Supplementary data to:

**Sustained Efficacy and Seroconversion with the Toll-Like Receptor 7 Agonist GS-9620 in the Woodchuck Model of Chronic Hepatitis B**

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Supplementary Methods

Quantitative RT-PCR. Pharmacodynamic (PD) response was determined by IFN-α, 2’5’-OAS and MxA RNA transcripts in whole blood collected at pretreatment and 24 hours following the first and last doses of GS-9620. Total RNA was isolated using the QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Following reverse transcription with the MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA) using random hexamers, cDNA were amplified using an ABI PRISM 7000 Sequence Detection instrument (Applied Biosystems, Foster City, CA) using SYBR GREEN Master Mix (Applied Biosystems, Foster City, CA) and woodchuck-specific primers (Supplementary Table 3). Woodchuck β-actin mRNA expression was used to normalize target gene expression. Levels of IFN-α, 2’5’-OAS (2’;5’-oligoadenylate synthetase) and MxA (myxovirus resistance A) mRNAs were calculated as a fold change relative to pre-treatment (2^-ΔΔCt).

RNA-Seq analysis of the woodchuck transcriptome. RNA-Seq and de novo transcriptome assembly was conducted by Expression Analysis (Durham, NC). Total RNA samples from liver biopsies collected at various time points (Supplementary Fig. 9) were converted into cDNA libraries using the TruSeq Stranded Total RNA-RiboZero Gold Sample Prep Kit (Illumina, San Diego, CA). The cDNA libraries were analyzed for size distribution by Agilent Bioanalyzer (DNA 1000 kit, Agilent, Santa Clara, CA), quantitated by qPCR (KAPA Library Quant Kit, KAPA Biosystems, Wilmington, MA), then normalized in preparation for sequencing. Pair-end sequencing was conducted using Illumina HiSeq 2000 with read length of 50 nucleotides. On average, approximately 60 million reads were generated per sample. Random sampling of sequencing reads across all samples was conducted to generate a combined dataset for de novo transcriptome assembly using Trinity [34]. The resulted transcriptome assembly consisted of a total of 231,332 contiguous transcripts (contigs), among which 100,824 were mapped to 46,700 NCBI Entrez genes (including 11,103 human genes). The non-annotated contigs likely resulted from unique woodchuck transcript sequences, non-coding sequences from DNA contamination or assembly artifacts due to the short read length of RNA-Seq. Using the transcriptome assembly as reference, quantification
of transcript abundance from all reads obtained from each sample was conducted using Bowtie [35] and RSEM (v 1.2.0) [36]. Transcript abundance of 11,013 human homologs was subjected to differential gene expression analysis. The Bioconductor package edgeR [37] was used to normalize sequence count data and conduct differential gene expression analysis. False discovery rate (FDR) was calculated using the Benjamini-Hochberg method [38]. All differentially expressed genes (DEGs) had an absolute fold change >1.5 and FDR <0.05, and passed a low-expression filter. Intrahepatic transcriptional responses were characterized by a gene module approach [23] and by Ingenuity® Pathway Analysis (IPA; QIAGEN, Redwood City, CA).

Supplementary References


Supplementary Fig. 1. Activation of human and woodchuck TLR7 by GS-9620. HEK293 cells were transfected with an expression vector for human TLR7 or woodchuck TLR7 together with an NF-κB luciferase reporter vector and stimulated with various concentrations of GS-9620. Luciferase activity was measured and expressed as relative fold-change above mock-treated cells (M). Data are expressed as mean ± standard deviation from three independent experiments.
Supplementary Fig. 2. Pharmacokinetics and pharmacodynamics of GS-9620 after oral administration in healthy adult male woodchucks. Plasma concentration of GS-9620 versus time (A) and pharmacodynamic response to a single GS-9620 administration to naïve woodchucks (n=3/group) as measured by mean fold increase in 2’5’-OAS (B) and MxA (C) transcript levels in whole blood samples collected over time.
A. Group 1: Placebo

