

Short communication

Abacavir, didanosine and tenofovir do not induce inflammatory, apoptotic or oxidative stress genes in coronary endothelial cells

Chul Kim¹, Samir K Gupta², Linden Green¹, Brian M Taylor¹, Maja Deuter-Reinhard¹, Zeruesenay Desta³, Matthias Clauss^{1*}

¹Department of Cellular & Integrative Physiology, Indiana Center for Vascular Biology & Medicine, Indiana University School of Medicine, Indianapolis, IN, USA

²Division of Infectious Diseases, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN, USA

³Division of Clinical Pharmacology, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN, USA

*Corresponding author e-mail: mclauss@iupui.edu

Background: The use of abacavir and didanosine in HAART has been associated with an increased risk of myocardial infarction in HIV-infected patients. The aim of this study was to address the development of endothelial dysfunction in cultivated coronary artery endothelial cells (HCAECs) in response to abacavir, didanosine and tenofovir. We examined the impact of these drugs on the expression levels of the proinflammatory, oxidative stress and apoptosis regulating genes in HCAECs.

Methods: We tested gene and protein expression changes in HCAECs in response to abacavir, didanosine and tenofovir using quantitative real-time reverse transcriptase PCR, FACS and ELISA. The assessed genes/proteins included the proinflammatory molecules VCAM-1, ICAM-1, MCP-1, RANTES and IL-6. In addition, we assessed the gene expression of the intracellular reactive

oxygen producing NADPH oxidase subunit gp91^{PHOX} and the apoptosis regulating molecules Bcl-2 and BAD.

Results: Exposure of HCAECs to abacavir, didanosine and tenofovir resulted in no statistically significant changes in any of the tested genes/proteins at any time point or at any concentration.

Conclusions: We found no evidence that abacavir, didanosine or tenofovir had direct *in vitro* effects on coronary endothelial cell gene transcription and protein expression of the selected mediators. If abacavir or didanosine increase cardiovascular risk, it is likely not through the direct endothelial activation pathways tested in these experiments. However, further studies are needed to completely exclude the toxicity of abacavir or didanosine on endothelial cells.

Introduction

HIV-infected individuals are at higher risk for cardiovascular events compared with the general population [1,2]. Part of this risk is likely due to the drugs used in HAART to treat HIV infection. Although protease inhibitors have been associated with metabolic dysregulation and consequently became candidates for increased risk for vascular events [3], there has been increasing attention given to the nucleoside reverse transcriptase inhibitor (NRTI) class of antiretrovirals. Specifically, the ‘Data Collection on Adverse Events of Anti-HIV Drugs’ (D:A:D) cohort study has suggested that the risk of myocardial infarction is higher with the use of abacavir and didanosine compared with other drugs in this class [4,5].

Results from the SMART trial group [6] and a separate, large, prospective, nationwide cohort study in Denmark [7] have also suggested an association between abacavir and cardiovascular disease. In addition, use of abacavir was associated with impaired *in vivo* endothelial function [8]. Much of the uncertainty surrounding the possible contribution of abacavir and didanosine on cardiovascular disease in HIV-infected patients is related to the lack of evidence for a biological mechanism that would support causality for the purported association. Because it is not possible to obtain *ex vivo* vascular samples from patients receiving the drugs in question, experimental *in vitro* models are required. Therefore, we examined the effects of

Table 1. List of primers employed for gene expression studies using reverse transcriptase PCR

Gene	Forward	Reverse
ICAM-1	CGCTGAGCTCCTCTGCTACT	GATGACTTTTGAGGGGGGACA
VCAM-1	TGTTGAGATCTCCCCTGGAC	GAATTGGTCCCCTCACTCCT
MCP-1	CCCCAGTCACCTGCTGTAT	TGGAATCCTGAACCCACTTC
RANTES	CTGCTTGCTCTTGCTCCTAGC	TGTAACCTGCTGCTGTGGT
IL-6	AAAGAGGCACTGGCAGAAAA	TTTTCACCAAGGCAAGTCTCC
gp91phox	GAAAACCTCTTGGGTACAGCA	AGCAGATTTCGACAGACTGG
BAD	GATCCGTGCTGTCTCCTTT	AAAACCCAAAACCTCCGATG
Bcl-2	AAGCTGCAGGCTGTTAAGA	CGTTTTGCCTGAAGACTGTT
TNF- α	TCTCTTCTGATCGTGGA	CCCAGATAGATGGGCTCATACC
eF1 α	TTGGAAGGGGAAGGAATATC	TTAGTGACAGGCAATCAGCA

