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Oral Cyclosporin A Inhibits CD4 T cell P-glycoprotein Activity in HIV-Infected Adults Initiating Treatment with Nucleoside Reverse Transcriptase Inhibitors

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Abstract

Purpose—P-glycoprotein limits tissue penetration of many antiretroviral drugs. We characterized effects of the P-glycoprotein substrate cyclosporin A on T cell P-glycoprotein activity in HIV-infected AIDS Clinical Trials Group study A5138 participants.

Methods—We studied P-glycoprotein activity on CD4 and CD8 T cells in 16 participants randomized to receive oral cyclosporin A (n=9) or not (n=7) during initiation antiretroviral therapy (ART) that did not include protease or non-nucleoside reverse transcriptase inhibitors.

Results—CD4 T cell P-glycoprotein activity decreased by a median of 8 percentage points with cyclosporin A/ART (difference between cyclosporin A/ART versus ART only *P*=0.001). Plasma trough cyclosporin A concentrations correlated with change in P-glycoprotein activity in several T cell subsets.

Conclusions—Oral cyclosporin A can inhibit peripheral blood CD4 T cell P-glycoprotein activity. Targeted P-glycoprotein inhibition might enhance delivery of ART to T cells.

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Keywords

HIV/AIDS; Antiretroviral therapy; P-glycoprotein; Cyclosporin A; T lymphocytes

Introduction

P-glycoprotein, the efflux transporter encoded by *ABCB1*, limits drug entry into tissues and cells [1,2]. Inhibition of P-glycoprotein may enhance penetration of substrate drugs [3,4]. There is interest in the role of P-glycoprotein during human immunodeficiency virus (HIV) therapy [5] because protease inhibitors and other antiretrovirals are P-glycoprotein substrates [6–9] and P-glycoprotein expression on CD4 T lymphocytes could decrease antiviral effects [10–12]. Protease inhibitors can inhibit P-glycoprotein activity [13–15] but increased cellular P-glycoprotein expression can be induced *ex vivo* by protease inhibitors or non-nucleoside reverse transcriptase inhibitors (NNRTIs) [16–19]. Limited data also suggest that P-glycoprotein overexpression in cell lines may influence HIV-associated effects, conferring relative resistance to infection [20,21] and apoptosis [22,23]. An *ABCB1* polymorphism that affects P-glycoprotein expression [24] may predict more favorable virologic responses to antiretroviral therapy (ART) [25–27] and/or decreased drug toxicity [28,29], although data are conflicting.

Targeted P-glycoprotein inhibition has been pursued as a strategy to restore susceptibility of multidrug resistant cancer cells to chemotherapy [30–33]. The immunosuppressant drug, cyclosporin A, inhibits P-glycoprotein activity [34,35]. This may explain in part the increased oral bioavailability of some chemotherapeutic agents with concomitant cyclosporin administration [36]. Oral cyclosporin A increased plasma levels (trough and area-under-the-curve) of the HIV protease inhibitor (PI) nelfinavir in a single study of seven HIV-infected subjects [37].

Immune activation during HIV infection predicts disease progression [38,39], suggesting that immunosuppressive therapy might paradoxically be beneficial during ART. AIDS Clinical Trials Group (ACTG) study A5138 tested the hypothesis that concomitant cyclosporin A would enhance immune reconstitution during ART initiation. Although no sustained effects were seen [40], A5138 provided an opportunity to assess the impact of cyclosporin A on P-glycoprotein activity during ART initiation. The ART regimens during the first 14 days of A5138 included neither PIs nor NNRTIs which could affect P-glycoprotein activity. We hypothesized that cyclosporin A would inhibit peripheral blood T cell P-glycoprotein activity. The objective of this study was to determine the effects of cyclosporin A on T cell P-glycoprotein efflux activity *ex vivo* using specimens from a clinical trial who were receiving PI and NNRTI-sparing ART in the presence and absence of cyclosporin A.

Methods

ACTG protocol A5138

Primary A5138 results have been reported [40]. Briefly, ART-naïve HIV-infected individuals initiated twice-daily co-formulated abacavir/zidovudine/lamivudine. Participants were randomized to also receive either cyclosporin A (Neoral; Sandoz) 4mg/kg twice daily, or no cyclosporin A during the first 14 days of ART. On day 15 all participants added efavirenz 600 mg once daily. Trough cyclosporin A concentrations were obtained at days 3, 7, 10, and 14. The primary study was registered as trial NCT00031070 (http://www.clinicaltrials.gov). The primary study protocol and all substudies were approved by local Institutional Review Boards at each clinical trial site. All participants in this substudy provided written informed consent under the A5138 protocol version that included P-glycoprotein assays.

