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Adipocytes Impair Efficacy of Antiretroviral Therapy

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Abstract

Adequate distribution of antiretroviral drugs to infected cells in HIV patients is critical for viral suppression. In humans and primates, HIV- and SIV-infected CD4 T cells in adipose tissues have recently been identified as reservoirs for infectious virus. To better characterize adipose tissue as a pharmacological sanctuary for HIV-infected cells, in vitro experiments were conducted to assess antiretroviral drug efficacy in the presence of adipocytes, and drug penetration in adipose tissue cells (stromal-vascular-fraction cells and mature adipocytes) was examined in treated humans and monkeys. Co-culture experiments between HIV-1-infected CD4 T cells and primary human adipocytes showed that adipocytes consistently reduced the antiviral efficacy of the nucleotide reverse transcriptase inhibitor tenofovir and its prodrug forms tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF). In HIV-infected persons, LC-MS/MS analysis of intracellular lysates derived from adipose tissue stromal-vascular-fraction cells or mature adipocytes suggested

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Conflicts of Interest

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that integrase inhibitors penetrate adipose tissue, whereas penetration of nucleoside/nucleotide reverse transcriptase inhibitors such as TDF, emtricitabine, abacavir, and lamivudine is restricted. The limited distribution and functions of key antiretroviral drugs within fat depots may contribute to viral persistence in adipose tissue.

Keywords

Adipose tissue; Antiretroviral therapy; CD4 T cells; Dolutegravir; HIV reservoirs; Tenofovir

Introduction

HIV persistence is facilitated by inadequate distribution of antiretroviral drugs in cellular and anatomic reservoirs. For example, incomplete suppression of viral replication in lymph nodes is associated with decreased drug penetration (Fletcher et al., 2014; Lorenzo-Redondo et al., 2016). Other pharmacological sanctuaries associated with HIV persistence include brain, intestine, bone marrow, and genital tissues (Cory et al., 2013). In conjunction with inadequate antiviral immunity, these pharmacological sanctuaries promote longevity of viral reservoirs.

Antiretroviral pharmacology is complex and regulated by a number of factors, including half-life, drug interactions, cellular transporters, hydro-phobicity/-philicity, and drug resistance mutations. Although a substantial number of studies have investigated the relationship between ART and AT health and metabolism, only one has examined ART distribution in AT (Dupin et al., 2002): Dupin et al. (2002) measured nucleoside/nucleotide reverse transcriptase inhibitors (N(t)RTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), and protease inhibitor (PI) concentrations in lysates of whole fat samples by liquid chromatography/mass spectrometry (LC-MS/MS). They observed that NNRTI concentrations were significantly higher in AT lysates compared to PI concentrations, whereas N(t)RTIs were undetectable. Others and we have recently demonstrated that latently-infected CD4 T cells accumulate in AT and establish replication-competent reservoirs during chronic HIV infection, indicating the need for better understanding of antiretroviral pharmacology in AT (Couturier et al., 2015; Damouche et al., 2015; Couturier et al., 2016; Damouche et al., 2017; Hsu et al., 2017; Koethe et al., 2017; Couturier et al., 2018).

In the present study, we assessed the in vitro influence of adipocytes upon antiretroviral function, and measured the distribution of N(t)RTIs and integrase strand transfer inhibitors (INSTIs) in AT cellular fractions (stromal-vascular-fraction cells and mature adipocytes) of HIV-1 infected persons. Our results suggest that adipocytes cross-talk with infected CD4 T cells to increase HIV replication and reduce ART effectiveness, and that AT poses a significant barrier to ART distribution in treated HIV infection.

Materials and Methods

Cells and compounds

Human CD4 T cells were obtained from de-identified buffy coat preparations from healthy donors (Gulf Coast Regional Blood Center, Houston, TX). Peripheral blood mononuclear cells (PBMC) were isolated from leukopaks with Ficoll-Paque Plus (GE Healthcare), from which CD4 T cells were purified with EasySep negative selection kits (Stemcell Technologies). Human subcutaneous pre-adipocytes and mature adipocytes were obtained from Zen-Bio. Caco-2 colon epithelial cell lines were obtained from American Type Culture Collection.

