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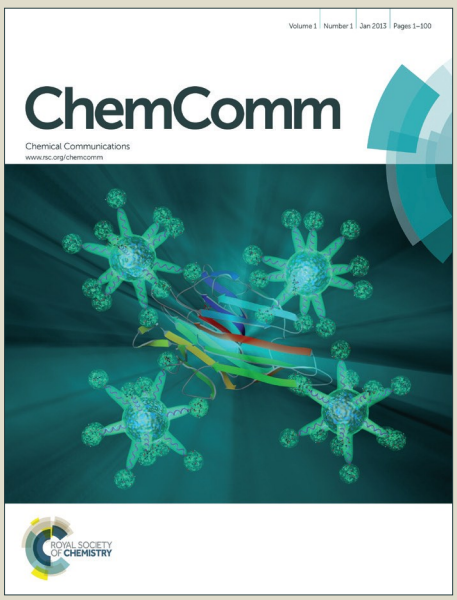

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Journal Name

COMMUNICATION

Targeting VEGF with LNA-Stabilized G-rich Oligonucleotide for Efficient Breast Cancer Inhibition

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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In this study, we investigated the efficacy of an LNA (locked nucleic acid)-modified DNA aptamer named RNV66 targeting VEGF against various breast cancer cell lines. Our results demonstrate that RNV66 efficiently inhibits breast cancer cell proliferation both *in vitro* and *in vivo*. Introduction of LNA nucleotides were crucial for higher efficacy. Furthermore, the binding interaction of RNV66 with VEGF was investigated using molecular dynamic simulations leading to the first computational model of the LNA aptamer-VEGF complex blocking its interaction with VEGF-receptor.

Chemically-modified nucleic acid analogues offer great promise towards the development of oligonucleotide-based therapeutics. FDA has approved three drugs of this class, namely Vitravene, a 21mer phosphorothioate-modified antisense oligonucleotide (ASO) against cytomegalovirus retinitis, Macugen, a 27mer aptamer oligonucleotide modified with 2'-*O*-methyl-RNA and 2'-fluoro-DNA nucleotides for the treatment of age-related macular degeneration, and Kynamro, another ASO modified with 2'-*O*-methoxyethyl-RNA chimera against familialhypercholesterolemia.[1] With four candidates currently in phase I and II clinical trials, locked nucleic acid (LNA, Figure 1)-modified oligonucleotides offer great potential towards therapeutic development.[2] LNA is one of the most prominent nucleotide analogues developed in recent years because of its unprecedented target binding affinity and remarkable nuclease resistance.[3] Since its invention, several independent laboratories have confirmed that LNA offers substantially increased potency *in vitro* and *in vivo* over other chemistries when applied as antisense constructs, siRNAs and LNA-antimiRs (miRNA targeting using LNA-DNA mixmers), and that LNA is well tolerated in animals.[4-6] Compared to LNA, unlocked nucleic acid (UNA, Figure 1) is a conformationally flexible analogue that recently showed great promise in gene silencing technologies and also to modulate aptamer binding affinity.[7]

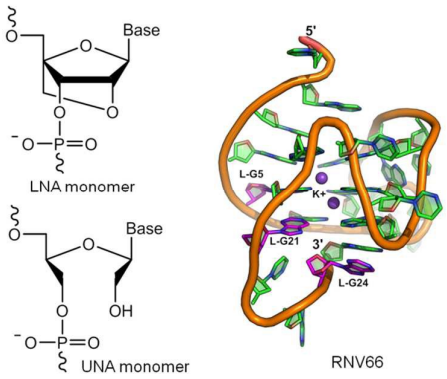

Figure 1. Structural representations of LNA and UNA monomers and an energy minimized structure of LNA-modified aptamer RNV66.

