**Supplementary Information for Dysregulation of Hepatitis B Virus Nucleocapsid Assembly *in vitro* by RNA-directed Small Ligands**

Nikesh Patel[1◊], Fardokht Abulwerdi[2*], Farzad Fatehi[3], Iain Manfield[1], Stuart Le Grice[2], John S. Schneekloth, Jr.[2], Reidun Twarock[3] & Peter G. Stockley[1◊]

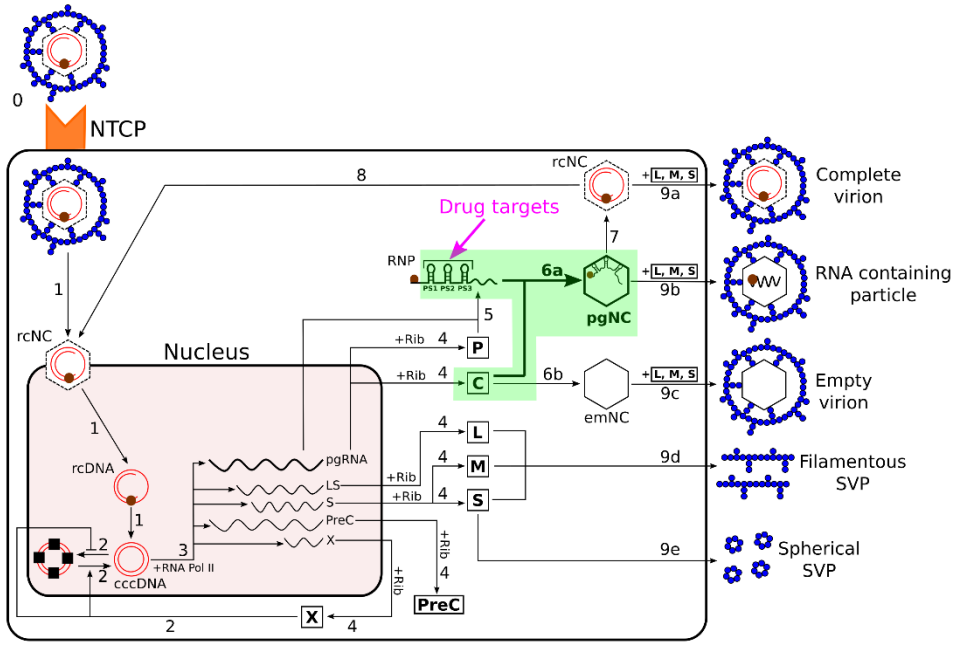
[1]Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, LS2 9JT, UK.

[2]Center for Cancer Research, National Cancer Institute, Frederick, MD 21702-1201

[3]Departments of Biology and Mathematics & York Centre for Complex Systems Analysis, University of York, York, YO10 5DD, UK

[◊]Joint communicating authors

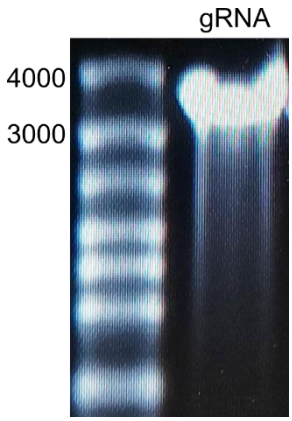
*Current Address: Center for Drug Evaluation and Research (CDER), US Food And Drug Administration, Washington DC, USA




