The histone deacetylase inhibitor panobinostat lowers biomarkers of cardiovascular risk and inflammation in HIV patients

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Objective: To investigate the effect of the histone deacetylase inhibitor panobinostat on HIV-associated inflammation.

Design: Sub-study of a single-arm, phase I/II clinical trial.

Methods: HIV-infected adults on suppressive antiretroviral therapy received oral panobinostat 20 mg three times per week, every other week, for 8 weeks, that is, four cycles of treatment. Plasma levels of high-sensitivity C-reactive protein, matrix metalloproteinase 9, soluble CD40 ligand and interleukin-6 were determined using human ELISA kits. Soluble endothelia selectin (E-selectin) was measured by a multiplex immunoassay. Total monocyte count, phenotype changes on monocytes and monocyte histone acetylation were analyzed using flow cytometry. Whole-genome expression in peripheral blood mononuclear cells was analyzed at baseline and on-panobinostat employing the Affymetrix Human Transcriptome Array 2.0 microarray assay. Changes from baseline were analyzed using Wilcoxon signed-rank test. For the gene-expression analyses, fold-changes, *P* values and false detection rate were computed using TAC software.

Results: Panobinostat treatment led to significant reductions in multiple established plasma markers of inflammation. Notably, high-sensitivity C-reactive protein decreased by a median of 58% during treatment and this change persisted for 4 weeks after treatment. Plasma levels of interleukin-6, matrix metalloproteinase 9, E-selectin and soluble CD40 ligand also significantly decreased on and/or postpanobinostat. Additionally, we observed a significant reduction in the proportions of intermediate monocytes and tissue factor-positive monocytes. This suppression of cardiovascular risk biomarkers was associated with a prominent reduction in the expression of genes related to inflammation and atherosclerosis.

Conclusion: Collectively, these data indicate that panobinostat may have therapeutic potential to target excess inflammation in HIV patients with high cardiovascular risk.

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Introduction

Although orally active histone deacetylase inhibitors (HDACis) are used for the treatment of hematological malignancies, HDACis may have therapeutic potential in several noncancer diseases [1]. HDACis reduce the production of cytokines *in vitro* and *in vivo* and exert anti-inflammatory properties at nanomolar concentrations, which is considerably less than the concentrations targeting neoplastic cells [1,2]. Excess inflammation plays a key role in the pathogenesis of cardiovascular disease (CVD) in the general population, and among persons with HIV in particular. Thus, even in virologically suppressed HIV patients, there is increased risk of non-AIDS morbidity, particularly CVD [3]. This is likely due to HIV-associated chronic inflammation, as evidenced by elevated levels of biomarkers of inflammation and coagulation [4]. Indeed, these biomarkers are predictive of the risk of cardiovascular events and all-cause mortality in persons with HIV [5]. Therefore, reducing HIV-associated chronic inflammation and cardiovascular risk is a therapeutic target.

Panobinostat is an orally active pan-HDACi undergoing development for the treatment of multiple myeloma. We recently conducted a clinical trial to investigate the ability of panobinostat to activate HIV from latency and impact the latent HIV reservoir [6]. Here, we show that 8 weeks of low-dose panobinostat treatment resulted in a significant suppression of established markers of inflammation and concomitantly down-regulated the expression of genes related to inflammation and atherosclerosis.

Methods

Between September 2012 and February 2014, we conducted an investigator-initiated, single-arm, phase I/II clinical trial as previously described [6]. Briefly, 15 aviremic HIV-infected adults with $CD4^+$ T-cell counts above 500/μl received oral panobinostat 20 mg three times per week, every other week, for 8 weeks, that is, four cycles of treatment, while maintaining combination antiretroviral therapy (cART) (Fig. 1a). Ethics committee approval and informed consent were obtained in accordance with the principles of the Declaration of Helsinki. This trial was registered with ClinicalTrial.gov, number NCT01680094.

Cryopreserved peripheral blood mononuclear cells (PBMCs) and plasma-isolated prepanobinostat (baseline), twice on-panobinostat (in the first and third treatment cycle, i.e. early and late) and 4 weeks postpanobinostat were subjected to the below described analyses.