Breast cancer is currently the most diagnosed cancer form in women globally[8] with approximately 1 in 10 women developing it during their lifetime despite great advances in our understanding of the cancer biology of breast cancer. Effective treatments with fewer side-effects are thus urgently required. Vascular endothelial growth factor (VEGF) plays a vital role in regulating angiogenesis, an important process for tumor growth and metastasis.[9] A higher density of the microvasculature and an over-expression observed in breast cancer makes VEGF an attractive therapeutic target.[10] A humanized monoclonal antibody, Bevacizumab (Avastin), targeting VEGF-165 is currently used for the treatment of metastatic colorectal cancer and renal cell carcinoma in combination with other drugs. It's use, however, has been reported to be affiliated with severe side effects.[11,12] Although Bevacizumab was also approved in some countries for the treatment of metastatic breast cancer in patients with HER2- negative tumors, the medical benefits were very modest in addition to severe side effects and it was later withdrawn from th e market for this indication.[13]

Nucleic acid aptamers are powerful alternative therapeutic molecules with the prospect of target specific therapy possessing several advantages over antibody-based approaches.[14] LNA

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Electronic Supplementary Information (ESI) available: Sequences, experimental protocol, additional figures and computational methods. See DOI: 10.1039/x0xx00000x

nucleotides are ideal candidates to circumvent the poor nuclease resistance and low target binding affinity of aptamers composed of natural nucleotide monomers.[3] We have extensively investigated the applicability of LNA nucleotides in this context.[15] Very recently, we have reported an NMR structure of RNV66 (5'-TGTG**G**^{L}GGGTGGACGGGCCGG**G**^{L}TA**G**^{L}A-3'), an LNA-modified DNA aptamer[16] variant of a DNA aptamer[17] targeting VEGF-165 and -121 isoforms (VEGF-A) with very high affinity and specificity. We observed that LNA nucleotide incorporations were important to induce a stable G-quadruplex structure with a single conformation unlike the multiple conformations adopted by the DNA aptamer V7t1.[17] Herein, we report the efficacy of RNV66 to inhibit the proliferation of breast cancer cells *in vitro* and *in vivo*. We further report the first computational model of an oligonucleotide-VEGF complex by molecular dynamic simulations.

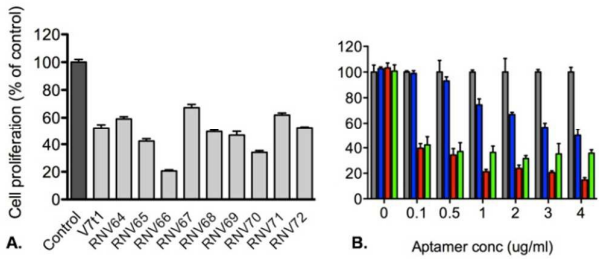

Figure 2. In vitro evaluation of various LNA and UNA-modified VEGF aptamers (4 µg/mL). A. MTT-based MCF-7 breast cancer cell proliferation assay using various candidates; B. HUVEC proliferation after the treatment with a non-targeting control sequence (grey), V7t1 (blue), RNV66 (red) and RNV70 (green), Y axis same as in A.

First, RNV66 and a series of other LNA and UNA (unlocked nucleic acid)[7] modified aptamer constructs (Table S1) were synthesized based on V7t1[17] and their therapeutic efficacy was investigated using MCF-7 breast cancer cell lines. We performed MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), a dye-based colorimetric cell viability assays[18] 48 h after treatment with the aptamer candidates. The results showed that RNV66, among other candidates tested, significantly inhibited the proliferation of breast cancer cells *in vitro* (Figure 2A). A UNA modified candidate RNV70 was also found to be effective but not at the level as RNV66. Notably, the unmodified DNA variant V7t1 was not as efficient as RNV66 (Figure 2A). In parallel, a non-VEGF targeting control sequence showed no inhibition (Control; Figure 2A). VEGF-ELISA experiments were also carried out to detect the level of VEGF in MCF-7 cell suspension after the treatment with RNV66 for 48 h. There was no detectable level of VEGF content which is indicative of efficient VEGF targeting, whereas significant amount of VEGF was detected after treatment with the control sequence and the unmodified DNA aptamer V7t1 (Figure S1). To draw additional insights on VEGF targeting specificity of RNV66, we conducted a cell proliferation assays using Human Umbilical Vein Endothelial Cells (HUVECs) as a model *in vitro* angiogenic system.[19] Results showed that RNV66 effectively blocked HUVEC proliferation whereas the non-targeting control sequence did not show any inhibition (Figure 2B).

