Supplementary Materials

Title: Off-target In Vitro Profiling Demonstrates that Remdesivir Is a Highly Selective Antiviral Agent

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Content

Table S1: *In vitro* effects of RDV and GS-441524 on mtDNA levels after 10-day treatment in HepG2 cells

Study	Compound	Concentration (μM)	Relative Amount of mtDNA (% Control)[a]	*p*-value Compared to Control[b]
1	DMSO (control)	—	100.0 ± 22.2	—
	RDV	0.04	88.9 ± 30.4	0.215
		0.2	98.1 ± 30.1	0.702
		1.0	93.4 ± 27.1	0.436
	ddC	0.2	76.9 ± 12.8	0.005
		2.0	10.0 ± 3.8	< 0.001
		20.0	0.7 ± 0.2	< 0.001
2	DMSO (control)	—	100.0 ± 20.3	—
	RDV	0.4	122.8 ± 23.9	0.067
		2.0	74.2 ± 11.7	0.008
		10.0	148.0 ± 25.6	0.001
	ddC	0.2	94.7 ± 3.4	0.005
		2.0	56.5 ± 10.5	< 0.001
		20.0	12.2 ± 2.8	< 0.001
3	DMSO (control)	—	100.0 ± 8.8	—
	GS-441524	1.0	96.1 ± 31.5	0.70
		10.0	90.3 ± 21.9	0.22
		100	83.3 ± 11.7	0.003
	ddC	0.2	57.0 ± 10.4	< 0.001
		2.0	25.1 ± 7.8	< 0.001
		20.0	6.9 ± 2.9	< 0.001

[a] Data represent average ± SD of three independent experiments performed in triplicate.

[b] Paired, two-tailed Student's t-test.

Table S2: Formation of active metabolite GS-443902 in HEp-2, PC-3, and PHH cells after 24-hr treatment of RDV and GS-441524 at 1 μM.

Cells	Formation of 5'-triphosphate Concentration[a] (in pmole/million cells) and in μM[b]	
	RDV	GS-441524
HEp-2	43 ± 6 (19 μM[b])	5.5 ± 1.1 (2.4 μM)
PC-3	153 ± 16 (81 μM)	6.6 ± 4.9 (3.5 μM)
PHH	45 ± 23 (13.6 μM)	1.9 ± 1.6 (0.56 μM)

[a] Represents average ± STD of three independent experiements. Data for PHH was from three donors.

[b] The cellular concentration was converted to μM using the following cell volumes measured using confocal imaging: HEp-2 = 2.3 ± 1.2 pL, PC-3 = 1.89 ± 0.95 pL, and PHH = 3.4 ± 0.2 pL based on measurements of > 50 cells. Data for PHH were measured using cells from three donors.

30 **Table S3:** Eighty-seven mammalian molecular targets tested against RDV-containing

31 diastereomeric mixture GS-466547 and the nucleoside analog GS-441524 at 10 μM

Category	Family	Name	Species
GPCR	Acetylcholine	M1, M2, M3, and M4	human
	Adenosine	A1, A2A	human
	Adrenoceptor	Alpha: 1A, 1B, 1D, 2A, 2B Beta: 1 and 2	human
	Angiotensin	AT1	human
	Bradykinin	B2	human
	Cannabinoid	CB1, CB2	human
	Chemokine	CCR1, CXCR2 (IL-8RB)	human
	Cholecysteokinin	CCK1 (CCKA), CCK2 (CCKB)	human
	Dopamine	D1, D2S, D2L	human
	Endothelin	ETA	human
	GABAB	B1b/B2	human
	Glutamate (metabotropic)	mGlu5	human
	Histamine	H1 and H2	human
	Leukotriene	CysLT1	human
	Melanocortin	MC1 and MC4	mouse
	Neuropeptide Y	Y1	human
	Opioid	Delta (DOP), Kappa (KOP), Mu (MOP)	human
	Platelet-activating factor	PAF	human
	Serotonin	5-HT1A, 5-HT2A, 5-HT2B, 5-HT2C	human
	Tachykinin	NK1	human
	Vasopressin/oxytocin	V1A	human
Nuclear receptor	Androgen	AR	human
	Estrogen	ERalpha	human
	Glucocorticoid	GR	human
	Progesterone	PR	human
	Retinoic acid	RARalpha (NR1B1)	human
Ion channel	Acetylcholine	nAChR (muscle-type)	human
	Calcium	Cav1.2, L-type: dihydropyridine site, diltiazem site, verapamil site; Cav2.2, N-type	rat
	GABAA	Non-selective: flunitrazepam-binding, TBOB-binding; Alpha1/beta2/gamma2 (human)	rat
	Glutamate	Non-selective: TCP-binding, AMPA-binding, kainate-binding, NMDA-binding, strychnine-insensitive	rat