BAD, Bcl-xL/Bcl-2-associated death promoter; Bcl-2, B-cell leukaemia/lymphoma 2; eF1 α , elongation factor- α ; gp91phox, glycosylated protein 91 phox; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated on activation normal T-cell expressed and secreted; TNF- α , tumor necrosis factor- α ; VCAM-1, vascular cell adhesion molecule-1.

abacavir and didanosine on endothelial cell production of multiple genes involved in several cardiovascular disease pathways, including endothelial leukocyte adhesion, inflammation, oxidative stress and apoptosis. We also examined tenofovir in this *in vitro* model in order to provide comparison with a drug from the NRTI class of antiretrovirals that is not implicated in cardiovascular disease.

Methods

Cell and drug preparation

Human coronary artery endothelial cells (HCAECs) from Lonza (Walkersville, MD, USA) were maintained in EMB-2MV medium at 37°C in 5% CO₂ and 95% air. All results in this study were confirmed with HCAECs derived from two independent donors. Culture medium was changed every 2 days. Abacavir and didanosine were obtained from Toronto Research Chemicals, Inc. (ON, Canada); tenofovir was obtained from Gilead Sciences, Inc. (Foster City, CA, USA).

Drug treatment

HCAECs were provided with fresh medium every second day and were split when reaching confluency. The HCAECs were treated at four different time points and at the concentration indicated in the text. Numbers of viable cell were assessed by using trypan blue exclusion staining and a Neubauer counting chamber. The effect of tumor necrosis factor- α (TNF- α ; 20 ng/ml) [9] as a positive control for endothelial activation was tested at 8 h after bolus application.

Quantitative real-time PCR

After the indicated treatments, RNA was purified using the NucleoSpin RNA isolation kit (Clontech Laboratories, Mountain View, CA, USA). For quantitative PCR analysis, the iScript one-step qRT-PCR kit with SYBR

Green (Biorad, Hercules, CA, USA) was used for complementary DNA (cDNA) synthesis and PCR amplification as per the manufacturer's instructions using gene-specific primer pairs (Table 1) in a Chromo4 Opticon analyser instrument (Bio-Rad). The thermal cycling conditions were as follows: 50°C for 10 min (cDNA synthesis) and 95°C for 5 min (reverse transcriptase inactivation), followed by 42 cycles of 95°C for 10 s and 58°C for 30 s. To compare the levels of gene expression between the experimental groups, a second derivative calibration method for relative quantification was employed. The amount of the target gene transcript was normalized to the elongation factor- α (eF1 α) as previously described [9]. All experiments were replicated at least 3 $\times$ and results were analysed using ANOVA with the Bonferroni correction for multiple comparisons. *P*-values <0.05 were considered statistically significant.

Flow-assisted cytometric analysis and ELISA

Vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) were determined by flow-assisted cytometric (FACS) analysis as previously described [9]. Briefly, HCAECs (1 $\times$ 10⁶) were treated at the indicated time points and subsequently stained with phycoerythrin-labelled monoclonal mouse anti-human VCAM-1 and unlabelled ICAM-1 antibodies or isotype matched control antibodies (all antibodies were obtained from ABCAM, Inc., [Cambridge, MA, USA]) for 1 h on ice. For detection of ICAM-1, additional incubation for 1 h with a secondary goat-anti-mouse antibody conjugated to Alexa 488 was performed. After washing with phosphate-buffered saline, the cells were fixed in 1% paraformaldehyde and FACS was performed with a FACS-Calibur flow cytometer (Becton Dickinson Labware, Mountain View, CA, USA).

