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SAMHD1 enhances nucleoside-analogue efficacy against HIV-1 in myeloid cells

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SAMHD1 is an intracellular enzyme that specifically degrades deoxynucleoside triphosphates into component nucleoside and inorganic triphosphate. In myeloid-derived dendritic cells and macrophages as well as resting T-cells, SAMHD1 blocks HIV-1 infection through this dNTP triphosphohydrolase activity by reducing the cellular dNTP pool to a level that cannot support productive reverse transcription. We now show that, in addition to this direct effect on virus replication, manipulating cellular SAMHD1 activity can significantly enhance or decrease the anti-HIV-1 efficacy of nucleotide analogue reverse transcription inhibitors presumably as a result of modulating dNTP pools that compete for recruitment by viral polymerases. Further, a variety of other nucleotide-based analogues, not normally considered antiretrovirals, such as the anti-herpes drugs Aciclovir and Ganciclovir and the anti-cancer drug Clofarabine are now revealed as potent anti-HIV-1 agents, under conditions of low dNTPs. This in turn suggests novel uses for nucleotide analogues to inhibit HIV-1 in differentiated cells low in dNTPs.

Sterile α-motif/histidine-aspartate domain-containing protein 1 (SAMHD1) is an antiretroviral protein that restricts HIV-1 infection in non-cycling cells such as macrophages[1], dendritic cells (DCs)[2] and resting $CD4^+$ T-cells[3,4]. Viruses from the HIV-2/SIV_{smm} and SIV_{rcm}/SIV_{mnd-2} lineages encode the accessory protein Vpx that overcomes this restriction by directing SAMHD1 for proteasomal degradation[1,2,5,6].

The prevailing hypothesis is that SAMHD1 restricts HIV-1 replication through its dNTP triphosphohydrolase activity by depleting the intracellular dNTP pool to levels that do not support viral reverse transcription[7–10]. More recently, it has been proposed that SAMHD1 nucleic acid binding and a nuclease activity might contribute to alternative mechanisms of restriction[11–14]. However, although measurements of nucleic acid binding support this notion[14,15], variability in the *in vitro* measurements of nuclease activity appear inconsistent with this idea[11,14]. In contrast, the nature of the allosteric regulation of SAMHD1 triphosphohydrolase activity through nucleotide binding and tetramerisation has been extensively characterised both structurally[10,16–19] and biochemically[19–22].

SAMHD1 restriction activity is also regulated by phosphorylation. In cycling THP_1 cells that are relatively permissive to HIV-1 infection, SAMHD1 is largely phosphorylated by cyclin A2/CDK1 at Threonine 592. By contrast, T592 phosphorylation is reduced in differentiated THP-1 cells that are restrictive to HIV-1 infection[23–25]. In other cell types and primary macrophages, CDK2 has been proposed to be the kinase that phosphorylates SAMHD1[26,27] controlled by upstream regulation through the cyclin D3/CDK6 complex[28,29]. Moreover, CyclinL2 has been proposed to be a negative regulator of SAMHD1 in macrophages[30], whereas a cyclin D2/CDK4/p21 complex has been proposed to be responsible for maintaining the non-phosphorylated form of SAMHD1 in GM-CSF derived macrophages[31].

The rate of HIV-1 proviral synthesis is limited by the intracellular dNTP concentration[32] and it can be accelerated in non-dividing cells by elevating intracellular dNTP levels[33]. Although SAMHD1 reduces the dNTP pool in non-cycling cells thereby decreasing HIV-1 infection[7–9], other reports showed that SAMHD1 depletion of dNTP levels in cells could also increase the susceptibility of HIV-1 to nucleoside reverse transcriptase inhibitors (NRTIs)

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Nucleoside analogue[a]	Generic name	Therapeutic use[b]	Base	References
Antiherpesvirus				
ACV	Aciclovir	HSV-1, HSV-2 and VSV treatment, EBV and CMV prevention	G	(37, 38)
GCV	Ganciclovir	Treatment of CMV infections	G	(39)
Anticancer				
CFB	Clofarabine	Treatment of ALL in children after failure.	A[c]	(40, 41)
NRTIs				
D4T	Stavudine	HIV/AIDS therapy	T	(42, 43)
ddI	Didanosine	HIV/AIDS therapy	H[d]	(44, 48, 49)
ddA[e]	Dideoxy A		A	
ABC	Abacavir	HIV/AIDS therapy	Φ[f]	(45, 50, 51)
CBV[g]	Carbovir		G	

Table 1. Nucleoside analogues selected for this study. [a]**ACV**: Acycloguanosine; **GCV**: 2′-Nor-2′-deoxyguanosine; **CFB**: 2-chloro-9-(2′-Deoxy-2′-fluoro-beta-D-arabinofuranosyl)adenine; d4T: 2′,3′-didehydro-2′,3′-dideoxythymidine; **ddI**: 2′,3′-dideoxyinosine; **ddA**: 2,3′-Dideoxyadenosine; **ABC**: (1S, 4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]cyclopent-2-en-1-yl}methanol; **CBV** (1S, 4R)-4-[2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl]cyclopent-2-en-1-yl]methanol. [b]HSV-1: Herpes simplex virus type I; HSV-2: Herpes simplex virus type II; VSV: Varicella zoster virus; EBV: Epstein-Barr virus; CMV: Cytomegalovirus; ALL: Acute Lymphoblastic Leukaemia. [c]2-Chloro-adenine. [d]Hypoxanthine. [e]Cellular converted form of ddI. [f]Non-natural (2-amino-6-(cyclopropylamino)purine). [g]Cellular converted active form of ABC.

used in antiretroviral therapy, likely by decreasing the levels of dNTPs that can compete with chain terminators during proviral synthesis[34–36].

Agents that modulate SAMHD1 function would have great value for studies of its anti-HIV effects. Since the triphosphohydrolase activity of SAMHD1 is regulated allosterically by nucleotide analogues whilst the efficacy of nucleotide analogues can be affected simultaneously by SAMHD1 activity, we used a combination of *in vitro* and cell-based assays to study the mutual effects of nucleotide analogues and SAMHD1 activity on HIV-1 replication.

We first used an *in vitro* enzyme-coupled assay to test the effect on SAMHD1 activity of the triphosphate derivatives of a panel of FDA-approved nucleoside analogues widely used in antiviral and anticancer therapy, detailed in Table 1. Aciclovir (ACV) and Ganciclovir (GCV) are acyclic guanosine analogues used as anti-herpesvirus agents[37–39]. The halogenated adenosine analogue Clofarabine (CFB) is employed in anticancer therapy[40,41]. The NRTIs Stavudine (d4T)[42,43], Didanosine (ddI)[44] and Abacavir (ABC)[45] are selective inhibitors of HIV-1 and HIV-2 replication used in HIV/AIDS therapy[46,47]. We next tested whether the presence of SAMHD1 caused changes in the anti-HIV-1 efficacy of these nucleoside analogues in phorbol myristate acetate (PMA)-treated and untreated human monocytoid cell lines. We also compared the efficacy of nucleoside analogues in U937 cells expressing SAMHD1 or the catalytically inactive mutant HD206–7AA, and in THP-1 cells expressing endogenous SAMHD1 or transduced with Vpx. Surprisingly, this analysis revealed anti-HIV-1 activities for ACV, GCV and CFB in addition to the NRTIs in PMA-treated cells; these were further enhanced in the presence of added SAMHD1.

