Rational Design Leads to More Potent RNA Interference Against Hepatitis B Virus: Factors Effecting Silencing Efficiency

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RNA interference (RNAi) can be an effective antiviral agent; however, overexpression of RNAi can be toxic through competition with the endogenous microRNA (miRNA) machinery. We used rational design to identify highly potent RNAi that is effective at nontoxic doses. A statistical analysis was conducted to pinpoint thermodynamic characteristics correlated with activity. Sequences were selected that conformed to a consensus internal stability profile (ISP) associated with active RNAi, and RNAi triggers were expressed in the context of an endogenous miRNA. These approaches yielded highly active hepatitis B virus (HBV) RNAi. A statistical analysis found a correlation between activity and nucleation by binding within the seed sequence to accessible regions in the target RNA. Guide strands were selected for favorable strand biasing, but increased strand biasing did not correlate with potency, suggesting a threshold effect. Exogenous short hairpin RNAs (shRNAs), but not miRNAs were previously reported to compete with miRNAs for the miRNA/RNAi machinery. In contrast, we show that exogenous Polymerase III- but not Polymerase II-driven miRNAs compete with exogenous miRNAs, at multiple steps in the miRNA pathway. Exogenous miRNAs also compete with endogenous miR-21. Thus, competition with endogenous miRNAs should be monitored even when using miRNA-based therapeutics. However, potent silencing was achieved at doses where competition was not observed.

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INTRODUCTION

Hepatitis B virus (HBV) is a DNA virus that replicates through an RNA intermediate. HBV infection is strongly associated with hepatocellular carcinoma[1] and is the ninth leading cause of death worldwide according to the World Health Organization.[2] Current treatments are costly, have signi cant side e ects[3,4] and are e ective in only ~50% of patients.[5] HBV produces four major classes of mRNAs. e approximate sizes of these mRNAs are 3.5, 2.4, 2.1, and 0.7 kb. e 3.5 kb pregenomic RNA is the template for replication of the viral DNA minus strand. e HBV genome contains extensive overlapping reading frames (**Figure 1d**).

RNA interference (RNAi) is an endogenous gene-silencing pathway that responds to double-stranded (ds)RNAs by silencing homologous genes.[6] Speci c silencing of a transcript can be directed by the transfection of cells or animals with short-interfering RNAs (siRNAs, **Figure 2**). Alternatively, short hairpin RNAs (shRNAs) can be expressed from plasmid or viral templates within cells or animals to trigger silencing (**Figure 2**). Recently, it has been appreciated that the RNAi machinery is also involved in normal gene regulation. MicroRNAs (miRNAs) are a family of ~22 nucleotide single-stranded noncoding RNAs that silence endogenous transcripts (reviewed in ref. 7). miRNAs are synthesized as part of a hairpin structure embedded within a primary miRNA (pri-miRNA, **Figure 2**). In the nucleus, Drosha and DGCR8 (DiGeorge syndrome critical region gene 8) cooperate to cleave pri-miRNAs into hairpin-like structures called pre-miRNAs. It is likely that exogenous shRNAs are substrates for the RNAi/miRNA machinery because they resemble pre-miRNAs. Pre-miRNAs are transported to the cytoplasm by Exportin-5 (ref. 8) and cleaved into mature miRNAs by Dicer. Mature miRNAs form duplexes with endogenous target RNAs and either prevent their translation or cause their degradation.[7] Again, it is likely that synthetic siRNAs are substrates for the RNAi/miRNA machinery because they resemble the duplex produced by Dicer cleavage of pre-miRNAs.

RNAi has been used to inhibit a wide variety of viruses in cultured cells and *in vivo*. Previously, we developed a transient model for testing HBV RNAi-based therapeutics in which HBV replication and RNAi were initiated by co-transfection of cultured hepatoma cells or mouse liver with HBV and RNAi expression plasmids encoding shRNAs.[9] Using this model, we and subsequently others, have shown that RNAi could be used to inhibit HBV replication (reviewed in ref. 10). Because no rational design rules were available at the time of our initial study, RNAi sequences were chosen based on conservation of sequences

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