Supplementary Information

Title:

The Acyclic Retinoid Peretinoin Inhibits Hepatitis C Virus Replication and Infectious Virus Release *in Vitro*

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Figure Legends for Supplementary Figures

Figure S1. Structures of Peretinoin, ATRA, 9-cis RA, and 13-cis RA

Figure S2. Full-length blots/gels for Figure 2B. In addition to Peretinoin, IFNα2b was added to cells replicating HJ3-5 virus with the indicated concentrations. At 72 h after IFNα2b treatment, the cell lysates were collected and subjected to western blot analysis using anti-core protein and anti-β-actin antibodies.

Figure S3. Effect of Peretinoin on protein expression directed by HCV IRES. (A) Schematic representation of HCV 5′-UTR-GLuc-HCV 3′-UTR RNA and cap-CLuc-polyA RNA (depicted left and right, respectively). **(B)** Both RNAs were transfected into Huh-7.5 cells that had been treated with the indicated concentrations of Peretinoin at 24 h prior to transfection. After transfection, the same concentrations of Peretinoin were added immediately, and the medium was collected and replaced every 3 h until 12 h. GLuc and CLuc activity was determined; GLuc activity was normalised to CLuc activity, and then the ratio was further normalised to that of DMSO-treated cells, which was set to 100%. Data represent the mean ratio ± SD from 2 independent experiments at each time point.

Figure S4. Effect of Peretinoin on cellular IFN signalling. Huh-7.5 cells **(A)** and HCV (H77S.3/GLuc2A)-replicating Huh-7.5 cells **(B)** were treated with 10–40 μM Peretinoin, accompanied with or without IFNα-2b (10 IU/mL). At 3 h later, the cell lysates were collected and subjected to western blot analysis with anti-pSTAT1, anti-STAT1, and anti-β-actin antibodies.

Figure S5. Reduction of LD signal by Peretinoin. Huh-7.5 cells were infected with HJ3-5 virus at an MOI of 1, and 72 h later, 250 μM OA and 0.5 % DMSO or 250 μM OA and 40 μM Peretinoin were added. **(A)** At 72 h later, the cells were fixed and stained for nuclei, LDs, and HCV core protein, and each image was taken under exactly the same condition (nuclei, blue; LDs, green; HCV core protein, red) and then merged. **(B)** The signal intensity of nuclei, HCV core protein, and LDs from 5 cells, which were stained for HCV core protein and LDs, was quantitated as described in the Methods. Data shown represent mean signal intensity ± SD from 5 cells, and the difference was analysed statistically using Student's t-test. N.S., not significant.

Figure S6. Full-length blots/gels for Figure 3D.

Figure S7. Full-length blots/gels for Figure 4C.

Figure S8. Correlation between infectious virus titre and GLuc activity. Huh-7.5 cells were seeded in 48-well plates at a density of 4.0×10^4 cells/well. At 24 h later, the cells were infected with serial dilutions of the HJ3-5/GLuc2A virus. The medium was replaced with fresh medium every 24 h until 72 h. At 72 h later, GLuc activity was determined, and at the same time, the infected cells were fixed and stained for the FFU assay. The correlation between GLuc activity and FFUs/mL was examined. R^2, coefficient of determination.

Figure S9. Intra- and extra-cellular infectivity assay after removing Peretinoin in extra-cellular medium. FT3-7 cells were transfected with HJ3-5/GLuc2A RNA, and 7 days later, 0.5% DMSO, or 20–40 μM Peretinoin, were added. At 72 h later, extra- and intra-cellular viruses were collected and used to infect naïve Huh-7.5 cells. Replication capacity was also determined by measuring secreted GLuc activity. To prepare intra-cellular infectious virus, cell pellets of HJ3-5/GLuc2A-replicating FT3-7 cells harvested after trypsinization were resuspended in complete medium, washed twice with PBS, and lysed by 4 cycles of freezing and thawing. The lysates were clarified by centrifugation at 2,300 × *g* for 5 min prior to inoculation onto naïve Huh-7.5 cells. To remove Peretinoin in the medium derived from extra-cellular cultures, we centrifuged the medium containing Peretinoin in an SW41 rotor (Beckman Coulter Inc., Brea, CA) at 209,678 × *g* for 1.5 h at 4 °C, and then the precipitated virus was re-suspended to fresh medium, with which we infected naïve Huh-7.5 cells. After 6 h inoculation of extra- and intra-cellular virus, medium containing virus was removed by extensive wash, and medium was replaced with fresh one every 24 h until 48 h. At 48 h after infection, we determined GLuc activity and used it as an indicator of the infectious virus titre. Intra- and extra-cellular infectivity was normalised to replication capacity at infection, and these were then normalised to those of DMSO-treated cells, which were set to 100%. The ratio of extracellular infectious virus to intracellular virus was calculated at the indicated conditions, and it was then normalised to DMSO-treated cells, which were set to 100%. Data show the mean ratio to that of DMSO-treated cells ± SD from 3 independent experiments.

Figure S10. Effect of Peretinoin on the entry of HCV into cells. (A) Huh 7.5 cells were treated with the indicated concentrations of Peretinoin for 72 h, and then they were infected with HJ3-5 virus at MOI of 1. The infected cells were incubated 2 h at 4 and 37 °C, and medium containing virus was extensively washed and removed. At 3h later, total cellular RNA was extracted, and the amount of HCV RNA and 18S rRNA was quantitated by RTD-PCR. Relative HCV RNA abundance normalised to the amount of 18S rRNA is presented as fold change ± SD

compared to DMSO-treated cells at 37 °C from 3 independent experiments. **(B)** Huh-7.5 cells were treated with the indicated concentrations of Peretinoin for 48 h, and then, they were infected with serial dilutions of HJ3-5 virus. The infected cells were maintained without Peretinoin, and the infectious virus titer was determined at 72 h later. Data show the mean FFUs/mL ± SD from at least 3 independent experiments.