Results

***In vitro* activity of nucleotide analogues.** Since the triphosphohydrolase activity of SAMHD1 is allosterically regulated by nucleotide analogues, and nucleotide analogues can also be hydrolysed by SAMHD1, we used a coupled-enzyme *in vitro* assay to assess their role as activators, substrates or inhibitors of SAMHD1. We evaluated the triphosphate forms of the panel of nucleoside analogues detailed in Table 1 as well as dideoxyguanosine triphosphate (ddATP) and Carbovir triphosphate (CBV-TP) that are active antiretroviral agents produced after cellular conversion of ddI to ddATP[48,49] and ABC to CBV-TP[50,51]. Our previous *in vitro* assays, demonstrated that Aciclovir triphosphate (ACV-TP) was a non-hydrolysable allosteric activator of SAMHD1 with comparable efficiency to GTP, Ganciclovir triphosphate (GCV-TP) was also non-hydrolysable but displayed no activation of SAMHD1 and Clofarabine triphosphate (CFB-TP) was a substrate hydrolysed by SAMHD1 at a rate comparable to natural dNTP substrates in the presence of the activator GTP[21]. Other studies have demonstrated SAMHD1 has little or no hydrolytic activity against stavudine triphosphate (d4T-TP)[35,36]. Our data confirm this observation (Fig. 1A) and in addition demonstrate that d4T-TP is not a SAMHD1 inhibitor or activator (Fig. 1B,C). By contrast, didanosine triphosphate (ddI-TP) is hydrolysed by SAMHD1 even in the absence of GTP. In the presence of activating GTP, ddI-TP hydrolysis increases three-fold, to around six-fold slower than the TTP hydrolysis rate (Fig. 1A). In addition, ddI-TP activates SAMHD1 triphosphohydrolase activity, but to a much lesser degree than the natural activator, GTP (Fig. 1B) and displays no significant inhibitory effects (Fig. 1C). Quantification of ddI-TP hydrolysis data, demonstrates cooperativity with respect to ddI-TP concentration (Fig. 1D). Data are best fit with a Hill equation with parameters $K_S = 226 \pm 12\,\mu M$, $n = 1.7 \pm 0.1$ and $k_{cat} = 0.24 \pm 0.04\,s^{-1}$. ddI-TP only weakly activates SAMHD1 and analysis of the ddI-TP concentration dependence of TTP hydrolysis (Fig. 1E) yields only lower estimates for the maximal rate and the ddI-TP concentration at half maximal activation, $k_{max} > 0.1\,s^{-1}$ and $K_a > 300\,\mu M$. By contrast, analysis of ddATP, the cellular active form of ddI, revealed no hydrolysis by SAMHD1, as has been shown previously[34,35] and additionally there was no measurable activation or inhibition of SAMHD1 by ddATP (Fig. 1F–H). Examination of Abacavir triphosphate (ABC-TP) and Carbovir

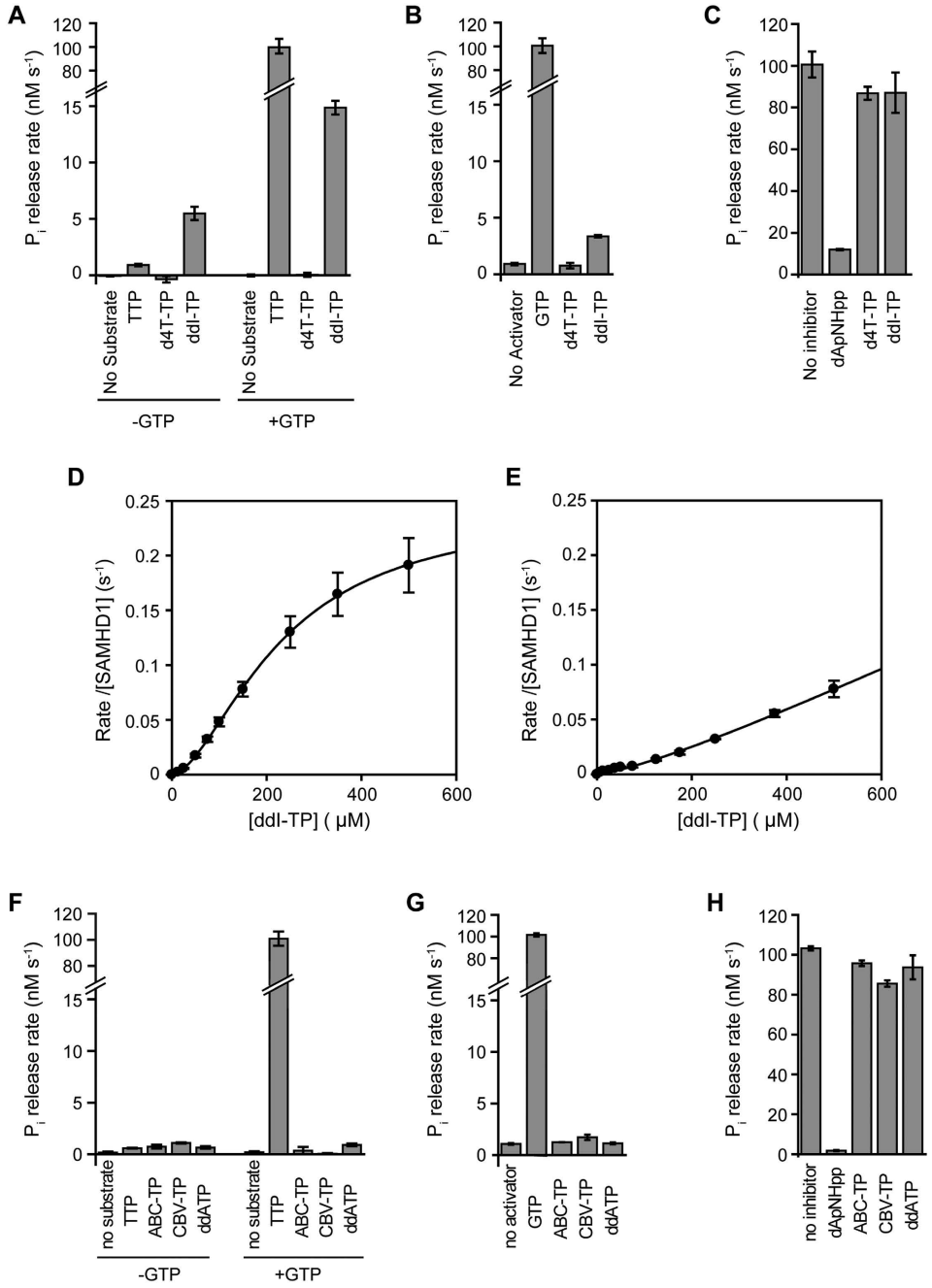


Figure 1. The effects of d4T-TP,ddI-TP,ddA-TP, ABC-TP and CBV-TP on SAMHD1 triphosphohydrolase activity. (**A**) Hydrolysis of 0.3 mM TTP, d4T-TP or ddI-TP in the absence (left) and presence (right) of 0.1 mM activator GTP. (**B**) Activation of SAMHD1 TTP hydrolysis. Triphosphohydrolase activity was measured with 0.3 mM TTP substrate upon addition of 0.1 mM GTP, d4T-TP or ddI-TP as activators. (**C**) Inhibition of SAMHD1 GTP-activated TTP hydrolysis. Triphosphohydrolase activity was measured with 0.3 mM TTP and 0.1 mM GTP activator alone and with addition of 0.3 mM dApNHpp positive control, d4T-TP or ddI-TP. Error bars are the standard error of the mean (SEM) of three independent measurements. (**D**) Concentration dependence of SAMHD1 ddI-TP hydrolysis in the presence of 0.2 mM GTP (saturating activator concentration). Nonlinear least squares fitting using a Hill equation gives the apparent binding constant $K_S = 226 \pm 12\,\mu M$, catalytic constant $k_{cat} = 0.24 \pm 0.04\,s^{-1}$ and the Hill coefficient $n = 1.7 \pm 0.1$ (Mean ± SEM). (**E**) ddI-TP allosteric activation of TTP hydrolysis. Rates were determined for 1 mM TTP at varying dd-ITP concentration. Nonlinear least squares fitting gives only lower estimates for the maximal rate and the ddI-TP concentration at half maximal activation of $k_{max} > 0.1\,s^{-1}$ and $K_a > 300\,\mu M$. (**F**) Hydrolysis of 0.3 mM TTP, ABC-TP, CBV-TP and ddATP in the absence (left) and presence (right) of 0.1 mM activator GTP. (**G**) Activation of SAMHD1 TTP hydrolysis. Triphosphohydrolase activity was measured with 0.3 mM TTP substrate upon addition of 0.1 mM GTP, ABC-TP, CBV-TP or ddATP as activators. (**H**) Inhibition of SAMHD1 GTP-activated TTP hydrolysis. Triphosphohydrolase activity was measured with 0.3 mM TTP and 0.1 mM GTP activator alone and with addition of 0.3 mM dApNHpp positive control, ABC-TP, CBV-TP or ddATP. Error bars are the range of data from two independent measurements.