***Supplementary Figure 1: The hepatitis B virus (HBV) life cycle (courtesy of*** [1]***).***

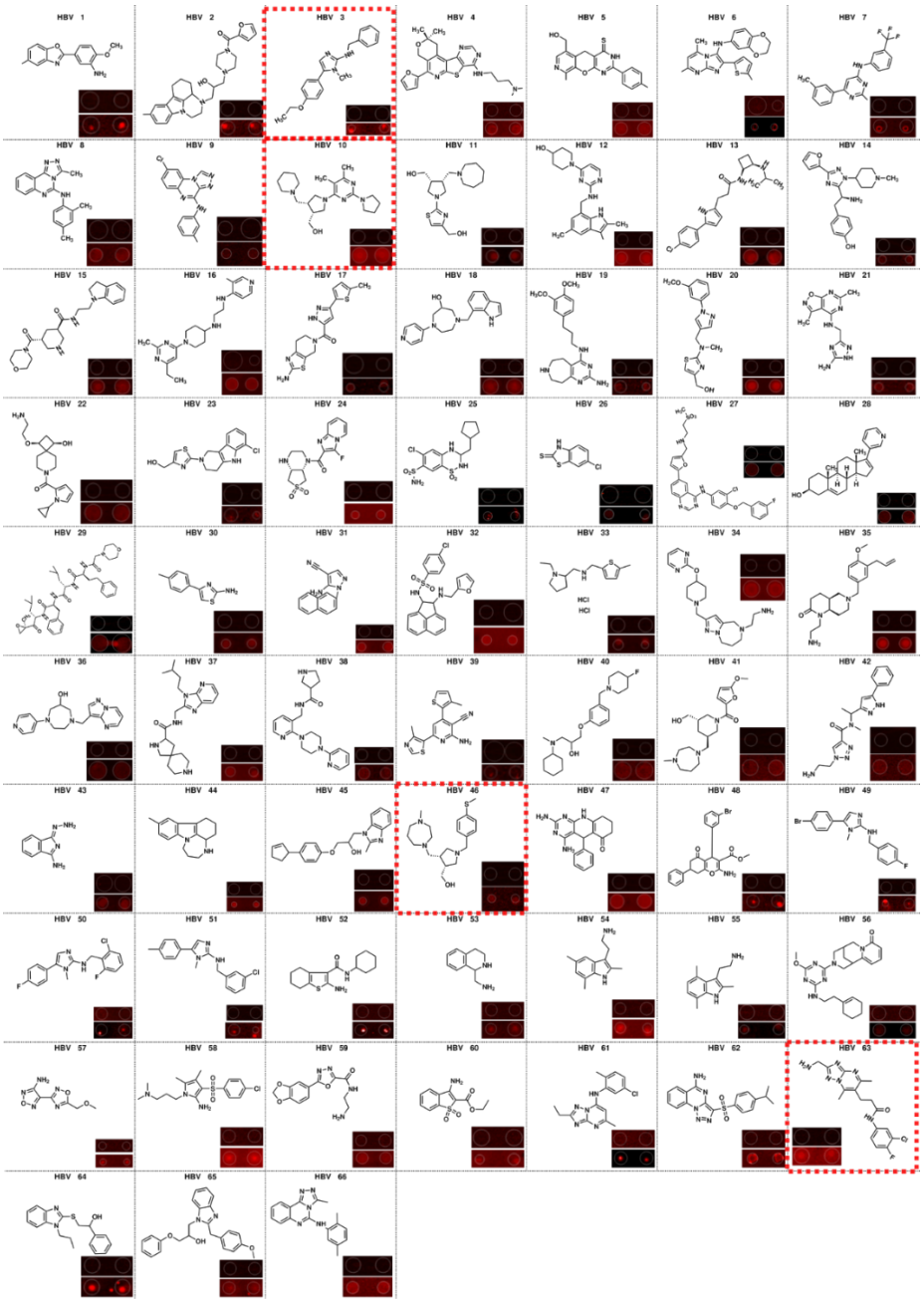
*The assembly stage of HBV nucleocapsids is highlighted green.* (0) Viral entry is mediated by the sodium taurocholate co-transporting polypeptide (NTCP) receptor, which allows the nucleocapsid (rcNC) containing relaxed circular DNA (rcDNA) to be released into the cell via endocytosis (not shown). (1) After attachment to the nucleus, the rcDNA is delivered. Host DNA repair factors convert the rcDNA into covalently closed circular DNA (cccDNA). (2) X protein blocks the silencing of cccDNA, allowing for transcription. (3) RNA pol II transcribes viral HBV RNAs, including the pgRNA; LS, S, PreC, and X mRNAs. The pgRNA encodes both the core (C) and polymerase (P) proteins; X mRNA the X protein; the LS and S mRNAs encode the L, M and S surface proteins; and PreC mRNA the precore (PreC) protein. (4) mRNAs are translated by ribosomes (Rib). (5) The pgRNA and P form a 1:1 assembly competent ribonucleoprotein (RNP) complex. (6a) Encapsidation of the RNP complex by C proteins to form a nucleocapsid containing pgRNA:P (pgNC). (6b) Assembly of C proteins into an empty nucleocapsid (emNC). (7) The reverse transcription of pgRNA by P within the pgNC resulting in the conversion of pgNC into an rcDNA-containing nucleocapsid (rcNC), which is also termed

the mature nucleocapsid. (8) rcDNA is recycled from some mature nucleocapsids to form more cccDNAs. (9a) Envelopment of mature nucleocapsids within a membrane layer containing the surface proteins L, M, and S, allowing for the secretion of a complete virion. (9b) Some RNA containing immature nucleocapsids can be enveloped, resulting in secretion of an RNA-containing particle. (9c) Envelopment of an empty nucleocapsid and secretion of an empty virion. (9d) L, M, and S proteins can form empty filaments and filamentous subviral particles (SVPs) via conventional tubular budding into late endosomes and exit the cell. (9e): S proteins assemble into octahedral spheres (spherical SVP) and exit the cell.



