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1 **Development of 2-deoxystreptamine-nucleobase conjugates for the inhibition of oncogenic**  
2 **miRNAs production**

3

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1 **Abstract**

2 The discovery of new original scaffolds for selective RNA targeting is one of the main challenges of  
3 current medicinal chemistry since therapeutically relevant RNAs represent potential targets for a number  
4 of pathologies. Recent efforts have been devoted to the search for RNA ligands targeting the biogenesis  
5 of oncogenic miRNAs whose overexpression has been directly linked to the development of various  
6 cancers. In this work, we developed a new series of RNA ligands for the targeting of oncogenic miRNAs  
7 biogenesis based on the 2-deoxystreptamine scaffold. This latter is part of the aminoglycoside neomycin  
8 and is known to play an essential role in the RNA interaction of this class of RNA binders. 2-  
9 deoxystreptamine was thus conjugated to natural and artificial nucleobases to obtain new binders of  
10 oncogenic miR-372 precursor (pre-miR-372). We identified some conjugates bearing a similar  
11 biological activity compared to previously synthesized neomycin analogs and studied their mode of  
12 binding with the target pre-miR-372.

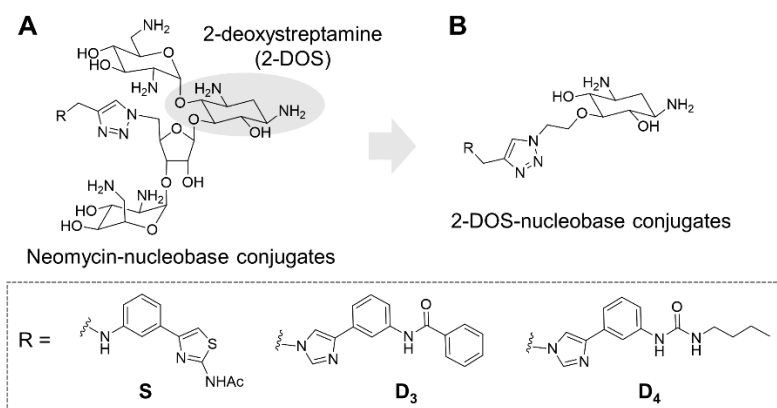
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## 1 **Introduction**

2 Targeting RNA using specific small molecules represents one of the great challenges of current  
3 medicinal chemistry and the first clinical successes of this paradigm are already on the market.<sup>1,2</sup> First  
4 of all, a number of antibiotics targeting prokaryotic ribosomal RNA have been employed in therapy for  
5 many decades.<sup>3</sup> Furthermore, a recent breakthrough has been made with the commercialization in 2020  
6 of Risdiplam as a modifier of RNA splicing in spinal muscular atrophy (SMA).<sup>4</sup> This compound acts  
7 upon binding to the SMN2 mRNA inducing the correction of splicing errors causing the deadly  
8 pathology and represents a major progress for treatment. Various therapeutically relevant RNAs are  
9 currently under study as innovative and original targets, such as viral RNAs and oncogenic RNAs.<sup>5</sup> In  
10 this last class, microRNAs (miRNAs or miRs) are of particular interest as major regulators of gene  
11 expression. MicroRNAs are short non-coding RNAs that recognize mRNAs and control protein  
12 expression upon inducing their degradation.<sup>6</sup> Each miRNA is responsible for the regulation of hundreds  
13 of proteins thus being part of a massive regulation process inside cells. Despite being essential, miRNAs  
14 can be deregulated (overexpressed or underexpressed) and variations in the expression of miRNAs have  
15 been directly correlated with various pathologies such as cancer.<sup>7</sup> For this reason, miRNAs have been  
16 studied as potential therapeutic targets.<sup>8</sup>

17 Various strategies have been developed in particular to tackle the overexpression of oncogenic miRNAs.  
18 Oligonucleotides can directly inhibit the function of oncogenic miRNAs upon complementary  
19 hybridization.<sup>9</sup> This specific approach is particularly successful, but still bears some limitations  
20 regarding its clinical application.<sup>10</sup> Small molecules were also developed to inhibit the biogenesis of  
21 oncogenic miRNAs upon binding to oncogenic miRNAs precursors such as primary miRNAs (pri-  
22 miRNAs) and pre-miRNAs.<sup>11</sup> This approach is based on the search for RNA binders selective for a  
23 particular secondary and/or tertiary structure present in the precursors of the targeted miRNA that are  
24 called pre-miRNAs and pri-miRNAs and that bear structures suitable for small-molecule binding.  
25 Among the most successful examples of this strategy, compounds from the group of Disney designed  
26 using the Inforna approach demonstrated to be very specific inhibitors of miRNAs biogenesis.<sup>12-15</sup>  
27 Screening of compound libraries containing a collection of RNA binders also gave interesting results.<sup>16-</sup>  
28 <sup>20</sup> Our group contributed to the field with the design of multimodal RNA ligands synthesized upon  
29 conjugation of various RNA binding domains bringing both affinity and selectivity for the target.<sup>21-24</sup>  
30 More specifically, we built conjugates, whose general structure is illustrated in Figure 1A, between  
31 aminoglycosides (such as neomycin) that are strong but non-selective RNA ligands with natural and  
32 artificial nucleobases that are heteroaromatic compounds able to selectively recognize RNA bases and  
33 RNA base pairs, respectively.<sup>25</sup>



**Figure 1.** General structure of previously synthesized neomycin-nucleobase conjugates (A) and of the newly designed 2-DOS-nucleobase conjugates (B). The detailed structure for nucleobases **S**, **D<sub>3</sub>** and **D<sub>4</sub>** is illustrated in the dashed square.

This led us to the identification of RNA ligands inducing the inhibition of the production of an oncogenic miRNA, miR-372, *in vitro* and in cancer cells overexpressing this miRNA. Some of the synthesized compounds were able to affect the biogenesis of a small set of miRNAs sharing common precursors or common binding sites with pre-miR-372 and, importantly, sharing the same protein targets. The main issue with these aminoglycoside conjugates is the size and the hydrophilicity of the obtained compounds. Both these features limit the perspectives for clinical applications because the physico-chemical properties are not drug-like and because they probably contribute to the lack of specificity. The analysis of aminoglycoside structures reveals that the vast majority contains the meso-1,3 diaminocyclitol 2-deoxystreptamine (2-DOS, Figure 1A), glycosylated at the 4-position, as well as the 5- (neomycin class) or 6- (kanamycin-gentamicin class) position with a variety of aminosaccharides.<sup>26</sup> In this work, we thus decided to replace the neomycin moiety with 2-deoxystreptamine, which is part of neomycin and is known to play an important role in the RNA binding ability of aminoglycosides. 2-DOS has been reported to bind weakly to two base units within a disrupted RNA helix. Solution studies carried out with 2-DOS have shown that it binds to 5'-3' two-base steps (including GU, UG, and GG), albeit with a very low affinity (>1 mM).<sup>27</sup> For this reason, various conjugates of 2-DOS have been previously reported as dimers for prokaryotic ribosomal RNA binding but also for the inhibition of miRNA maturation.<sup>28-30</sup> Here, we report the synthesis of a series of 2-DOS conjugates (Figure 1B) analogs of a previous neomycin-conjugated series in order to study their biological activities and structure-activity relationships in the context of the inhibition of miR-372 biogenesis. We found that some conjugates bear a very good affinity for pre-miR-372 target and that, although the affinity is lower than the one of their neomycin counterpart, the inhibition activity is maintained. After the evaluation of binding affinity, selectivity, inhibition activity and site of interaction, we identified one compound with very promising binding and inhibition properties compared to its neomycin counterpart and bearing much better physico-chemical properties for future development.