Nucleoside analogue	EC_{50} (μM)[a] [95% CI][b]			
	U937		THP1	
	PMA^-	PMA^+	PMA^-	PMA^+
ACV	>200	52.2 [49.5–54.9]	>200	0.657 [0.476–0.838]
GCV	>200	>200	>200	0.367 [0.322–0.412]
CFB	0.119 [0.101–0.137]	0.037 [0.012–0.061]	0.132 [0.055–0.210]	0.003 [0.002–0.004]
d4T	6.55 [1.83–11.3]	7.04 [6.97–7.11]	6.21 [5.91–6.52]	3.95 [3.05–4.85]
ddI	45.8 [29.2–62.5]	2.45 [0.563–4.34]	2.03 [1.74–2.32]	0.093 [0.065–0.121]
ABC	14.1 [5.07–23.0]	7.47 [6.67–8.27]	0.493 [0.142–0.844]	0.031 [0.022–0.040]
NVP	0.505 [0.126–0.883]	0.118 [0.064–0.171]	0.369 [0.232–0.506]	0.219 [0.201–0.237]
RAL	0.013 [0.012–0.014]	0.063 [0.038–0.088]	0.044 [0.035–0.053]	0.037 [0.021–0.046]

Table 2. Anti-HIV-1 activity of nucleoside analogues in monocytoid cell lines. [a]EC_{50} is the effective concentration of drug that inhibits infection of cells by 50% measured as percentage of GFP^+ cells. Values were calculated by non-linear regression for at east two independent experiments. [b]95% confidence interval. Non-ambiguous values were subject to a replicate test.

triphosphate (CBV-TP) also showed they were not hydrolysed by SAMHD1 and neither ABC-TP nor CBV-TP displayed any activation or inhibition of SAMHD1 (Fig. 1F–H).

Anti-HIV-1 activities of nucleoside analogues. The anti-HIV-1 activities of the six selected nucleoside-analogues and non-nucleoside RT inhibitor (NVP) and integrase inhibitor (RAL) controls were evaluated in proliferating H9 and Jurkat T cell lines (Supplementary Table S1). As would be expected for T cell lines, the anti-herpesvirus agents ACV and GCV showed no anti-HIV-1 activity when tested at concentrations up to 200 μM. The anticancer drug CFB did have anti-HIV-1 activity with EC_{50} values of 0.094 and 0.663 μM in H9 and Jurkat cells, respectively. The three NRTIs (d4T, ddI and ABC) showed anti-HIV-1 activities with EC_{50} values below 10 μM; NVP and RAL inhibited HIV-1 at nanomolar concentrations (Supplementary Table S1).

By contrast, when the anti-HIV-1 effects of these compounds were assessed in undifferentiated (PMA^-) and PMA-differentiated (PMA^+) U937 and THP-1 lines a different pattern emerged (Table 2). Here, although the anti-herpesvirus agents ACV and GCV did not show anti-HIV-1 activity in PMA^- cells ($EC_{50} > 200$ μM), ACV showed an increased activity in PMA^+ U937 cells ($EC_{50} = 52.2$ μM), and both ACV and GCV had extremely significant activities in PMA^+ THP-1 cells (EC_{50} 0.657 and 0.367 μM; p values < 0.0001) (Fig. 2A,B,D,E). The anti-HIV-1 activity of CFB also increased in PMA^+ cells. In U937, the increase was 3-fold and in THP-1 cells the increase was highly significant at 44-fold ($EC_{50} = 0.003$ μM) (Fig. 2C,F). Measurements of the established NRTIs also revealed increases of anti-HIV-1 activities in PMA^+ cells, with the greatest effects observed in THP-1 cells. Thus, ddI and ABC had greatly increased anti-HIV-1 activities in PMA^+ cells, down to nanomolar EC_{50} in THP-1 cells (22- and 16-fold, respectively) (Table 2). By contrast, d4T had similar anti-HIV-1 activity in PMA^+ and PMA^- U937 cells, with only a small increased activity (2-fold) when comparing PMA^+ to PMA^- THP-1 cells (Table 2). As might be expected, the NVP and RAL controls showed only minor, non-significant differences (p values > 0.005) in their activities in PMA^- and PMA^+ cells (Table 2).

Increased anti-HIV-1 activities of NRTIs have been reported previously in PMA^+ treated THP-1 cells and monocyte-derived macrophages (MDM), where dNTP levels are reduced[34–36]. However, our observations reveal that compounds with no apparent anti-HIV-1 activity in T-cells or undifferentiated monocytoid cell lines (ACV and GCV) can display marked anti-HIV-1 effects in differentiated monocytoid cells. Moreover, this observation is particularly marked in the differentiated THP-1 cells where SAMHD1 is expressed in its highly active non-phosphorylated form[24].

Cytotoxicity of nucleotide analogues. To compare the anti-HIV-1 activities and cytotoxic effects of the nucleoside analogues in T- and monocytoid cell lines, we determined the cytotoxic concentration (CC_{50}) by flow cytometry and calculated the selectivity index (SI) for each compound (Supplementary Tables S1 and S2). With the exception of CFB, all nucleoside analogues displayed no toxicity in any of the cell lines when evaluated at concentrations up to 200 μM ($CC_{50} > 200$ μM). In T cells, where ACV and GCV showed no anti-HIV-1 activity they also did not exhibit any cytotoxic effect ($CC_{50} > 200$ μM). The anti-HIV-1 activity of CFB in T cells was only observed at greater than cytotoxic concentrations ($SI < 1$). In contrast, NRTIs (d4T, ddI, ABC) showed $SIs > 30$ (Supplementary Table S1).

In monocytoid U937 and THP-1 cells, SI values increased significantly upon differentiation (Supplementary Table S2). ACV showed SI values of >4 and >304 in PMA^+ U937 and THP-1 cells, respectively. Similarly, the SI of GCV increased to >545 in PMA^+ THP-1 cells. As observed in T cells, the anti-HIV-1 activity of CFB in PMA^- cells was only observed at greater than cytotoxic concentrations ($SI < 1$). However, upon differentiation cytotoxicity was greatly decreased ($CC_{50} > 1.6$ μM) resulting in SI values of >43 and >533 in PMA^+ U937 and THP-1 cells, respectively. The NRTIs (d4T, ddI, ABC) did not show cytotoxicity ($CC_{50} > 200$ μM) thus increasing the SIs, particularly in PMA^+ THP-1 cells (Supplementary Table S2).

Effect of SAMHD1 on the anti-HIV-1 activity of nucleoside analogues. To test whether the anti-HIV-1 activities of the selected nucleoside analogues could be further increased through dNTP depletion

