Precise excision of targeted RNA by third-generation antisense (3GA) oligonucleotides

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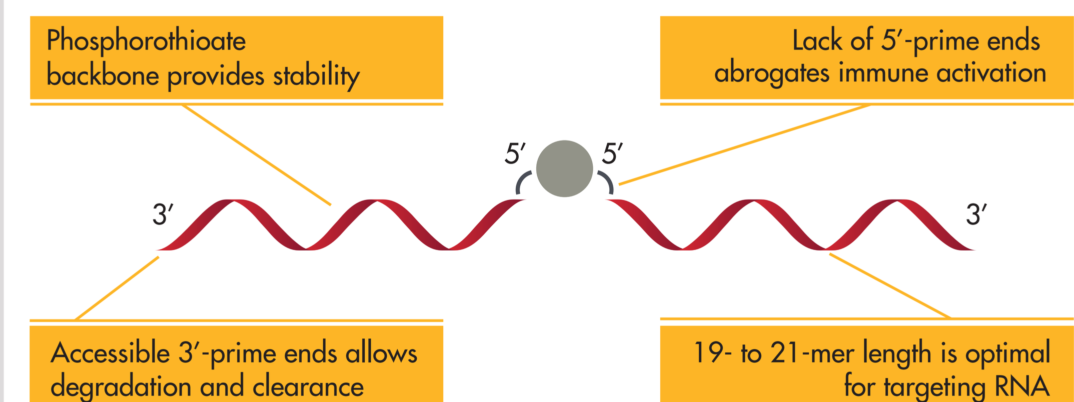
INTRODUCTION

The development of antisense technology is in its fourth decade.[1] Over the years, a large number of publications have reported the use of antisense oligonucleotides to silence genes in cell-based experiments, *in vivo* disease models, and clinical proof-of-concept studies. However, to date, the technology has had limited commercial success largely due to limitations including off-target effects[2] and unintended immune activation.[3]

Insights gained from our earlier work with first-generation antisense (1GA)[4] and second-generation antisense (2GA)[5] oligonucleotides and from our understanding of the interaction of nucleic acids with endogenous Toll-like receptors[6,7] have allowed us to design third-generation antisense (3GA)[8] oligonucleotides to address the limitations of previous antisense generations.

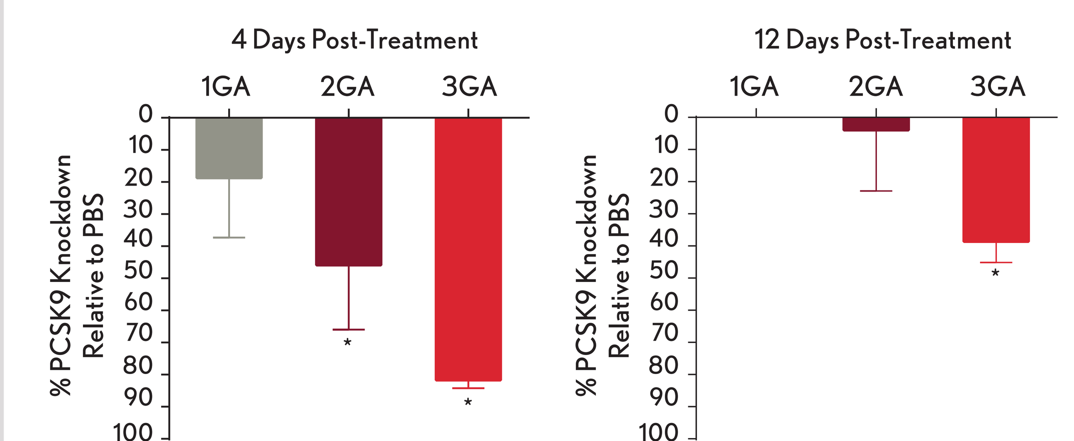
One of our key observations was that an accessible 5′ end is required for immune activation.[9] In the 3GA design, two antisense segments are linked together via their 5′ ends, thereby blocking the accessibility of the 5′ end. Moreover, the two 3′ ends of 3GA are exposed, allowing the compound to be degraded, thereby limiting tissue accumulation.[10] Previous studies have also shown that 3GA has an optimal length of 19- to 21-mer for effective gene silencing.[8]

Rational design of 3GA



Comparative activity of 3GA vs 1GA and 2GA

We had previously compared the gene-silencing activity of 3GA versus 1GA and 2GA *in vivo*. C57BL/6 mice were injected subcutaneously (s.c.) with 15 mg/kg of antisense compounds in 100 µL PBS for 5 consecutive days. Liver tissues were collected 4 and 12 days after the last dose and analyzed for PCSK9 RNA expression.



