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Antiretroviral compounds and cholesterol efflux from macrophages

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Abstract

Objective: HIV infection is associated with elevated risk of cardiovascular disease. The effect of anti-retroviral drugs on metabolism of atherogenic very low and low density lipoproteins is well studied, but a possible effect of these drugs on reverse cholesterol transport is still unclear. The objective of this study was to assess the effect of various classes of anti-HIV drugs on cellular cholesterol efflux.

Methods: The effect of pharmacological concentrations of seven commonly used antiretroviral compounds, Stavudine, Efavirenz, Nevirapine, Lopinavir, Amprenavir, Nelfinavir and Ritonavir, on cholesterol efflux from RAW 264.7 mouse macrophages and human monocyte-derived macrophages to apolipoprotein A-I and high density lipoprotein was tested.

Results: At high pharmacological concentration Nelfinavir and Ritonavir inhibited cholesterol efflux, while other compounds had no effect. However, the same concentrations of Nelfinavir and Ritonavir induced apoptosis, suggesting that the effect of these compounds on cholesterol efflux most likely resulted from their cytotoxicity. When tested in non-cytotoxic concentrations, Nelfinavir and Ritonavir did not affect cholesterol efflux from RAW 264.7 cells, human monocyte-derived macrophages, or human macrophages infected with HIV-1.

Conclusions: We conclude that tested antiretroviral compounds do not have a specific effect on cholesterol efflux.

Keywords

Cholesterol efflux; reverse cholesterol transport; anti-retroviral compounds; atherosclerosis

1. Introduction

HIV-1 infection is associated with elevated risk of atherosclerosis and coronary artery disease (CAD) [1]. The key elements in pathogenesis of atherosclerosis are disturbances in lipid and

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lipoprotein metabolism resulting in accumulation of cholesterol in vascular cells. There are two major contributors to this process. Hypercholesterolemia leads to increased delivery of cholesterol to cells. Diminished ability of vascular cells to release excessive cholesterol to plasma acceptors, due to either low level of these acceptors or impairment of cellular pathways of cholesterol efflux, is another mechanism of cholesterol accumulation. We have recently demonstrated that HIV-1 infection dramatically inhibits cholesterol efflux from macrophages, leading to accumulation of cholesterol in these cells [2]. HIV infection also affects plasma lipoprotein metabolism causing hypoalphalipoproteinemia [3,4]. The effects of HIV infection, however, usually coexist with the effects of the drugs used to treat this infection. Highly Active Antiretroviral Therapy (HAART), in particular its protease inhibitor component, affects plasma lipoprotein metabolism causing elevation of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) levels [5–7]. Combination of hypercholesterolemia caused by HAART and deficiency of cholesterol efflux and hypoalphalipoproteinemia caused by the infection is likely to be a major contributor to enhanced atherosclerosis in HIV-infected patients [8]. While the role of HIV in impairment of reverse cholesterol transport (RCT) has been established [2,9], it is also possible that components of HAART may have an independent effect on cellular stages of RCT, thus further increasing the risk of atherosclerosis in HIV patients. For example, it was shown recently that Ritonavir, one of the widely used protease inhibitors, inhibits cholesterol efflux from macrophages [10].

In this study, we tested the effect on cholesterol efflux of pharmacological concentrations of seven compounds belonging to different classes of antiretroviral agents and widely used as components of HAART.

2. Materials and Methods

Materials

Nelfinavir was obtained from Hoffman La Roche. All other antiretroviral compounds were a kind gift from NIH AIDS Research and Reference Reagent Program. Stock solution ($\times 100$) of Stavudine was prepared in saline; stock solutions of all other compounds were prepared in DMSO.

Cells and HIV infection

RAW 264.7 mouse macrophage cells were obtained from ATTC. When indicated RAW 264.7 cells were transfected with pcDNA3 containing FoxO gene (kind gift of Dr. E. Woodcock) as described previously [11]. Monocyte-derived macrophages (MDMs) were prepared from peripheral blood mononuclear cells of normal donors using adherence to plastic, and differentiated in the presence of macrophage colony-stimulating factor. Macrophage-tropic HIV-1 strains ADA was used for infection. All infections were performed using 3.5×10^6 cpm of RT activity per 10^6 cells.