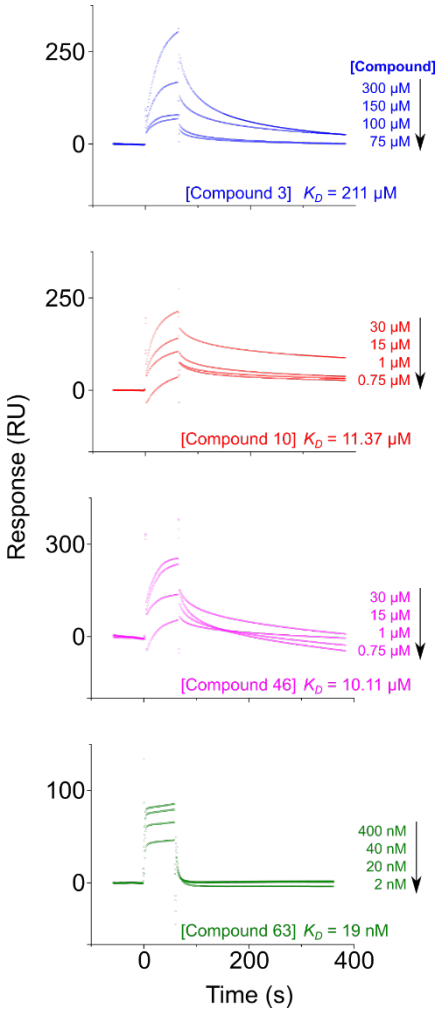

***Supplementary Figure 2: Analysis of in vitro transcribed gRNA.***

**(C)** *In vitro* transcribed gRNA, was separated on a denaturing 1% (w/v) agarose gel and visualised by staining with EtBr. *Left,* RiboRuler High Range RNA Ladder (ThermoFisher Scientific), sizes shown in nt. *Right,* gRNA.



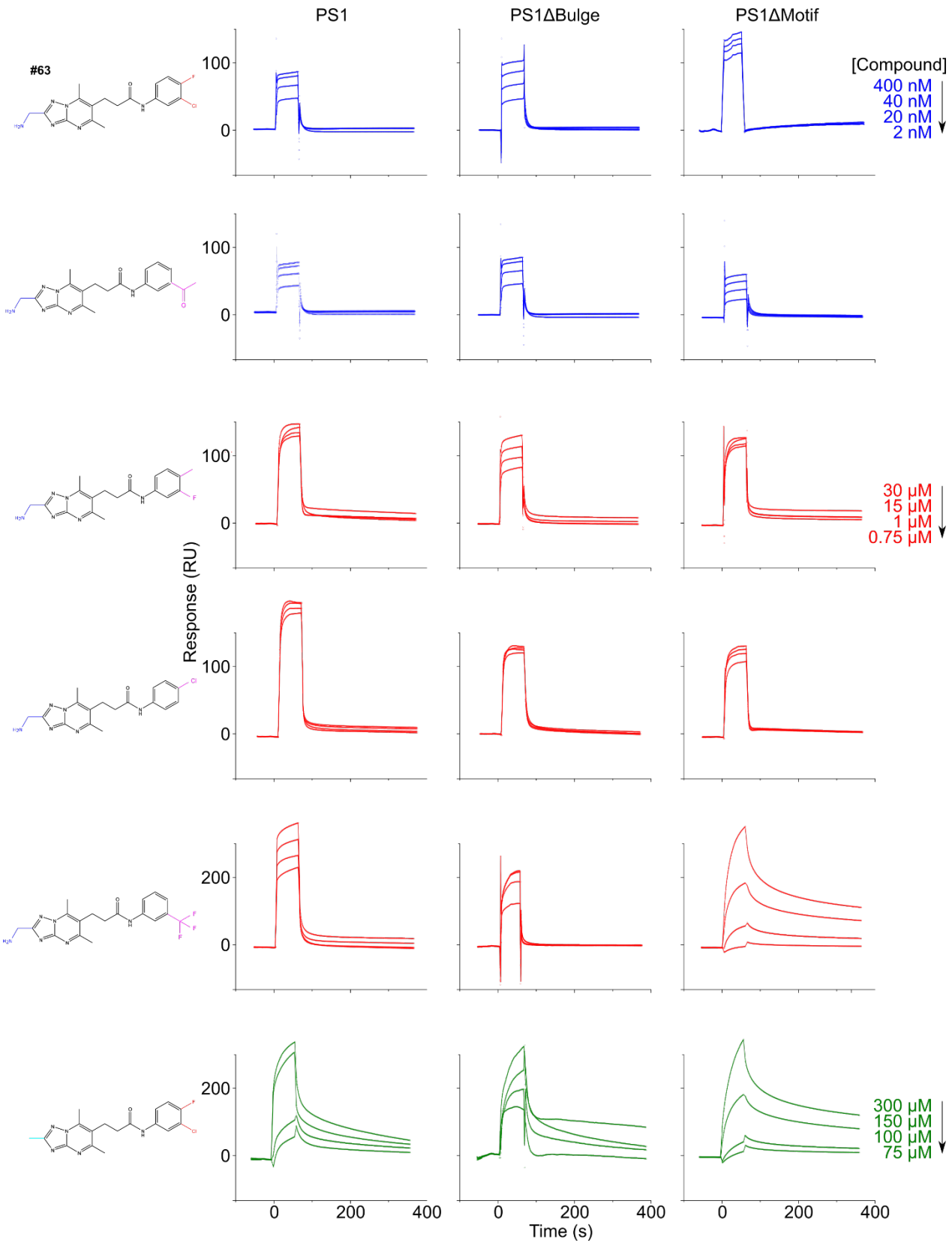
***Supplementary Figure 3: PS1 binding compounds.***