After the initial experiments, a colony formation assay was performed using MCF-7 cells for 14 days after the treatment with RNV66, V7t1 and a non-targeting control sequence. The result upon visual observation of the methylene-blue stained cells, demonstrated that RNV66 efficiently inhibited breast cancer cell proliferation whereas the effect was very minimal with V7t1 and there was no inhibition with the control sequence (Figure S2). We then examined the efficacy of RNV66 in triple negative breast cancer cell lines such as MDA-MB-231 and HS578T. Cell viability assays 48 h after the treatment revealed that RNV66 efficiently inhibited proliferation of both MDA-MB-231 and HS578T cells (Figure S3). As observed with MCF-7 cells, V7t1 was not as efficient as RNV66 and the control sequence did not show any inhibition (Figure S3). Confocal-microscopy-based experiments were also initiated to detect the level of VEGF. First, MDA-MB-231 cells were incubated with anti-VEGF antibody after treating with RNV66, V7t1 and non-targeting control. Later, a secondary antibody labelled with a fluorescent dye was added and incubated for 1h. The results clearly showed that RNV66 substantially reduced intracellular VEGF levels (Figure S4). In addition to transfecting aptamers into cells, we have added RNV66 directly to the media of different cell lines for 48 h then measured cell proliferation (Figure S5). These experiments also showed that RNV66 inhibited proliferation of breast cancer and HUVEC cell lines (Figure S5). Collectively, our results suggest that RNV66-derived inhibition of cell proliferation likely occurs through multiple mechanisms including both extracellular and intracellular inhibition of VEGF. Extracellular targeting of VEGF is well documented[11], but how RNV66 can alter the intracellular VEGF pool is less clear. It is possible that RNV66 can internalize in a receptor independent manner, but the exact pathway needs to be further elucidated.

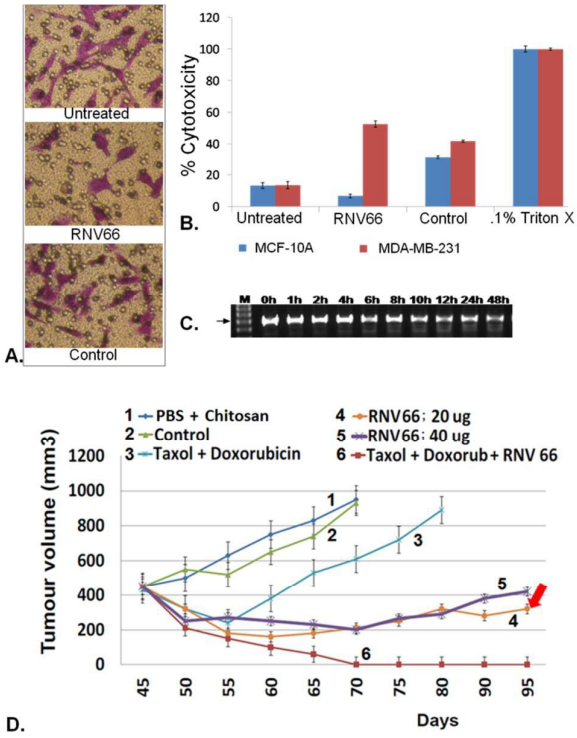

Figure 3. *In vitro* and *in vivo* analysis of RNV66. A. Migration assay; B. Cytotoxicity assay; C. Serum stability; D. *In vivo* efficacy evaluation by intratumoral injection.