## 1 **Methods**

### 2 **Chemistry**

3 Reagents and solvents were purchased from Merck or Carlo Erba and used without further purification.  
4 All reactions that involved air- or moisture-sensitive reagents or intermediates were performed under  
5 argon atmosphere. Flash column chromatography was carried out on silica gel (Merck; SDS 60 Å, 40-  
6 63 µM, VWR). Analytical TLC was conducted on pre-coated silica gel plates (60F254; Merck) and  
7 compounds were visualized by irradiation ( $\lambda = 254$  nm) or by staining with ninhydrin stain.  $^1\text{H}$  and  $^{13}\text{C}$   
8 NMR spectra were recorded on Bruker AC 200, 400 and 500 MHz spectrometers. Chemical shifts are  
9 reported in parts per million (ppm,  $\delta$ ) referenced to the residual  $^1\text{H}$  resonance of the solvent ( $\text{CDCl}_3$   $\delta$   
10 7.26;  $\text{CD}_3\text{OD}$   $\delta$  3.31;  $\text{DMSO}-d_6$   $\delta$  2.50;  $\text{acetone}-d_6$   $\delta$  2.05 ppm). Splitting patterns are designed as  
11 follows: s (singlet), d (doublet), t (triplet), m (multiplet), and br (broad). Coupling constant ( $J$ ) are listed  
12 in hertz (Hz). High-resolution mass spectrometry (HRMS) was carried out on a LTQ Orbitrap hybrid  
13 mass spectrometer with an electrospray ionization probe (ThermoScientific, San Jose, CA) by direct  
14 infusion from a pump syringe, to confirm the correct molar mass and high purity of the compounds.  
15 HPLC analyses were performed using a Water ARC UHPLC pump coupled to a Water 2998 photodiode  
16 array detector and Waters Cortex® C18+ column (50 x 4.6 mm, 2.7 µm). Analysis were run out at room  
17 temperature by using a gradient of  $\text{CH}_3\text{CN}$  containing 0.1% TFA (eluent B) in water containing 0.1%  
18 TFA (eluent A) at a flow rate of 1.5 mL/min. The employed gradient was: 5 → 40% eluent B over 5  
19 min, and 40 → 100 % eluent B over 2 min.

### 20 **Synthetic procedures**

21 The preparation of Boc-protected 2-DOS derivatives **1**, **2**, **3** and **4a-h** as well as the synthesis of modified  
22 nucleobases containing the alkyne chain **10a-h** are described in the Supporting Information.

23 **General procedure for the 1,3-dipolar cycloaddition (General procedure A).** 1,3-Dipolar  
24 cycloaddition was performed starting from the azido derivative of 2-DOS **3** and appropriately alkyne-  
25 modified nucleobases in order to obtain desired 1,2,3-triazole derivative of 2-DOS. To a solution **3** or **7**  
26 (100 mg, 0.196 mmol) and alkynes **10a-h** (0.216 mmol, 1.1 eq.) in  $\text{CH}_3\text{CN}$  (11 mL), CuI (74.5 mg, 0.4  
27 mmol, 2eq.) and DIPEA (205 µL, 1.18 mmol) were added and the reaction mixture was stirred at room  
28 temperature or under reflux for the reported times. The solvent was then removed under reduced pressure  
29 and the crude residue was purified by flash chromatography on a silica gel column leading to the desired  
30 1,4-disubstituted 1,2,3-triazole as a white solid.

### 31 **General procedure for deprotection of Boc and acetal groups (General procedure B)**

32 To a solution of protected compounds **4a-h** or **8** in  $\text{CH}_2\text{Cl}_2$  and  $\text{H}_2\text{O}$  (3/1). TFA (50 eq.) was then added  
33 and the reaction mixture was stirred at room temperature overnight. The solvent and the residues of TFA  
34 were then removed under reduced pressure. Precipitation in ethyl ether led to pure compounds as white

1 solid (TFA salts). Final removal of copper was performed upon stirring 1h at r.t. in the presence of  
2 Chelex resin.

3 **2-deoxystreptamine-4-O-[adenyl-N-ethyl]triazol (5a)**. General procedure **B** was applied to solution  
4 of **4a** (100 mg, 0.146 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (3 mL) and water (1 mL) in the presence of TFA (557 μL, 7.32  
5 mmol). Pure compound **5a** was obtained after precipitation in CH<sub>2</sub>Cl<sub>2</sub> and Chelex resin treatment as a  
6 white solid in 60% yield (55.3 mg). Retention time 0.91 min; <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ ppm: 8.30-  
7 8.10 (m, 3H), 5.63 (br s, 2H), 4.65 (s, 2H), 4.45-4.50 (m, 1H), 4.15-4.10 (m, 1H), 3.39-3.20 (m, 4H),  
8 3.15-3.10 (m, 1H), 2.40-2.30 (m, 1H), 1.55-1.45 (m, 1H); <sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD) δ ppm: 163.1,  
9 162.8, 156.7, 143.1, 140.3, 129.9, 126.9, 82.5, 77.2, 74.5, 71.7, 55.8, 51.9, 51.4, 50.8, 29.8; MS (ESI)  
10 *m/z* 405.21063 [M+H]<sup>+</sup> (C<sub>16</sub>H<sub>25</sub>O<sub>3</sub>N<sub>10</sub> requires 405.21056).

11 **2-deoxystreptamine-4-O-[uracil-N-ethyl]triazol (5b)**. General procedure **B** was applied to a solution  
12 of **4b** (27 mg, 0.0408 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (0.6 mL) and water (0.2 mL) in the presence of TFA (149 μL,  
13 2.04 mmol). Pure compound **5b** was obtained after precipitation in CH<sub>2</sub>Cl<sub>2</sub> and Chelex resin treatment  
14 as a white solid in 98% yield (24.3 mg). Retention time 0.82 min; <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ ppm:  
15 8.06 (s, 1H), 7.72 (d, *J* = 3.2 Hz, 1H), 5.68 (d, *J* = 3.2 Hz, 1H), 5.02 (s, 2H), 4.64 (t, *J* = 2.2 Hz, 2H),  
16 4.50-4.45 (m, 1H), 4.15-4.10 (m, 1H), 3.40-3.35 (m, 4H), 3.20-3.10 (m, 1H), 2.40-2.30 (m, 1H), 1.80-  
17 1.70 (m, 1H); <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>OD) δ ppm: 166.9, 153.1, 147.4, 126.5, 103.1, 83.0, 77.7, 75.0,  
18 72.3, 52.2, 51.8, 51.2, 44.6, 30.3; MS (ESI) *m/z* 382.18375 [M+H]<sup>+</sup> (C<sub>15</sub>H<sub>24</sub>O<sub>5</sub>N<sub>7</sub> requires 382.18334).

19 **2-deoxystreptamine-4-O-[cytosyl-N-ethyl]triazol (5c)**. General procedure **B** was applied to a solution  
20 of **4c** (25.5 mg, 0.0385 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (0.6 mL) and water (0.3 mL) in the presence of TFA (149 μL,  
21 1.93 mmol). Pure compound **5c** was obtained after precipitation in CH<sub>2</sub>Cl<sub>2</sub> and Chelex resin treatment  
22 as a white solid in 70% yield (10.2 mg). Retention time 0.76 min; <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ ppm:  
23 8.17 (s, 1H), 8.04 (d, *J* = 3.1 Hz, 1H), 6.11 (br s, 1H), 5.13 (s, 2H), 4.67 (t, *J* = 2.2 Hz, 2H), 4.50-4.40  
24 (m, 1H), 4.20-4.10 (m, 1H), 3.50-3.35 (m, 4H), 3.20-3.10 (m, 1H), 2.45-2.40 (m, 1H), 1.90-1.80 (m,  
25 1H); <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>OD) δ ppm: 162.8, 150.9, 150.6, 126.5, 95.2, 82.4, 77.3, 74.5, 71.8, 51.8,  
26 51.5, 50.9, 45.4, 29.8; MS (ESI) *m/z* 381.19952 [M+H]<sup>+</sup> (C<sub>15</sub>H<sub>25</sub>O<sub>4</sub>N<sub>8</sub> requires 381.19933).

27 **2-deoxystreptamine-4-O-[4-((3-benzamidophenyl)-1H-imidazol-1-yl)ethyl]triazol (5d)**. General  
28 procedure **B** was applied to a solution of **4d** (60 mg, 0.0739 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (1.5 mL) and H<sub>2</sub>O (0.5  
29 mL) in the presence of TFA (854 μL, 3.69 mmol). Pure compound **5d** was obtained after precipitation  
30 in CH<sub>2</sub>Cl<sub>2</sub> and Chelex resin treatment as a white solid in 97% yield (55.0 mg). Retention time 2.78 min;  
31 <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ ppm: 8.28 (s, 1H), 8.00-7.95 (m, 3H), 7.70-7.50 (m, 8H), 5.60 (s, 2H),  
32 4.72 (t, *J* = 2.0 Hz, 2H), 4.50-4.45 (m, 1H), 4.20-4.15 (m, 1H), 3.45-3.35 (m, 4H), 3.15-3.10 (m, 1H),  
33 2.45-2.40 (m, 1H), 1.80-1.75 (m, 1H); <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>OD) δ ppm: 169.5, 141.4, 136.4, 133.6,  
34 131.4, 130.9, 130.1, 129.1, 123.5, 83.0, 77.7, 75.1, 73.5, 72.2, 49.9, 49.7, 49.6, 30.2; MS (ESI) *m/z*  
35 533.26215 (C<sub>27</sub>H<sub>33</sub>O<sub>4</sub>N<sub>8</sub> 533.26193).

