Antisense oligonucleotides linked to an immunomodulatory dinucleotide are highly potent anti-HBV agents

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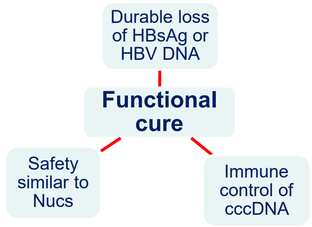
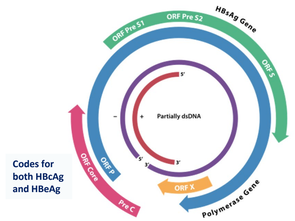
BACKGROUND

Over 250 million patients worldwide are chronically infected with the Hepatitis B virus (HBV). There remains a significant unmet need for effective combination therapies with favorable safety profiles to achieve functional cure rates, given the substantial heterogeneity in chronic HBV (CHB) patients. Towards this goal, we have designed novel chimeric antisense oligonucleotides (CASOs) targeting all HBV viral transcripts, by attaching the immunomodulatory dinucleotide SB 9000, the active metabolite of Inarigivir, via a cleavable linker to ASO. Inarigivir is an orally available, hepatic-selective RIG-I agonist, which is currently in global Phase II clinical trials against CHB. It is converted to the active dinucleotide metabolite, SB 9000, and has both direct-acting and immune-mediated antiviral responses characterized by reductions in HBV DNA, RNA, HBsAg and HBcrAg as revealed in the recently completed Phase IIa Achieve® trials. We hypothesize that this two-pronged approach of combining gene-silencing and immune modulation to generate CASOs will achieve simultaneous inhibition of HBV DNA, RNA and antigen production and activate RIG-I *in vivo* for immune-mediated viral clearance in CHB



Combining Inarigivir with Nuc and SBP CASO can result in functional cure

CASO strategy to inhibit all HBV viral transcripts

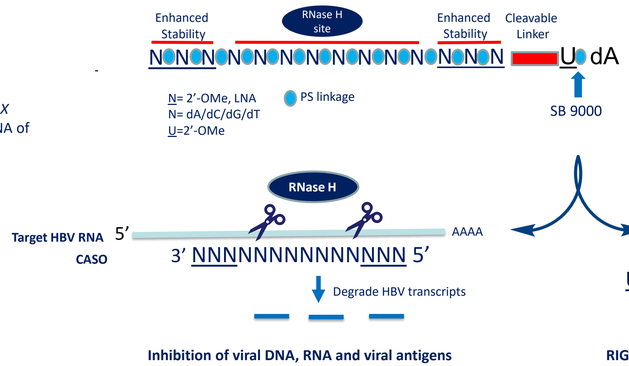


- The viral genome encodes four overlapping ORFs: *S*, *C*, *P*, and *X*
- >99% Sequence homology in HBx gene enables targeting mRNA of all HBV genotypes and subtypes

HBx protein is critical for HBV replication cycle
- Inhibits SMC5/6 that repress cccDNA transcription
- Integrates into host genome; enhances oncogenic potential
- Involved in the development of HCC

CASO is an ASO linked to SB 9000, a RIG-I agonist

Designed to achieve simultaneous inhibition of HBV DNA, RNA and antigen production and also activate RIG-I in vivo

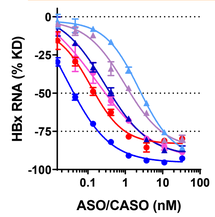


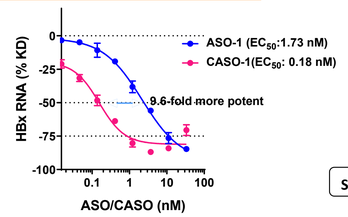
Design and synthesis of CASOs and sequence homology to HBV genotypes A-H

A series of 8- to 20-mer phosphorothioate ASOs having sugar and base modifications, targeting different regions of the HBx RNA, were synthesized with or without SB 9000 attached by a cleavable linker using standard phosphoramidite chemistry. The target mRNA regions of SB 527 and SB 539 have >98% homology to HBV genotypes A-H.

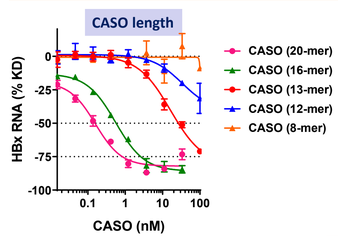
RESULTS

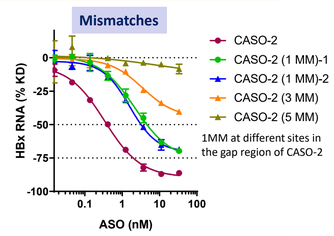
Addition of SB 9000 to ASO results in enhanced RNA knockdown activity in luciferase reporter assays





Decreasing the length of ASO or introduction of mismatches in the central region and 5'- and 3'-ends of CASO completely abrogates the RNA knockdown activity