	Glycine	Strychnine-binding (non-selective)	rat
	Potassium	KV (non-selective), hERG dofetilide-binding	rat
	Serotonin	5-HT1A, 5-HT1B, 5-HT2A, 5-HT2B, 5-HT2C, 5-HT3	human
	Sodium	Batrachotoxinin binding (site 2)	rat
Transporter	Nucleoside	Adenosine	Guinea pig
	ATase	Na^+/K^+, brain	pig
	Dopamine	DAT	human
	GABA	GABA (non-selective)	rat
	Norepinephrine	NET	human
	Serotonin	SET	human
Lipid metabolism	Cyclooxygenase	COX1, COX2	human
Neurotransmitter metabolism	Monoamine oxidation	Monoamine oxidase MAO-A, MAO-B	rat
	Acetylcholine turnover	Acetylcholinesterase	human
	Peroxisome proliferator-activated receptors	PPARgamma	human
Kinase	Insulin receptor kinase	IRK	human
	Tyrosine kinase	p56lck kinase (LCK)	human
	PKC kinase	PKCalpha	human
Phosphodiesterase	Cyclic nucleotide signaling	PDE3A, PDE4D2	
Protease	Chymotrypsin serine peptidase	Cathepsin G	human
	Zinc metalloprotease	Angiotensin converting enzyme (ACE)	human

Table S4: Effect of compounds on mitochondrial respiration after 4-hour treatment in human primary hepatocytes

Compounds	CC_{50} (µM)[a]		
	Spare Respiratory Capacity	**DNA Level**	**ATP**
RDV	> 30	> 30	> 30
GS-704277	> 100	> 100	> 100
GS-441524	> 100	> 100	> 100
Phenformin	15.0 ± 5.2	> 100	> 100

[a] CC_{50} values were reported as an average of three or more independent experiments

Table S5: Validation of the $2^{-\Delta\Delta C_T}$ Method for Cytochrome b and β-actin Target Genes

Amount of Total Cellular DNA (ng/reaction)	C_T Value[a]		ΔC_T Value
	Cytochome b	β-actine	
1.56	23.41	32.74	-9.33
3.13	22.43	31.82	-9.39
6.25	21.48	30.8	-9.32
12.5	20.31	29.9	-9.59
25	19.36	29.14	-9.78
50	18.63	28.5	-9.87

[a] The data represent the mean ± SD of three independent experiments performed in triplicate.

Table S6: DNA and RNA primers and templates used in single-nucleotide incorporation assays

Oligonucleotides	Sequences[a]
Primer (D19)	5'-GGTCCCTGTTCGGGCGCAC-3'
Templates (D36)	3'-CGAAAGTCCAGGGACAAGCCCGCGTG**T**GTATCTCT-5'
	3'-CGAAAGTCCAGGGACAAGCCCGCGTG**G**CTATCTCT-5'
	3'-CGAAAGTCCAGGGACAAGCCCGCGTG**A**CTATCTCT-5'
	3'-CGAAAGTCCAGGGACAAGCCCGCGTG**C**GTATCTCT-5'
Primer (R12)	5'-UUUUGCCGCGCC-3'
Templates (D18)	3'-CGGCGCGG**T**ACGTAAGGG-5'
	3'-CGGCGCGG**G**TACTAAGGG-5'
	3'-CGGCGCGG**A**CGTTAAGGG-5'
	3'-CGGCGCGG**C**ATGTAAGGG-5'

[a] The bold letter in the template sequences denotes the bases that pair with the incoming dNTP or NTP.

