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Short Communication

Antiviral interactions of combinations of highly potent 2,4(1H,3H)-pyrimidinedione congeners and other anti-HIV agents

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ABSTRACT

Structure–activity relationship evaluation of seventy-four 2,4(1H,3H)-pyrimidinedione derivatives identified seven lead compounds based on anti-HIV-1 potency, extended range of action to include HIV-2, virus entry inhibition, reverse transcriptase inhibition, and lack of cytotoxicity to human cells. The selected pyrimidinedione congeners are highly active inhibitors of HIV-1 with EC_{50} values ranging from 0.6 to 2 nM in CEM-SS cells infected with laboratory derived viruses, 11–20 nM in fresh human PBMCs infected with subtype B (HT/92/599) virus, and 2–7 nM in PBMCs infected with the clinical subtype C (ZA/97/003) virus. Combination antiviral assays were performed using the laboratory adapted RF strain of HIV-1 in CEM-SS cells and with a clade B and C low passage clinical isolate in fresh human peripheral mononuclear cells and the compound interactions were analyzed using MacSynergy II. The seven pyrimidinedione compounds resulted in additive to synergistic interactions in combination with entry and fusion inhibitors, nonnucleoside and nucleoside reverse transcriptase inhibitors, and the protease inhibitors. No evidence of antagonistic antiviral activity or synergistic cytotoxicity was detected with the combinations of compounds tested. The dual mechanism of action of the pyrimidinediones resulting in inhibition of both virus entry and reverse transcription suggests excellent potential of these lead pyrimidinediones as candidates for combination therapy with other approved HIV inhibitors of varying mechanism of action.

Highly active anti-retroviral therapy (HAART) was an important breakthrough in HIV treatment, significantly reducing morbidity and mortality due to HIV infection (Carpenter et al., 2000; UNAIDS/WHO, 2009). HAART, however, still suffers from the emergence of multi-drug resistant virus strains, drug–drug interactions, and adherence to the prescribed regimens. We have been actively developing 2,4(1H,3H)-pyrimidinedione (PYD) derivatives, which target two early steps of HIV replication including virus entry and reverse transcription (Buckheit et al., 2008). The parent compound, IQP-0410 (SJ-3366; 1-(3-cyclopenten-1-ylmethyl)-5-ethyl-6-(3,5-dimethylbenzoyl)-2,4(1H,3H)-pyrimidinedione) is a member of the HEPT (6-substituted acyclouridine derivative) class of molecules (Baba et al., 1994) and has been reported as a highly potent NNRTI with an extended range of action, including HIV-2 (Buckheit et al., 2008). Cell-based evaluations with both HIV-1 and HIV-2 suggest that the PYDs inhibit virus entry through recognition of a pre-fusion conformational structure involving both envelope and Gag determinants (manuscript in preparation). The dual mechanism of action of the PYD congeners renders these compounds ideal for continued development. From the series of seventy-four PYDs those with cyclopropyl, phenyl, and 1- or 3-cyclopenten-1-yl substitutions at the N-1, a methyl linker between the cyclic moiety and the N-1, and a benzoyl group at the C6 of the PYD possessed the greatest antiviral activity (Buckheit et al., 2001). IQP-0405, IQP-0406, IQP-0407, IQP-0528, IQP-0558, IQP-0410, and IQP-1187 (Table 2) were defined as highly potent inhibitors of HIV-1 with EC_{50} values ranging from 0.6 to 2 nM in a CEM-SS-based CPE assay, 11–20 nM against the subtype B (HT/92/599) HIV in PBMCs, 2–7 nM against the subtype C (ZA/97/003) HIV in PBMCs, and inhibit HIV entry to uninfected target cells with EC_{50} values ranging from 0.17 to 2 μM. Each of the seven lead candidates possess anti-HIV activity that is greater than other HEPT-like inhibitors reported in the literature (Requejo, 2006) and no other HEPT-like NNRTI has been reported to demonstrate HIV-2 inhibitory activity. The dual mechanism of action resulting in inhibition of virus entry and reverse transcription suggests that each of these congeners might be expected to represent a potential candidate for further development with an additional rationale to be defined by their interaction with other approved HIV inhibitors. We have evaluated the lead pyrimidinedione congeners (Table 1; Buckheit et al., 2008) using *in vitro* anti-HIV combination therapy assays using both CEM-SS-based CPE inhibition assays and PBMC-based virus replication inhibition assays with other HIV inhibitors targeting virus entry and fusion (Chicago Sky Blue,

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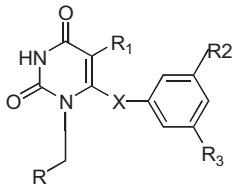
Table 1
Chemical structure of lead PYD congeners.