Dye efflux assay

Acid-citrate-dextrose anticoagulated whole blood was shipped to Vanderbilt University at ambient temperature, and assayed within 24 hours of phlebotomy. Three specimens not processed within 24 hours were not analyzed. The activity of P-glycoprotein in peripheral blood T cells was determined by measuring the cellular efflux of the fluorescent P-glycoprotein substrate 3,3′-diethyloxacarbocyanine iodide [$DiOCB_2(3)$] as described elsewhere [13,41]. The cellular efflux of $DiOC_2(3)$, which is a specific P-glycoprotein substrate [42], has been demonstrated to be directly related to functional P-glycoprotein in these cells [43,44]. Briefly, $CD4^+$ and $CD8^+$ T cells were loaded with $DiOC_2(3)$ by incubating 1 mL of ACD anti-coagulated whole blood with 1 mL of 100 nM $DiOC_2(3)$ in PBS for 15 minutes at 37°C. Cells were collected by centrifugation, washed with ice-cold PBS and resuspended in 600 μL of RPMI 1640 plus 50 mM HEPES (pH 7.4). Verapamil was added to one-half of each sample to a final concentration of 19 μM resulting in greater than 95% inhibition of the cellular efflux of $DiOC_2(3)$ by P-glycoprotein. Then 100 μL aliquots of each sample, plus or minus verapamil, were added in duplicate to each well of a 96 deep-well microplate. For each microplate, a sample from one of six healthy adults with previously determined P-gp activity was included as an assay control. The microplate was then incubated at 37°C for 60 minutes. After incubation, T cells were labeled with fluorochrome-conjugated monoclonal antibodies (CD62L-PE, CD45RA-APC, and CD4-PerCP-Cy5.5 or CD8-PerCP-Cy5.5) for 30 minutes on ice. Erythrocytes were lysed with Optilyse B essentially as described by the manufacturer (Immunotech, Marseille, France), leukocytes were pelleted by centrifugation, washed with ice-cold PBS, and fixed with 2% paraformaldehyde in PBS prior to analysis by flow cytometry.

Flow cytometry analysis

T cell $DiOC_2(3)$ content was quantified using a FACSCalibur flow cytometer fitted with a Multiwell AutoSampler (Becton Dickinson, San Jose, CA). For each sample, data from at least 10,000 electronically gated lymphocytes were collected. Fluorescence of $DiOC_2(3)$ was detected following excitation at 488 nm, through a 530 nm band-pass filter. Data were analyzed with WinMDI software version 2.8 (J. Trotter, The Scripps Institute). Naive T cells were defined by surface staining for both CD45RA and CD62L (L-selectin). The percentage of the total and naïve $CD4^+$ or $CD8^+$ T cells that were dim (dye-efflux positive) was determined by gating on parallel control samples with near complete P-glycoprotein inhibition (>95%) by verapamil as illustrated in Figure 1. For each subject net dye efflux (P-glycoprotein activity) was reported as the percentage dim cells after subtracting background dim cell percentage in verapamil control incubations.

Plasma cyclosporin A concentrations

Plasma for cyclosporin A trough assay was obtained immediately prior to the morning dose, and concentrations determined by standard monoclonal antibody or fluorescent polarization radioimmunoassay at commercial laboratories. Results are presented as nanograms/mL of plasma.

Statistical analysis

Distributions of baseline, week 2 and week 4 laboratory values are summarized with median values and interquartile ranges (IQR). Exact Wilcoxon rank-sum and sign tests compared distribution of P-glycoprotein efflux changes from baseline between and within arms, respectively, and Spearman's correlation assessed the association between efflux changes and cyclosporin A trough levels. The median-unbiased point estimate of the shift parameter between the two distributions and exact 95% confidence interval was estimated from the Hodges-Lehmann procedure. For example, a positive shift means efflux values from the cyclosporin A/ART arm are shifted lower than those in the ART alone arm. Subjects who did

not complete two weeks of cyclosporin A (in the cyclosporin A arm) and four weeks of ART were excluded from these analyses; results presented are for the as-treated patient study population based on ART and cyclosporin A administration. Separate inference for changes from baseline to day 14 and day 28 was performed, and no adjustments were made for multiple comparisons across time.