Lipoproteins

LDL and HDL were isolated from human plasma (pooled plasma supplied by Red Cross) by sequential centrifugation. Apolipoprotein A-I was isolated from HDL as described previously [12]. LDL was acetylated as described by Basu et al. [13].

Cholesterol efflux

Cellular cholesterol was labeled by incubation in serum-containing medium with [1α , 2α (n)- ^3H]-cholesterol (GE Health-Amersham, final radioactivity 0.5 MBq/ml) for 48 h in a CO_2 incubator. Cells were then washed and incubated for 18 h at 37°C in serum-free medium in the presence of indicated concentrations of the compounds or a vehicle and in the presence

or absence of the LXR agonist TO-901317 (4 $\mu\text{mol/L}$). When indicated, acetylated LDL was also added at the final concentration of 50 $\mu\text{g/ml}$ to load cells with cholesterol. Cells were washed and incubated for another 3 h at 37°C in serum-free medium containing 30 $\mu\text{g/ml}$ of lipid-free apoA-I or HDL. Cholesterol efflux was expressed as the proportion of [^3H] cholesterol transferred from cells to medium. Non-specific efflux (i.e. the efflux in the absence of an acceptor) was subtracted. Cholesterol efflux from HIV-infected MDMs was measured at the peak of infection (7–10 days after infection).

Cell death assays

Cells were incubated with the indicated concentrations of antiretroviral compounds in serum-free medium for 18 h in the presence or absence of acetylated LDL (final concentration 50 $\mu\text{g/ml}$). Cells were analyzed for apoptosis by Cell Death Detection ELISA (Roche) measuring nucleosome enrichment in the cells. Cytotoxicity was also assayed using a commercial lactate dehydrogenase (LDH) cytotoxicity assay kit (Cayman, Ann Arbor, MI) following manufacturer's instructions. For MDMs, Annexin V-FITC Apoptosis Detection kit (Calbiochem) was used, and results were analyzed by flow cytometry following manufacturer's instructions.

Statistics

All experiments were reproduced 2–4 times and representative experiments are shown. Unless otherwise indicated, experimental groups consisted of quadruplicates; means $\pm$ SEM are presented. The Student's *t*-test was used to determine statistical significance of the differences.

3. Experimental Results

Seven commonly used antiretroviral drugs belonging to three different classes were tested for their effect on cholesterol efflux from murine macrophages, RAW 264.7. At a concentration of 10 $\mu\text{mol/L}$, which is higher than plasma levels found in patients treated with these drugs [14], Stavudine (NRTI), Efavirenz (NNRTI), Nevirapine (NNRTI), Lopinavir (PI) and Amprenavir (PI) did not affect the efflux of cholesterol from macrophages to apolipoprotein A-I (apoA-I) (Fig. 1A, B) and HDL (Fig. 1C). On the other hand, Nelfinavir (PI) and Ritonavir (PI) used at the same concentration, caused a 50% inhibition of the efflux to apoA-I (Fig. 1A). A similar effect was observed when cells were stimulated with LXR agonist, TO-901317, conditions where ABCA1-dependent cholesterol efflux becomes the predominant pathway (Fig. 1B). When cholesterol efflux to HDL was tested, Nelfinavir caused a 30% inhibition of cholesterol efflux, while Ritonavir did not affect the efflux (Fig. 1C). Thus, the only compounds affecting cholesterol efflux were Nelfinavir and Ritonavir.