Figure S11. Selection of Peretinoin-resistant HCV mutants or cells. Huh-7.5 cells were transfected with H77S.3/GLuc2A RNA. At 48 h later, Peretinoin was added at the indicated concentrations. The medium was collected and replaced with fresh medium every 24 h for 13–15 days. When the cells became confluent, they were passaged at a 1:3 ratio, and GLuc activity was determined every day. Two sets of experiments were performed independently, depicted as Sets 1 and 2. Data show the mean GLuc activity ± SD from 3 different plates.

Supplementary Methods

RNA transcription

To synthesise cap-CLuc-polyA RNA, pCMVCLuc (New England Biolabs, Inc., Tokyo, Japan) was amplified by PCR using 2 primers (5′-TAA TAC GAC TCA CTA TAG GGC CAC CAT GAA GAC CTT AAT TCT TGC CGT T-3′ and 5′-AGA CAC ACA AAA AAC CTA CAC ACA GAT GTA ATG-3′), and the resulting PCR product was used as a template for the following RNA transcription. Capped RNA was synthesised using a MEGAscript T7 Kit (Life Technologies, Carlsbad, CA) by following a modified protocol for capped RNA transcription. Synthesised RNA was purified using an RNeasy Mini Kit.

Antibodies for western blot analysis

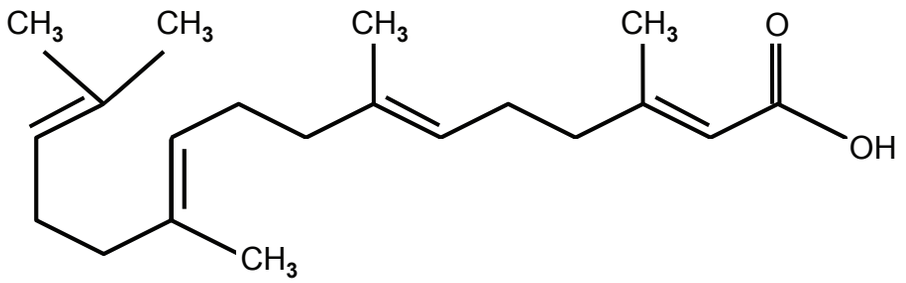
The expression of phosphorylated STAT1 (pSTAT1) and STAT1 was evaluated by using rabbit anti-pSTAT1 and rabbit anti-STAT1 antibodies, respectively.

Secreted luciferase activity

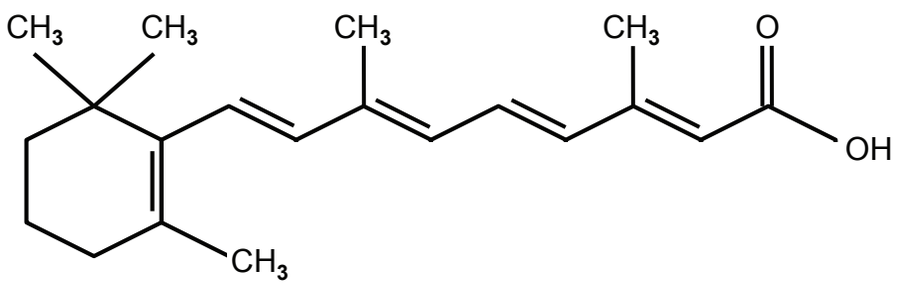
The activity of secreted CLuc was determined using a BioLux *Cypridina* Luciferase Assay Kit.