3GA exerted more potent knockdown of PCSK9 mRNA at 4 days post-treatment compared to 1GA and 2GA. At 12 days post-treatment, there was no knockdown observed with 1GA and 2GA while 3GA continued to show sustained PCSK9 mRNA knockdown. *$P<0.05$ vs PBS, n=3, mean + SEM are shown.

Aim of the study

The 3GA structure consists of two 19-mer oligonucleotide segments linked via their 5′ ends, with each segment identical to 1GA. Both 1GA and 3GA are phosphorothioate oligodeoxynucleotides expected to activate RNase H-mediated excision. Therefore, it is intriguing that 3GA exerts more potent gene-silencing activity than 1GA and silences for a longer duration.

We conducted the current study to gain insight into the mechanisms underlying the increased potency and duration of 3GA activity. In these studies, we employed RLM-RACE to identify excision sites in the target RNA and discern any differences between 1GA and 3GA.

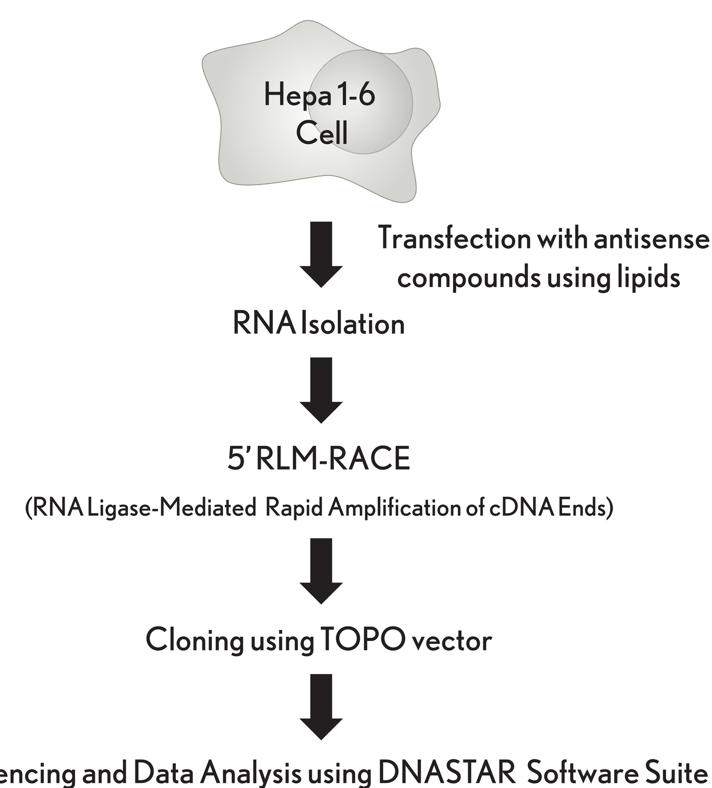
Structure and chemistry of compounds used in this study

Compound	Sequence
PCSK9 mRNA	651 - GAACCUACAUUGUGGUGCUGAUGGAGGAGACCCAGAGGCUACAGAUUGAA - 700
1GA	3′- CCACGACTACCTCCTCTGG -5′
2GA	3′- CCACGACTACCTCCTCUGG-5′
3GA	3′- CCACGACTACCTCCTCTGG -X-GGTCTCCTCCATCAGCACC-3′
Control 1	5′-GGTCTCCTCCATCAGCACC-X- CCACGACTACCTCCTCTGG -5′
Control 2	3′- CCACGACTACCTCCTCTGG - CCACGACTACCTCCTCTGG-3′
siRNA	5′-GGUGCUGAUGGAGGAGACCdTdT-3′ 3′-dTdT CCACGACUACCUCCUCUGG -5′