36 **2-deoxystreptamine-4-O-[4-(3-aminophenyl)-1H-imidazol-1-yl)ethyl]triazol (5e)**. General  
37 procedure **B** was applied to a solution of **4e** (49 mg, 0.0692 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (0.5 mL) and H<sub>2</sub>O (0.2

1 mL) in the presence of TFA (265  $\mu$ L, 3.46 mmol). Pure compound **5e** was obtained after precipitation  
2 in  $\text{CH}_2\text{Cl}_2$  and Chelex resin treatment as a white solid in 97% yield (49.7 mg). Retention time 2.77 min;  
3  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  ppm: 9.08 (s, 1H), 8.27 (s, 1H), 7.90-7.80 (m, 2H), 7.39 (t,  $J = 5.0$  Hz,  
4 1H), 7.60-7.45 (m, 2H), 5.61 (s, 2H), 4.75-4.70 (m, 2H), 4.50-4.40 (m, 1H), 4.30-4.20 (m, 1H), 3.40-  
5 3.30 (m, 3H), 3.22 (t,  $J = 9.8$  Hz, 2H), 3.40-3.30 (m, 1H), 2.40-2.30 (m, 1H), 1.80-1.70 (m, 1H), 1.60-  
6 1.50 (m, 2H), 1.40-1.30 (m, 2H), 0.97 (t,  $J = 5.0$  Hz, 3H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  ppm: 156.8,  
7 143.1, 141.7, 140.1, 137.4, 134.3, 128.6, 118.7, 117.5, 115.6, 109.0, 98.4, 82.6, 77.2, 74.5, 71.8, 67.0,  
8 51.4, 50.8, 49.6, 40.5, 33.3, 21.0, 14.1; MS (ESI)  $m/z$  528.30426 ( $\text{C}_{25}\text{H}_{38}\text{O}_4\text{N}_9$  528.30413).

9 **2-deoxystreptamine-4-O-[4-(3-(2-naphthamido)phenyl)-1H-imidazol-1-yl]ethyl]triazol (5f).**  
10 General procedure **B** was applied to a solution of **4f** (29 mg, 0.0336 mmol) in  $\text{CH}_2\text{Cl}_2$  (0.5 mL) and  $\text{H}_2\text{O}$   
11 (0.2 mL) in the presence of TFA (129  $\mu$ L, 1.68 mmol). Pure compound **5f** was obtained after  
12 precipitation in  $\text{CH}_2\text{Cl}_2$  and Chelex resin treatment as a white solid in 87% yield (23.0 mg). Retention  
13 time 2.45 min;  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  ppm: 8.54 (s, 1H), 8.29 (s, 1H), 8.20-7.90 (m, 5H), 7.75-  
14 7.50 (m, 5H), 5.61 (s, 2H), 4.75-4.70 (m, 2H), 4.50-4.40 (m, 1H), 4.25-4.15 (m, 1H), 3.40-3.30 (m, 4H),  
15 3.20-3.10 (m, 1H), 2.45-2.35 (m, 1H), 1.80-1.70 (m, 1H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  ppm: 141.0,  
16 136.6, 133.2, 134.1, 133.2, 131.0, 130.1, 129.5, 129.3, 128.9, 128.0, 125.1, 122.8, 82.7, 77.3, 74.6, 71.8,  
17 54.8, 51.8, 49.6, 31.3, 17.2.

18 **2-deoxystreptamine-4-O-[4-(3-pentanamidophenyl)-1H-imidazol-1-yl]ethyl]triazol (5g).** General  
19 procedure **B** was applied to a solution of **4g** (30 mg, 0.0372 mmol) in  $\text{CH}_2\text{Cl}_2$  (0.5 mL) and  $\text{H}_2\text{O}$  (0.2  
20 mL) in the presence of TFA (143  $\mu$ L, 1.86 mmol). Pure compound **5g** was obtained after precipitation  
21 in  $\text{CH}_2\text{Cl}_2$  and Chelex resin treatment as a yellow solid in 87% yield (28.0 mg). Retention time 1.10  
22 min;  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  ppm: 8.93 (s, 1H), 8.26 (s, 1H), 7.90 (s, 1H), 7.40-7.30 (m, 1H),  
23 7.25-7.15 (m, 2H), 7.00-6.90 (m, 1H), 5.58 (s, 2H), 4.75-4.65 (m, 2H), 4.50-4.40 (m, 1H), 4.25-4.15 (m,  
24 1H), 3.50-3.35 (m, 4H), 3.25-3.15 (m, 1H), 2.50-2.40 (m, 1H), 1.80-1.70 (m, 1H);  $^{13}\text{C}$  NMR (125 MHz,  
25  $\text{CD}_3\text{OD}$ )  $\delta$  ppm: 135.9, 130.1, 125.1, 118.5, 118.1, 117.4, 114.2, 81.2, 75.8, 73.1, 70.4, 50.4, 50.0, 49.4,  
26 43.4, 28.4; MS (ESI)  $m/z$  429.23608 ( $\text{C}_{20}\text{H}_{29}\text{O}_3\text{N}_8$  429.23571).

27 **1,3-Bis-*N*-(*tert*-butyloxycarbonyl)-5,6-*O*-cyclohexylidene-2-deoxystreptamine-4-*O*-[4-**  
28 **phenylthiazol-2-yl]amino)butyl)-1H-imidazol-1-yl]ethyl]triazol (5h).** General procedure **B** was  
29 applied to a solution of **4h** (16 mg, 0.033 mmol) in  $\text{CH}_2\text{Cl}_2$  (0.5 mL) and water (0.2 mL) in the presence  
30 of TFA (129  $\mu$ L, 1.65 mmol). Final compound **5h** was obtained as a white solid in 98% yield (12 mg).  
31 Retention time min 10.5 min (Phenomenex Synergi 4  $\mu\text{m}$  Fusion-RP 80 $\text{\AA}$  column (250 x 4.6 mm); flow  
32 rate of 1 mL/min, gradient 5  $\rightarrow$  40% eluent B over 20 min);  $^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  (ppm): 8.00-  
33 7.85 (m, 3H), 7.40-7.30 (m, 3H), 7.30-7.20 (m, 1H), 4.60-4.50 (m, 2H), 4.50-4.40 (m, 1H), 4.20-4.10  
34 (m, 3H), 3.50-3.35 (m, 4H), 3.20-3.00 (m, 3H), 2.70-2.60 (m, 2H), 2.40-2.30 (m, 1H), 1.80-1.70 (m,  
35 1H);  $^{13}\text{C}$  NMR (100MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  (ppm): 169.7, 159.1, 151.3, 135.8, 130.1, 129.5, 127.1, 108.8, 82.6,  
36 77.3, 74.5, 72.1, 51.8, 51.4, 50.9, 43.4, 35.9, 30.0; MS (ESI)  $m/z$  545.22974 ( $\text{C}_{24}\text{H}_{33}\text{O}_5\text{N}_8\text{S}$  545.22891).

