Premature Senescence of Vascular Cells Is Induced by HIV Protease Inhibitors
Implication of Prelamin A and Reversion by Statin

Chloé Lefèvre, Martine Auclair, Franck Boccara, Jean-Philippe Bastard, Jacqueline Capeau, Corinne Vigouroux, Martine Caron-Debarle

Objective—To determine whether and how protease inhibitors (PIs) could affect vascular aging.

Methods and Results—HIV therapy with PIs is associated with an increased risk of premature cardiovascular disease. The effect of ritonavir and a combination of lopinavir and ritonavir (for 30 days) on senescence, oxidative stress, and inflammation was evaluated in human coronary artery endothelial cells (HCAECs). These HCAECs were either cotreated or not cotreated with pravastatin or farnesyl transferase inhibitor (FTI)-277 or with 2 antioxidants (manganese [III] tetrakis [4-benzoic acid] porphyrin [MnTBAP] and N-acetyl cysteine). Senescence markers were evaluated in peripheral blood mononuclear cells (PBMCs) from HIV-infected patients under PI treatment. PIs induced senescence markers, prelamin A accumulation, oxidative stress, and inflammation in HCAECs. Senescence markers and prelamin A were also observed in PBMCs from HIV-infected patients under ritonavir-boosted PIs. Pravastatin, FTI-277, and antioxidants improved PI adverse effects in HCAECs. Senescence markers were lower in PBMCs from PI-treated patients cotreated with statins.

Conclusion—PIs triggered premature senescence in endothelial cells by a mechanism involving prelamin A accumulation. Accordingly, circulating cells from HIV-infected patients receiving PI therapy expressed senescence markers and prelamin A. Statin was associated with improved senescence in endothelial cells and patient PBMCs. Thus, PIs might promote vascular senescence in HIV-infected patients; and statins might exert beneficial effects in these patients. (Arterioscler Thromb Vasc Biol. 2010;30:2611-2620.)

Key Words: endothelial cell ■ PBMC ■ HIV protease inhibitor ■ aging ■ prelamin A ■ statin ■ antioxidants

The increased risk of premature myocardial infarction observed in HIV-infected patients has been attributed to antiretroviral therapy, HIV infection itself, and a synergistic interaction between these factors and other classic cardiovascular risk factors.1–4 Antiretroviral therapy may promote premature cardiovascular disease through endothelial dysfunction either indirectly, via protease inhibitor (PI)–induced metabolic disturbances; or directly, via alterations of endothelial cells.5–8 Accordingly, several endothelial cell functions, including production of reactive oxygen species (ROS) and secretion of inflammatory cytokines, can be deregulated by short-term exposure (24 to 72 hours) of cultured endothelial cells to ritonavir or other PIs.8–12

The risk of premature cardiovascular events might also result from the accelerated biological aging imposed by antiretroviral therapy and/or HIV itself.13–16 Indeed, vascular endothelial cell dysfunction is a feature of the human physiological aging process,17,18 and syndromes of premature aging are associated with precocious cardiovascular disease. The most striking findings are observed in progeroid syndromes linked to molecular alterations in the prelamin A maturation process.19–22 In these genetically determined accelerated aging phenotypes, both vascular endothelial and smooth muscle cells (VSMCs) are altered.20,23 They exhibit senescent morphological features at the cellular and nuclear levels and aging-related dysfunctions linked to prelamin A accumulation.23–25 Prelamin A accumulation is also observed in VSMCs during physiological aging and in atherosclerotic lesions, where it often colocalized with senescent and degenerate VSMCs.26

The physiological posttranslational processing of prelamin A is complex, leading to the production of mature lamin A, a ubiquitous protein of the nuclear lamina meshwork. The 2 major steps are catalyzed by a farnesyl transferase, which
adds a farnesyl moiety at the carboxyl terminus of prelamin A; and by the metalloprotease zinc metalloproteinase Ste 24 (Zmpste24), which removes the C-terminal 15-amino acid farnesylated polypeptide, resulting in the production of the nonfarnesylated mature lamin A.27,28 Several studies have shown that accumulation of a permanently farnesylated form of prelamin A, as the result of mutations in lamin A/C or Zmpste24 impairing the farnesylation-dependent proteolysis of prelamin A, leads to progeroid phenotypes. Indeed, decreasing the amount of farnesylated prelamin A or blocking the farnesylation of prelamin A ameliorates cellular senescence in human and murine phenotypes of accelerated ageing.25,29–31

