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Suppression of Pre Adipocyte Differentiation and Promotion of Adipocyte Death by Anti-HIV Drugs

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Abstract. *In the present study, we investigated the ability of anti-HIV drugs to interfere with normal cell cycle progression and to induce oxidative stress by perturbing the redox environment. Our results provide evidence that anti-HIV drugs have a differential effect on adipocyte cell cycle and differentiation, being able to modify the response to oxidative stress through an increase of reactive oxygen species (ROS) that compromises the induction of phase-2 and antioxidant enzymes. In detail, saquinavir, efavirenz, and stavudine exert antiadipogenic influences on the model 3T3-L1 cell line, perturbing the oxidative response and inducing of apoptosis. When considered together, the effects of anti-HIV drugs on 3T3-L1 pre adipocytes are distinct but commonly antiadipogenic, thus suggesting another additional possible mechanism by which antiretroviral therapies could contribute to lipoatrophy.*

The introduction of highly active antiretroviral therapy (HAART) has significantly changed the clinical course of HIV disease, with prolonged survival and better quality of life for HIV-infected patients. However, these combination therapy regimens have been demonstrated to cause a metabolic syndrome consisting of subcutaneous adipose tissue atrophy and increased visceral and dorsocervical adipose tissue (1). Other symptoms include dyslipidemia (2-4), hyperglycemia (5, 6), and insulin resistance (7). The complex of these metabolic alterations is commonly indicated by the term lipodystrophy syndrome.

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The cause of the lipodystrophy syndrome may be a complex physiological response to multiple factors, including one or more components of combination drug regimens, viral infection, or effective viral suppression. Currently, the cause of this syndrome, referred to hereafter as HIV/HAART-associated syndrome (HAS), is unknown.

Previous studies have shown that in human aortic endothelial cells (HAECs), protease inhibitors (PIs) alone and as part of a HAART regimen, increased generation of reactive oxygen species (ROS) (8). Nelfinavir significantly increased ROS generation and suppressed cytosolic superoxide dismutase (SOD) levels, which can also lead to necrosis of adipocytes (9).

In a previous report (10), our research group evaluated five antiretroviral drugs (indinavir, amprenavir, efavirenz, stavudine and saquinavir), belonging to the three main classes of anti-HIV drugs, were able to affect adipocyte differentiation in our experimental model. We showed that these antiviral agents inhibit pre adipocyte differentiation. Saquinavir, efavirenz and stavudine were able to drastically inhibit the increase in protein expression of both CCAAT/enhancer-binding proteins (C/EBPα) and peroxisome proliferator-activated receptors (PPARγ), while indinavir and amprenavir were only able to partially affect the protein expression of these adipocyte differentiation markers (10).

Our previous study only evaluated the effect of antiviral drugs on the adipocyte differentiation. However, pre adipocyte proliferation is another relevant topic because cells undergo clonal expansion prior to differentiation (11). Inhibition of pre adipocyte proliferation may represent an additional mechanism by which antiviral agents contribute to lipoatrophy, by reducing the number of precursor cells available for differentiation. Another aspect not to be underestimated is that several antiviral drugs promote adipocyte cell death (12). Thus, antiretroviral drugs might promote adipose tissue atrophy by promoting adipocyte loss and preventing replacement of lost adipocytes by inhibiting pre adipocyte differentiation.

In the present study, we analyzed whether these same antiviral drugs are able to modify cell cycle of pre adipocyte

Table I. *Human primer sense and anti-sense sequences and expected PCR products (bp).*

Gene	Sense and antisense sequences	Conditions	bp
HO1	5' GTGTAAGGACCCATCGGAGA-3'	40 cycles at 94˚C for 5 s	153
	5' ATGCACCAAGGACCAGAGC-3'	60˚C for 3 s, 72˚C for 6 s	
SOD 1	5' ACATTGCCCAAGTCTCCAAC-3'	45 cycles at 94˚C for 5 s	271
	5' AGGGCATCATCAATTCGAG-3'	60˚C for 6 s, 72˚C for 11 s	
NQO1	5' GGCTGAACAAAAGAAGCTGG-3'	40 cycles at 94˚C for 5 s	246
	5 AATGACATTCATGTCCCCGT-3'	60˚C for 5 s, 72˚C for 10 s	

HO1: Heme oxygenase-1; SOD1: superoxide dismutase-1; NQO1: phase II detoxifying enzyme NAD(P)H:quinone oxidoreductase-1.

and to induce apoptosis. Moreover we compared the mRNA expression of genes encoding antioxidant and phase II detoxifying enzymes in a cell line treated with antiretroviral drugs during differentiation.

