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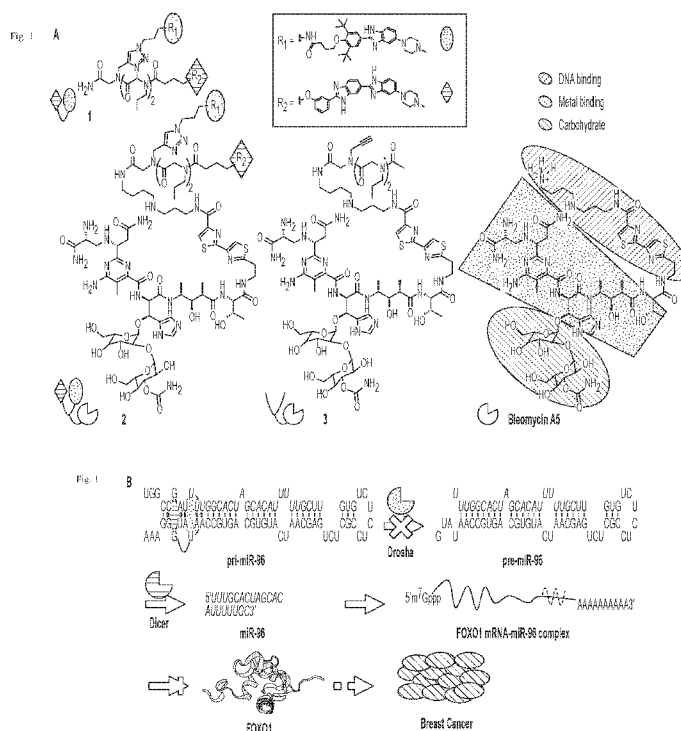
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## (54) Title: TARGET VALIDATION AND PROFILING OF THE RNA TARGETS OF SMALL MOLECULES



(57) **Abstract:** A method for the precise cellular destruction of an oncogenic non-coding RNA with a RNA-binding small molecule conjugated with bleomycin A5 is described. The method affords reversal of phenotype. Bleomycin A5 was coupled to an RNA-binding molecule that selectively binds the microRNA-96 hairpin precursor (pri-miR-96). By coupling of bleomycin A5's free amine to the RNA-binding molecule, its affinity for binding to pri-miR-96 is >100-fold stronger than to DNA. The conjugate compound selectively cleaves pri-miR-96 in triple negative breast cancer (TNBC) cells. Selective cleavage of pri-miR-96 enhances expression of FOXO1 protein, a pro-apoptotic transcription factor that miR-96 silences, and triggers apoptosis in TNBC cells. No effects were observed in healthy breast epithelial cells. This method provides programmable control for targeting RNA through the selection of an RNA-binding molecule/bleomycin A5 conjugate and provides a facile method of mapping the cellular binding sites of an RNA-binding molecule.

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## **Target Validation and Profiling of the RNA Targets of Small Molecules**

### **STATEMENT OF GOVERNMENT SUPPORT**

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### **BACKGROUND**

ENCODE showed that over 70% of our genome is transcribed into RNA<sup>1</sup>. These RNAs have diverse functions, particularly those that are non-coding<sup>1</sup>. Small molecule targeting of RNA, however, has been challenging, except for RNAs that fold into highly three-dimensional (protein-like) structures such as ribosomes and riboswitches<sup>2-4</sup>. More recently, it has been shown that pre-mRNAs in complexes can be targeted and stabilized with small molecules<sup>5-6</sup>. Most RNAs, however, do not have highly complex long-range folds but do have extensive two-dimensional (secondary) structure that that could be targeted with small molecules<sup>7-8</sup>. Indeed, small molecules have been discovered that bind microRNAs (miRNAs) and inhibit their biogenesis<sup>9-11</sup>.

### **SUMMARY**

The invention is directed, in various embodiments, to a method of mapping an RNA binding site of an RNA-binding small molecule, the binding site being disposed within an RNA sequence library, comprising contacting the RNA of the sequence library and an effective amount of a compound comprising a conjugate of an RNA-cleaving moiety and an RNA-binding small molecule to cleave the RNA of the sequence library comprising the binding site to provide a cleaved RNA fragment;

then, amplifying and sequencing the cleaved RNA fragment, thereby identifying the site of binding within the RNA sequence library of the small RNA-binding molecule associated with the site of cleavage;

to identify the sequence of the RNA binding site of the small RNA-binding molecule.

In various cases, the methods disclosed herein comprise contacting a library of RNA sequences and an effective amount of a compound comprising a conjugate of an RNA-cleaving moiety and an RNA-binding small molecule to cleave the RNA of the library at a binding site of the compound to the RNA to provide a cleaved RNA fragment; and amplifying and sequencing the cleaved RNA fragment, thereby identifying the site of binding

of the small RNA-binding molecule to the RNA of the library which is associated with the site of cleavage.

For example, the RNA-binding small molecule can comprise an N-methyl-piperazinyl-bis-benzimidazole group. For example, the conjugate of the RNA-binding molecule can comprise bleomycin, for instance bleomycin A5, as the RNA-cleaving moiety. In some embodiments, the RNA-binding molecule can be Targaprimir-96.

In various embodiments, a method of the invention comprises precisely targeting cellular destruction of an oncogenic non-coding RNA precursor, comprising contacting a cell expressing the non-coding RNA precursor and an effective amount of the compound. For instance, the oncogenic non-coding RNA precursor can comprise oncogenic primary microRNA-96 (pri-miR-96). More specifically, the compound can be a covalent conjugate of Targaprimir-96 and bleomycin A5.

In various embodiments, a method comprises enhancing expression of FOXO1 protein in breast cancer cells, comprising contacting the cells with an effective amount of the conjugate. For example, the contacting can be via administration to a human patient. More specifically, the conjugate can be a covalent conjugate of Targaprimir-96 and bleomycin A5.

In various embodiments, a method of the invention can comprise triggering apoptosis in triple negative breast cancer cells, comprising contacting the cells with an effective amount of the conjugate. The breast cancer cells can be present in a human patient. The conjugate can be a covalent conjugate of Targaprimir-96 and bleomycin A5.

In various embodiments, methods comprise treating triple negative breast cancer, comprising administering to a patient afflicted therewith an effective dose of the compound. For example, the breast cancer can comprise expression of oncogenic primary microRNA-96 (pri-miR-96). The compound can be a covalent conjugate of Targaprimir-96 and bleomycin A5.

In various embodiments, the RNA sequence library can comprise a transcriptome. For example, the transcriptome can be viral, or can be mammalian, or can be bacterial. The RNA sequence library can comprise one or more of synthetic, semi-synthetic, or natural RNA. The RNA sequence library can comprise the genome of an RNA virus.

In various embodiments, a method of the invention can be carried out in vitro, or can be carried out in living cells, e.g., in virally- or bacterially-infected cells.

In various embodiments of a method of the invention, a set of RNA sequences and a set of compounds comprising candidate RNA-binding small molecules can be assayed in a 2-dimensional parallel array.

The invention further provides a compound comprising a conjugate of an RNA-cleaving moiety and an RNA-binding small molecule. The RNA-cleaving moiety can comprise bleomycin. The RNA-binding small molecule can comprise N-methyl-piperazinyl-bis-benzimidazole group, e.g., Targaprimir-96.

## BRIEF DESCRIPTION OF THE FIGURES

### **Figure 1. Pri-miR-96 is oncogenic and suppresses apoptosis in cancer cells via repression of the pro-apoptotic transcription factor Forkhead box protein O1 (FOXO1).**

(A) structures of the compounds used in these studies. Compound **1** was designed via Informa and selectively targets pri-miR-96. Compound **2** is a version of **1** conjugated to bleomycin A5 while compound **3** is a version of **2** that lacks RNA-binding modules. (B) secondary structure of pri-miR-96 and the miR-96-FOXO1 pathway. Compound binding sites are indicated in the secondary structure, and mature miR-96 is indicated in red lettering. The red “X” indicates inhibition of Drosha processing by a small molecule; likewise the red arrow indicates that inhibition of pri-miR-96 processing inhibits repression of FOXO1 and hence breast cancer. Compound **1** inhibited the production of mature miR-96, de-repressed a downstream target, pro-apoptotic transcription factor FOXO1, and triggered apoptosis.

**Figure 2. DNA cleavage by 2 and 3 in vitro and in cells.** (A) studying the DNA cleaving activity of **2** and **3** in vitro. Appending RNA-binding modules onto the bleomycin core (affording **2**) decreased the amount of DNA cleavage as compared to **3**, which lacks RNA-binding modules. (B) Visualization and quantification of DNA damage in MDA-MB-231 cells treated with 500 nM **1**, **2** or **3** for 12 h. Data are expressed as mean  $\pm$  s.e.m. ( $n \geq 3$ ). \*  $p < 0.05$ , as determined by a two-tailed Student *t* test by comparison to untreated DNA (A) or cells (B).

**Figure 3. Studying the effect of 1 and 2 on mature miR-96 and pri-miR-96 levels and on miRNA-mediated biology.** (A) the effect of **1** and **2** on mature miR-96 levels in MDA-MB-231 TNBC cells. (B) the effect of **1** and **2** on pri-miR-96 levels in MDA-MB-231 cells. As expected based on their modes of action, **1** (simple binding) increased pri-miR-96 levels, while **2** (cleavage) reduced them. (C) co-addition of increasing concentrations of **1** (5 to 500 nM) and a constant concentration of **2** (500 nM) to MDA-MB-231 cells increased levels of pri-miR-96, diminishing the cleaving capacity of **2** as expected. (D) effect of **2** on expression of FOXO1 protein, a direct target of miR-96, as determined by Western blot. (E) effect of **2** on apoptosis in MDA-MB-231 cells (red), MDA-MB-231 cells that overexpress pri-miR-96 via a plasmid (green), and in MCF-10a healthy breast cells (blue), as determined by Caspase

assays. Data are expressed as mean  $\pm$  s.e.m. ( $n \geq 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , as measured by a two-tailed Student *t* test by comparison to untreated cells.

**Figure 4. An unbiased miRNA-profiling approach shows that **2** most significantly affects mature miR-96 levels in MDA-MB-231 cells and amplification of cleavage products identifies the small molecule binding site within pri-miR-96.** (A) Volcano plot for profiling the effect of **2** on all expressed miRNAs in MDA-MB-231 cells; miR-96 is the most affected. (B) a scheme of the amplification approach to identify small molecule binding sites via cleavage. (C) Top, representative Sanger sequencing results from cDNA of the cleaved RNA. The cleavage site is indicated with a red box. Bottom, analysis of several clones reveals three cleavage sites (indicated with a box and arrow in the pri-miR-96 secondary structure); 40% of reads stop at the first C (5'); ~30% of reads stop at the A; and ~30% of reads stop the second C (3'). Data are expressed as mean  $\pm$  s.e.m. ( $n \geq 3$ ).