The concentration of the two PIs affecting cholesterol efflux was similar to that used in previous studies and considered pharmacologically relevant [10,15], but was above the concentrations found in patients' plasma in another study [14]. Therefore, dose-dependence of the effects of Nelfinavir and Ritonavir on cholesterol efflux from LXR agonist-activated RAW 264.7 cells to apoA-I was studied (Fig. 2A). Concentrations of the drugs from 0.1 $\mu\text{mol/L}$ to 30 $\mu\text{mol/L}$ were tested, however, at 30 $\mu\text{mol/L}$ the viability of cells dropped dramatically making results un-interpretable. Ritonavir slightly increased cholesterol efflux at concentrations 1 and 5 $\mu\text{mol/L}$, the reasons for this effect are unclear. Both Nelfinavir and Ritonavir inhibited cholesterol efflux at concentrations above 5 $\mu\text{mol/L}$ (Fig. 2A).

The dramatic effect of high dose of Nelfinavir and Ritonavir on cell viability raised a possibility that the effect of these drugs on cholesterol efflux may be a result of cytotoxicity. The toxicity of all compounds was assessed using the cell death assay (nucleosome enrichment). Incubation of RAW 264.7 cells for 18 h with 10 $\mu\text{mol/L}$ of Ritonavir or Nelfinavir led to pronounced

cytotoxic effect, while incubation with the same concentration of other compounds did not (Fig. 2B). Although these findings suggest that inhibition of cholesterol efflux by Ritonavir and Nelfinavir may be caused by toxicity of these compounds, an alternative explanation is also possible. Excessive concentration of cellular cholesterol is toxic [16] and toxicity may be a consequence, rather than a cause, of inhibition of cholesterol efflux. To test this possibility, we repeated the efflux experiments with cells loaded with cholesterol by incubation with AcLDL, a condition similar to that used by others when testing the effect of antiretroviral compounds on cholesterol efflux [10]. If cytotoxicity of the drugs was secondary to cholesterol efflux impairment, one would expect to see reduced cholesterol efflux and increased cytotoxicity in cholesterol-loaded cells. When cholesterol loaded cells were tested, Ritonavir affected cholesterol efflux only at the highest concentration (30 $\mu\text{mol/L}$), while Nelfinavir did not affect cholesterol efflux at concentrations up to 10 $\mu\text{mol/L}$, with dramatic drop in cell viability at 30 $\mu\text{mol/L}$ (Fig. 2C). A dose-response analysis demonstrated that Ritonavir and Nelfinavir induced cell death at concentrations above 1 $\mu\text{mol/L}$ in unloaded cells and only modest effect on cell death at highest concentration in cholesterol-loaded cells (Fig. 2D), i.e. at the same concentrations where the effect on cholesterol efflux was observed. When toxicity of the compounds was assessed by measuring LDH activity in cell culture medium (which reflects compromised integrity of the plasma membrane), Ritonavir did not cause changes in LDH release across the range of concentrations used, while Nelfinavir caused dramatic release of LDH at 30 $\mu\text{mol/L}$ (Fig. 2E). Thus, loading cells with cholesterol moderated the effects of Nelfinavir and Ritonavir on cholesterol efflux, arguing against the possibility that cytotoxicity was the consequence of cholesterol efflux impairment and suggesting that direct toxicity of the drugs is likely the main cause of reduced cholesterol efflux.

To further examine the relationship between apoptosis and cholesterol efflux we induced apoptosis in RAW 264.7 cells by transfecting the cells with an established mediator of apoptosis, FoxO. FoxO induced cell death and inhibited cholesterol efflux to apoA-I (Table 1). This finding supports our conclusion that the effect of Nelfinavir and Ritonavir on cholesterol efflux results from cytotoxicity of the drugs.