Plasma levels of high-sensitivity C-reactive protein (hsCRP; Invitrogen, Waltham, Massachusetts, USA; catalog KHA0031), matrix metalloproteinase 9 (MMP-9; Invitrogen, KHC3061), soluble CD40 ligand (sCD40L; Invitrogen, KHS4001), D-dimer (American Diagnostica, 602) and interleukin (IL)-6 (Quansys, cytokine 8 plex array; Logan, Utah, USA) were determined using human ELISA kits. Soluble endothelia selectin (E-selectin) was measured by a multiplex immunoassay (R&D Systems, Minneapolis, Minnesota, USA; LKT007). Assays were performed as described by the manufacturer and were analyzed in a single batch.

Total monocyte count was determined using fluorescence flow cytometry (Sysmex, Kobe, Japan). Phenotype changes on monocytes were analyzed by flow cytometry on a BD FACSVerse flow cytometer. For each sample, two million PBMCs were stained with Live/Dead Fixable Near-IR (Invitrogen) and fluorophore-labeled antibodies to CD3-BV421 (clone SK7), CD20-BV421 (2H7), CD16-PerCP-Cy5.5 (3G8) (all BD Biosciences, Franklin Lakes, New Jersey, USA). CD56-BV421 (HCD56), CD14-PE (M5E2), CD11b-PE-Cy7 (M1/70) (all Biolegend, San Diego, California, USA) and tissue factor-FITC (VIC-7) (BioNordica). All flow samples had above 96% viability [median 98.2%, interquartile range (IQR) 97.7–98.6] and only live cells and singlets were included in the analyses.

Monocytes were identified by size, granularity, and by lack of CD3, CD20 and CD56 expression. Median fluorescence intensity (MFI) of CD14 and CD16 expression and isotype controls were used to divide monocytes into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and nonclassical ($CD14^{+}CD16^{++}$) monocytes (Fig. 2b and Supplementary figure S1, http://links.lww.com/QAD/A678) [7,8].

As a cellular measure of the pharmacodynamic response to panobinostat, levels of monocyte histone acetylation were determined using histone H3 intracellular staining on a BD FACSCanto flow cytometer. Flow cytometry for histone acetylation levels was based on the method of Rigby *et al.* [9] and performed as described previously [6]. Flow cytometry data were analyzed in Flow Jo (v10.0.7; FlowJo LLC, Ashland, Oregon, USA).

Whole-genome expression in PBMCs was analyzed at baseline and on-panobinostat (early) employing the Affymetrix Human Transcriptome Array 2.0 cartridge microarray assay [10].

Changes from baseline were analyzed using Wilcoxon signed-rank test in GraphPad Prism (version 6; GraphPad Software, San Diego, California, USA). For the gene-expression analyses, fold-changes, *P* values and false detection rate (FDR) were computed using TAC software.

Results

Fifteen patients were enrolled in the clinical study, all of whom completed full panobinostat dosing and follow-up.