These 66 compounds shown are the commercially available subset of the 72 immobilized ligands in the SMM that showed red fluorescence (bottom panels) above the background (top panels) when washed with an AlexaFluor 647-labelled oligonucleotide encompassing annealed PS1 or buffer (RNase-free 1× PBS (12 mM $NaH_2PO_4$, 137 mM NaCl, 3 mM KCl, pH 7.4)) alone.



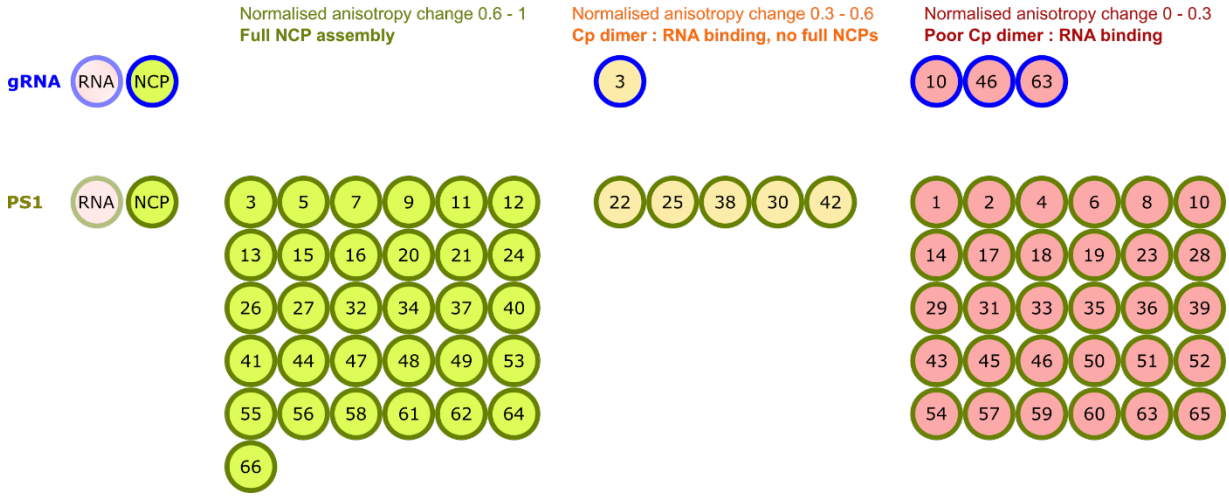

***Supplementary Figure 4: SPR Traces for hit compounds.***

*Top to bottom,* Raw SPR traces for Compound #3 (blue), #10 (red), #46 (magenta) and #63 (green) binding to immobilized PS1(NC_003977.1): Fits from titrations, detailed right, are shown. Measured $K_D$ values are shown right. Compounds were washed over immobilized RNA oligonucleotides in a buffer containing 20 mM HEPES (pH 7.5), 250 mM NaCl, 2%(v/v) DMSO and 0.1%(v/v) Tween20, at a rate of 10 μL/min for 6 min at 37 °C.