Atazanavir sulfate, raltegravir, emtricitabine, efavirenz, tenofovir, and tenofovir disoproxil fumarate (TDF) were obtained from the NIH AIDS Reagent Program. Tenofovir alafenamide (TAF) was donated by Gilead Sciences.

Co-culture experiments and measurement of HIV replication and intracellular ART concentrations

Most in vitro experiments involved co-cultures of HIV-1-infected CD4 T cells with mature adipocytes with or without individual antiretroviral agents added. Mature adipocytes were first differentiated from preadipocytes in accordance with manufacturer protocols (5×10^4 preadipocytes/well). For HIV infections, purified CD4 T cells were first stimulated with $1 \mu\text{g/ml}$ plate-bound anti-CD3 antibodies and 100 ng/ml recombinant IL2 (Biolegend) for 48 hrs in complete RPMI medium (10% FBS, 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids, 2 mM sodium pyruvate, 25 mM HEPES, and $1 \times$ antibiotic-antimycotic) at $37^\circ\text{C}/5\% \text{ CO}_2$. Cells were washed, then incubated with R5X4 dual-tropic HIV-1 (strain 89.6) at 0.1 multiplicity of infection in complete RPMI medium and 100 ng/ml IL2 for 48 hrs (these infection conditions yielded baseline intracellular p24 levels of 10-20% seven days post-infection). Cells were then washed and 5×10^5 cells seeded into transwells (Corning, $0.4 \mu\text{m}$ pore size) with 1 ml media in 12-well plates with mature adipocytes in lower wells (3 ml media/well) in Zen-Bio DMEM/Ham's F-12 adipocyte maintenance medium (FBS, HEPES, biotin, pantothenate, dexamethasone, insulin, amphotericin B, and pen/strep). Immediately prior to addition of transwells and CD4 T cells to adipocytes, antiretroviral compounds at indicated concentrations were added to the lower wells. HIV-infected CD4 T cells and adipocytes were then co-cultured in the presence of 100 ng/ml IL-2 and indicated compounds for 6-7 days. For direct contact co-cultures, HIV-infected CD4 T cells (5×10^5) were seeded directly onto mature adipocytes, and co-cultured in transwells with 5×10^4 undifferentiated pre-adipocytes or Caco-2 cells in lower wells in DMEM medium. Co-culture experiments were conducted in singlet wells for each condition and repeated at least three times with different donor cells.

At indicated time points, HIV replication was measured by intracellular p24 flow cytometry or extracellular p24 ELISA. For measurement of surface proteins and intracellular p24, CD4 T cells were harvested from transwells and first stained with CD69-APC mabs and Zombie Violet viability dye (Biolegend). Cells were then stained with intracellular p24-PE mabs (Beckman-Coulter clone KC57) with BD Biosciences Cytotfix/Cytoperm and Perm/Wash

solutions. Data was acquired with a Gallios Flow Cytometer and analyzed with Kaluza software (Beckman-Coulter). Extracellular p24 was measured in culture supernatants by ELISA (Advanced Bioscience Laboratories). For measurement of intracellular ART concentration, CD4 T cells were washed 3× with PBS, then incubated in 1ml 70% methanol at −20°C for twelve hours to permeabilize cells. Cells were then pelleted and supernatants stored at −20°C for measurement of drugs by LC-MS/MS as previously described (King et al., 2006; Podany et al., 2014). For measurement of ART in adipocytes, media was removed, cells gently rinsed 3× with PBS. One ml 70% methanol was then added to wells and incubated with adipocytes at −20°C for twelve hours. Methanol was then collected from wells, centrifuged to pellet cells and debris, and supernatants stored at −20°C for measurements by LC-MS/MS.

Tissue processing and measurement of intracellular ART concentrations

Human AT samples, lymph nodes, or blood were obtained from deceased de-identified donors through the National Disease Research Interchange (Pts 03, 04, 06, 07, and 08; NDRI -Philadelphia, PA), or from live donors undergoing elective surgeries (Pts 01, 02, and 05). For tissue acquisition from live donors, protocols were approved by the Baylor College of Medicine Institutional Review Board and informed consent was obtained from each donor. PBMC and AT samples (subcutaneous and visceral) were also acquired from three recycled uninfected rhesus macaques at necropsy that had received emtricitabine and TAF via experimental subcutaneous nano-channel implants for approximately ten weeks.