Secreted interleukin (IL)-6, monocyte chemoattractant protein-1 (MCP-1) and regulated on activation normal

T-cell expressed and secreted (RANTES) were assessed by ELISA kits according to the instructions of the manufacturers (R&D, Minneapolis, MN, USA) using supernatant from HCAECs at the indicated time points and conditions.

Results

Abacavir, didanosine and tenofovir do not increase leukocyte adhesion molecule and inflammatory gene expression

The median and maximal *in vivo* physiological concentrations for abacavir (2 and 4 μ M, respectively), didanosine (1.1 and 7.3 μ M, respectively) and tenofovir (0.65 and 1.3 μ M, respectively) in plasma [10–12] were tested in all experiments assessing gene expression changes after 2, 8 and 24 h of incubation. Because endothelial cell adhesion molecules play a pivotal role in inflammation, we first addressed VCAM-1 and ICAM-1 expression. As shown in Figure 1A, VCAM-1 and ICAM-1 gene expression levels were not induced by any of the three drugs at their median *in vivo* concentrations when incubated for 8 h. Furthermore, treatment for 2 and 24 h or with the maximal *in vivo* concentration did not result in gene expression. By contrast, treatment with TNF- α (20 ng/ml) as a positive control resulted in strong induction of VCAM-1 and ICAM-1 in HCAECs (Figure 1A). These data were confirmed by FACS using monoclonal antibodies against human VCAM-1 and ICAM-1 (Figure 1B).

We then examined the effects of these three drugs on the endothelial gene expression levels of the chemokines MCP-1 and RANTES. Again, none of the drugs induced gene expression of MCP-1 or RANTES at any analysed time point or concentration (Figure 1C shows the values for the median *in vivo* concentrations at 8 h of incubation), whereas treatment with TNF- α showed strong induction. Again, these results were confirmed on protein levels using specific ELISA kits for MCP-1 and RANTES (Figure 1D). In addition, gene expression and secreted protein levels of the proinflammatory cytokine (IL-6) were not changed significantly at the two concentrations and the three time points tested (CK *et al.*, data not shown).

Abacavir, didanosine and tenofovir do not increase apoptosis or oxidative stress gene expression

Because oxidative stress and cell death in endothelial cells are mechanism leading to vascular dysfunction and atherosclerosis, we next tested for level changes in the genes regulating apoptosis and reactive oxygen species (ROS) after incubation with abacavir, didanosine and tenofovir. Again, neither the gene expression of pro-apoptotic Bcl-xL/B-cell leukaemia/lymphoma 2 (Bcl-2)-associated death promoter (BAD) nor the

expression of anti-apoptotic Bcl2 or the ROS-inducing gp91phox subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase were affected by the three administered NRTI drugs at any given time point or concentration (Figure 1E for the 8 h induction and median concentrations, data for 2 and 24 h and maximal concentration are not shown).

Long-term incubation with abacavir does not increase leukocyte adhesion molecule, pro-apoptotic BAD or proinflammatory IL-6 or MCP-1 gene expression