PS1
PS1ΔBulge
PS1ΔMotif
#63
[Compound]
400 nM
40 nM
20 nM
2 nM
30 μM
15 μM
1 μM
0.75 μM
300 μM
150 μM
100 μM
75 μM
Response (RU)
Time (s)

***Supplementary Figure 5: SPR Traces for SAR investigation.***

*Top to bottom,* Raw SPR traces for Compounds shown in figure 6 (*one compound per row – compounds shown left*) binding to immobilized PS1(JQ707375.1), PS1ΔBulge and PS1ΔMotif (*columns from left to right*): Fits from titrations, detailed right, are shown. Measured $K_D$ values are shown in figure 6. Compounds were washed over immobilized RNA oligonucleotides in a buffer containing 20 mM HEPES (pH 7.5), 250 mM NaCl, 2%(v/v) DMSO and 0.1%(v/v) Tween20, at a rate of 10 µL/min for 6 min at 37 $^{o}$C.

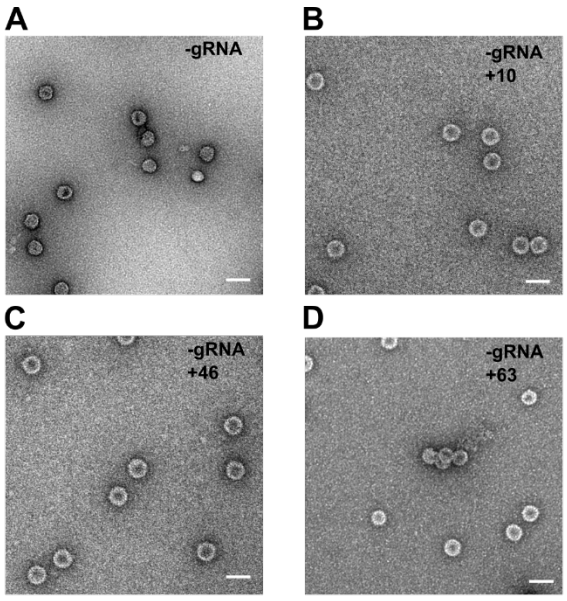



***Supplementary Figure 6: Summary of plate-based assembly inhibition assays.***

Heat annealed 1 nM gRNA (top) or 15 nM PS1 (bottom) oligo (NC_003977.1, Fig 3) were dispensed into a 96-well plate (blue, green outlines respectively). 10 µM compound were added to wells as labelled. 1.2 µM Cp dimer was titrated as described in Methods [2] and any assembled material then challenged with 1 µM RNase A. The final normalised fluorescence anisotropy was calculated, and control reactions ± Cp used to denote full NCP (1) and degraded RNA (0).

Anisotropy change is displayed here by the colour fill in each well. Left to right: *light green* 0.6-1, suggestive of efficient NCP assembly; *light yellow* = 0.3-0.6, suggestive of some Cp dimer: RNA binding; and *light red* = 0-0.3, suggestive of poor Cp dimer: RNA binding.

Assay was performed in triplicate, and the average anisotropy change shown, full data with associated standard error of the mean available in Supplementary Tables 4 and 5, for PS1 and gRNA reassemblies, respectively.




***Supplementary Figure 7: Cp self-association is unaffected by PS-binding compounds.***

(A) Self-assembly of Cp dimer in the absence of ligands; (B) as in (A) with 10 μM Compound #10 added; (C) as in (B) with 10 μM Compound #46; and (D) as in (B) with 10 μM Compound #63. Negatively stained images of these assembly mixes.