ART intracellular concentrations were determined in single cell preparations by LC-MS/MS. For measurement of ART in PBMCs, PBMCs were first isolated from whole blood via Ficoll-Paque Plus, washed, then incubated in 1ml 70% methanol at −20°C for twelve hours. Cells were pelleted and supernatants stored at −20°C. For measurement of drugs in lymph node cells, lymph nodes were minced and digested with collagenase into single cells, washed, then incubated in 1ml 70% methanol at −20°C for twelve hours, pelleted, and supernatants stored at −20°C.

For ART measurement from AT stromal-vascular-fraction cells (AT-SVF), fat samples were minced and digested with collagenase, filtered to remove large debris, and centrifuged to pellet AT-SVF cells but leave adipocytes suspended in the floater fraction. The adipocytes were collected and stored at −20°C. AT-SVF cells were washed then incubated in 1ml 70% methanol at −20°C for twelve hours, pelleted, and supernatants stored at −20°C. For measurement of ART in the adipocytes, 1ml floater fraction was incubated in 2ml 100% methanol for twelve hours at −20°C. The number of adipocytes in the floater fraction was estimated with a hemacytometer. 1ml chloroform was then added to tubes, vortexed, and centrifuged to pellet cells. Supernatants were collected and stored at −20°C. Validation of the method for drug measurements in the floater fraction adipocytes was conducted by incubating 1ml floater fractions (isolated from AT samples of uninfected human donors obtained from NDRI) with a drug from each class (N(t)RTI, NNRTI, INSTI, or PI) for twelve hours at 37°C, followed by methanol/chloroform extraction as described above (shown as Fig. 4A).

Measurement of intracellular ART compounds by LC-MS/MS

All drug concentrations were detected using an LC-MS/MS system consisting of a Shimadzu Nexera liquid chromatography system coupled to an AB Sciex 6500 triplequadrupole mass spectrometer. Chromatography for the nucleosides was 98:2:0.1 water:acetonitrile:formic acid v:v:v (TFV/FTC) or 87.5:12.5:0.1 water:acetonitrile:formic acid (CBV/3TC) for mobile phase with a flow rate of 0.25 mL/min and a Phenomenex Synergi Polar RP 2.5 μ m 100 $\times$ 2mm column for solid phase. Separation for other analytes was accomplished with 70:30:0.1 methanol:water:formic acid v:v:v (ATV, DTG, EFV, EVG, RTV) or 60:40:0.1 water:acetonitrile:formic acid v:v:v (NVP) and a ACE 3 C18 3 μ m 100 $\times$ 3mm column for solid phase. Detection by mass spectrometer was done in positive ion mode for all of the analytes except EFV, which had to be in negative ion mode. Multiple reaction mode was used to increase analyte selectivity by fragmenting the “parent” ions into smaller “daughter” ions and detecting these transitions.

Standard calibrators were prepared and the methods monitored with validated quality control samples for all of the extraction methods. Drug extraction from adipocytes was performed by protein precipitation and addition of stable-isotope labeled internal standards for all of the analytes except the triphosphate metabolites of the nucleosides. Triphosphate metabolites were extracted from the adipocyte matrix by loading the prepared samples onto solid phase extraction (SPE) anion exchange columns to separate triphosphates from any mono or di phosphorylated metabolites. Once eluted from the SPE, triphosphates were dephosphorylated with phosphatase. Standard curves were prepared from the parent compounds corresponding to the appropriate triphosphate (i.e. TFV for TFVDP, FTC for FTCTP, etc.). Stable-isotope labeled internal standards for each analyte was added to all of the samples to track the desalting step which was done on reversed-phase SPE cartridges. Salt was separated from the analytes which were then subsequently eluted with methanol and dried. After reconstitution in water, the nucleosides were ready for LC-MS/MS analysis.

Human sample viral outgrowth assay

Viral outgrowth assays were conducted to determine the presence of infectious HIV in CD4 T cells in peripheral blood or AT of HIV-infected patients (Laird et al., 2016). PBMC and AT-SVF cells were first isolated from blood or fat, as described above. CD4 T cells were then purified from PBMC and AT-SVF cells via EasySep negative selection kits (Stemcell Technologies), and activated with 10 μ g/ml PHA-L + 1 μ g/ml IL2 in complete RPMI medium for 48 hours to induce viral replication. Medium was replenished with IL-2 medium, then MOLT4-CCR5 cells added to propagate infectious virus. Cells were cultured for up to four weeks with medium replenishment every four days. HIV replication was measured by p24 ELISA.

