic miRNAs. We studied in particular miR-372 and miR-373, which are implicated in some tumor cell growth using a gastric cancer cell system, the AGS cell line, the growth of which is dependent on high levels of miR-372 and miR-373. Some of these multimodal ligands, composed of the aminoglycoside neomycin conjugated to natural and artificial nucleobases, were revealed to be able to inhibit the cleavage of pre-miRNAs by Dicer enzyme at the extracellular level probably by binding to these stem-loop structured RNAs. Compound **3e**, containing neomycin conjugated to nucleobase **S**, also demonstrated the ability to inhibit AGS cells proliferation in a dose-dependent manner. Its cytostatic effect has been directly correlated to the decrease in the production of oncogenic miR-372 and miR-373 as shown by qRT-PCR and luciferase reporter experiments and the de-repression of LATS-2 protein, which is the target of these miRNAs, although it also affects in some manner the production of other miRNAs. Altogether these results demonstrate that this compound is very promising for further studies of its mechanism of action and optimization of its specificity and biological activity. The approach presented here, consisting of the conjugation of different RNA binding motives, was demonstrated to be successful for the targeting of oncogenic miRNAs and open the way for the rational design of pre-miRNAs' ligands. Other RNA binding motives, such as intercalators or other antibiotics, could be envisaged in order to synthesize RNA ligands with improved activity. The discovery of pre-miRNA ligands and in particular inhibitors of oncogenic miRNAs' production could be not only a great advance in anticancer therapy studies but also a valuable tool in order to better understand miRNA pathways and mechanisms.

METHODS

Chemistry. *Materials.* Reagents and solvents were purchased from Aldrich or Alfa Aesar and used without further purification. All reactions involving air- or moisture-sensitive reagents or intermediates were performed under an argon atmosphere. Flash column chromatographies were carried out on silica gel (Merck, SDS 60 Å, 40−63 μm, VWR). Analytical thin-layer chromatography (TLC) was conducted on Macherey-Nagel precoated silica gel 60F254 plates, and compounds were visualized by irradiation (254 nm) or by staining with ninhydrin stain or anisaldehyde stain. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 200 MHz or a Bruker AC 500 MHz spectrometer. Chemical shifts are reported in parts per million (ppm, δ) referenced to the residual ^{1}H resonance of the solvent ($CDCl_3$, δ 7.26; CD_3OD δ 3.31; DMSO-d_6 δ 2.50). Splitting patterns are

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designated as follows: s (singlet), d (doublet), t (triplet), m (multiplet), br (broad). Coupling constants (*J* values) are listed in hertz (Hz). High resolution mass spectra (HRMS) were obtained with a LTQ Orbitrap hybrid mass spectrometer with an electrospray ionization probe (Thermoscientific, San Jose, CA) by direct infusion from a pump syringe to confirm correct molar mass and high purity of compounds. HPLC was performed using a Water Alliance 2695 pump coupled with Waters 996 photodiode array detector and a Thermo Scientific RP-C_{18} column (250 mm × 4.6 mm, 5 μm for analytical HPLC and 250 mm × 10 mm, 5 μm for semipreparative HPLC). All HPLC analyses were run at RT. A gradient of CH_3CN containing 0.1% TFA (eluent B) in water containing 0.1% TFA (eluent A) from 5% to 100% was used at a flow rate of 1 mL/min for analytical HPLC and 3.5 mL/min for semipreparative HPLC. Analytical HPLC method A: from 5% to 100% of eluent B in 20 min; HPLC method B: 5% eluent B during 5 min, from 5% to 40% eluent B in 20 min; HPLC method C: from 5% to 15% eluent B in 5 min, 15% eluent B during 5 min, from 15% to 40% eluent B in 20 min.

The synthesis of neomycin azide **1** and of all alkyne intermediates of natural and artificial nucleobases (compounds **4**, **5**, **7**, **11**, **12**, **20a,b**, **26**, **29**, and **32**) is reported in the Supporting Information file.

General Procedure for the 1,3-Dipolar Cycloaddition (General Procedure A). 1,3-Dipolar cycloaddition was performed between neomycin azide **1** and appropriately modified compounds **4**, **5**, **7**, **11**, **12**, **20a,b**, **26**, **29**, and **32** in order to obtain desired conjugates **2a**–**j**. CH_3CN was used when performing the reaction at RT, whereas toluene was used when performing the reaction under heating.

To a solution of neomycin azide **1** (50 mg, 0.04 mmol) and alkynes **4**, **5**, **7**, **11**, **12**, **20a**, **20b**, **26**, **29**, or **32** (1.1 equiv) in acetonitrile or toluene (4 mL) were added copper iodide (3.2 mg, 0.4 equiv) and *N*,*N*-diisopropylethylamine (42 μL, 6 equiv) at RT or under reflux, respectively, and the mixture was stirred overnight. The solvent was then removed under reduced pressure. The crude residue was purified by flash chromatography on a silica gel column using a CH_2Cl_2/MeOH 95:5 mixture leading to the desired 1,4-disubstited 1,2,3-triazole as a white solid.

*Boc-Neomycin-A (**2a**).* General procedure A was employed for the reaction between compound **1** (100 mg, 0.08 mmol) and alkyne **4** (15.4 mg, 1.1 equiv) in CH_3CN in the presence of CuI (6.2 mg, 0.4 equiv) and DIPEA (85 μL, 6 equiv) at RT leading to compound **2a** as a white solid: yield 99 mg (87%); R_f = 0.25 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (500 MHz, CD_3OD) δ 8.35–8.23 (m, 3H), 6.75–6.42 (m, 2H), 5.75–5.56 (m, 2H), 5.39 (s, 1H), 5.06 (s, 1H), 4.96 (s, 1H), 4.75–4.65 (m, 1H), 4.40–4.08 (m, 3H), 3.97 (t, *J* = 6.1 Hz, 1H), 3.90 (s, 1H), 3.82–3.68 (m, 3H), 3.61–3.25 (m, 9H), 3.11–3.05 (m, 1H), 1.96–1.90 (m, 1H), 1.50–1.36 (m, 55H); ^{13}C NMR (125 MHz, CD_3OD) δ 159.2, 158.7, 158.5, 158.2, 158.1, 158.0, 157.9, 157.3, 154.3, 150.5, 143.1, 142.7, 127.7, 120.0, 111.6, 100.7, 98.9, 86.0, 80.9, 80.7, 80.6, 80.5, 80.4, 80.3, 80.2, 80.1, 79.8, 79.7, 75.4, 74.6, 74.5, 73.1, 72.9, 72.8, 71.7, 69.2, 56.6, 55.9, 53.7, 52.7, 51.1, 51.0, 43.9, 42.4, 42.3, 39.5, 35.9, 29.0, 28.9, 28.8, 28.7; MS (ESI), *m*/*z* 1413.7108 (M + H)$^+$ ($C_{61}H_{101}N_{14}O_{24}$ requires 1413.7108).