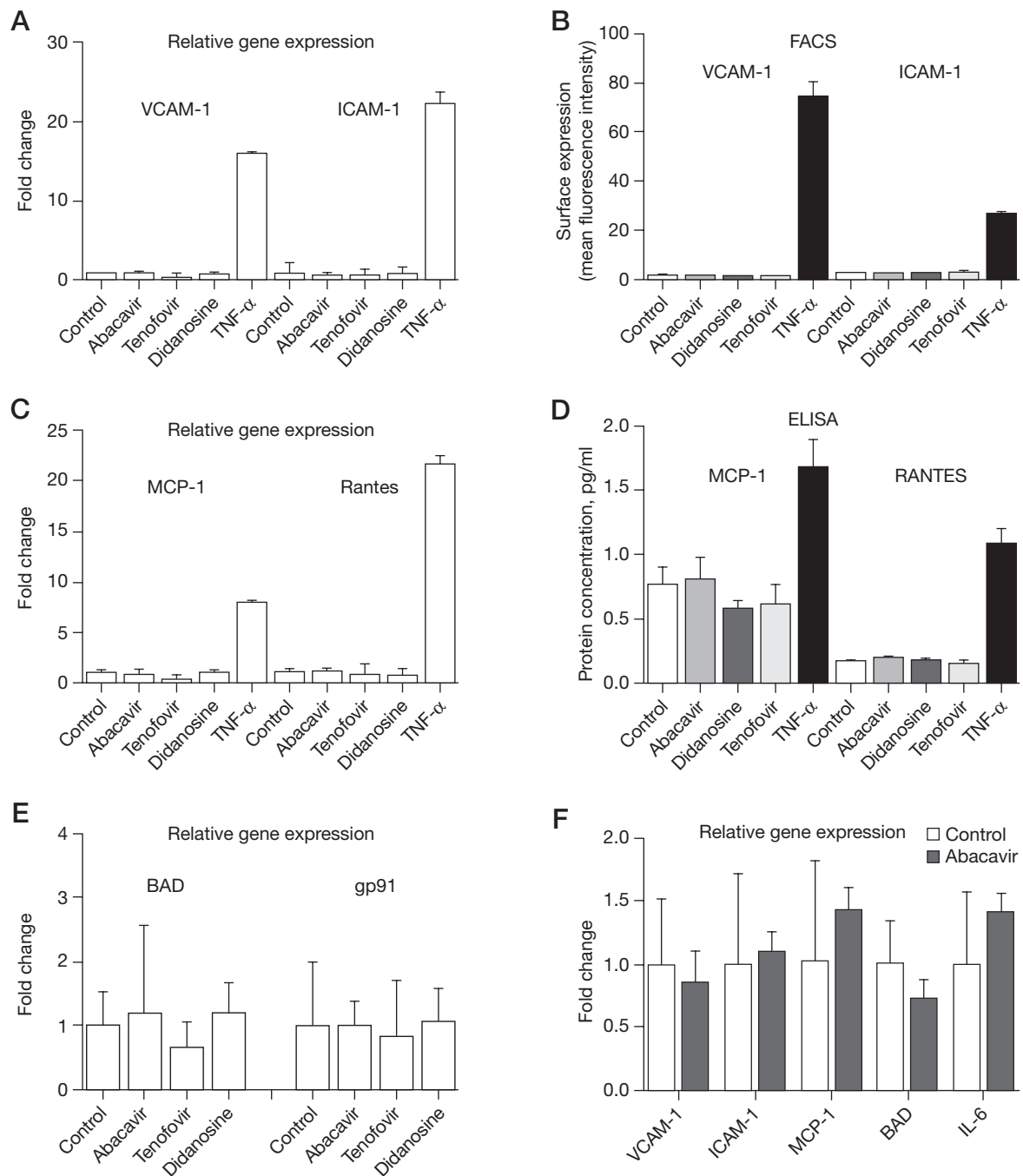
To more closely address *in vivo* conditions, when endothelial cells are constantly exposed to drugs, we tested prolonged treatment with abacavir for up to 10 days by providing fresh drug with a medium exchange every second day. However, abacavir did not induce VCAM-1 and ICAM-1 gene expression after constant exposure (Figure 1F). In addition, we tested some of the proinflammatory and proapoptotic genes for long-term effects of abacavir in HCAECs including MCP-1, BAD and IL-6, which also did not result in significantly increased gene expression (Figure 1F).

Discussion

This study is to the best of our knowledge the first to address the possibility that direct endothelial activation can explain potentially increased risk of cardiovascular events in HIV-infected patients treated with abacavir or didanosine. Neither these compounds or tenofovir were able to induce coronary arterial endothelial cell expression of the specific candidate genes chosen to present several atherosclerotic mechanistic pathways, including endothelial leukocyte adhesion, inflammation, oxidative stress and apoptosis.