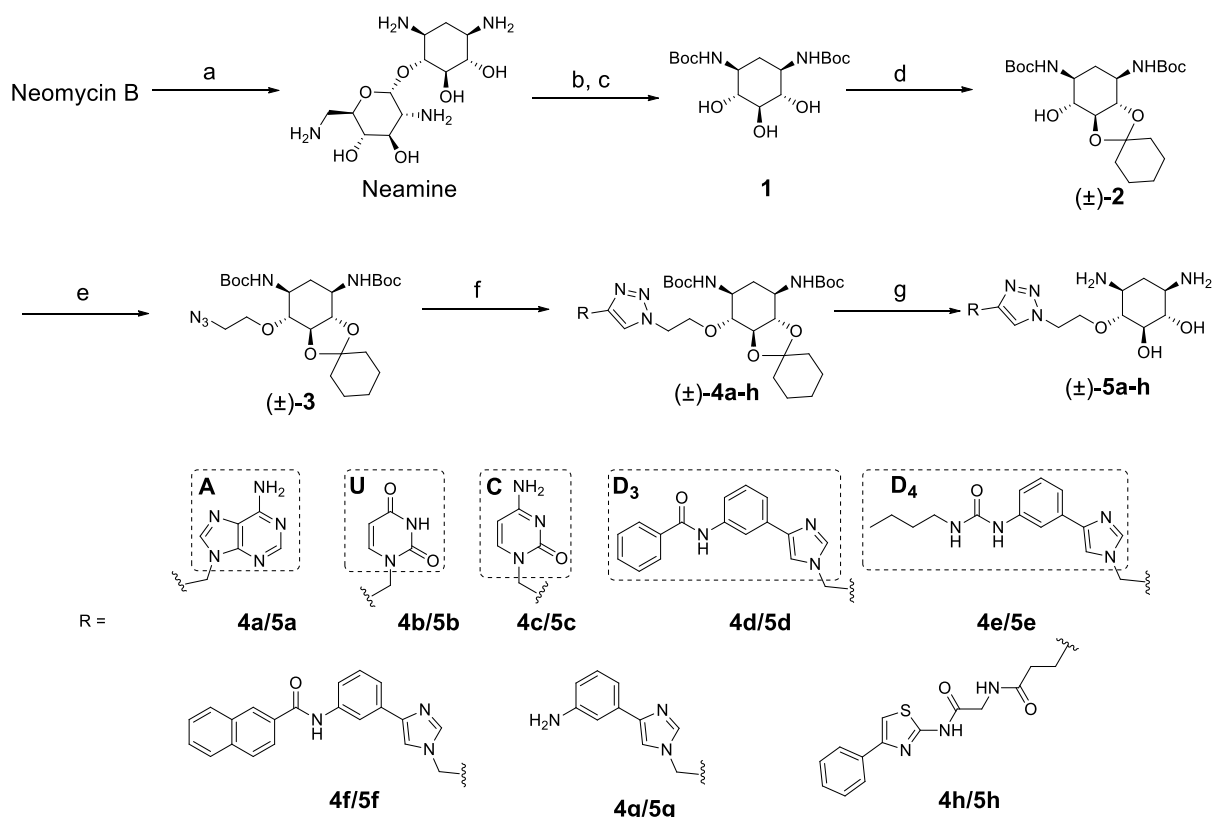


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## Results and Discussion

### Design of 2-DOS conjugates

The disubstituted 2-DOS-containing aminoglycosides represent a vast majority of this class of antibiotics and are strong RNA binders. The aim of this work is to assess if 2-DOS moiety could replace neomycin in our previously synthesized pre-miR-372 ligands.<sup>23</sup> These latter contained neomycin conjugated with natural and artificial nucleobases and some of them were efficient and selective inhibitors of oncogenic miR-372 maturation *in vitro* and in cells.<sup>23 25</sup> Neomycin is known to bind to unpaired RNA bases at sites where the double helix is distorted by the presence of bulges or loops. Natural nucleobases should interact with unpaired bases *via* the formation of Watson-Crick-Franklin H-bonds and artificial nucleobases are meant to interact with paired bases *via* Hoogsteen H-bonds thus forming triplets.<sup>25</sup> So, the conjugation of neomycin to natural nucleobases would direct the compounds on single-stranded regions of bulges and loops, and the conjugation of neomycin to artificial nucleobase conjugates should direct the ligands close to stem-bulge and stem-loop junctions. In our previous studies, we observed that artificial nucleobase conjugates were more efficient and selective binders of pre-miR-372 than natural bases conjugates and some of them were able to inhibit its processing by Dicer enzyme *in vitro*.<sup>23</sup> A specific inhibition of proliferation of adenocarcinoma cancer cells (AGS) overexpressing miR-372 was also observed and this activity was linked to the inhibition of Dicer processing for miR-372. The study of the site of interaction of the best binders and inhibitors showed that they indeed bound at stem-bulge junctions and close to the site of cleavage of Dicer enzyme.<sup>21, 24</sup> Even with these promising results in hand, as well as with a complete structure-activity characterization of this series of conjugates,<sup>21, 22, 24</sup> the size of the obtained compounds as well as their high hydrophilicity represent relevant drawbacks for the physico-chemical properties of this kind of conjugates. Furthermore, neomycin is known to be a toxic drug when administered and to lack specificity when used against a particular RNA. We thus decided to prepare new analogs of these conjugates by replacing the neomycin moiety by its substructure 2-DOS and to study the activity of these compounds against miR-372 maturation that we took as a model for comparison with our previously reported neomycin conjugates. 2-DOS represents a key structural feature of most aminoglycosides and occupies a central position in their structure as well as an essential role for their interaction with prokaryotic ribosomal RNA. We thus wondered if the small 2-DOS moiety could be sufficient for binding yet leading to more favorable physico-chemical properties in the final conjugates. The most active compound in the neomycin series was the one containing nucleobase **S**.<sup>23</sup> However, the 2-DOS analog of this conjugate was previously reported and did not show activity for miR-372 maturation inhibition.<sup>24</sup> As illustrated in Scheme 1, we thus chose to conjugate 2-DOS with natural nucleobases adenine (A), cytosine (C) and uracil (U) that were not studied previously.



1  
 2 **Scheme 1.** Synthesis of the new series of 2-DOS conjugates **5a-h**. Reagents: a) HCl, reflux, overnight;  
 3 b) 48% HBr aq., reflux, 2 days; c) Boc<sub>2</sub>O, NaOH, dioxane/H<sub>2</sub>O, overnight; d) 1,1-  
 4 dimethoxycyclohexane, pTsOH, DMF, 50°C, 55 mbar, 1h; e) 2-Azidoethyl-p-toluensulfonate, NaH,  
 5 THF, 30°C, 3h; f) **10a-h**, CuI, DIPEA, CH<sub>3</sub>CN, from r. t. to 50°C, from 1h to overnight; g) TFA, CH<sub>2</sub>Cl<sub>2</sub>,  
 6 H<sub>2</sub>O, r. t., overnight.

7  
 8 We also chose 4-(3-benzamidophenyl)imidazole (nucleobase **D<sub>3</sub>**) that can form two hydrogen bonds  
 9 with T-A and C-G pairs and 4-(3-butylamidophenyl)imidazole (nucleobase **D<sub>4</sub>**) that was found to  
 10 recognize G-C base pairs with 3 hydrogen bonds (Figure S1 in Supporting Information) used for the  
 11 previously prepared neomycin analogs. Furthermore, we decided to study two analogs of **D<sub>3</sub>** and **D<sub>4</sub>**. To  
 12 this aim, we used the unsubstituted 3-aminophenylimidazole and the 4-(3-  
 13 naphthylamidophenyl)imidazole in order to assess the influence of the substitution on the  
 14 phenylimidazole moiety on biological activity. A new analog of the S-conjugate was also synthesized  
 15 using a phenylthiazole moiety for comparison.<sup>24</sup> For the synthesis of these conjugates, we employed 1,3-  
 16 dipolar cycloaddition leading to the formation of a triazole linker suited for direct comparison with the  
 17 previously reported compounds but also to take advantage of a straightforward synthesis of these new  
 18 analogs (Figure 1B).

19

20 **Synthesis of 2-DOS conjugates**

1 2-DOS is a meso compound that contains 5 contiguous stereogenic centers that pose a challenge for the  
2 preparation of enantiomeric pure conjugates. To date, the most practical method to obtain 2-  
3 deoxystreptomycin is by a straightforward acidic degradation of neomycin.<sup>26</sup> A more complex synthetic  
4 route was previously reported for the preparation of enantiomerically pure compounds.<sup>31</sup> In this work,  
5 we first decided to employ the fastest synthetic pathway leading to desired conjugates as racemic  
6 mixtures.

7 As illustrated in Scheme 1, 2-DOS moiety was prepared starting from commercially available neomycin  
8 upon acidic hydrolysis in the presence of a concentrated solution of HCl. This led in quantitative yields  
9 to neamine that was submitted to a further acidic treatment of aqueous HBr. The protection of the amino  
10 groups with Boc protecting group was then performed on the crude product in the presence of tert-  
11 butoxycarbonyl anhydride and NaOH in a mixture H<sub>2</sub>O/dioxane 1:1 leading to compound **1** in 50% yield  
12 over two steps. Next step was the protection of one vicinal diol in the presence of 1,1-  
13 dimethoxycyclohexane and pTsOH in DMF that led to racemic compound ( $\pm$ )-**2** in 98% yield. Following  
14 substitution of the remaining hydroxyl group with 2-azidoethyltosilate led to compound ( $\pm$ )-**3** ready for  
15 1,3 dipolar cycloaddition in 33% yield. This latter was performed using appropriately modified  
16 heteroaromatic compounds **10a-h** (Figure S2 in Supporting Information) containing the alkyne essential  
17 for the coupling reaction. The cycloaddition reaction was performed in CH<sub>3</sub>CN in the presence of CuI  
18 and DIPEA and led to desired compounds ( $\pm$ )-**4a-h** in 46 to 87% yields. Final removal of the acetal and  
19 Boc protecting groups in CH<sub>2</sub>Cl<sub>2</sub> in the presence of H<sub>2</sub>O and TFA led to desired compounds ( $\pm$ )-**5a-h** in  
20 60 to 97% yields as TFA salts. Complete removal of copper traces possibly remaining in the final  
21 product was performed upon treatment with Chelex resin. All synthesized compounds were also fully  
22 characterized by NMR, HRMS and HPLC for purity and these data are shown in the Supporting  
23 Information. The purity of the final products was included between 95% and 99%.