*Boc-Neomycin-U (**2b**).* General procedure A was employed for the reaction between compound **1** (50 mg, 0.04 mmol) and alkyne **5** (6.6 mg, 1.1 equiv) in CH_3CN in the presence of CuI (3.2 mg, 0.4 equiv) and DIPEA (42 μL, 6 equiv) at RT leading to compound **2b** as a white solid: yield 34.5 mg (61%); R_f = 0.31 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (500 MHz, CD_3OD) δ 8.13 (s, 1H), 7.87 (d, *J* = 7.8 Hz, 1H), 5.60 (d, *J* = 7.8 Hz, 1H), 5.46 (s, 1H), 5.20–4.99 (m, 3H), 4.95 (d, *J* = 1.5 Hz, 1H), 4.70–4.56 (m, 1H), 4.29 (s, 1H), 4.16 (s, 1H), 3.98 (t, *J* = 6.2 Hz, 1H), 3.84–3.62 (m, 3H), 3.54–3.35 (m, 12H), 3.12 (t, *J* = 8.8 Hz, 1H), 1.99–1.88 (s, 1H), 1.50–1.38 (m, 55H); ^{13}C NMR (125 MHz, CD_3OD) δ 166.7, 159.2, 158.8, 158.5, 158.2, 158.0, 157.9, 157.8, 157.7, 152.7, 147.1, 143.3, 127.4, 127.3, 102.9, 100.6, 98.8, 98.7, 86.4, 86.3, 81.1, 80.9, 80.7, 80.5, 80.4, 80.3, 80.2, 79.3, 75.5, 74.7, 74.5, 73.1, 73.0, 72.6, 71.7, 69.1, 56.4, 53.6, 53.1, 52.7, 51.0, 44.0, 42.6, 42.2, 35.8, 29.0, 28.9, 28.8; MS (ESI), *m*/*z* 1390.6836 (M + H)$^+$ ($C_{60}H_{100}N_{11}O_{26}$ requires 1390.6836).

*Boc-Neomycin-C (**2c**).* General procedure A was employed for the reaction between compound **1** (100 mg, 0.08 mmol) and alkyne **7** (13.4 mg, 1.1 equiv) in CH_3CN in the presence of CuI (6.2 mg, 0.4 equiv) and DIPEA (85 μL, 6 equiv) at RT leading to compound **2c** as a white solid: yield 88 mg (78%); R_f = 0.25 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (500 MHz, CD_3OD) δ 8.13 (s, 1H), 7.77 (d, *J* = 7.2 Hz, 1H), 5.89 (d, *J* = 7.2 Hz, 1H), 5.42 (s, 1H), 5.20–4.99 (m, 3H), 4.95 (d, *J* = 1.2 Hz, 1H), 4.72–4.63 (m, 1H), 4.38–4.18 (m, 3H), 3.98 (t, *J* = 6.3 Hz, 1H), 3.79–3.66 (m, 3H), 3.55–3.27 (m, 13H), 3.17–3.11 (m, 1H), 1.95–1.92 (s, 1H), 1.46–1.36 (m, 55H); ^{13}C NMR (125 MHz, CD_3OD) δ 167.9, 159.3, 158.8, 158.6, 158.5, 158.3, 158.2, 158.1, 157.9, 150.9, 149.3, 143.8, 127.4, 123.5, 111.6, 102.9, 100.6, 98.7, 96.4, 86.4, 80.8, 80.7, 80.4, 80.3, 75.4, 74.8, 74.6, 73.2, 73.1, 72.8, 71.7, 69.2, 56.6, 56.5, 53.7, 52.9, 51.0, 45.2, 42.6, 42.5, 42.2, 35.4, 29.0, 28.9, 28.8; MS (ESI), *m*/*z* 1389.6990 (M + H)$^+$ ($C_{60}H_{101}N_{12}O_{25}$ requires 1389.6995).

*Boc-Neomycin-G (**2d**).* General procedure A was employed for the reaction between compound **1** (100 mg, 0.08 mmol) and alkyne **11** (17.0 mg, 1.1 equiv) in toluene in the presence of CuI (6.2 mg, 0.4 equiv) and DIPEA (85 μL, 6 equiv) and PPh_3 (17 mg, 0.8 equiv) at 110 °C leading to compound **2d** as a white solid: yield 42 mg (36%); R_f = 0.15 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (500 MHz, CD_3OD) δ 8.37 (s, 1H), 8.05–7.73 (m, 1H), 5.58 (d, *J* = 14.6 Hz, 1H), 5.40 (s, 1H), 5.32 (d, *J* = 14.6 Hz, 1H), 5.05 (s, 1H), 4.98 (s, 1H), 4.78–4.71 (m, 1H), 4.57 (s, 1H), 4.23–3.91 (m, 5H), 3.62–3.27 (m, 11H), 3.13–3.07 (m, 1H), 1.93–1.90 (m, 1H), 1.47–1.40 (m, 55H); ^{13}C NMR (125 MHz, CD_3OD) δ 159.6, 158.7, 158.5, 158.3, 158.2, 158.0, 155.0, 150.9, 149.4, 143.5, 128.3, 123.5, 112.0, 101.0, 98.8, 88.1, 80.9, 80.7 80.4, 80.3, 80.2, 80.0, 79.6, 78.3, 76.0, 75.5, 74.5, 73.1, 72.6, 72.5, 71.7, 69.2, 56.3, 53.7, 52.6, 51.5, 50.8, 42.4, 42.3, 42.2, 39.3, 35.7, 29.0, 28.9, 28.8, 28.7, 28.6; MS (ESI), *m*/*z* 1429.7048 (M + H)$^+$ ($C_{61}H_{101}N_{14}O_{25}$ requires 1429.7057).