B. Group 2: 5/2.5 mg/kg QOD Uninfected

C. Group 3: 5/2.5 mg/kg QOD

D. Group 4: 5/2.5 mg/kg QOD QOW

E. Group 5: 5 mg/kg QW

F. Group 1: Placebo
G

**Group 2: 5/2.5 mg/kg QOD Uninfected**

<table>
<thead>
<tr>
<th>1st Dose</th>
<th>Last Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
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</table>

2'5'-OAS Fold Increase from Pretreatment


H

**Group 3: 5/2.5 mg/kg QOD**

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
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</tbody>
</table>

2'5'-OAS Fold Increase from Pretreatment


I

**Group 4: 5/2.5 mg/kg QOD QOW**

<table>
<thead>
<tr>
<th>1st Dose</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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<tr>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
</tr>
</tbody>
</table>

2'5'-OAS Fold Increase from Pretreatment


J

**Group 5: 5 mg/kg QW**

<table>
<thead>
<tr>
<th>1st Dose</th>
<th>Last Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>7933</td>
<td>7950</td>
</tr>
<tr>
<td>7950</td>
<td>7969</td>
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<td>7989</td>
<td>7994</td>
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<tr>
<td>7994</td>
<td>7998</td>
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</table>

2'5'-OAS Fold Increase from Pretreatment


K

**First Dose**

<table>
<thead>
<tr>
<th>No Viral Response</th>
<th>Vira Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-α</td>
<td>MxA</td>
</tr>
</tbody>
</table>

Mean ± SEM Fold Change from Pretreatment

**Last Dose**

<table>
<thead>
<tr>
<th>No Viral Response</th>
<th>Vira Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAS</td>
<td></td>
</tr>
</tbody>
</table>

**No Viral Response**

| 0 | 2 | 4 | 6 | 8 | 10 |

**Viral Response**

| 7883 | 7962 | 7967 | 7977 | 7979 | 7992 | 7997 |

**First Dose**

| 7883 | 7962 | 7967 | 7977 | 7979 | 7992 | 7997 |

**Last Dose**

<table>
<thead>
<tr>
<th>IFN-α</th>
<th>MxA</th>
</tr>
</thead>
</table>

**OAS**
Supplementary Fig. 3. Individual animal pharmacodynamic response after administration of GS-9620. Fold increases in MxA (A-E) and 2’5’-OAS (F-J) transcript levels in whole blood samples at 24 hours following the first and last dose in each animal as compared to pretreatment. In group 4, four animals had a lower pharmacodynamic response to GS-9620 (animals 7999, 7985, 7955 and 7949) and these animals also did not have reduction in viral load (Fig. 2d). The means of these 4 animals compared to the other three in the group are shown (K).
Supplementary Fig. 4. Liver WHV replicative intermediates (RI) levels in WHV-infected woodchucks. The vertical outer dotted lines represent the treatment period and, when present, the inner vertical dotted lines represent dose holiday.
A  Group 1: Placebo

B  Group 3: 5/2.5 mg/kg QOD

C  Group 4: 5/2.5 mg/kg QOD QOW

D  Group 5: 5 mg/kg QW

**Supplementary Fig. 5. Liver WHV cccDNA levels in WHV-infected woodchucks.**

The vertical outer dotted lines represent the treatment period and, when present, inner vertical dotted lines represent dose holiday.
Supplementary Fig. 6. Liver WHV RNA levels in WHV-infected woodchucks. The vertical outer dotted lines represent the treatment period and, when present, inner vertical dotted lines represent dose holiday.
**A Group 1: Placebo**

**B Group 3: 5/2.5 mg/kg QOD**

**C Group 4: 5/2.5 mg/kg QOD QOW**

**D Group 5: 5 mg/kg QW**

**Supplementary Fig. 7. Serum WHsAg levels in WHV-infected woodchucks.** The vertical outer dotted lines represent the treatment period and, when present, inner vertical dotted lines represent dose holiday.
(8A-1) Group 1: Placebo, Animal 7875  
(Male)  

No HCC was noted at necropsy for this animal.

(8A-3) Group 1: Placebo, Animal 7957  
(Female)  

HCC was noted at necropsy for this animal.