Results

Of 42 A5138 participants, our analysis comprises 16 individuals. The other 26 were excluded because they either enrolled to A5138 before the P-glycoprotein study was implemented (n=16) or were not in the as-treated study population (n=10). Baseline characteristics (age, race/ethnicity, sex, CD4 T cells, and plasma HIV-1 RNA) did not differ by group (9 randomized to cyclosporin A/ART; 7 to ART alone; Table), and were similar to the other 26 participants (data not shown). Of the 16 participants, 14 had CD4 T cell P-glycoprotein assay data at day 14 (6 in the cyclosporin A/ART arm; 8 in the ART alone arm), and 13 had data at day 28 (5 and 8, respectively). Median baseline P-glycoprotein activities, expressed as the percentage of dye efflux positive (dim) cells, within T cell subsets were: 23% in total CD4 T cells, 28% in naïve CD4 T cells, 47% in total CD8 T cells, and 76% in naive CD8 T cells.

Median baseline P-glycoprotein activity in total CD4 T cells was 24% in the cyclosporin A/ART arm and 22% in the ART alone arm (Figure 2A). Median changes from baseline to week 2 were an 8 percentage point decrease in the cyclosporin A/ART arm (relative decrease of 42% in proportion of dim cells), and no change in the ART alone arm (95% CI for shift in distributions= 5–12 percentage points; P=0.001). At 28 days (two weeks after discontinuing cyclosporin A and initiating efavirenz), median changes from baseline in CD4 T cell P-glycoprotein activity no longer differed between arms. In naïve CD4 T cells, median baseline P-glycoprotein activity was 28% in the cyclosporin A/ART arm and 33% in the ART alone arm. The median changes from baseline to week 2 were a 9 percentage point decrease in the cyclosporin A/ART arm, and no change in the ART alone arm (Figure 2B; 95% CI for shift in distributions= 3–22 percentage points; P=0.01). At 28 days the median change from baseline in naïve CD4 T cell P-glycoprotein activity no longer differed between arms. In total and naïve CD8 T cells there were no significant changes in P-glycoprotein activity from baseline to day 14 (Figures 2C and D).

We next assessed correlations between day 14 trough plasma cyclosporin A concentrations and change in P-glycoprotein activity from baseline to day 14. In total CD4 T cells we found no significant correlation (Spearman's rho= 0.00; *P*=1.00; Figure 3A), whereas in naïve CD4 T cells there was an inverse correlation (−0.89; *P*=0.02; Figure 3B). In CD8 T cells there was a trend toward an inverse correlation (−0.77; *P*=0.07), which in naive CD8 T cells reached statistical significance (−0.94; *P*=0.005; Figure 3C and D).

Discussion

This study demonstrates that oral cyclosporin A inhibits peripheral blood T cell P-glycoprotein activity in HIV-infected adults, and quantifies the magnitude of this inhibition. An inhibitory effect was demonstrable on total CD4 T cells, less so on naïve CD4 T cells, and not on CD8 T cells (Figure 2). This difference among cell types may reflect the lower baseline P-glycoprotein activity on total CD4 T cells [41], which potentially allows detection of inhibition by cyclosporin A even at relatively low drug concentrations. This would be consistent with the lack of observed correlation between cyclosporin A concentrations and P-glycoprotein inhibition in total CD4 T cells (Figure 3A). In contrast, a correlation was seen in naïve CD4 and CD8 T cell subsets (Figure 3B–D), suggesting that the greater absolute P-glycoprotein activity in these cell populations may have made a concentration-dependent inhibition more

demonstrable (i.e. only the highest cyclosporin A concentrations provided an inhibitory effect). It was notable that in the total CD4 T cell population (Figure 3A), all subjects had a >5% decrease in P-glycoprotein activity, regardless of trough cyclosporin A concentration, without an apparent dose response. In contrast, in the naïve CD8 T cell subsets (with greatest baseline P-glycoprotein activity) >5% decreases in P-glycoprotein activity were only seen in two subjects with the highest cyclosporin A trough concentrations (Figure 3D). We did not perform cyclosporin A dose-response experiments using our assay; previous studies have demonstrated full inhibition of P-glycoprotein function in lymphocytes when exposed to much higher concentrations of cyclosporin A than the trough levels observed in these study subjects [43].