Markers of premature senescence, including prelamin A, are observed in adipose tissue from HIV-infected patients receiving treatment with PIs and in cultured adipocytes and fibroblasts exposed for a long time to some PIs.15,32 This has been linked to the ability of some PIs, including ritonavir and lopinavir, to directly inhibit Zmpste24,33,34 thus inducing the accumulation of a toxic permanently farnesylated form of prelamin A.15

Statins, which inhibit 3-hydroxy-3-methylglutaryl–coenzyme A reductase (an enzyme required for the synthesis of the farnesyl moiety and, thus, for the farnesylation of prelamin A25), have beneficial effects on premature aging resulting from genetic laminopathies,25,30,31 PI exposure15 or physiological vascular aging.26 The farnesyl transferase inhibitor FTI-277 acts downstream of statins to block prelamin A farnesylation.27,28,35 Whether farnesylation inhibitors could prevent PI toxicity in cultured endothelial cells has never been investigated.

Our objective was to study whether ritonavir-boosted PIs could induce accelerated senescence and associated dysfunctions in vascular endothelial cells, thus potentially contributing to cardiovascular complications observed prematurely in HIV-infected patients. We investigated the effects of long-term exposure of human coronary artery endothelial cells (HCAECs) to a frequently used PI combination (lopinavir associated with ritonavir) on a series of markers of cellular aging, oxidative stress, and inflammation; and evaluated the effects of pravastatin and antioxidants. We then tested senescence markers in peripheral blood mononuclear cells (PBMCs) of HIV-infected patients receiving treatment with a ritonavir-boosted PI, cotreated or not with statins.

### Methods

#### Cell Culture and Treatment

Commercially obtained HCAECs and endothelial cell complete growth medium were used in this study. Cells were exposed to ritonavir (SC-208310), 7.5 μmol/L, or lopinavir (SC-207831), 10 μmol/L, plus ritonavir, 2 μmol/L, at clinically relevant concentrations30 or to the solvent (0.05% dimethyl sulfoxide) for 30 days, from passage 2 to 8. The cells were also incubated with pravastatin (25 μmol/L) or FTI-277 (20 μmol/L) during the last 72 or 48 hours of PI treatment, respectively. They were incubated with MtbTAP (100 μmol/L) or N-acetyl cysteine (NAC) (1 mmol/L) for the last 24 hours of PI treatment.

PBMCs were extracted from EDTA-collected blood by density-gradient centrifugation (Biocoll separating solution) and analyzed by Western blotting. Informed consent was obtained from patients who had blood collected simultaneously for other biological analyses.

#### Western Blotting

Cell extracts were subjected to SDS-PAGE and Western blotting. We used antibodies against lamin A/C (SC-7292), prelamin A (SC-6214 and ANT0045), p53 (clone DO-1, ab80645), p21\(^{WAF-1}\) (556431), HDJ-2/DNAJ (clone KA2A5.6), and β-actin (A-5441). Anti-lamin A/C antibodies SC-7292 also recognize prelamin A. Protein bands were visualized with enhanced chemiluminescence Western blotting substrate. Quantification was performed versus β-actin with an image analyzer (ChemiGenius2) and computer software.

#### Cellular Senescence

Cellular senescence was evaluated by the cell population doubling level (PDL), the senescence-associated (SA) β-galactosidase activity, and the protein expression of cell cycle arrest markers, as previously described.15 At passages 2 and 8, the cumulative PDLs in control cells were 15 and 35, respectively. Dividing cells were identified by 5-bromodeoxyuridine incorporation.15 Nuclear morphological features were assessed by fluorescence microscopy after 4',6-diamidino-2-phenylindole staining.15 Nuclear structure was assessed as either normal (eg, ovoid nuclei) or dysmorphic (eg, lobulated or sticklike nuclei) by an observer blinded for treatment. Cell morphological features were evaluated by phase-contrast microscopy on X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside)-stained cells.