24

### 25 **Evaluation of the affinity, selectivity and inhibition activity**

26 Once the new series of 2-DOS conjugates **5a-h** prepared, we evaluated these compounds for their ability  
27 to bind to pre-miR-372 and inhibit its processing by Dicer enzyme *in vitro* and compared the results to  
28 the corresponding neomycin analogs when available. First of all, we studied the dissociation constants  
29 ( $K_D$ ) of all compounds for pre-miR-372 using a fluorescence-based assay where the targeted RNA is  
30 labeled with a fluorophore (FAM) at the 5'-end. Binding of an efficient ligand induces a change in the  
31 fluorophore environment that quenches fluorescence and the variation of the fluorescence signal in  
32 function of the concentration of ligand allows for the measurement of  $K_D$ .<sup>32</sup> As illustrated in Table 1, all  
33 newly synthesized compounds **5a-h** are good pre-miR-372 ligands with  $K_D$  in the low  $\mu$ M range (from  
34 1.62 to 17.1  $\mu$ M) except for **5c** that bears a higher  $K_D$  of 101  $\mu$ M. It should be noted that 2-DOS and  
35 nucleobases alone have no affinity for the target. A closer look at the obtained  $K_D$  suggests that **D<sub>3</sub>** and  
36 **D<sub>4</sub>** analogs (**5d** and **5e**, respectively) are the strongest binders closely followed by the unsubstituted  
37 phenylimidazole analog **5g**. Introduction of a naphthalene substituent as in **5f** and of the phenylthiazole

1 substituent as in **5h** induces a slight loss of affinity while the natural nucleobases conjugates **5a-c** are  
 2 the weakest binders.

3

4 **Table 1.** Dissociation constants values in  $\mu\text{M}$  for synthesized compounds **5a-h** toward pre-miR-372  
 5 alone ( $K_D$ ) and in the presence of tRNA (100 eq.,  $K_D'$ ) or DNA (100 eq.  $K_D''$ ).

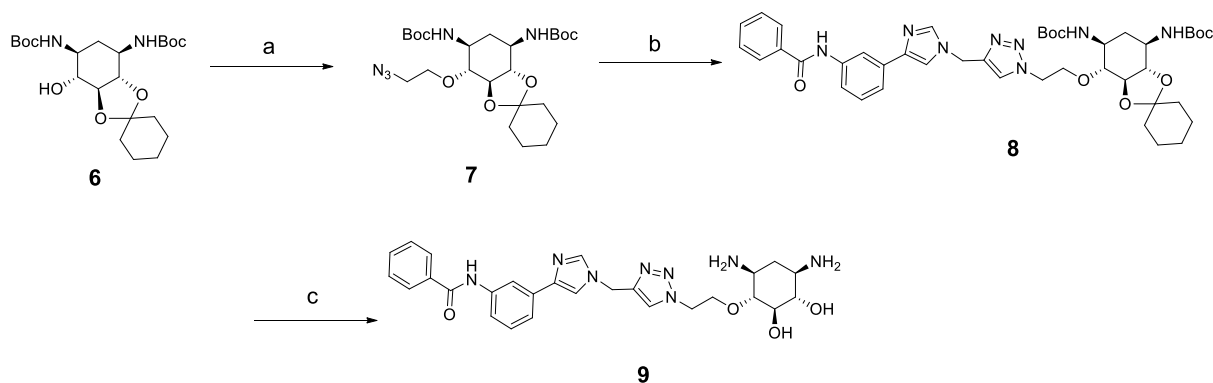
ID	$K_D$	$K_D'$	$K_D'/K_D$	$K_D''$	$K_D''/K_D$	$IC_{50}$	$IC_{50}'$
<b>5a</b>	$11.1 \pm 2.3$	> 1 mM	-	47.1	4.2	no inhibition	no inhibition
<b>5b</b>	$17.1 \pm 0.1$	> 1 mM	-	> 1 mM	-	no inhibition	no inhibition
<b>5c</b>	$101 \pm 18$	> 1 mM	-	> 1 mM	-	no inhibition	no inhibition
<b>5d</b>	$2.52 \pm 0.58$	$2.64 \pm 0.52$	1.0	$2.96 \pm 0.85$	1.2	$15.9 \pm 1.8$	$29.5 \pm 9.1$
<b>5e</b>	$1.62 \pm 0.49$	$3.65 \pm 0.85$	2.2	$2.11 \pm 0.81$	1.3	$72.4 \pm 1.0$	no inhibition
<b>5f</b>	$6.78 \pm 0.46$	$8.51 \pm 1.3$	1.3	$9.02 \pm 1.2$	1.3	> 100 $\mu\text{M}$	no inhibition
<b>5g</b>	$3.53 \pm 0.89$	$3.92 \pm 0.52$	1.1	$4.27 \pm 0.35$	1.2	$81.1 \pm 2.3$	> 500 $\mu\text{M}$
<b>5h</b>	$5.13 \pm 0.41$	$8.56 \pm 2.0$	1.7	$11.0 \pm 0.90$	2.1	$74.9 \pm 12$	no inhibition
<b>Neo-D<sub>3</sub></b>	$0.0189 \pm 0.0017$	$0.0208 \pm 0.0019$	1.1	$0.0244 \pm 0.0018$	1.3	$8.30 \pm 1.1$	20.4
<b>Neo-D<sub>4</sub></b>	$0.0461 \pm 0.0038$	$0.0164 \pm 0.0012$	1.1	$0.0222 \pm 0.0024$	1.5	$25.6 \pm 1.1$	
<b>2-DOS</b>	> 1 mM	-	-	-	-	no inhibition	no inhibition

6

7 Selectivity is of course another important parameter to evaluate. To this aim, we chose to measure  $K_D$   
 8 in the presence of two different competitors, tRNA and DNA, that are particularly abundant in cells. In  
 9 both cases, 100 equivalents of competitor were added to the mixture during  $K_D$  measurement. An  
 10 increase in  $K_D$  (ratio  $K_D'/K_D$  for tRNA or  $K_D''/K_D$  for DNA higher than one) suggests a lack of selectivity  
 11 since the compound can bind to other nucleic acid structures. The obtained results are illustrated in Table  
 12 1 and suggest that natural nucleobase conjugates **5a-c** were not selective in the presence of tRNA and  
 13 DNA except for **5a** that maintained some affinity in the presence of DNA ( $K_D''/K_D = 4.2$ ). Compounds  
 14 **5d-5h** maintained their affinity and seemed thus to be selective for a pre-miRNA structure such as pre-  
 15 miR-372, compared to other nucleic acid structures.