| RNA Oligonucleotide | Sequence 5' → 3' |
|---|---|
| PS1(NC_003977.1) | GGGUUUUGGGUUUGUUUAAAGACUGGGAGGAGUUGGGGGAGGAGCCC |
| PS1(JQ707375.1) | UGUGUGUUUAAGGACUGGGAGGAGCUGGGGGAGGAGAUUAGGUUAAAGG |
| PS1ΔBulge | UGUGUGUUUAAGGACUGGGAGGAGCUGGGGGAGGAGACCCGGCUAAGG |
| PS1ΔMotif | UGUGUGUUUAAGGACUGGGAGGAGCUGGGGGAUUUUACUAGGCUCAAGG |
| PS2 | GGGUUUUGGGGUGACAGUUAAUGAAAAAAGGAGAUUAAAAUUACCCC |
| PS3 | GGGUUUUGGGGCUGGCAUUCUAUAUAAGAGAGAAACUACACGCCCCC |
| ΔPS | UCGGACAGCAAAGGUACAAGGACCAGGGUUCGA |
| US | UUUUUACCUAUCUCUUUUUCUCU |
| Epsilon | UGUUCAUGUCCUACUGUUCAAGCCUCCAAGCUGUGCCUUGGGUGGCUUUGGGGCAUGGAC A |

***Supplementary Table 1. RNA Oligonucleotides used in SPR binding assays.***

| | Affinities / nM | | | |
|---|---|---|---|---|
| Compound | PS1 (NC_003977.1) | PS1 (JQ707375.1) | PS2 | PS3 |
| 1 | 165 ± 2 | | 141 ± 4 | 1750 ± 125 |
| 2 | 10000 ± 119 | | 86400 ± 101 | 44100 ± 28 |
| 3 | 212000 ± 1179 | 224000± 455 | 320 ± 5 | 99100 ± 591 |
| 4 | 171000 ± 24900 | | 1910 ± 83 | 59200 ± 3220 |
| 5 | 239 ± 1 | | 50300 ± 1890 | 312 ± 1 |
| 6 | 61 ± 2 | | 112 ± 1 | 615 ± 22 |
| 7 | 456 ± 5 | | 174000 ± 248 | 491 ± 5 |
| 8 | 31 ± 2 | 92 ± 12 | 385000 ± 1040 | 1720 ± 1 |
| 9 | 30 ± 10 | | 2890 ± 45 | 37 ± 3 |
| 10 | 11800 ± 284 | 11400 ± 169 | 10800 ± 691 | 8530 ± 797 |
| 11 | 11400 ± 220 | | 147 ± 35 | 27100 ± 1234 |
| 12 | 61800 ± 4538 | | 46300 ± 271 | 94700 ± 8466 |
| 13 | 20100 ± 20 | | 26200 ± 375 | 206 ± 17 |
| 14 | 345000 ± 22 | | 65000 ± 167 | 161000 ± 1010 |
| 15 | 32 ± 3 | | 6 ± 4 | 1 ± 2 |
| 16 | 18300 ± 92 | | 22900 ± 135 | 21500 ± 159 |
| 17 | 135 | 421 ± 54 | 199000 ± 402 | 9960000 ± 2000 |
| 18 | 83500 ± 700 | | 124000 ± 5250 | 58500 ± 2180 |
| 19 | 19300 ± 900 | 12100 ± 231 | 16800 ± 565 | 16820 ± 1140 |
| 20 | 11 ± 5 | | 66000 ± 3479 | 2280 ± 23 |
| 21 | 20 ± 2 | | 30300 ± 79 | 3930 ± 526 |
| 22 | 18500 ± 264 | | 37800 ± 29 | 932000 ± 4200 |
| 23 | 429000 ± 50 | | 31 ± 6 | 368 ± 3 |