*Boc-Neomycin-S (**2e**).* General procedure A was employed for the reaction between compound **1** (100 mg, 0.08 mmol) and alkyne **12** (27.9 mg, 1.1 equiv) in CH_3CN in the presence of CuI (6.2 mg, 0.4 equiv) and DIPEA (85 μL, 6 equiv) at RT leading to compound **2e** as a slightly yellow solid: yield 108 mg (86%); R_f = 0.36 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (500 MHz, Acetone-d_6) δ 11.22 (br, 1H), 9.34 (br, 1H), 8.29 (s, 1H), 7.97 (s, 1H), 7.59 (d, *J* = 7.5 Hz, 1H), 7.55 (d, *J* = 6.9 Hz, 1H), 7.38 (s, 1H), 7.31 (d, *J* = 7.9 Hz, 1H), 6.60–5.87 (m, 6H), 5.34–4.78 (m, 6H), 4.73–4.15 (m, 6H), 4.10–3.21 (m, 19H), 3.21–3.05 (m, 2H), 2.91–2.80 (m, 2H), 2.28 (s, 3H), 2.04–1.98 (m, 1H), 1.42–1.37 (m, 55H); ^{13}C NMR (125 MHz, Acetone-d_6) δ 172.6, 170.1, 160.0, 159.2, 159.0, 158.5, 158.4, 158.0, 157.9, 157.2, 157.1, 151.2, 148.3, 141.5, 137.1, 130.7, 125.1, 122.8, 120.9, 119.3, 111.8, 109.4, 102.0, 100.4, 87.0, 82.1, 81.6, 80.7, 80.6, 80.4, 80.3, 80.2, 80.1, 80.0, 76.4, 75.7, 74.9, 74.2, 73.8, 72.0, 69.3, 57.7, 54.3, 53.5, 52.9, 52.8, 52.3, 43.5, 42.2, 38.1, 36.6, 29.8, 29.7, 29.6, 23.9, 23.2; MS (ESI), *m*/*z* 1553.7290 (M + H)$^+$ ($C_{69}H_{109}N_{12}O_{26}S$ requires 1553.7291).

*Boc-Neomycin-HB-Ar (**2f**).* General procedure A was employed for the reaction between compound **1** (50 mg, 0.04 mmol) and alkyne **20a** (13.6 mg, 1.1 equiv) in toluene in the presence of CuI (3.2 mg, 0.4 equiv) and DIPEA (42 μL, 6 equiv) at 110 °C leading to compound **2f** as a white solid: yield 42 mg (67%); R_f = 0.47 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (500 MHz, CD_3OD) δ 7.99 (s, 1H), 7.87–7.84 (m, 1H), 7.49 (d, *J* = 8.7 Hz, 1H), 7.44–7.42 (m, 2H), 7.30–7.27 (m, 3H), 7.04–6.99 (m, 1H), 5.42 (s, 1H), 5.11 (s, 1H), 4.93 (d, *J* = 1.3 Hz, 1H), 4.71–4.61 (m, 1H), 4.35–4.09 (m, 3H), 4.03–3.90 (m, 3H), 3.80–3.33 (m, 15H), 3.29–3.10 (m, 3H), 1.94–1.92 (m, 1H), 1.47–1.39 (m, 55H). ^{13}C NMR (125 MHz, CD_3OD) δ 159.2, 158.9, 158.5, 158.3, 158.2, 158.0, 155.6, 150.0, 142.3, 140.5, 137.5, 129.9, 123.9, 120.5, 118.7, 111.5, 100.5, 98.9, 80.8, 80.7, 80.4, 80.3, 80.2, 75.6, 74.7, 74.6, 74.5, 73.2, 73.0, 72.7, 71.6, 69.1, 58.3, 56.6, 53.6, 51.0, 42.5, 42.1, 35.8, 29.3, 29.2, 29.0, 28.9, 28.8, 23.6, 18.4; MS (ESI), *m*/*z* 1545.7577 (M + H)$^+$ ($C_{71}H_{109}N_{12}O_{26}$ requires 1545.7570).

*Boc-Neomycin-HB (**2g**).* General procedure A was employed for the reaction between compound **1** (40 mg, 0.032 mmol) and alkyne **20b** (10.2 mg, 1.1 equiv) in toluene in the presence of CuI (2.5 mg, 0.4 equiv) and DIPEA (34 μL, 6 equiv) at 110 °C leading to compound **2g** as a white solid: yield 30 mg (60%); R_f = 0.47 (CH_2Cl_2/MeOH

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9:1); ^{1}H NMR (500 MHz, CD_3OD) δ 7.99 (s, 1H), 7.79 (s, 1H), 7.44 (d, J = 8.6 Hz, 1H), 7.23 (d, J = 8.6 Hz, 1H), 5.42 (s, 1H), 5.11 (s, 1H), 4.93 (s, 1H), 4.69−4.63 (m, 1H), 4.36−4.09 (m, 3H), 3.95 (t, J = 6.3 Hz, 1H), 3.90 (s, 1H), 3.83−3.72 (m, 2H), 3.66−3.61 (m, 1H), 3.56−3.33 (m, 16H), 3.30−3.12 (m, 5H), 2.00−1.93 (m, 1H), 1.55−1.36 (m, 57H), 0.96 (t, J = 7.3 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 168.7, 159.2, 158.9, 158.5, 158.4, 158.3, 158.2, 158.0, 147.9, 142.3, 138.2, 125.8, 118.5, 111.4, 111.0, 100.5, 98.9, 86.2, 80.9, 80.7, 80.4, 80.3, 80.1, 79.4, 74.7, 74.5, 73.2, 73.0, 72.7, 71.7, 69.1, 56.7, 55.9, 53.6, 53.0, 52.6, 51.1, 43.8, 42.5, 42.1, 40.6, 35.9, 33.4, 29.3, 29.0, 28.9, 28.8, 23.5, 21.1, 14.2; MS (ESI), m/z 1525.7882 $(M + H)^+$ ($C_{69}H_{113}N_{12}O_{26}$ requires 1525.7884).