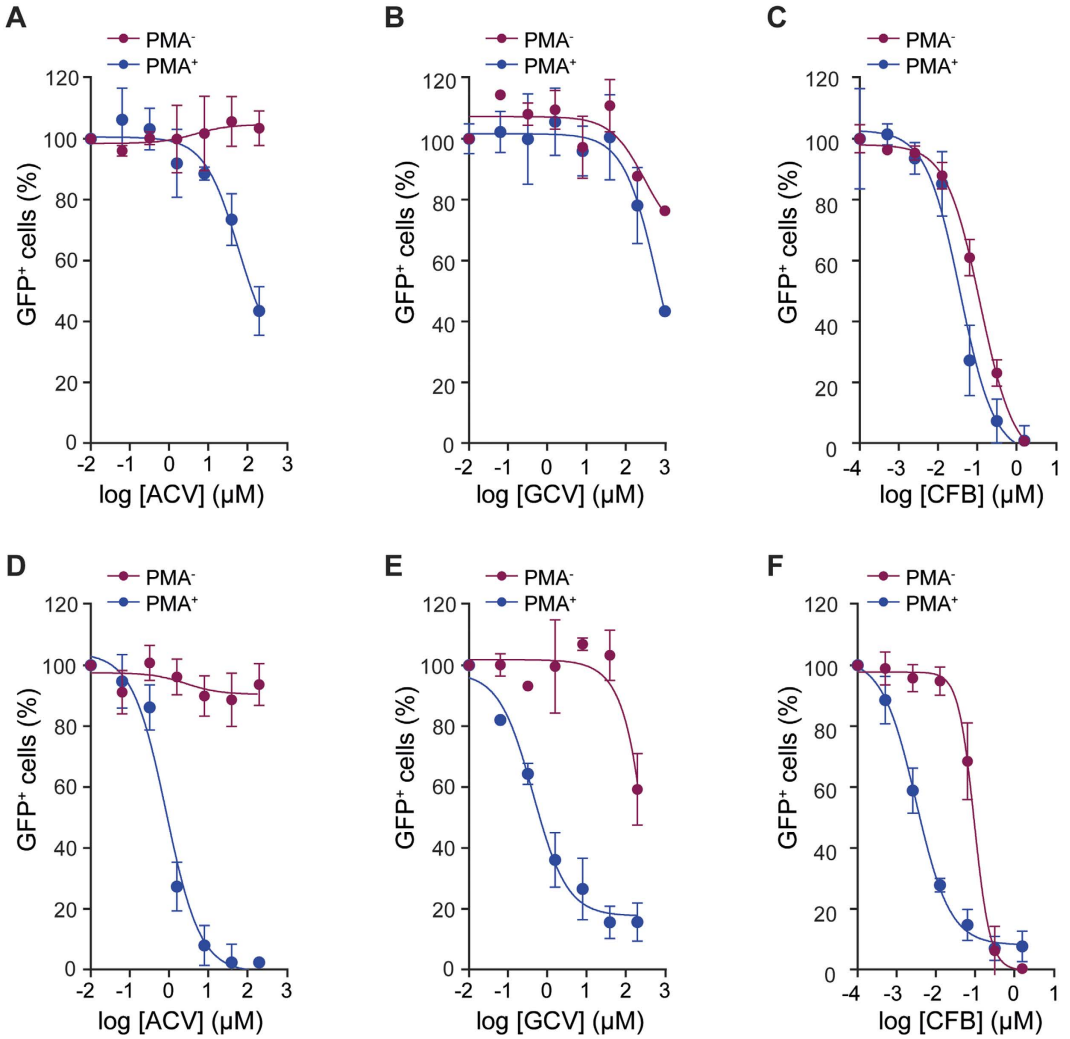


Figure 2. Anti-HIV-1 activities of ACV, GCV and CFB in monocytoid cell lines. PMA^- (red) and PMA^+ (blue) U937 (**A**–**C**) and THP-1 (**D**–**F**) cells were cultured in the presence of increasing concentration of Aciclovir (ACV) (**A**,**D**), Ganciclovir (GCV) (**B**,**E**) and Clofarabine (CFB) (**C**,**F**). Cells were incubated with HIV-1-GFP and the percentage of infected GFP^+ cells, measured by flow cytometry. To account for differences in overall infectivity of PMA^- and PMA^+ cells, in each panel the data is plotted as the normalised percentage of GFP^+ cells against drug concentration. Error bars represent the standard deviation from at least two independent experiments.

by SAMHD1, we evaluated antiviral activities in undifferentiated (PMA^-) and differentiated (PMA^+) U937 cells transduced with catalytically active SAMHD1 (*wt*) or an inactive mutant (HD206-7AA). Comparative EC_{50} data and statistical significance test results from these assays are summarised in Table 3.

Inspection of these data showed that ACV and GCV had no anti-HIV-1 activity in PMA^- cells transduced with either *wt* SAMHD1 or HD206-7AA. In PMA^+ cells expressing HD206-7AA, ACV had anti-HIV-1 activity with $EC_{50} = 50.9\,\mu M$ (Fig. 3A) comparable to that observed in untransduced U937 PMA^+ cells (Fig. 2A). However, in PMA^+ cells expressing *wt* SAMHD1, ACV anti-HIV-1 activity was enhanced a further 78 fold ($EC_{50} = 0.652\,\mu M$; $p < 0.0001$) (Fig. 3A). Similarly, GCV had no significant anti-HIV-1 activity in PMA^+ cells expressing HD206-7AA ($EC_{50} > 200\,\mu M$) but in cells expressing *wt* SAMHD1 anti-HIV-1 activity of GCV was also greatly enhanced, >200 fold ($EC_{50} = 0.876\,\mu M$; $p < 0.0001$) (Fig. 3B). As was observed for endogenous SAMHD1 expression in THP-1 cells, these data demonstrate that exogenous expression of SAMHD1 in other differentiated monocytoid cells also greatly enhances the anti-HIV activities of ACV and GCV anti-herpesvirus agents.

Analysis of CFB data showed transduction of *wt* SAMHD1 or HD206-7AA did not affect the EC_{50} values of CFB in PMA^- cells, which remained higher than the cytotoxic concentrations (Supplementary Table S2, SI < 1). Upon introduction of HD206-7AA into PMA^+ U937 cells a small reduction in the CFB EC_{50} was observed (Fig. 3C) but only to a level comparable with untransduced PMA^+ U937 cells (Table 2). In contrast, there was a 22-fold increase in the anti-HIV-1 activity of CFB in PMA^+ cells expressing *wt* SAMHD1 over those expressing HD206-7AA (Fig. 3C). Although, not as large as the effects observed with ACV and GCV these data demonstrate

Nucleoside analogue	PMA$^-$				PMA$^+$			
	EC_{50} (μM)[a] [95% CI][b]		Fold	p[c]	EC_{50} (μM) [95% CI]		Fold	p
	SAMHD1	HD206/7AA			SAMHD1	HD206/7AA		
ACV	>200	>200	—	—	0.652 [0.175–1.13]	50.9 [46.1–55.8]	78	<0.0001
GCV	>200	>200	—	—	0.876 [0.823–0.929]	>200	>228	<0.0001
CFB	0.138 [0.021–0.255]	0.108 [0.081–0.135]	1	0.0878†	0.0013 [0.000–0.002]	0.029 [0.022–0.036]	22	0.0010
d4T	0.801 [0.531–1.07]	2.71 [1.90–3.52]	3	0.0013	0.528 [0.317–0.739]	5.82 [5.671–5.969]	11	<0.0001
ddI	23.7 [10.6–36.7]	55.1 [44.2–65.9]	2	0.0018	0.109 [0.030–0.188]	8.79 [7.53–10.1]	81	<0.0001
ABC	17.5 [15.9–19.1]	22.1 [7.12–37.1]	1	0.0590†	0.173 [0.164–0.182]	7.06 [4.72–9.40]	41	<0.0007
NVP	0.112 [0.000–0.223]	0.124 [0.000–0.248]	1	0.6442†	0.097 [0.000–0.195]	0.145 [0.113–0.177]	1	0.1297†
RAL	0.011 [0.002–0.020]	0.012 [0.003–0.021]	1	0.2115†	0.070 [0.013–0.127]	0.075 [0.007–0.143]	1	0.7548†

Table 3. Anti-HIV-1 activity of nucleoside analogues in SAMHD1 expressing U937 cells. [a]EC_{50} is the effective concentration of drug that inhibits infection of cells by 50% measured as the percentage of GFP$^+$ cells in SAMHD1+ or HD206/7AA+ populations. Values were calculated by non-linear regression for at least two independent experiments. [b]95% confidence interval. Non-ambiguous values were subject to a replicate test. [c]EC_{50} values for each replicate were used to perform an unpaired two-tailed t-test to determine differences between SAMHD1+ and HD206/7AA+ populations. †Difference not statistically significant.

that whilst differentiation of U937 cells alone reduces the EC_{50} of CFB to below cytotoxic levels, additional expression of SAMHD1 in differentiated monocytoid cells enhances the anti-HIV activity of CFB even further.

The NRTIs d4T and ddI showed only slightly greater anti-HIV-1 activities in PMA$^-$ cells expressing *wt* SAMHD1 over those transduced with HD206/7AA (3- and 2-fold, respectively) and ABC showed no significant difference (Table 3). In PMA$^+$ HD206-7AA expressing cells the antiviral activities of all NRTIs (d4T, ddI and ABC) were increased but only to EC_{50} values comparable with those observed in untransduced cells (Table 2). However, in PMA$^+$ cells expressing *wt* SAMHD1 the antiviral activities of all NRTIs (d4T, ddI and ABC) were increased significantly (11-, 81- and 41-fold, respectively) compared to cells expressing HD206-7AA (Fig. 3D–F). These data support the notion that SAMHD1 specifically enhances the efficacy of NRTIs through triphosphohydrolase activity acting on the cellular dNTP pool. This argument is strengthened further by the observations that the non-nucleoside RT inhibitor NVP and integrase inhibitor RAL did not show any significant difference in anti-HIV-1 activities upon *wt* SAMHD1 expression in PMA$^-$ or PMA$^+$ cells (Table 3).

Vpx knockdown of SAMHD1 activity. Since we found significant differences in the anti-HIV-1 activities of nucleoside analogues in U937 cells expressing exogenous *wt* SAMHD1 or HD206-7AA, we further evaluated their anti-HIV-1 activities in undifferentiated (PMA$^-$) and differentiated (PMA$^+$) THP-1 cells expressing endogenous SAMHD1 after transduction with the HIV-2 accessory viral protein Vpx, which reduces SAMHD1 levels by targeting it for proteasomal degradation[1,2,5,6]. These data and statistical significance test results are summarised in Table 4.