#### Oxidative Stress and Inflammation

The production of ROS was assessed by the oxidation of 5-[and 6]-chloromethyl-2,7-dichlorodihydrofluorescein diacetate, acetyl ester (CMH2DCFDA), C6827 and the reduction of nitroblue tetrazoium, as previously described.15 Results were normalized to DNA or protein level, respectively.

Interleukin (IL)-6, IL-8, and monocyte chemoattractant protein-1 were analyzed on 24-hour culture supernatant using commercially available technology (Luminox), devices (Bioplex 200), and multiplex human analyte assay kits (Procarta). Data analysis was performed using computer software (Bio-Plex Manager 4.1). The results were normalized to the protein content in the well. The sensitivity of the test was 10 pg/mL for all markers.

#### Statistics

The experiments were repeated 3 to 8 times. Results are expressed as mean±SEM, and statistical significance was determined using ANOVA and the Kruskal-Wallis nonparametric test, followed by a Fisher protected least-significant difference test for pairwise differences. \(P<0.05\) was considered significant. Statistical analysis was performed with computer software (StatView SAS software, version 5.0).

### Results

#### Long-Term PI Exposure Triggered Endothelial Cell Senescence

Ritonavir or lopinavir-ritonavir progressively decreased PDL values between passages 2 and 8 (30 days of treatment), up to values representing 55% and 27% of control, respectively, whereas PDL values in untreated and solvent (dimethyl sulfoxide)–treated cells did not vary (Figure 1A). DNA replication also decreased in response to PIs (Figure 1B): on day 30, 5-bromodeoxyuridine incorporation decreased by 4- to 5-fold compared with untreated or solvent-treated cells, whichever the PI used. Long-term PI exposure also increased the protein expression of the cell cycle arrest markers p53 and p21\(^{WAF-1}\) (Figure 1C).

Premature senescence of PI-treated endothelial cells was also demonstrated by morphological alterations (namely, increased size and cell flattening) (Figure 1D) and by the marked increase in SA–β-galactosidase activity (an up to 6-
Figure 1. PIs induced endothelial cell senescence and prelamin A accumulation. A, PDL values were evaluated from 3 to 4 experiments. B, 5-Bromodeoxyuridine (BrdU)-positive nuclei were examined by immunofluorescence microscopy (×20 magnification) and counted relative to total nuclei (4',6-diamidino-2-phenylindole [DAPI] staining, 3 experiments). C, Cell cycle arrest markers were identified by Western blotting. Representative blots (4 experiments) are shown, and quantification was performed. D, Cell morphological features were assessed in X-Gal-labeled cells examined by phase contrast microscopy (×20 magnification). Representative pictures (3 experiments) are shown. The scale bar indicates 50 μm. E, SA-β-galactosidase activity was assessed by the ratio of pH 6- to pH 4-positive blue staining (5 experiments). F, Lamin A, lamin C, and prelamin A (arrow) were revealed on day 30 by Western blotting. Representative blots (3 to 4 experiments) are shown, and quantification of prelamin A was performed. Similar pictures were obtained using anti–prelamin (ANT0045) antibodies. The scale bar indicates 10 μm. H, Dysmorphic nuclei were counted in control and PI-treated cells stained with DAPI at ×40 magnification (130 to 200 cells, 3 to 5 experiments). Nuclei showing 1 or several blebs or stick-like morphological features were considered dysmorphic. β-Actin was used as an index of protein loading. Results are expressed as the mean±SEM. *P<0.05 vs dimethyl sulfoxide (dmsO)-treated cells. lopi indicates lopinavir; rito, ritonavir; OD, optic density.
Adverse Effects
Pravastatin and FTI-277 Reversed PI Adverse Effects
The ability of pravastatin and FTI-277 to decrease the amount of farnesylated prelamin A and, thus, its cellular toxicity was assessed by the accumulation of prelamin A and the ratio of unfarnesylated to farnesylated HDJ-2 (Figure 3A and supplemental Figure, A [available online at http://atvb.ahajournals.org]). They increased the ratio of unfarnesylated to farnesylated HDJ-2 and reverse senescence markers, remained too brief to allow a significant recovery of nuclear membrane morphological features.

Pravastatin and FTI-277 almost normalized PI-induced oxidative stress and inflammation markers (IL-6, IL-8, and monocyte chemoattractant protein-1) (Figure 4C).