We further examined the effects of Ritonavir and Nelfinavir on apoptosis and cholesterol efflux from human monocyte-macrophages (MDM) infected or not with HIV-1. After a 24 h incubation with Ritonavir, the proportion of apoptotic cells increased dose-dependently within a concentration range of 0.1–30 $\mu\text{mol/L}$ (Fig. 3A). Incubation of MDM with Nelfinavir for 24 h increased apoptosis only at the highest concentration of 30 $\mu\text{mol/L}$ (Fig. 3A). Consistent with our previous finding [2], HIV-1 infection reduced cholesterol efflux from human macrophages to apoA-I by over 50% (Fig. 3B, D). When tested at concentration of 1 $\mu\text{mol/L}$, Ritonavir did not affect cholesterol efflux to apoA-I from uninfected or HIV-infected human macrophages (Fig. 3B). Incubation of uninfected MDM with Nelfinavir (0.1–30 $\mu\text{mol/L}$, 24 h) affected cholesterol efflux to apoA-I only at highest concentration 30 $\mu\text{mol/L}$ (Fig. 3C). Nelfinavir did not affect cholesterol efflux from HIV-infected macrophages at concentration 10 $\mu\text{mol/L}$ (Fig. 3D).

4. Discussion

The main finding of this paper is that seven commonly used antiretroviral compounds belonging to three different classes of drugs used for anti-HIV therapy did not affect cholesterol efflux from macrophages at non-cytotoxic concentrations. This conclusion is in direct contradiction to the findings of Wang et al. [10] who reported that Ritonavir inhibited cholesterol efflux from macrophages. In our experiments, Ritonavir and another PI, Nelfinavir, also inhibited cholesterol efflux from macrophages, however, this effect correlated with cytotoxicity of these two compounds. The mechanisms of cholesterol efflux inhibition described by Wang et al. included decreased expression of several proteins, increased

production of superoxide anion, decrease of mitochondrial membrane potential and ATP production, and ERK 1/2 activation [10]. All these changes are also consistent with apoptosis [17]. The toxic effect of Ritonavir has been observed for several cell types [15,18]. In this study, both Ritonavir and Nelfinavir induced apoptosis at concentrations inhibiting cholesterol efflux, whereas non-toxic concentrations did not inhibit cholesterol efflux. Interestingly, while Ritonavir was toxic for all cell types tested, toxicity of Nelfinavir toward human MDM was significantly lower than toward RAW 264.7.

Our results argue against the possibility that apoptosis was a consequence, rather than a cause, of cholesterol efflux inhibition by the drugs. Indeed, cholesterol efflux from RAW 264.7 cells activated with LXR agonist and treated with Nelfinavir or Ritonavir was higher than in untreated non-activated cells, but still the drugs caused apoptosis. Further, loading of cells with cholesterol did not exaggerate the effects of the compounds as would be expected if the cause of toxicity was inhibition of cholesterol efflux. We therefore conclude that the effect of Ritonavir and Nelfinavir on cholesterol efflux is likely due to toxicity of the drugs and that at non-toxic concentration none of the tested compounds affected cholesterol efflux from macrophages *in vitro*.

HIV infection and its treatment are associated with a significantly increased risk of development of atherosclerosis and CAD [4]. The potential mechanisms include enhanced delivery of cholesterol to vessel wall due to hypercholesterolemia and enhanced uptake of lipoproteins by macrophages; both of these pathogenic effects have been attributed to anti-retroviral compounds. The mechanism of compensatory removal of excess cholesterol from macrophages is also impaired, but this impairment is apparently caused mainly by HIV itself [2] with no evidence for specific contribution of antiretrovirals to the cellular pathways of reverse cholesterol transport. Thus, antiretroviral therapy contributes to the high risk of atherosclerosis in HIV-infected patients mainly by enhancing forward cholesterol transport, while HIV itself seems to be responsible for the impairment of protective pathway, reverse cholesterol transport.

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Figure 1. The effect of antiretroviral compounds on cholesterol efflux from RAW 264.7 murine macrophages

RAW 264.7 cells were labeled by incubation in serum-containing medium with [3 H]-cholesterol for 48 h. Cells were then washed and incubated with the compounds at a final concentration of 10 μ mol/L or a vehicle, in the absence (A, C) or presence (B,) of the LXR agonist TO-901317 (4 μ mol/L), followed by 3 h incubation with 30 μ g/ml of lipid-free apoA-I (A, B), HDL (C) or no acceptor. The radioactivity of the medium and cells was counted in a β -counter. Cholesterol efflux was expressed as the proportion of [3 H]cholesterol transferred from cells to medium after subtraction of non-specific efflux (i.e. efflux in the absence of an acceptor). PI- protease inhibitors; NNRTI – non-nucleoside reverse transcriptase inhibitors;

NRTI – nucleoside analog reverse transcriptase inhibitors. Mean $\pm$ SEM of quadruplicate determination of a representative experiment out of three performed is shown, * $p < 0.01$ versus vehicle.