Structure	IQP #	R1	R2	R3	X	R
	0405	Et	Me	Me	O	Cyclopropyl
	0406	iPr	Me	Me	O	Cyclopropyl
	0407	Et	Me	Me	C=O	Cyclopropyl
	0528	iPr	Me	Me	C=O	Cyclopropyl
	0558	iPr	Me	Me	C=O	1-Cyclopenten-1-yl
	0410	Et	Me	Me	C=O	3-Cyclopenten-1-yl
	1187	iPr	Me	Me	C=O	3-Cyclopenten-1-yl

Cyanovirin, ISIS 5320, and T20), reverse transcriptase (Efavirenz, UC781, AZT, and Tenofovir), and protease (Ritonavir), to evaluate their combination antiviral interactions.

HIV-1 inhibitory activity of the compounds was evaluated in CEM-SS cells and fresh human PBMCs as described previously (Nara et al., 1987; Foley et al., 1965) in microtiter assays using the RF laboratory strain of HIV-1 and CXCR4-tropic low passage subtype B (HT/92/599) and CCR5-tropic subtype C (ZA/97/003) clinical isolates (AIDS Research and Reference Reagent Program, NIAID). Antiviral and toxicity data are reported as the quantity of drug required to inhibit 50% of virus-induced cell killing or virus production [EC_{50}] and the quantity of drug required to reduce cell viability by 50% [TC_{50}]. The standard combination anti-HIV assays (Buckheit et al., 1995) were performed using 5 concentrations of test compound A tested in all possible combinations with 9 concentrations of test compound B in a checkerboard pattern and analyzed for compound interactions at the 95% and 99% confidence intervals using the Prichard and Shipman MacSynergy II software program (Prichard and Shipman, 1990). Our extensive experience with the evaluation of combination assay data has resulted in the following interpretations of synergy plot data: >100 μM^2% is defined as a highly synergistic interaction; 51–99 μM^2% is defined as synergistic; 0–50 μM^2% and −50–0 μM^2% is defined as additive; −99–−51 μM^2% is defined as antagonistic; and <−100 μM^2% is defined as highly antagonistic.

A summary of the results of combination assay evaluations are presented in Table 3 (CEM-SS), Table 4 (fresh human PBMCs with subtype B (HT/92/599)) and Table 5 (fresh human PBMCs with subtype C (ZA/97/003)). At effective concentrations of these two drug combinations, no synergistic toxicity was observed with the exception of toxicity at a single combination concentration of IQP-1187 and either ISIS 5320 or AZT (data not shown). Importantly, no antagonistic antiviral interactions of the tested compounds were observed. Though there were significant differences in the defined interactions of the compounds depending on the anti-HIV assay employed (CEM-SS cells versus PBMCs), as well as differences observed in the PBMC assays when using a subtype B or subtype C clinical isolate, several two-drug combinations stood out as being highly synergistic. Specifically, tenofovir in combination with six of the seven PYD congeners yielded synergistic interactions when evaluated versus the subtype C (ZA/97/003) in PBMCs, but primarily additive interactions versus the subtype B (HT/92/599) isolate. Of the seven lead molecules evaluated, IQP-0405 yielded the most synergistic interactions in combination with the tested anti-HIV inhibitors followed by IQP-0407 and IQP-0528. Interestingly, each of these three congeners possesses a cyclopropyl substitution at the N-1 of the PYD. Though additive to slightly synergistic in drug combinations with the seven lead compounds versus the subtype C isolate, AZT yielded synergistic interactions with all seven compounds in both the CEM-SS CPE assay and the subtype B PBMC assay. In the CEM-SS CPE assay and subtype B PBMC based assay, IQP-0405 demonstrated moderate to highly synergistic interactions with the entry inhibitors Chicago Sky Blue, Cyanovirin, ISIS 5320, the fusion inhibitor T20, the NRTI AZT and the PI Ritonavir. These studies suggest the combination of the NNRTI PYD derivatives with HIV entry inhibitors, NRTIs or PIs would each represent an effective combination therapeutic product. These results would also suggest that the prioritization of combination therapeutic strategies for infected individuals in the United States (predominantly subtype B strains) would be different from strategies employed for the treatment of subtype C infections in sub-Saharan Africa. Subtype C is the most rapidly expanding HIV-1 subtype and predominates in Eastern and Southern Africa and India, accounting for almost 60% of HIV cases worldwide (Requejo, 2008). However, no significant differences have been observed in the disease progression or pathogenicity of infection in individuals infected by subtype C versus patients infected by other group M subtypes (Taylor et al., 2008). Despite relative conservation of the RT sequence among the HIV-1 subtypes, studies have shown that viruses carrying RT from subtype C isolates had decreased levels of viral cDNA accumulation, which correlated with reduced

Table 2
Anti-HIV-1 efficacy of PYD congeners and antiviral agents of varying MOA.