Figures legends

Figure S1: Effect of GS-441524, parent nucleoside of RDV, on mitochondrial respiration (spare respiratory capacity) (● blue, solid lines), ATP levels (■ red, dotted lines), and total DNA (♦ green, dashed lines) after a 3-day treatment in PC-3, HepG2, PHH, and RPTEC. The spare respiratory capacity was normalized by cell numbers. GS-441524 showed minimal inhibition of mitochondrial respiration, ATP levels, and total DNA in all cells tested.

Figure S2: Known mitochondrial toxin chloramphenicol showed a specific inhibition of mitochondrial respiration (spare respiratory capacity) (● blue, solid lines), but no effect on ATP levels (■ red, dotted lines) and total DNA (♦ green, dashed lines) after a 3-day treatment in PC-3. The spare respiratory capacity was normalized by cell numbers.

Figure S3: Acute effect of RDV and its metabolites GS-704277 and GS-441524 on PHH after a 4-hour treatment. The effects on mitochondrial respiration (spare respiratory capacity) (● blue, solid lines), ATP levels (■ red, dotted lines), and total DNA (♦ green, dashed lines) are shown as % of DMSO-treated control. The spare respiratory capacity was normalized by cell numbers. None of the compounds tested showed specific inhibition of mitochondrial respiration, ATP levels, and total DNA, while the positive control phenformin, a known mitochondrial toxin, showed specific inhibition of mitochondrial respiration.

Figure S4: Effect of GS-704277, a metabolite of RDV, on mitochondrial respiration (spare respiratory capacity) (● blue, solid lines), ATP levels (■ red, dotted lines), and total DNA (♦ green, dashed lines) after a 3-day treatment in PHH. The spare respiratory capacity was normalized by cell numbers. GS-704277 showed no inhibition of the mitochondrial respiration, ATP levels, and total DNA at the highest concentration tested (100 μM). Phenformin, a known mitochondria toxin, specifically inhibited mitochondrial respiration.

Materials and Methods

HPLC-MS/MS method

HPLC parameters

Column: Phenomenex Luna C18 HST 2.5 μm 2.0 x 50 mm column (part No. 00B-4446-B0)

Mobile phases: Mobile phase A containing 3 mM ammonium formate (pH 5) with 10 mM dimethylhexylamine (DMHA) in water; Mobile phase B containing 3 mM ammonium formate (pH 5) with 10 mM DMHA in 50% acetonitrile.

HPLC pump and Autosampler: Shimadzu LC-30AD binary pump system was used for elution and separation, with an HTC PAL autosampler from LEAP Technologies.

Flow rate: 0.35 mL/min

HPLC elution program:

Time (min)	Mobile Phase B (%)
0	18
0.5	18
4.2	50
5.2	70
6.0	100
6.2	18

Sciex QTrap 6500+ mass spectrometer was used for metabolite analysis in multiple reaction monitoring (MRM) mode.