16 With these experiments we assessed that all compounds were promising binders and that compounds  
 17 **5d-5h** were also selective for pre-miRNAs. We thus decided to verify if the compounds were also able  
 18 to inhibit Dicer processing of pre-miR-372 by measuring the  $IC_{50}$  using a previously published cell-free  
 19 assay where the pre-miR-372 was double labeled with a fluorophore (FAM) and a quencher (DAB) at  
 20 the 5'- and at the 3'-end, respectively.<sup>23</sup> When Dicer cleaves the pre-miRNA appropriately the  
 21 fluorescence signal increases, while when Dicer cleavage is inhibited no fluorescence is observed. As  
 22 shown by  $IC_{50}$  values reported in Table 1, compounds **5a-5c** were not able to induce inhibition and  
 23 compounds **5e-5h** had a weak inhibition activity starting at 72.4  $\mu\text{M}$  for **5e** to more than 100  $\mu\text{M}$  for **5f**.  
 24 Noteworthy, **5d** was the most active compound with an  $IC_{50}$  of 15.9  $\mu\text{M}$ . This value is in the same range  
 25 of the one of compound **Neo-D<sub>3</sub>** while this latter bears a much higher affinity for the target. This could

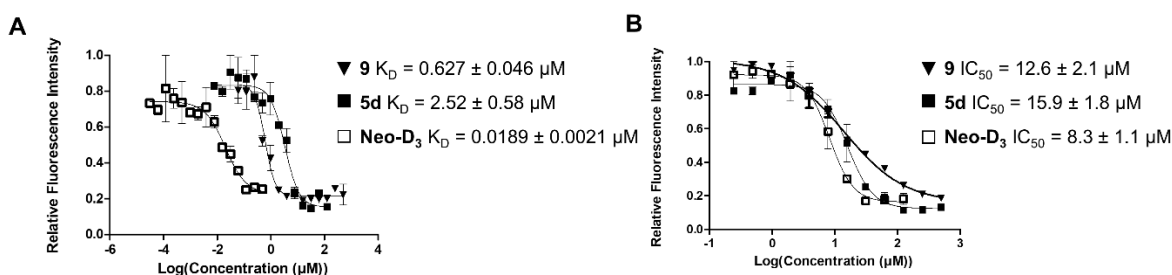
1 be explained by a difference in the binding site that could be more favorable for inhibition in the case  
 2 of compound **5d** than of compound **Neo-D<sub>3</sub>** despite a loss in binding affinity.  
 3 Selectivity remains a major concern when designing new RNA ligands. We thus evaluated IC<sub>50</sub>s in the  
 4 presence of HEK293 cells lysates instead of using the recombinant enzyme. These conditions mimic the  
 5 intracellular complexity and give hints about the potential selectivity in cells. With these experiments  
 6 we demonstrated that all compounds lost their ability to inhibit miR-372 maturation except for  
 7 compound **5d** whose IC<sub>50</sub>' is 29.5 μM instead of 15.9 μM in the presence of pure enzyme.  
 8 Despite the binding selectivity for all compounds against tRNA and DNA as well as the inhibition  
 9 selectivity in the case of compound **5d**, one could wonder what would be the affinity and the inhibition  
 10 activity of these compounds against other pre-miRNAs. We thus measured K<sub>D</sub> and IC<sub>50</sub> of the newly  
 11 synthesized compounds for three supplementary pre-miRNAs: pre-miR-21, pre-miR-18a and pre-miR-  
 12 148a. These three miRNAs are oncogenic and overexpressed in many types of cancers.<sup>33</sup> Although their  
 13 sequences and structures differ from those of pre-miR-372, the general shape of pre-miRs remains  
 14 similar, i.e. hairpin stem-loops with bulges. The obtained results (Table S1 in the Supporting  
 15 Information) show that compounds **5a-c**, the weakest binders of pre-miR-372, remain weak binders or  
 16 loose affinity for the other miRNAs. Furthermore, **5a-c** did not show any ability to inhibit Dicer  
 17 cleavage. Compounds **5d-h** instead maintain similar affinity for all pre-miRNAs, but show major  
 18 differences in inhibition activity. Indeed, compound **5d** inhibits weakly pre-miR-21 maturation and is  
 19 not active on pre-miR-18. Inhibition activity is maintained for pre-miR-148a but is lower than the one  
 20 for pre-miR-372. The weak inhibitors of pre-miR-372 maturation **5e-h** remain weak for the other tested  
 21 miRNAs except for **5e** that inhibits preferentially pre-miR-18 cleavage (IC<sub>50</sub> 20.3 μM) and pre-miR-  
 22 148a (IC<sub>50</sub> 18.2 μM). Compound **5d** thus seems to be the most promising of the series for the inhibition  
 23 of pre-miR-372 processing with IC<sub>50</sub> in the low μM range similar to one of its neomycin counterpart  
 24 and yet having a much higher K<sub>D</sub> value.  
 25 As mentioned above, compounds **5a-h** are racemic mixture. We thus wanted to verify if the enantiopure  
 26 analog of the most promising compound of the series **5d** showed any difference in activity, affinity and  
 27 selectivity. The synthesis of this compound is similar to the one of **5d** but starts from the enantiopure  
 28 compound **6** prepared following a previously reported procedure (Scheme 2).<sup>31</sup>



1 **Scheme 2.** Synthesis of enantiopure compound **9**. Reagents: a) 2-Azidoethyl-p-toluensulfonate, NaH,  
2 THF, 30°C, 3h; b) **10h**, CuI, DIPEA, CH<sub>3</sub>CN, r. t., 5h; c) TFA, CH<sub>2</sub>Cl<sub>2</sub>, H<sub>2</sub>O, r. t., overnight.

3  
4 The introduction of the 2-azidoethyl linker led to compound **7** in 20% yield and the 1,3-dipolar  
5 cycloaddition in the presence of 4-(3-benzamidophenyl)imidazole led to compound **8** in 56% yield. Final  
6 deprotection in acidic conditions allowed desired derivative **9** in 98% yield. The comparison of the  
7 affinity for pre-miR-372 between **9** and **5d** revealed that compound **9** was a stronger binder with a K<sub>D</sub>  
8 of 0.627 μM. This improved affinity was then reflected in a better inhibition activity since **9** showed a  
9 IC<sub>50</sub> of 12.6 μM. This enantiomer thus seems more favorable for binding and inhibition.

10 Altogether these results show that there is a good correlation between K<sub>D</sub> and IC<sub>50</sub> suggesting that RNA  
11 binding is the underlying mechanism of inhibition. Furthermore, the result obtained with compound **9**  
12 is particularly interesting since comparison of IC<sub>50</sub> between **9** and compound **Neo-D<sub>3</sub>** shows that these  
13 are within the same range (8.3 μM vs 12.6 μM, Figure 2). Neomycin could thus be replaced by 2-DOS  
14 without losing activity.



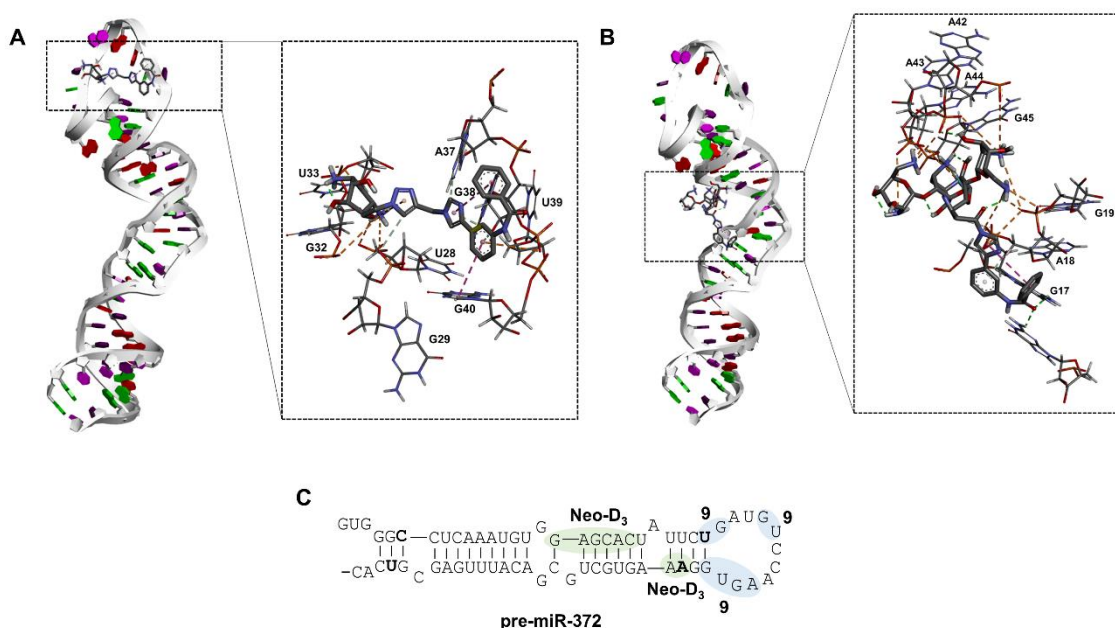
15  
16 **Figure 2.** Comparison of the dissociation constants (K<sub>D</sub>, A) and of inhibition activities, (IC<sub>50</sub>, B) for  
17 compounds **9**, **5d** and **Neo-D<sub>3</sub>** against pre-miR-372.

18  
19 The explanation for the good inhibition activity of compound **9** despite a higher K<sub>D</sub> value could come  
20 from a more favorable binding site for pre-miR-372 compared to the one of **Neo-D<sub>3</sub>**. This parameter  
21 could also explain the differences observed in the inhibition activities of other pre-miRNAs while  
22 keeping the affinity for them. Indeed, binding to a particular RNA is not sufficient to induce an inhibition  
23 activity since it is essential to bind to a functional site that in this case would be preferentially the  
24 cleavage site of Dicer enzyme.