Importantly, during the first 14 days the ART regimen did not include protease inhibitors or NNRTIs, medications reported to inhibit and/or induce P-glycoprotein activity, and which could therefore confound analyses focused on cyclosporin A. After day 14, all A5138 participants received efavirenz, which can induce P-glycoprotein expression under some conditions. Exposing a colon adenocarcinoma cell line to high concentrations of efavirenz for three days caused a two-fold increase in P-glycoprotein expression but somewhat decreased P-glycoprotein activity [18]. In contrast, administering oral efavirenz to rats for six days did not increase intestinal P-glycoprotein activity [45]. In the present study, 14 days of efavirenz (days 14 to 28) did not markedly alter peripheral blood T cell P-glycoprotein activity in the ART alone arm. We cannot exclude modest induction of P-glycoprotein by efavirenz following discontinuation of cyclosporine A. Recent data in cell culture [46,47] and murine [48] models have suggested that abacavir may also serve as a P-glycoprotein substrate. Zidovudine and lamivudine did not affect P-glycoprotein [47]. Although we cannot exclude an inhibitory effect of abacavir in this study, all subjects received the same dose of abacavir for the same length of time, and only as-treated data were included in analyses, so any differences between groups would be most likely due to cyclosporin A exposure.

Our study has several limitations, and these results should be considered preliminary. Since specimen collection for this study did not begin until after A5138 began enrolling, not all A5138 participants were eligible for inclusion in this analysis. Demographics, CD4 and HIV-1 RNA levels at baseline did not differ between A5138 subjects included and not included in this analysis (data not shown). This study required overnight shipment of specimens to the P-glycoprotein assay laboratory. Specimens assayed within 24 hours at ambient temperature have not shown substantial changes in efflux activity compared to fresh specimens (authors' unpublished data), but we cannot exclude additional effects resulting from overnight shipping. Certainly, the *in vivo* pharmacodynamics of these relationships are complicated, and cannot be fully characterized by P-glycoprotein activity and cyclosporin A concentrations alone. Factors we did not assess in this study such as other cellular drug transporters and hepatic metabolism likely influence these relationships. Although PI- and NNRTI-sparing regimens are not currently recommended ART regimens in clinical practice, this study design provided a unique opportunity to investigate the effects of short-term cyclosporin A in this context, and the insights provided are relevant. Nonetheless, our data cannot be used to determine effects of cyclosporin A or other P-glycoprotein inhibitors used for longer periods of time or with ART regimens containing ritonavir or other PI. In addition, the absolute changes in P-glycoprotein efflux activity are small, and the clinical relevance of such changes is unknown. Finally, because of the small sample size in this study, there was not adequate statistical power to detect modest or moderate differences between all groups.

Optimizing pharmacokinetic profiles of long-term ART remains a priority. Adjunctive pharmacologic manipulation is well established in HIV therapeutics, primarily involving targeted inhibition of CYP3A isoforms to enhance plasma exposure for protease inhibitors [49]. While cyclosporin A may not be well suited to this purpose because of its immunosuppressive properties, studies should continue to evaluate targeted inhibition of P-

glycoprotein and other drug transporters, and associated pharmacokinetic and clinical consequences.