Statistics

Data were analyzed with GraphPad Prism. Comparisons between conditions for in vitro experiments were conducted with Student’s t-test and $p < 0.05$ was considered significant.

Results

Adipocytes reduce the efficacy of tenofovir in vitro

As a critical “backbone” component of ART, N(t)RTIs are typically administered in conjunction with NNRTIs, INSTIs, or PIs. To determine if adipocytes affect the functions of these key drugs, HIV-infected CD4 T cells were co-cultured with primary human adipocytes for up to seven days in the presence of antiretroviral compounds: tenofovir and its prodrug forms TDF or TAF, emtricitabine, efavirenz, raltegravir, or atazanavir.

Fig. 1A shows representative graphs of HIV replication (intracellular p24) by CD4 T cells after seven days of culture in control medium alone or co-culture with adipocytes, +/- indicated drug. Fig. 1B shows HIV replication percent changes from ART-untreated (UT) specimens. The capacity of tenofovir and its more bioavailable prodrug forms TDF and TAF to suppress HIV replication at physiological concentrations ($<1\mu\text{g/ml}$) was consistently and significantly mitigated in the presence of adipocytes, whereas the antiviral action of the other drugs was mostly unaffected. In medium alone, $1\text{-}5\mu\text{g/ml}$ tenofovir reduced HIV replication $\sim 65\text{-}91\%$, but reduced HIV replication $\sim 5\text{-}43\%$ in the presence of adipocytes ($p<0.05$ comparing Medium alone to +Adipocytes, $n=4$). $0.5\text{-}2\mu\text{g/ml}$ TDF reduced HIV replication $\sim 60\text{-}95\%$ in medium alone, but reduced HIV replication $\sim 15\text{-}38\%$ in the presence of adipocytes ($p<0.05$, $n=4$). $0.1\text{-}1\mu\text{g/ml}$ TAF reduced HIV replication $\sim 40\text{-}98\%$ in medium alone, but reduced HIV replication $\sim 1\text{-}64\%$ in the presence of adipocytes ($p<0.05$, $n=4$). Additionally, the more potent activity of TAF at lower doses relative to tenofovir and TDF in these in vitro experiments is consistent with patient studies reporting equivalent efficacy and less toxicity of TAF compared to TDF (Sax et al., 2014; Sax et al., 2015; Wohl et al., 2016; Gallant et al., 2016). The attenuation of tenofovir antiviral activity was also confirmed by measuring extracellular p24 by ELISA in which $0.1\text{-}1\mu\text{g/ml}$ TDF reduced HIV replication $\sim 57\text{-}85\%$ in medium alone, but reduced HIV replication $\sim 4\text{-}41\%$ in the presence of adipocytes ($p<0.01$, $n=5$). (Fig. 1C-D).

HIV-infected CD4 T cells were also co-cultured in direct contact with adipocytes in the presence of tenofovir, TDF, emtricitabine, or efavirenz, with results similar to those of the transwell experiments (Supplemental Fig. 1A). In medium alone with $1\mu\text{g/ml}$ tenofovir or TDF, HIV production was $\sim 15\text{-}16\%$ p24+, but was increased to $\sim 55\text{-}60\%$ during direct contact co-culture with adipocytes and $1\mu\text{g/ml}$ tenofovir or TDF ($p<0.05$, $n=3$).

To determine if other relevant cell types affect antiretroviral efficacy, HIV-infected CD4 T cells were co-cultured with primary pre-adipocytes (undifferentiated large fibroblastic adipocyte precursor cells) or with Caco-2 intestinal epithelial cell lines for seven days in the presence of TDF. Supplemental Fig. 1B-C shows a representative experiment of HIV replication by CD4 T cells (intracellular p24) and percent changes from UT controls during co-culture with pre-adipocytes, and Supplemental Fig. 1D-E shows results during co-culture with Caco-2 cells ($n=6$). In contrast to co-culture experiments with adipocytes, at $0.1\text{-}1\mu\text{g/ml}$ TDF, neither preadipocytes or Caco-2 cells significantly affected the antiviral activity of TDF. These results suggest that adipocytes specifically reduce the antiviral functions of tenofovir, TDF, and TAF in vitro.