Mass spectrometry parameters

Ion source	Probe height (mm)	Spray voltage (V)	Temperature (°C)	Entrance Potential (V)	Curtain gas (psi)	GS1 (psi)	GS2 (psi)	Collision gas (psi)
ESI +	4	5500	550	10	30	30	30	9

MRM Channels

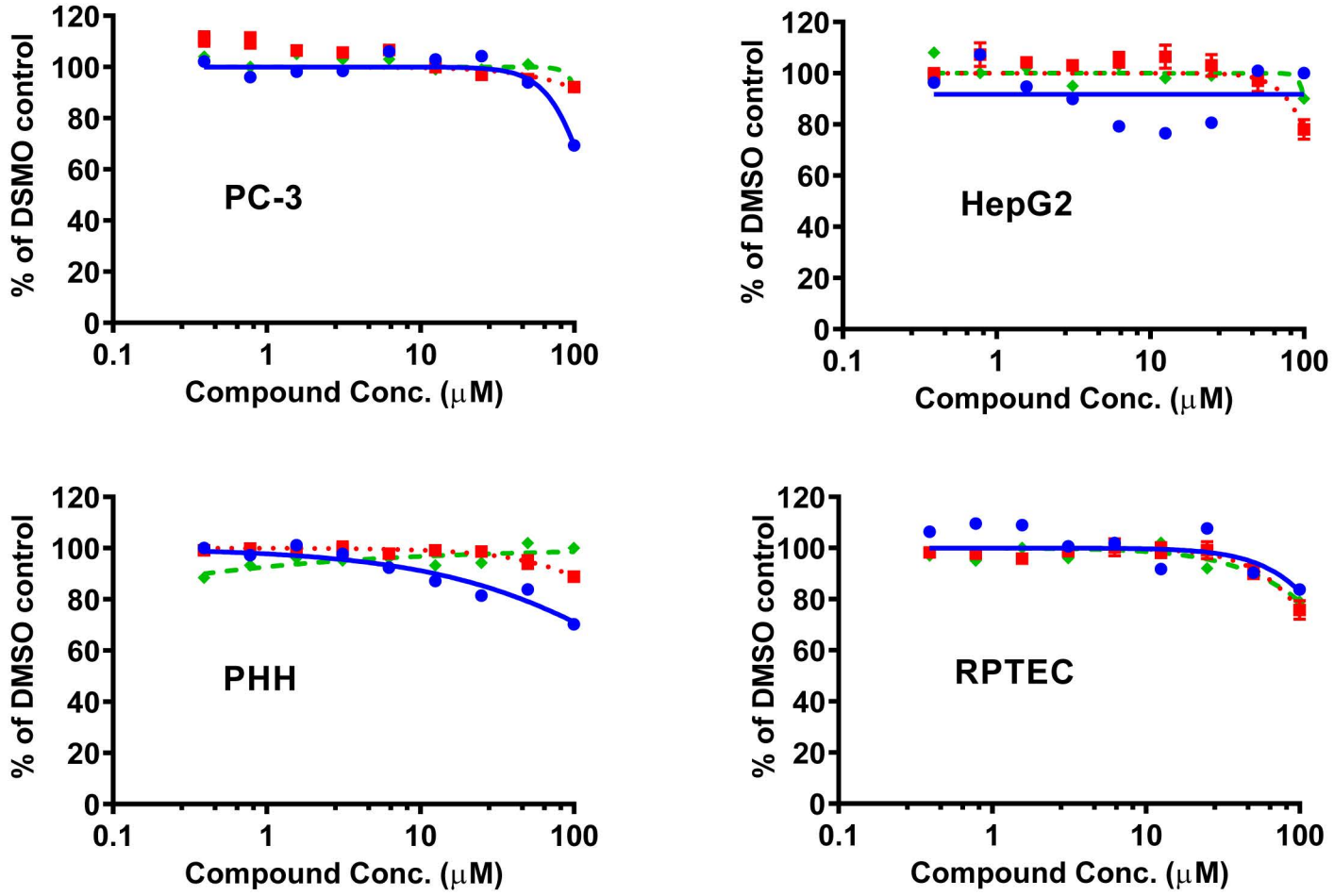
Analyte	Description	Parent Mass (m/z)	Product Mass (m/z)	Declustering Potential (V)	Collision Energy (volts)	CXP (volts)
GS-441524	Parent	292.0	202.1	120	17	14
GS-441524-MP	Parent-monophosphate	372.0	202.1	120	22	20
GS-441524-DP	Parent-diphosphate	452.0	202.1	120	23	20
GS-443902	Parent-triphosphate	532.0	202.1	120	29	13
Chloro-ATP	Internal standard	541.9	169.9	120	39	36