MnTBAP and NAC Reversed PI Adverse Effects
To further examine the mechanism of PI action, we checked for the impact of 2 antioxidants (MnTBAP and NAC). As expected, they reversed the long-term PI effects on oxidative stress (Figure 5A and B). More important, PI-induced protein expression of p53 and p21WAF-1 and SA–β-galactosidase activity were partly reversed by the antioxidants (Figure 5C and D and supplemental Figure, C and D), whereas prelamin A accumulation and nuclear dysmorphism were unaffected by MnTBAP and NAC (Figure 5E and F and supplemental Figure, A). The data suggest that oxidative stress plays a role in premature senescence of endothelial cells.

Long-Term PI Exposure Induced Prelamin A Accumulation and Nuclear Dysmorphism
Ritonavir and lopinavir-ritonavir induced the accumulation of prelamin A, which was progressive (data not shown) and maximal on day 30 (10- to 12-fold increase) (Figure 1F). Prelamin A accumulated at the nuclear periphery, in intranuclear membrane invaginations and nuclear blebs, whereas it was not observed in control or solvent-treated cells (Figure 1G). PIs also progressively (data not shown) altered the nuclear morphological features: on day 30, 18% to 22% of nuclei were dysmorphic in cells treated with ritonavir and lopinavir-ritonavir, compared with 7% to 10% in untreated or solvent-treated cells (Figure 1H).

Long-Term PI Exposure Induced Oxidative Stress and Inflammation
Long-term PI exposure markedly increased ROS production compared with control or solvent-treated cells. On day 30, PIs increased the oxidation of CM-H$_2$DCFDA and the reduction of nitroblue tetrazolium by 8- to 10-fold and 7- to 13-fold, respectively (Figure 2A and B). The 2 PIs also increased the secretion into the culture medium of the inflammatory mediators IL-6, IL-8, and monocyte chemoattractant protein-1 (2- to 3-fold) compared with control cells (Figure 2C).

MnTBAP and NAC Reversed PI Adverse Effects
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Figure 2. PIs induced oxidative stress and inflammation. A and B, ROS production was assessed by the oxidation of CM-H$_2$DCFDA (A) and the reduction of nitroblue tetrazolium (NBT) (B). The results (5 to 8 experiments) are expressed as the mean ± SEM. C, IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) were analyzed using 24-hour culture supernatants. Results (3 to 5 experiments) are expressed as the mean ± SEM. *P < 0.05 vs dimethyl sulfoxide (dms)–treated cells. lopi indicates lopinavir; rito, ritonavir.
Ritonavir-Boosted PI Therapy Increased Prelamin A and Senescence Markers in Human PBMCs From Well-Controlled HIV-Infected Patients

PBMCs from HIV-infected patients, aged 40 to 55 years, were evaluated and compared with those from 7 HIV-noninfected controls, aged 25 to 57 years, not receiving statin treatment (Table). As shown in Figure 6, prelamin A and senescence markers (p53 and p21<sup>WAF-1</sup>) were significantly increased in PBMCs from HIV-infected patients receiving treatment with a ritonavir-boosted PI (patients 3 to 6) compared with PBMCs from noninfected controls or from HIV-infected patients who did not receive PI treatment (patients 1 and 2). Interestingly, senescence markers were lower in PBMCs from PI-treated patients who received statin therapy (patients 5 and 6) compared with those who did not receive statin therapy (patients 3 and 4).

Discussion

To our knowledge, we demonstrate herein for the first time that 2 PIs frequently used in HIV therapy triggered premature senescence in human primary endothelial cells and that these effects might result from the accumulation of farnesylated prelamin A. We also detected prelamin A and senescence markers in mononuclear cells from HIV-infected patients receiving PI treatment.

