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Short communication

Rapamycin enhances aplaviroc anti-HIV activity: Implications for the clinical development of novel CCR5 antagonists

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ABSTRACT

Maraviroc, the only CCR5 antagonist HIV inhibitor currently approved, has potent antiviral activity in treatment-experienced individuals infected with CCR5-using HIV-1 (R5 HIV-1). However, recent data from the MOTIVATE trials indicate that R5 HIV-1 can develop resistance to Maraviroc, underscoring the need for additional CCR5 antagonists. The CCR5 antagonist aplaviroc (APL) is active against Maraviroc-resistant viral strains but its clinical development has ended because of dose-related toxicity. Here we demonstrate that reduction of CCR5 density (receptors/cell) with the immunomodulatory drug rapamycin (RAPA) enhances the antiviral activity of APL, allowing lower, non-toxic effective doses. In the presence of RAPA, the concentration of APL required for 90% inhibition of R5 HIV-1 in primary CD4 lymphocytes was reduced by as much as 25-fold. We conclude that low doses of RAPA may reduce the anti-HIV effective dose of APL-derivatives currently in development and thus minimize their potential toxicity. Combinations of RAPA and CCR5 antagonists could provide an effective means to control drug-resistant R5 HIV in patients, most notably those infected with Maraviroc-resistant viruses.

Current treatment guidelines recommend the use of at least two, and preferably three, fully active agents in new drug regimens in patients with evidence of HIV-1 resistance (Hammer et al., 2008). Maraviroc inhibits multidrug-resistant R5 HIV-1, but is the only licensed drug from the CCR5 antagonist class (Hammer et al., 2008). Development of HIV-1 resistance to Maraviroc has been reported in recent clinical trials (Fätkenheuer et al., 2008; Gulick et al., 2008), suggesting that additional drugs from the CCR5 antagonist class will be needed. Several CCR5 antagonists are currently in development but dose-related toxicity remains a concern (Clotet, 2007). Since the antiviral activity of CCR5 antagonists increases at lower CCR5 densities (Ketas et al., 2007; Platt et al., 2005), we hypothesized that reduction of CCR5 levels with rapamycin (RAPA) (Heredia et al., 2003, 2007) could lower the anti-HIV effective dose of the antagonist and thus reduce potential toxicity. We tested this hypothesis using aplaviroc (APL), a CCR5 antagonist whose clinical development was ended due to hepatotoxicity (Currier et al., 2008; Lalezari et al., 2005; Nichols et al., 2008).

We first correlated CCR5 density with APL inhibition of R5 HIV-1 ADA pseudovirus infection of CD4 lymphocytes from different donors (Fig. 1a). Coreceptor densities ranged between 4053 and 10 046 CCR5s/cell, within previously reported ranges (Hladik et al., 2005; Lee et al., 1999; Reynes et al., 2000). APL EC_{50} values varied ~14-fold, in agreement with variability of APL potency among donors (Maeda et al., 2004). Importantly, APL EC_{50} values positively correlated with CCR5 density (Spearman correlation, $r = 1.0$, $P = 0.016$). In contrast, EC_{50} values for efavirenz, which acts intracellularly, varied only <3-fold among donors and did not correlate with CCR5 density (not shown). These data demonstrate that CCR5 density impacts the antiviral activity of APL as judged by pseudovirus infection.

We next assessed whether reduction of CCR5 density on PBMCs could increase the antiviral activity of APL. Based on previous studies of CCR5 downregulation by RAPA treatment (Heredia et al., 2003), we evaluated the antiviral activity of APL in RAPA-treated lymphocytes. We performed these experiments using replication-competent R5 HIV ADA and determined antiviral activity by p24 production. In the representative experiment shown in Fig. 1b, cell treatment with RAPA concentrations of 0.1, 0.3 and 1 nM reduced CCR5 density from 7.6×10^3 to 4.5-, 3.9- and 3.5×10^3 CCR5 receptors/CD4 lymphocyte, respectively. RAPA inhibited HIV-1 by 20, 35 and 65%, respectively, with an EC_{50} of 0.53 nM. The observed reductions on CCR5 density and viral replication by RAPA are in agreement with previous studies (Heredia et al., 2007). Consistent with the idea that a decreased surface CCR5 density would make