Figure S1

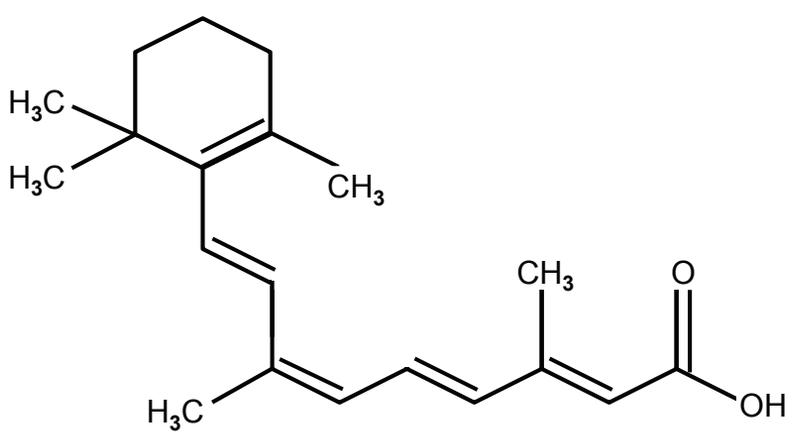
Peretinoin



ATRA



9-cis RA



13-cis RA

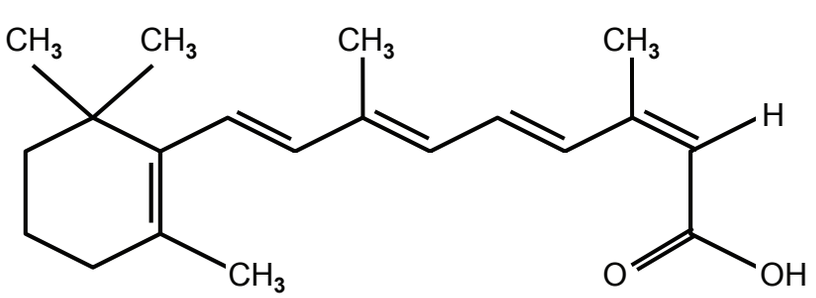


Figure S2

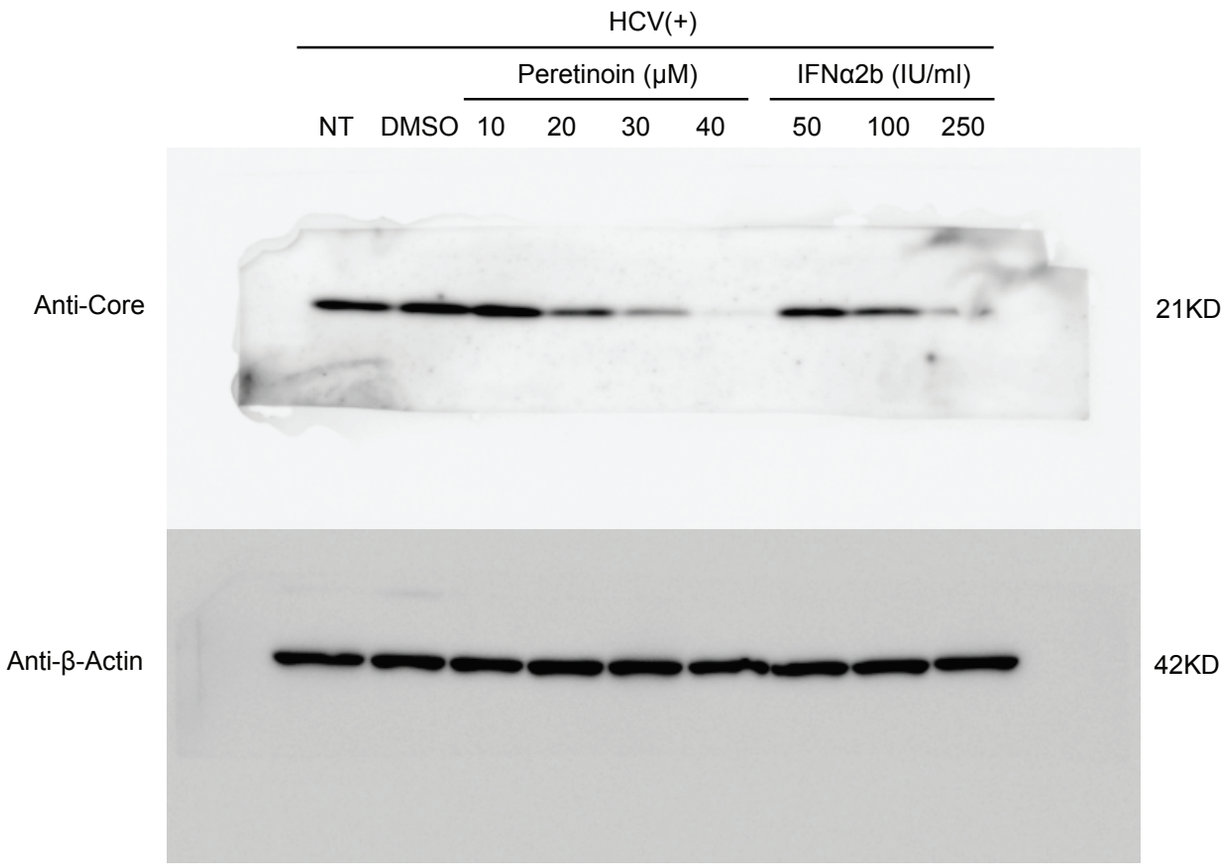

HCV(+)
Peretinoin (μM)
IFNα2b (IU/ml)
NT
DMSO
10
20
30
40
50
100
250
Anti-Core
21KD
Anti-β-Actin
42KD