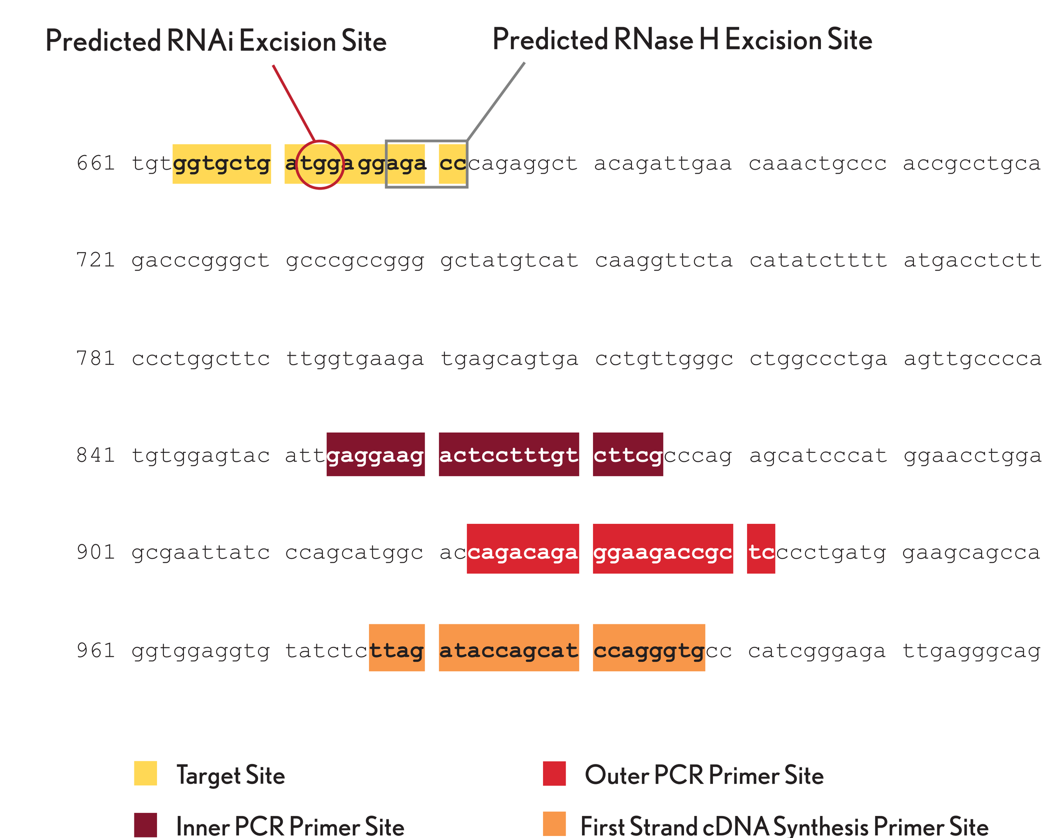
1GA = phosphorothioate oligodeoxynucleotide; 2GA = phosphorothioate oligodeoxynucleotide with four 2′-OMe ribonucleotides at both 5′ and 3′ ends; 3GA = 38-mer compound which consists of two 19-mer phosphorothioate oligodeoxynucleotide segments linked via their 5′ ends with a glycerol linker (X); Control 1 = 38-mer compound similar to 3GA except linkage is via 3′ ends; Control 2 = 38-mer compound forming a tandem repeat of the 19-mer sequence; siRNA = 19-mer double stranded oligoribonucleotides with two nucleotide overhangs on each end.

MECHANISM OF ACTION OF 3GA

RLM-RACE assay: detection of excision sites on target RNA



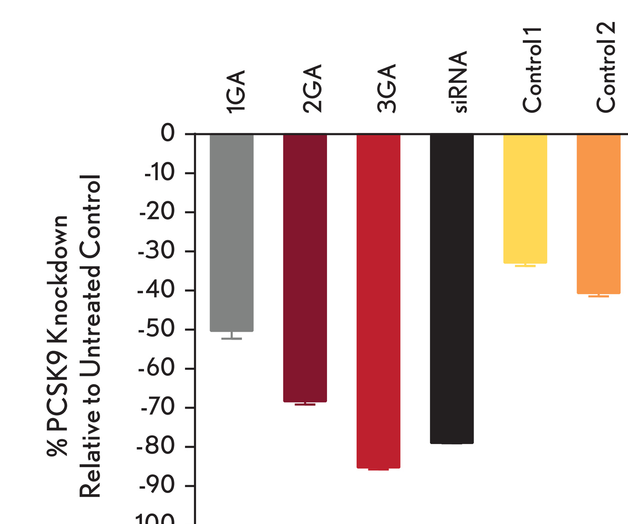
RLM-RACE primer sites and predicted sites for RNase H or RNAi-mediated excision