Compound	Antiviral assay[a]		
	CEM-SS/HIV-1_{RF} EC_{50} (μM)	PBMC/HIV-1 clade B (HT/92/599) EC_{50} (μM)	PBMC/HIV-1 clade C (ZA/97/003) EC_{50} (μM)
IQP-0405	0.001	0.013	0.007
IQP-0406	0.0009	0.012	0.004
IQP-0407	0.002	0.019	0.002
IQP-0528	0.0002	0.014	0.005
IQP-0558	0.0005	0.02	0.006
IQP-0410	0.0002	0.011	0.005
IQP-1187	0.0004	0.02	0.003
T20	0.002	0.016	0.003
Chicago Sky Blue (μg/mL)	3.5	5.4	4.6
Cyanovirin (μg/mL)	0.002	0.7	0.7
UC781 (ng/mL)	0.01	1.5	0.06
ISIS 5320	0.7	0.9	2.0
Tenofovir	1.2	0.02	0.7
AZT	0.005	0.01	0.002
Efavirenz	0.0009	0.002	0.1

[a] The results presented were obtained from representative antiviral assays with appropriate control compounds evaluated in parallel selected from a minimum of three antiviral assays. HIV cytoprotection were obtained by XTT dye reduction endpoint and virus replication in PBMCs with clinical HIV isolates was measured by RT incorporation. We have demonstrated that the standard error among multiple antiviral assays averaged less than 10% of the respective mean EC_{50} and TC_{50}. In each individual assay, mean efficacy, and toxicity values are derived from a minimum of three replicate wells.

Table 3
Combination anti-HIV therapy results using CEM-SS cells infected with the RF strain of HIV-1.

IQP compound tested in combination with:	IQP-0405 Synergy volume[a]	IQP-0406 Synergy volume	IQP-0407 Synergy volume	IQP-0528 Synergy volume	IQP-0558 Synergy volume	IQP-0410 Synergy volume	IQP-1187 Synergy volume
Chicago Sky Blue (EI)	**171.7** ± 80.9	38.9 ± 47.8	**91.6** ± 45.5	**124.9** ± 67.7	31.4 ± 19.7	12.8±0.3	27.5 ± 3.4
Cyanovirin (EI)	**146.9** ± 101.5	42.0 ± 32.6	**173.5** ± 101.8	**140.4** ± 87.1	**102.6** ± 21.6	24.9 ± 35.0	**99.8** ± 8.0
Efavirenz (NNRTI)	16.5 ± 5.7	**63.3** ± 73.8	**113.5** ± 28.3	**106.8** ± 49.6	19.6 ± 8.7	35.4 ± 8.6	**86.2** ± 14.4
ISIS 5320 (EI)	**182.1** ± 114.1	**68.2** ± 80.2	**206.4** ± 96.3	**90.9** ± 47.3	**151.7** ± 51.5	**65.3** ± 17.2	**125.5** ± 39.5
UC781 (NNRTI)	**59.8** ± 8.6	17.5 ± 26.4	28.9 ± 8.2	**63.3** ± 9.7	30.2 ± 4.0	39.8 ± 13.8	18.6 ± 13.3
T20 (FI)	**139.2** ± 88.7	25.6 ± 37.1	25.3 ± 12.1	15.3 ± 18.2	13.1 ± 18.7	44.4 ± 3.2	15.3 ± 13.4
AZT (NRTI)	**153.2** ± 54.0	**88.7** ± 66.8	**232.9 ± 16.3**	**188.1** ± 173.4	**133.7** ± 89.6	**179.0** ± 89.4	**83.5** ± 9.5
Tenofovir (NRTI)	20.7 ± 5.9	16.9 ± 19.7	**118.3** ± 22.7	30.2 ± 0.7	5.3 ± 7.5	35.0 ± 10.9	7.8 ± 11.0
Ritonavir (PI)	**74.6** ± 16.5	18.7 ± 21.1	**69.1** ± 14.2	12.9 ± 7.3	20.5 ± 8.6	6.4 ± 8.6	28.2 ± 17.4