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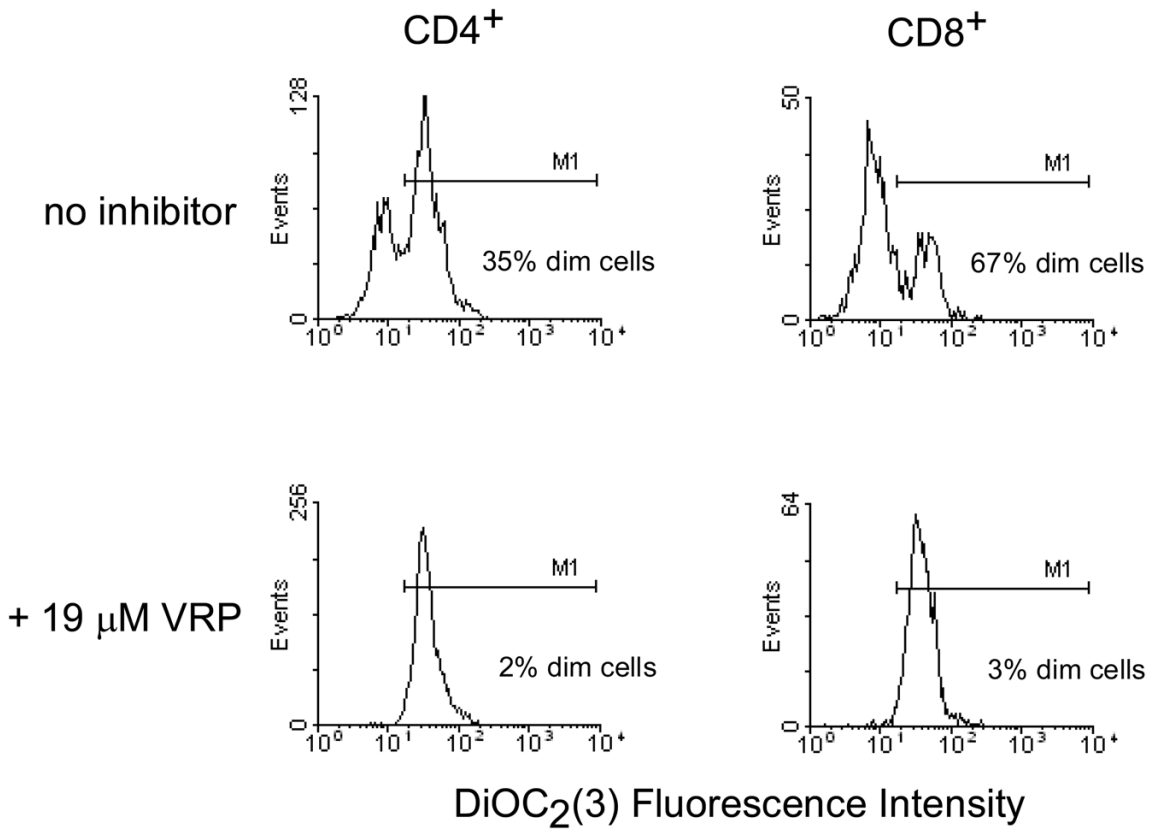

Figure 1.
Flow cytometry histograms illustrating 3,3′-diethyloxacarbocyanine iodide [$DiOC_2(3)$] efflux by total $CD4^+$ and $CD8^+$ T cells and near complete inhibition of dye efflux by verapamil (VRP). M1 delineates the cells within the population that are dye efflux negative (bright).

