

In Vivo Effects of Tenofovir-DF/Emtricitabine and Abacavir/Lamivudine with Atazanavir-R on Platelet Activating Factor Metabolism in HIV Naive Patients

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Abstract

Antiretroviral therapy (ART) has successfully decreased AIDS morbidity and mortality and increased the lifespan of HIV patients to several decades. However, numerous factors contribute with unknown mechanisms to chronic immune activation and inflammation leading to severe "non-AIDS morbidities". Platelet Activating Factor (PAF) is a potent lipid inflammatory mediator with important role in the "non-AIDS morbidities". The purpose of this study was to investigate whether tenofovir-DF/emtricitabine and abacavir/lamivudine with atazanavir boosted ritonavir (ART_A and ART_B, respectively) affect *in vitro* PAF activity and *in vivo* PAF levels and metabolism.

In this intent, the two ART regimens were examined *in vitro* against platelet aggregation induced by PAF. In addition, PAF levels and PAF metabolic enzymes were determined in HIV-1 infected volunteers before and after the initiation of antiretroviral therapy for a 12-month period.

The *in vitro* results showed that ritonavir was the most potent inhibitor against PAF induced platelet aggregation while abacavir presented the less potent action. The *in vivo* results showed that tenofovir-DF/emtricitabine with atazanavir-r seems not to affect PAF levels and metabolism while abacavir/lamivudine with atazanavir-r increased bound and total PAF blood levels, PAF biosynthesis in platelets and also decreased Lp-PLA2 activity. In addition, ART_B revealed higher lyso-PAF-AT specific activity at 3rd, 6th and 9th month ($p_3=0.04$, $p_6=0.04$ and $p_9=0.03$) compared to ART_A.

In conclusion, there is a direct relation between *in vitro* and *in vivo* effect of antiretrovirals on PAF and abacavir-containing regimen activates PAF biosynthesis leading to elevated PAF levels.

Keywords: Platelet activating factor; Inflammation; Human immunodeficiency virus; non-AIDS morbidities; Tenofovir-DF; Emtricitabine; Abacavir; Lamivudine; Atazanavir; Ritonavir

Abbreviations: PAF: Platelet Activating Factor; HIV: Human Immunodeficiency Virus; ART: Antiretroviral Therapy; CVD: Cardiovascular Disease; PAF-CPT: PAF-Cholinephosphotransferase; Lyso-PAF AT: Lyso-PAF Acetyltransferase; PAF-AH: PAF-Acetylhydrolases; Lp-PLA₂: Lipoprotein-Phospholipase; HLs: Human Leukocytes; HPs: Human Platelets; ERCs: Erythrocytes; BSA: Bovine Serum Albumin

Background

According to WHO, there were approximately 36.9 million people living with HIV at the end of 2015 [1]. The effectiveness of highly active antiretroviral therapy in suppressing viral replication and reducing HIV-related morbidity and mortality has been consistently demonstrated [2,3]. However, low-level HIV replication, high levels of other copathogens, persistent immune dysfunction, bacterial translocation and antiretroviral drug toxicity increase several

inflammatory mediators leading to chronic immune activation and inflammation which are correlated to severe "non-AIDS morbidities" [4,5] including cardiovascular disease (CVD) [6].

Platelet Activating Factor is a potent lipid inflammatory mediator, originally identified as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine [7,8]. In humans, PAF is mainly synthesized in the inflammation-implicated cells such as neutrophils, basophils, eosinophils, monocytes, macrophages, platelets and endothelial cells and also in the cells of many organs (e.g. kidney) [9-11]. PAF exerts its autocrine and paracrine actions through binding to a G-protein coupled receptor located on the plasma membrane and nuclear membrane of a wide variety of mammalian cells. Under physiological conditions, PAF levels are under strict enzymatic control [12,13]. Referring to PAF metabolism, there are three key metabolic enzymes, two biosynthetic ones namely PAF-cholinephosphotransferase (PAF-CPT) and lyso-PAF-acetyltransferase (lyso-PAF-AT) as well as two different isoforms of PAF-acetylhydrolases, PAF-AH and Lipoprotein-phospholipase A2 (Lp-PLA₂) in plasma [7].

Limited evidence suggests that PAF may be a crucial link between systemic inflammation, immune activation and HIV infection and

thus contributing to the “non-AIDS morbidities” [14]. Our research team has shown that specific antiretroviral drugs exert *in vitro* antagonistic effect against PAF action [15] and they also affect PAF metabolism *in vivo* [16].

A number of studies also suggest that treatment with tenofovir-DF/emtricitabine with atazanavir-r decreases inflammatory and hypercoagulation markers [17-19] but tenofovir-DF has been associated with renal impairment especially when combined with PIs such as atazanavir-r [20-23]. On the other hand, conflicting results occur concerning the effect of abacavir/lamivudine/atazanavir-r regimen on markers of systemic and/or chronic inflammation [17,18,24,25].

The objective of the present work was to investigate whether tenofovir-DF/emtricitabine and abacavir/lamivudine with atazanavir boosted ritonavir may exert *in vitro* antagonistic effect against PAF action and also affect *in vivo* its levels and metabolic enzymes in HIV naive patients.