Adipocytes increase CD4 T cell activation and HIV replication and sequester ART in vitro

We next examined adipocyte regulation of HIV replication in conjunction with CD4 T cell viability, activation, and antiretroviral sequestration. Viability of HIV-infected CD4 T cells cultured in medium alone or with adipocytes was generally increased in the presence of 1µg/ml of ART, concomitant with suppression of viral replication (Fig. 2A, n=6). Compared to medium alone, adipocytes generally increased expression of the T cell activation marker CD69 in conjunction with intracellular p24 (CD69+p24+ double-positives) in CD4 T cells after seven days, even in the presence of 1µg/ml ART, but significantly in the presence of tenofovir, TDF, and TAF (Fig. 2B). In medium alone, CD69+p24+ expression was <2% in the presence of tenofovir, TDF, or TAF, but was increased to ~8-12% during co-culture with adipocytes (p<0.05, n=4).

To determine if reduced antiviral activity of tenofovir during co-culture experiments is associated with significant sequestration of drugs by adipocytes, intracellular concentrations of phosphorylated tenofovir (TFV-diphosphate, TFV-DP) in CD4 T cells and adipocytes were quantified by LC-MS/MS. After seven days of co-culture in the presence of 0.1-10µg/ml TDF, adipocytes accumulated 10-fold more TFV-DP vs CD4 T cells (Fig. 2C, p<0.05, n=3). The substantial accumulation of TFV-DP by adipocytes was associated with increased HIV production (Fig. 2D) and reduced intracellular TFV-DP (Fig. 2E) in CD4 T cells. In control medium alone, intracellular TFV-DP in CD4 T cells was 2.6×10^3 fmol/million cells with 0.1µg/ml TDF and 2.6×10^4 fmol/million cells with 1µg/ml TDF, but during co-culture with adipocytes was significantly less (6.4×10^2 fmol/million cells with 0.1µg/ml TDF, 5.3×10^3 fmol/million cells with 1µg/ml TDF; Fig. 3C, p<0.05, n=3). Thus, the capacity of adipocytes to upregulate HIV replication in CD4 T cells and for TFV to penetrate or become phosphorylated pose significant obstacles for the adequate distribution of drugs within AT.

AT of HIV-infected persons harbor latently infected CD4 T cells

To determine if AT constitute a pharmacological sanctuary for HIV-infected CD4 T cells, we examined AT cells for infectious virus and antiretroviral distribution. For three HIV-infected specimens, AT-SVF CD4 T cells infected with infectious provirus was first determined by a viral outgrowth assay (Laird et al., 2016). Fig. 3 shows HIV latently infected CD4 T cells producing infectious HIV for patients 01 and 03, with input CD4 cell numbers indicated in parentheses (PBMC for Pt.03 was unavailable). Infectious virus was not detectable from AT-SVF CD4 T cells of Pt.02. These results indicate that AT harbors latently infected CD4 T cells capable of initiating new infections upon proviral induction, consistent with previous reports demonstrating the presence of replication-competent HIV and SIV in AT CD4 T cells of infected humans and monkeys (Damouche et al., 2015, Couturier et al., 2016).

INSTIs penetrate AT

Drug concentrations in intracellular lysates of separate SVF and adipocyte fractions of seven HIV-infected patients' adipose tissue were next measured by LC-MS/MS. Other tissues such as PBMC, lymph nodes, and serum were also examined depending on patient availability. NNRTIs, PIs, and INSTIs directly inhibit viral enzyme function and these compounds were measured in cell lysates, whereas N(t)RTIs are prodrugs that require intracellular

phosphorylation (diphosphates-DP or triphosphates-TP) to function and these phosphorylated forms were measured in cell lysates. To first validate the methods of lysate preparation and intracellular drug measurement in adipocytes derived from the floater fraction of digested adipose tissue samples, Fig. 4A shows a representative result of two experiments showing detection of ART class representatives in adipocytes following spike-in treatment of floater fractions isolated from adipose tissue samples of uninfected donors.