Figure S3

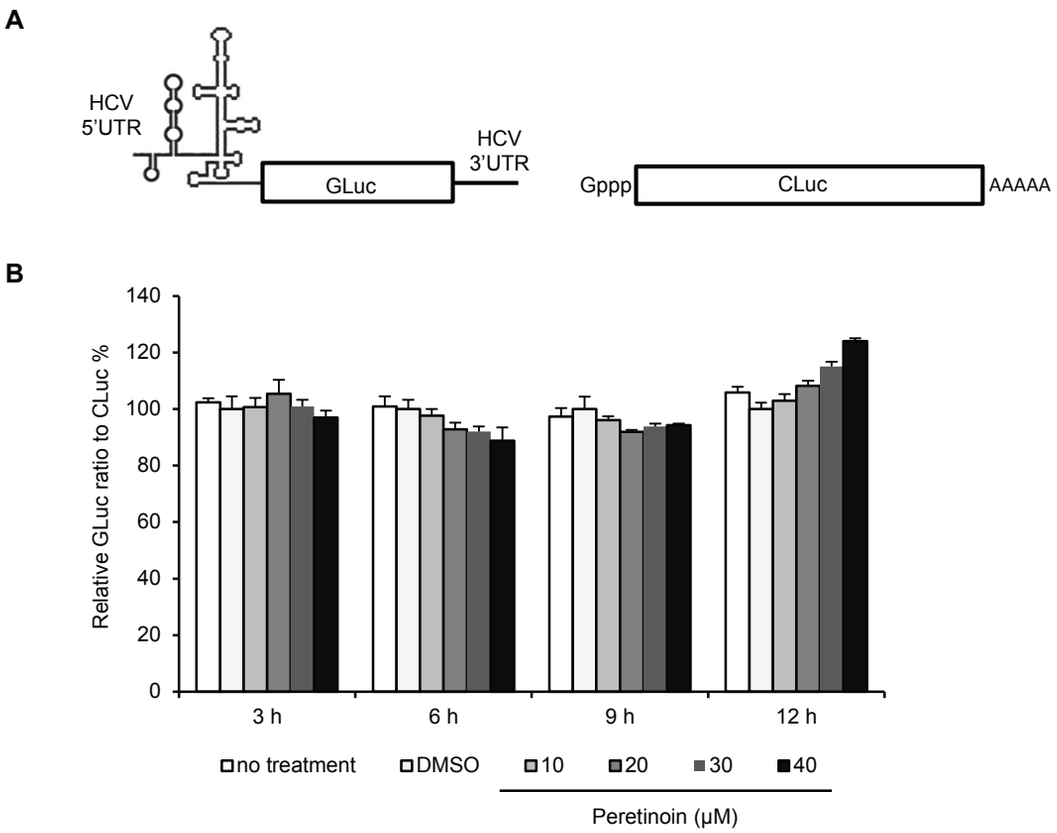

A
HCV
5'UTR
GLuc
HCV
3'UTR
Gppp
CLuc
AAAAA
B
Relative GLuc ratio to CLuc %
140
120
100
80
60
40
20
0
3 h
6 h
9 h
12 h
no treatment
DMSO
10
20
30
40
Peretinoin (μM)

Figure S4

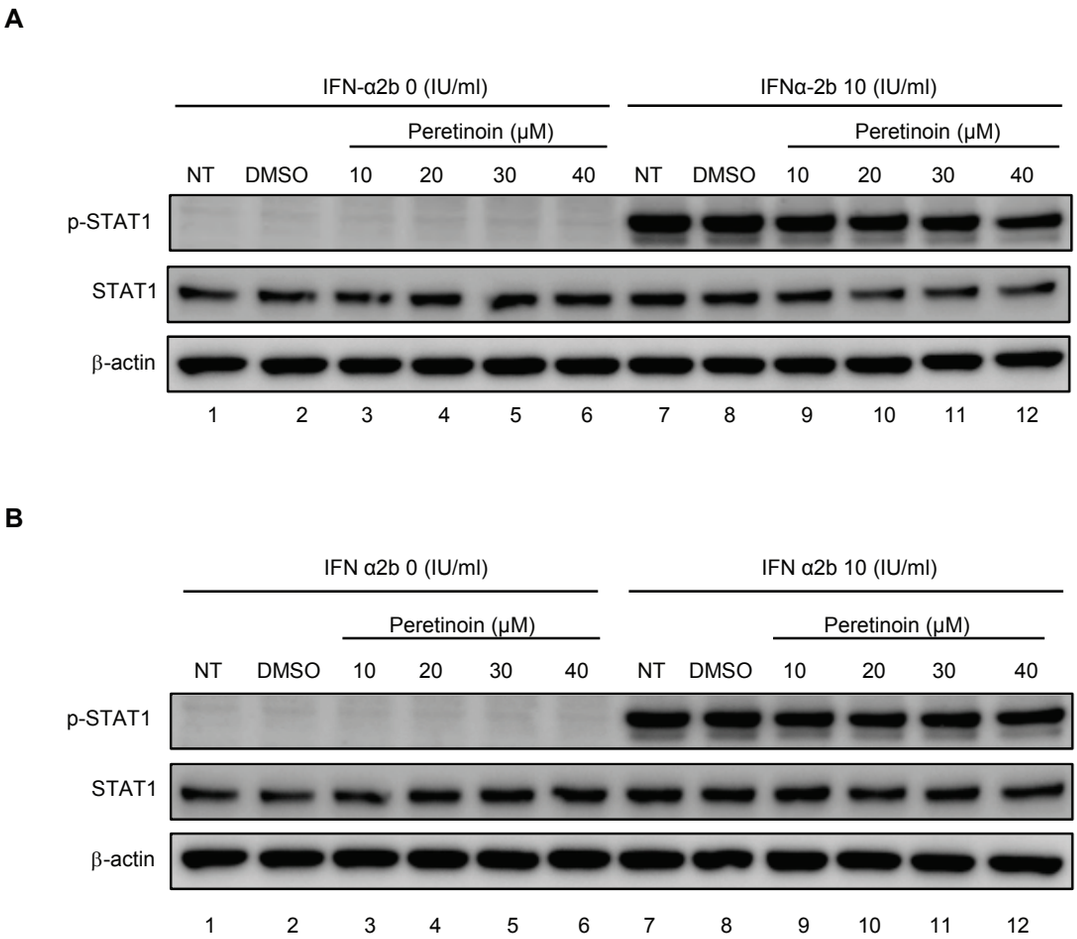
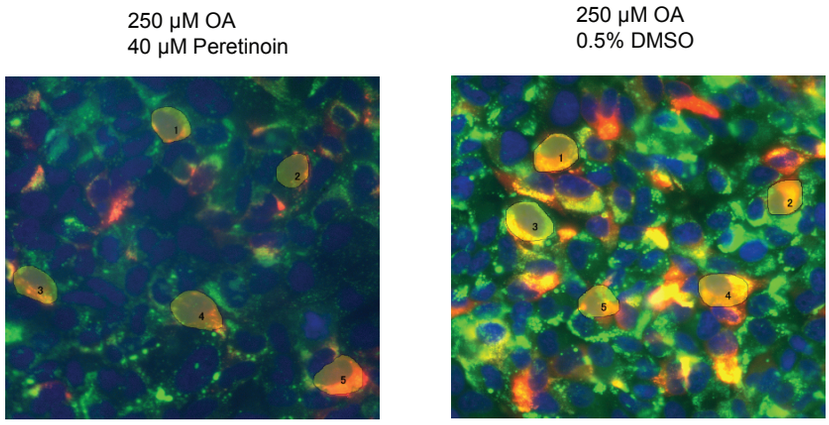