Methods

Study design

Study patients (n=20) were recruited from the 3rd Internal Medicine Department Infectious Diseases Unit, Red Cross General Hospital, Athens, Greece. Informed consent was obtained before the study enrollment as well as approval from the Red Cross General Hospital ethics committee according to the Declaration of Helsinki. All participants were male, treatment naïve and asymptomatic HIV-infected individuals at CDC A2 clinical stage fulfilling the criteria for ART initiation according to the European [26] and International guidelines [27]. Patients were randomly assigned in 2 groups. ART_A group was consisted of 10 patients who received co-formulated tenofovir-DF/emtricitabine with atazanavir boosted ritonavir and ART_B group was also consisted of 10 patients who received coformulated abacavir/lamivudine with atazanavir boosted ritonavir. The mean age in ART_A group was 34 ± 8 years (75% smokers) while in ART_B group the mean age was 35 ± 10 years (40% smokers). Exclusion criteria were the presence of inflammatory or other diseases (renal disorders, periodontal or autoimmune disease, diabetes, and hypertension), allergies or any medication other than ART. In ART_A group, 2 patients were excluded from the analysis due to a concurrent disease during the study period. The study lasted for 12 months and blood samples were collected before (baseline, defined as 0 months) and after 1, 3, 6, 9 and 12 months of ART initiation.

Materials and Instrumentation

Centrifugations were performed in a Heraeus Multifuge 3L-R, a Heraeus Labofuge 400R, a Jouan C312 and a refrigerated Micro 22R Hettich centrifuge. Homogenizations were conducted at 30% of power of a supersonic Bandelin Sonoplus HD 2070 sonicator (Heinrichstraze 3-4, D-12207 Berlin, Germany). The liquid scintillation counter used was a 1209 Rackbeta (Pharmacia, Wallac, Finland) coupled to a Facit B3100 recorder. Platelet aggregation assay was performed on a model 400 VS aggregometer of Chrono-Log (Havertown, PA, USA) coupled to a Chrono-Log recorder at 37°C with constant stirring at 1200 rpm. High Performance Liquid Chromatography (HPLC) was conducted on a Hewlett Packard series 1100, supplied with an 1100 HP UV detector, connected to a Hewlett Packard model HP-3396A integrator-plotter. Separation of lipids was carried out on a particil 10 SCX WCS

Analytical column, 4.6 mm × 250 mm Whatman at room temperature. The determination of the inflammatory biomarkers was conducted on an automatic analyzer Siemens Center 60 MacPherson Road Singapore 348615 and a BD FACS Canto II Flow cytometer. CD4+ were measured by a Tetra One System on the EPICS XL flow cytometer (Beckman Coulter, Nyon, Switzerland) and viral load was determined using the Versant HIV-1 RNA 3.0 assay (bDNA).

Reagents were obtained from Sigma (St. Louis, MO, USA), Biomol International LP (Palatine House, Matford Court, Exeter, UK) and New England Nuclear (Dupont, Boston, MA, USA). Solvents were purchased from Merck KGaA (Darmstadt, Germany).

In vitro experiments on washed rabbit platelets

PAF and antiretroviral drugs were dissolved in Bovine Serum Albumin (BSA) and the induced aggregation was examined with washed rabbit platelets according to the method of Demopoulos et al. [8]. The antiretrovirals were added 1 min prior to the addition of PAF (final concentration 1.13×10^{-11} mol/L). The PAF induced platelet aggregation was measured before (considered as 0% inhibition) and after the addition of various concentrations of the examined antiretroviral. Consequently, the plot of percentage inhibition (ranging from 20% to 80%) versus different concentrations of the antiretroviral was linear. From this curve, the concentration of the antiretroviral that inhibited 50% PAF induced aggregation was calculated, and this value was defined as IC₅₀. The experiments were performed in duplicates.

Quantification of PAF

The isolation and purification of PAF was performed as previously described [16]. Briefly, 10 mL of blood were collected from each patient and poured into 40 mL of absolute ethanol. Bound and Free PAF were extracted separately according to the Blich and Dyer method. After its extraction, PAF was firstly purified by silicic acid column chromatography and secondly by HPLC (Hewlett-Packard series 1100) on a cation-exchange column. The final samples were dissolved in BSA (1.25% in saline) and PAF levels were determined by measuring the aggregatory activity towards washed rabbit platelets. The quantification of PAF was based on a standard curve constructed with the use of known concentrations of synthetic PAF. Total PAF levels occur from the sum of Bound and Free PAF and are expressed as fmol/mL of blood.

Isolation of plasma, platelets, leukocytes and erythrocytes

A total amount of 9 mL blood was obtained from each volunteer in 1 mL of sodium citrate/ citrate acid anticoagulant solution. The sample was centrifuged at 194 xg for 10 min at 25°C and the isolation of plasma, leukocytes, platelets and erythrocytes was carried out as previously described [28].