Materials and Methods

Cell culture. 3T3-L1 pre adipocytes were cultured and differentiated with standard protocol as previously described (10). Briefly, 3T3-L1 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in humidified atmosphere of 5% CO_2 and 95% air at 37˚C to confluence. Two days later, the induction of adipocyte differentiation was initiated by treatment of the cells with the differentiation medium containing 1 μM insulin, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine for two days, followed by two days of treatment with the medium containing 1 μM insulin alone. The medium was replaced every two days for the following six days.

Chemicals and treatments. Indinavir was kindly provided by Merck Sharp and Dohme ltd (Hoddesdon, Hertfordshire, UK), and amprenavir was kindly provided by Glaxo Group Ltd (Greenford, Middlesex, UK). Saquinavir and efavirenz and stavudine were purchased from Roche Farma S.A. (Leganes, Spain), and Bristol-Myers Squibb (Meymac France) respectively. Saquinavir, efavirenz, stavudina and amprenavir were dissolved in dimethyl sulfoxide, while indinavir was dissolved in water at a final concentration of 30 or 10 mM respectivly. Drug treatment was performed as described previously (10). Briefly, 3T3-L1 pre adipocytes were cultured and differentiated with standard protocol. The induction of adipocyte differentiation was initiated by treatment of the cells with the differentiation medium and with the drug.

Flow cytometry for cell cycle analysis. Following treatment with antiviral agents and the induction of differentiation, the 3T3-L1 cells were collected and centrifuged at 2000 × g at 4˚C. The pellets were fixed in 1 ml of 70% ethanol for 4 h at -20˚C. Then cells were centrifuged at 2000 × g at 4˚C, washed with phosphate buffered saline (PBS) and incubated in 500 ml of a hypotonic buffer (0.1% TritonX-100, 0.1% sodium citrate, 50 mg/ml propidium iodide, and 100 mg/ml RNase). Twenty thousand cells were analyzed each sample using a Becton Dickinson FACSCalibur flow cytometer (Franklin Lakes, NJ, USA) and the percentages of G_1, S, G_2/M, and sub-G_1 (apoptotic cells) populations were calculated by ModFit version III software (Verity Software House, Topsham, ME, USA), respectively.

Real-time polymerase chain reaction (PCR). Total RNA was isolated with the High Pure RNA Isolation Kit (Roche Diagnostics, Milan, Italy). One hundred nanograms of total cellular RNA were reverse-transcribed (Expand Reverse Transcriptase; Roche Diagnostics, Milan, Italy) into complementary DNA (cDNA) using random hexamer primers (random hexamers, Roche Diagnostics, Milan, Italy), at 42˚C for 45 min, according to the manufacturer's instructions. The resulting cDNA was subjected to real-time PCR analysis by rapid cycling in glass capillaries with a thermocycler (Light-Cycler; Roche Molecular, Milan, Italy). The reaction was performed in a final volume of 20 μl with Light-Cycler-DNA Master SYBR Green I (Roche Diagnostics Milan, Italy), which contains nucleotides, *Taq* DNA polymerase, buffer, SYBR Green I dye and 10 mM $MgCl_2$; cDNA (2 μl), primers and sterile water were added. For primer sequences, PCR conditions and size of products see Table I. Each real-time PCR was carried out three times for each sample. SYBR Green I fluorescence was monitored at the end of each cycle to assess the amount of PCR product formed. After completion of the amplification protocol, a melting curve analysis was performed to confirm the specificity of amplification by cooling the sample to 65˚C at a rate of 20˚C/s, maintaining a temperature of 65˚C for 10 s and then heating at a rate of 0.2˚C/s to 95˚C, with continuous measurement of fluorescence. Cycle to cycle fluorescence emission readings were monitored and analysed by Light Cycler Data Software (Roche Molecular Biochemicals, Milan, Italy). The specificity of the amplification products was verified by electrophoresis on a 1.4% agarose gel stained with ethidium bromide (1 μg/ml). Sequence-specific standard curves were generated using serial dilution of specific DNA standards. All quantifications were normalised to the quantity of the housekeeping gene β-actin. The data are presented as the ratio of the target gene expression and the gene expression in unstimulated conditions.

Statistical analysis. Each experiment was performed in triplicate. The results are expressed as means±standard deviations (SD). Student's *t*-test was used to determine statistical differences between the means, and $p<0.05$ was considered a significant difference.

