A novel sirtuin 2 modulator reduces hepatitis B virus infectivity *in vitro*

Liudi Tang[1], Stacy Remiszewski[1], John Kulp[2], Matthew Todd[1], Kate Sullivan[1], James R. Tonra[1]
Thomas Shenk[1,3], Lillian Chiang[1],

1 Evrys Bio, 3805 Old Easton Road, Doylestown, PA 18902

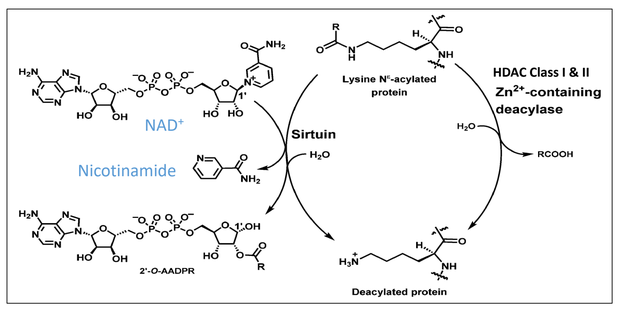
2 Conifer Point Consulting, 3805 Old Easton Road, Doylestown, PA 18902

3 Department of Molecular Biology, Princeton University, Princeton, NJ 08544

Abstract

Sirtuins (SIRT1-7) are NAD+-dependent protein deacylases that catalyze the removal of distinct protein-acyl groups. SIRTs are particularly responsive to changes in the metabolic capacity of the host cell. In part because viral infection often regulates cellular metabolism to its benefit, SIRT modulators are being considered in antiviral drug development strategies. Using genetic and pharmacological approaches, several studies have demonstrated that SIRT2 activity contributes to the hepatitis B virus (HBV) life cycle. We have utilized in silico modeling to drive medicinal chemistry efforts to advance our drug-like small molecules. The compounds bind adjacent to the catalytic pocket to allosterically modulate SIRT2 enzymatic activity in association with broad-spectrum antiviral activity. An early SIRT2-targeting lead molecule, namely FLS-359, selectively inhibits SIRT2 over SIRT1 and SIRT3 in biochemical assays. Addition of FLS-359 to HepG2.2.15 cultures rapidly reduces extracellular HBsAg, HBeAg and intracellular viral replication intermediates at non-toxic doses. In addition, treatment of HepG2-NTCP cells with FLS-359 prevented the establishment of covalently closed circular (ccc) DNA upon HBV infection, indicating a role of SIRT2 early in HBV life cycle. FLS-359 did not affect HBV cccDNA levels once viral establishment was complete, however even then FLS-359 reduced viral antigen production. Importantly, these effects of FLS-359 were also found with HBV infection of primary human hepatocytes from two different donors. Mechanistically, FLS-359 reduces HBV promoter activity, which correlates with its ability to decrease the deposition of the active histone marker Ac-H3K27 on HBV DNA. Thus, FLS-359 perturbs HBV RNA accumulation, likely through its ability to inhibit the de-acylation function of SIRT2. The anti-HBV effect of FLS-359 was similar across varying HBV genotypes, indicating the potential for broad effects on HBV patients. In conclusion, the potential of FLS-359 to reduce HBsAg load as well as to prevent new infection supports the further development of SIRT2 inhibitors as HBV antivirals.

Sirtuins are NAD+ dependent deacylases



SIRT1-7 are evolutionarily conserved from yeast to humans.

NAD+/NADH — *Nutrients high* — **Sirtuin Activity** — NAD+/NADH — *Nutrients low*

- Virus Infection
- Tumorigenesis

- SIRT1-7 are categorized into four groups based on their sequence homology.
- SIRT1-7 have varied enzymatic activities.
- SIRT1-7 have varied subcellular localizations

Herskovits, 2013, Cell Research

Evidence for SIRT2 involvement in HBV life cycle

- SIRT2 enhances HBV RNA transcription and DNA synthesis through the AKT/GSK-3β/β-Catenin Signaling Pathway (Piracha, 2018, JVI)
- AGK-2, a small molecule SIRT2 inhibitor, inhibits HBV replication *in vitro* and in transgenic mice (Yu, 2018, Int J Med Sci)
- HBx upregulates the expression of SIRT2 in HCC cell lines (Cheng, 2018, Biochem Biophys Res Commun)