No differences were observed in the anti-HIV-1 activities of ACV and GCV in PMA$^-$ THP-1 cells upon Vpx transduction (Table 4). By contrast, in PMA$^+$ cells, transduction with Vpx reduced ACV anti-HIV-1 activity 27-fold ($p = 0.0004$) (Fig. 4A) and GCV anti-HIV-1 activity was entirely lost ($EC_{50} > 200\,\mu M$), ($p < 0.0001$) (Fig. 4B). The anti-HIV-1 activity of CFB decreased in both PMA$^-$ and PMA$^+$ THP-1 cells upon Vpx expression (Table 4). However, in the PMA$^-$ cells the EC_{50} was still greater than the observed CFB CC_{50} (Supplementary Table S2). In PMA$^+$ cells, introduction of Vpx reduced CFB anti-HIV-1 activity 4-fold (Fig. 4C). This maintained the EC_{50} at lower than cytotoxic levels but the reduction was significantly lower than that observed for ACV and GCV. Nevertheless, the data show that removal of SAMHD1 activity in differentiated THP-1 cells by Vpx-mediated proteasomal degradation results in reduction of anti-HIV-1 activity of these anti-herpes and anti-cancer agents.

Evaluation of the NRTIs showed that Vpx transduction of THP-1 PMA$^-$ cells resulted in only small effects on the anti-HIV-1 activities of d4T and ddI (2–3 fold lower) and no difference with ABC (Table 4). By contrast, in THP-1 PMA$^+$ cells, the anti-HIV-1 activities of all NRTIs decreased significantly when cells were transduced with Vpx, d4T 35-fold, ddI 55-fold and ABC 187-fold (Fig. 4D–F). By comparison, Vpx transduction of PMA$^-$ or PMA$^+$ THP-1 cells had no effect on anti-HIV-1 activities of control NVP and RAL (Table 4) suggesting that the decreased anti-HIV-1 activities of the NRTIs is a result of competition from an increased cellular dNTP pool following removal of SAMHD1.

Discussion

This study was initiated to identify nucleotide analogue drugs that would activate or inhibit SAMHD1 to facilitate an improved understanding of the effect of SAMHD1 on HIV-1 replication. To this end, we had two alternative but not mutually exclusive hypotheses. First that a drug affecting SAMHD1 activity, either positively or negatively, would impact dNTP pool sizes thereby affecting HIV-1 reverse transcription. Second, that cell dependent changes in SAMHD1 activity and the resulting changes in dNTP levels might influence the effects of the drugs themselves on HIV-1 replication. The data obtained and discussed below, provided clear evidence supporting the second hypothesis, at least in the majority of cases. This would suggest that altering SAMHD1 activity might provide a

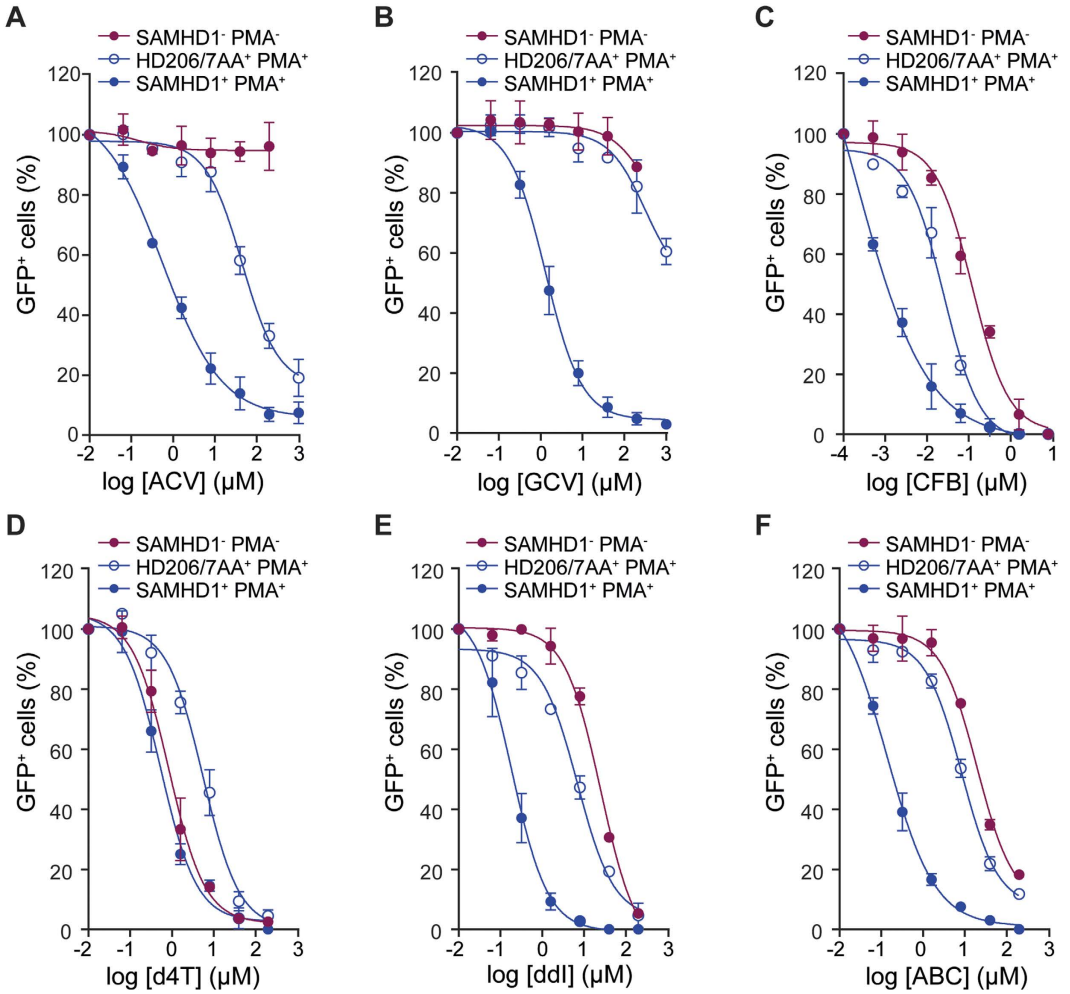


Figure 3. Anti-HIV-1 activities of nucleoside analogues in U937 cells expressing SAMHD1. Antiviral activities were determined in undifferentiated (PMA^-) and differentiated (PMA^+) U937 cells expressing SAMHD1 or the mutant HD206-7AA. Cells were cultured in the presence of increasing concentration of Aciclovir (ACV) (**A**), Ganciclovir (GCV) (**B**), Clofarabine (CFB) (**C**), Stavudine (d4T) (**D**), Didanosine (ddI) (**E**) and Abacavir (ABC) (**F**). Cells were incubated with HIV-1-GFP and the percentage of infected GFP^+ cells was measured by flow cytometry. PMA^- cells expressing SAMHD1 (red filled circles), PMA^+ cells expressing the mutant HD206-7AA (blue open circles) and PMA^+ cells expressing SAMHD1 (blue filled circles) are shown. Infectivity data is plotted as the normalised percentage of GFP^+ cells against drug concentrations. Error bars represent the standard deviation from at least 2 independent experiments.

means for enhancing drug effectiveness. Surprisingly our data also provided unambiguous evidence for direct effects of ACV, GCV and CFB not normally thought of as antiretroviral drugs, on HIV-1 replication in certain non-proliferating cell types.

Control of dNTP pools in cycling and non-cycling cells. Cellular dNTP concentrations vary by a factor of at least 100 between rapidly proliferating cells typified by activated lymphocytes or tumour cells, where they are required by DNA polymerases to actively replicate the genome, and non-proliferating, terminally-differentiated cells such as monocyte-derived macrophages where they are important for DNA repair and mitochondrial DNA synthesis[52–54]. These levels are set by controlling the activity of the enzymes ribonucleotide reductase (RNR), crucial for dNTP biosynthesis, and SAMHD1, that catalyses dNTP degradation[55]. Regulation of RNR and SAMHD1 activity is provided by a range of mechanisms including synthesis, degradation, allostery and phosphorylation[56–58] that allow control of dNTPs at levels ranging from 20 nM to 2 μM[32]. Given the requirement for dNTPs in HIV-1 replication, such a range of concentrations might be expected to have profound effects both on the apparent efficiency of HIV-1 reverse transcription in different cell types as well as on interactions between RT and NRTIs.