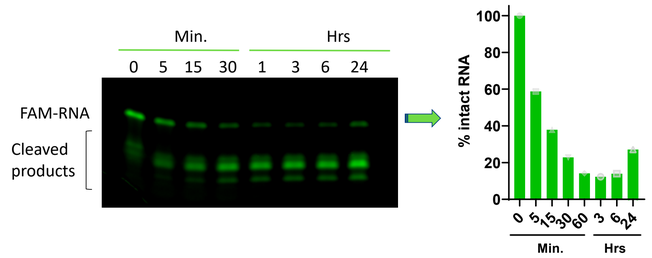


CASO length	EC_{50} (nM)	% RNA knockdown (11 nM)
20-mer	0.18	84.1
16-mer	0.33	82.5
13-mer	26.5	36
12-mer	>100	8.78
8-mer	-	11.1

CASO (Mismatches)	EC_{50} (nM)	% RNA knockdown (11 nM)
CASO-2	0.4	87
CASO-2 (1 MM)-1	4.54	63.2
CASO-2 (1 MM)-2	3.22	65.2
CASO-2 (3 MM)	-	33.8
CASO-4 (5 MM)	-	-

For luciferase reporter screening, the HBx gene was cloned into the dual *Renilla* and firefly (housekeeping) luciferase psiCHECK™-2 Vector (Promega). ASOs/CASOs were transfected in Hepa 1-6 cells along with the plasmid and luciferase activities were measured after 24 hours.

SB 527 elicits robust RNase H-mediated cleavage of target HBV RNA



RNase H assay with single strand FAM-RNA complementary to SB 527. FAM-labeled RNA was incubated at room temperature with SB 527 and RNase H1 in RNase H assay buffer. An aliquot of the assay mixture was taken out at each time point, fixed in formamide and run on a 15% TBE/urea gel. The gel was observed with Azure gel imager to visualize full length FAM-labeled RNA and its cleavage products. The relative intensity of the intact RNA bands was quantitated using ImageJ software and normalized to the intensity of the band at time zero.

CASOs are highly potent in HBV antiviral assays

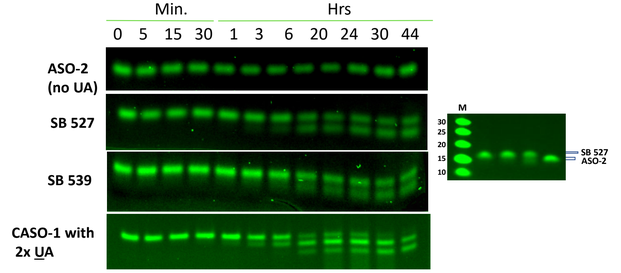
	ASO / CASO	Reporter assays RNA knockdown EC_{50} (nM)	Reporter assays % RNA knockdown at 11 nM)	Anti-HBV activity (HBV DNA) EC_{50} (nM) HepG2 assays	Anti-HBV activity (HBV DNA) EC_{90} (nM) HepG2 assays	Cytotoxicity CC_{50} (nM) HepG2 assays
	ASO-1	1.73	76.5	>500	>500	>500
	CASO-1	0.18	84.1	131	366	>500
SB 527	CASO-2	0.28	87.3	90	242	>500
	CASO-3	0.027	95.3	43	93	>500
SB 539	CASO-4	0.17	85.1	10	22	>500
	Con. ASO	-		>500	>500	>500

CASO	Anti-HBV activity (HBsAg) EC_{50} (nM) PHH assays	Anti-HBV activity (HBeAg) EC_{50} (nM) PHH assays
CASO-1	44.6	16.0
CASO-2	<10	<10
CASO-3	<10	<10
CASO-4	<10	<10

Anti-HBV assays: (i) HepG2.2.15 cells: Antiviral activity of ASOs/CASOs was evaluated in HepG2.2.15 cells following lipid transfection of ASOs. Extracellular HBV DNA levels were monitored by dot-blot hybridization and toxicity assessed by neutral red dye uptake 72 hours post transfection. (ii) Primary Human Hepatocytes (PHH): CASOs were transfected on Day 1 and cells were infected with HBV (Genotype D, MOI 500) the following day. Culture supernatants and cells were collected at 11 days post infection to quantitate HBV DNA (qPCR), and HBsAg, HBeAg (ELISA).

Nuclease-mediated release of SB 9000 from the parent ASOs

SB 9000 starts dissociating at 6 hrs whereas end modifications render ASOs resistant to serum nuclease degradation up to 48 hrs



ASO or CASOs were incubated at 37° C in 25% fetal bovine serum. An aliquot of the assay mixture was taken out at each time point, fixed in formamide and run on a 20% TBE gel, stained with SYBR Safe and observed with Azure gel imager..

CONCLUSIONS:: The lead, SB 527 and SB 539, knock down HBV viral transcripts by an RNase H-mediated mechanism and were found to be potent anti-HBV agents in cell-based assays. Further studies are ongoing for the development of the lead CASOs for inclusion in a combinatorial approach to elevate functional cure rates in CHB.

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