Fig. 4B shows results of ART in intracellular lysates of HIV-infected patients' tissues; the intracellular NRTI-triphosphates were measured for abacavir (CBV-TP), lamivudine (3TC-TP), and emtricitabine (FTC-TP), and N(t)RTI-diphosphates were measured for tenofovir, TDF, and TAF (TFV-DP). In Pt.01, CBV-TP, 3TC-TP, and dolutegravir were undetectable in visceral AT-SVF cells. Tissues for drug measurements were unavailable for Pt.02. In Pt.03, dolutegravir was present in lymph node cells (793 fmol/million cells) and subcutaneous AT-SVF cells (129 fmol/million cells) and adipocytes (151 fmol/million cells), whereas CBV-TP and 3TC-TP were undetectable. In Pt.04, dolutegravir was present in lymph node cells (749 fmol/million cells) and subcutaneous AT-SVF cells (1,439 fmol/million cells) and adipocytes (382 fmol/million cells), whereas TFV-DP was present in lymph node cells (68 fmol/million cells) and FTC-TP was undetectable. In Pt.05, elvitegravir was present in subcutaneous AT-SVF cells (339 fmol/million cells) but undetectable in PBMC, whereas FTC-TP and TFV-DP were undetectable. In HIV Pt.06, abacavir was present in PBMC (27 fmol/million cells) and undetectable in lymph node cells and visceral adipocytes, whereas nevirapine was present only in visceral adipocytes (139 fmol/million cells). In Pt.07, dolutegravir was present in subcutaneous AT-SVF cells (4 fmol/million cells), whereas CBV-TP, atazanavir, and ritonavir were undetectable, and 3TC-TP was detectable in serum (37 ng/ml). In Pt.08, PBMC contained FTC-TP (14,682 fmol/million cells), TFV-DP (633 fmol/million cells), and dolutegravir (46 fmol/million cells), lymph node cells contained FTC-TP (1,053 fmol/million cells), TFV-DP (58 fmol/million cells), and dolutegravir (17 fmol/million cells), subcutaneous AT-SVF cells contained FTC-TP (4,459 fmol/million cells), TFV-DP (724 fmol/million cells), and dolutegravir (449 fmol/million cells), subcutaneous adipocytes contained FTC-TP (11 fmol/million cells), TFV-DP (7 fmol/million cells), and dolutegravir (363 fmol/million cells), and serum contained FTC-TP (2,687 ng/ml), TFV-DP (246 ng/ml), and dolutegravir (1,324 ng/ml).

Lastly, we examined the distribution of N(t)RTIs in AT cells and PBMC of three recycled uninfected rhesus macaques receiving emtricitabine and TAF for approximately ten weeks via experimental subcutaneous nano-channel implants. PBMC contained FTC-TP (700-1,400 fmol/million cells) and TFV-DP (80-770 fmol/million cells), whereas both compounds were undetectable in lysates of AT-SVF and adipocyte fractions of all three monkeys (Supplemental Fig. 2). These results show that dolutegravir penetrates fat, whereas the capacity of N(t)RTIs to penetrate adipose tissue appears more variable.

Discussion

Efficient penetration and maintenance of therapeutic concentrations of ART in cells and tissues that harbor HIV is essential to maintenance of viral suppression and preventing rebound viremia. The results presented here demonstrate that adipocytes have significant

impact on ART distribution and activity within AT, which is newly believed to constitute a reservoir for latently infected HIV-/SIV-infected CD4 T cells (Couturier et al., 2015; Damouche et al., 2015; Couturier et al., 2016; Damouche et al., 2017; Hsu et al., 2017; Koethe et al., 2017; Couturier et al., 2018). In particular, these data suggest that adipocytes markedly diminish the antiretroviral effectiveness of NRTIs, particularly tenofovir, which constitutes an important “backbone” component of many antiretroviral regimens and is a first line agent for the treatment of HIV infection. Additionally, the INSTI dolutegravir penetrates AT and is likely important for inhibiting viral replication in AT.