Acknowledgement

This study was supported by NIH SBIR 5R44AI114079-03

FLS-359 modulates SIRT2 deacetylase activity

A

Drug (μM)	SIRT1 dAcetyl IC_{50}	SIRT2 dAcetyl IC_{50}	SIRT3 dAcetyl IC_{50}	SIRT5 dSucc IC_{50}	SIRT6 dMyr IC_{50}
FLS-359	33	3.2	12.3	>100	>100

B

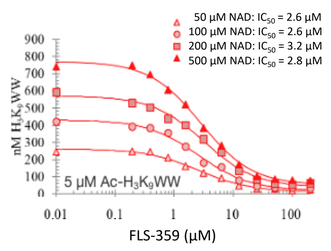


Figure 1. **(A)** FLS-359 shows moderate SIRT2 and weak SIRT1, SIRT3 inhibition. (**B)** Dose response curves of SIRT2 inhibition by FLS-359 measured by the conversion of acetylated to de-acetylated peptide substrate H3K9WW at increasing concentrations of NAD^+ . Enzyme inhibition is not sensitive to substrate concentrations demonstrating non-competitive inhibition as the mode of action.

SIRT2 inhibition reduces HBV infectivity in HepG2-NTCP cells

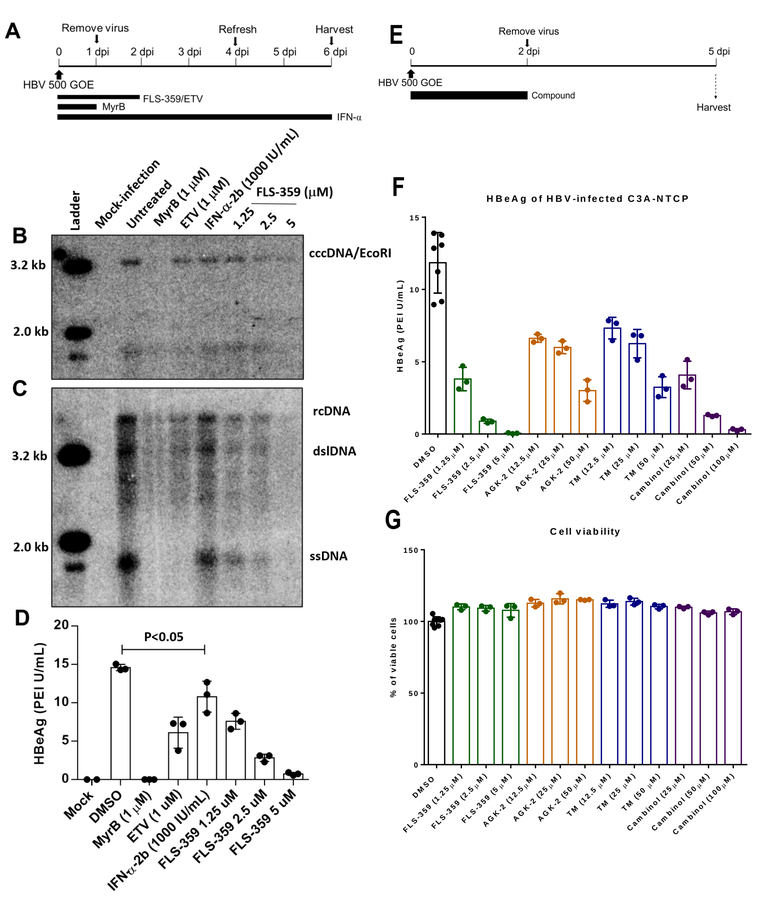


Figure 2. (A-D) C3A-NTCP cells were infected with HBV at MOI of 500 GOE, and treated with the indicated compounds **(A)** At 6 days post infection, Hirt DNA **(B)** and intracellular coreDNA **(C)** were measured by Southern blot analysis. Extracellular HBeAg **(D)** was quantified by ELISA; Mean ± SD are presented (n = 3). **(E-G)** C3A-NTCP cells were infected with HBV at MOI of 500 GOE, and treated with different classes of SIRT2 inhibitors for the initial 2 days before harvesting at 5 dpi **(E)**. Extracellular HBeAg was quantified by ELISA **(F)**, and cells were fixed and DAPI stained for nuclei count as a readout for cell viability **(G)**. Mean ± SD are presented (n = 3).