Enzymatic assays

PAF-CPT activity assay: The assay was performed on the homogenates of leukocytes and platelets as previously described [16]. Briefly, the reaction was carried out at 37°C for 20 min in a final volume of 200 µL containing as final concentrations: 100 mM Tris-HCl (pH 8.0), 15 mM dithiothreitol (DTT), 0.5 mM EDTA, 20 mM MgCl₂, 1 mg/mL BSA, 100 µM CDP-Choline, 100 µM 1-O-alkyl2-sn-acetyl-glycerol (AAG, added in the assay mixture in ethanol), and the sample (0.05 and 0.1 mg/mL final concentration of protein for both leukocytes

and platelets). The mixture of the buffer solution and the cofactors were incubated at 37°C for 5 min. Initially, the homogenized sample was added in the mixture followed after 30s by AAG and 30s later the reaction was started by the addition of CDP-Choline while the reaction was stopped by 0.5 mL of methanol.

Lyso-PAF-AT activity assay: The assay was performed on the homogenates of leukocytes and platelets as previously described [16]. Briefly, the reaction was carried out at 37°C for 30 min in a final volume of 200 μ L containing 50 mM Tris-HCl (pH 7.4), 0.25 mg/mL BSA, 20 μ M lyso-PAF and 200 μ M acetyl-CoA and the sample (0.125 mg/mL final concentration of protein for both leukocytes and platelets). The reaction was started by the addition of the homogenized sample and was stopped after 30 min by adding 0.5 mL of methanol.

Determination of enzyme assays derived-PAF: After the assays of PAF-CPT and lyso-PAF-AT, PAF was extracted according to the Bligh-Dyer method and was separated by thin-layer chromatography (TLC) on Silica Gel G coated plates with a development system consisted of chloroform:methanol:acetic acid:water (100:57:16:8,v/v/v/v). PAF band was scrapped off, extracted using Bligh-Dyer and finally quantified by the washed rabbit platelet aggregation assay [8]. Enzymatic activities for both PAF-CPT and lyso-PAF-AT were expressed as specific activities in pmol/min/mg of protein.

PAF-acetylhydrolase activity assay: PAF-AH in HLs, HPs, ERCs as well as Lp-PLA₂ in plasma were determined by the trichloroacetic acid precipitation method using [³H] PAF as a substrate, as previously described [16]. Briefly, the reaction took place for 30 min at 37°C in a final volume of 200 μ L. Initially 50 mM of Tris/HCl buffer (pH 7.4) was incubated with 4 nmol of [³H]-PAF (20 Bq per nmol) [³H]-acetyl PAF/PAF solution in BSA (1% in saline) for 5 min. The reaction started by the addition of homogenized samples (0.25 mg/mL in the case of HLs, 0.5 mg/mL in the case of HPs, 2.5 mg/mL in the case of ERCs or 2 μ L in the case of plasma). The reaction was terminated by the addition of BSA solution (0.75 mg/mL) followed by precipitation with trichloroacetic acid (TCA, 9.6% v/v). The samples were then placed in an ice bath for 30 min and subsequently centrifuged at 16,000 xg for 5 min. The [³H]-acetate released into the aqueous phase was measured on a liquid scintillation counter. The enzyme activity was expressed as pmol of PAF degraded per min per μ L of plasma or pmol of PAF degraded per min per mg of protein.

Biochemical markers and immunological analysis

Clinical biochemical markers were measured by a Siemens Dimension RxL automatic analyzer. CD4+ cell counts were defined using Tetra One System on the EPICS XL flow cytometer, while viral load was determined using the Versant HIV-1 RNA 3.0 assay.

Statistical analysis

Normal distribution was tested with the Shapiro-Wilk criterion. The results are expressed as median values and interquartile range (25-75) for non-parametric values and as mean and standard deviation using % change from the baseline value for parametric values. Difference among antiretrovirals' *in vitro* activity was tested with one-way ANOVA for each chemical substance with post hoc analysis for multiple comparisons and t-test was used to compare the two ART combinations. Mann Whitney test was used for the baseline differences between the two groups. Differences within each group during the 12-month treatment were determined by one-way ANOVA with post hoc analysis for multiple comparisons compared to baseline value. The

comparison of the two groups was made using repeated measure ANOVA (P_{time} , P_{trial} , $P_{time*trial}$). Viral load changes are reported in a logarithmic scale. Statistical significance was considered as $p < 0.05$. The analysis was performed using IBM SPSS Statistics 20.

Results

Anthropometric and biochemical characteristics

Baseline values of anthropometric and biochemical characteristics of patients are shown in Table 1 and are expressed as medians and interquartile range (25-75). There are baseline differences in VL, total cholesterol, HDL, LDL and glucose between the 2 groups.

For this reason, the biochemical characteristics of ART_A and ART_B groups after ART administration are shown as % change from baseline in Table 2 and are expressed as means and standard deviation. In both groups, the viral load was progressively reduced during the study period ($p_s < 0.001$), while CD4+ cell counts were gradually increased ($p_s < 0.001$) even from the 1st month of treatment. In ART_A group, γ GT, ALP and PLT were significantly increased, while glucose was decreased. In ART_B group total cholesterol, HDL, triglycerides, ALP and WBC were significantly increased, while RBC, hemoglobin and hematocrit were decreased.