## DETAILED DESCRIPTION

To provide rational approaches to target RNA with small molecules, we developed a sequence-based approach dubbed Informa<sup>12</sup>. In particular, Informa enabled the design of a small molecule (Targaprimir-96, **1**, Fig. 1A) that selectively targets the Drosha endonuclease-processing site of oncogenic primary microRNA-96 (pri-miR-96)<sup>13</sup>. MiRNAs are non-coding RNAs that play pervasive roles in biology, and their aberrant expression or mutation can be causative of disease. They are initially produced as precursors (pri-miRNA) that are processed by the nuclease Drosha followed by translocation to the cytoplasm as precursor microRNAs (pre-miRNAs). Pre-miRNAs are cleaved by the cytoplasmic nuclease Dicer to produce mature miRNAs that bind to the 3' untranslated regions (UTRs) of mRNAs and repress translation. Application of **1** to triple negative breast cancer (TNBC) cells inhibited the production of mature miR-96, de-repressed pro-apoptotic transcription factor Forkhead box protein O1 (FOXO1) that the miRNA repressed, and triggered apoptosis (Fig. 1B)<sup>13</sup>.

To expand the functional repertoire of small molecules that target RNA from simple binding to selective cleavage, bleomycin A5 was conjugated to **1**, affording **2** (Fig. 1). Bleomycin, a natural product used for treatment of cancer, cleaves DNA<sup>14, 15</sup> but also cleaves RNA, as pioneered by the Hecht group<sup>16, 17</sup>. Bleomycin contains four domains: (i) a metal ion-binding domain that activates O<sub>2</sub> and leads to nucleic acid cleavage<sup>18-21</sup>; (ii) a DNA-binding domain, which affects cleavage efficiency<sup>22</sup>; (iii) a linker region between the metal ion-binding and DNA-binding domains, which also affects cleavage efficiency<sup>23-27</sup>; and (iv) a carbohydrate domain which facilitates cellular uptake<sup>20</sup>. We chose bleomycin A5 as the

cationic dimethyl sulfonium in the C-terminal DNA-binding domain has been replaced with a butyl-1,4-diamine side chain. This modification serves two purposes: (i) facile conjugation of the terminal primary amine to RNA-binding modules containing carboxylates. Notably, acylation of the butyl-1,4-diamine side chain (cationic) with a small molecule affords an uncharged linkage; and (ii) reduction of DNA binding affinity<sup>28-30</sup> as the cationic side chain, known to drive binding to DNA, has been acylated and is no longer charged. In addition, it has been shown that increasing the size and hydrophobicity of bleomycin A5's butyl-1,4-diamine side chain further decreases DNA binding affinity and the extent of cleavage<sup>28-30</sup>. Conjugation of an RNA-binding module is therefore likely to alter bleomycin A5's binding and cleavage preferences toward RNA, as we have observed in the selective cleavage of expanded r(CUG) repeats<sup>31</sup>.

The secondary structure of oncogenic pri-miR-96<sup>32</sup> was analyzed to determine if it might be a suitable target for bleomycin-mediated cleavage (Fig. 1B). Previously, it has been shown that bleomycin can cleave AU pairs in RNA<sup>33</sup>, and indeed AU pairs are present adjacent to pri-miR-96's Drosha site. Thus, conjugation of bleomycin to compound **1** could provide a selective cleaving small molecule, provided that the bleomycin is positioned towards these AU pairs. As previous studies have shown that the 3,5-di-*tert*-butylbenzyl benzimidazole module in **1** binds the 1×1 UU internal loop in the Drosha site (teal oval; Fig. 1)<sup>12</sup>, conjugation of bleomycin A5 to **1** (**2**; Fig. 1A) places the cleaving module near the neighboring AU pairs. Control compound **3** (Fig. 1A), which lacks the RNA-binding modules, was also synthesized.

The sites of cleavage by **2** and **3** were studied *in vitro* by using primer extension with a radioactively labeled primer after reaction. In the presence of **2** and Fe<sup>2+</sup>, a site of selective cleavage was observed adjacent to the Drosha site, as predicted; that is, cleavage at this site was not observed when pri-miR-96 was treated with Fe<sup>2+</sup> alone or **3** and Fe<sup>2+</sup>. To further assess the ability of **2** and **3** to cleave nucleic acids, they were tested for cleaving DNA. As shown in Fig. 2A, **2** cleaved DNA with 5-fold lower efficiency than **3** at concentrations  $\geq$  500 nM, as calculated by comparing the percentage of DNA plasmid cleaved by both compounds ( $p < 0.05$ ). That is, conjugation of bleomycin to an RNA binder significantly reduced its ability to cleave DNA *in vitro*, as expected based on previous reports<sup>30</sup>. To confirm our *in vitro* cleavage results, we measured the affinities of **1**, **2**, **3** and bleomycin A5 for pri-miR-96 and DNA by microscale thermophoresis (MST)<sup>34, 35</sup> (Fig. 2B). In agreement with *in vitro* cleavage studies, **1** and **2** bound avidly to pri-miR-96 with K<sub>ds</sub> of 39 ± 18 nM

and  $64 \pm 11$  nM, respectively, while saturable binding to **3** and bleomycin A5 was not observed ( $K_d > 30$   $\mu$ M). In contrast, no saturable binding of **1**, **2**, or **3** to DNA was observed ( $K_d > 30$   $\mu$ M); however, bleomycin A5 bound DNA with a  $K_d$  of  $\sim 1$   $\mu$ M. Collectively, these results indicate that modification of bleomycin A5's side chain, whether by an RNA-binding module or a peptoid linker, greatly reduced its affinity for DNA as expected<sup>30</sup> and that **2** selectively bound pri-miR-96 in the low nM concentration range.

This phenomenon was also observed in cells, as studied by assessing DNA damage using an antibody for gamma H2A histone family, member X ( $\gamma$ -H2A.X)<sup>36</sup>, a marker for DNA double stranded breaks visualized as nuclear foci. In agreement with *in vitro* DNA cleavage (Fig. 2A), **3** caused  $\sim 2.3$ -fold more DNA damage than **2** (500 nM of **2** or **3**; Fig. 2C), as calculated by comparing the average foci number per cell for each compound. Collectively, these data show that pri-miR-96 can be cleaved to a greater extent by **2** than DNA is cleaved within a concentration window; that is, the targets that are cleaved by bleomycin can be attenuated by addition of an RNA-binding module at mid-nanomolar concentrations both *in vitro* and in cells, *vide infra*.

Next, the effect of **2** on pri-miR-96 and mature miR-96 levels in MDA-MB-231 TNBC cells was measured via RT-qPCR. To study the cleaving effects of **2**, the compound was first complexed with  $\text{Fe}^{2+}$  (1 equivalent), diluted into growth medium, and then added to cells. While both compounds reduced mature miR-96 levels (Fig. 3A), **1** increased level of pri-miR-96 while **2** decreased them (Fig. 3B), as expected based on their designed mode of action, simple binding and cleavage, respectively. When **2** is prepared in the absence of  $\text{Fe}^{2+}$ , no statistically significant effect was observed on pri-miR-96 levels; mature miR-96 levels were reduced upon treatment with 500 nM compound as expected based on **2**'s binding properties ( $p < 0.05$ ). These data suggest that the cleavage of pri-miR-96 contributes to the downregulation of mature miR-96. Control compound **3** had no effect on mature or pri-miR-96 levels. To further confirm **2**'s mode of action, a competition cleavage experiment was completed in which increasing concentrations of **1** and a constant concentration of **2** were delivered to MDA-MB-231 cells. Both compounds show similar levels of cell permeability at 500 nM as determined by flow cytometry. Results show that the cleavage caused by **2** is effectively competed off when **1** was added (Fig. 3C). Each of these results supports the hypothesis that **2** is targeting pri-miR-96 for destruction.

In cancer cells, miR-96 suppresses apoptosis by silencing the production of pro-apoptotic transcription factor FOXO1<sup>32</sup>. Thus, inhibition of miR-96 by **2** should increase the

amount of FOXO1 and trigger apoptosis. Indeed, the amount of FOXO1 protein in MDA-MB-231 cells was increased by ~1.8-fold when treated with 500 nM of **2** (Fig. 3D). The effect of **2** on phenotype (suppression of apoptosis)<sup>12, 13, 32</sup> was then assessed by using both Annexin/PI staining and Caspase assays. Importantly, **2** induced apoptosis in MDA-MB-231 cells (Fig. 3E (red)) and had no effect on MCF-10a healthy breast epithelial cells in which pri-miR-96 is not expressed in measurable amounts (Fig. 3E, blue). Further, the apoptotic effect of **2** was reduced in cells in which pri-miR-96 was overexpressed from a plasmid (Fig. 3E, green). We also measured the effect of **2** on other miRNAs, including miR-10b, which was previously shown to be a target of bleomycin A5<sup>33</sup>, oncogenic miR-21<sup>37</sup>, and all other miRNAs predicted to target the FOXO1 3' untranslated region (UTR) by TargetScan<sup>38</sup>. None of these targets was affected.

One of the beauties of antisense is that the oligonucleotide's on- and off-targets can be inferred by depletion of an RNA's levels. To determine whether **2** can be used in target profiling studies akin to antisense, an unbiased profiling experiment (RiboSNAP; small molecule nucleic acid profiling by cleavage applied to RNA) on the 349 miRNAs expressed in MDA-MB-231 cells was completed. The data from these studies are presented as a volcano plot, a logarithmic plot of fold change vs. statistical significance (Fig. 4A). Importantly, these studies show: (i) miR-96 levels were affected to the greatest extent and were the most statistically significant, illustrating **2**'s remarkable selectivity. This result is of great interest considering that **2**'s mode of action is RNA cleavage. Evidently, conjugation of bleomycin A5 to **1** does not alter **1**'s selectivity<sup>13</sup>; (ii) small molecule-bleomycin conjugates can be used in cellular target profiling studies; and (iii) the RNA targets cleaved in cells by bleomycin can be precisely programmed by conjugation to a selective RNA small molecule binder. One challenge in developing chemical probes targeting RNA has been the perception that compounds cannot be selective, and these studies suggest that small molecules, even those that cleave, can be selective for an RNA target.

