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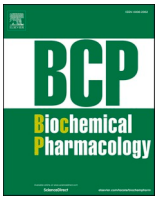

Divergent effects of the antiretroviral drugs, dolutegravir, tenofovir alafenamide, and tenofovir disoproxil fumarate, on human adipocyte function

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ABSTRACT

Combined antiretroviral therapy (cART) has been associated with increased body weight accompanied by metabolic alterations in people living with human immunodeficiency virus (PLWH). To gain insight into the combined effects of cART components on adipocyte dysfunction, we assessed whether and how treatment of human adipocytes with dolutegravir (DTG) and the nucleotide-analog reverse-transcriptase inhibitors (NRTIs), tenofovir alafenamide (TAF) and tenofovir disoproxil fumarate (TDF), alone and in combination, altered biological processes related to adipose tissue dysfunction. DTG, TAF, and TDF were applied to human Simpson-Golabi-Behmel syndrome (SGBS) adipose cells during differentiation (day 10) and ensuing differentiation (day 14). Expression of selected marker genes was determined by qPCR, the release of adipokines and inflammatory cytokines to the culture media was assessed, and cell respiration was measured. Adipogenesis was not altered by the combined treatment of human adipocytes. However, DTG at the highest dose repressed adipogenesis marker genes expression, and TAF and TDF appeared to mitigate this effect. DTG repressed the expression of adiponectin and the release of adiponectin and leptin in differentiating adipocytes, and these effects were mantained in combination with TAF and TDF. DTG plus TAF or TDF on human adipocytes enhanced inflammation and stress and increased the release of proinflammatory cytokines to the culture media. Together, our results show that combined therapy with these drugs can alter inflammation, cellular stress, and fibrosis in human adipocytes. These findings may improve our understanding and management of the effects of cART on body adiposity and metabolic dysregulation in PLWH.

1. Introduction

The success of combined antiretroviral therapy (cART) in reducing the morbidity and mortality that are associated with infection by the human immunodeficiency virus (HIV) has turned HIV into a manageable chronic disease. However, this success has been overshadowed by side effects that decrease wellness among people living with HIV (PLWH).

Currently, integrase chain transfer inhibitor antiretroviral drugs

Abbreviations: INSTIs, integrase strand-transfer inhibitors; NRTIs, nucleotide/nucleoside-analog reverse transcriptase inhibitors; cART, combined antiretroviral therapy; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil fumarate; DTG, dolutegravir; HIV, human immunodeficiency virus; PLWH, people living with HIV; SGBS, Simpson-Golabi-Behmel syndrome; PPAR, peroxisome proliferator-activated receptor; LPL, lipoprotein lipase; TNF, tumor necrosis factor; MCP1, monocyte chemoattractant protein-1; FBS, fetal bovine serum; CCL2, chemokine (C-C motif) ligand 2; IL-6, interleukin-6; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

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(INSTIs) are recommended for initial therapy and for exchange and rescue from previous treatments in PLWH [1]. They are always used in combination with other antiretroviral drugs, such as the nucleotide-analog reverse transcriptase inhibitor (NRTI), tenofovir alafenamide (TAF). Several studies reported that the use of INSTIs, mainly dolutegravir (DTG), in combination with TAF, is associated with weight gain and even clinical obesity [2,3]. WHO has identified obesity, which is defined as an excessive accumulation of fat that presents health risks, including a reduced life expectancy, as an epidemic health problem [4]. Thus, there is growing concern that the widespread use of regimens based on INSTIs and TAF may fuel an epidemic of weight gain and obesity among PLWH.

A greater weight gain was first observed in PLWH treated with INSTIs, both in cohort studies and clinical trials [5–9]. Weight gain after cART often occurs because of the so-called 'return to health' phenomenon. However, significant specific associations were observed between weight gain and the use of DTG and bictegravir (BIC) compared to the other INSTIs [10]. As reported for large-cohort studies, cART-naïve patients treated with DTG presented a higher level of obesity after 48 weeks and this gain was enhanced in women receiving a combined ART comprising DTG and TAF compared to a combination of DTG and TDF. [11,12]. In fact, a recent study on substitution of TDF by TAF in INSTI-containing cART regimes indicated that TAF was particularly involved in eliciting weight gain [13]. A recent report further indicated that excessive weight gain occurred in PLWH not only when starting INSTI-containing cART but also when switching to INSTI-based cART after previous non-INSTI-based treatments [15].

Nucleotide or non-nucleotide transcriptase inhibitor (principally TAF), gender (exacerbated in women), ethnicity (aggravated in sub-Saharan patients), viral load, and CD4 count are the described added factors contributing to the gain in weight observed in in INSTI-containing treatments [12,13,15]. All the observed pro-obesogenic events in PLWH have the obvious consequence of increasing metabolic risk and cardiovascular disease [14,16–18]. However, it is not known whether this is caused directly by toxic effects of INSTI and TAF, indirectly by the increased weight, or by a combination of both factors.