Results

Anti-HIV drugs induce apoptosis of 3T3-L1 cells. To assess whether anti-HIV drugs induce alteration in cell cycle progression, we evaluated their effect on cell cycle phase distribution. Different anti-HIV drugs were added to culture

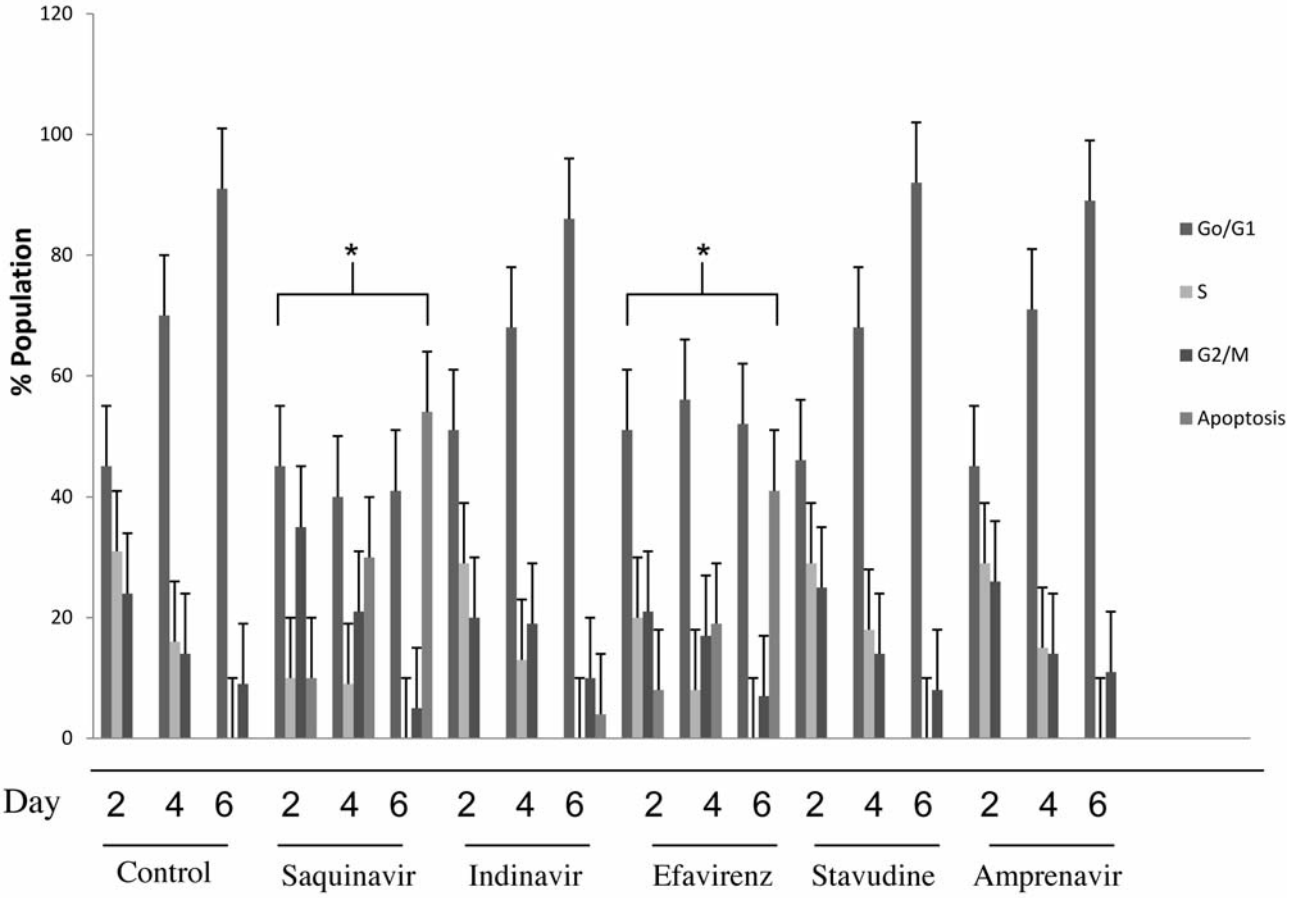

Figure 1. *Cell cycle distribution of 3T3-L1 cells treated with anti-HIV drugs compared to untreated cells. The effect of anti-HIV drug treatment in 3T3-L1 pre-adipocytes on cell cycle distribution and apoptosis. The quantified values are the reported as means of three independent experiments; asterisks indicate significant difference ($p<0.05$) vs. the respective control as calculated by Student's t-test.*

medium as previously described (10), and cells were induced to differentiate using the standard 6 days protocol. 3T3-L1 cells was collected at 2, 4 and 6-day at start differentiation course and the FACS analysis were performed.