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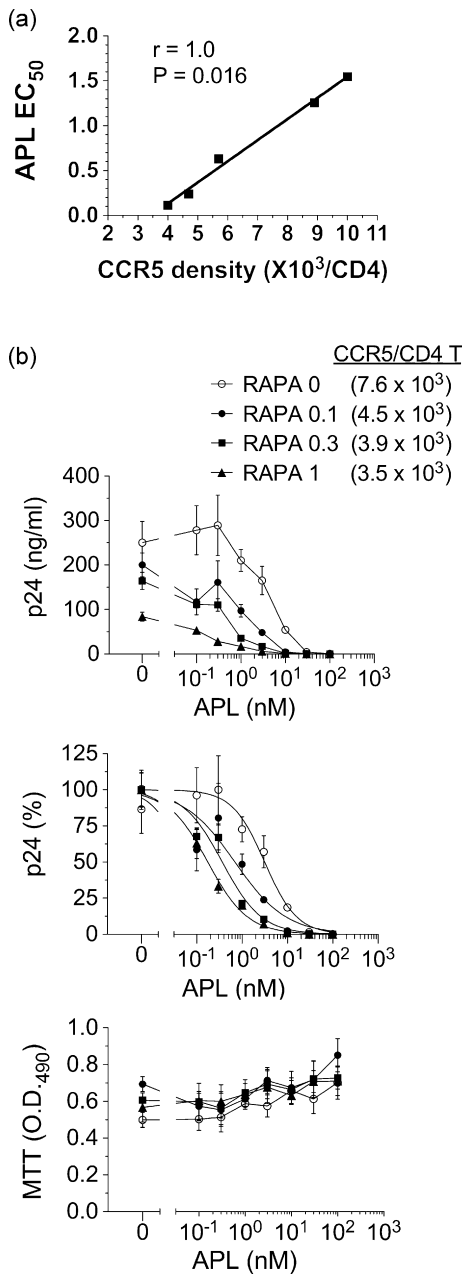

Fig. 1. Impact of CCR5 density and RAPA treatment on the antiviral activity of APL. (a) CCR5 density inversely correlates with APL activity. Donor PBMCs were subjected to quantitative flow cytometry analysis and R5 HIV-1 ADA pseudovirus infection. Replication-defective HIV-1 reporter viruses were produced by cotransfecting 2×10^6 293T cells with 10 μg of pNL4.3-env$^-$-luc3 and 10 μg of pSV-ADAenv using the calcium phosphate protocol, as described (Abrahamyan et al., 2005). For infection, cells were mixed with pseudovirus at a ratio of 10 ng p24/2×10^5 cells. Infectivity was determined by measuring luciferase activity in cell lysates on day 3 after infection. EC_{50} values were derived from a non-linear sigmoidal dose–response curve performed in Prism software. CCR5 densities were plotted against APL EC_{50}, and the association tested using the Spearman's correlation test (*r*, Spearman correlation coefficient). (b) RAPA enhances APL antiviral activity in the absence of cell toxicity. Seven-days RAPA-treated PBMCs were subjected to CCR5 density quantification and infection with replication-competent HIV-1 ADA (M.O.I = 0.001) in the presence of RAPA and APL. Infected cells were cultured in the presence of drugs. Infectivity was determined by p24 production on day 5 after infection (upper panel). Infectivity data at each RAPA concentration were normalized to viral inhibition in the absence of APL (middle panel). Cell viability was determined by the MTT assay on day 5 after infection (lower panel). Data (means ± S.D.) are from an experiment representative of 3 independent experiments using different donors.