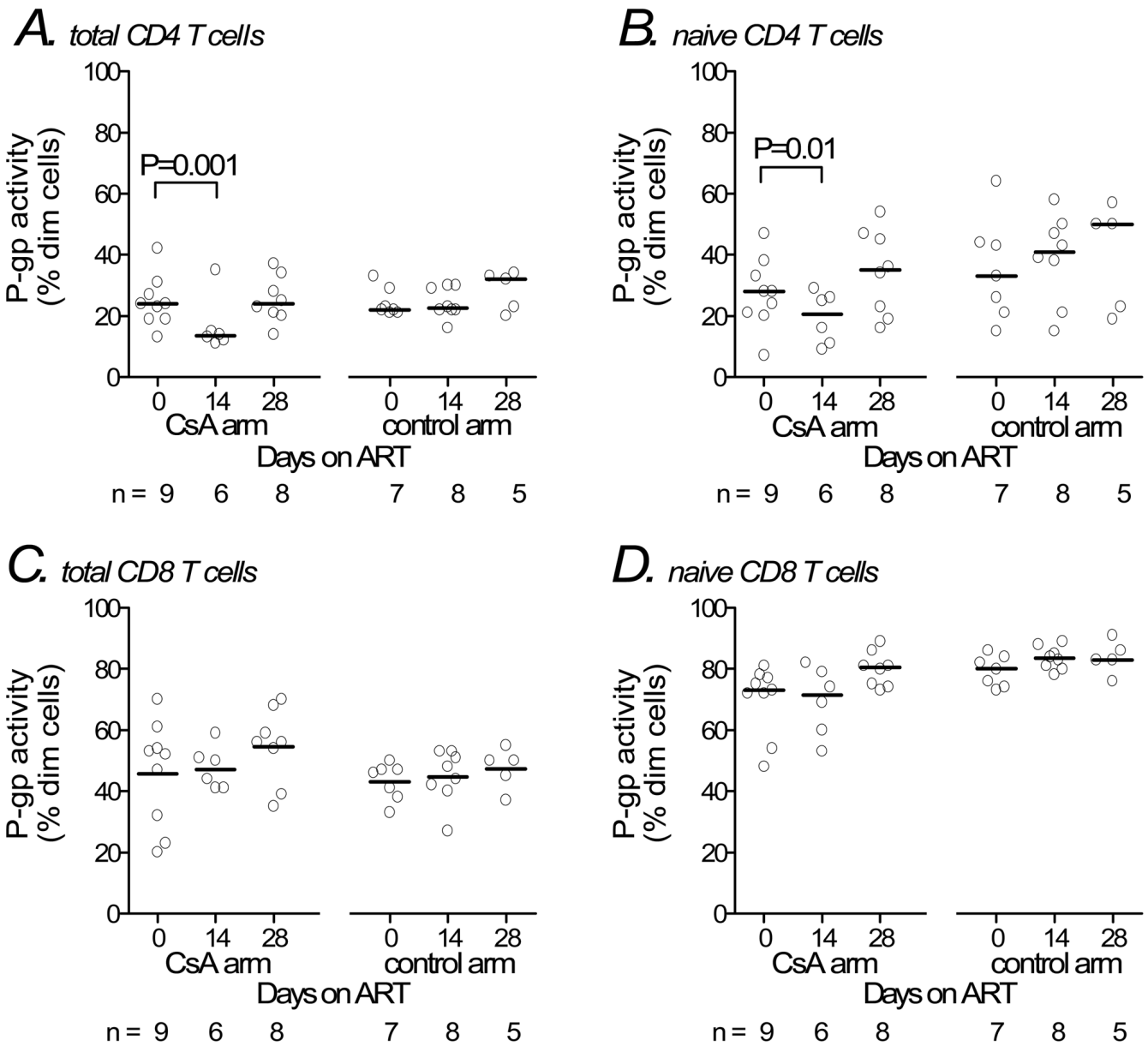

Figure 2.

P-glycoprotein activity in T cell subsets among study participants. Samples were analyzed at baseline (receiving neither ART nor cyclosporin A), study day 14 (all receiving zidovudine/lamivudine/abacavir, with or without cyclosporin A), and study day 28 (all receiving zidovudine/lamivudine/abacavir/efavirenz without cyclosporin A). Markers represent individual study participants. Horizontal lines represent median values. Sample sizes for groups at each time-point are shown at the bottom of each panel. Panels represent total CD4 T cells (A), naïve CD4 T cells (B), total CD8 T cells (C), and naïve CD8 T cells (D). P-values shown are for the Hodges-Lehmann shift parameter and correspond to the change in distribution of P-glycoprotein activities within the cyclosporin A arm during the first 14 days of therapy. ART = antiretroviral therapy; CsA = cyclosporin A; P-gp = P-glycoprotein.