FLS-359 inhibits HBV cccDNA establishment rather than promoting its decay in primary human hepatocytes

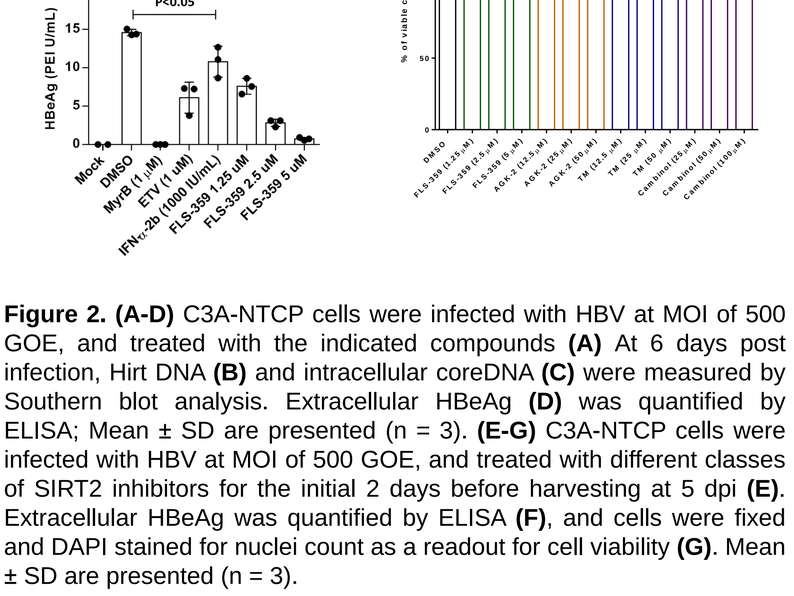
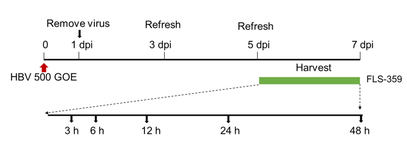


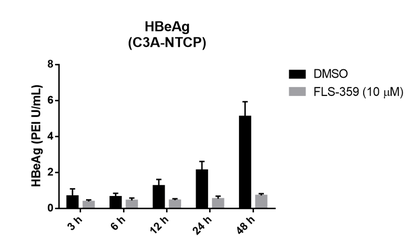
Figure 3. Primary human hepatocytes (from Phoenix Bio) were infected with HBV at MOI = 500 GOE. Drug treatment started either 4h before viral inoculation **(A-E)**, or 4d after HBV infection **(F-J).** 6-days after drug treatment, cells were harvested for cccDNA and RNA quantification by qPCR. Culture supernatant was collected for HBsAg and HBeAg quantifications by ELISA.

FLS-359 rapidly reduces HBV RNA and HBsAg

A



B



C

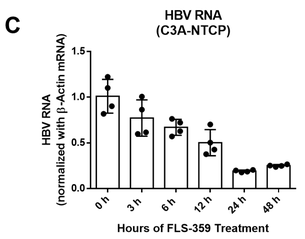


Figure 4. HBV-infected C3A-NTCP cells were treated with DMSO or FLS-359 (10 μM). At indicated time points, the culture supernatant was harvested to measure HBeAg **(B)**, and the cells were harvested for total HBV RNA quantification using qRT-PCR **(C)**. Mean ± SD are presented (n = 4).