Figure 2. The effect of Nelfinavir and Ritonavir on cholesterol efflux and cell death

A, C - RAW 264.7 cells were labeled by incubation in serum-containing medium with [^3H]-cholesterol for 48 h. Cells were then washed and incubated with indicated concentrations of Nelfinavir (circles) or Ritonavir (triangles), in the presence of the LXR agonist TO-901317 (4 $\mu\text{mol/L}$) and in the presence (C) or absence (A) of acetylated LDL (final concentration 50 $\mu\text{g/ml}$). Cells were then washed and incubated for 3 h with 30 $\mu\text{g/ml}$ of lipid-free apoA-I or no acceptor. The radioactivity of the medium and cells was counted in a β -counter. Cholesterol efflux was expressed as the proportion of [^3H]-cholesterol transferred from cells to medium after subtraction of non-specific efflux (i.e. efflux in the absence of an acceptor). Mean $\pm$ SEM of quadruplicate determination of a representative experiment is shown.

B - RAW 264.7 macrophages were incubated with the indicated compounds at concentration 10 $\mu\text{mol/L}$ in serum-free medium for 18 h. Cells were analyzed for apoptosis by Cell Death Detection assay measuring nucleosome enrichment in the cells.

D - RAW 264.7 macrophages were incubated with the indicated concentrations of Nelfinavir (circles) or Ritonavir (triangles) in the presence (open symbols) or absence (closed symbols) of acetylated LDL (final concentration 50 $\mu\text{g/ml}$) in serum-free medium for 18 h. Cells were analyzed for apoptosis by Cell Death Detection assay measuring nucleosome enrichment in the cells.

E - RAW 264.7 macrophages were incubated with the indicated concentrations of Nelfinavir (circles) or Ritonavir (triangles) in the presence (open symbols) or absence (closed symbols) of acetylated LDL (final concentration 50 $\mu\text{g/ml}$) in serum-free medium for 18 h. Cytotoxicity was assayed using a commercial LDH cytotoxicity assay kit.

Figure 3. The effect of Nelfinavir and Ritonavir on apoptosis (A) and cholesterol efflux (B–D) from human monocyte-derived macrophages

A- MDM were incubated with indicated concentrations of Ritonavir or Nelfinavir for 24 h and the proportion of apoptotic cells was determined by flow cytometry using Annexin V-FITC Apoptosis Detection kit.

B–D - Triplicate cultures of MDMs were incubated with Ritonavir (**B**, 1 μmol/L) or Nelfinavir (**C**, indicated concentrations, and **D**, 10 μmol/L) for 24 h and used for analysis of cholesterol efflux as described in Materials and Methods. In experiments presented in panels B and C, cells were infected or not with HIV-1_{ADA} (note, cells from different donors were used for panels B and D) and compounds were added to cells at the peak of infection (7–10 days after infection). Filled bars – vehicle, hatched bars – compound. Results are mean ± SD and show one representative experiment out of two performed. * $p < 0.01$ (B, D - versus uninfected; C - versus control).

Table 1**Induction of apoptosis and cholesterol efflux**

RAW 264.7 cells were transfected with cDNA containing FoxO gene or mock-transfected. After 24 h nucleosome enrichment and cholesterol efflux were measured as described in “Materials and Methods”

Cells	Nucleosome enrichment (fold of control)	Cholesterol efflux (%)
RAW/Mock	1	2.34±0.25
RAW/FoxO	2.1	1.85±0.17*

*
p<0.05