Long-term exposure of HCAECs to ritonavir or lopinavir-ritonavir triggered the senescence program. These PIs were

Figure 3. Pravastatin and FTI-277 altered senescence markers in PI-treated HCAECs. Pravastatin or FTI-277 was added for the last 72 or 48 hours in cells incubated for 30 days with or without PIs. A and B, Prelamin A (antibodies SC-6214) and HDJ-2 (A) and p53 and p21<sup>WAF-1</sup> (B) were identified by Western blotting in cell lysates. The ratio of unfarnesylated to farnesylated HDJ-2 was determined and used as a marker of cellular farnesylation processes. β-Actin was used as an index of protein loading. Representative blots (3 experiments) are shown, and quantifications were presented in the supplemental Figure, A through D. C, SA–β-galactosidase activity was measured (3 experiments) as described in the legend to Figure 1. D, Dysmorphic nuclei were evaluated as described in the legend to Figure 1. Results (3 to 5 experiments) are expressed as the mean±SEM of the percentage of total nuclei. *P<0.05 vs dimethyl sulfoxide (dmso)–treated cells for the indicated treatment. #P<0.05 vs the effect of the respective PI in control cells. ns indicates not significantly different from the effect of the respective PI in control cells. lopi indicates lopinavir; rito, ritonavir.
selected because they display proatherogenic lipid profiles in HIV-infected patients\textsuperscript{37,38} and induce short-term endothelial cell dysfunction in experimental studies.\textsuperscript{7–11} In HCAECs, PIs decreased cell proliferation and division, increased protein expression of cell cycle blockers and SA–\textbeta–galactosidase activity, and altered cell and nuclear morphological features. They also increased ROS production and induced oversecretion of inflammation markers. These data are consistent with the high plasticity of endothelial cells that can rapidly be activated, enter into senescence, and induce vascular damages. Accordingly, senescent vascular endothelial cells are present in human atherosclerotic lesions of patients with age-related cardiovascular disease\textsuperscript{17,18} and in injured blood vessels of patients with severe progeroid syndromes,\textsuperscript{19,23} in which both endothelial and muscular vascular cells were damaged.\textsuperscript{20,23} Senescent endothelial cells exhibit features of oxidative stress\textsuperscript{17,39} and inflammation.\textsuperscript{40,41}

Our data showing that the antioxidants MnTBAP and NAC could improve PI-induced senescence markers in endothelial cells are consistent with the hypothesis that PI effects might be mediated by ROS overproduction. Many studies have shown that oxidative stress plays a major role in endothelial damage leading to cardiovascular complications and atherosclerosis.\textsuperscript{39} ROS have pleiotropic effects in endothelial cells and are key mediators of signaling pathways that underlie vascular inflammation in atherosclerosis.\textsuperscript{39,40} Oxidative stress can trigger the aging program in endothelial cells\textsuperscript{42} and other cell types\textsuperscript{15,16} via mitochondrial dysfunction. Mitochondria-derived ROS are increased by PI treatment coincident with mitochondrial dysfunction.\textsuperscript{15,16} Otherwise, mitochondrial dysfunction has been considered an initiating event in atherogenesis.\textsuperscript{43}

The beneficial effect of the farnesylation inhibitors, pravastatin and FTI-277, on senescence markers and aging-associated dysfunctions in PI-treated cells indicates the toxicity of farnesylated prelamin A. It was already known that genetically determined accumulation of permanently farnesylated forms of prelamin A was responsible for severe syndromes of accelerated aging with precocious cardiovascular diseases.\textsuperscript{19–21,44} PI treatment, via the inhibition of Zmpste24, also results in the accumulation of farnesylated prelamin A,\textsuperscript{34} retained at the nuclear rim and in intranuclear/transnuclear membrane invaginations.\textsuperscript{30,31,44,45} Decreasing the amount of farnesylated prelamin A with pravastatin or FTI-277 im-
Figure 5. Impact of MnTBAP and NAC on PI-induced effects. MnTBAP and NAC were added for the last 24 hours in cells incubated for 30 days with or without PIs. A and B, ROS production was assessed (3 to 5 experiments) as described in the legend to Figure 2. C and E, p53, p21WAF-1, and prelamin A were analyzed on Western blots. β-Actin was taken as an index of protein loading. Representative blots (3 to 4 experiments) are shown, and quantification was presented in the supplemental Figure, A, C, and D. D, SA–β-galactosidase activity (3 experiments) was measured as in the legend to Figure 1. F, Dysmorphic nuclei were evaluated as described in the legend to Figure 1. Results (3 to 5 experiments) are expressed as the mean±SEM. *P<0.05 vs dimethyl sulfoxide (dmso)–treated cells for the indicated treatment. #P<0.05 vs the effect of the respective PI in control cells. ns indicates not significantly different from the effect of the respective PI in control cells. lopi indicates lopinavir; rito, ritonavir.
proven cell cycle and senescence markers, production of ROS, and inflammatory cytokines. However, the farnesylation inhibitors failed to reverse PI-induced nuclear dysmorphies, probably resulting from their short-term use (24 to 72 hours); this use was probably too brief to reverse the progressive effect of PIs. Age-related inhibition of Zmpste24 was recently implicated in vascular cell senescence associated with physiological aging. In senescent VSMCs, pravastatin and FTI-277, which inhibit the farnesylation of prelamin A, could also improve cell function at different levels. The ability of MnTBAP and NAC to improve PI-induced senescence markers without decreasing either prelamin A or nuclear dysmorphic level strongly suggests that oxidative stress resulted from the toxicity of farnesylated prelamin A. This reinforced the idea that prelamin A accumulation is the initial toxic event involved in PI effects in endothelial cells and that antioxidants were acting downstream of statins.