[a] Synergy volumes were calculated by the Prichard and Shipman MacSynergy II Program at the 95% confidence interval. The results of the MacSynergy II evaluation quantify the volume of the synergistic, additive, or antagonistic surface and are expressed in units of $\mu M^2\%$ (or μMμg/mL% for Chicago Sky Blue and Cyanovirin). Synergistic results are highlighted in bold. The mean and standard deviation from at least two replicate results are presented for each combination of products.

Table 4
Combination anti-HIV therapy results using PBMCs infected with the subtype B (HT/92/599) HIV-1 strain.

IQP compound tested in combination with:	IQP-0405 Synergy Volume[a]	IQP-0406 Synergy Volume	IQP-0407 Synergy volume	IQP-0528 Synergy volume	IQP-0558 Synergy volume	IQP-0410 Synergy volume	IQP-1187 Synergy volume
Chicago Sky Blue (EI)	19.6 ± 14.5	**109.4** ± 123.7	19.9 ± 23.6	**75.3** ± 12.9	16.1 ± 18.5	13.3 ± 6.0	32.1±20.3
Cyanovirin (EI)	**107.8** ± 57.8	7.8 ± 12.2	32.3 ± 8.4	**84.6** ± 25.7	27.7 ± 27.2	**78.5** ± 33.7	**81.1** ± 41.6
Efavirenz (NNRTI)	**71.6** ± 12.1	12.6 ± 20.0	**56.7** ± 5.9	31.1 ± 22.7	27.7 ± 27.2	19.0 ± 8.9	**177.4** ± 0.2
ISIS 5320 (EI)	28.0 ± 1.3	**146.0** ± 143.3	**100.0** ± 44.5	**235.9** ± 149.9	**87.0** ± 24.3	24.6 ± 34.6	15.1 ± 11.7
UC781 (NNRTI)	**102.4** ± 18.4	41.0 ± 32.3	**117.5** ± 39.8	30.8 ± 25.7	15.9 ± 12.2	13.3 ± 13.7	**102.6** ± 44.3
T20 (FI)	**139.1** ± 47.1	**150.7** ± 203.1	13.2 ± 6.0	**98.8 ± 42.4**	14.1 ± 19.9	**84.4** ± 45.5	**83.3** ± 39.7
AZT (NRTI)	**77.5 ± 7.8**	**317.6** ± 194.4	**111.8** ± 30.1	**206.0** ± 70.9	**81.7** ± 1.4	**216.1** ± 9.1	**75.6** ± 5.1
Tenofovir (NRTI)	**125.0** ± 6.4	23.9 ± 11.0	**146.9** ± 2.8	16.8 ± 16.5	31.4 ± 1.4	14.9 ± 6.7	23.0 ± 9.4
Ritonavir (PI)	**96.0** ± 36.6	**51.6** ± 50.0	**103.2** ± 5.4	**123.9** ± 34.0	**56.9** ± 5.0	**132.7** ± 53.7	**91.8** ± 10.7

[a] Synergy volumes were calculated by the Prichard and Shipman MacSynergy II program at the 95% confidence interval. The results of the MacSynergy II evaluation quantify the volume of the synergistic, additive, or antagonistic surface and are expressed in units of $\mu M^2\%$ (or μMμg/mL% for Chicago Sky Blue and Cyanovirin). Synergistic results are highlighted in bold. The mean and standard deviation from at least two replicate results are presented for each combination of products.

Table 5
Combination anti-HIV therapy results using PBMCs infected with the subtype C (ZA/97/003) HIV-1 strain.