A
IFN-α2b 0 (IU/ml)
IFNα-2b 10 (IU/ml)
Peretinoin (μM)
Peretinoin (μM)
NT DMSO 10 20 30 40 NT DMSO 10 20 30 40
p-STAT1
STAT1
β-actin
1 2 3 4 5 6 7 8 9 10 11 12
B
IFN α2b 0 (IU/ml)
IFN α2b 10 (IU/ml)
Peretinoin (μM)
Peretinoin (μM)
NT DMSO 10 20 30 40 NT DMSO 10 20 30 40
p-STAT1
STAT1
β-actin
1 2 3 4 5 6 7 8 9 10 11 12

Figure S5

A


250 μM OA
40 μM Peretinoin
250 μM OA
0.5% DMSO

B

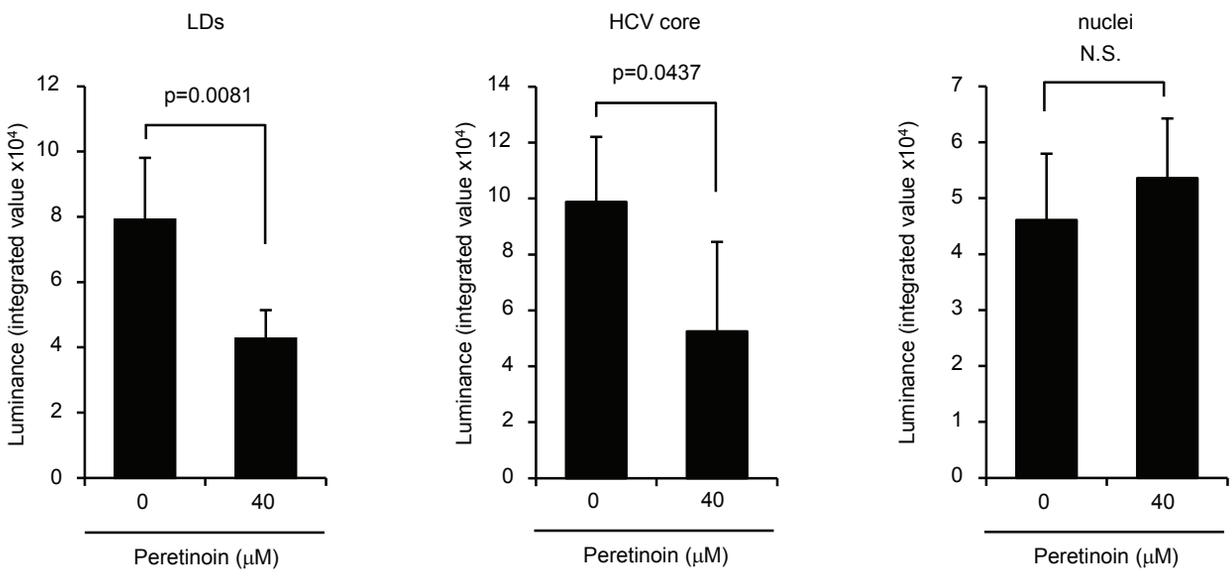

LDs
p=0.0081
Luminance (integrated value x10⁴)
Peretinoin (μM)
HCV core
p=0.0437
nuclei
N.S.