***In vitro* measurements of SAMHD1 activity.** It is possible that any concentration dependent drug effects on SAMHD1 activity might complicate interpretation of cell-based assays to examine nucleoside analogue effects on HIV-1 replication. Our *in vitro* experiments, summarized in Table 1 now allow us to exclude this possibility in the majority of cases. No evidence for SAMHD1 inhibition was seen with any analogue tested. Similarly

Nucleoside analogue	PMA$^-$				PMA$^+$			
	EC_{50} (μM)[a] [95% CI][b]		Fold	p[c]	EC_{50} (μM) [95% CI]		Fold	p
	Vpx$^-$	Vpx$^+$			Vpx$^-$	Vpx$^+$		
ACV	>200	>200	—	—	1.08 [0.735–1.43]	29.6 [2.62–56.5]	27	0.0004
GCV	>200	>200	—	—	0.632 [0.596–0.668]	>200	>316	<0.0001
CFB	0.340 [0.118–0.563]	>1.6	>5	<0.0001	0.003 [0.002–0.004]	0.012 [0.003–0.021]	4	0.0017
d4T	4.39 [3.76–5.02]	13.3 [12.1–14.4]	3	0.0001	1.49 [0.501–2.48]	52.2 [33.0–71.5]	35	0.0009
ddI	1.04 [0.863–1.22]	2.73 [1.40–4.07]	3	0.0039	0.079 [0.061–0.096]	4.34 [3.19–5.50]	55	0.0005
ABC	0.459 [0.399–0.519]	0.622 [0.356–0.888]	1	0.0619†	0.038 [0.036–0.040]	7.050 [4.67–9.43]	187	0.0002
NVP	0.244 [0.240–0.248]	0.292 [0.067–0.517]	1	0.1146†	0.223 [0.196–0.250]	0.235 [0.217–0.253]	1	0.0379†
RAL	0.035 [0.017–0.053]	0.043 [0.016–0.070]	1	0.0683†	0.038 [0.020–0.056]	0.037 [0.002–0.072]	1	0.9071†

Table 4. Anti-HIV-1 activity of nucleoside analogues in Vpx expressing THP1 cells. [a]EC_{50} is the effective concentration of drug that inhibits infection of cells by 50% measured as the percentage of GFP$^+$ cells in Vpx$^-$ or Vpx$^+$ populations. Values were calculated by non-linear regression for at least two independent experiments. [b]95% confidence interval. Non-ambiguous values were subject to a replicate test. [c]EC_{50} values for each replicate were used to perform an unpaired two-tailed t-test to determine differences between Vpx$^-$ and Vpx$^+$ populations. †Difference not statistically significant.

GCV-TP, d4T-TP, ABC-TP and CBV-TP showed no activity as activator or substrate, implying no direct effects on SAMHD1. By contrast both ACV-TP and ddI-TP were allosteric activators of SAMHD1 though ddI-TP was much less efficient than the natural activator GTP. Thus for these two compounds a role for activation cannot formally be excluded. However, ddI and ddI-MP are poor substrates for further phosphorylation by cellular kinases[59] and ddI is rapidly metabolized to ddA-TP in cells[49] making a direct effect of ddI-TP on SAMHD1 activity unlikely.

Efficacy of NRTIs in cycling and non-cycling cells. NRTIs compete with endogenous dNTPs for incorporation into newly reverse transcribed HIV-1 DNA and act as chain terminators. Therefore, given equal cellular uptake and comparable conversion efficiency to triphosphorylated forms, their efficacy would be predicted to depend on the level of competing natural dNTPs. Thus in non-dividing cells further depletion of the dNTP pool by catalytically active SAMHD1 might be expected to profoundly reduce the doses of NRTIs required to inhibit HIV-1 infectivity. This notion is supported by our observations of increased NRTI efficacy in PMA-differentiated U937 cells transduced with active SAMHD1 (Table 3) and deceased NRTI efficacy in THP-1 cells were SAMHD1 has been targeted for degradation through expression of Vpx (Table 4). In addition, although minor, the presence of SAMHD1 in proliferating myeloid cells results in enhancement of d4T and ddI anti-HIV-1 activity (Tables 3 and 4).

Anti HIV-1 activity of CFB. Evaluation of our data also showed that SAMHD1 could enhance the anti-HIV-1 activity of the anticancer agent CFB (Tables 3 and 4). The anticancer agents 5-azacytidine, CFB, and resveratrol have previously been shown to exhibit potent anti-HIV-2 activity with EC_{50} values for 5-azacytidine, clofarabine and resveratrol being significantly lower for HIV-2 compared to HIV-1[60]. More recently CFB was shown to have significant anti-HIV-1 activity in primary macrophages acting both to reduce dNTP concentrations by inhibiting RNR and HIV-1 viral DNA via RT[61]. Our data support these previous observations and also that concentration of CFB for full anti-HIV-1 activity was significantly decreased upon differentiation of monocytes (Table 2). Moreover, CFB anti-HIV-1 activity was enhanced 22-fold in the presence of SAMHD1 in PMA-differentiated monocytes (Table 3), consistent with a mechanism involving chain termination of HIV-1 DNA synthesis in a manner analogous to NRTIs.

Anti HIV-1 activity of ACV and GCV. ACV and GCV are normally considered as herpes virus specific drugs. Their incorporation into growing DNA results in chain termination; selectivity is determined by the viral thymidine kinase, required for the efficient conversion into the mono and subsequent triphosphorylated derivatives needed for incorporation into DNA[62]. However they have also been shown to reduce HIV-1 load and/or delay disease progression[63–65] with ACV acting as a chain terminator of reverse transcriptase[66]. If so, how does phosphorylation occur in HIV-1 infected individuals? One school of thought postulates that kinases from co-infecting human herpesviruses are involved[67,68]. Against this idea are studies that show that herpesvirus co-infection is not required for ACV activity against HIV-1[69] implying that as yet uncharacterised human kinases must be involved[70].

Our data now show that SAMHD1 has a pronounced effect on the anti-HIV-1 activity of the drugs ACV and GCV when tested in myeloid derived cell lines. ACV and GCV do not display substantive anti-HIV-1 activities in proliferating monocytes (Table 2). However, in differentiated cells expressing SAMHD1, where the dNTP pool levels are much lower[52,53,71] ACV and GCV anti-HIV-1 activities were greatly enhanced (Figs 2 and 3) and this enhancement could be abrogated by introduction of Vpx to reduce SAMHD1 triphosphohydrolase activity (Fig. 4). These data indicate that the anti-herpes virus drugs are most active in non-proliferating cells with low dNTP levels and are fully consistent with the observation that the anti-HIV-1 activity of ACV can be potentiated by ribavirin, a drug that depletes intracellular dGTP pools[68] and that ACV can inhibit HIV-1 replication in resting T-cells[69]. Our observations also support the idea that a cellular kinase, expressed in THP-1 and U937 cells, is