We selected our candidate genes based on their known involvement in atherosclerotic disease development in the general population and in HIV-infected patients. MCP-1 is implicated in atherosclerotic lesion formation [13] as it recruits monocytes and T-cells to the sites of plaque initiation [14] and IL-6 is a prognostic marker for patients being at cardiovascular risk [15]. VCAM-1 and ICAM-1 are up-regulated during proinflammatory processes, including HIV infection, and mediate adhesion and transmigration of leukocytes to atherosclerotic plaques. In addition, cellular programmed cell death (apoptosis), linked to the deregulation of anti-apoptotic proteins, such as Bcl-2 [16], and pro-apoptotic proteins, such as BAD [17], is not induced by the antiretroviral drugs addressed in this study. One further mechanism by which endothelial inflammation is believed to be linked to endothelial dysfunction is via induction of intracellular endothelial oxidative stress caused by ROS, which are produced by endothelial NADPH oxidase in response to proinflammatory

Figure 1. Abacavir, didanosine and tenofovir do not increase endothelial genes and proteins for leukocyte adhesion, inflammation, oxidative stress or apoptosis



(A) Gene expression assessed by reverse transcriptase (RT)-PCR and (B) protein expression changes of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) assessed by flow-assisted cytometric analysis (FACS) in response to incubation for 8 h with tumor necrosis factor- α (TNF- α ; 20 ng/ml) as a positive control, abacavir (2 μ M), tenofovir (0.65 μ M) and didanosine (1.06 μ M). Values were normalized to controls. Significant increase was only observed with TNF- α . (C) Gene and (D) protein expression changes of endothelial produced chemokines monocyte chemoattractant protein-1 (MCP-1) and regulated on activation normal T-cell expressed and secreted (RANTES) in response to incubation for 8 h with TNF- α (20 ng/ml), abacavir (2 μ M), tenofovir (0.65 μ M) or didanosine (1.06 μ M). Significant increase was only observed with TNF- α . (E) Gene expression assessed by RT-PCR for Bcl-xL/B-cell leukaemia/lymphoma 2 (Bcl-2)-associated death promoter (BAD) and glycosylated protein 91 phox (gp91phox) 8 h after stimulation with abacavir (2 μ M), tenofovir (0.65 μ M) or didanosine (1.06 μ M). (F) Gene expression assessed by RT-PCR for VCAM-1, ICAM-1, MCP-1, BAD and interleukin (IL)-6 at 10 days after continuous stimulation with abacavir (2 μ M), which was maintained with the medium exchange every second day. No significant differences were found between the negative control and the drug-related gene expression changes. Shown are the SEM of triplicate values as assessed by real-time PCR (A, C, E and F), FACS (B) and ELISA (D). All RT-PCR experiments were performed in three independent experiments and reproduced at least 2 $\times$. FACS and ELISA measurements were performed in three independent experiments.

cytokines via up-regulation of its regulatory subunit glycosylated protein 91 phox (gp91^{phox}) [18].

Taken together, our *in vitro* data do not support any direct biological effects of abacavir, didanosine or tenofovir on endothelial cell function as measured using the candidate genes examined in these experiments. As such, our results corroborate the findings from Martínez *et al.* [19] and Hammond *et al.* [20], both of whom did not find increased levels of circulating IL-6, high-sensitivity C-reactive protein (hsCRP), MCP-1, soluble VCAM-1, or soluble ICAM-1 in patients receiving abacavir. One limitation of our study is that by design it does not address the possibility that long-term exposure over years can result in accumulation of stress or endothelial damage. Nevertheless, we did not see changes in expression of proinflammatory and apoptosis regulating genes when HCAECs were incubated with the median *in vivo* concentrations of abacavir for up to 10 days, indicating that prolonged exposure of the drug for at least the here tested extended time period is unlikely to activate endothelial cells.

In summary, we conclude that if abacavir and didanosine are causally associated with cardiovascular events in HIV-infected patients, they likely do not do so by direct endothelial involvement in the atherosclerotic pathways investigated in the current study.

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Disclosure statement

The authors declare no competing interests.

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