In vitro, adipocytes reduced tenofovir efficacy and increased T cell activation and HIV replication, a finding associated with sequestration of substantial amounts of ART within the adipocytes (Figs. 1–2). Upregulation of CD4 T cell activation and HIV production in AT may further increase the necessary tissue concentration required for suppression of local viral replication; in this context, it is important to note that HIV-infected, tissue-resident immune cells have higher activation states and HIV viral loads than circulating cells. In ART-treated, HIV-infected patients, viral persistence in lymph nodes is associated with greater CD4 T cell activation, low-level HIV replication, and decreased ART penetration (Fletcher et al., 2014; Lorenzo-Redondo et al., 2016). The significant uptake of tenofovir by adipocytes in vitro is consistent with previous findings demonstrating rapid accumulation of NRTIs and PIs by murine and human adipocytes (Janneh et al., 2003; Vernochet et al., 2005; Janneh et al., 2010). However, these in vitro results need to be replicated in the more complex in vivo environment. Damouche et al. (2015) showed that CD4 T cells are distal to blood vessels (compared to CD8 T cells which are closer to blood vessels) in AT of SIV-infected rhesus macaques, suggesting additional barriers for ART to contact infected immune cells in AT. Notably, adipocytes promote cancer progression, in part by enhancing metastases and protecting tumor cells from chemotherapeutic agents (Behan et al., 2009; Nieman et al., 2011; Pramanik et al., 2013; Ye et al., 2016; Sheng et al., 2017). Thus, the unique morphology and metabolic activity of adipocytes, and their interactions with pharmacologic compounds, may confer survival advantages to HIV-infected immune cells or malignant cells in AT depots.

Pharmacokinetics within AT depots and intracellular metabolism of drugs in adipocytes are poorly characterized. We observed that latently infected CD4 T cells in AT of HIV-infected persons can be reactivated to produce infectious HIV (Fig. 3), corroborating previous reports demonstrating that HIV and SIV reservoirs in AT are replication-competent (Damouche et al., 2015; Couturier et al., 2016). We also observed that the INSTI dolutegravir penetrates AT, whereas penetration or activation of N(t)RTIs such as abacavir, lamivudine, emtricitabine, and tenofovir may be more limited (Fig. 4 and Supplemental Fig. 2). Additionally consistent with previous reports, lymph node cells contained less ART drugs compared to PBMC (Pts 06 and 08), and N(t)RTIs were less consistently detectable in adipose tissue (Dupin et al., 2002; Fletcher et al., 2014). However, these in vivo data are highly variable and low in number, and limited conclusions can be made as detailed patient clinical data and posology were unavailable, as well as patient samples being derived from two different sources (live subjects undergoing surgeries or recently deceased cadavers) with different sample acquisition and processing times, and from different depots. Thus, these data present more qualitative information regarding ART distribution in adipose tissue.

Although interpretation is difficult, it is clear that dolutegravir penetrates adipose tissue. Despite the lack of posology, in which ART intake for the cadaver subjects would likely have been at least seven days prior to tissue harvest and processing, it is intriguing that dolutegravir was still readily detectable in adipose cells, suggesting the possibility for long-term retention in fat depots and re-release similar to that of other compounds such as cannabis (Muniyappa et al., 2013).

Differential distribution of NNRTIs and INSTIs in AT could be due to a variety of pharmacological factors. Lipophilicity of drugs may be important variables for AT penetration, as N(t)RTIs such as tenofovir and emtricitabine are relatively hydrophilic compounds and were less consistently detectable in AT cells (as shown in Fig. 4 and Supplemental Fig. 2). By contrast, the more hydrophobic dolutegravir (as indicated by its higher partition coefficient logP value) did enter AT in vivo. Additionally, the intracellular concentrations of dolutegravir in AT-SVF cells shown in Fig. 4B could be in both CD4 T cells and non-CD4 T cells (primarily preadipocytes, macrophages, and CD8 T cells), as CD4 T cells are usually <5% of the AT-SVF (Couturier et al., 2015; Couturier et al., 2016). Expression of drug transporters by adipocytes and drug-drug interactions also likely influence ART penetration into AT. It will be important to understand how the selective penetration of these agents into AT is associated with viral persistence in AT, as well as the fat accumulation, weight gain and obesity observed in ART-treated, HIV-infected patients (Crum-Cianflone et al., 2010; Guehi et al., 2016; Koethe et al., 2016; Menard et al., 2017; Norwood et al., 2017).