As shown in Figure 1, the treatment with indinavir, amprenavir and stavudine did not produce significant differences in the cell cycle distribution with respect to the untreated 3T3-L1 control cells. In contrast, treatment with saquinavir and efavirenz caused an increase of the sub-G1 peak compared to 3T3-L1 control cells, indicative of apoptotic cell death.

Anti-HIV drugs modified mRNA levels of superoxide dismutase-1 (SOD1), heme oxygenase-1 (HO-1) and phase II detoxifying enzyme NAD(P)H:quinone oxidoreductase-1 (NQO1). In order to investigate whether anti-HIV drugs act on the adipose tissue *via* oxidative stress, we evaluated mRNA expression of the antioxidant enzymes-1 (SOD1), heme oxygenase-1 (HO1) and phase II detoxifying enzyme NAD(P)H:quinone oxidoreductase-1 (NQO1).

RNAs both of 3T3-L1 control cells and anti-HIV drug-treated cells were collected and extracted at days 2, 4 and 6 after induction and examined by real time PCR. Saquinavir-, efavirenz- and stavudine-treated 3T3-L1 cells exhibited down regulation of induction of *SOD1* and *NQO1* mRNA compared both to indinavir- and amprenavir- treated and control 3T3-L1 cells. However, *HO1* mRNA levels decreased slightly during differentiation in response to saquinavir, efavirenz and stavudine treatment compared to treatment with indinavir and amprenavir and 3T3-L1 control cells where the *HO1* mRNA levels were lower (Figure 2a, 2b and 2c).

Discussion

In the present study, we investigated the ability of five anti-HIV drugs to interfere with normal cell cycle progression and to induce oxidative stress by perturbing the redox environment.

We did not find any significant differences in the cell cycle distribution of 3T3-L1 cells treated with indinavir, amprenavir and stavudine compared to the 3T3-L1 control