| 24 | 15 ± 4 | | 14300 ± 20 | 44 ± 7 |
| 25 | 732 ± 57 | 1240 ± 87 | 42200 ± 29 | 12 ± 4 |
| 26 | 66 ± 9 | | 2760 ± 2 | 52 |
| 27 | 816 ± 12 | | 3960000 ± 7012 | 50700000 ± 1330 |
| 28 | 883 ± 4 | | 57 ± 3 | 677 ± 8 |
| 29 | 16 ± 8 | 28 ± 9 | 182 ± 2 | 73 ± 17 |
| 30 | 177 | | 169000 ± 8600 | 11 ± 1 |
| 31 | 154000 ± 6999 | | 65400 ± 191 | 18 ± 1 |
| 32 | 64300 ± 32 | | 547000 ± 4680 | 36 |
| 33 | 751000 ± 186 | | 113000 ± 1950 | 1750 |
| 34 | 374000 ± 1500 | | 116000 ± 3280 | 60400 ± 1600 |
| 35 | 1220 ± 150 | 1940 ± 65 | 348 ± 51 | 4730 ± 378 |
| 36 | 585 ± 37 | | 239 ± 2 | 358000 ± 1000 |
| 37 | 37 ± 2 | | 258000 ± 1350 | 3740 ± 3 |
| 38 | 55 ± 2 | 184 ± 31 | 124 | 1 ± 5 |
| 39 | 3 ± 7 | | 467 ± 2 | 4540 ± 328 |
| 40 | 832000 ± 2750 | | 58000 ± 7090 | 22700 ± 2500 |
| 41 | 532 ± 12 | | 241000 ± 1380 | 697000 ± 53 |
| 42 | 18 ± 1 | | 2090 ± 1 | 388 ± 32 |
| 43 | 17 ± 1 | | 74 | 19 ± 1 |
| 44 | 519000 ± 546 | | 173 ± 27 | 589000 ± 14600 |
| 45 | 248000 ± 371 | | 17100 ± 798 | 162 ± 3 |
| 46 | 10100 ± 119 | 10200 ± 224 | 16500 ± 336 | 32700 ± 227 |
| 47 | 2 | | 99 ± 2 | 73100 ± 4740 |
| 48 | 384 ± 5 | | 112000 ± 572 | 90 ± 7 |
| 49 | 256 ± 24 | | 83800 ± 1840 | 311000 ± 432 |
| 50 | 7 ± 3 | | 170 ± 2 | 1240000 ± 850 |
| 51 | 133 ± 9 | | 403000 ± 63500 | 336000 ± 100 |
| 52 | 7 ± 6 | | 4660000 ± 7960 | 9 ± 2 |
| 53 | 751 ± 12 | 621 ± 56 | 299000 ± 7970 | 935 ± 10 |
| 54 | 37900 ± 189 | | 7120 ± 1970 | 3590000 ± 26 |
| 55 | 24200 ± 1071 | | 456000 ± 7680 | 1160000 ± 3233 |
| 56 | 1310 ± 3 | 768 ± 98 | 11 ± 2 | 57 ± 7 |
| 57 | 9 ± 5 | | 227 ± 1 | 20 ± 11 |
| 58 | 23400 ± 243 | | 13600 ± 8190 | 24600 ± 2230 |
| 59 | 0.1 ± 2 | | 69 ± 1 | 29 ± 3 |
| 60 | 5 ± 3 | | 49 ± 5 | 59300 ± 620 |
| 61 | 2 ± 4 | | 33900 ± 232 | 217000 ± 1400 |
| 62 | 2000 ± 234 | | 516000 ± 22700 | 4610 ± 392 |
| 63 | 12 | 19 | 20 ± 1 | 15 |
| 64 | 10 ± 7 | | 46 | 79 ± 3 |
| 65 | 14 | | 1 | 28 ± 4 |
| 66 | 272 ± 6 | | 100 ± 1 | 2 |