Calibration Standard Curve

Standard	*Concentration (fmol/sample)*
1	40,000
2	10,000
3	2,500
4	625
5	156
6	39.1

Retention Times

Analyte	*Retention Time (min)*
Chloro-ATP	4.8
GS-443902	4.7
GS-441524-DP	4.1
GS-441524-MP	3.1
GS-441524	1.5



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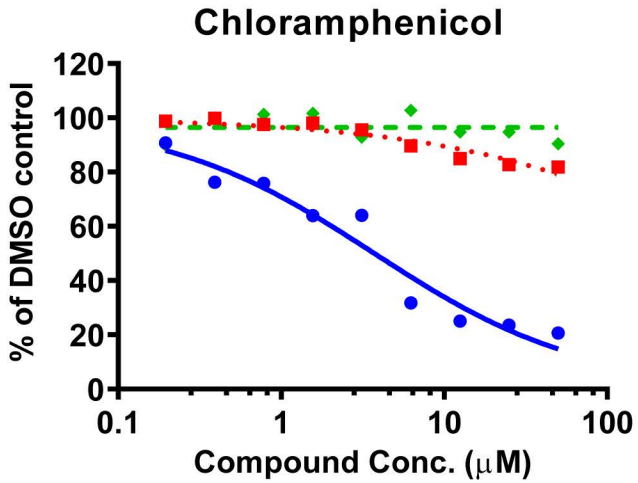

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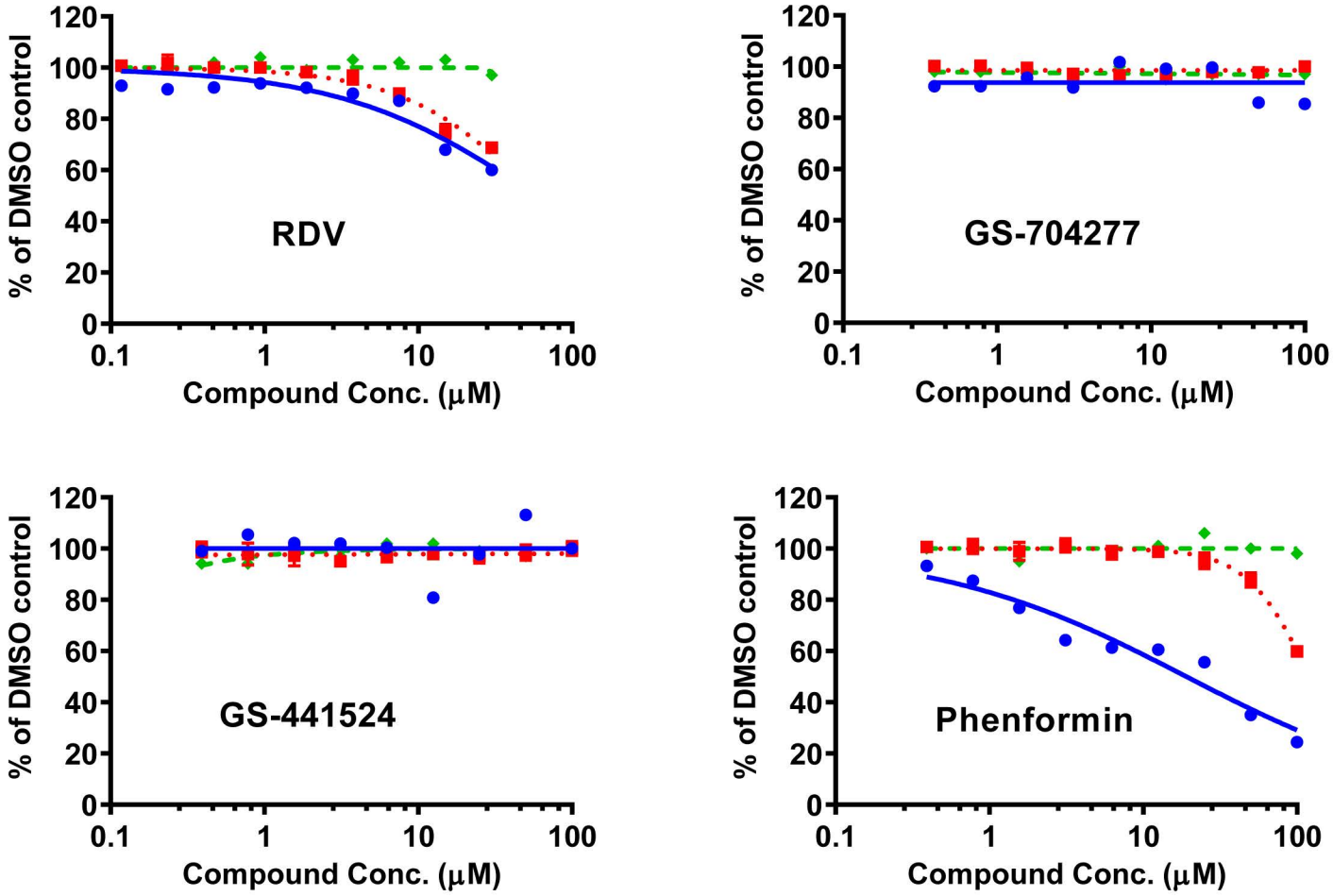