*Boc-Neomycin-D$_3$ (**2h**).* General procedure A was employed for the reaction between compound **1** (100 mg, 0.081 mmol) and alkyne **26** (26.7 mg, 1.1 equiv) in CH_3CN in the presence of CuI (6.2 mg, 0.4 equiv) and DIPEA (85 μL, 6 equiv) at RT leading to compound **2h** as a white solid: yield 95 mg (76%); R_f = 0.47 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (500 MHz, CD_3OD) δ 8.20 (s, 1H), 8.05−7.92 (m, 4H), 7.70−7.50 (m, 6H), 7.38 (t, J = 7.8 Hz, 1H), 5.45−5.36 (m, 3H), 5.06 (s, 1H), 4.96 (s, 1H), 4.76−4.67 (m, 1H), 4.36−4.10 (m, 3H), 3.98 (t, J = 6.3 Hz, 1H), 3.91 (s, 1H), 3.82−3.62 (m, 3H), 3.56−3.35 (m, 10H), 3.30−3.22 (m, 2H), 2.96−2.86 (m, 1H), 1.91−1.84 (m, 1H), 1.50−1.34 (m, 55H); ^{13}C NMR (125 MHz, CD_3OD) δ 168.9, 159.2, 158.8, 158.5, 158.3, 158.2, 158.1, 158.0, 144.0, 142.6, 140.2, 139.3, 136.3, 135.8, 133.0, 130.3, 129.7, 128.8, 127.0, 122.4, 121.1, 119.0, 117.4, 111.8, 100.5, 98.7, 86.4, 80.9, 80.7, 80.4, 79.9, 79.1, 75.4, 74.6, 74.5, 73.2, 73.0, 72.6, 71.7, 69.1, 56.7, 56.6, 55.9, 53.6, 52.9, 52.6, 50.9, 43.8, 42.8, 42.6, 42.2, 35.6, 29.0, 28.9, 28.8; MS (ESI), m/z 1541.7622 $(M + H)^+$ ($C_{72}H_{109}N_{12}O_{25}$ requires 1541.7621).

*Boc-Neomycin-D$_4$ (**2i**).* General procedure A was employed for the reaction between compound **1** (100 mg, 0.08 mmol) and alkyne **29** (26.2 mg, 1.1 equiv) in CH_3CN in the presence of CuI (6.2 mg, 0.4 equiv) and DIPEA (85 μL, 6 equiv) at RT leading to compound **2i** as a white solid: yield 98 mg (79%); R_f = 0.47 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (500 MHz, CD_3OD) δ 8.17 (s, 1H), 7.92 (s, 1H), 7.71 (s, 1H), 7.62 (s, 1H), 7.37−7.22 (m, 3H), 5.54 (s, 1H), 5.41−5.34 (m, 2H), 5.04 (s, 1H), 4.95 (s, 1H), 4.71−4.69 (1H), 4.56 (s, 1H), 4.22−4.12 (m, 2H), 3.98 (t, J = 5.6 Hz, 1H), 3.91 (s, 1H), 3.87−3.64 (m, 3H), 3.57−3.31 (m, 10H), 3.30−3.14 (m, 3H), 2.89−2.73 (m, 1H), 1.91−1.76 (m, 1H), 1.55−1.36 (m, 57H), 0.96 (t, J = 7.3 Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 159.2, 158.8, 158.4, 158.3, 158.2, 158.1, 144.1, 142.9, 141.4, 139.1, 135.7, 130.2, 129.9, 129.2, 126.9, 120.0, 118.9, 117.2, 117.0, 111.8, 100.5, 98.4, 86.8, 80.9, 80.7, 80.4, 80.3, 79.7, 78.5, 75.4, 74.7, 74.5, 73.2, 72.9, 72.5, 71.6, 69.1, 56.6, 55.9, 53.6, 52.8, 52.5, 50.8, 43.9, 42.8, 42.2, 40.6, 35.5, 33.5, 29.0, 28.8, 21.1, 14.3; MS (ESI), m/z 1536.8048 $(M + H)^+$ ($C_{70}H_{114}N_{13}O_{25}$ requires 1536.8043).

*Boc-Neomycin-BIG (**2j**).* General procedure A was employed for the reaction between compound **1** (50 mg, 0.04 mmol) and alkyne **32** (12.0 mg, 1.1 equiv) in toluene in the presence of $CuSO_4{\cdot}5H_2O$ (2 mg, 0.2 equiv) and 0.1 M of sodium ascorbate solution (160 mL, 0.4 equiv) at 110 °C leading to compound **2j** as a white solid: yield 35 mg (57%); R_f = 0.19 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (500 MHz, CD_3OD) δ 7.95 (s, 1H), 7.56−7.42 (m, 2H), 7.26−7.09 (m, 2H), 5.44 (s, 1H), 5.13 (s, 1H), 4.94 (s, 1H), 4.70−4.52 (m, 1H), 4.38−4.10 (m, 4H), 4.03−3.87 (m, 2H), 3.84−3.35 (m, 13H), 3.22−3.00 (m, 3H), 2.82−2.68 (m, 2H), 2.00−1.89 (m, 1H), 1.45−1.38 (m, 55H); ^{13}C NMR (125 MHz, CD_3OD) δ 175.4, 171.4, 171.3, 159.2, 159.0, 158.9, 158.5, 158.3, 158.2, 157.9, 147.6, 125.4, 123.2, 111.6, 100.5, 98.9, 86.2, 81.0, 80.8, 80.7, 80.5, 80.3, 80.2, 79.8, 79.7, 75.6, 74.7, 74.5, 73.2, 73.1, 72.7, 71.7, 69.1, 56.7, 55.2, 53.6, 53.0, 52.6, 51.1, 44.2, 42.6, 42.1, 42.0, 36.4, 35.9, 30.8, 28.9, 28.8, 22.6; MS (ESI), m/z 1510.7530 $(M + H)^+$ ($C_{67}H_{108}N_{13}O_{26}$ requires 1510.7523).

General Procedure for Boc Deprotection of Compounds 3a−j (General Procedure B). *tert*-Butoxycarbonyl (Boc) removal was obtained after treatment of compounds **2a−j** with TFA (3 mL, large excess) overnight at RT. The solvent and the residues of TFA were then removed under reduced pressure. Final precipitation in a mixture 49:1 Et_2O/MeOH led to pure compounds **3a−j** as white solids (TFA salts).