### 25 26 **Molecular docking of compound **9** on pre-miR-372**

27 To gain a better insight into the mode of binding of **9** and suggest an explanation for the good inhibition  
28 activity of this compound similar to the one of the **Neo-D<sub>3</sub>** analog despite a much higher K<sub>D</sub>, we  
29 performed a molecular docking study of both compounds on the pre-miR-372 sequence. The sequence  
30 of the pre-miR-372 hairpin loop was generated from the miRBase database and then input to the MC-  
31 Fold/MC-Sym pipeline to construct a 3D model. For this model, we analyzed energy optimization using

1 TINKER. Next, we conducted molecular docking for pre-miR-372 in the presence of compound **9** using  
 2 the AutoDock program without defining any preferred binding site but performing the docking study on  
 3 the entire sequence.<sup>34</sup> As shown in Figure 3, docking suggested that both compounds **9** and **Neo-D<sub>3</sub>** bind  
 4 to the cleavage site of Dicer even if differently. Compound **Neo-D<sub>3</sub>** seems to interact with residues A45  
 5 and A46 corresponding to the cleavage site on one strand of pre-miR-372 stem, while **9** with U31 and  
 6 G32 corresponding to the cleavage site on the opposite strand. Regarding the other interactions, **Neo-D<sub>3</sub>**  
 7 interacts with other residues of the stem region (G20 to C25) while **9** with residues of the loop region  
 8 (G35-U36 and A40-G43). Both these binding sites involving the cleavage site of the enzyme, the  
 9 compounds behave similarly in terms of inhibition activity.



10  
 11 **Figure 3.** Molecular docking of compound DOS-D<sub>3</sub> **9** (A) and **Neo-D<sub>3</sub>** (B) performed on pre-miR-372  
 12 structure using Autodock 4 software. Primary and secondary structure of pre-miR-372 (C) and binding  
 13 sites of compound **9** (light blue) and **Neo-D<sub>3</sub>** (light green).

14  
 15 In conclusion, we were able to synthesize a new series of 2-desoxystreptamine analogs conjugated to  
 16 various heteroaromatic compounds such as natural and artificial nucleobases as new RNA binders. We  
 17 performed a comparison of affinity, selectivity, inhibition activity and site of interaction for oncogenic  
 18 pre-miR-372 with a previously synthesized series of ligands containing the neomycin aminoglycoside  
 19 instead of 2-DOS. The aim of this work was to reduce the overall size and hydrophilicity of the RNA  
 20 ligands and to verify if the biological properties could be maintained. We observed that all synthesized  
 21 compounds are good RNA binders with selectivity when tested in competition with other nucleic acid  
 22 structures. Some compounds were able to inhibit miR-372 maturation upon inhibiting Dicer cleavage of  
 23 pre-miR-372 but only compound **5d** showed an IC<sub>50</sub> in the low micromolar range. Some selectivity was  
 24 also observed once the compound tested against other pre-miRNAs. However, we already observed that

1 compounds that are selective for a small set of pre-miRNAs can have a very specific biological effect  
2 and this could be an advantage for a comprehensive biological activity. The same evaluations performed  
3 on the enantiopure analog of **5d**, compound **9**, showed that this latter was an even better ligand and  
4 inhibitor concerning pre-miR-372. Finally, it was observed that both **5d** and **9** are weaker binders of pre-  
5 miR-372 compared to the neomycin counterpart **Neo-D<sub>3</sub>**, but that they maintained a similar inhibition  
6 activity. Molecular docking suggested that this is due to the particular binding site that is located on the  
7 nucleotides that are cleaved by Dicer enzyme thus being functional for efficient inhibition. Further  
8 chemical optimization of these 2-DOS conjugates could thus lead to even better inhibition activity and  
9 to study their intracellular activity.

10

### 11 **Acknowledgments**

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16 fellowship to A.R. was supported by Université Côte d'Azur.

17



1 **Figures and Schemes**

2 **Figure 1.** General structure of previously synthesized neomycin-nucleobase conjugates (A) and of the  
3 newly designed 2-DOS-nucleobase conjugates (B). The detailed structure for nucleobases **S**, **D<sub>3</sub>** and **D<sub>4</sub>**  
4 is illustrated in the dashed square.

5 **Figure 2.** Comparison of the dissociation constants ( $K_D$ , A) and of inhibition activities, ( $IC_{50}$ , B) for  
6 compounds **9**, **5d** and **Neo-D<sub>3</sub>** against pre-miR-372.

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8 structure using Autodock 4 software. Primary and secondary structure of pre-miR-372 (C) and binding  
9 sites of compound **9** (light blue) and **Neo-D<sub>3</sub>** (light green).

10 **Scheme 1.** Synthesis of the new series of 2-DOS conjugates **5a-h**. Reagents: a) HCl, reflux, overnight;  
11 b) 48% HBr aq., reflux, 2 days; c) Boc<sub>2</sub>O, NaOH, dioxane/H<sub>2</sub>O, overnight; d) 1,1-  
12 dimethoxycyclohexane, pTsOH, DMF, 50°C, 55 mbar, 1h; e) 2-Azidoethyl-p-toluensulfonate, NaH,  
13 THF, 30°C, 3h; f) **10a-h**, CuI, DIPEA, CH<sub>3</sub>CN, from r. t. to 50°C, from 1h to overnight; g) TFA, CH<sub>2</sub>Cl<sub>2</sub>,  
14 H<sub>2</sub>O, r. t., overnight.

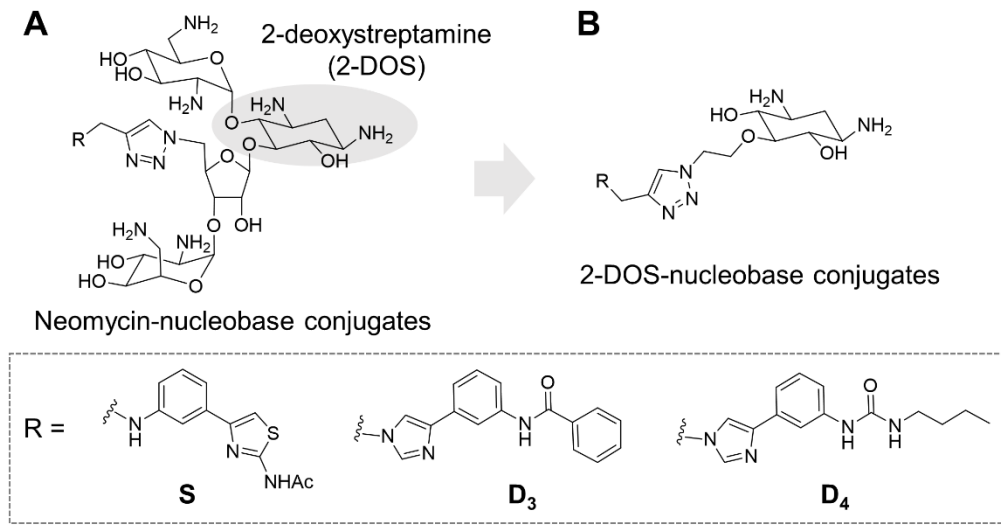
15 **Scheme 2.** Synthesis of enantiopure compound **9**. Reagents: a) 2-Azidoethyl-p-toluensulfonate, NaH,  
16 THF, 30°C, 3h; b) **10h**, CuI, DIPEA, CH<sub>3</sub>CN, r. t., 5h; c) TFA, CH<sub>2</sub>Cl<sub>2</sub>, H<sub>2</sub>O, r. t., overnight.