Target Site · Outer PCR Primer Site · Inner PCR Primer Site · First Strand cDNA Synthesis Primer Site

Gene-silencing activity of different antisense compounds

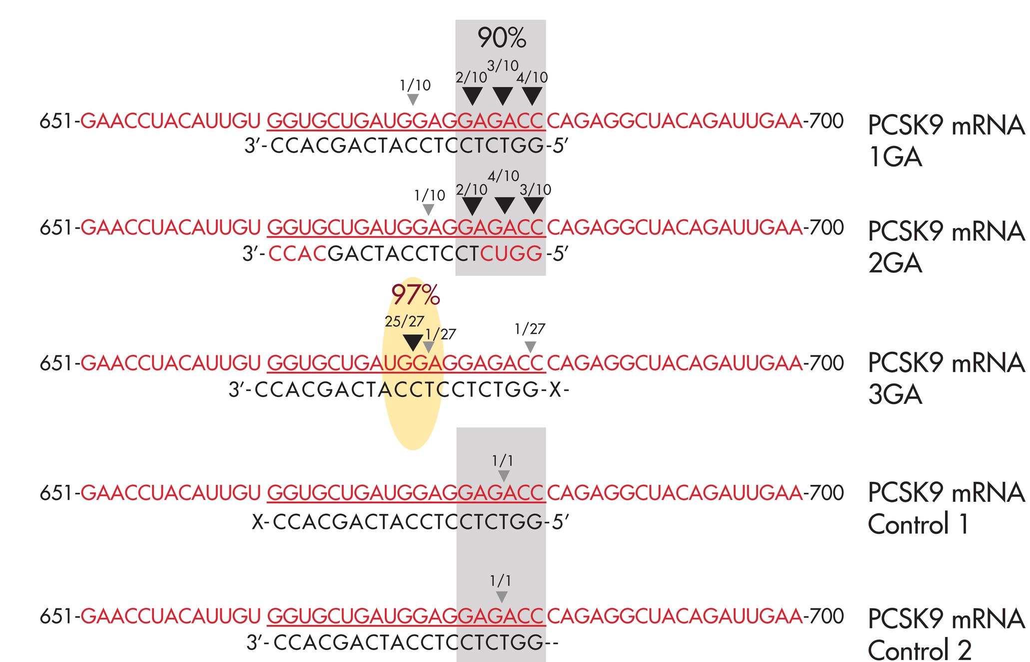
Hepa 1-6 cells were transiently transfected with 25 nM of different antisense compounds for 16 hours. Total RNA was isolated and knockdown levels were assessed via qPCR. n=3, mean + SD are shown.



RLM-RACE results

The RLM-RACE data that follow show excision sites within the PCSK9 hybridization sequence, generated either by RNase H or the RNAi machinery. Black triangles indicate major sites of excision while gray triangles indicate minor sites of excision. Numerators indicate the number of clones exhibiting excision at the specified sites; denominators indicate the total number of clones exhibiting excision within the hybridization sequence; percentages of clones within specified regions are shown. Regions consistent with RNase H-mediated excision are represented by gray rectangles while regions consistent with RNAi-mediated excision are represented by yellow ovals. Data for 1GA, 2GA, 3GA, and siRNA are collated from multiple independent experiments.

Excision sites observed with various antisense compounds



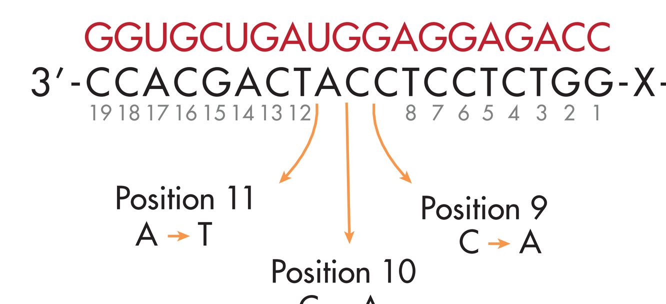
Excision sites observed with siRNA and 3GA were in a similar region