In vitro effect of antiretrovirals against PAF aggregation

The *in vitro* effects of antiretrovirals and their combinations against PAF induced aggregation on washed rabbit platelets are shown in Table 3. Concerning the IC₅₀ of each chemical substance, ritonavir displayed the most potent inhibition against PAF action with a significant lower IC₅₀ value compared to all other antiretrovirals except from atazanavir ($p^c = 0.03$, $p^d = 0.004$, $p^e = 0.001$ and $p^f < 0.001$). Atazanavir was significantly more effective against PAF action compared to lamivudine and tenofovir ($p^e = 0.01$ and $p^f < 0.001$). Emtricitabine, tenofovir and lamivudine were significantly more effective than abacavir ($p^s < 0.001/0.003/0.02$, respectively) which demonstrated the less potent inhibition against PAF action. Regarding ART regimen, the IC₅₀ of the ART combinations were at the same order ($p = 0.18$).

PAF levels and specific activity of metabolic enzymes

Baseline values of PAF levels and specific activity of metabolic enzymes of patients are shown in Table 4 and are expressed as medians and interquartile range (25-75). There are baseline differences in Bound PAF, Total PAF, lyso-PAF-AT in leukocytes and platelets as well as in Lp-PLA₂. For this reason, the PAF levels as well as the specific activity of its metabolic enzymes in ART_A and ART_B groups after ART administration are shown as % change from baseline in Table 5. and are expressed as means and standard deviation.

PAF levels

A significant time effect was observed in the levels of Bound PAF ($P_{time} = 0.002$) and Total PAF ($P_{time} = 0.003$) without significant difference between the two ART regimens ($P_{trial} = 0.84$, $P_{trial} = 0.33$). Within the same group, no change on PAF levels (Bound, Free and Total) in ART_A group was observed throughout the study period. Concerning ART_B group, Bound and Total PAF levels were gradually increased ($P_{bound} = 0.004$ and $P_{total} = 0.005$) throughout the study period, while Free PAF levels remained stable. Bound and Total PAF levels reached their maximum value at the 12th month ($p_{s0-12} = 0.005$).

Specific activity of PAF biosynthetic enzymes

The specific activity of lyso-PAF-AT in leukocytes and platelets was increased compared to baseline values ($p_{\text{time}}=0.02$ and $p_{\text{time}}=0.05$ respectively) presenting a significant time effect. Most important in the case of leukocytes' lyso-PAF-AT specific activity, a significant trial effect was also observed ($p_{\text{trial}}=0.04$) with the ART_B group revealing higher lyso-PAF-AT specific activity at 3rd, 6th and 9th month ($p_3=0.04$, $p_6=0.04$ and $p_9=0.03$) compared to the ART_A group. Within the same ART group, there was no significant change in any biosynthetic enzyme in leukocytes or platelets in ART_A group. On the contrary, in ART_B group lyso-PAF-AT specific activity in platelets was increased ($p_{\text{time}}=0.006$) and displayed its maximum value at the 12th month ($p_{0-12}=0.011$). In addition, a marginally increase in PAF-CPT specific activity in platelets in the same group was also observed ($p_{\text{time}}=0.051$).

Specific activity of PAF catabolic enzymes

A significant time effect was only observed in the specific activity of Lp-PLA₂ ($p_{\text{time}}=0.02$). No change was depicted in PAF catabolic enzymes during the study period in both groups with the exception of Lp-PLA₂ activity in ART_B group which was decreased during the 12month treatment ($p_{\text{time}}=0.007$).

Discussion

PAF is a potent lipid inflammatory mediator implicated in the pathogenesis of HIV infection and "non-AIDS comorbidities", especially those with an inflammatory background. Our research team has previously proposed that PAF may be implicated in increased cardiovascular risk associated with abacavir use [16]. In the present study, we investigated whether tenofovir-DF/emtricitabine and abacavir/lamivudine with atazanavir boosted ritonavir affect *in vitro* the activity of PAF on platelets and *in vivo* its levels and metabolic enzymes in HIV naïve patients.

In both groups, raise in CD4⁺ cell counts and decline in viral load was observed as it was expected after ART initiation [29]. An increase in ALP within the normal range, has been detected in both groups and this is in accordance with previous reports on atazanavir containing regimens [30]. In addition, previous studies have shown that abacavir use in combination with atazanavir-r may result in abnormal fasting lipid profile as was the case with ART_B group [31-33].

Baseline Anthropometric and Biochemical characteristics	ART_A	ART_B	p
CD4+ (cells/ μ L)	305.5 (254.8-381.0)	268.0 (241.0-380.5)	0.74
Viral Load (log copies/mL)	2.8 (4.5-5.5)	4.3 (3.8-5.0)	0.03
BMI (Kg/cm ²)	24.7 (22.5-26.9)	23.8 (22.3-26.3)	0.74
Total Cholesterol (mg/dL)	147.0 (125.0-168.0)	182.0 (155.5-203.0)	0.04
HDL (mg/dL)	26.5 (25.3 - 35.5)	43.0 (33.5-55.0)	0.002