The most common method to identify small molecule binding sites within an RNA is to monitor sites of protection from nuclease cleavage or reaction with chemical modification reagents. Indeed, this approach identified sites in the ribosome that bound antibiotics<sup>39, 40</sup>. However, some binding sites can be silent due to lack of reactivity with a chemical modifier and can require long residence times of the small molecule to prevent reactivity (irreversible). Thus, careful tuning of the experimental conditions is often necessary. Although laborious, these types of experiments are invaluable to validate or identify the target(s) of small molecules, which is essential to establish a compound's mode of action.

We posited that analyzing the cleavage footprints of **2** from RNA harvested from treated cells could identify the precise binding site, an approach we named Ribo-SNAP-Map. If cleavage sites could be amplified, both the small molecule's RNA target (Ribo-SNAP) and the binding site within the RNA (Ribo-SNAP-Map) could be identified quickly after compound treatment.

To implement Ribo-SNAP-Map, we developed a procedure to enrich the partial cleavage products of pri-miR-96 (Fig. 4B) using a gene specific forward primer and a universal reverse primer in an RT-qPCR experiment<sup>41</sup>. Gel analysis showed a new band at ca. 130 base pairs only when cells were exposed to **2**, not to **1** or **3**. Sequencing analysis confirmed that the cleavage sites were proximal to the predicted and *in vitro* mapped binding sites for **2** (Fig. 4C). Molecular modeling of **2** binding with pri-miR-96 also showed that **2** positions the cleaving moiety towards the AU sequence that is cleaved rather than other regions in the RNA that are distant from the ligand's binding site. Thus, Ribo-SNAP-Map can indeed be used to map binding sites in cells. Notably, AU pairs proximal to a small molecule's binding site is not requisite for selective cleavage of bleomycin A5 conjugates, as observed for r(CUG) repeats<sup>31</sup>.

Previous studies have provided small molecules that cleave RNA by using light<sup>42</sup>, are nuclease mimics<sup>43</sup>, or recruit endogenous nucleases to an RNA target. In the first approach, applicability can be limited because of the necessity of light to penetrate cells and tissue. Compounds that act as nuclease mimics interact with an expanded repeating RNA and have a mixed mode of inhibition (transcriptional inhibition, inhibition of protein binding, and cleavage)<sup>43</sup>. Notably, expanded repeating RNAs are atypical targets; due to its repeating nature and hence multiple small molecule binding sites, inefficient cleavage could afford a significant biological effect. More recently, we developed an approach named Ribonuclease targeting chimeras (RIBOTACs) to recruit endogenous RNase L to cleave a desired RNA target<sup>44</sup>. However, cleavage patterns on the RNA target using these methods can be complex and may not be proximal to the binding site. In contrast, cleavage with **2** is proximal and not complex, allowing straightforward identification of RNA sequences nearby ligand binding sites.

Herein, we showed that small molecules can be engendered with antisense-like properties in cells using chimeric compounds comprised of a selective RNA-binding small molecule and bleomycinA5 as a cleaving moiety. Indeed, these studies and others suggest that: (i) the targets cleaved by bleomycin can be tuned by conjugation to an RNA-binding small molecule; (ii) these capacities are likely programmable; and (iii) the ability to cleave

RNAs with small molecules could expand the target scope of ligands that modulate the biology of RNA, akin to the revolution that PROTACs<sup>45</sup> initiated in the protein targeting field. Most RNAs' biology may not be affected by simple binding and engendering a small molecule with the ability to cleave will likely expand the number of RNAs that can be targeted with organic compounds.

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**Table 1.** Sequences of primers used in this study.

| Primer            | Sequence (5' to 3')                      |
|-------------------|------------------------------------------|
| miR-96            | TTTGGCACTAGCACATTTTGTGCT (SEQ. ID NO:1)  |
| pri-miRNA-96-F    | AGAGAGCCCGCACCAGT (SEQ. ID NO:2)         |
| pri-miRNA-96-R    | CTTGAGGAGGAGCAGGCT (SEQ. ID NO:3)        |
| RNU6              | ACACGCAAATTCGTGAAGCGTTC (SEQ. ID NO:4)   |
| Universal reverse | GAATCGAGCACCAGTTACGC (SEQ. ID NO:5)      |
| miR-10b           | TACCCTGTAGAACCGAATTTGTG (SEQ. ID NO:6)   |
| miR-21            | TAGCTTATCAGACTGATGTTGA (SEQ. ID NO:7)    |
| miR-27a           | TTCACAGTGGCTAAGTTCCGC (SEQ. ID NO:8)     |
| miR-9             | TCTTTGGTTATCTAGCTGTATGA (SEQ. ID NO:9)   |
| miR-194           | TGTAACAGCAACTCCATGTGGA (SEQ. ID NO:10)   |
| miR-15a           | TAGCAGCACATAATGGTTTGTG (SEQ. ID NO:11)   |
| miR-16            | TAGCAGCACGTAAATATTGGCG (SEQ. ID NO:12)   |
| miR-139           | TCTACAGTGCACGTGTCTCCAGT (SEQ. ID NO:13)  |
| miR-182           | TTTGGCAATGGTAGAACTCACACT (SEQ. ID NO:14) |
| miR-196a          | TAGGTAGTTTCATGTTGTTGGG (SEQ. ID NO:15)   |
| miR-128a          | TCACAGTGAACCGGTCTCTTT (SEQ. ID NO:16)    |
| miR-142           | TGTAGTGTTCCTACTTTATGGA (SEQ. ID NO:17)   |
| miR-223           | TGTCAGTTTGTCAAATACCCC (SEQ. ID NO:18)    |
| miR-101           | TACAGTACTGTGATAACTGAA (SEQ. ID NO:19)    |
| miR-132           | TAACAGTCTACAGCCATGGTCG (SEQ. ID NO:20)   |
| miR-212           | TAACAGTCTCCAGTCACGGCC (SEQ. ID NO:21)    |
| miR-135a          | TATGGCTTTTATTCTATGTGA (SEQ. ID NO:22)    |
| miR-1271          | CTTGGCACCTAGCAAGCACTCA (SEQ. ID NO:23)   |

## **Examples**

### **Experimental Procedures**

**RNA preparation:** The single-stranded DNA template (5'-GGGTGGCCGATTTTGGCACTAGCACATTTTGTCTGTCTCTCCGCTCTGAGCAATCATGTGCAGTGCCAATATGGGAAA-3') (**SEQ. ID NO:24**) for PCR amplification was purchased from Integrated DNA Technologies, Inc. (IDT) and used without further purification. PCR amplification was performed in 1× PCR Buffer (10 mM Tris, pH 9.0, 50 mM KCl, and 0.1% (v/v) Triton X-100), 2 μM T7 promoter forward primer (5'-GGCCGGATCCTAATACGACTCACTATAGGGTGGCCGATTTTGGC-3') (**SEQ. ID NO:25**), 2 μM reverse primer (5'-TTTCCCATATTGGCA-3') (**SEQ. ID NO:26**), 4.25 mM MgCl<sub>2</sub>, 330 μM dNTPs, and 1 μL of Taq DNA polymerase in a 300 μL reaction. The cycling conditions used for PCR were 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The resulting double stranded DNA template was transcribed by T7 RNA polymerase in 1× Transcription Buffer (40 mM Tris HCl, pH 8.1, 1 mM spermidine, 0.001% (v/v) Triton X-100 and 10 mM DTT) containing 2.25 mM of each rNTP and 5 mM MgCl<sub>2</sub> at 37 °C overnight. The RNA product was purified on a denaturing 15% polyacrylamide gel, and concentrations were determined by UV absorbance at 260 nm.

**In vitro RNA cleavage and primer extension:** The pri-miR-96 RNA (1 μM) was folded as previously described<sup>2</sup> by heating at 65 °C for 5 min and slowly cooling to room temperature. Different concentrations of **2** or **3** (5, 50, 500 nM) were pre-activated by the addition of 1 eq Fe<sup>2+</sup> and added to the folded RNA in a total volume of 20 μL. Next, 1 eq Fe<sup>2+</sup> was added 30 min and then 60 min later, and the reaction mixtures were incubated at 37 °C overnight.<sup>3</sup> After ethanol precipitation and quantification by Nanodrop, reverse transcription was performed by using SuperScript™ III Reverse Transcriptase (ThermoFisher Scientific) per the manufacturer's protocol using 5' <sup>32</sup>P-labeled primer (~10,000 counts). The A, T, G and C sequencing ladders were generated by using a ratio of ddNTP/dNTP of 3:1. The RNA was digested by the addition of RNase A and RNase H and incubated at 37 °C for 30 min. Then, an equal volume of Loading Buffer (95% formaldehyde, 50 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol) was added to each reaction. The final mixture was resolved on a denaturing 15% polyacrylamide gel.

**DNA cleavage in vitro:** Different concentrations of **2** or **3** (5, 50, 500, 5000 and 10000 nM) were pre-activated by the addition of 1 eq Fe<sup>2+</sup> and then 2 μL of a plasmid encoding GFP (150 ng/μL) was added in a final volume of 20 μL. An additional equivalent of Fe<sup>2+</sup> was

added 30 min and then 60 min later, and the reaction mixture was incubated at 37 °C overnight. The reaction mixture was loaded on 0.8% agarose gel with 6×Gel Loading Dye, Purple (NEB) and stained with ethidium bromide for 10 min.

**Visualization of DNA damage:** MDA-MB-231 cells were grown in a glass bottom 96-well plate and treated with compound for 24 h. Cells were washed with 1× DPBS three times and then fixed with 100 µL of 4% paraformaldehyde for 10 min at 37 °C. Cells were washed with 1× DPBS three times and then with 0.1% Triton X-100 in 1× DPBS three times for 5 min at 37 °C. Cells were then incubated with a 1:500 dilution of anti-γH2AX (Abcam)<sup>4</sup> at 37 °C for 1 h, washed three times with 0.1% Triton X-100 in 1× DPBS for 5 min at 37 °C, and incubated with a 1:200 dilution of goat anti-mouse IgG-DyLight 488 conjugate (Thermo Scientific) at 37 °C for 1 h. After washing the cells with 0.1% Triton in 1× DPBS and twice with 1× DPBS for 5 min at 37 °C, nuclei were stained with DAPI (100 µL of 1 µg/mL), and cells were imaged.