(8A-2) Group 1: Placebo, Animal 7896  
(Female)  

Animal was euthanized in Week 27 and HCC was noted at necropsy.

(8A-4) Group 1: Placebo, Animal 7968  
(Male)  

Animal was euthanized in Week 24 and HCC was noted at necropsy.
**Group 1: Placebo, Animal 7972**

(Female)

- No HCC was noted at necropsy for this animal.

**Group 1: Placebo, Animal 7995**

(Male)

- HCC was noted at necropsy for this animal.

**Group 1: Placebo, Animal 7993**

(Female)

- HCC was noted at necropsy for this animal.

**Group 2: 5/2.5 mg/kg QOD**

Uninfected, Animal 6854

(Male)
(8B-2) Group 2: 5/2.5 mg/kg QOD
Uninfected, Animal 6856 (Female)

(8B-4) Group 2: 5/2.5 mg/kg QOD
Uninfected, Animal 6864 (Male)

(8B-3) Group 2: 5/2.5 mg/kg QOD
Uninfected, Animal 6860 (Female)

(8B-5) Group 2: 5/2.5 mg/kg QOD
Uninfected, Animal 6865 (Male)
**Group 2: 5/2.5 mg/kg QOD**

Uninfected, Animal 6866
(Female)

---

**Group 3: 5/2.5 mg/kg QOD, Animal 7883 (Male)**

No HCC was noted at necropsy for this animal.

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**Group 2: 5/2.5 mg/kg QOD**

Uninfected, Animal 6868
(Male)

---

**Group 3: 5/2.5 mg/kg QOD, Animal 7962 (Female)**

No HCC was noted at necropsy for this animal.
(8C-3) Group 3: 5/2.5 mg/kg QOD,
Animal 7967 (Male)

(8C-5) Group 3: 5/2.5 mg/kg QOD,
Animal 7979 (Female)

(8C-4) Group 3: 5/2.5 mg/kg QOD,
Animal 7977 (Female)

(8C-6) Group 3: 5/2.5 mg/kg QOD,
Animal 7992 (Male)
(8C-7) Group 3: 5/2.5 mg/kg QOD, Animal 7997 (Female)

No HCC was noted at necropsy for this animal.

(8D-2) Group 4: 5/2.5 mg/kg QOD, QOW, Animal 7955 (Male)

No HCC was noted at necropsy; animal had no viral response to treatment.

(8D-1) Group 4: 5/2.5 mg/kg QOD, QOW, Animal 7949 (Male)

HCC was noted at necropsy; animal had no viral response to treatment.

(8D-3) Group 4: 5/2.5 mg/kg QOD, QOW, Animal 7959 (Female)

Animal was euthanized in Week 15; no HCC was noted at necropsy.
(8D-4) Group 4: 5/2.5 mg/kg QOD
QOW, Animal 7963
(Female)

No HCC was noted at necropsy for this animal.

(8D-6) Group 4: 5/2.5 mg/kg QOD
QOW, Animal 7985 (Male)

No HCC was noted at necropsy; animal had no viral response to treatment.

(8D-5) Group 4: 5/2.5 mg/kg QOD
QOW, Animal 7980
(Female)

No HCC was noted at necropsy for this animal.

(8D-7) Group 4: 5/2.5 mg/kg QOD
QOW, Animal 7999
(Female)

No HCC was noted at necropsy; animal had no viral response to treatment.
(8E-1) Group 5: 5 mg/kg QW, Animal 7933 (Female)
Animal died after the last dose administration; no HCC was noted at necropsy.
(8E-3) Group 5: 5 mg/kg QW, Animal 7969 (Male)
No HCC was noted at necropsy for this animal.