Figure S6

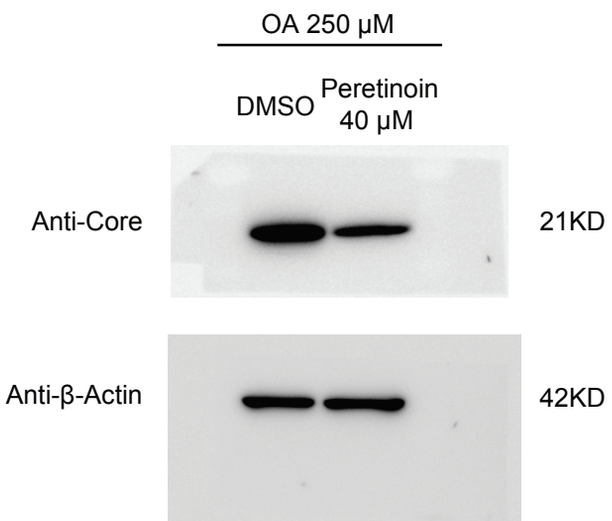

OA 250 μM
DMSO
Peretinoin
40 μM
Anti-Core
21KD
Anti-β-Actin
42KD

Figure S7

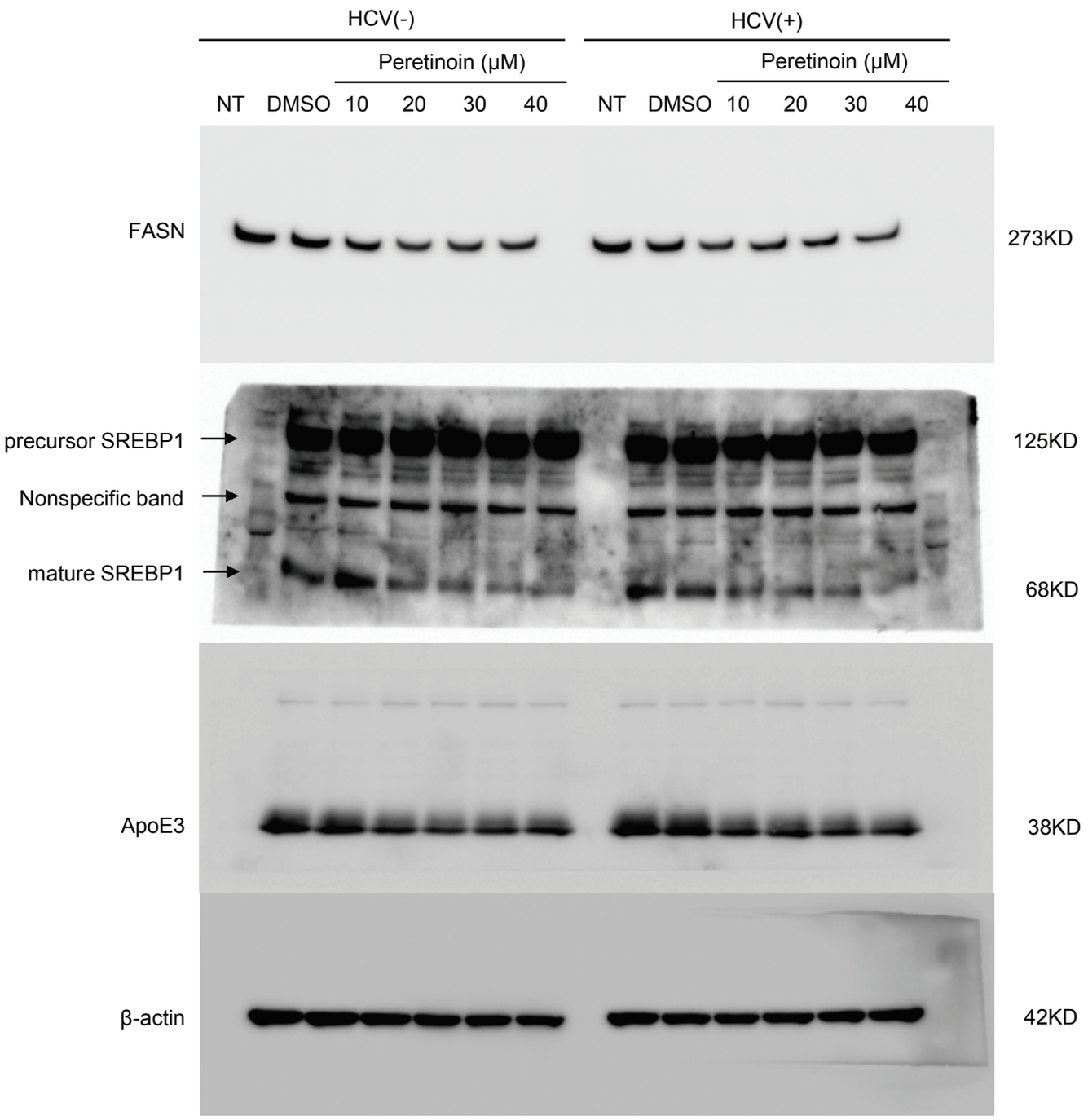

HCV(-)
HCV(+)
Peretinoin (μM)
Peretinoin (μM)
NT
DMSO
10
20
30
40
NT
DMSO
10
20
30
40
FASN
273KD
precursor SREBP1
Nonspecific band
mature SREBP1
125KD
68KD
ApoE3
38KD
β-actin
42KD

Figure S8

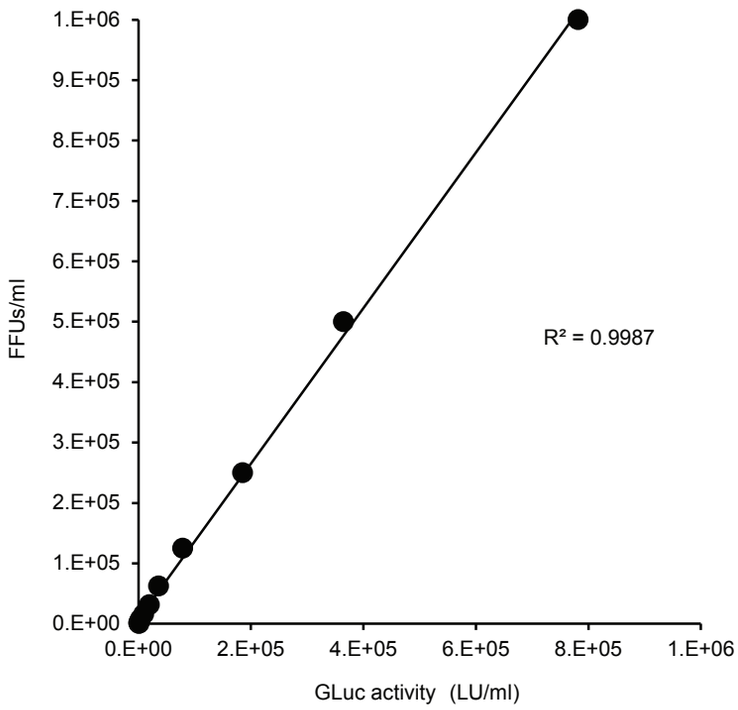

1.E+06
9.E+05
8.E+05
7.E+05
6.E+05
5.E+05
4.E+05
3.E+05
2.E+05
1.E+05
0.E+00
FFUs/ml
R² = 0.9987
0.E+00
2.E+05
4.E+05
6.E+05
8.E+05
1.E+06
GLuc activity (LU/ml)