LDL (mg/dL)	88.5 (76.8-106.8)	118.0 (100.0-127.0)	0.03
Triglycerides (mg/dL)	131.5 (88.5 - 177.0)	62.0 (50.0-132.0)	0.11
Glucose (mg/dL)	90.5 (88.3-92.8)	82.0 (72.0-90.5)	0.05
Blood Urea Nitrogen (mg/dL)	13.5 (12.3-17.0)	13.0 (12.0-18.0)	0.96
Creatinine (mg/dL)	0.85 (0.80-0.90)	0.90 (0.80-1.00)	0.74
Serum Glutamic Oxaloacetic Transaminase (U/L)	21.5 (17.8-33.3)	28.0 (19.5-32.5)	0.54
Serum Glutamic Pyruvic Transaminase(U/L)	19.5 (17.5-51.3)	25.0 (16.5-33.5)	0.67
γ - Glutamyl Transferase (U/L)	22.0 (14.5-42.8)	21.0 (16.0-25.5)	0.67
Alkaline Phosphatase (U/L)	65.5 (56.8-70.8)	58.0 (50.0-77.5)	0.67
White Blood Cells Count ($10^3/\mu$ L)	6.30 (5.13-7.13)	5.20 (4.95-7.00)	0.42
Platelet Count ($10^3/\mu$ L)	197.0 (167.8-224.8)	172.0 (153.5-214.0)	0.48
Red Blood Cells Count ($10^6/\mu$ L)	5.04 (4.66-5.26)	4.80 (4.71-5.28)	0.74
Hemoglobin (g/dL)	14.25 (13.43-15.08)	14.30 (13.90-15.40)	0.67
Hematocrit (%)	43.0 (40.4-45.0)	42.5 (41.1-46.3)	0.74

Results are expressed as median values and interquartile range (25th -75th). Non-parametric Mann Whitney test was used for the baseline differences between the two groups

Table 1: Baseline anthropometric and biochemical characteristics of ART_A and ART_B groups.

The *in vitro* results showed that ritonavir and, to a lesser extent, atazanavir were the most potent inhibitors against PAF induced platelet aggregation among the chemical substances tested. Emtricitabine and tenofovir-DF were following whereas lamivudine and abacavir were the least potent PAF inhibitors. It should be noted that the antiretrovirals with intermediate inhibitory activity (emtricitabine and tenofovir) are part of the ART_A regimen while the less potent inhibitors (lamivudine and abacavir) are part of the ART_B regimen. In addition, the two most potent agents (ritonavir and atazanavir) are included in both combinations and this may explain a modest inhibitory effect of both ART regimens.

The *in vivo* study showed that treatment with tenofovir-DF/emtricitabine with atazanavir boosted ritonavir does not significantly affect PAF metabolism according to published data suggesting that this

combination decreases inflammatory and coagulation markers [17-19]. On the contrary, abacavir/lamivudine with atazanavir boosted ritonavir exhibited a gradual increase in bound and total PAF levels with a pronounced effect at the end of the study. This effect can be attributed to the stimulation of the biosynthetic enzymes and also to the decrease in plasma catabolic enzyme activity, both observed in our study. The persistent excess of PAF signaling may lead to disorders associated with chronic inflammation. The decline of Lp-PLA₂ is in accordance with previous studies in subjects treated with ATV or ATV-r [25]. Additionally, our previous results have shown that administration of tenofovir-DF/emtricitabine with efavirenz down-regulates PAF levels and metabolism while abacavir/lamivudine with efavirenz [16] has the opposite effect. Studies investigating the effect of abacavir containing regimens on inflammation resulted in conflicting results. Some of those have demonstrated that plasma levels of inflammatory markers significantly fell while others have reported no

significant change. In most cases, hsCRP remains unchanged or even increases [17,18,25]. Mechanism by which abacavir containing regimens affects inflammatory pathways is not clear. It has already been reported that abacavir containing regimens can induce a low-level hypercoagulable state by increasing platelet aggregation which is in accordance with our findings, since PAF is a potent inducer of platelet aggregation [34]. Our data reveal the action of ART in PAF metabolic pathways and also indicate the stimulating effect of abacavir/lamivudine in PAF levels and metabolism that demands further investigation. Although statistically significant differences were demonstrated, the small sample size is a limitation of this study. Herein, since the available data regarding the effects of ART on the PAF pathway are limited in retrospective and *in vitro* studies, future prospective studies are needed in order to explore optimal therapeutic interventions that might improve long term prognosis of HIV infection.