To further evaluate the applicability of RNV66, we also tested its efficacy using HeLa cervical cancer cells *in vitro*. The experiment revealed that RNV66 efficiently inhibited the proliferation of HeLa cells (Figure S5), indicating that the effect is not limited only to breast cancer cell lines, and that RNV66 can also be useful for the treatment of other solid cancers. To assess the effect of RNV66 on

cancer metastasis, the migration potential of MDA-MB-231 cells was investigated. The experiment revealed that RNV66 reduced the migration capability of MDA-MB-231 breast cancer cells whereas the non-targeting control sequence was not effective (Figure 3A).

We also performed *in vitro* lactate dehydrogenase (LDH) release-based cytotoxicity assays[20] in normal breast epithelial cells (MCF-10A) and in breast cancer cells (MDA-MB-231). The results showed that RNV66 has no significant toxic effects to normal breast epithelial cells where VEGF is generally expressed at normal levels, whereas it is toxic to breast cancer cells where VEGF is highly over-expressed (Figure 3B). Similar results were observed in normal intestinal epithelial cells and colon adenocarcinoma cells (Figure S7). As a prelude to *in vivo* studies in breast cancer mice models, the stability of RNV66 was first investigated in serum for 48 h. remarkably, we found that RNV66 was stable even after 48 h of incubation (Figure 3C). We speculated that this might be due to a combination of two distinct structural features, namely its G-quadruplex structure (Figure 1) and the incorporation of LNA nucleotides (known to induce high nuclease resistance).[3]

After the successful series of *in vitro* studies, the *in vivo* efficacy of RNV66 was investigated using a mouse breast cancer model (see supplementary information for animal ethics policy and experimental details). The experiment included six treatment groups with six mice in each and all mice were inoculated with 4T1 cells. After 45 days of tumor growth, individual groups were treated with PBS+chitosan, a non-VEGF targeting control sequence, taxol+doxorubicin (known drug combination as positive control), RNV66 (20 ug), RNV66 (40 ug) and RNV66+Taxol+doxorubicin by intra-tumoral injection. Chitosan-based nano-formulation was performed for all test candidates. The results were truly remarkable in that RNV66 efficiently inhibited breast cancer progression (Figure 3D, lines 4 and 5) and the combination of RNV66+Doxorubicin+Taxol (Figure 3D, line 6) completely resolved the tumor burden. These data provide evidence that RNV66 is very promising candidate for elaborate pre-clinical investigations.

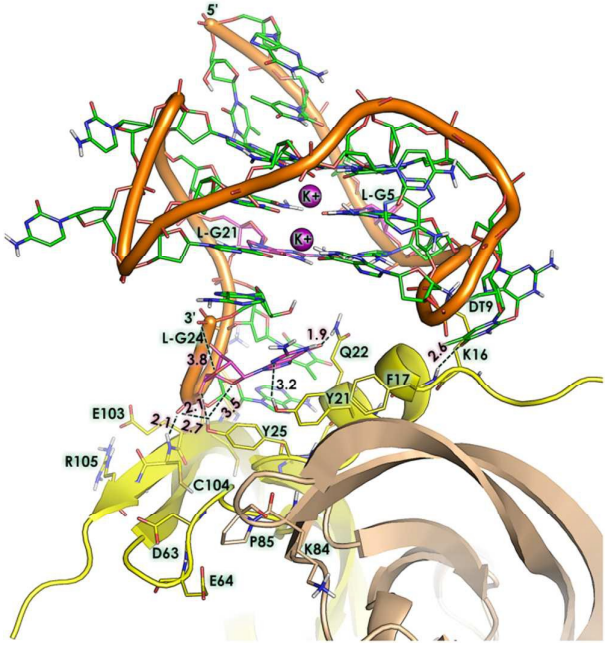

Figure 4. Energy minimised binding interactions of RNV66 with VEGF as determined by molecular dynamic simulations.