We used PBMCs from HIV-infected patients receiving ritonavir-boosted PI therapy to address clinical relevance to our in vitro studies. A few studies performed with human circulating cells linked HIV infection and therapy with cardiovascular disease and premature senescence. HIV infection has been associated with accelerated aging of PBMCs, and senescence markers on CD4+ and CD8+ lymphocytes have been recently related to altered cardiovascular function in patients with well-controlled HIV infection; otherwise, ritonavir can modulate several genes implicated in lipid metabolism, inflammation, and atherosclerosis in isolated PBMCs. We observed that ritonavir-boosted PI (but not nucleoside reverse transcriptase inhibitor) therapy could induce prelamin A accumulation and senescence markers in PBMCs from middle-aged HIV-infected patients with an undetectable viral load. Whether an older age and/or a higher viral load would promote premature senescence of PBMCs remains to be investigated. Interestingly, senescence markers were improved in patients cotreated with PI and statin, in keeping with the negative role for farnesylated prelamin A.

Our experimental study has some limitations. It may not account for many factors involved in the accelerated atherosclerotic process in HIV-infected patients, including active metabolites, pharmacokinetic parameters, HIV infection itself, immune balance, environmental factors, and genetic predisposition. Moreover, endothelial cells are outside their usual environment and cannot recruit macrophages or other inflammatory cells, which participate in the formation and leakage of the atherosclerotic plaque. Our results in isolated patients’ PBMCs are descriptive and do not demonstrate a mechanism. However, when examined together with our in vitro results on endothelial cells, they strongly suggest that statins may be beneficial in preventing or delaying premature...

Table. PBMC Studies: Characteristics of Patients

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<th>Patient No./Age, y</th>
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<th>Statin Treatment During the Previous Year</th>
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<td>None, 15</td>
<td>Lopinavir, 15; ritonavir, 15</td>
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HIV viral load, copies/mL, <20.

ABC indicates abacavir; DDI, didanosine; FTC, emtricitabine; NA, not available; NRTI, nucleoside or nucleotide reverse transcriptase inhibitor; PI, protease inhibitor; TDF, tenofovir; ZDV, zidovudine.

Figure 6. Senescence markers in PBMCs from HIV-infected or noninfected patients. PBMCs were isolated from blood collected from the indicated HIV-infected patients (1 to 6) and from 7 controls (Table). Cell lysates were subjected to Western blot with antibodies against lamin A/C, prelamin A, p53, and p21WAF-1. β-Actin was used as an index of protein loading. Representative blots from 3 experiments are shown, and quantification was performed. Results from noninfected control subjects are expressed as the mean ± SEM.
senescence. More clinical data are required to confirm this proposition.

In conclusion, these data provide the first experimental support that ritonavir-boosted PIs are associated with premature senescence markers in both cultured endothelial cells and PBMCs of HIV-infected patients. This suggests that PI therapy might participate in the early development of cardiovascular disease in HIV-infected patients. The ability of statins to improve PI-induced vascular senescence could be beneficial in HIV-infected patients.

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Supplement material
Figure I: Effects of farnesylation inhibitors and antioxidants on PI effects. Quantification of the results presented in figures 5E (a), 3A (a,b) and 3B and 5C (c,d).