FLS-359 also hampers HBV replication derived from integrated HBV genome

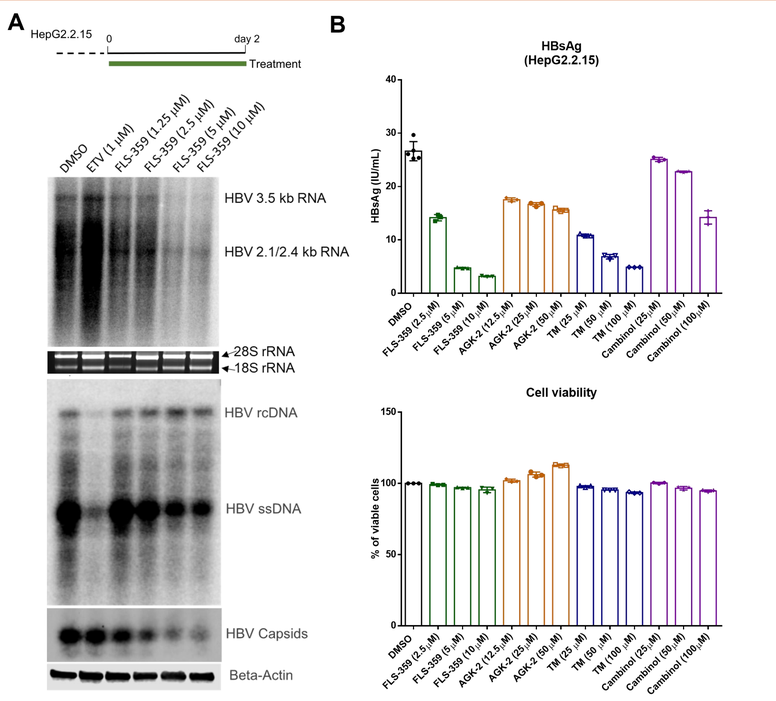


Figure 5. **(A)** Compound treated HepG2.2.15 was harvested for HBV RNA Northern blot analysis, intracellular HBV coreDNA Southern blot analysis, and intracellular viral capsid particle gel. The β-Actin Western blot serves as a loading control. **(B)**. HepG2.2.15 cells were treated with different classes of SIRT2 inhibitors for 2 days. HBeAg was determined by ELISA, and cells were fixed and DAPI stained for nuclei count as a readout for cell viability. Mean ± SD are presented (n = 3).

FLS-359 epigenetically silences HBV transcriptional activity

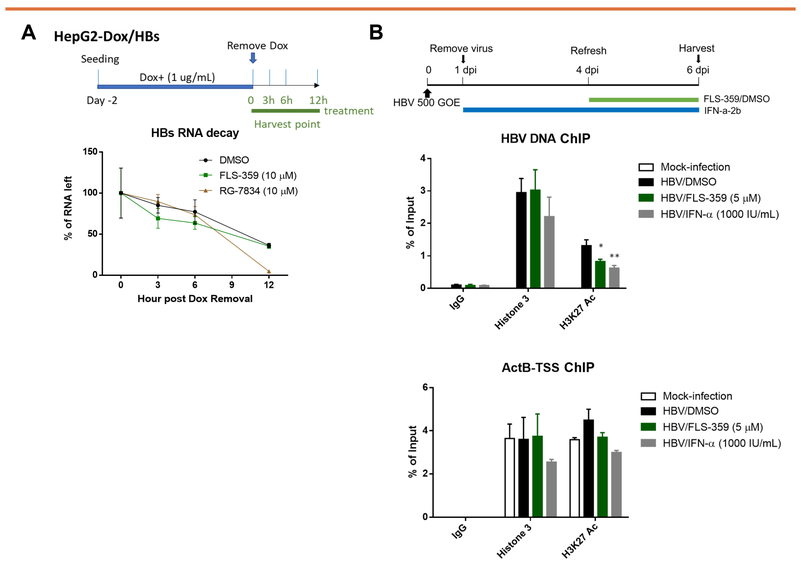


Figure 6 (A) A doxycycline-inducible HBsAg expressing cell line (HepG2-Dox/HBs) was allowed to produce HBs transcripts, followed by shutting down the promoter and treating with the indicated compounds for 3, 6, and 12 hours. HBs transcripts were measured by qRT-PCR. Mean ± SD are presented (n = 3). **(B)**. HBV-infected C3A-NTCP cells were treated with indicated drugs. Chromatin immunoprecipitation was performed using antibody against IgG, Histone 3, or Ac-H3K27. The enrichment of HBV DNA and actin transcription starting site (TSS) were quantified by qPCR. Mean ± SD are presented (n = 2).

Conclusions

- FLS-359, a drug-like small molecule SIRT2 inhibitor, hampers HBV cccDNA establishment *in vitro*
- FLS-359 reduces the accumulation of HBV RNA transcripts derived from infected hepatocytes as well as from integrated HBV genome.
- Reduction of HBV RNA correlates with decreased deposition of the active histone marker Ac-H3K27 on HBV DNA