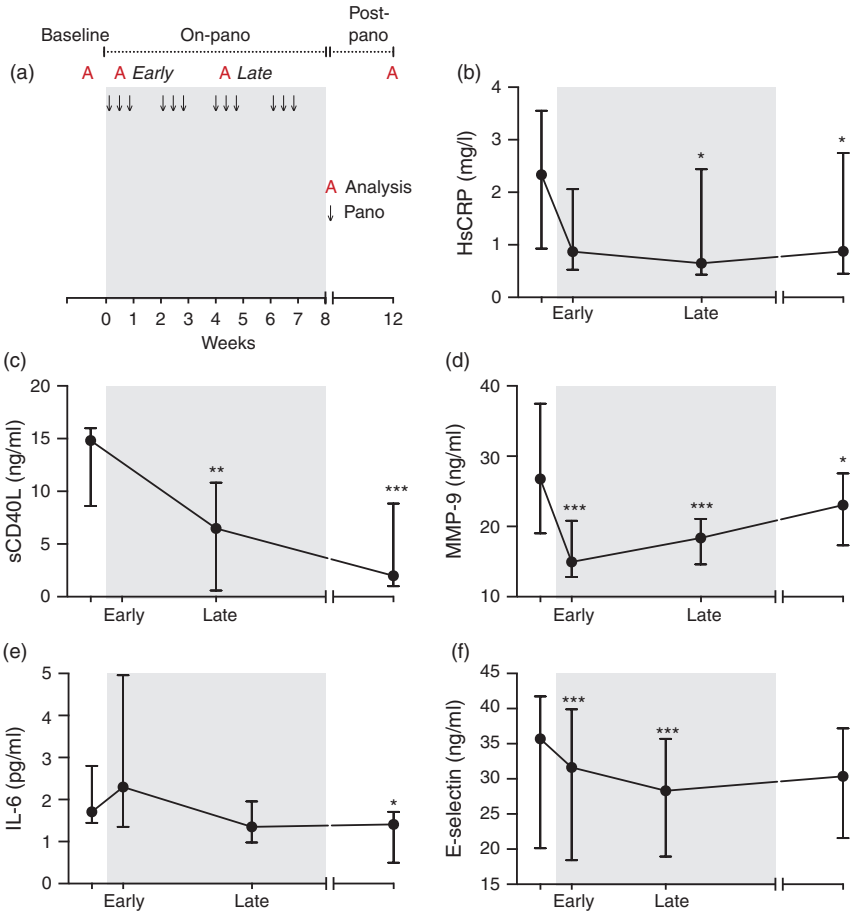
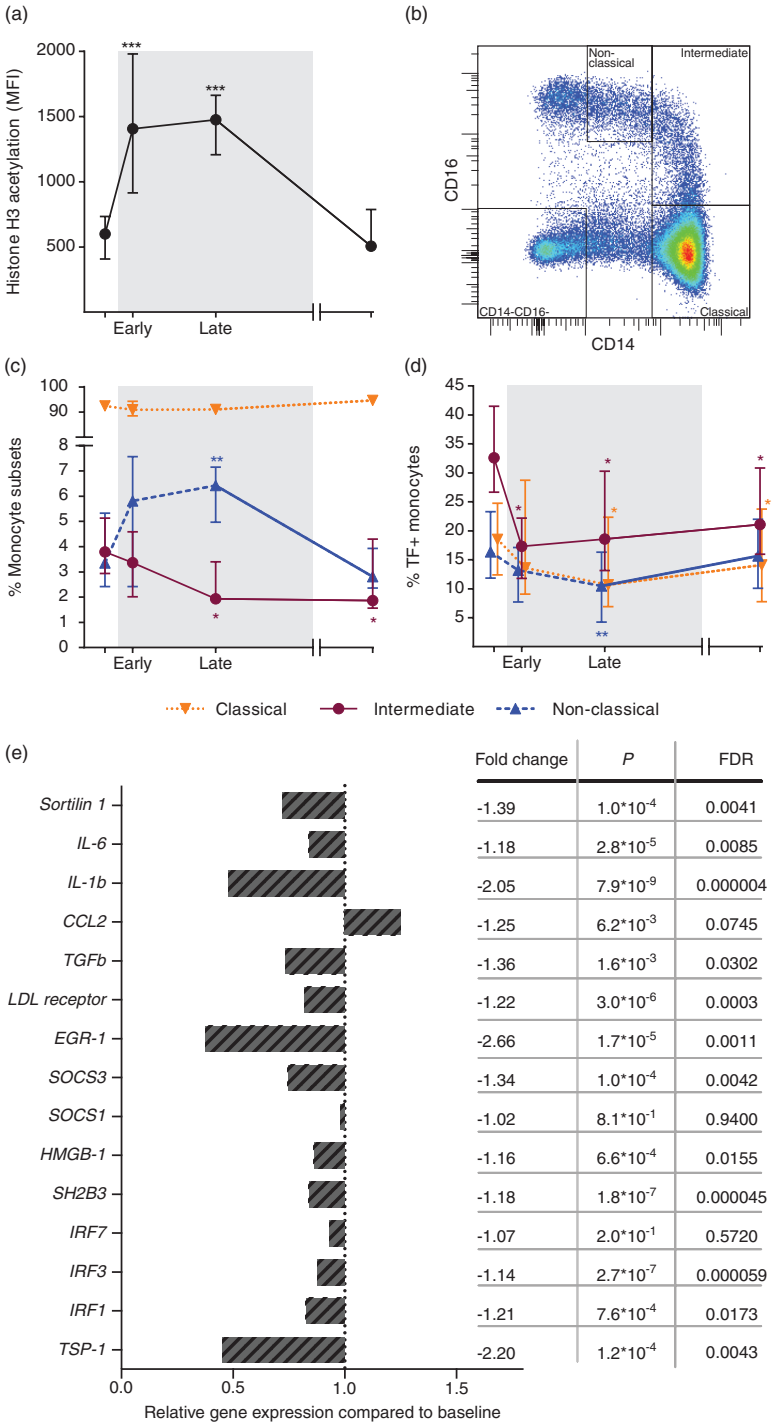