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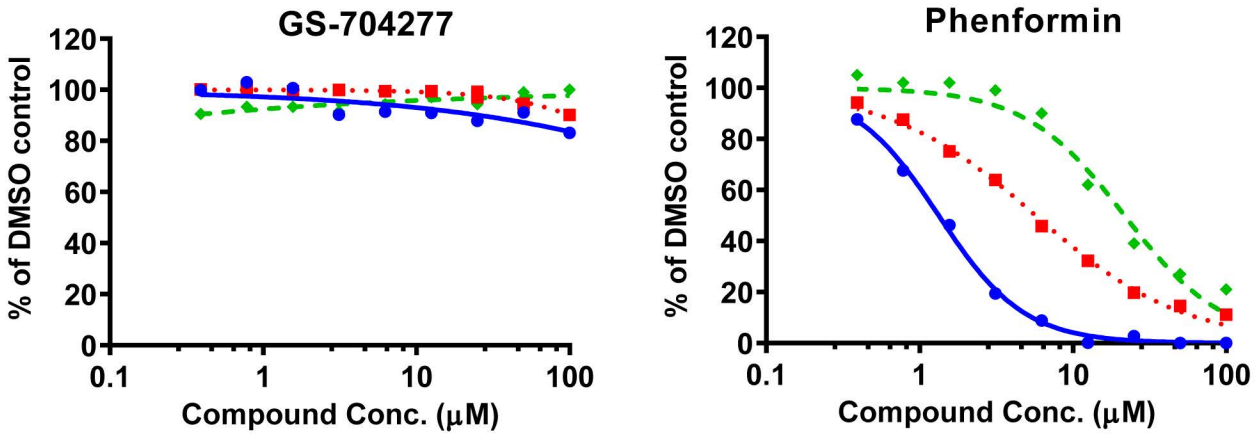

Figure S4. Effect of GS-704277, a metabolite of RDV, on mitochondrial respiration (spare respiratory capacity) (● blue, solid lines), ATP level (■ red, dotted lines), and total DNA (♦ green, dashed lines) after 3-day treatment in PHH. The spare respiratory capacity was normalized by cell numbers. GS-704277 showed no inhibition of the mitochondrial respiration, ATP level, and total DNA at the highest concentration tested (100 µM). Phenformin, a known mitochondria toxin, specifically inhibited mitochondrial respiration.