**RT-qPCR of miRNAs:** Cells (~70% confluency) were treated with various concentrations of compound for 24 h. Total RNA was extracted using a Quick-RNA Miniprep Kit (Zymo Research) per the manufacturer's protocol. Approximately 200 ng of total RNA, as determined by Nanodrop, was used for reverse transcription using a miScript II RT Kit (Qiagen) per the manufacturer's protocol. RT-qPCR was performed on a 7900HT Fast Real Time PCR System (Applied Biosystem) using Power SYBR Green Master Mix (Applied Biosystems). All primers were purchased from IDT and listed in Table S1. The primers for unbiased miRNA profiling were purchased from Eurofins Genomics in a 384-well plate. The expression levels of miRNAs were normalized to U6 small nuclear RNA.<sup>5</sup>

**Western blotting:** Cells in 6-well plates (~70% confluency) were treated with 500 nM of **2** for 48 h. Total protein was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology) following the manufacturer's protocol and quantified using a Micro BCA Protein Assay Kit (Pierce Biotechnology). Approximately 20 µg total protein was separated on a 10% SDS-polyacrylamide gel, and then transferred to a PVDF membrane. The membrane was washed with 1× Tris-buffered saline (TBS) and then blocked in 5% milk in 1× TBST (1× TBS containing 0.1% Tween-20) for 1 h at room temperature. After incubation in 1:1000 FOXO1 primary antibody (Cell Signaling Technology) in 1× TBST containing 5% milk overnight at 4 °C, the membrane was washed with 1× TBST and incubated with 1:2000 anti-rabbit IgG horseradish-peroxidase secondary antibody conjugate (Cell Signaling Technology) in 1×TBS for 1 h at room temperature. The membrane was washed with 1× TBST and protein expression was quantified using SuperSignal West Pico Chemiluminescent

Substrate (Pierce Biotechnology) per the manufacturer's protocol. To quantify  $\beta$ -actin expression, the membrane was stripped using  $1\times$  Stripping Buffer (200 mM glycine, pH 2.2 and 0.1% SDS) followed by washing in  $1\times$  TBST. The membrane was blocked and probed for  $\beta$ -actin similarly using 1:5000  $\beta$ -actin primary antibody (Cell Signaling Technology) in  $1\times$  TBST containing 5% milk at room temperature for 1 h. The membrane was washed with  $1\times$  TBST and incubated with 1:10,000 antirabbit IgG horseradish-peroxidase secondary antibody conjugate (Cell Signaling Technology) in  $1\times$  TBS for 1 h at room temperature.  $\beta$ -actin protein expression was quantified using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) per the manufacturer's protocol. The fold change of FOXO1 expression was calculated by normalizing FOXO1 band intensity to  $\beta$ -actin band intensity.

**Caspase assay:** Cells in 96-well plates (~60% confluency) were treated with various concentrations of compound for 48 h. In the case of miR-96 overexpression, cells were transfected with a plasmid encoding pri-miR-96 with Lipofectamine 2000 (Thermo Fisher Scientific) per the manufacturer's protocol, split into 96-well plates, and treated with compounds for 48 h. MCF 10A cells were cultured in Dulbecco's modified eagle medium/F12 (DMEM/F12) supplemented with 20 ng/ $\mu$ L epidermal growth factor (EGF), 0.5  $\mu$ g/mL hydrocortisone, cholera toxin 0.1  $\mu$ g/mL, bovine insulin 10  $\mu$ g/mL, 1% penicillin/streptomycin and 10% FBS (complete growth medium). Caspase-3/7 activities were measured by using Caspase-Glo® 3/7 Assay Systems (Promega) per the manufacturer's protocol. Luminescence of compound-treated wells was normalized to untreated cells, and the fold change of caspase activities was calculated.

**Annexin V/PI Assay:** Cells in 6-well plates (~60% confluency) were incubated with **2** or **3** for 48 h. As a positive control, cells were treated with 10  $\mu$ M camptothecin for 24 h. The cells were detached from the surface by using accutase and washed twice with ice-cold  $1\times$  DPBS and then three times with  $1\times$  Annexin Binding Buffer (50 mM Hepes (pH 7.4), 700 mM NaCl and 12.5 mM  $\text{CaCl}_2$ ). The cells were suspended in 100  $\mu$ L  $1\times$  Annexin Binding Buffer containing 5  $\mu$ L Annexin V-APC (eBioscience). The cells were incubated for 10 min at room temperature followed by washing with  $1\times$  Annexin Binding Buffer. The cells were then stained with 1  $\mu$ g/mL propidium iodide in 300  $\mu$ L of  $1\times$  Annexin Binding Buffer for 15 min at room temperature. Flow cytometry was performed using a BD LSRII instrument (BD Biosciences). For data analysis, the appropriate quadrant was assigned, and the early and late apoptosis percentages, as shown by Annexin V and PI staining, were calculated.

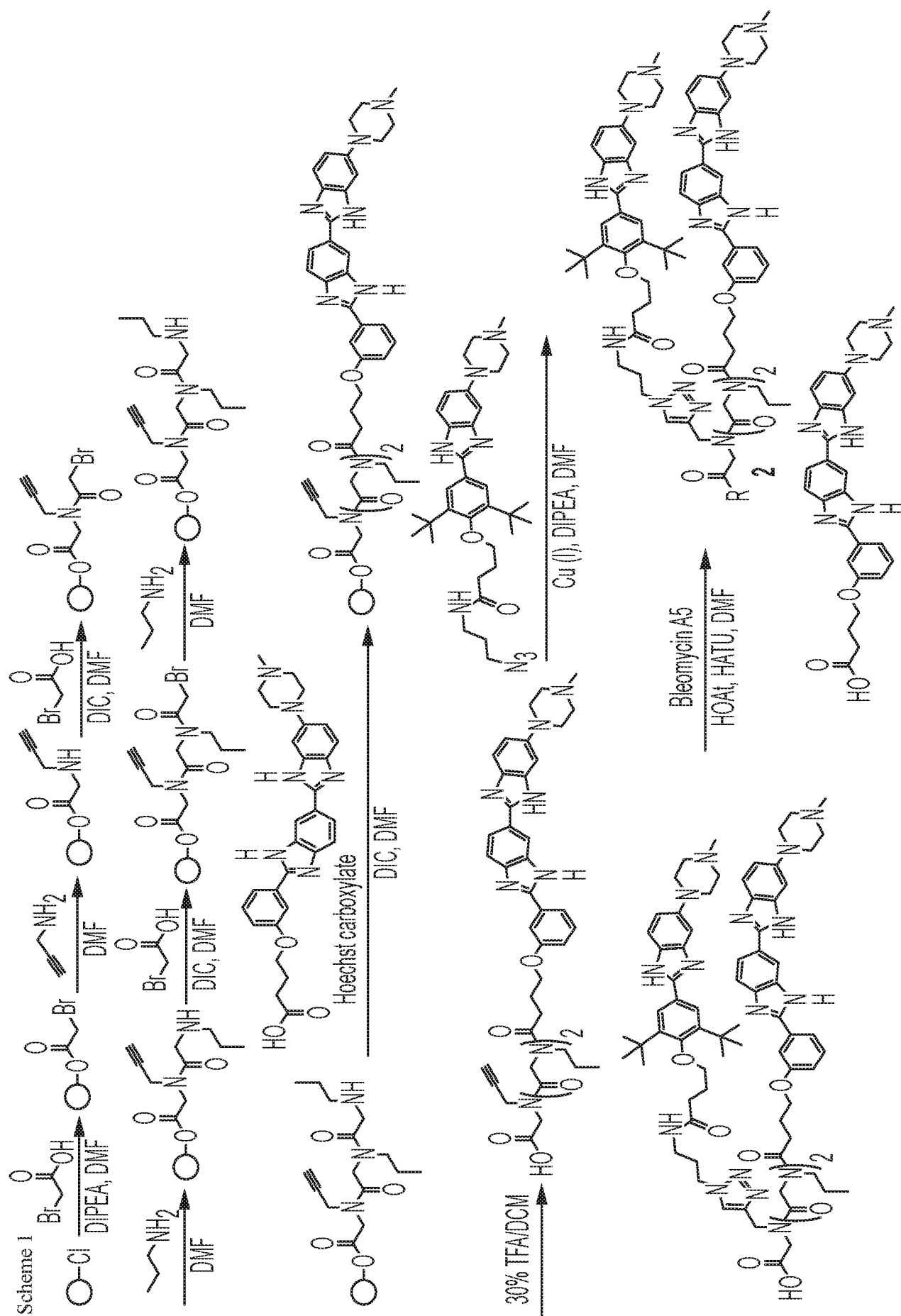
**Molecular modeling:** Modeling of the binding of **2** to pri-miR-96 was generated as previously reported<sup>6</sup>. Briefly, we first modeled the binding of **1** to pri-miR-96. The target RNA structure was modeled in RNAComposer (<http://rnacomposer.cs.put.poznan.pl/>) using the secondary structure predicted with ViennaRNA (<http://ma.tbi.univie.ac.at/>). The RNA-binding small molecule was energy minimized using MacroModel (Schrodinger, LLC, NY) and placed proximal to the RNA. Energy minimization of the RNA-small molecule complex was performed until the gradient of energy was less than 0.01 kcal/mol/Å. The bleomycin A5 moiety was then manually added and allowed flexibility during an additional energy minimization to model the binding of **2** to pri-miR-96. Energy minimization was performed until the gradient of energy was less than 0.01 kcal/mol/Å. The conformation with lowest energy is shown in Figure S8.