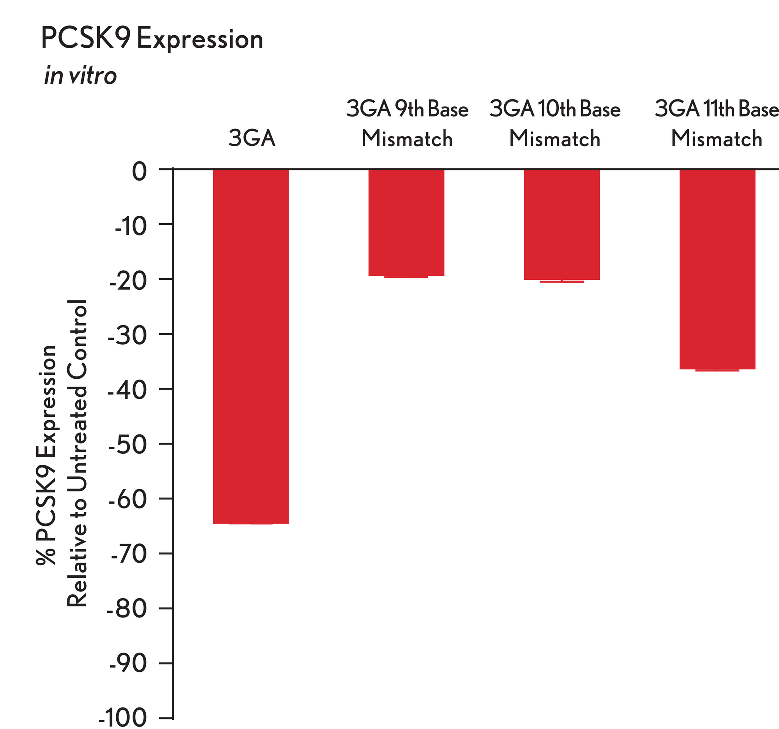
The aforementioned data suggest that 3GAs generate excision sites in a region similar to that of siRNAs and different from those of 1GA, 2GA, and control compounds.

Further insights into the mechanism of action of 3GA

Incorporation of mismatches at critical sites in siRNAs leads to reduction in gene-silencing activity.[11] These critical sites span the central region of the target RNA, where excision takes place. To further elucidate the mechanism of 3GAs, we introduced mismatches in the 3GA at the 9th, 10th, and 11th positions and evaluated the activity of these compounds in cell-based assays. We hypothesized that, similar to siRNAs, nucleotides in the central region of the 3GA are critical for the gene-silencing activity of 3GA.

Mismatches at the 9th, 10th, or 11th positions led to loss of gene-silencing activity *in vitro*

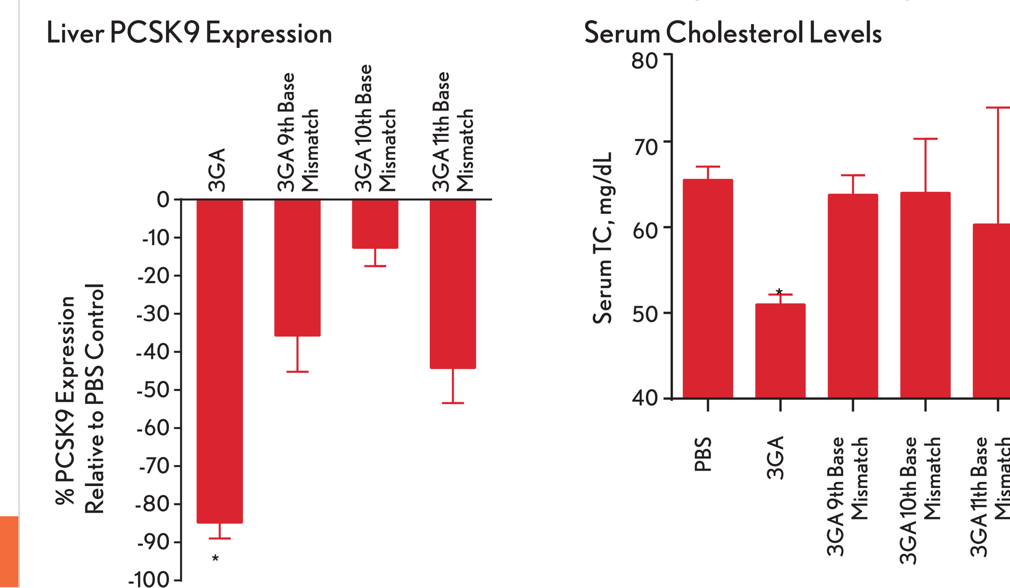




Hepa 1-6 cells were treated with 5 nM 3GA or mismatched compounds for 16 hours. PCSK9 expression was analyzed by qPCR.