Anthropometric & biochemical characteristics	Groups	Months					P
		1	3	6	9	12	
CD4+ (cells/ μ L)	ART_A	137.3 \pm 22.9	161.7 \pm 42.5	*175.6 \pm 24.9	*198.2 \pm 56.2	*230.3 \pm 85.4	<0.001
	ART_B	*142.3 \pm 25.8	*153.6 \pm 22.5	*176.6 \pm 36.6	*163.8 \pm 21.9	*205.2 \pm 41.4	<0.001
Viral Load (log copies/mL)	ART_A	*0.9 \pm 0.7	*0.1 \pm 0.2	*0.0 \pm 0.1	*0.0 \pm 0.0	*0.0 \pm 0.0	<0.001
	ART_B	*1.9 \pm 1.9	*0.1 \pm 0.2	*0.0 \pm 0.0	*0.0 \pm 0.0	*0.0 \pm 0.0	<0.001
BMI (Kg/cm ²)	ART_A	101.8 \pm 3.1	103.4 \pm 6.2	104.5 \pm 10.0	105.1 \pm 12.5	105.7 \pm 12.2	0.78
	ART_B	101.4 \pm 1.6	100.8 \pm 2.5	101.9 \pm 3.3	102.8 \pm 2.9	102.8 \pm 4.6	0.21
Total Cholesterol (mg/dL)	ART_A	106.1 \pm 16.5	107.8 \pm 24.6	118.6 \pm 30.4	113.9 \pm 28.0	115.4 \pm 26.1	0.63
	ART_B	*113.8 \pm 7.7	*115.0 \pm 10.3	*115.5 \pm 11.3	*120.7 \pm 9.5	*116.8 \pm 11.8	<0.001
HDL (mg/dL)	ART_A	112.7 \pm 17.8	115.8 \pm 24.2	126.9 \pm 26.7	119.4 \pm 30.1	128.5 \pm 26.8	0.12
	ART_B	108.3 \pm 9.7	109.8 \pm 9.9	*117.1 \pm 9.2	*116.8 \pm 13.5	*117.9 \pm 8.7	<0.001
LDL (mg/dL)	ART_A	101.8 \pm 27.9	96.2 \pm 20.5	117.0 \pm 44.8	111.9 \pm 36.3	111.7 \pm 40.0	0.75
	ART_B	111.3 \pm 7.1	106.1 \pm 12.9	106.4 \pm 10.6	104.1 \pm 24.2	106.9 \pm 16.0	0.62
Triglycerides (mg/dL)	ART_A	125.0 \pm 25.7	147.6 \pm 81.6	133.5 \pm 54.0	*133.0 \pm 71.5	156.4 \pm 122.2	0.69
	ART_B	154.6 \pm 50.5	194.1 \pm 71.4	169.8 \pm 61.5	201.9 \pm 89.1	192.6 \pm 94.0	0.02
Glucose (mg/dL)	ART_A	98.4 \pm 6.3	94.4 \pm 6.3	99.0 \pm 5.7	101.4 \pm 5.5	92.8 \pm 7.4	0.03
	ART_B	104.8 \pm 8.1	106.2 \pm 7.4	106.8 \pm 12.4	103.9 \pm 8.9	106.8 \pm 12.2	0.54
Blood Urea Nitrogen (mg/dL)	ART_A	104.8 \pm 25.9	112.5 \pm 36.5	98.3 \pm 22.2	91.3 \pm 25.2	115.6 \pm 35.5	0.48
	ART_B	105.8 \pm 24.2	95.4 \pm 13.3	108.8 \pm 33.2	104.4 \pm 16.4	105.7 \pm 26.2	0.77
Creatinine (mg/dL)	ART_A	100.2 \pm 11.4	96.9 \pm 8.3	98.9 \pm 11.5	103.2 \pm 12.2	101.7 \pm 15.4	0.9
	ART_B	97.4 \pm 15.1	92.4 \pm 13.8	95.9 \pm 16.3	96.8 \pm 12.9	93.2 \pm 11.4	0.79
Serum Glutamic Oxaloacetic Transaminase (U/L)	ART_A	102.4 \pm 26.7	87.7 \pm 18.2	92.6 \pm 24.3	84.7 \pm 16.7	85.9 \pm 21.9	0.36
	ART_B	86.4 \pm 15.9	80.7 \pm 21.7	82.3 \pm 22.3	85.6 \pm 25.3	81.8 \pm 26.8	0.33
Serum Glutamic Pyruvic Transaminase (U/L)	ART_A	118.6 \pm 51.5	94.8 \pm 28.1	109.9 \pm 50.2	83.0 \pm 33.4	82.2 \pm 35.6	0.32
	ART_B	77.0 \pm 27.6	71.81 \pm 32.8	74.6 \pm 38.4	80.1 \pm 39.0	79.8 \pm 43.1	0.49