IQP compound tested in combination with:	IQP-0405 Synergy volume[a]	IQP-0406 Synergy volume	IQP-0407 Synergy volume	IQP-0528 Synergy volume	IQP-0558 Synergy volume	IQP-0410 Synergy volume	IQP-1187 Synergy volume
Chicago Sky Blue (EI)	25.0 ± 32.7	20.5 ± 40.0	23.9 ± 24.0	25.2 ± 13.7	11.5 ± 15.8	35.1 ± 13.2	40.3 ± 3.7
Cyanovirin (EI)	**84.6** ± 24.7	29.9 ± 27.1	**85.9** ± 32.0	**100.6** ± 51.1	12.2 ± 1.4	**60.9** ± 4.0	5.9 ± 6.0
Efavirenz (NNRTI)	41.3 ± 4.2	**86.2** ± 117.4	23.8 ± 3.8	11.9 ± 16.8	**74.1** ± 0.6	**74.0** ± 26.2	**116.2** ± 20.5
ISIS 5320 (EI)	22.7 ± 18.0	1.0 ± 0	11.1 ± 15.7	5.6 ± 0.1	6.8 ± 7.1	2.3±0.9	9.4 ± 12.6
UC781 (NNRTI)	18.7 ± 16.1	22.3 ± 12.1	**68.0** ± 6.3	24.7 ± 29.6	23.5 ± 31.1	**87.4** ± 11.5	**92.5** ± 54.3
T20 (FI)	**73.3** ± 33.0	**88.1** ± 56.6	11.4 ± 16.1	**63.4** ± 1.8	1.1 ± 0.2	29.0 ± 28.1	**186.6** ± 41.1
AZT (NRTI)	48.0 ± 2.5	**109.7** ± 50.5	34.6 ± 4.9	16.2 ± 0	**61.1** ± 4.2	28.3 ± 4.0	32.6 ± 18.3
Tenofovir (NRTI)	**88.3** ± 41.1	**227.7 ± 308.1**	**210.7** ± 107.8	**259.2** ± 244.9	**272.3** ± 273.4	**96.4** ± 21.3	32.7 ± 22.1
Ritonavir (PI)	**127.3** ± 82.2	**130.0** ± 112.7	**82.6** ± 31.0	**132.9** ± 51.2	13.4 ± 17.4	**62.2** ± 13.4	**55.9** ± 6.5

[a] Synergy volumes were calculated by the Prichard and Shipman MacSynergy II program at the 95% confidence interval. The results of the MacSynergy II evaluation quantify the volume of the synergistic, additive, or antagonistic surface and are expressed in units of $\mu M^2\%$ (or μMμg/mL% for Chicago Sky Blue and Cyanovirin). Synergistic results are highlighted in bold. The mean and standard deviation from at least two replicate results are presented for each combination of products.

integration and lower levels of virus replication (Grossman et al., 2001; Iordanskiy et al., 2010). Since subtype C HIV isolates predominantly use the CCR5 co-receptor for viral entry, even in late infection, this may reduce the viral cytopathogenicity and affect the spread of virus. The decrease in overall replication of subtype C viruses may contribute to differences observed in the combination assays using a subtype B or subtype C clinical isolate. It has also been reported that differences in the selection of resistant viruses occurs when subtype C viruses are utilized, suggesting other important biological differences between subtype C and subtype B viruses which need to be considered when designing combination therapies (Martinex-Cajas et al., 2009).

The most common medications given in first-line HAART treatment are the NNRTIs in combination with two NRTIs in HIV-infected individuals without prior exposure to antiretroviral therapy or in patients whom experienced virological breakthroughs on at least one prior PI-based regimen (Boyd, 2011; Mbuagbaw and Irlam, 2010). Second generation NNRTIs, Delavirdine, Etravirine and Rilpivirine, have demonstrated a higher genetic barrier to resistance than Efavirenz and Nevirapine, in addition to long plasma half-lives, low pill burdens, increased tolerability, and the ability to be safely combined with other commonly used medications (Azijn et al., 2010; De Clercq, 2009; Seminari et al., 2008). *In vitro* combination studies of FDA approved NNRTIs with other classes of HIV inhibitors typically result in additive to synergistic interactions (Azijn et al., 2010; De Clercq, 2004; Seminari et al., 2008). Many variations in HAART regimens are available, some of which differ in toxicity, adverse effects, suppression of virus replication, development of viral resistance, and patient adherence (Deek and Perry, 2010; Palella et al., 1998; Yazdanpanah et al., 2004). The two-drug combination anti-HIV-1 results indicate PYD congeners, which alone target two steps in virus replication, may

be clinically promising as additions to primary or salvage HAART therapy. A co-formulated product of a PYD with an approved HIV drug essentially yields three possible mechanisms of antiviral action in one product with potentially fewer drug–drug interactions. The PYDs with a cyclopropyl R group (IQP-0405, IQP-0407 and IQP-0528) have been identified as lead candidates in combination therapy evaluations, as well as range of action evaluations in PBMCs, selection of drug resistant virus, cross resistance evaluations, metabolic stability in liver microsomes and stability in fresh human hepatocytes.

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