*Neomycin-A (**3a**).* General procedure **B** was applied to compound **2a** (86 mg, 0.061 mmol) leading to the desired product **3a** as a white solid: yield 85 mg (93%); t_R 6.1 min (analytical HPLC method A); ^{1}H NMR (500 MHz, CD_3OD) δ 8.38 (s, 1H), 8.38 (s, 1H), 8.29 (s, 1H), 5.77 (d, J = 3.6 Hz, 1H), 5.65 (br, 2H), 5.37 (d, J = 3.7 Hz, 1H), 5.34 (d, J = 1.4 Hz, 1H), 4.76 (dd, J = 15.0, 4.5 Hz, 1H), 4.49 (t, J = 4.9 Hz, 1H), 4.48−4.44 (m, 1H), 4.36−4.33 (m, 1H), 4.16 (t, J = 3.1 Hz, 1H), 4.06−4.02 (m, 2H), 3.99 (td, J = 8.4, 3.3 Hz, 1H), 3.83−3.80 (m, 2H), 3.73−3.70 (m, 1H), 3.58−3.54 (m, 2H), 3.45−3.37 (m, 4H), 3.35−3.31 (m, 1H), 3.29−3.20 (m, 1H), 2.51−2.40 (m, 1H), 2.04 (q, J = 12.6 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 154.4, 150.4, 149.6, 144.4, 126.3, 111.4, 109.6, 96.9, 96.5, 86.6, 81.6, 77.7, 76.4, 74.7, 74.0, 72.9, 72.2, 72.0, 69.4, 69.3, 69.2, 55.9, 55.0, 53.1, 52.9, 51.3, 50.2, 41.8, 41.6, 29.5; MS (ESI), m/z 813.3976 $(M + H)^+$ ($C_{31}H_{53}O_{12}N_{14}$ requires 813.3967).

*Neomycin-U (**3b**).* General procedure **B** was applied to compound **2b** (34.5 mg, 0.025 mmol). The crude product was subjected to semipreparative HPLC purification leading to desired product **3b** as a white solid: yield 25 mg (59%); t_R 6.7 min (analytical HPLC method B); ^{1}H NMR (500 MHz, CD_3OD) δ 8.18 (s, 1H), 7.76 (d, J = 7.9 Hz, 1H), 5.82 (d, J = 3.5 Hz, 1H), 5.72 (d, J = 7.9 Hz, 1H), 5.33 (d, J = 3.3 Hz, 1H), 5.32 (d, J = 1.4 Hz, 1H), 5.12−4.98 (m, 2H), 4.78 (dd, J = 14.9, 4.8 Hz, 1H), 4.47−4.44 (m, 1H), 4.39 (t, J = 5.1 Hz, 1H), 4.34−4.32 (m, 1H), 4.16 (t, J = 3.1 Hz, 1H), 4.12−4.05 (m, 3H), 3.79−3.75 (m, 2H), 3.72−3.71 (m, 1H), 3.64−3.58 (m, 2H), 3.52−3.37 (m, 7H), 3.35 (s, 1H), 3.27−3.19 (m, 1H), 2.46−2.39 (m, 1H), 2.05 (q, J = 12.6 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 166.6, 152.8, 147.2, 126.7, 111.7, 103.0, 96.9, 96.2, 86.5, 81.2, 77.5, 77.3, 74.7, 73.8, 73.1, 72.2, 72.1, 69.4, 69.2, 58.4, 54.6, 52.9, 52.2, 51.4, 50.3, 44.4, 41.7, 41.6, 29.4; MS (ESI), m/z 790.3690 $(M + H)^+$ ($C_{30}H_{52}O_{14}N_{11}$ requires 790.3690).

*Neomycin-Cytosin (**3c**).* General procedure **B** was applied to compound **2c** (78 mg, 0.056 mmol) leading to desired product **3c** as a white solid: yield 78 mg (94%); t_R 5.9 min (analytical HPLC method A); ^{1}H NMR (500 MHz, CD_3OD) δ 8.21 (s, 1H), 7.96 (d, J = 7.5 Hz, 1H), 6.07 (br, 1H), 5.91 (d, J = 3.3 Hz, 1H), 5.31 (d, J = 2.8 Hz, 1H), 5.30 (d, J = 1.5 Hz, 1H), 5.16−5.01 (m, 2H), 4.78 (dd, J = 14.9, 4.8 Hz, 1H), 4.47−4.44 (m, 1H), 4.36−4.32 (m, 2H), 4.17−4.09 (m, 4H), 3.91 (dd, J = 4.6, 2.9 Hz, 1H), 3.76 (t, J = 9.0 Hz, 1H), 3.72−3.71 (m, 1H), 3.62 (dd, J = 8.8, 3.4 Hz, 1H), 3.52−3.43 (m, 7H), 3.36 (td, J = 8.2, 3.5 Hz, 2H), 3.25−3.19 (m, 1H), 2.48−2.41 (m, 1H), 2.05 (q, J = 12.6 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 164.4, 151.4, 143.7, 127.3, 123.7, 111.8, 96.8, 95.7, 86.0, 80.9, 77.5, 77.4, 74.5, 73.7, 73.5, 72.1, 71.9, 69.4, 69.3, 69.2, 54.4, 53.0, 52.3, 51.4, 50.4, 45.9, 41.7, 41.4, 29.4; MS (ESI), m/z 789.3863 $(M + H)^+$ ($C_{30}H_{53}O_{13}N_{12}$ requires 789.3855).

*Neomycin-G (**3d**).* General procedure **B** was applied to compound **2d** (36 mg, 0.025 mmol) leading to desired product **3d** as a white solid: yield 35 mg (92%); t_R 12.1 min (analytical HPLC method B); ^{1}H NMR (500 MHz, CD_3OD) δ 8.18 (s, 1H), 8.12 (br, 1H), 5.40 (s, 1H), 5.39 (s, 1H), 5.36 (d, J = 4.9 Hz, 1H), 5.15 (d, J = 2.9 Hz, 1H), 4.48−4.43 (m, 2H), 4.36−4.34 (m, 1H), 4.17 (t, J = 3.0 Hz, 1H), 3.98 (t, J = 9.6 Hz, 1H), 3.96−3.90 (m, 1H), 3.74 (t, J = 8.9 Hz, 1H), 3.72−3.69 (m, 1H), 3.67 (t, J = 9.6 Hz, 1H), 3.62 (t, J = 9.5 Hz, 1H), 3.53 (dd, J = 10.5, 3.4 Hz, 1H), 3.45−3.32 (m, 8H), 3.25−3.18 (m, 1H), 2.45−2.40 (m, 1H), 2.03 (q, J = 12.6 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 160.1, 155.6, 151.5, 149.4, 145.0, 134.9, 125.4, 123.7, 97.3, 97.1, 86.6, 81.8, 77.8, 76.9, 75.2, 74.0, 72.5, 72.2, 71.7, 69.4, 69.3, 69.2, 55.0, 52.9, 52.1, 51.1, 50.0, 41.6, 29.5; MS (ESI), m/z 829.3924 $(M + H)^+$ ($C_{31}H_{53}O_{13}N_{14}$ requires 829.3917).