(8E-2) Group 5: 5 mg/kg QW, Animal 7950 (Female)
HCC was noted at necropsy for this animal.
(8E-4) Group 5: 5 mg/kg QW, Animal 7978 (Male)
Animal died following liver biopsy. HCC was noted at necropsy.
Supplementary Fig. 8. Individual animal viral load, AST, ALT, GGT and WHsAg levels over time. The left y-axis represents serum WHV DNA (vge/mL) and serum WHs Ag (ng/mL) levels and the right y-axis represents serum liver enzymes level (U/L). The vertical outer dotted lines represent the treatment period and, when present, the inner vertical dotted lines represent dose holiday.
Supplementary Fig. 9. Study design and liver biopsy timing. Liver biopsies (Bx) were taken at the indicated timepoints and analyzed by RNA-Seq. Gene expression data was expressed relative to pre-treatment (day -14). The number of animals analyzed at each time-point for each group was as follows: group 1 (placebo) n=7 for day 43, n=6 for day 71, n=6 for day 120, n=7 for day 250, group 5 (5 mg/kg QW) n=6 for day 23, n=5 for day 57, n=4 for day 78, n=5 for day 250. Tx: treatment.
Supplementary Fig. 10. Intrahepatic transcriptional signature associated with GS-9620 treatment. Top canonical pathways identified by Ingenuity Pathway Analysis from group 5 (5 mg/kg QW) on-treatment (day 27) differentially expressed genes (DEGs, n=1,238) relative to pre-treatment. Pathway enrichment was calculated with the Fisher’s exact test with multiple testing correction by the Benjamini and Hochberg method [38]. The \(-\log(p\text{-value})\) for \(p=0.05\) and \(p=0.01\) significance levels are indicated. TCR: T cell receptor, BCR: B cell receptor, PLC: phospholipase C.
Supplementary Fig. 11. Intrahepatic expression of select genes induced by GS-9620 treatment. Heatmap columns represent mean of n=4-7 animals on different study days, and rows represent different genes. Red and blue coloring of cells represents over- and under-expression compared to pre-treatment, respectively, as indicated by the scale bars for log2 fold-change values. Expression of these genes was only significantly induced (FDR<0.05 relative to pre-treatment) during GS-9620 treatment (group 5, day 23). It is important to note that while no liver tissue was available for qRT-PCR confirmation of mRNA levels, there is high concordance between RNA-Seq and qRT-PCR data (Wang et al. Nat Biotechnol 2014;32:926-932).
## Supplementary Table 1. Comparison of GS-9620 exposure and induced pharmacodynamic response after a single oral dose in normal healthy adult woodchucks, humans, chimpanzees and cynomolgus monkeys.

<table>
<thead>
<tr>
<th>Species (normal healthy)</th>
<th>GS-9620 Oral Dose</th>
<th>Mean Serum GS-9620 Cmax (nM)</th>
<th>Mean Serum GS-9620 Cmax (ng/mL)</th>
<th>MxA Mean Fold Increase in PBMCs</th>
<th>2’5’-OAS Mean Fold Increase in PBMCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Woodchuck</td>
<td>1 mg/kg</td>
<td>4.8</td>
<td>1.9</td>
<td>30.1</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>2.5 mg/kg</td>
<td>6.2</td>
<td>2.5</td>
<td>13.5</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>5 mg/kg</td>
<td>23.6</td>
<td>9.5</td>
<td>35.6</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>660</td>
<td>266</td>
<td>59.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Human*</td>
<td>0.3 mg</td>
<td>0.46</td>
<td>0.184</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>1 mg</td>
<td>1.09</td>
<td>0.440</td>
<td>2.7</td>
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</tr>
<tr>
<td></td>
<td>2 mg</td>
<td>1.57</td>
<td>0.633</td>
<td>10.6</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>4 mg</td>
<td>7.26</td>
<td>2.929</td>
<td>17.0</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>6 mg</td>
<td>18.01</td>
<td>7.261</td>
<td>16.9</td>
<td>7.6</td>
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<tr>
<td></td>
<td>8 mg</td>
<td>20.67</td>
<td>8.336</td>
<td>22.7</td>
<td>11.1</td>
</tr>
<tr>
<td></td>
<td>12 mg</td>
<td>29.68</td>
<td>11.969</td>
<td>30.5</td>
<td>13.1</td>
</tr>
<tr>
<td>Chimpanzee*</td>
<td>0.3 mg/kg</td>
<td>3.6</td>
<td>1.5</td>
<td>8.6</td>
<td>4.4</td>
</tr>
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<td></td>
<td>1.0 mg/kg</td>
<td>55.4</td>
<td>22.3</td>
<td>13</td>
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<td>Cynomolgus monkey*</td>
<td>0.05 mg/kg</td>
<td>0.35</td>
<td>0.14</td>
<td>4.1</td>
<td>6.7</td>
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<td></td>
<td>0.15 mg/kg</td>
<td>0.89</td>
<td>0.36</td>
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<td>0.5 mg/kg</td>
<td>10.42</td>
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<td>1.5 mg/kg</td>
<td>81.84</td>
<td>33</td>
<td>34.5</td>
<td>82.9</td>
</tr>
</tbody>
</table>