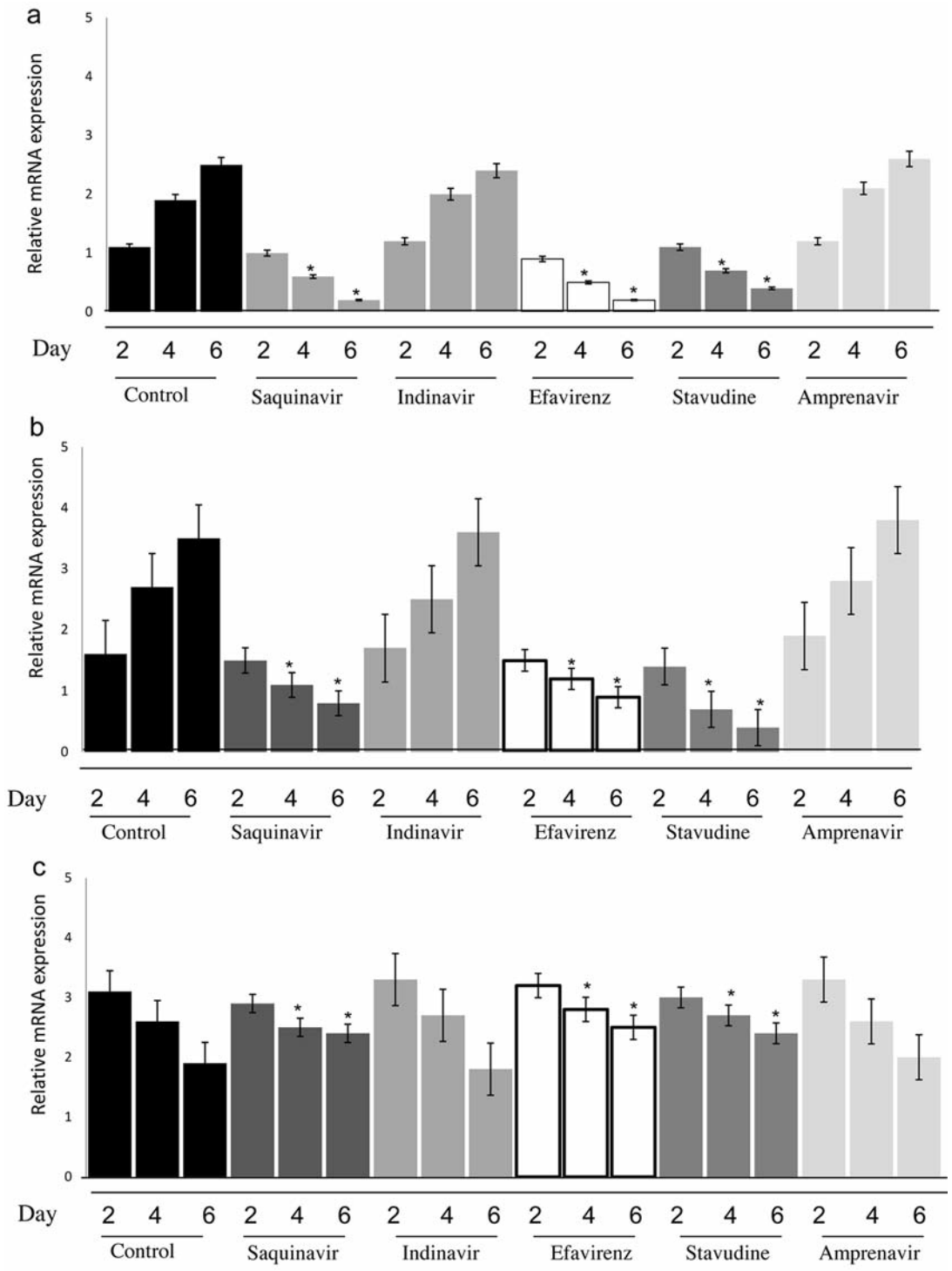

Figure 2. *a. Real-time PCR analysis using specific primers for superoxide dismutase-1 (SOD1). Expression of SOD1 in 3T3-L1 pre-adipocytes treated with anti-HIV drugs. The quantified values are reported as the means of three independent experiments; asterisks indicate significant difference ($p<0.05$) vs. the respective control calculated as by Student's t-test. b. Real-time PCR analysis using specific primers for phase II detoxifying enzyme NAD(P)H:quinone oxidoreductase-1 (NQO1). Expression of NQO1 in 3T3-L1 pre-adipocytes treated with anti-HIV drugs. The quantified values are reported as the means of three independent experiments; asterisks indicate significant difference ($p<0.05$) vs. the respective control as calculated by Student's t-test. c: Real-time PCR analysis using specific primers for heme oxygenase-1 (HO-1). Expression of HO1 in 3T3-L1 pre-adipocytes treated with anti-HIV drugs. The quantified values were reported as the means of three independent experiments; asterisks indicate significant difference ($p<0.05$) vs. the respective control as calculated by Student's t-test.*

cells, however, we did observe that 3T3-L1 cells treated with saquinavir and efavirenz presented an apoptotic peak, in accordance with a recent report suggesting that another drug, nelfinavir, may contribute to adipose tissue atrophy by promoting adipocyte cell death and inhibiting pre adipocyte differentiation (12).

The expression levels of SOD have been reported to increase through differentiation, while its down regulation is able to induce or promote the pathogenesis of metabolic disorders, such as diabetes and and atherosclerosis (13). In contrast, an increased level of NQO1 seems to correlate with an inactivation of antioxidant enzymes (14). Therefore, we performed an analysis of 3T3-L1 cells treated or not treated with anti-HIV drugs to evaluate their capacity to induce oxidative stress during differentiation. We showed, for the first time, the stronger down regulation of *SOD* and *NQO1* in 3T3-L1 cells treated with saquinavir, efavirenz and stavudine compared to 3T3-L1 untreated control cells.

Interestingly, HO1 was not modulated in the saquinavir-, efavirenz- and stavudine- treated 3T3-L1 cells. Literature date show that HO1 expression decreased during differentiation, while peroxisome proliferator-activated receptors (PPARγ) levels increased. Again up regulation of *HO1* in obesity reduces adiposity and increases adiponectin expression (15). Moreover, previous studies have shown that *HO1* expression negatively regulates adipocyte stem cell differentiation (16) and plays an important physiological role in obesity, and diabetes which are chronic inflammatory conditions with peculiarities similar to lipodystrophy syndrome (17).

Our findings provide evidence that anti-HIV drugs have a differential effect on adipocyte cell cycle and differentiation, being able to modify the response to oxidative stress through modulation of expression of phase-2 and antioxidant enzymes.

In summary, we can conclude from our studies that saquinavir, efavirenz, and stavudine exert antiadipogenic influences on the model 3T3-L1 cell line, perturbing the oxidative response and inducing apoptosis. When considered together, the effects of anti-HIV drugs on 3T3-L1 pre adipocytes are distinct but commonly antiadipogenic, thus suggesting another additional possible mechanism by which antiretroviral therapies could contribute to lipoatrophy.

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