*Neomycin-S (**3e**).* General procedure **B** was applied to compound **2e** (87 mg, 0.056 mmol) leading to desired product **3e** as a white solid: yield 82.6 mg (90%); t_R min 7.9 (analytical HPLC method A); ^{1}H NMR (500 MHz, CD_3OD) δ 8.23−7.91 (m, 2H), 7.63 (d, J = 6.5 Hz, 1H), 7.47 (s, 1H), 7.41−7.24 (m, 2H), 5.95 (s, 1H), 5.40 (s, 1H), 5.31 (s, 1H), 4.72 (s, 1H), 4.53−3.93 (m, 7H), 3.91−3.31 (m, 12H), 3.27−3.03 (m, 4H), 2.92−2.75 (m, 2H), 2.60−2.35 (m, 1H), 2.22 (s, 3H), 2.12−1.90 (m, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 171.1, 159.6, 150.8, 140.1, 136.8, 130.2, 123.2, 120.9, 120.0, 111.4, 109.1, 96.9, 96.8, 81.6, 77.7, 74.8, 74.0, 72.9, 72.8, 72.1, 71.9, 69.5, 69.3, 69.2,

55.1, 53.0, 51.2, 41.7, 36.9, 30.8, 29.5, 22.7; MS (ESI), *m/z* 953.4159 $(M + H)^+$ ($C_{39}H_{61}O_{14}N_{12}S$ requires 953.4151).

*Neomycin-HBAr (**3f**).* General procedure **B** was applied to compound **2f** (25 mg, 0.016 mmol) leading to desired product **3f** as a white solid: yield 20 mg (76%); t_R 22.1 min (analytical HPLC method C); ^{1}H NMR (500 MHz, CD_3OD) 7.97 (s, 1H), 7.89 (d, *J* = 2.0 Hz, 1H), 7.51 (d, *J* = 8.8 Hz, 1H), 7.44−7.41 (m, 2H), 7.32−7.27 (m, 3H), 7.06−7.02 (m, 1H), 5.83 (d, *J* = 3.7 Hz, 1H), 5.40 (d, *J* = 4.4 Hz, 1H), 5.32 (d, *J* = 1.5 Hz, 1H), 4.85−4.81 (m, 1H), 4.72 (dd, *J* = 15.0, 5.0 Hz, 1H), 4.45 (dd, *J* = 7.8, 4.4 Hz, 1H), 4.40 (t, *J* = 4.6 Hz, 1H), 4.33−4.27 (m, 1H), 4.14 (t, *J* = 3.1 Hz, 1H), 4.06−3.95 (m, 3H), 3.80 (t, *J* = 9.0 Hz, 1H), 3.71−3.67 (m, 1H), 3.61−3.33 (m, 13H), 3.30−3.19 (m, 3H), 2.47−2.39 (m, 1H), 2.07−1.96 (m, 1H); ^{13}C NMR (125 MHz, CD_3OD) *δ* 168.6, 163.1, 156.0, 148.3, 147.7, 142.3, 140.3, 137.5, 130.0, 125.0, 124.2, 120.8, 119.2, 111.6, 110.9, 97.1, 96.8, 86.6, 81.9, 77.8, 76.7, 74.9, 74.1, 72.7, 72.2, 71.8, 69.4, 69.3, 69.2, 55.0, 52.9, 52.6, 51.2, 50.2, 49.0, 41.6, 29.7, 29.1, 23.4; MS (ESI), *m/z* 945.4444 $(M + H)^+$ ($C_{41}H_{61}O_{14}N_{12}$ requires 945.4430).

*Neomycin-HB (**3g**).* General procedure **B** was applied to compound **2g** (30 mg, 0.020 mmol) leading to desired product **3g** as a white solid: yield 24.5 mg (77%); t_R 17.1 min (analytical HPLC method C); ^{1}H NMR (500 MHz, CD_3OD) *δ* 8.00 (s, 1H), 7.79 (s, 1H), 7.47 (d, *J* = 8.5 Hz, 1H), 7.19 (d, *J* = 8.5 Hz, 1H), 5.72 (s, 1H), 5.39 (d, *J* = 3.4 Hz, 1H), 5.33 (s, 1H), 4.90−4.82 (m, 1H), 4.75−4.69 (m, 1H), 4.48−4.26 (m, 3H), 4.18−4.12 (m, 1H), 4.05−3.90 (m, 3H), 3.77 (t, *J* = 8.6 Hz, 1H), 3.69 (s, 1H), 3.61−3.35 (m, 12H), 3.31−3.16 (m, 4H), 2.52−2.37 (m, 1H), 2.03−1.90 (m, 1H), 1.56−1.50 (m, 2H), 1.45−1.37 (m, 2H), 0.97 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD) *δ* 158.7, 142.3, 138.0, 119.0, 111.5, 110.8, 97.1, 96.9, 86.6, 81.9, 77.8, 76.8, 74.9, 74.1, 72.6, 72.2, 71.7, 69.5, 69.3, 55.1, 52.9, 52.6, 51.1, 50.0, 41.6, 41.5, 40.8, 33.3, 29.8, 29.2, 23.1, 21.1, 14.2; MS (ESI), *m/z* 925.4754 $(M + H)^+$ ($C_{39}H_{65}O_{14}N_{12}$ requires 925.4743).