The molecular basis of the obesogenic effects of INSTIs in combination with other drugs, such as TAF, is unknown. It has been hypothesized that central effects of INSTIs disturb central mechanisms of food satiety control [19], but growing evidence from experimental animal models and *in vitro* adipocyte cell systems suggests that INSTIs directly impact adipose tissue. Human adipose cell models are endorsed strategies for the identification of molecular perturbances marked using the antiretroviral drugs composing cART [20–24] while dissecting the distinct effects of distinct INSTIs to directly impact human adipocytes [25].In general, the *in vitro* studies agreed that DTG does not cause massive effects on adipocyte differentiation, but rather elicits specific effects, such as promotion of a profibrotic gene expression pattern and marked downregulation of adiponectin expression and secretion [25,26]. Consistent results were obtained in DTG-treated PLWH [27]. DTG also appears to impair the acquisition of a thermogenic beige/brown phenotype by cultured adipocytes [28,29]. The above-listed findings were overall consistent with observations reported for murine and simian animal models treated with DTG [26,28,29].

Despite growing awareness that TAF may be involved in the obesogenic response to current INSTI-containing cART treatments, the effects of TAF alone or in combination with DTG have not been fully analyzed in human adipocytes. One recent study indicated that TAF antagonizes the profibrotic effects of DTG in a mouse adipocyte cell line [30], but little is known beyond that. Therefore, we herein report a comprehensive analysis of the potential effects of DTG, alone and in combination with TAF or its related molecule, TDF, on human adipocytes, with the goal of understanding the potential additive effects of DTG + TAF or TDFcomponents on potential adipocyte dysfunction.

2. Materials and methods

2.1. Reagents

Reagents for cell culture and subsequent sample analysis were purchased from Sigma-Aldrich (Saint Louis, MI, USA). Rosiglitazone was acquired from Alexis Biochemicals (Enzo Life Sciences, Farmingdale, NY, USA). DMEM/F12 medium and fetal bovine serum (FBS) were provided by Life Technologies (Carlsbad, CA, USA). DTG was acquired from MedChemExpress (Monmouth Junction, NJ, USA). TAF and TDF were provided by Selleckchem Chemicals (Houston, TX, USA). Initial tests on antiretroviral drugs comprised their use at Cmax concentrations observed in patients [32] that confirmed not to cause cytotoxicity SGBS cells. Solvent to the drugs was dimethyl sulfoxide (DMSO) or ethanol (EtOH), that were added to the controls when applicable (DMSO/EtOH $\leq$0.1 % of cell medium).

2.2. Cell culture

To assess the effects of the antiretroviral drugs, DTG, TAF, and tenofovir disoproxil fumarate (TDF) on adipogenesis and adipocytes, we used human SGBS (Simpson-Golabi-Behmel syndrome) cells. SGBS cells are recognized as accurate for studying human adipogenic differentiation [31] and the effects of antiretroviral drugs on human fat cell function [20,23,24]. Cultured human SGBS preadipocytes were differentiated to adipocytes as formerly reported [25]. These cells were cultivated in 10 % FBS DMEM/F12 medium. Once confluent, adipogenesis was triggered with a differentiation medium (QuickDiff). QuickDiff media is serum-free medium and incorporates 20 nM insulin, 100 μg/ml human apo-transferrin, 0.2 nM triiodothyronine (T3), and 100 nM cortisol, supplemented with 25 nM dexamethasone, 500 μM 3-isobutyl-methylxantine, and either 2 μM rosiglitazone (to achieve full differentiation) or 0.5 μM rosiglitazone (substandard for adipogenic differentiation, to assess differential effects over adipogenesis). After 7 days, QuickDiff was changed for a lipgenic medium (3FC) incorporating 20 nM insulin, 100 μg/ml human apo-transferrin, 0.2 nM triiodothyronine (T3), and 100 nM cortisol without further supplementation, and maintained for an additional 3 days (chronic treatment) or 7 days (for completely differentiated adipocytes). The utilized drug antiretroviral concentrations were as follows: For DTG, SGBS preadipocytes and adipocytes were exposed to doses of 1 and 10 μM, which represented concentrations between the minimum and maximum circulating concentrations (Cmin and Cmax, respectively) observed in patients [33]. SGBS cells were treated with drugs all through the 10-day adipogenesis process or acutely (24 h) when the cells were completely differentiated (14 days).

2.3. Cytotoxicity assessment

Cytotoxicity was determined using a CyQUANT LDH Cytotoxicity Assay Kit (C20300, Invitrogen, Waltham, MA, USA) complying with protocol instructions facilitated by the manufacturer. Drugs were added to SGBS cells in culture for 24 h. Lactate dehydrogenase activity was used to estimate cytotoxicity relative to the maximum activity (100 %) seen after total cell lysis.