Fig. 1. Plasma levels of soluble biomarkers decrease during panobinostat treatment. The gray-scaled box represents panobinostat treatment period. (a) Study design. The black arrows (↓) indicate the dosing of 20 mg panobinostat three times/week every other week for 8 weeks while maintained on ART. Analyses were done at baseline, on-panobinostat during the first (early) and third (late) treatment cycle, and four weeks postpanobinostat. (b–f) Plasma levels of five soluble biomarkers are shown for all 15 patients. Baseline is a mean of two measurements separated by 4 weeks. Data are shown as median and error bars as interquartile range. ART, antiretroviral therapy; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; MMP-9, matrix metalloproteinase 9; Pano, panobinostat; sCD40L, soluble CD40 ligand. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

We recorded statistically significant changes from baseline in multiple biomarkers of cardiovascular risk. The median concentration of hsCRP decreased on-panobinostat compared to baseline (2.3 mg/l, IQR 0.9–3.6 versus 0.65 mg/l, IQR 0.4–2.4; $P = 0.01$), corresponding to a 58% (IQR −86.2 to 10.5) decrease. This suppression persisted up to 4 weeks postpanobinostat (0.88 mg/l, IQR 0.45–2.8; $P = 0.01$) (Fig. 1b). Similar significant decreases on-panobinostat and postpanobinostat were recorded for sCD40L ($P = 0.003$, $P < 0.0001$) and MMP-9 ($P < 0.0001$, $P = 0.018$) (Fig. 1c, d and Supplementary Table S1, http://links.lww.com/QAD/A678). Moreover, IL-6 levels were significantly lower at the follow-up 4 weeks postpanobinostat than at baseline ($P = 0.011$; Fig. 1e). In contrast, E-selectin levels were significantly suppressed only on-panobinostat ($P < 0.0003$) (Fig. 1f). For D-dimer, there was a tendency towards a decline at the postpanobinostat follow-up, but this change remained nonsignificant ($P = 0.06$; Supplementary Table S1, http://links.lww.com/QAD/A678). A total overview of all assayed biomarkers is provided in the supplemental data (Supplementary Table S1, http://links.lww.com/QAD/A678).

In addition to soluble mediators of inflammation, much attention has been given to monocytes due to their role in atherosclerosis [11]. Therefore, we explored specific characteristics of monocytes related to cardiovascular risk. First, we assayed monocyte histone H3 acetylation to confirm panobinostat's epigenetic effect on monocytes and observed highly significant increases similar to that described for lymphocytes (Fig. 2a) [6]. Second, we detected a significant decrease in the total number of circulating monocytes at the early ($P = 0.0003$) and late treatment ($P = 0.048$) time points as compared to baseline (data not shown). Third, the proportion of intermediate monocytes decreased significantly on-panobinostat ($P = 0.01$) and postpanobinostat ($P = 0.04$) as compared to baseline levels, with a concurrent increase in the proportion of nonclassical monocytes on-panobinostat