γ- Glutamyl Transferase (U/L)	ART_A	95.7 ± 17.5	118.5 ± 25.1	141.2 ± 39.5	114.9 ± 32.3	115.7 ± 27.7	0.02
	ART_B	117.1 ± 27.5	107.5 ± 37.5	111.7 ± 39.4	121.6 ± 37.7	125.1 ± 36.1	0.55
Alkaline Phosphatase (U/L)	ART_A	118.3 ± 6.2	*136.7 ± 14.3	*154.0 ± 20.4	*158.5 ± 26.5	*154.3 ± 14.0	<0.001
	ART_B	103.8 ± 7.6	116.7 ± 12.2	*127.6 ± 13.8	*131.6 ± 17.6	*130.2 ± 16.4	<0.001
White Blood Cells Count (10 ³ /μL)	ART_A	107.8 ± 10.3	111.4 ± 20.7	107.5 ± 21.3	111.0 ± 23.0	124.7 ± 29.4	0.27
	ART_B	104.8 ± 15.9	114.2 ± 22.4	118.4 ± 21.0	118.3 ± 24.7	*144.4 ± 55.8	0.02
Platelet Count (10 ³ /μL)	ART_A	115.5 ± 10.4	129.1 ± 23.4	120.4 ± 19.2	121.8 ± 24.5	128.8 ± 20.9	0.03
	ART_B	113.1 ± 9.5	113.7 ± 18.3	108.7 ± 18.2	106.3 ± 14.2	116.3 ± 19.0	0.17
Red Blood Cells Count (10 ⁶ /μL)	ART_A	99.5 ± 4.7	98.7 ± 4.6	100.5 ± 4.5	98.3 ± 7.2	99.2 ± 7.6	0.96
	ART_B	*95.5 ± 2.0	*92.0 ± 3.4	*91.4 ± 3.3	*91.9 ± 2.5	*91.7 ± 2.8	<0.001
Hemoglobin (g/dL)	ART_A	101.0 ± 3.6	102.5 ± 6.2	106.5 ± 4.1	102.9 ± 7.2	105.9 ± 6.2	0.09
	ART_B	96.0 ± 3.5	98.0 ± 3.3	99.2 ± 4.0	100.6 ± 4.0	100.4 ± 4.0	0.03
Hematocrit (%)	ART_A	100.7 ± 4.4	102.3 ± 4.8	105.8 ± 4.3	103.3 ± 7.1	105.4 ± 7.3	0.16
	ART_B	96.4 ± 1.5	97.6 ± 4.1	98.6 ± 3.1	99.5 ± 2.7	99.8 ± 4.0	0.05

The results are expressed as % change from baseline values in mean values (± sd). One way ANOVA was used for the difference within each group during the overall 12-month study (p) with post hoc analysis for multiple comparisons compared to baseline value (*p<0.05)

Table 2: % change of anthropometric and biochemical characteristics of ART_A and ART_B groups after ART administration.

Chemical substances	IC50 (± SD) (mol)	ART Combination	IC50 (± SD) (mg/mL)	f. Abacavir a,b,c,d,e
a. Ritonavir c,d,e,f	0.6 10 ⁻⁶ (± 0.08 10 ⁻⁶)	Truvada®:Reyataz®:Norvir®	2.68 10 ⁻³ (± 0.8 10 ⁻³)	8.1 10 ⁻⁶ (± 1.8 10 ⁻⁶)
b. Atazanavir e,f	1.8 10 ⁻⁶ (± 0.2 10 ⁻⁶)	1:01:01		
c. Emtricitabine a,f	3.4 10 ⁻⁶ (± 0.3 10 ⁻⁶)			
d. Tenofovir-DF a,f	4.3 10 ⁻⁶ (± 0.5 10 ⁻⁶)	Kivexa®:Reyataz®:Norvir®	3.55 10 ⁻³ (± 0.1 10 ⁻³)	
e. Lamivudine a,b,f	5.1 10 ⁻⁶ (± 1.1 10 ⁻⁶)	1:01:01		

The results are expressed as mean values (± sd) of three separate experiments. One way ANOVA was used to compare IC 50 values of chemical substance with Post hoc analysis for multiple comparisons and t-test was used to compare the two ART combinations. a-f indicate significance with the corresponding antiretroviral.

Table 3: *In vitro* effect of antiretrovirals and their combinations against PAF induced aggregation on washed rabbit platelets.

Baseline PAF metabolism	ART_A Group	ART_B Group	p
Bound PAF levels (fmol/mL)	4.5 (2.3-7.7)	37.4 (25.0-54.7)	<0.001
Free PAF levels (fmol/mL)	1.2 (1.0-2.2)	0.2 (0.1-3.8)	0.10
Total PAF levels (fmol/mL)	5.5 (3.5-14.2)	38.0 (25.1-59.5)	<0.001
PAF-CPT in leukocytes (pmol/min/mg)	194.6 (134.7-374.8)	103.0 (69.9-157.8)	0.08
PAF-CPT in platelets (pmol/min/mg)	20.0 (19.2-25.2)	52.7 (22.7-66.1)	0.02
Lyso-PAF-AT in leukocytes (pmol/min/mg)	6.6 (4.2-14.5)	11.0 (7.9-15.4)	0.24
Lyso-PAF-AT in platelets (pmol/min/mg)	5.5 (2.0-12.0)	5.5 (3.54-8.53)	0.08
PAF-AH in leukocytes (pmol/min/mg)	91.0 (65.3-110.2)	66.1 (44.5-93.6)	0.12
PAF-AH in platelets (pmol/min/mg)	385.8 (297.4-468.2)	279.5 (245.8-378.0)	0.08

PAF-AH in erythrocytes (pmol/min/mg)	8.0 (4.6-9.4)	11.9 (8.4-13.8)	0.03
Lp-PLA2 in plasma (pmol/min/μL)	29.2 (21.6-33.2)	27.3 (23.8-41.0)	1.00
The results are expressed as median values and interquartile range (25th-75th). Non-parametric Mann Whitney test was used for the baseline differences between the two groups (p).			

Table 4: Baseline PAF metabolism of ART_A and ART_B groups.