***Supplementary Table 2: SPR binding affinities (nM) of PS binding compounds for RNA oligonucleotides PS1, PS2 and PS3.***

Left to right: Binding affinities (nM) and associated standard errors of the mean of the interaction between PS1(NC_003977.1)/PS1(JQ707375.1)/PS2/PS3 RNA oligonucleotides and compounds identified in the SMM. Errors <1 are omitted. Sensorgrams at 5 different compound concentrations were performed in triplicate. Control oligonucleotides show no significant binding and have therefore been omitted for clarity. Where binding to controls was detected, affinities were several orders of magnitude higher than those for the PSs 1-3 sites. Figures are given to 3 significant figures.

| Compound | $K_D$ / nM | | |
|---|---|---|---|
| | PS1(JQ707375.1) | PS1ΔMotif | PS1ΔBulge |
| 3 | 224000 ± 455 | 421000 ± 367 | 312000 ± 391 |
| 10 | 11400 ± 169 | 48200 ± 258 | 10300 ± 525 |
| 46 | 10200 ± 225 | 35500 ± 126 | 9650 ± 227 |
| 63 | 19 ± 0.5 | 242 ± 3 | 21 ± 0.43 |

***Supplementary Table 3: Binding affinities (nM) and associated standard error of the mean of PS binding compounds used throughout, for RNA oligonucleotides PS(JQ707375.1) and its variants, PS1ΔMotif and PS1ΔBulge. Figures are given to 3 significant figures.***

| Compound | Normalised Anisotropy Change | Standard Error |
|---|---|---|
| PS1 alone | 0.069 | 0.007 |
| PS1 NCP | 1.000 | 0.033 |
| 1 | 0.633 | 0.066 |
| 2 | -0.167 | 0.050 |
| 3 | 0.606 | 0.033 |
| 4 | -0.349 | 0.005 |
| 5 | 0.716 | 0.029 |
| 6 | -0.375 | 0.055 |
| 7 | 0.914 | 0.017 |
| 8 | 0.549 | 0.030 |
| 9 | 1.014 | 0.021 |
| 10 | 0.668 | 0.154 |
| 11 | 1.182 | 0.057 |
| 12 | 2.075 | 0.093 |
| 13 | 1.205 | 0.040 |
| 14 | 0.563 | 0.031 |
| 15 | 0.716 | 0.026 |
| 16 | 0.745 | 0.021 |
| 17 | 0.388 | 0.025 |
| 18 | -0.141 | 0.027 |
| 19 | 0.167 | 0.051 |
| 20 | 0.838 | 0.025 |
| 21 | 0.770 | 0.026 |
| 22 | 0.311 | 0.009 |
| 23 | 0.582 | 0.020 |
| 24 | 1.032 | 0.012 |
| 25 | 0.414 | 0.090 |
| 26 | 0.507 | 0.060 |
| 27 | 0.718 | 0.028 |
| 28 | 0.121 | 0.048 |
| 29 | 0.232 | 0.019 |
| 30 | 0.497 | 0.018 |
| 31 | 0.144 | 0.027 |
| 32 | 0.665 | 0.014 |
| 33 | 1.265 | 0.012 |
| 34 | 0.623 | 0.016 |
| 35 | 0.110 | 0.029 |
| 36 | -0.393 | 0.033 |

| | | |
|---|---|---|
| 37 | 0.649 | 0.040 |
| 38 | 0.585 | 0.009 |
| 39 | -0.544 | 0.005 |
| 40 | 1.188 | 0.016 |
| 41 | 0.994 | 0.029 |
| 42 | 0.254 | 0.038 |
| 43 | -0.131 | 0.015 |
| 44 | 0.965 | 0.025 |
| 45 | 0.367 | 0.034 |
| 46 | 0.705 | 0.200 |
| 47 | 0.728 | 0.029 |
| 48 | 0.938 | 0.005 |
| 49 | 0.885 | 0.011 |
| 50 | 1.311 | 0.059 |
| 51 | 0.129 | 0.026 |
| 52 | 0.040 | 0.028 |
| 53 | 0.596 | 0.005 |
| 54 | 1.104 | 0.042 |
| 55 | 0.807 | 0.063 |
| 56 | 0.918 | 0.026 |
| 57 | 0.141 | 0.014 |
| 58 | 0.903 | 0.036 |
| 59 | 0.314 | 0.018 |
| 60 | 0.235 | 0.041 |
| 61 | 0.647 | 0.014 |
| 62 | 0.931 | 0.058 |
| 63 | 0.496 | 0.036 |
| 64 | 1.125 | 0.027 |
| 65 | 1.451 | 0.018 |
| 66 | 0.938 | 0.085 |

***Supplementary Table 4: Data associated with plate-based assembly inhibition assay using PS1 RNA oligonucleotide.*** Left to right – the compound used, the normalized anisotropy change with respect to the no compound control and standard error of the mean for the triplicate measurements.

| Compound | Normalised Anisotropy Change | Standard Error |
|---|---|---|
| gRNA | 0.031 | 0.006 |
| gRNA NCP | 0.765 | 0.030 |
| 3 | 0.916 | 0.016 |
| 10 | 1.219 | 0.035 |
| 46 | 1.594 | 0.011 |
| 63 | 0.477 | 0.021 |

***Supplementary Table 5: Data associated with plate-based assembly inhibition assay using gRNA.*** Left to right – the compound used, the normalized anisotropy change with respect to the no compound control and standard error of the mean for the triplicate measurements.

[1] F. Fatehi, R.J. Bingham, E.C. Dykeman, N. Patel, P.G. Stockley, & R. Twarock, An intracellular model of hepatitis b viral infection: An in silico platform for comparing therapeutic strategies, Viruses. 13 (2021).

[2] N. Patel, S. Clark, E.U. Weiß, C.P. Mata, J. Bohon, E.R. Farquhar, D.P. Maskell, N.A. Ranson, R. Twarock, & P.G. Stockley, In vitro functional analysis of gRNA sites regulating assembly of hepatitis B virus, Commun. Biol. 2021 41. 4 (2021) 1–12.