Fold change	*P*	FDR
-1.39	$1.0*10^{-4}$	0.0041
-1.18	$2.8*10^{-5}$	0.0085
-2.05	$7.9*10^{-9}$	0.000004
-1.25	$6.2*10^{-3}$	0.0745
-1.36	$1.6*10^{-3}$	0.0302
-1.22	$3.0*10^{-6}$	0.0003
-2.66	$1.7*10^{-5}$	0.0011
-1.34	$1.0*10^{-4}$	0.0042
-1.02	$8.1*10^{-1}$	0.9400
-1.16	$6.6*10^{-4}$	0.0155
-1.18	$1.8*10^{-7}$	0.000045
-1.07	$2.0*10^{-1}$	0.5720
-1.14	$2.7*10^{-7}$	0.000059
-1.21	$7.6*10^{-4}$	0.0173
-2.20	$1.2*10^{-4}$	0.0043

Fig. 2. Monocyte phenotype changes and gene expression in peripheral blood mononuclear cells. (a) Levels of histone H3 acetylation in monocytes as determined by flow cytometry and expressed by mean fluorescence intensity indicating the monocyte-specific pharmacodynamic effect of panobinostat ($n = 15$). (b) Representative flow cytometry dot plot and gating strategy for defining classical, intermediate and nonclassical monocyte subsets. (c, d) Proportion of each of the defined monocyte subsets and the proportion of tissue factor-positive cells within the individual monocyte subsets ($n = 10$). The gray-scaled box represents the panobinostat treatment period. Data are shown as median and corresponding interquartile range. (e) Summarized changes in the expression of selected genes in PBMCs 4 days after initiating panobinostat (on-panobinostat early) compared to baseline. ADAMTS7, adam metallopeptidase with thrombospondin type motif 1; CCL2, monocyte chemoattractant protein-1; EGR, early growth response; FDR, false detection rate; HMGB-1, high-mobility protein group B; IL-1b, interleukin-1 beta; IL-6, interleukin-6; IRF, interferon regulatory factor; LDL, low-density lipoprotein; SOCS, suppressors of cytokine signaling; TF, tissue factor; TGFb, transforming growth factor beta; TSP1, thrombospondin. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

($P = 0.008$; Fig. 2c). Fourth, compared to baseline, the proportion of monocytes expressing tissue factor decreased significantly on-panobinostat in all three subsets ($P < 0.03$) and postpanobinostat for classical and intermediate monocytes ($P = 0.02$, $P = 0.049$) (Fig. 2d). This finding was supported by a decrease in MFI among tissue factor-positive monocytes ($P = 0.02$, median −3.2%, IQR −6.5 to −0.8).

In addition to these effects on protein level, panobinostat treatment was associated with significant changes in the expression of several inflammatory genes. Thus, the expression of the key pro-inflammatory genes [IL-6, IL-1b, early growth response (EGR)-1, high-mobility protein group B (HMGB-1)] was significantly down-regulated, as was the expression of genes related to coronary artery disease such as sortilin, low-density lipoprotein receptor, thrombospondin and SH2B adaptor protein-3 (Fig. 2e) [12].

Discussion

In this study, we explored the effect of the highly potent HDACi, panobinostat, on biomarkers of cardiovascular risk. Eight weeks of panobinostat treatment in HIV-positive patients on suppressive ART was associated with a significant reduction in inflammatory biomarkers. Moreover, we observed a concurrent decrease in the expression of genes related to inflammatory pathways and cardiovascular risk. Collectively, these data indicate that panobinostat may have potential to target excess inflammation in HIV-positive patients with high cardiovascular risk.

Soluble biomarkers are frequently used to assess cardiovascular risk and, therefore, their predictive value in this regard is well characterized [13]. Specifically, elevated biomarkers such as CRP, IL-6 and D-dimer are strongly associated with cardiovascular risk and all-cause mortality in persons with HIV [5,14,15]. Therefore, the prominent and simultaneous decrease in these indicators of cardiovascular risk during and after panobinostat treatment is of considerable interest. Notably, panobinostat suppressed hsCRP levels below 1 mg/l, which is the recommended cut-off for distinguishing low versus average cardiovascular risk [13].