Mismatches at the 9th, 10th, or 11th positions led to loss of gene-silencing activity *in vivo*

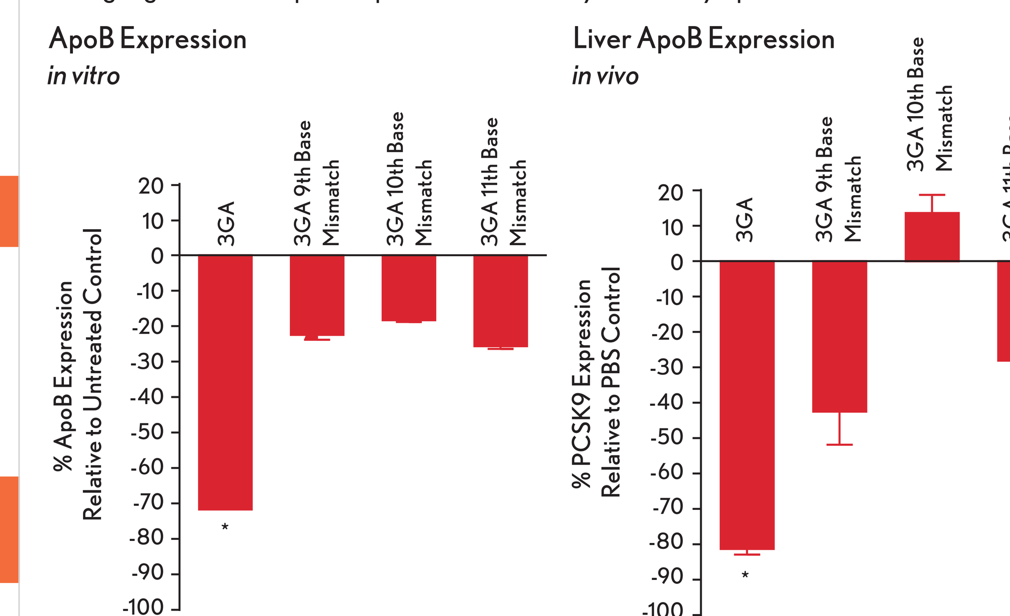
We evaluated the gene-silencing activity of 3GAs with mismatches at the 9th, 10th, and 11th positions *in vivo*. Mice were injected with 3GA or mismatched compounds daily for five days at 15 mg/kg s.c. Four days post-treatment, blood samples were collected to evaluate serum cholesterol levels, and liver tissues were collected and analyzed for PCSK9 expression.



3GAs with mismatches at the 9th, 10th, and 11th positions showed a significant reduction in PCSK9 gene-silencing activity *in vivo* that correlated with serum cholesterol levels. *$P<0.05$ vs PBS, n=3, mean + SEM are shown.

Mismatches in ApoB 3GA also led to loss of gene-silencing activity *in vitro* and *in vivo*

Hepa 1-6 cells were transfected with 12.5 nM 3GAs targeting ApoB (2709 – 2727) or mismatched compounds for 16 hours. ApoB expression was analyzed by qPCR. For *in vivo* studies, 3GAs or mismatched 3GAs were injected s.c. into mice daily for 5 days at 15 mg/kg s.c. Liver ApoB expression was analyzed 4 days post-treatment.



CONCLUSIONS

- 3GA exerts potent gene-silencing activity in cell-based assays and *in vivo*
- 3GAs generate excision sites in the target RNA at a region similar to that observed with siRNA, suggesting a novel mechanism of action
- This mechanism may be key to the increased potency and sustained activity of 3GA compared to 1GA and 2GA
- Structure-activity relationship studies of 3GAs with mismatches in the region of excision indicate specificity of 3GAs
- Further studies are in progress to harness the potential of 3GAs in targeting diseases with point mutations

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