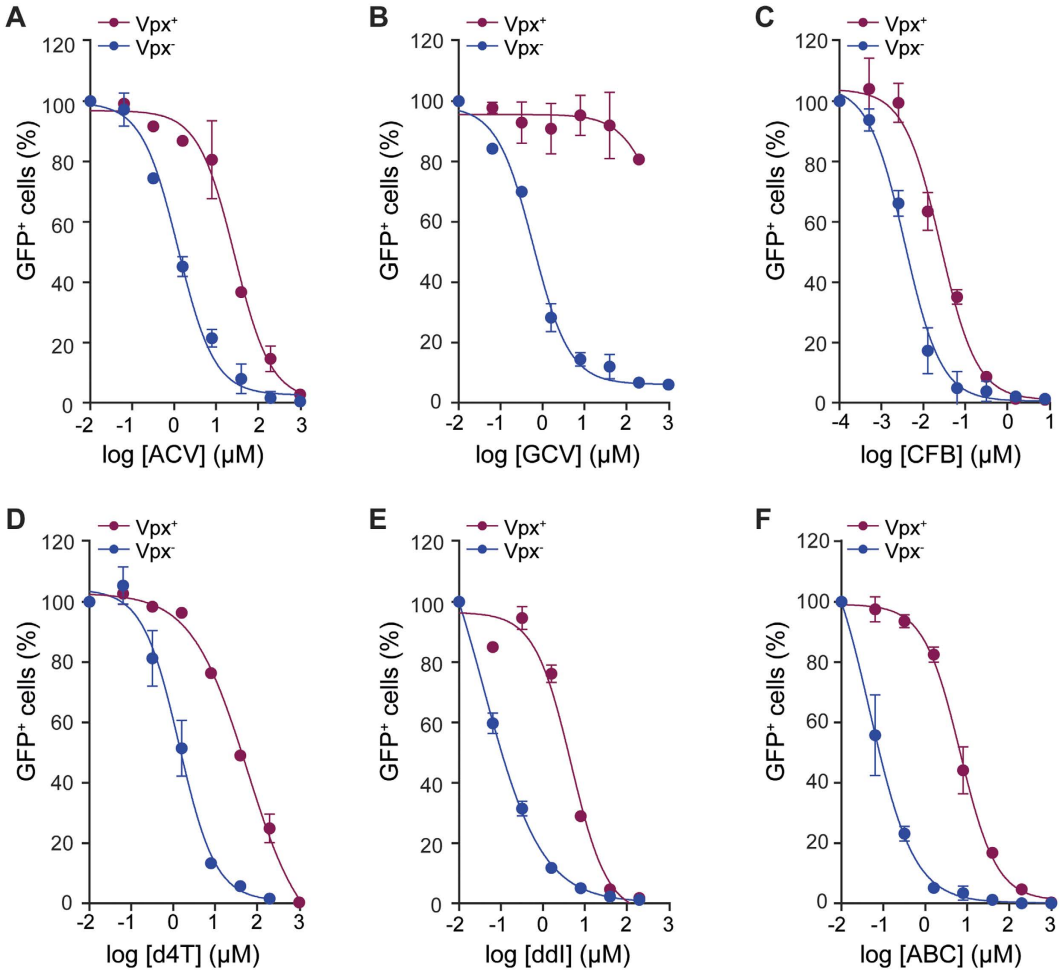


Figure 4. Anti-HIV-1 activities of nucleoside analogues in differentiated THP-1 cells after Vpx knockdown of endogenous SAMHD1 expression. Antiviral activities were determined in differentiated (PMA^+) THP-1 cells expressing endogenous SAMHD1 and after transduction with Vpx. Cells were cultured in the presence of increasing concentrations of Aciclovir (ACV) (**A**), Ganciclovir (GCV) (**B**), Clofarabine (CFB) (**C**), Stavudine (d4T) (**D**), Didanosine (ddI) (**E**) and Abacavir (ABC) (**F**). Cells were incubated with HIV-1-GFP and the percentage of infected GFP^+ cells was measured by flow cytometry. Cells expressing SAMHD1 (Vpx^-) (blue filled circles), cells transduced with Vpx (Vpx^+) (red filled circles) are shown. Infectivity data is plotted as the normalised percentage of GFP^+ cells against drug concentrations. Error bars represent the standard deviation from at least 2 independent experiments.

responsible for initial phosphorylation. Such cells might provide starting material for the purification and further characterization of this enzyme.

In light of these results, the potential utility of nonstandard antiviral and anticancer drugs to target HIV-1 in differentiated cells low in dNTPs might also be considered. For example, our data, coupled with the low cost and favourable safety profile[72] of ACV prompt the question of whether ACV might find a niche role in the treatment or prevention of HIV-1. Thus, it is tempting to speculate that ACV might be useful for the prevention of mother to child transmission, or for reducing reservoir size, in resource poor settings where viral load suppression of the expectant mother may be less than complete and treatment of the new-born infant could be initiated before results of tests for transmission become available. We are currently testing this idea in a variety of primary culture systems with low dNTP concentrations such monocyte-derived macrophages or resting T-cells, aiming to identify important HIV-1 target cells that are susceptible to ACV treatment.

Methods

Synthesis of abacavir-5′-triphosphate and carbovir-5′-triphosphate. The synthesis of the ammonium salt of abacavir-5′-triphosphate (ABC-TP) was performed as described[73] with minor modifications. Under a nitrogen atmosphere 364 mg (1.27 mmol) of Abacavir was dissolved in 9.5 mL anhydrous pyridine and cooled to −35 °C. Then 1.65 mL (1.65 mmol, 1.3 eq) of a 1 M solution of 5-chloro-*cyclo*Saligenyl-phosphorchloridate in THF was added portion-wise over a period of 4 h. The reaction mixture was slowly warmed to room temperature and all volatiles were evaporated. The residue was co-evaporated three times with toluene (3 × 5 mL) and finally once with CH_2Cl_2. The crude product was purified by flash column chromatography ($CH_2Cl_2/CH_3OH + 1\%$

of acetic acid, 95:5 to 90:10) to give 490 mg of the *cyclo*Saligenyl-triester with minor impurities. Then 47.5 mg (97.2 μmol) of this phosphate triester were dissolved in 1 mL anhydrous DMF. A solution of 96.3 mg (146 μmol) bis-tetrabutylammonium hydrogen pyrophosphate in 0.7 mL DMF, which had been stirred for 1 h over 4 Å molecular sieves, was added in one aliquot. After 72 h the solvent was removed under reduced pressure and the residue was purified by reverse phase flash column chromatography (H_2O/CH_3CN, 99:1 to 0:100), ion exchange (NH_4^+) on Dowex (50WX8) and again reversed phase flash column chromatography (H_2O/CH_3CN, 100:1 to 0:100) to give 20.5 mg (34.5 μmol) of the triphosphate as a colourless solid after freeze drying. **^{1}H NMR:** (600 MHz, D_2O): δ [ppm] = 7.99 (s, 1H), 6.19–6.26 (m, 1H), 5.93–5.89 (m, 1H), 5.52–5.48 (m, 1H), 4.10–4.06 (m, 1 H), 4.03–3.98 (m, 1H), 3.23–3.17 (m, 1H), 2.90–2.76 (m, 2H), 1.78–1.71 (m, 1H), 0.98–0.93 (m, 2H), 0.76–0.72 (m, 2H). **^{13}C NMR:** (151 MHz, D_2O): δ [ppm] = 148.9, 139.3, 138.5, 129.2, 68.3, 59.9, 45.5, 33.6, 22.9, 6.7, 6.7. **^{31}P NMR:** (243 MHz, D_2O): −10.73, −10.91, −23.27. **HRMS** (ESI^-, m/z): calcd. for $C_{14}H_{21}N_6O_{10}P_3$: 525.0459 $[M-H]^-$, found 525.0360 $[M-H]^-$. **IR (ATR):** wavenumber [cm^{-1}] = 2988, 2884, 1644, 1445, 1407, 1210, 1118, 891, 832, 484.

Synthesis of abacavir-5′-triphosphate and carbovir-5′-triphosphate. Carbovir 5′-triphosphate (CBV-TP) was prepared similarly. Amounts used were: 308 mg (1.25 mmol) carbovir, 9.5 mL anhydrous pyridine and 1.62 mL (1.62 mmol, 1.3 eq) of 1 M 5-chloro-*cyclo*Saligenyl-phosphorcloridate solution (THF). The crude product was purified by flash column chromatography (CH_2Cl_2/CH_3OH + 1% of acetic acid, 95:5 to 90:10) to give 219 mg of the *cyclo*Saligenyl-triester with minor impurities. In the second step 47.7 mg (106 μmol) *cyclo*Sal-triester of carbovir in 1 mL dry DMF and 105 mg (159 μmol) of bis-tetrabutylammonium hydrogen pyrophosphate in 0.8 mL DMF were reacted. After purification and freeze-drying 12.3 mg (22.1 μmol) of the triphosphate was obtained as a colorless solid. **^{1}H NMR:** (600 MHz, D_2O): δ [ppm] = 8.00 (s, 1H), 6.29–6.26 (m, 1H), 5.94–5.92 (m, 1H), 5.54–5.49 (m, 1H), 4.08–3.98 (m, 2 H), 3.23–3.17 (m, 1H), 2.85–2.77 (m, 1H), 1.78–1.71 (m, 1H). **^{13}C NMR:** (151 MHz, D_2O): δ [ppm] = 158.6, 153.7, 151.0, 138.4, 138.3, 129.3, 115.2, 68.6, 60.1, 45.4, 33.8. **^{31}P NMR:** (243 MHz, D_2O): −10.87, −10.98, −23.31. **HRMS** (ESI^-, m/z): calcd. for $C_{11}H_{16}N_5O_{11}P_3$: 485.9986 $[M-H]^-$, found 486.0020 $[M-H]^-$. **IR (ATR):** wavenumber [cm^{-1}] = 2988, 1686, 1610 1407, 1214, 1056, 1004, 948, 893, 473.