CCR5 antagonists more effective, RAPA increased APL inhibition of p24 production (Fig. 1b, upper panel). After normalizing p24 values to viral inhibition by RAPA (Fig. 1b, middle panel), the EC_{50} values for APL were 3.92, 1.16, 0.39 and 0.12 nM at 0, 0.1, 0.3 and 1 nM RAPA, respectively. Cell viability was not affected (Fig. 1b, lower panel); toxicity CC_{50} values could not be determined because neither RAPA nor APL gave dose response curves at the assayed concentrations. The antiviral activity of APL was less potent in the multi-cycle assay compared with single-cycle pseudovirus infections, as shown by the higher EC_{50} (compare Fig. 1a, which corresponds to single-cycle infection, and EC_{50} data obtained in multi-cycle infection at equivalent CCR5 densities), in agreement with differences in assay sensitivities (Heyndrickx et al., 2008). Regardless, the observed reductions of APL EC_{50} in the presence of RAPA suggested a synergistic viral inhibition by the drug combination.

To better understand the nature of the antiviral inhibition by the RAPA/APL combination, we evaluated possible drug interactions by determining Combination Index (CI) values (Chou and Talalay, 1984). When drugs were used in equimolar amounts (i.e., 1:1 ratio) against HIV-1 ADA, CI values for 50, 75 and 90% inhibition were 0.35, 0.26 and 0.22, respectively (Table 1). As CI values of <0.9 indicate synergy and values are proportional to the amount of synergy, the RAPA/APL combination clearly has considerable antiviral synergy. Importantly, the calculated Dose Reduction Index (DRI) indicated that ~17-fold less APL was required to inhibit HIV-1 ADA replication by 90% when used in combination with RAPA. In contrast, the combination of RAPA and efavirenz was strictly additive (CI values ranged from 0.9 to 1.2) (data not shown).

We next assessed the mode of antiviral interaction between RAPA and APL in early steps of the virus life cycle by determining CI values in a previously described cell–cell fusion assay (Abrahamyan et al., 2005; Heredia et al., 2007, 2008). Because preliminary experiments demonstrated that higher amounts of APL were required to inhibit cell–cell fusion compared to p24 production, we tested the drugs at a RAPA:APL ratio of 1:3. The obtained CI values for 50, 75 and 90% cell–cell fusion inhibition were 0.58, 0.22 and 0.09, respectively, demonstrating strong synergy between RAPA and APL (data not shown). The corresponding APL DRI values at 50, 70 and 90% inhibition were 3.6, 7.1 and 13.9, respectively, which are consistent with the drug reductions observed in the viral replication assays. Together, the results from the replication and cell–cell fusion assays clearly demonstrate that the combination of RAPA and APL has antiviral synergy against the reference HIV-1 ADA strain.

We finally evaluated the antiviral activity of the RAPA/APL combination against R5 HIV-1 primary isolates in multi-cycle PBMC infection assays as described above. Because drug sensitivities of primary isolates may differ from those of HIV-1 ADA, we tested RAPA:APL molar ratios of 1:1, 1:3 and 10:1 for each virus. Regardless of the molar ratio used, RAPA and APL had antiviral synergy. Representative results, at different ratios, are shown (Table 1). RAPA EC_{50} values ranged from 0.1 to 0.5 nM and APL EC_{50} values ranged from 0.04 to 1.67 nM in drug-alone treatments between viruses (Table 1, and data not shown). This synergy translated into APL dose reductions of up to ~25-fold. Thus, similarly to the situation with lab-adapted HIV-1, RAPA enhanced the antiviral activity of APL against primary isolates of HIV-1.

In summary, we have demonstrated that the antiviral activity of the CCR5 antagonist APL is impacted by CCR5 density in donor cells, consistent with similar findings on the CCR5 antagonist Vicriviroc (Heredia et al., 2008; Ketas et al., 2007). RAPA, which reduced CCR5 density on lymphocytes (Heredia et al., 2003, 2007), synergistically enhanced the antiviral activity of APL against both reference and primary strains of R5 HIV-1. This antiviral synergy correlated directly with the RAPA-mediated reductions in cell surface CCR5 and translated into APL dose reductions of up to 25-fold in drug combinations inhibiting R5 HIV by 90%. The mech-

Table 1
Combination Index (CI) values and APL dose reduction in the inhibition of HIV-1 infection by the RAPA/APL combination in PBMC infectivity assays.