Figure S9

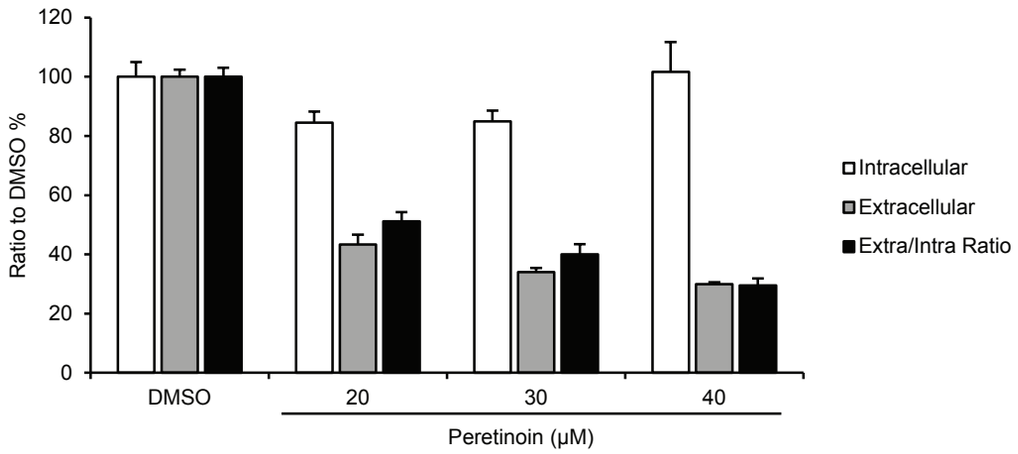
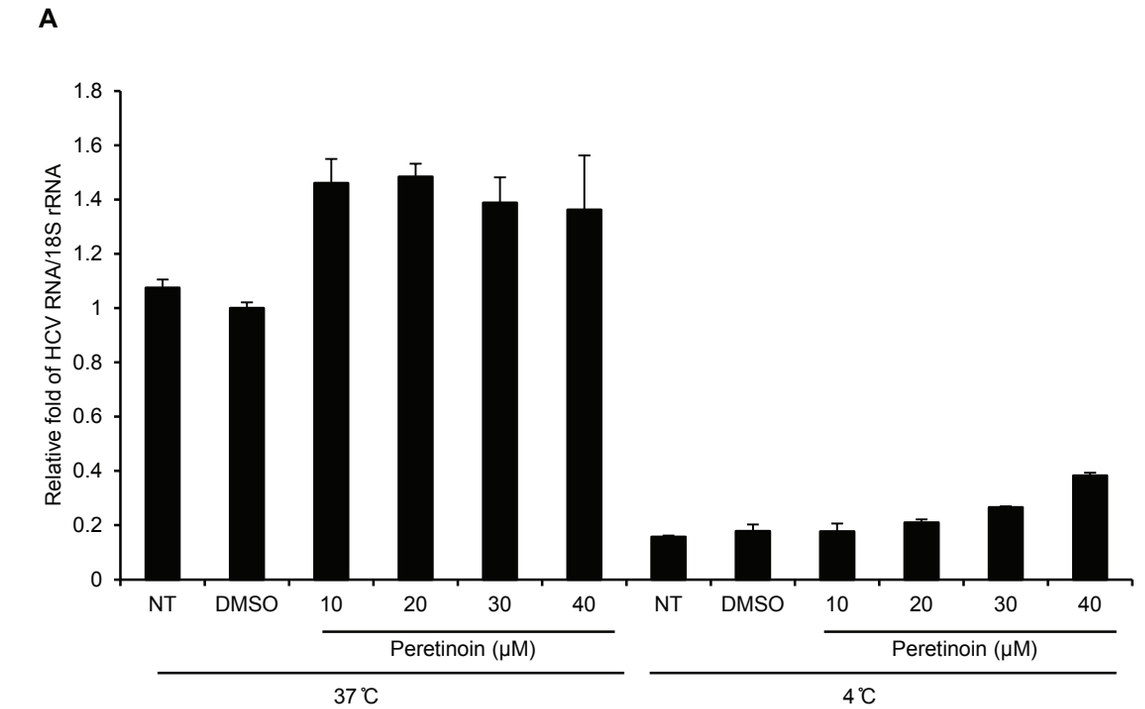

120
100
80
60
40
20
0
Ratio to DMSO %
DMSO
20
30
40
Peretinoin (μM)
Intracellular
Extracellular
Extra/Intra Ratio

Figure S10


A
Relative fold of HCV RNA/18S rRNA
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NT
DMSO
10
20
30
40
NT
DMSO
10
20
30
40
Peretinoin (μM)
Peretinoin (μM)
37℃
4℃