Next, we initiated a computational investigation to gain molecular insights of the binding interactions between RNV66 and VEGF. The NMR structure of RNV66[15] and the crystal structure of VEGF[21] were used for molecular docking experiments followed by molecular dynamics (MD) simulations. Interactions between RNV66 and VEGF were assessed using MM-GBSA (Molecular Mechanics Generalized Born Solvent Accessibility) binding free energies (See supplementary material). Of the 50 best poses obtained by docking, the majority of the poses show that RNV66 binds to VEGF at the site where the X-ray structure of the VEGF/VEGFR2 complex showed the proteins interactions. It is thus appealing to surmise that RNV66 acts by sterically blocking interactions between VEGF and VEGF-R2. Analyzing interactions between RNV66 and VEGF in detail show that in the top 50 poses there is a nearly equal number of poses showing interactions between two LNA residues of RNV66 (LNA-G5 or LNA-G24) and VEGF. When interacting with LNA-G24, the G-quadruplex binds end-on to VEGF while interactions with LNA-G5 are obtained in a side-on binding mode. Representative poses of either type were investigated using the MM-GBSA method. The MM-GBSA results revealed that a pose interacting with LNA-G24 of RNV66 binds to VEGF (ΔG_{Bind}= −60.26 ± 6.52 kcal^{-1}) more strongly when compared to other possible poses (Table S2). The complex where LNA-G24 of RNV66 interacts with VEGF reveals that LNA-G24 contacts residues such as Y21 and Q22 that are shown to be vital for VEGF receptor binding.[21,22] Moreover, residues such as D63, E64, K84 and P85 also interact with RNV66 through backbone phosphate groups or nucleotide moieties (Figure 4). In addition to RNV66 binding to VEGF, we also investigated possible RNV66-VEGF complex binding mode to VEGFR-2[22] using protein-protein docking experiments. These docking experiments showed that when RNV66 is bound to VEGF, proper positioning of VEGF at the VEGFR-2 active site is completely lost, primarily due to loss of shape complementarity (Figure S5). Similar docking experiments with free VEGF reproduced the native VEGF-VEGFR2 complex well (see Supp. Mater) so the loss of proper binding can be attributed to interactions with RNV66.

This experiment was further extended to analyze the importance of LNA nucleotide (LNA-G24) of RNV66 in VEGF binding. For this purpose, LNA-G24 was replaced by its natural counterpart, DNA-G in the RNV66 structure and docking experiments were performed as mentioned above. Results revealed that the DNA-G24 nucleotide reduced the affinity between RNV66 and VEGF considerably (ΔG_{Bind} = −60.26 ± 6.52 kcal^{-1} to −27.58 ± 10.76 kcal^{-1}). This observation strongly supports that the conformationally constrained LNA-G nucleotides not only stabilize the structure of the RNV66 G-quadruplex, but is also contributing to its VEGF binding.

To summarize, an LNA-modified G-quadruplex forming oligonucleotide (RNV66) was found to be very effective at inhibiting breast cancer cell proliferation *in vitro* and *in vivo*. Molecular docking followed by molecular dynamic simulations suggested possible interactions between RNV66 and VEGF and demonstrated that positioning of LNA nucleotides are important for high VEGF binding affinity, thus reinforcing the experimental observations. Based on the results, we firmly believe that RNV66 is a potential therapeutic candidate that offers great promise for future pre-clinical investigations.

RNV greatly acknowledges the financial support from the McCusker Charitable Foundation, Western Australian Neuroscience Research Institute and The University of Queensland and also from the Nucleic Acid Center, University of Southern Denmark (EVOLNA). JW

acknowledge support from the European Research Council under the European Union's Seventh Framework program (FP7/2007-2013)/ERC Grant agreement no. 268776. The QIMR Berghofer group was supported by a National Health and Medical Research Council of Australia project grant (1058415). SLE is supported by a Fellowship from the National Breast Cancer Foundation (NBCF) Australia.

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