Several studies have emphasized that innate immune activation contributes to morbidity and mortality in HIV-infected individuals, with particular attention given to monocytes due to their role in atherosclerosis [14,16–19]. Therefore, we explored the specific characteristics of monocytes related to cardiovascular risk and found not only a significant decline in the total number of circulating monocytes but also a reduction in the proportion of intermediate monocytes. Of note, others have shown that HIV-positive patients with CVD have higher numbers of circulating $CD14^+$ monocytes compared to HIV-positive patients without CVD [15]. Moreover, intermediate monocytes are found at an increased frequency in inflammatory disease [7] and predict cardiovascular events and progression of coronary atherosclerosis [11,16]. Thus, decreasing the proportion of intermediate monocytes could be of clinical benefit. The expression of tissue factor is increased on monocytes in HIV-infected individuals [20]. Tissue factor is an initiator of the extrinsic coagulation pathway, and promotes thrombus formation [21]. We observed a significant decrease in the proportion of tissue factor-positive monocytes on-panobinostat and, importantly, this was evident for each of the three individual subsets. The expression of tissue factor on monocytes is increased by inflammatory stimuli like CRP, CD40L, low-density lipoprotein (LDL) and LPS [21]; thus tissue factor may link inflammation and coagulation. However, although the suppressive effect of panobinostat on tissue factor expression across all three subsets is potentially important, it is unknown whether such an effect is associated with any clinical benefit.

In addition to these effects on protein level, panobinostat treatment was associated with significant changes in the expression of several inflammatory genes, which have previously been shown to play a role for atherosclerosis and CVD risk [12,22,23]. The epigenetic effects of HDACis are exerted through inhibition of HDACs, which promotes histone acetylation and induces changes in the chromatin organization [24]. Thus, by describing the chain of events from panobinostat dosing to histone modification, gene regulation and, finally, protein expression, we have attempted to closely map the effects of panobinostat on biological processes related to cardiovascular risk. Still, there may be post-transcriptional or post-translational modifications, which are not captured by our analyses.

Some additional limitations of our study should be noted. In the absence of a control group, we were unable to control for longitudinal variation in inflammatory markers. However, given the rapid and concurrent decreases in several biomarkers, the observed responses to panobinostat were unlikely due to natural variations. Still, our results need confirmation in larger placebo-controlled trials, which may also include investigations of the medium and long-term anti-inflammatory effects of panobinostat. Also, we evaluated risk markers and not disease outcome, and, thus, our data do not inform of the clinical benefit. Finally, as reported elsewhere [6], it should be noted that panobinostat treatment induced plasma viremia, which may in itself increase inflammation. Such an effect could have caused us to underestimate the anti-inflammatory effect of panobinostat.

Finding ways to impact chronic inflammation and decrease the risk of cardiovascular events are important goals in many diseases characterized by pathological inflammation, such as auto-inflammatory disorders, autoimmune diseases, atherosclerosis and HIV infection.

Similar to other studies, our data have considerable implications for the use of HDACis to target excess inflammation. However, the optimal and best tolerated dose of a particular HDACi remains unclear. For example, the dose of vorinostat in graft-versus-host disease was reduced by 50% and was nevertheless effective [2]. Future studies may provide additional information on implicated pathways and alternative dosage strategies to optimize anti-inflammatory effect relative to clinical tolerability.

In conclusion, treatment with panobinostat significantly lowered soluble and cell-based measures known to be associated with all-cause mortality and cardiovascular events. These changes were consistent with a prominent reduction in steady-state mRNA levels of genes related to inflammation and cardiovascular risk. Collectively, this suggests a potential role for panobinostat and other HDACis in HIV patients with high cardiovascular risk.

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Contribution: O.S.S., M.T., T.A.R., C.A.D., L.Ø. and A.S.K. conceived and designed the study; C.A.D. and A.S.K. measured soluble markers; C.R.B. and A.S.K. designed and performed the monocyte flow experiments with input from M.T. and O.S.S.; R.O. designed and performed the histone acetylation flow experiment; M.T. performed the gene expression analysis with input from A.S.K. All authors participated in data analysis and interpretation. A.S.K. drafted the manuscript with input from T.A.R., M.T. and O.S.S.. All authors provided input to the manuscript and approved the final version.

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Conflicts of interest

Aarhus University has filed a patent application covering the use of panobinostat in HIV-infected patients on which T.A.R., M.T., C.R.B., L.Ø. and O.S.S. are inventors. For the remaining authors none were declared.

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