Measurement of *in vitro* dNTP triphosphohydrolase activity. SAMHD1 hydrolysis, activation and inhibition by nucleotide analogues was quantified using a continuous coupled assay employing the fluorescent phosphate biosensor MDCC-PBP to measure phosphate release from combined SAMHD1 triphosphohydrolase and *S. cerevisiae* Ppx1 exopolyphosphatase activity, described previously[21]. SAMHD1 and Ppx1 were expressed as strep-tagged fusion proteins and purified as described previously[19,21]. Didanosine triphosphate (ddI-TP) and dideoxy ATP (ddATP) were from Trilink Biotechnologies, Stavudine triphosphate (d4T-TP) was from Jena biosciences and Chemcyte. Abacavir triphosphate and Carbovir triphosphate were chemically synthesised in house from abacavir and carbovir as described above. Experiments were performed in 20 μL reaction volumes in 384-well low-protein-binding microplates (Corning, USA). In typical experiments, solutions containing SAMHD1, Ppx1, MDCC-PBP and activator (GTP, ddI-TP or d4T-TP), or activator plus inhibitor, were incubated for 5 min in assay buffer (20 mM Tris pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$ and 2 mM TCEP) at 25 °C before the reaction was initiated by the addition of substrate (TTP, ddI-TP or d4T-TP). The final concentrations were 100 nM SAMHD1(115–626), 10 nM Ppx1, 2.5 μM BSA, 40 μM MDCC-PBP (80 μM in ABC-TP and CBV-TP assays). Activator, substrate and inhibitor were added at concentrations described below. The fluorescence intensity increase resulting from the phosphate-product binding to MDCC-PBP was recorded at 430 nm excitation and 465 nm emission over 10–30 min in a Clariostar multiwell plate reader (BMG). Steady-state rates were obtained from the fluorescence time courses by fitting a linear function with maximal slope using the Mars software (BMG). Apparent dissociation constant of ddI-TP binding (K_S), catalytic constant (k_{cat}), maximal rate (k_{max}) and the ddI-TP concentration at half maximal activation (K_a) were determined by nonlinear least squares fitting using a Hill-function in the software package Grafit 7.0.3 (Erithacus software, UK). All measurements were performed at least in triplicate and data are represented as mean ± SEM.

Cell lines and reagents. Cell lines were obtained from the Programme EVA Centre for AIDS Reagents and tested free for mycoplasma contamination. Jurkat[74], H9[75], U937[76] and THP-1 cells[77] were maintained in RPMI-1640 medium containing [L]-glutamine (Life Technologies), supplemented with heat-inactivated 10% foetal bovine serum (Labtech), 100 U penicillin-G/mL and 10 μg streptomycin/mL. 293T cells were maintained in DMEM medium containing [D]-glucose, [L]-glutamine and sodium pyruvate (Life Technologies), supplemented with 10% foetal bovine serum (Labtech), 100 U penicillin-G/mL and 10 μg streptomycin/mL. ACV and GCV were from Sequoia Research products (UK), CFB from Sigma-Aldrich (USA), d4T from Santa Cruz Biotechnology (USA), ddI and ABC from Stratech Scientific (UK), and Nevirapine (NVP) and Raltegravir (RAL) from Cayman Chemical (USA). All compounds were prepared at 100 mM in dimethyl sulphoxide and stored at −20 °C until use.

Virus production. SAMHD1 and HIV-2 Vpx DNA sequences were inserted into pLGatewayIeYFP[78]. The active site mutation HD206-7AA was created by PCR-based site-directed mutagenesis (QuickChange-II XL, Agilent). Virus-like particles (VLPs) were produced by co-transfection of pVSV-G[79], pKB4[80] and the pLGatewayIeYFP plasmids (SAMHD1, HD206-7AA or Vpx) into 293T cells and harvesting 48 h after transfection. HIV-1-GFP was made by co-transfection of pVSV-G, p8.91[81] and pCSGW-GFP[82]. Viruses were titrated in U937 cells and stocks stored at −80 °C until use.

Cytotoxicity assays. H9, Jurkat and undifferentiated U937 and THP-1 (PMA^-) cells (6×10^5 cells/mL) were treated with increasing concentrations of nucleoside analogues (0–200 μM; except CFB, 0–1.6 μM) and incubated at 37 °C in 5% CO_2 for 48 h. Medium was then replaced and cells were further incubated for 72 h.

Non-dividing U937 and THP-1 (PMA^+) cells (6×10^5 cells/mL) were differentiated by addition of 100 nM phorbol-12-myristate-13-acetate (PMA^+) for 48 h and then treated with increasing concentrations of nucleoside analogues as described above. The percentage of live cells was determined by flow cytometry using Live/Dead fixable blue-fluorescent dyes (Invitrogen) and a Fortessa X20 analyser (BD Biosciences). Data were analysed using the FlowJo software suite.

Antiviral assays. H9 and Jurkat cells (6×10^5 cells/mL) were treated with increasing concentrations of nucleoside analogues (0–200 μM; except CFB, 0–1.6 μM) and incubated for 4 h at 37 °C in 5% CO_2. Cells were then infected with HIV-1-GFP by spinoculation at $800 \times g$ for 90 min in the presence of 1 μg/mL polybrene, and further incubated at 37 °C in 5% CO_2 for 72 h. Undifferentiated U937 and THP-1 (PMA^-) cells (6×10^5 cells/mL) were treated with increasing concentrations of nucleoside analogues (0–200 μM; except CFB, 0–1.6 μM) and incubated at 37 °C in 5% CO_2 for 48 h. Cells were then infected with HIV-1-GFP by spinoculation at $800 \times g$ for 90 min in the presence of 1 μg/mL polybrene, and further incubated at 37 °C in 5% CO_2 for 72 h. Non-dividing U937 and THP-1 (PMA^+) cells (6×10^5 cells/mL) were differentiated by addition of 100 nM PMA (PMA^+) for 48 h and treated with increasing concentrations of nucleoside analogues as described above. Cells were then infected with HIV-1-GFP in the presence of 1 μg/mL polybrene and further incubated at 37 °C in 5% CO_2 for 72 h. The percentage of GFP^+cells was determined by one-colour flow cytometry using a Fortessa X20 analyser (BD Biosciences) and the data analysed using the FlowJo software suite.

Antiviral assays were further performed in monocytoid cells transduced with YFP-expressing VLPs containing WT SAMHD1, HD206-7AA or Vpx. Briefly, U937 cells (6×10^5 cells/mL) were transduced with WT SAMHD1-YFP or HD206-7AA-YFP VLPs by spinoculation at $800 \times g$ for 90 min in the presence of 1 μg/mL polybrene. Similarly, THP-1 cells (6×10^5 cells/mL) were transduced with Vpx-YFP VLPs by spinoculation at $800 \times g$ for 90 min in the presence of 1 μg/mL polybrene. Cells were incubated at 37 °C in 5% CO_2 and after 72 h, PMA^+ cells (6×10^5 cells/mL) were treated with 100 nM PMA and PMA^- cells (7.5×10^4 cells/mL) were left untreated. Cells were then incubated at 37 °C in 5% CO_2 for 48 h. Nucleoside analogues were added at increasing concentrations (0–200 μM; except CFB, 0–1.6 μM) and cells were further incubated for 48 h. PMA^- cells were then infected with HIV-1-GFP by spinoculation at $800 \times g$ for 90 min in the presence of 1 μg/mL polybrene, PMA^+ cells were infected with HIV-1-GFP in the presence of 1 μg/mL polybrene only. Cells were incubated for 72 h at 37 °C in 5% CO_2 and then analysed by two-colour flow cytometry using a Fortessa X20 analyser (BD Biosciences). Data were analysed using the FlowJo software suite.

Statistical analysis. The cytotoxic concentration of nucleoside analogue that inhibits cell viability by 50% (CC_{50}) and the effective concentration of nucleoside analogue that inhibits infection of cells by 50% (EC_{50}) were calculated for each independent experiment by non-linear regression using GraphPad Prism v6.00 for Windows (GraphPad Software, San Diego, CA). Non-ambiguous values were subjected to a replicate test to calculate confidence intervals of 95% (95% CI). CC_{50} and EC_{50} values were determined for at least two independent experiments for each nucleoside analogue and a selectivity index (SI) also calculated, ($SI = CC_{50}/EC_{50}$). EC_{50} values for each replicate were used to perform an unpaired two-tailed t-test to determine significant differences between U937 cells expressing SAMHD1 *wt* or HD206-7AA mutant and THP-1 cells expressing Vpx or untransduced cells. P values of <0.005 were considered statistically significant.

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Author Contributions

P.O., S.K., H.C.T.G., M.W.Y. and S.W. performed experiments. P.O., S.K., C.M., K.N.B., I.A.T. and J.P.S. contributed to experimental design, data analysis and manuscript writing.

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