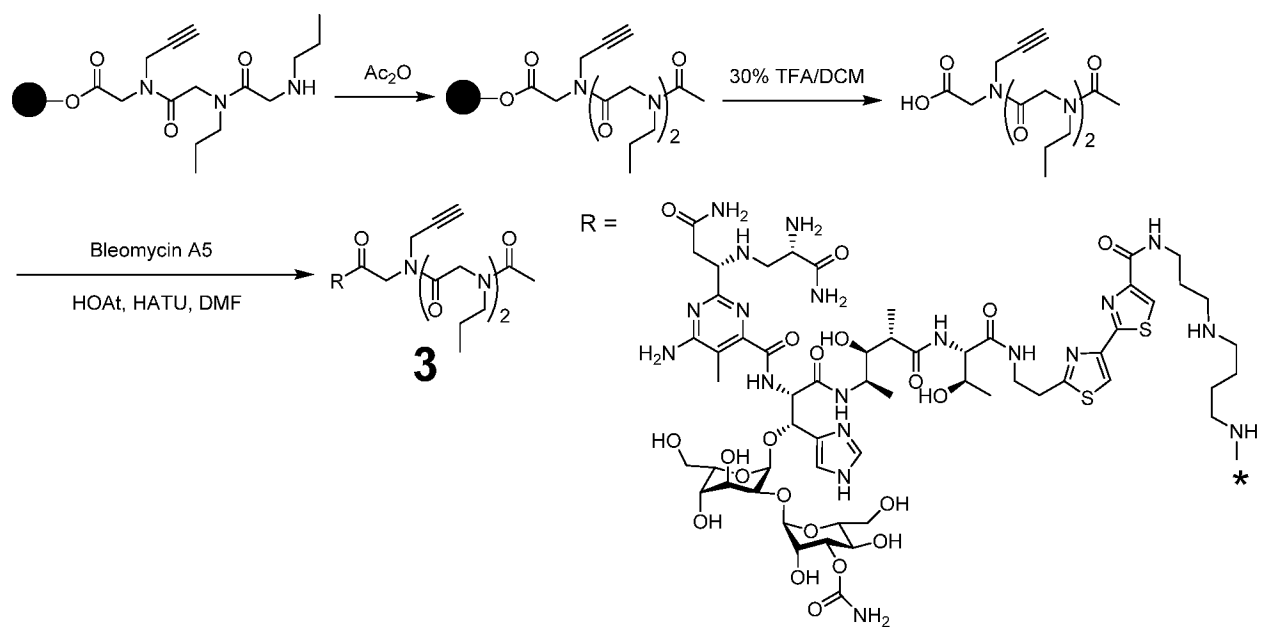
**Ribo-SNAP-Map**<sup>7-8</sup>: Cells were grown in 100 mm dishes to ~70% confluency and treated with **1** (control), **2**, or **3** (control) for 6 h. Total RNA was then extracted by treatment with TRIzol (ThermoFisher Scientific) and quantified by Nanodrop. Approximately 10 µg of total RNA was used for reverse transcription with a pri-miR-96 specific primer (5'-CAGACGTGTGCTCTTCCGATCTCGCAGCTGCGGGTCCT-3'; (**SEQ. ID NO:27**) 2 pmol) using Superscript III (SSIII; Life Technologies). 10 µg RNA with 2 pmol of gene-specific primer and 1 µL 10 mM dNTP Mix in total 13 µL was kept at 65°C for 5 min and in ice for 5 min. Then 4 µL 5X First-Strand Buffer, 1 µL 0.1 M DTT, 1 µL RNaseOUT and 1 µL SuperScript™ III RT were added and incubated at 50°C for 1 h and then 85°C for 10 min. After digesting the RNA with RNase A and RNase H, the cDNA was purified by using RNAClean XP beads (Beckman Coulter; 1.8 volumes of beads and 3 volumes of isopropanol).

The purified cDNA was ligated with a 3' adapter (/5Phos/AGATCGGAAGAGCGTCGTGTAG/3Bio/)(**SEQ. ID NO:28**) by T4 RNA ligase 1 (New England BioLabs; NEB) following the manufacturer's recommended protocol (2 µL 10× T4 RNA ligase buffer, 1 µL of 1 mM ATP, 10 µL 50% PEG 8000, 5 µL cDNA, 1 µL of 20 µM ssDNA adaptor, and 1 µL of T4 RNA ligase). Then, the cDNA ligated to the adaptor was purified with RNAClean XP beads as described above. PCR amplification was performed with the ligated cDNA by using Phusion polymerase (NEB) with cycles of 98 °C for 20 s, 64 °C for 20 s and 72 °C for 90 s and the following primers: forward - (5'-CAGACGTGTGCTCTTCCGATC-3') (**SEQ. ID NO:29**); reverse - (5'-CTACACGACGCTCTTCCGATCT-3') (**SEQ. ID NO:30**). The PCR products were then 5'-

end labeled with  $^{32}\text{P}$  as described above and separated on a denaturing 15% polyacrylamide gel. A 100 bp ladder (NEB) was used as a marker. For Sanger sequencing, the PCR products were separated on a denaturing 15% polyacrylamide gel, and the target band was excised from gel and ethanol precipitated. The purified DNA was ligated into a vector by using NEB's PCR Cloning Kit per the manufacturer's protocol. Antibiotic-resistant colonies were selected and subjected to Sanger sequencing by Genewiz.

**Scheme 1. Synthetic route for 2 and 3:**





**Synthesis of compound 1:** Compound 1 was synthesized as previously described.<sup>5</sup> Briefly, Rink amide resin (500 mg, 345  $\mu$ mol) with a substitution level of 0.69 mmol/g was shaken in *N,N*-dimethyl formamide (DMF) for 5 min and then deprotected with 20% piperidine in DMF (10 mL, 2x 30 min) at room temperature. The resin was washed with 10 mL DMF three times. Next, 5 eq of bromoacetic acid in 10 mL DMF solution and 5 eq of DIC (*N,N'*-diisopropylcarbodiimide) were added, and the resin was shaken at room temperature for 30 min. After washing with 10 mL DMF three times, 5 eq of propargylamine in 10 mL DMF was added, and the reaction mixture was shaken at room temperature for 2 h. The bromoacetic acid coupling step was repeated and 5 eq propylamine in 10 mL DMF was added, and the reaction mixture was shaken at room temperature for 2 h. The bromoacetic acid coupling step and propylamine step were repeated. The peptoid in the resin was then treated with a solution of 2 eq Hoechst carboxylate (synthesized as previously described)<sup>9</sup>, 2 eq DIC and 2 eq DIPEA (*N,N'*-diisopropylethylamine) in 10 mL DMF. The reaction mixture was shaken at room temperature for 2 h and washed with 10 mL DMF three and then 10 mL dichloromethane (DCM) three times. The product was cleavage from resin by adding 10 mL 30% trifluoroacetic acid (TFA) in DCM and shaken 30 min at room temperature. The eluate was concentrated under vacuum, and the product was precipitated with excess amounts of ether.

The resulting yellow solid was directly treated with a solution of 1.1 eq azide<sup>2</sup>, 0.2 eq Cu(I) catalyst and 2 eq DIEA in 2 mL DMF. The reaction mixture was kept at 65 °C overnight, and the reaction process was monitored by MALDI-TOF MS. After the starting material was no longer detectable, the product was precipitated with excess amounts of ether and dissolved in 50% methanol in water with 0.1%TFA. HPLC purification was performed with a linear gradient from 0% to 100% B (methanol or acetonitrile + 0.1%TFA) in A (water + 0.1%TFA) over 60 min and a flow rate of 5 mL/min. The pure fractions were collected, and the solvent was concentrated under vacuum. Excess amounts of ether were added, and the resulting solid was obtained as product. Purity was evaluated on a reverse phase Waters Symmetry C18 5  $\mu$ m 4.6  $\times$  150 mm column at room temperature with a flow rate of 1 mL/min and a linear gradient of 0–100% B in A. Absorbance was monitored at 254 nm and 345 nm. Mass spectra were recorded on a 4800 plus MALDI-TOF/TOF analyzer. MALDI:  $[M+H]^+$  calculated: 1391.8179;  $[M+H]^+$  observed: 1391.9153.

**Synthesis of compound 2:** Chlorotrityl resin (500 mg, 580  $\mu$ mol) was activated in 1 M HCl in 10 mL DCM for 10 min at room temperature, followed by washing with DCM three times and DMF three times. Next, 5 eq of bromoacetic acid and 5 eq DIPEA in 10 mL DMF were

added, and the resin shaken at room temperature for 30 min. After washing with 10 mL DMF three times, 5 eq propargylamine in 10 mL DMF were added, and the reaction mixture was shaken at room temperature for 2 h. The resin was washed with 10 mL DMF three times and 5 eq of bromoacetic acid in 10 mL DMF solution and 5 eq DIC were added. The resin was shaken at room temperature for 30 min followed by addition of 5 eq propylamine in 10 mL DMF. The reaction mixture was shaken at room temperature for 2 h. The bromoacetic acid coupling step and propylamine step were repeated. The peptoid on the resin was then treated with a solution of 2 eq Hoechst carboxylate, 2 eq DIC and 2 eq DIPEA in 10 mL DMF solution. The reaction mixture was shaken at room temperature for 2 h and washed with 10 mL DMF three times and then 10 mL DCM three times. The product was cleaved from resin by adding 10 mL of 30% TFA in DCM and shaking the resin for 30 min at room temperature. The eluate was concentrated under vacuum, and the product was precipitated with an excess amount of ether.

The resulting solid was directly treated with a solution of 1.1 eq azide, 0.2 eq Cu(I) catalyst and 2 eq DIEA in 2 mL DMF. The reaction mixture was kept 65 °C overnight, and the reaction process was monitored by MALDI MS. After the starting material was no longer detectable, the product was precipitated with an excess amount of ether.

The Bleomycin A5 coupling reaction was performed by adding 2 eq HOAt (1-Hydroxy-7-azabenzotriazole), 2 eq HATU (hexafluorophosphate azabenzotriazole tetramethyl uronium), 5 eq DIEA, and 2 eq Copper-bleomycin A5. The reaction mixture was shaken at room temperature overnight and the product was precipitated with an excess amount of ether. The solid was dissolved in 50% acetonitrile in water with 0.1%TFA and subjected to HPLC purification. After injection of the solution, the column was washed with 50 mM EDTA (pH 6.7) for 30 min to remove the copper ion and then washed with water for another 30 min. Then the target product was separated with a linear gradient from 0% to 100% B (acetonitrile +0.1%TFA) in A (water +0.1%TFA) over 60 min and a flow rate of 5 mL/min. The pure product was obtained and characterized as described above. MALDI:  $[M+H]^+$  calculated: 1775.7767;  $[M+H]^+$  observed: 1775.4158.

**Synthesis of compound 3:** Chlorotriyl resin (500 mg, 580  $\mu$ mol) was activated in 1 M HCl in 10 mL DCM for 10 min at room temperature, followed by washing with DCM three times and DMF three times. Then, 5 eq bromoacetic acid and 5 eq DIPEA in 10 mL of DM were added, and the resin was shaken at room temperature for 30 min. After washing with 10 mL DMF three times, 5 eq propargylamine in 10 mL DMF was added, and the reaction mixture was shaken at room temperature for 2 h. The resin was washed with 10 mL DMF three times

followed by addition of 5 eq of bromoacetic acid in 10 mL DMF solution and 5 eq DIC. The resin was shaken at room temperature for 30 min and then 5 eq propylamine in 10 mL DMF were added. After shaking the mixture at room temperature for 2 h, the bromoacetic acid coupling step and propylamine step were repeated. The peptoid on the resin was then treated with a solution of 2 eq acetic anhydride ( $\text{Ac}_2\text{O}$ ) and 2 eq DIPEA in 10 mL DMF. The reaction mixture was shaken at room temperature for 30 min and washed with 10 mL DMF three times and then 10 mL DCM three times. The product was cleaved from the resin by adding 10 mL 30% TFA in DCM and shaking at room temperature for 30 min. The eluate was concentrated under vacuum, and the product was precipitated with excess amount of ether.