PAF metabolism	groups	months					P _{time}	P _{time*trial}	P _{trial}
		1	3	6	9	12			
Bound PAF levels (fmol/mL)	ART_A	145.8 ± 107.6	226. ± 174.1	353.1 ± 253.8	292.3 ± 241.2	261.7 ± 243.6	0.002	0.15	0.84
	ART_B	147.9 ± 104.2	180.2 ± 153.8	244.5 ± 169.3	322.1 ± 210.2	*468.9 ± 408.4			
Free PAF levels (fmol/mL)	ART_A	86.0 ± 33.6	85.1 ± 38.4	105.5 ± 51.2	125.6 ± 121.2	90.8 ± 56.7	0.61	0.09	0.46
	ART_B	152.9 ± 87.6	167.2 ± 180.1	77.0 ± 55.1	64.2 ± 50.6	149.1 ± 159.9			
Total PAF levels (fmol/mL)	ART_A	123.0 ± 72.9	177.8 ± 121.9	263.9 ± 165.9	226.3 ± 179.7	198.1 ± 153.1	0.003	0.09	0.33
	ART_B	154.2 ± 102.2	191.6 ± 149.7	243.0 ± 167.1	312.2 ± 204.8	*462.0 ± 403.8			
PAF-CPT in leukocytes (pmol/min/mg)	ART_A	171.4 ± 102.2	186.3 ± 145.0	200.6 ± 205.7	180.1 ± 187.0	270.9 ± 478.7	0.18	0.79	0.68
	ART_B	120.5 ± 70.7	173.8 ± 172.0	148.2 ± 131.6	173.7 ± 125.2	222.3 ± 294.8			
PAF-CPT in platelets (pmol/min/mg)	ART_A	135.3 ± 104.1	156.1 ± 110.4	170.1 ± 127.3	212.5 ± 167.0	219.7 ± 148.5	0.08	0.44	0.13
	ART_B	102.4 ± 64.0	169.0 ± 98.6	112.1 ± 63.6	106.1 ± 57.0	187.4 ± 127.3			
Lyso-PAF-AT in leukocytes (pmol/min/mg)	ART_A	254.2 ± 284.3	522.1 ± 547.5	546.4 ± 524.2	670.5 ± 674.3	658.8 ± 761.8	0.02	0.1	0.04
	ART_B	123.8 ± 83.5	143.0 ± 85.7	171.8 ± 163.6	171.1 ± 153.7	230.9 ± 166.1			*(p3,p6,p9)
Lyso-PAF-AT in platelets (pmol/min/mg)	ART_A	122.0 ± 45.3	160.6 ± 158.6	149.7 ± 91.3	165.5 ± 152.6	149.8 ± 138.4	0.05	0.24	0.73
	ART_B	103.2 ± 50.5	144.4 ± 90.4	116.4 ± 59.9	179.6 ± 130.9	*269.0 ± 189.1			
PAF-AH in leukocytes (pmol/min/mg)	ART_A	128.5 ± 47.5	171.1 ± 101.7	132.7 ± 46.4	133.5 ± 50.4	120.7 ± 56.2	0.43	0.19	0.29
	ART_B	104.1 ± 52.8	93.7 ± 51.5	113.2 ± 63.1	124.7 ± 75.2	125.4 ± 92.4			
PAF-AH in platelets (pmol/min/mg)	ART_A	77.6 ± 30.0	79.6 ± 26.2	97.7 ± 37.8	91.5 ± 25.9	77.6 ± 24.8	0.53	0.47	0.17
	ART_B	98.3 ± 30.6	94.6 ± 32.3	97.3 ± 35.3	112.8 ± 65.7	112.0 ± 57.6			
PAF-AH in erythrocytes (pmol/min/mg)	ART_A	89.1 ± 14.8	102.5 ± 36.4	90.8 ± 32.8	102.1 ± 49.1	102.6 ± 41.2	0.5	0.7	0.55
	ART_B	90.0 ± 47.3	77.4 ± 21.6	84.0 ± 24.9	99.6 ± 42.0	96.7 ± 50.9			
Lp-PLA2 in plasma (pmol/min/μL)	ART_A	92.0 ± 17.8	91.6 ± 22.8	88.4 ± 27.7	91.4 ± 22.2	88.9 ± 22.0	0.02	0.37	0.79
	ART_B	94.8 ± 9.1	100.6 ± 17.0	83.0 ± 15.7	80.5 ± 15.8	84.2 ± 22.3			

The results are expressed as % change from baseline values in mean values (\pm sd). One way ANOVA was used for the difference within each group during the overall 12-month study (p) with post hoc analysis for multiple comparisons compared to baseline value ($*p < 0.05$) p_{time} displays difference between the two groups against the 12 months of the study, p_{trial} displays difference between the two groups against different ART and $p_{\text{time}^* \text{trial}}$ displays their combination.

Table 5: % change of PAF levels and PAF metabolism enzymes specific activity of TDF and ABC groups during ART administration.

Conclusion

This study indicates a relation between *in vitro* and *in vivo* action of antiretrovirals and also displays an activating effect of abacavir/lamivudine with atazanavir boosted ritonavir in PAF levels and metabolism.

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