In summary, we have shown that adipocytes significantly impact antiretroviral functions and that INSTIs penetrate adipose tissue more consistently than N(t)RTIs. Drug penetration into AT is important not only for controlling AT HIV reservoirs, but also because other major HIV reservoirs - such as lymph nodes, GALT and bone marrow - are lipid-rich or intricately associated with adipocytes/AT function. Complete targeting of viral reservoirs with ART should take into consideration the role of adipose tissue penetration and lipid-rich milieus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Human adipocytes increase CD4 T cell activation and HIV replication.
- Human adipocytes sequester substantial amounts of antiretroviral drugs.
- Integrase inhibitors penetrate adipose tissue of HIV patients.

Figure 1. Adipocytes reduce antiviral functions of tenofovir in vitro

HIV-infected CD4 T cells were seeded into transwells and cocultured with adipocytes or in medium alone in lower wells of 12-well plates for seven days with antiretroviral drugs. CD4 T cells were then measured for intracellular p24 by flow cytometry. Shown are representative experiments (A) and mean±sem percent p24 changes relative to untreated (UT) conditions (B) (*p<0.05 comparing medium alone to adipocytes, n=4). (C-D) Shown are a representative experiment of extracellular p24 measurements and mean±sem percent

changes relative to untreated conditions during coculture of infected CD4 T cells with adipocytes in the presence of TDF (* $p < 0.05$ comparing medium alone to adipocytes, $n=5$).

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Figure 2. Adipocytes increase CD4 T cell activation and HIV replication, and sequester substantial amounts of antiretroviral drugs

(A-B) HIV-infected CD4 T cells were cultured in transwells with adipocytes or medium alone in lower wells of 12-well plates in the presence of antiretroviral drugs for seven days. CD4 T cells were then measured for viabilities, and CD69 and intracellular p24 expression by flow cytometry. Shown are mean±sem percent viabilities (A, n=6) and percent CD69+/p24+ double-positives (B, n=4) of infected CD4 T cells during cocultures (*p<0.05 comparing medium alone to adipocytes). (C) Comparison of tenofovir uptake by infected CD4 T cells and adipocytes. HIV-infected CD4 T cells were cocultured with adipocytes in

the presence of TDF for seven days. Cells were then harvested and intracellular lysates prepared for TFV-DP measurement by LC-MS/MS. Shown are mean $\pm$ sem intracellular TFV-DP concentrations in CD4 T cells or adipocytes (* p <0.05, n =3). (D-E) Suppression of TDF efficacy in conjunction with significant drug uptake by adipocytes. HIV-infected CD4 T cells were cultured with adipocytes or medium alone in lower wells of 12-well plates in the presence of TDF. CD4 T cells were then harvested and measured for intracellular p24 by flow cytometry and intracellular TFV-DP concentrations by LC-MS/MS. Shown are mean $\pm$ sem intracellular p24 levels (D) and intracellular TFV-DP concentrations (E) in CD4 T cells during coculture with adipocytes (* p <0.05 comparing medium alone to adipocytes, n =3).

Figure 3. Presence of infectious HIV in CD4 T cells of adipose tissue

PBMC were isolated from peripheral blood of HIV patients (Patients 01 and 02), and AT-SVF cells were isolated from adipose tissue samples as described in Materials and Methods. CD4 T cells were then purified and activated with PHA+IL2 for 48 hrs (approximate input CD4 T cell numbers are shown in parentheses). MOLT4-CCR5 cells were then added and cells cultured for 3-4 weeks. Extracellular p24 was measured by ELISA.

Figure 4. Penetration of dolutegravir in adipose tissue of HIV patients

(A) Validation of method for measurement of antiretroviral drugs in floater fraction adipocytes. Floater fractions were isolated from adipose tissue of uninfected donors, then incubated with drugs for twelve hrs at 37°C. Lysates were then prepared using methanol/chloroform as described in Materials and Methods, and drugs measured by LC-MS/MS. Shown are intracellular drug concentrations of (fmol/million cells) of one experiment representative of two separate experiments. Intracellular phosphorylated N(t)RTIs are denoted as FTC-TP (emtricitabine), TFV-DP (tenofovir), CBV-TP (abacavir), and 3TC-TP

(lamivudine). (B) Detection of antiretroviral drugs in tissues of HIV patients. Intracellular lysates were processed from single preparations of lymph nodes, PBMC, subcutaneous (SC) or visceral (VS) AT-SVF cells or adipocytes, or serum, and measured for drug concentrations by LC-MS/MS.

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