The Bleomycin A5 coupling reaction was performed by adding 2 eq HOAt, 2 eq HATU, 5 eq DIEA and 2 eq Copper-Bleomycin A5. The reaction mixture was shaken at room temperature overnight, and the product was precipitated with an excess amount of ether. The solid was dissolved in 50% acetonitrile in water with 0.1%TFA and subjected to HPLC purification. After injection of the solution, the column was washed with 50 mM EDTA (pH 6.7) for 30 min to remove the copper ion and then washed with water for another 30 min. Then the target product was separated with a linear gradient from 0% to 100% B (acetonitrile +0.1%TFA) in A (water +0.1%TFA) over 60 min and a flow rate of 5 mL/min. The pure product was obtained and characterized as described above. MALDI:  $[\text{M}+\text{H}]^+$  calculated: 1928.7767;  $[\text{M}+\text{H}]^+$  observed: 1928.8219.

#### Documents Cited in Examples Section

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2. Agarwal, V., Bell, G. W., Nam, J. W., and Bartel, D. P. (2015) Predicting effective microRNA target sites in mammalian mRNAs. *Elife* 4, e05005.
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4. Angelbello, A. J., and Disney, M. D. (2018) Bleomycin can cleave an oncogenic noncoding RNA. *Chembiochem* 19, 43-47.
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6. Velagapudi, S. P., Cameron, M. D., Haga, C. L., Rosenberg, L. H., Lafitte, M., Duckett, D. R., Phinney, D. G., and Disney, M. D. (2016) Design of a small molecule against an oncogenic noncoding RNA. *Proc. Natl. Acad. Sci. U. S. A.* 113, 5898-5903.

7. Costales, M. G., Matsumoto, Y., Velagapudi, S. P., and Disney, M. D. (2018) Small molecule targeted recruitment of a nuclease to RNA. *J. Am. Chem. Soc.* 140, 6741-6744.

8. Fang, R., Moss, W. N., Rutenberg-Schoenberg, M., and Simon, M. D. (2015) Probing Xist RNA structure in cells using targeted structure-seq. *PLoS Genet.* 11, e1005668.

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10. Pushechnikov, A., Lee, M. M., Childs-Disney, J. L., Sobczak, K., French, J. M., Thornton, C. A., and Disney, M. D. (2009) Rational design of ligands targeting triplet repeating transcripts that cause RNA dominant disease: application to myotonic muscular dystrophy type 1 and spinocerebellar ataxia type 3. *J. Am. Chem. Soc.* 131, 9767-9779.

All patents and publications referred to herein are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference in its entirety.

**CLAIMS**

What is claimed is:

1. A method comprising  
contacting a library of RNA sequences and an effective amount of a compound comprising a conjugate of an RNA-cleaving moiety and an RNA-binding small molecule to cleave the RNA of the library at a binding site of the compound to the RNA to provide a cleaved RNA fragment; and  
amplifying and sequencing the cleaved RNA fragment, thereby identifying the site of binding of the small RNA-binding molecule to the RNA of the library which is associated with the site of cleavage.
2. The method of claim 1, wherein the RNA-binding small molecule comprises an N-methyl-piperazinyl-bis-benzimidazole group.
3. The method of claim 1 or 2, wherein the RNA-cleaving moiety comprises bleomycin.
4. The method of claim 3, wherein the bleomycin is bleomycin A5.
5. The method of claim 2, 3, or 4, wherein the RNA-binding small molecule is Targaprimir-96.
6. The method of any one of claims 1 to 5, wherein the RNA sequence is from an oncogenic non-coding RNA precursor, and the compound binds to the oncogenic non-coding RNA precursor.
7. The method of claim 6, further comprising contacting a cell expressing the non-coding RNA precursor with an effective amount of the compound.
8. The method of claim 6 or 7, wherein the oncogenic non-coding RNA precursor comprises oncogenic primary microRNA-96 (pri-miR-96).

9. The method of any one of claims 6 to 8, wherein the compound is a covalent conjugate of Targaprimir-96 and bleomycin A5.
10. The method of claim 1, wherein the RNA sequence of the library is from FOXO1.
11. The method of claim 10, further comprising enhancing expression of FOXO1 protein in breast cancer cells by contacting the cells with an effective amount of the compound.
12. The method of claim 11, wherein the contacting comprises administering to a human patient.
13. The method of any one of claims 10 to 12, wherein the compound is a covalent conjugate of Targaprimir-96 and bleomycin A5.
14. A method of triggering apoptosis in triple negative breast cancer cells, comprising contacting the triple negative breast cancer cells with an effective amount of the compound identified in the method of claim 1.
15. The method of claim 14, wherein the contacting comprises administering to a human patient.
16. The method of claim 14 or 15, wherein the compound is a covalent conjugate of Targaprimir-96 and bleomycin A5.
17. A method of treating triple negative breast cancer, comprising administering to a patient afflicted therewith an effective dose of a compound identified in the method of claim 1.
18. The method of claim 17, wherein the breast cancer comprises expression of oncogenic primary microRNA-96 (pri-miR-96).
19. The method of claim 17 or 18, wherein the compound is a covalent conjugate of Targaprimir-96 and bleomycin A5.

20. The method of claim 1, wherein the RNA sequence library comprises a transcriptome.
21. The method of claim 20, wherein the transcriptome is viral.
22. The method of claim 20, wherein the transcriptome is mammalian.
23. The method of claim 20, wherein the transcriptome is bacterial.
24. The method of claim 1, wherein the RNA sequence library comprises one or more of synthetic, semi-synthetic, or natural RNA.
25. The method of claim 1, wherein the RNA sequence library comprises the genome of an RNA virus.
26. The method of claim 1 carried out in vitro.
27. The method of claim 1 carried out in living cells.
28. The method of claim 27, wherein the cells are virally- or bacterially-infected cells.
29. The method of claim 1, wherein a set of RNA sequences and a set of compounds comprising candidate RNA-binding small molecules are assayed in a 2-dimensional parallel array.
30. A compound comprising a conjugate of an RNA-cleaving moiety and a RNA-binding small molecule.
31. The compound of claim 30, wherein the RNA-cleaving moiety comprises bleomycin.
32. The compound of claim 30 or 31, wherein the RNA-binding small molecule comprises N-methyl-piperaziny1-bis-benzimidazole group.
33. The compound of claim 32, wherein the RNA-binding small molecule is Targaprimir-96.