*Neomycin-D_3 (**3h**).* General procedure **B** was applied to compound **2h** (51 mg, 0.033 mmol) leading to desired product **3h** as a white solid: yield 50.0 mg (93%); t_R 8.6 min (analytical HPLC method A); ^{1}H NMR (500 MHz, CD_3OD) *δ* 8.93 (br, 1H), 8.40 (s, 1H), 8.22 (br, 1H), 7.97−7.95 (m, 3H), 7.67−7.48 (m, 6H), 5.98 (d, *J* = 3.7 Hz, 1H), 5.66−5.58 (m, 2H), 5.41 (d, *J* = 3.5 Hz, 1H), 5.35 (d, *J* = 1.4 Hz, 1H), 4.88−4.75 (m, 1H), 4.53 (t, *J* = 5.0 Hz, 1H), 4.51−4.47 (m, 1H), 4.35−4.32 (m, 1H), 4.17−4.00 (m, 4H), 3.89 (t, *J* = 4.1 Hz, 1H), 3.84 (t, *J* = 9.0 Hz, 1H), 3.73−3.69 (m, 1H), 3.58−3.34 (m, 8H), 3.30−3.19 (m, 3H), 2.48−2.45 (m, 1H), 2.02 (q, *J* = 12.6 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) *δ* 169.2, 143.4, 140.7, 136.0, 133.2, 130.9, 129.7, 128.7, 126.7, 122.9, 122.7, 119.3, 111.2, 96.9, 96.4, 86.5, 81.7, 77.9, 76.5, 74.8, 73.9, 72.9, 72.1, 72.0, 69.4, 69.3, 69.2, 55.8, 55.0, 53.4, 52.9, 51.2, 50.2, 44.4, 43.8, 41.8, 41.6, 29.5; MS (ESI), *m/z* 941.4490 $(M + H)^+$ ($C_{42}H_{61}O_{13}N_{12}$ requires 941.4481).

*Neomycin-D_4 (**3i**).* General procedure **B** was applied to compound **2i** (58 mg, 0.038 mmol) leading to desired product **3i** as a white solid: yield 58.0 mg (95%); t_R 8.5 min (analytical HPLC method A); ^{1}H NMR (500 MHz, CD_3OD) *δ* 8.70 (br, 1H), 8.35 (s, 1H), 7.87 (s, 1H), 7.83 (s, 1H), 7.40−7.33 (m, 2H), 7.22 (d, *J* = 7.6 Hz, 1H), 5.90 (d, *J* = 3.6 Hz, 1H), 5.61−5.53 (m, 2H), 5.40 (d, *J* = 3.7 Hz, 1H), 5.35 (d, *J* = 1.4 Hz, 1H), 4.86−4.75 (m, 1H), 4.52 (t, *J* = 4.9 Hz, 1H), 4.51−4.47 (m, 1H), 4.34−4.32 (m, 1H), 4.16 (t, *J* = 3.0 Hz, 1H), 4.08−3.97 (m, 3H), 3.86−3.77 (m, 2H), 3.71−3.69 (m, 1H), 3.55 (dd, *J* = 10.2, 3.7 Hz, 1H), 3.49−3.34 (m, 6H), 3.29−3.16 (m, 5H), 2.47−2.38 (m, 1H), 1.98 (q, *J* = 12.6 Hz, 1H), 1.56−1.50 (m, 2H), 1.44−1.37 (m, 2H), 0.96 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD) *δ* 158.8, 143.8, 141.9, 130.8, 126.5, 120.6, 120.4, 117.1, 111.0, 96.9, 96.4, 86.6, 81.8, 77.8, 76.5, 74.9, 73.9, 72.9, 72.2, 71.9, 69.5, 69.3, 69.2, 55.9, 55.0, 53.3, 52.9, 51.2, 50.2, 44.2, 43.8, 41.8, 41.6, 40.6, 33.3, 29.6, 21.1, 14.2; MS (ESI), *m/z* 936.4916 $(M + H)^+$ ($C_{40}H_{66}O_{13}N_{13}$ requires 936.4898).

*Neomycin-BIG (**3j**).* General procedure **B** was applied to compound **2j** (34 mg, 0.022 mmol) leading to desired product **3j** as a white solid: yield 33 mg (92%); t_R 7.6 min (analytical HPLC method A); ^{1}H NMR (500 MHz, CD_3OD) *δ* 7.97 (s, 1H), 7.75−6.95 (m, 4H), 5.93 (s, 1H), 5.41 (s, 1H), 5.34 (s, 1H), 4.74−4.66 (m, 1H), 4.56−4.28 (m, 3H), 4.22−3.95 (m, 5H), 3.86−3.65 (m, 3H), 3.60−3.33 (m, 7H), 3.27−3.21 (m, 1H), 3.13−2.98 (m, 2H), 2.83−2.64 (m, 2H), 2.46−2.39 (m, 1H), 2.03 (q, *J* = 12.2 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) *δ* 175.7, 175.1, 148.5, 125.0, 124.8, 112.8, 111 0.7, 97.4, 97.1, 87.1, 82.1, 78.2, 77.1, 75.2, 74.5, 73.1, 72.6, 72.3, 69.8, 69.7, 69.6, 56.4, 53.3, 53.2, 51.6, 50.6, 42.0, 36.2, 30.0, 22.7; MS (ESI), *m/z* 910.4392 $(M + H)^+$ ($C_{37}H_{60}O_{14}N_{13}$ requires 910.4377).

Biology. *RNA and Biochemicals.* All buffers and solutions to be used in the fluorescence experiments were filtered through 0.22 *μ*m Millipore filters (GP ExpressPLUS membrane). The synthesis of the double labeled 67-mer pre-miR-372 and 69-mer pre-miR-373 sequences has been obtained following a previously reported procedure.[32]

Oligonucleotides were purchased from Eurogentec (Seraing, Belgium) as follows:

For pre-miR-372:

5′-FAM-GUGGGCCUCAAAUGUGGAGCACUAUUCUGAU-GU-3′ (ODN1)

5′-phosphate-CCAAGUGGAAAGUGCUGCGACAUUUGAG-CGUCAC-3′-DABCYL (ODN2)

5′-FAM-GUGGGCCUCAAAUGUGGAGCACUAUUCUGAUG-UCCAAGUGGAAAGUGCUGCGACAUUUGAGCGUCAC-3′ (ODN3)

For pre-miR-373:

5′-FAM-GGGAUACUCAAAAUGGGGGCGCUUUCCUUUUU-GU-3′ (ODN4)