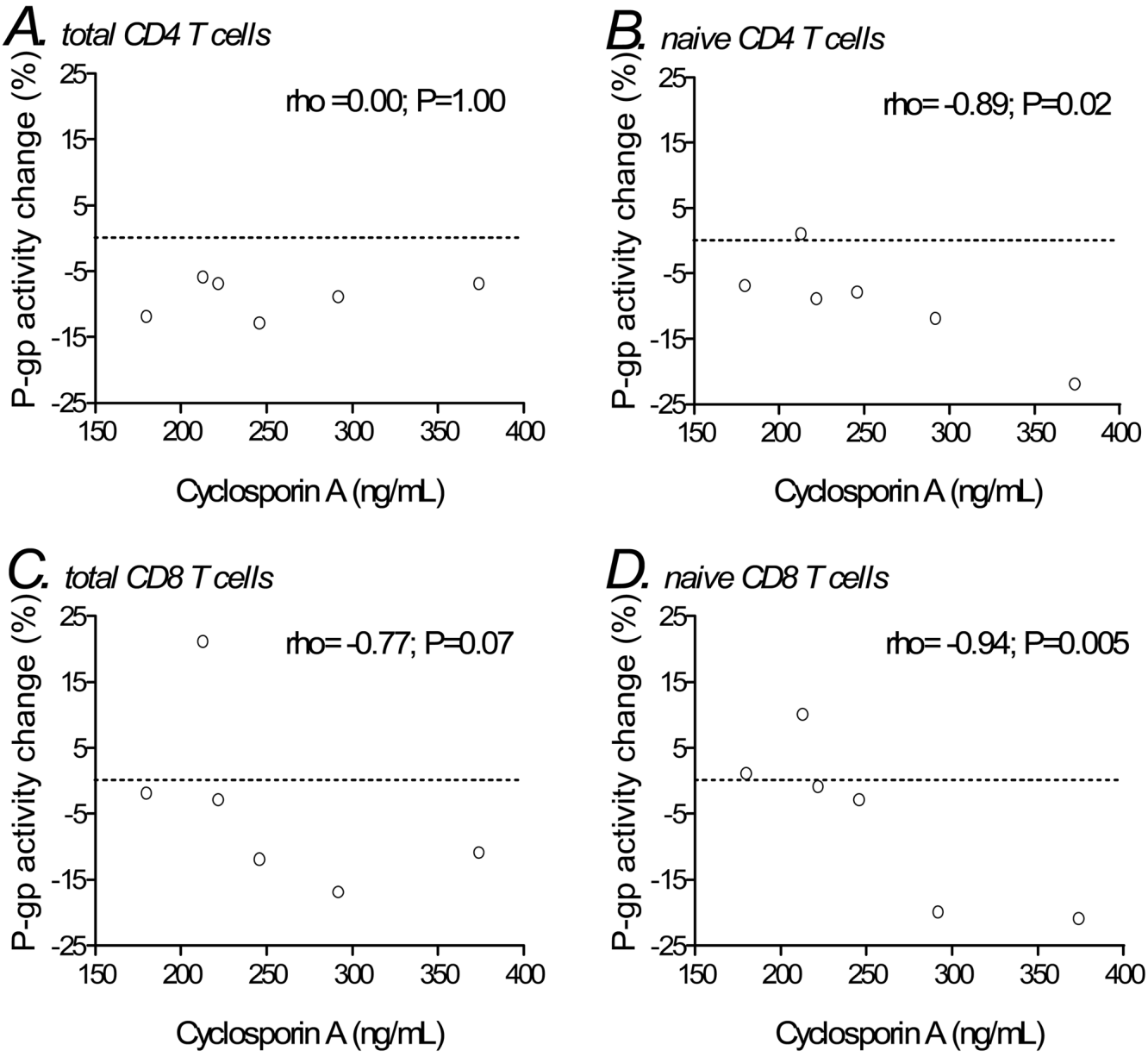

Figure 3.

Associations between P-glycoprotein activity (change from baseline to week 14) and plasma trough cyclosporin A concentrations at day 14 in T cell subsets among individuals in the cyclosporin A arm. Panels represent total CD4 T cells (A), naïve CD4 T cells (B), total CD8 T cells (C), and naïve CD8 T cells (D). Correlation coefficients (rho) and P-values shown are for Spearman correlations. The horizontal dotted lines represent no change in P-glycoprotein activity from baseline.

ART = antiretroviral therapy; CsA = cyclosporin A; P-gp = P-glycoprotein.

Table

Baseline demographics and HIV disease parameters in total group and by treatment arm

	Total (N = 16)	ART alone (n = 7)	ART + cyclosporin A (n = 9)
Age, median (range)	40 (21–55)	32 (21–49)	43 (23–55)
Race/ethnicity, n (%)			
Black non-Hispanic	4 (25)	1 (14)	3 (33)
White non-Hispanic	11 (69)	5 (71)	6 (67)
Hispanic	1 (6)	1 (14)	0 (0)
Male sex, n (%)	15 (94)	7 (100)	8 (89)
CD4 T cells/mm^3, median (interquartile range)	326 (261–397)	276 (263–404)	340 (229–392)
HIV-1 RNA (log_{10} copies/mL plasma), median (interquartile range)	4.9 (4.3–5.5)	5.1 (4.3–5.5)	4.9 (4.4–5.0)

ART = antiretroviral therapy

P>0.05 for all between-arm comparisons, by Fisher's exact or Kruskal-Wallis tests.