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Fig. 1

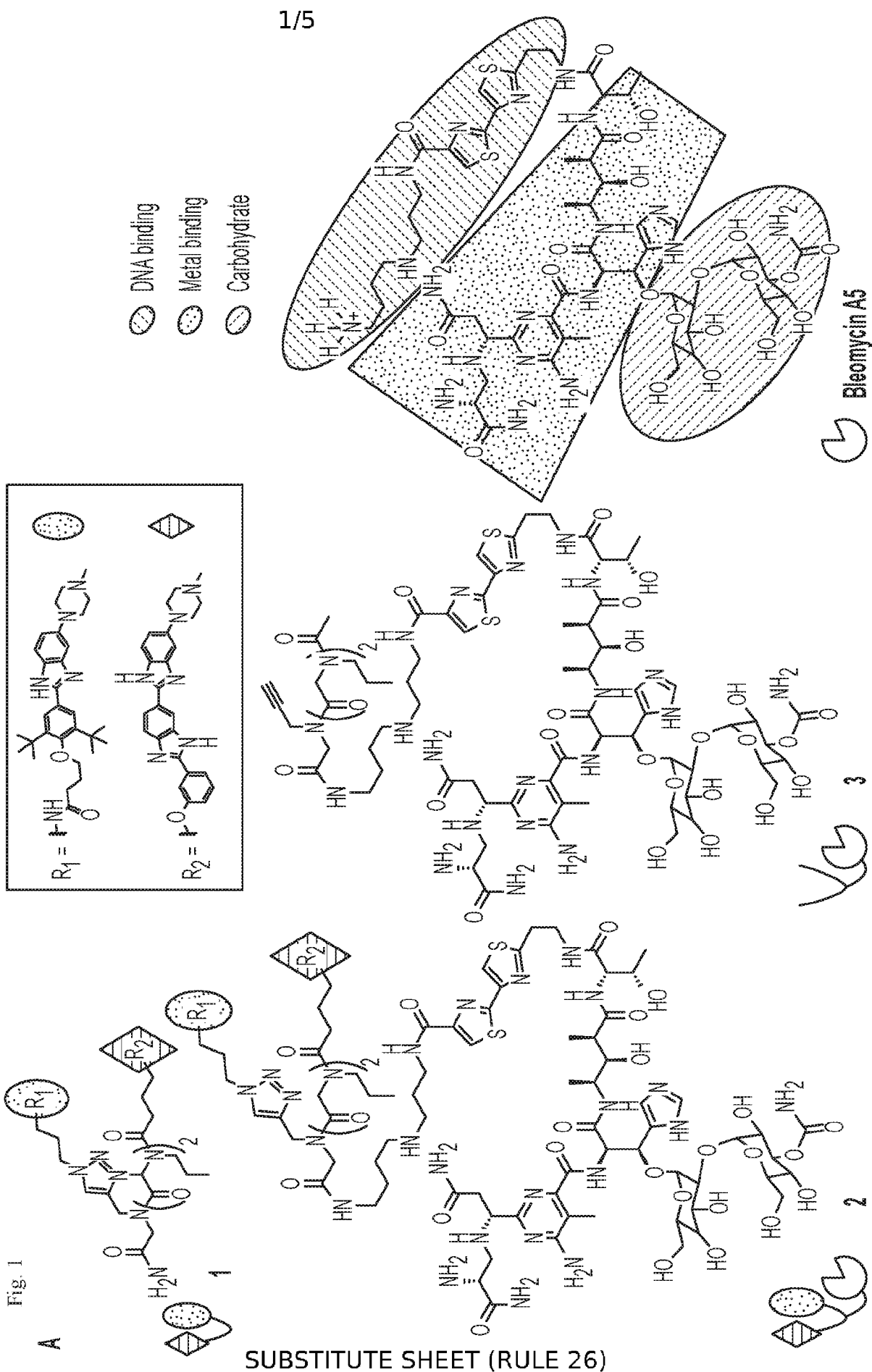
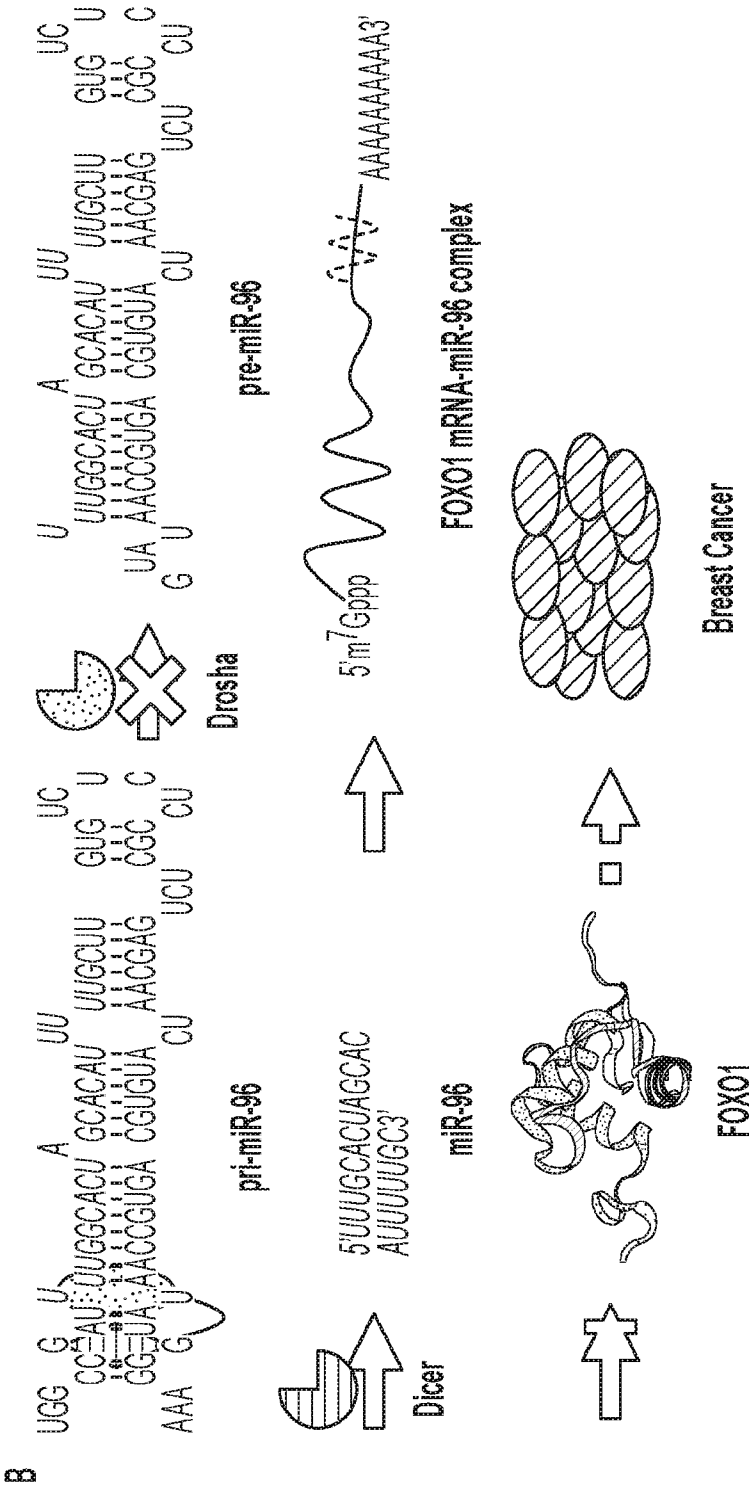
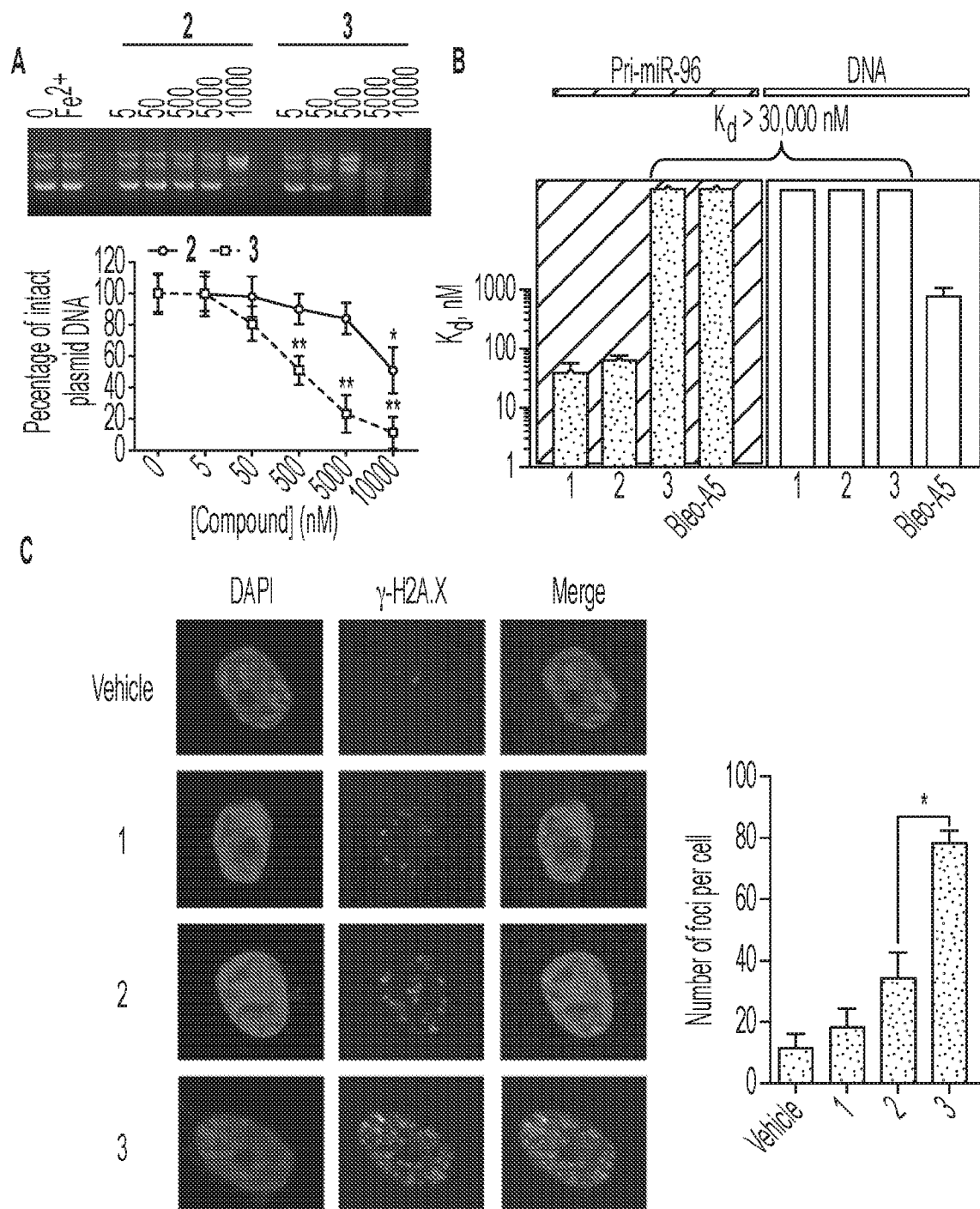