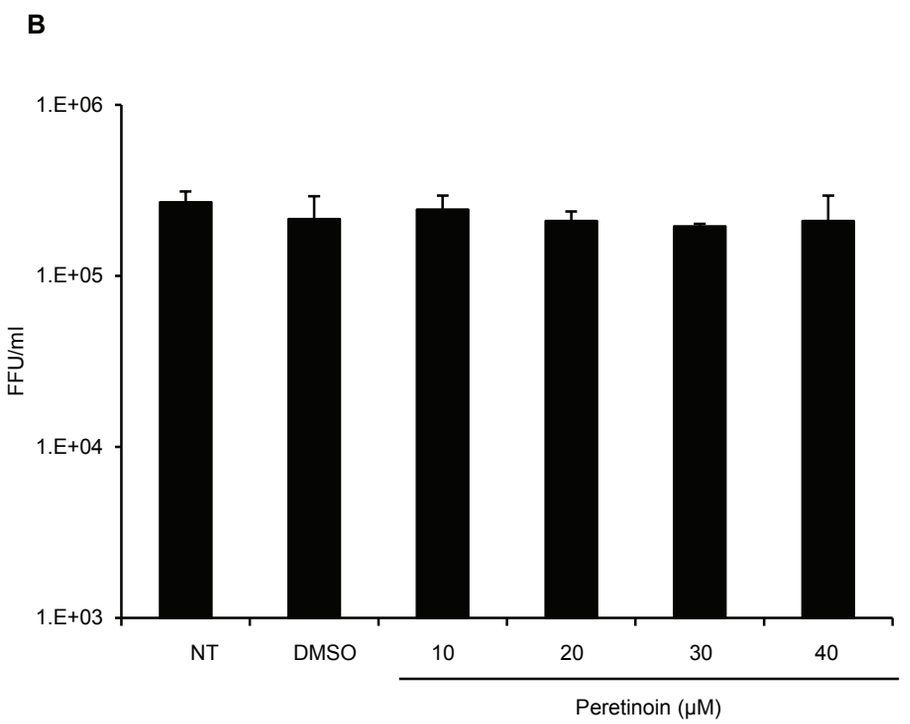
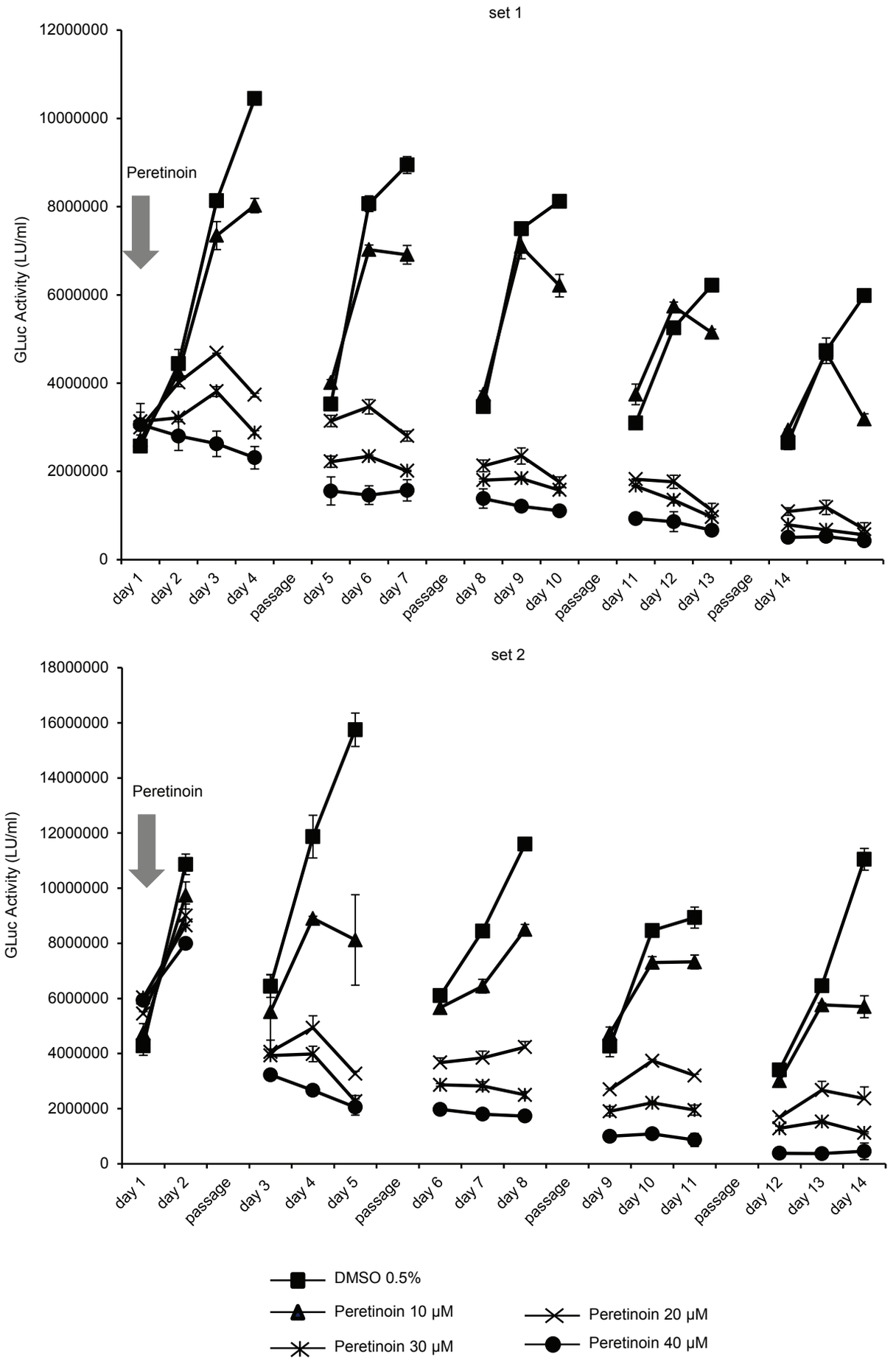

B
FFU/ml
1.E+06
1.E+05
1.E+04
1.E+03
NT
DMSO
10
20
30
40
Peretinoin (μM)

Figure S11


set 1
GLuc Activity (LU/ml)
12000000
10000000
8000000
6000000
4000000
2000000
0
Peretinoin
day 1
day 2
day 3
day 4
passage
day 5
day 6
day 7
passage
day 8
day 9
day 10
passage
day 11
day 12
day 13
passage
day 14
set 2
GLuc Activity (LU/ml)
18000000
16000000
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
Peretinoin
day 1
day 2
passage
day 3
day 4
day 5
passage
day 6
day 7
day 8
passage
day 9
day 10
day 11
passage
day 12
day 13
day 14
DMSO 0.5%
Peretinoin 10 μM
Peretinoin 20 μM
Peretinoin 30 μM
Peretinoin 40 μM