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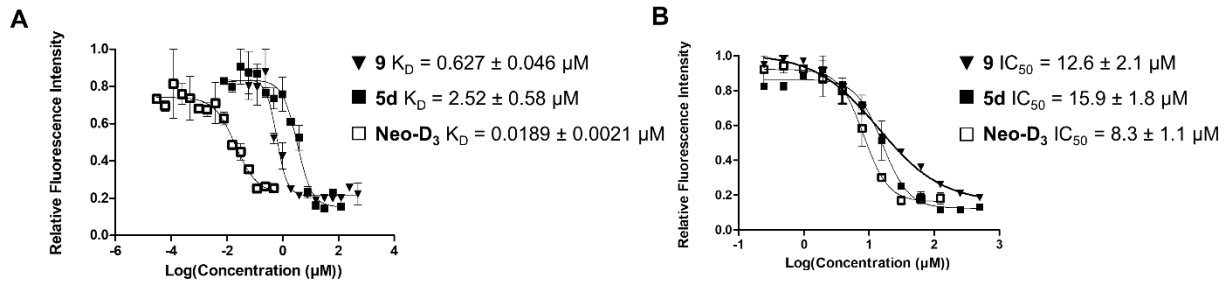
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1 **Figure 1.**



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1 **Figure 2.**

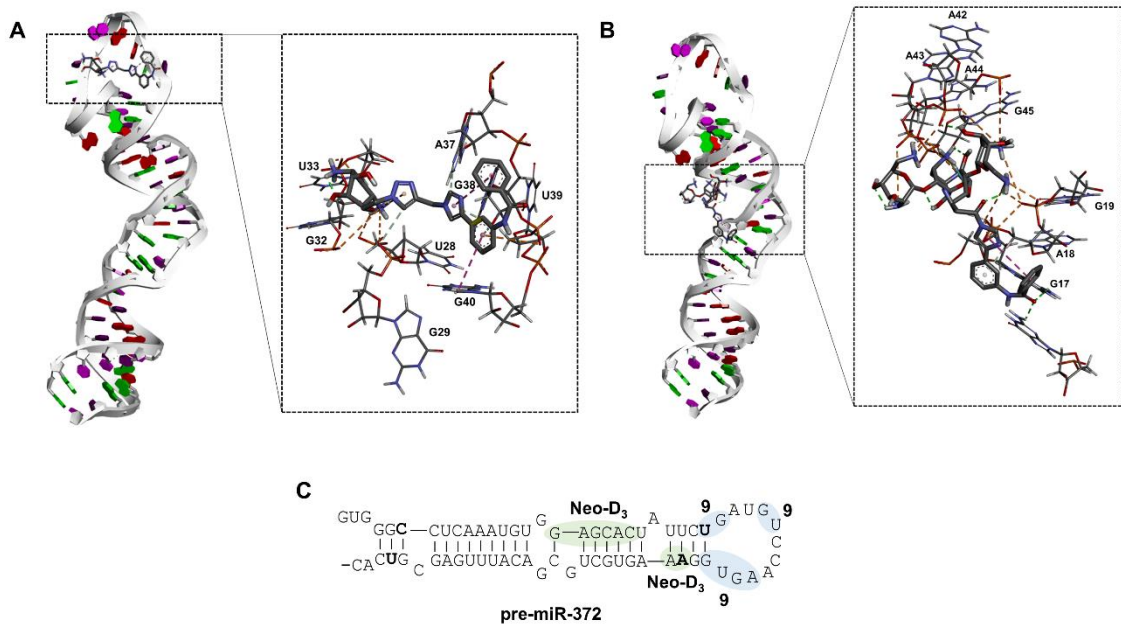


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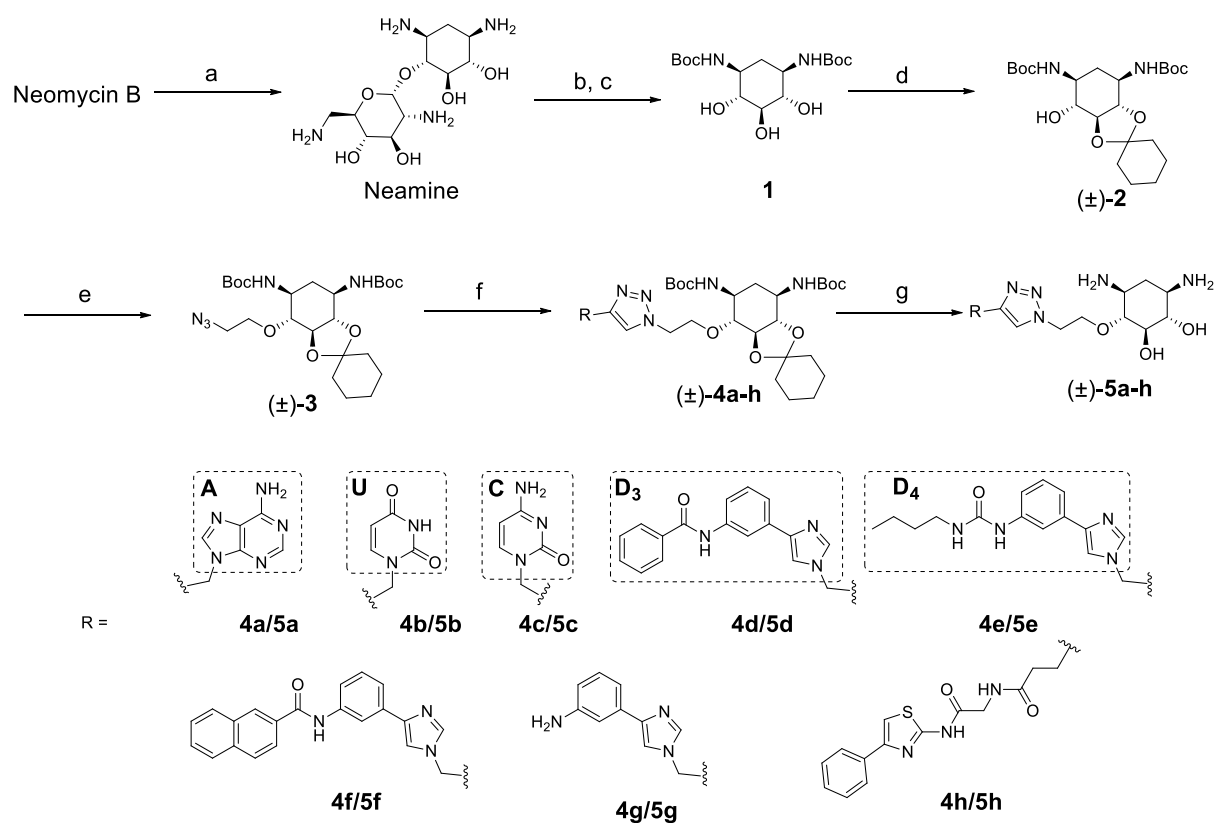
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1 **Figure 3.**



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1 **Scheme 1.**



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- 1 **Table 1.** Dissociation constants values in  $\mu\text{M}$  for synthesized compounds **5a-h** toward pre-miR-372  
 2 alone ( $K_D$ ) and in the presence of tRNA (100 eq.,  $K_D'$ ) or DNA (100 eq.  $K_D''$ ).

ID	$K_D$	$K_D'$	$K_D'/K_D$	$K_D''$	$K_D''/K_D$	$IC_{50}$	$IC_{50}'$
<b>5a</b>	$11.1 \pm 2.3$	> 1 mM	-	47.1	4.2	no inhibition	no inhibition
<b>5b</b>	$17.1 \pm 0.1$	> 1 mM	-	> 1 mM	-	no inhibition	no inhibition
<b>5c</b>	$101 \pm 18$	> 1 mM	-	> 1 mM	-	no inhibition	no inhibition
<b>5d</b>	$2.52 \pm 0.58$	$2.64 \pm 0.52$	1.0	$2.96 \pm 0.85$	1.2	$15.9 \pm 1.8$	$29.5 \pm 9.1$
<b>5e</b>	$1.62 \pm 0.49$	$3.65 \pm 0.85$	2.2	$2.11 \pm 0.81$	1.3	$72.4 \pm 1.0$	no inhibition
<b>5f</b>	$6.78 \pm 0.46$	$8.51 \pm 1.3$	1.3	$9.02 \pm 1.2$	1.3	> 100 $\mu\text{M}$	no inhibition
<b>5g</b>	$3.53 \pm 0.89$	$3.92 \pm 0.52$	1.1	$4.27 \pm 0.35$	1.2	$81.1 \pm 2.3$	> 500 $\mu\text{M}$
<b>5h</b>	$5.13 \pm 0.41$	$8.56 \pm 2.0$	1.7	$11.0 \pm 0.90$	2.1	$74.9 \pm 12$	no inhibition
<b>Neo-D<sub>3</sub></b>	$0.0189 \pm 0.0017$	$0.0208 \pm 0.0019$	1.1	$0.0244 \pm 0.0018$	1.3	$8.30 \pm 1.1$	20.4
<b>Neo-D<sub>4</sub></b>	$0.0461 \pm 0.0038$	$0.0164 \pm 0.0012$	1.1	$0.0222 \pm 0.0024$	1.5	$25.6 \pm 1.1$	
<b>2-DOS</b>	> 1 mM	-	-	-	-	no inhibition	no inhibition

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