HIV-1 strain	RAPA:APL molar ratio	% of inhibition[a]	CI[b]	APL (nM)		APL dose reduction
				Alone	Combined	
ADA	1:1	50	0.35	1.67	0.16	10.3
		75	0.26	3.7	0.27	13.2
		90	0.22	8.2	0.50	16.9
JV1083	1:1	50	1.15	0.68	0.14	5
		75	0.38	1.62	0.28	5.8
		90	0.19	3.8	0.54	7
93RW008	1:3	50	1.13	0.23	0.18	1.3
		75	0.59	1.33	0.33	4
		90	0.41	7.6	0.60	12.6
92UG031	1:3	50	0.25	0.19	0.03	6.2
		75	0.19	0.81	0.06	12
		90	0.16	3.47	0.15	23
93UG082	10:1	50	0.67	0.05	0.005	8.7
		75	0.56	0.15	0.010	14.7
		90	0.48	0.42	0.016	24.8
93BR029	10:1	50	0.73	0.04	0.007	5.4
		75	0.73	0.14	0.017	8
		90	0.75	0.44	0.037	11.9

[a] Determined by measuring p24 levels on day 5 after infection.
[b] CI < 0.9 indicates synergy; 0.9 < CI < 1.1 indicates additivity; CI > 1.1 indicates antagonism. Results for each virus are representative of at least 3 independent experiments.

anism of antiviral synergy by RAPA and CCR5 antagonists is not currently known. Previous evidence suggests that HIV requires the concerted engagement of approximately 4 to 7 CCR5 receptors for successful formation of a fusion pore (Kuhmann et al., 2000; Sougrat et al., 2007). We postulate that RAPA reduction of CCR5 density prevents HIV virions from engaging a minimum number of CCR5s necessary for fusion. In the RAPA/APL combination, reduction of CCR5 density and partial blocking of CCR5 by APL would effectively prevent CD4 bound viral envelopes from engaging sufficient CCR5 molecules. In turn, CD4 triggered envelopes unable to progress in the entry cascade would be exposed for prolonged periods of time and subjected to inactivation (Platt et al., 2005). These mechanisms are consistent with the observed differences on CI synergy values at different percentages of viral inhibition and between different viruses (Table 1). Specifically, synergy may be higher (lower CI) at increased RAPA concentrations because CCR5 density is lower (Fig. 1b), with reduced probability of engaging sufficient CCR5. In addition, envelopes from different viruses vary in their affinities for CCR5, with fusion efficiencies affected differently by changes in CCR5 levels (Platt et al., 2005; Reeves et al., 2002).

The clinical development of APL was ended because of idiosyncratic hepatotoxicity (Currier et al., 2008; Nichols et al., 2008). This is unfortunate because APL is active against viruses resistant to Maraviroc (Westby et al., 2007), the only licensed CCR5 antagonist. Our results suggest that RAPA may provide an effective means to reduce the antiviral effective dose, and thus potential toxicity, of novel CCR5 antagonists. Despite the in vitro (Heredia et al., 2003, 2008; Roy et al., 2002) and in vivo (Aquaro et al., 2009; Gilliam et al., 2007) anti-HIV activity of RAPA, its potential immunosuppressive activity may argue against using it in HIV infection. However, it should be noted that RAPA inhibits HIV and enhances entry inhibitors at concentrations of <1 nM in the absence of toxicity (Heredia et al., 2003, 2008; Roy et al., 2002). These RAPA concentrations are lower than the 1–10 nM plasma levels in transplant patients (Augustine et al., 2007). Moreover, RAPA alongside antiretrovirals is safely used in HIV-infected organ-transplant recipients (Ballarin et al., 2008; Kumar et al., 2005; Moreno et al., 2008). Ultimately, the use of low-dose RAPA and CCR5 antagonists in SIV-infected macaques will help determine the safety and efficacy of this potential therapeutic approach.

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