*Data from previously published work [10-12].
Supplementary Table 2. GS-9620 serum concentration in woodchucks. GS-9620 serum levels (mean ± standard deviation) evaluated 4 hours after administration of 2.5 mg/kg dose for groups 2, 3 and 4 and after 5 mg/kg for group 5 during week 4 of the study. No PK analyses were performed for group 1, the placebo control group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animals</th>
<th>Intended Regimen</th>
<th>GS-9620 Dose (mg/kg)a</th>
<th>Number of Animals</th>
<th>GS-9620 Exposure at 4 hrs Post Dose</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>nM</td>
<td>ng/mL</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Uninfected Control</td>
<td>QOD x 4 weeks</td>
<td>2.5</td>
<td>7</td>
<td>6.2 ± 3.3 2.5 ± 1.4</td>
</tr>
<tr>
<td>3</td>
<td>WHV Infected</td>
<td>QOD x 4 weeks</td>
<td>2.5</td>
<td>4b</td>
<td>5.0 ± 2.4 2.0 ± 1.0</td>
</tr>
<tr>
<td>4</td>
<td>WHV Infected</td>
<td>QOD every other week for 8 weeks</td>
<td>2.5</td>
<td>7</td>
<td>7.4 ± 6.0 3.0 ± 2.5</td>
</tr>
<tr>
<td>5</td>
<td>WHV Infected</td>
<td>Once weekly for 8 weeks</td>
<td>5</td>
<td>6c</td>
<td>11.9 ± 9.7 4.9 ± 4.0</td>
</tr>
</tbody>
</table>

a GS-9620 exposure levels were evaluated at the 2.5 mg/kg dose for groups 2-4 and at 5 mg/kg for group 5 during Week 4. Starting dose was 5 mg/kg for each GS-9620 treatment group; due to the occurrence of thrombocytopenia a 9-10 day dose holiday was initiated in groups 2 and 3, followed by dose reduction to 2.5 mg/kg for all groups except for group 5 which was maintained at 5 mg/kg. Group 1 is the placebo control group and no PK analyses for this group were performed.

b Only 4 samples were available for analysis.

c One animal in group 5 had died prior to pharmacokinetic sampling.
Supplementary Table 3. Oligonucleotides used for qRT-PCR. F: forward primer; R: reverse primer.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primers</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>IFN-α</td>
<td>F</td>
<td>5’-TGCTCTCTGGGCTGTGACCTGCCT-3’</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5’-GGAAGGTGTCCAGGAGGGTCTTGT-3’</td>
</tr>
<tr>
<td>2′5′-OAS</td>
<td>F</td>
<td>5’-GACCCAGCCCCCAGATCT-3’</td>
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<tr>
<td></td>
<td>R</td>
<td>5’-AACTCGCCCTCTAGCTGCC-3’</td>
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<td>MxA</td>
<td>F</td>
<td>5’-AGCCGCTCCTTCAGGAACCTTC-3’</td>
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<tr>
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<td>R</td>
<td>5’-CAGATTGTCTACTGCCAGGACCAGGT-3’</td>
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<tr>
<td>β-actin</td>
<td>F</td>
<td>5’-GATCACAGCCCTGGCACC-3’</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5’-TGGACAGAGGAGGCCAGGATG-3’</td>
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