Fig. 1



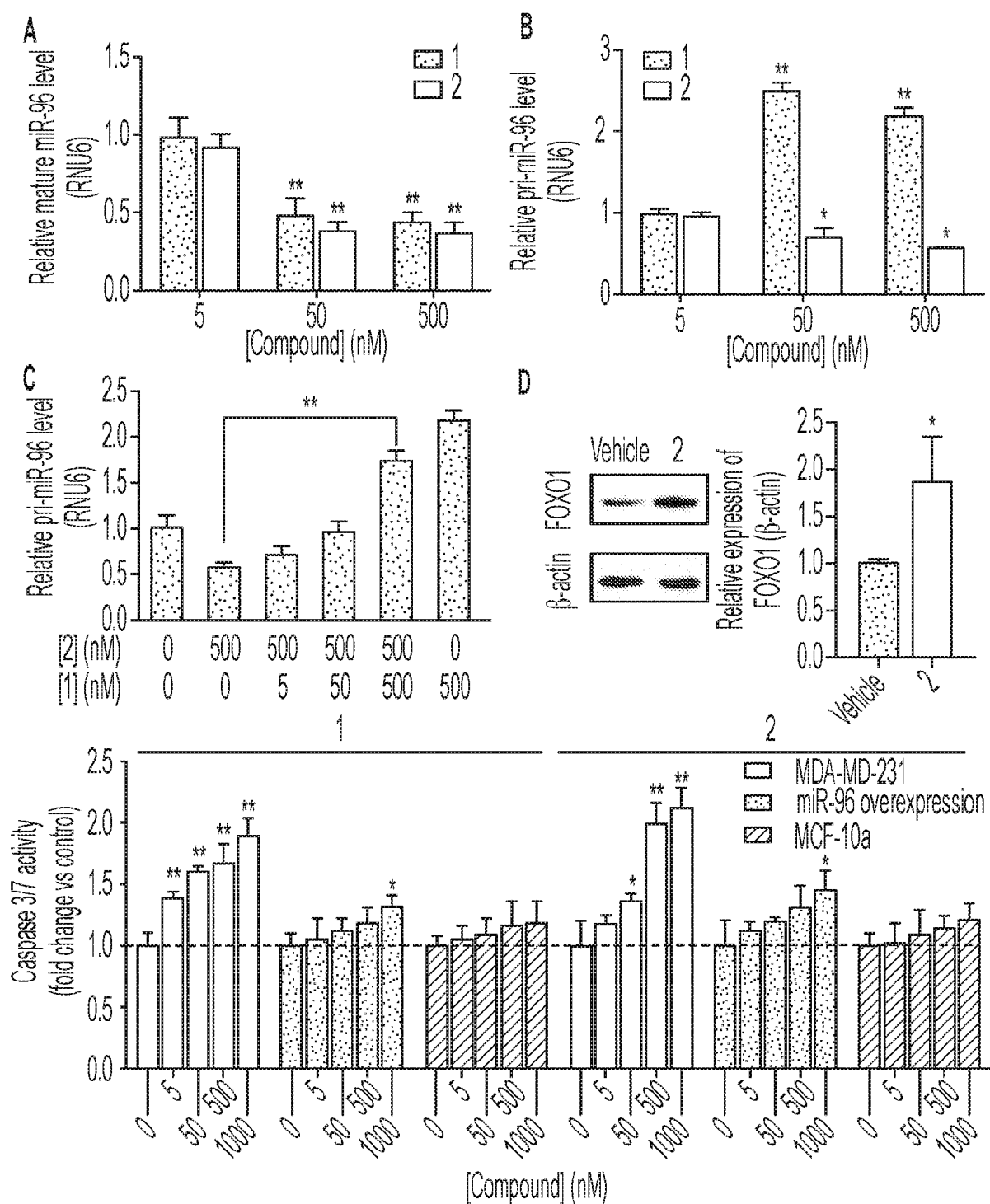
3/5

Fig. 2



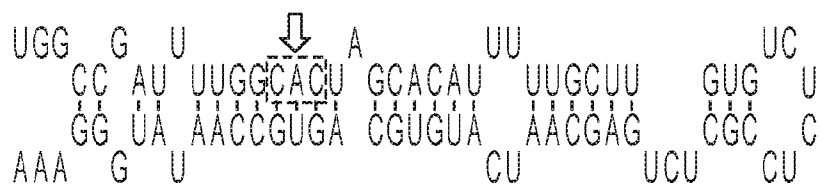
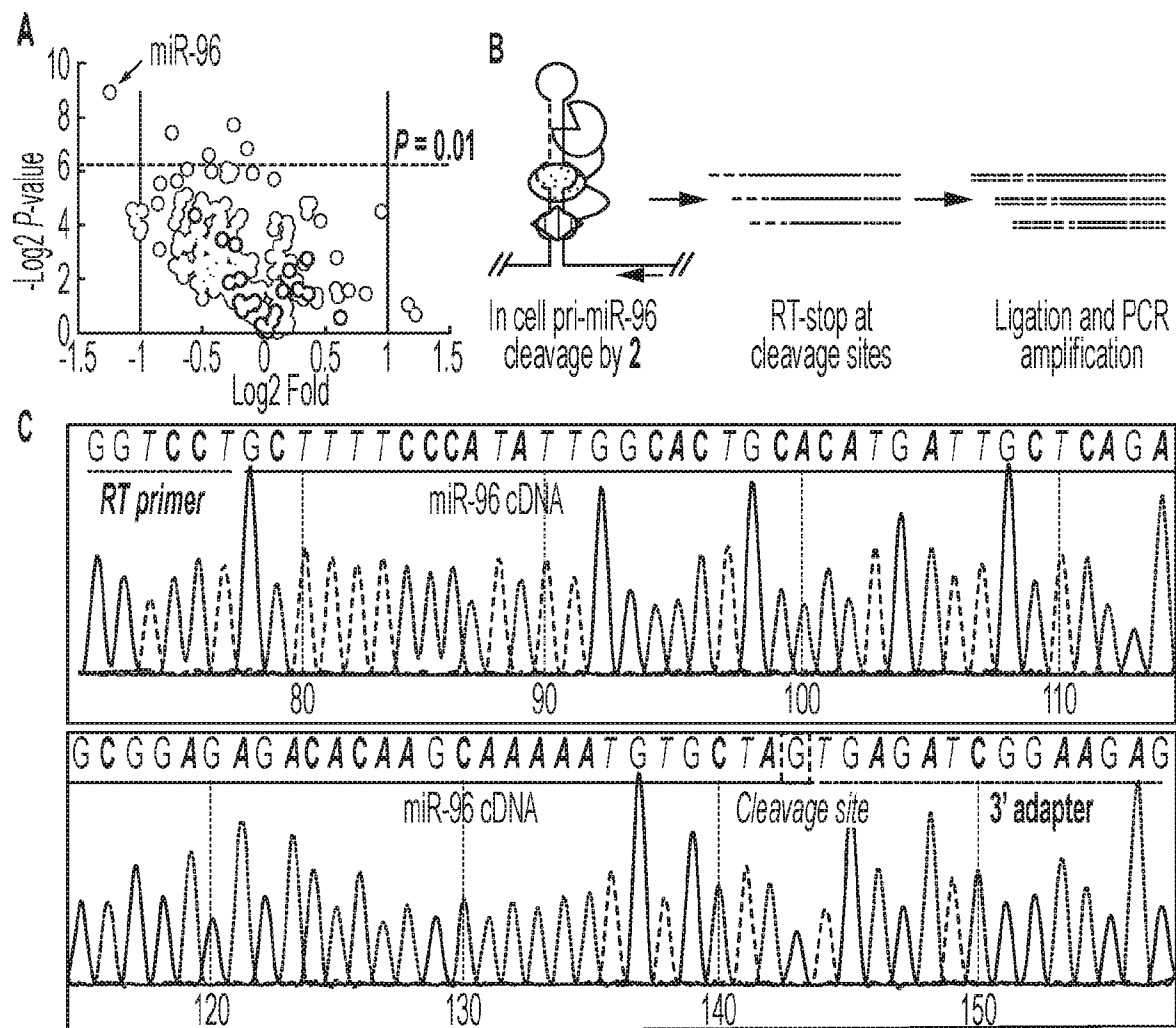
4/5

Fig. 3



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Fig. 4



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/53179

## A. CLASSIFICATION OF SUBJECT MATTER

IPC - C40B 30/00; C40B 40/00; C40B 40/06; C12N 15/113; A61K 47/54; A61K 47/55 (2019.01)

CPC - C40B 30/00; C40B 40/00; C40B 40/06; C12N 15/113; A61K 47/545; A61K 47/55

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category*     | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                            | Relevant to claim No.             |
|---------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------|
| X<br>---<br>Y | WO 2018/098297 A1 (THE SCRIPPS RESEARCH INSTITUTE) 31 May 2018 (31.05.2018).<br>Especially para [0015], [0030], [0036], [0110].                                                               | 1-4, 24, 26, 29<br>-----<br>10-13 |
| Y             | VELAGAPUDI et al. Design of a small molecule against an oncogenic noncoding RNA. Proc Nat Acad Sci 24 May 2016, Vol 113, No 21, Pages 5898-5903. Especially abstract, pg 5898 col 2 para 1-2. | 10-13                             |

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

30 December 2019

Date of mailing of the international search report

28 FEB 2020

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Lee Young

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/53179

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely: |
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 5-9  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-----Go to Extra Sheet for continuation-----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
  - 1-4, 10-13, 24, 26, 29 limited to the library of RNA sequences is from FOXO1 (claim 10) and the method is performed in vitro (claim 26)

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT  
Information on patent family members

International application No.

PCT/US 19/53179

Continuation of Box III: Observations where Unity of Invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I+: Claims 1-4, 10-13, 20-29, drawn to a method comprising contacting a library of RNA sequences with a compound to identify a site(s) of cleavage.

The method to identify the site of cleavage in a library of RNA sequences will be searched to the extent that the library is the first named library, the RNA library sequence is from the gene FOXO1 (claim 10) and the method is carried out in vitro (claim 26). It is believed that claims 1-4, 10-13, 24, 26, 29 read on this first named invention and thus these claims will be searched without fee to the extent that they encompass an RNA library of FOXO1 transcript sequences and the method is performed in vitro. Additional libraries of RNA sequences and methods of carrying out the method will be searched upon payment of additional fees. Applicant must specify the claims that encompass any additional elected RNA sequence libraries or methods of carrying out the method. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be: RNA sequence library is a genome of an RNA virus (claim 25) and the method is carried out in vitro (claim 26) (claims 1-4, 24-26, 29).

Group II: Claims 14-19, drawn to a method of treating triple negative breast cancer cells

Group III: Claims 30-33, drawn to a composition comprising a conjugate of an RNA -cleaving moiety and an RNA-binding small molecule.

The inventions listed as Groups I+, II, III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I+ has the special technical feature of contacting a library of RNA sequences with a compound to identify a site of cleavage, not required by Groups II or III.

Group II has the special technical feature of a method of treating triple negative breast cancer cells by administering or contacting with a compound, not required by Group I+ or III.

Group III has the special technical feature of a composition of a compound comprising a conjugate of an RNA-cleaving moiety and an RNA-binding small molecule, not required by Groups I+ or II.

Common Technical Features:

1. Group I+ and Group III share the common technical feature of a compound comprising a conjugate of an RNA-cleaving moiety and an RNA-binding small molecule.

2. Group I+ claim 1 is shared by Group II.

However, said common technical features do not represent a contribution over the prior art, and are disclosed by WO 2018/098297 A1 to The Scripps Research Institute (hereinafter "TSRI") [published 31 May 2018].

As to common technical feature #1, TSRI discloses a compound comprising a conjugate of an RNA-cleaving moiety and an RNA-binding small molecule (para [0036]; "Small molecules that selectively cleave RNAs could have broad applicability and extend targeting by such probes to a variety of cellular RNAs. Therefore, small molecules that cleave r(CUG).sup.exp in cells were developed. The natural product bleomycin cleaves RNA. in vitro and was conjugated to [compound] 1, affording 2H-K4NMeS [compound 3] (Fig. 3a), in vitro, a model of r(CUG).sup.exp was cleaved by [compound] 3 between U and G nucleotides"; para [0030]; "Initial Compound Design. A compound was designed to target r(CUG).sup.exp using a bis-benzimidazole H as a ligand for the 1\*1 nucleotide UU internal loops found in r(CUG).sup.exp (Fig. 1a). Dimeric display of H modules on an N-methyl peptide backbone generated compound 2H-K4NMeS (1), allowing recognition of two adjacent UU loops and the distance between them (Fig. 1a). [Compound] 1 has improved metabolic stability compared to a previously reported compound").

-----continued on next sheet-----

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/53179

-----continued from previous sheet-----

As to common technical feature #2 (claim 1), TSRI discloses a method comprising contacting a library of RNA sequences (para [0015]; "Such interactions are identified by screening a small molecule library against a library of RNA motifs. Inforna has successfully -facilitated the design of potent small molecule modulators of several RNA repeat expansion disorders and cancer related miRNAs"), and an effective amount of a compound comprising a conjugate of an RNA-cleaving moiety and an RNA-binding small molecule (para [0036], [0030]) to cleave the RNA of the library at a binding site of the compound to the RNA (para [0036]) to provide a cleaved RNA fragment (para [0036]); and amplifying (para [0159]; "Primer sets represent the positions of the DMPK mRNA that were amplified after the mRNA was digested to separate the r(CUG).sup.exp-containing fragments from the rest of the mRNA") and sequencing the cleaved RNA fragment (para [0162]; "Sequencing analysis of the samples was also performed"), thereby identifying the site of binding of the small RNA-binding molecule to the RNA of the library which is associated with the site of cleavage (para [0036]).

As the common technical features were known in the art at the time of the invention, they cannot be considered common special technical features that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I+, II, III lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Item 4 (cont.) Claims 5-9 are multiple dependent claims and are not drafted according to the second and third sentences of PCT Rule 6.4(a).