5′-phosphate-CUGUACUGGGAAGUGCUUCGAUUUUGGG-GUGUCCC-3′-DABCYL (ODN5)

5′-FAM-GGGAUACUCAAAAUGGGGGCGCUUUCCUUUUU-GUCUGUACUGGGAAGUGCUUCGAUUUUGGGGUGUCCC-3′ ODN6

ODN1 and ODN2 or ODN3 and ODN4 (100 *μ*L, 10 nmol) were incubated in 50 mM Tris·HCl, pH 7.4, 10 mM MgCl2, 10 mM DTT, 1 mM ATP, 200 U RNasin, and 200 U T4 ligase (Applichem Lifescience) in a total volume of 239 *μ*L for 18h at 37 °C. The crude product was then purified by reverse phase HPLC using a gradient of CH_3CN in 0.1 M TEAA from 3 to 40% at a flow rate of 1 mL/min over 40 min (t_R = 27 min). Pure fractions were lyophilized. Prior to use in Dicer experiments, the beacons were taken up in 20 mM Tris·HCl buffer, pH 7.4, 2.5 mM $MgCl_2$, 12 mM NaCl, and 1 mM DTT, heated for 2 min at 90 °C, then placed for 10 min at 4 °C and finally allowed to return at RT.

FRET Dicer Assay. The Dicer assay was performed in 384-well plates (Greiner bio-one) in a final volume of 40 *μ*L using a 5070 EpMotion automated pipetting system (Eppendorf). Each experiment was performed in triplicate. Beacon at 50 nM concentration was used in each well, and reaction mixtures containing inhibitors were preincubated at RT for 30 min. Recombinant Dicer (0.25 U) was then added, and the fluorescence increase was measured every minute for 5 h. For IC_{50} experiments, each ligand was added in 12 dilutions (from 0.244 *μ*M to 500 *μ*M), and the fluorescence increase was measured after 4 h. The fluorescence was measured on a GeniosPro (Tecan) with an excitation filter of 485 ± 10 nm and an emission filter of 535 ± 15 nm. Each point was measured 10 times with a 500 *μ*s integration time and averaged. Inhibition data were analyzed using Graphpad Prism 5 software. Binding data (KD and FRET experiments) were analyzed using Prism 5 (GraphPad Software) by nonlinear regression following the equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/1 + 10^{[(\log IC_{50} - X)\cdot \text{Hills Slope}]}$.

Binding Experiments and K_d Determination. Binding experiments were performed in 384-well plates (Greiner bio-one) in a final volume of 60 *μ*L using a 5070 EpMotion automated pipetting system (Eppendorf). Each experiment was performed in duplicate. Ten nanomolar beacon (ODN3 and ODN6) was used in each well. Each ligand was added in 15 dilutions (from 0.030 nM to 0.5 *μ*M), and the fluorescence increase was measured after 4 h. The fluorescence was measured on a GeniosPro (Tecan) with an excitation filter of 485 ± 10 nm and an emission filter of 535 ± 15 nm. Each point was measured 10 times with a 500 *μ*s integration time and averaged. Binding data were analyzed using Graphpad Prism 5 software. Unless otherwise

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stated, binding profiles were well modeled using a simple model assuming the one to one stoichiometry.

Cell Culture and Treatment. All tissue culture reagents were from Invitrogen (France). AGS cells (ATCC CRL 1739) were routinely grown in Dulbecco's modified Eagle medium (DMEM)/F-12 (Ham's) medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine at 37 °C in a humidified 5% CO_2 atmosphere. For the experiments assessing the activity of the conjugates, the cells were plated into 24-well plates at a density of 5×10^4 cells/well. The compounds dissolved in sterile water were directly added in the growth medium at the indicated final concentrations.

Cell Viability. Cells were plated in 96-well plates at a density of 2×10^3 cells/well and treated with the compounds at the indicated concentrations. Cell viability was measured using the Cell Titer reagent (Promega) as indicated by the manufacturer.

Quantitative RT-PCR. Total RNA was extracted using Trizol reagent (Invitrogen), according to the manufacturer's protocol. RNA concentrations were determined by a NanoDrop spectrophotometer (NanoDrop Technologies, Inc.). The miScript Reverse Transcription and miScript SyBR Green PCR systems (Qiagen) were used to quantify the expression of mature miRNA on 2.5 ng of total RNA according to the manufacturer's instructions. MiRNAs levels were normalized to SNORD25 and RNU1A noncoding RNAs. Real time qPCR was performed on a RotorGene cycler (Qiagen). Relative expressions were calculated using the comparative Ct method.

Reporter Luciferase Assays. Mir-372-mediated inhibition of translation was assessed with the luciferase reporter plamsid pGL4-PM372, which contains the perfectly matched antisense sequence to miR-372 downstream to the firefly luciferase gene driven by the SV40 promoter in the pGL4 vector backbone (Promega, France). The pGL4-mm372, which contains a mismatched anti-miR-372 sequence, was used as negative control. All transfections were performed in 24-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were seeded into 24 well plates at a density of 5×10^4 cells/well and cotransfected with pGL4-PM372 or pGL4-mm372 plasmids at 100 ng/well in the presence of 3 ng/well pRL-SV40 vector to assess transfection efficiency (Promega). Firefly and Renilla luciferases were measured at 48 h post-transfection using the Dual Luciferase Assay (Promega). Firefly and Renilla luciferases were measured 48h post-transfection on cell lysates using the Dual Luciferase Assay (Promega). Firefly luciferase activities were normalized for transfection efficiency by *Renilla* luciferase.

Immunofluorescence. The cells were grown on glass coverslips, fixed with 3% paraformaldehyde in phosphate buffered saline (PBS) and permeabilized in Triton 0.2% in PBS. After blocking with 3% bovine serum albumine (BSA) in PBS, they were incubated overnight at 8 °C with anti-LATS2 antibody (1/200 dilution in PBS/BSA, Bethyl Laboratories, Texas, USA) and then, after 4 washes, with a secondary antirabbit IgG antibody coupled to Alexafluor488 (1/2,000 in PBS/BSA, Invitrogen, France) and Hoechst 33342 100 ng/mL, for 30 min at RT. The coverslips were mounted on glass slides using Slowfade Gold antifade reagent (Invitrogen) prior to imaging in epifluorescence on a Zeiss microscope.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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