2.4. Cell proliferation assays

To assess the effects of the drugs in SGBS preadipocytes, cells were cultured to 50 % confluency and maintained in a proliferation media (OF-medium) containing 10 % FBS and 1 % penicillin/streptomycin supplemented DMEM/F12. The cultures were exposed to antiretroviral drugs alone or in combination (DTG, TAF, and TDF) for 72 h, which was the average time needed to reach confluence. For staining, medium was removed, and cells were washed with ice-cold 1X PBS and stained for 30 min using crystal violet (0.02 % in deionized water). The excess dye was

Table 1
Assay-on-Demand probes from Life Technologies.

Gene name	Gene symbol	Reference
Peroxisome proliferator-activated receptor-gamma (PPARγ)	*PPARG*	Hs00234592_m1
Leptin	*LEP*	Hs00174877_m1
Adiponectin	*ADIPOQ*	Hs00605917_m1
Lipoprotein lipase	*LPL*	Hs00173425_m1
Solute carrier family 2, facilitated glucose transporter member 1 (GLUT1)	*SLC2A1*	Hs00892681_m1
Solute carrier family 2, facilitated glucose transporter member 4 (GLUT4)	*SLC2A4*	Hs00168966_m1
NADH:ubiquinone oxidoreductase subunit B8	*NDUFB8*	Hs00922353_g1
Mitochondrially encoded NADH dehydrogenase 6	*MT-ND6*	Hs02596879_g1
Succinate dehydrogenase complex iron sulfur subunit B	*SDHB*	Hs00268117_m1
Ubiquinol-cytochrome *c* reductase core protein 1	*UQCRC1*	Hs00163415_m1
Mitochondrially encoded cytochrome *b*	*MTCYB*	Hs025968677_s1
Cytochrome *c* oxidase subunit IV	*COX4I1*	Hs00266371_m1
ATP synthase-coupling factor 6, mitochondrial	*ATP5J*	Hs00365888_m1
Uncoupling protein 1	*UCP1*	Hs00222453_m1
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)	*PPARGC1A*	Hs00173304_m1
Iodothyronine deiodinase 2	*DIO2*	Hs00255341_m1
Bone morphogenetic protein 8b	*BMP8B*	Hs01629120_s1
Chemokine (C-C motif) ligand 2 (MCP-1)	*CCL2*	Hs00234140_m1
Interleukin-6 (IL-6)	*IL-6*	Hs00174131_m1
Interleukin-8 (IL-8)	*IL-8*	Hs00174103_m1
Fibroblast growth factor 21	*FGF21*	Hs00173927_m1
Growth differentiation factor 15	*GDF15*	Hs00171132_m1
Heat shock protein family A (BIP, GRP 78)	*HSPA5*	Hs99999174_m1
DNA-damage inducible transcript 3 (CHOP-10)	*DDIT3*	Hs99999172_m1
Hypoxia inducible factor 1 subunit alpha (MOP1)	*HIF1A*	Hs00153153_m1
Collagen type III alpha 1 chain	*COL3A1*	Hs00943809_m1
Transforming growth factor beta 1	*TGFB1*	Hs00998133_m1
Actin alpha 1	*ACTA1*	Hs05032285_s1
Ribosomal protein lateral stalk subunit P0	*RPLP0*	Hs99999902_m1

cleared away, ice-cold water was applied to wash the and the cell-bound dye was recovered in 70 % EtOH. To estimate differences, the optical density was measured at 570 nm using Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Assessment of lipid accumulation in adipocytes

Differences in lipogenesis capacity was estimated as previously described [25] through the analysis of micrographs of cell cultures were captured using a Motic AE2000 microscope with a Moticam 5.0 MP camera (Motic, Xiamen, China). Subsequently, these images were quantified using ImageJ software as previously reported [25].

2.6. RNA isolation, conventional reverse transcription, and quantitative polymerase chain reaction (qRT-PCR)

RNA was purified by the usage of Macherey-Nagel affinity column-based method (Thermo Fisher Scientific) as previously described [24,25]. 0.5 μg of total RNA of these samples were kept for reverse transcription using random hexamer primers (Applied Biosystems, Waltham, MA, USA) in a final volume of 20uL. The real time PCR quantification was then performed in the Applied Biosystems QuantStudio 3/5 using 20 μl of a reaction mixture (1 μl of cDNA, 10 μl of TaqMan Universal PCR master mix, 250 nM probes, and 900 nM primers from an Assays-on-Demand gene expression assay mix (TaqMan; Applied Biosystems). Assay-on-Demand probes were acquired from Life Technologies and are listed in Table 1. Relative expression of mRNAs was calculated using the comparative ($2^{-\Delta CT}$) method normalized to the housekeeping gene (RPLP0 mRNA).

2.7. Western blot assay

SGBS human adipocytes were cultured in 6-well plates and differentiated as described in M&M 2.2. At the end of each test, cells were collected using a scrapper. Then, they were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer. Concentration of the protein was estimated with Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). For quantification, proteins were separated through a 12 % SDS/PAGE and transferred to PVDF membranes. After blocking of the membrane with 5 % skim milk (in Tris-buffered saline with 0.1 % Tween® 20 Detergent (TBST)) and incubated with primary antibodies [OXPHOS (MS601, Mitosciences, Abcam; Cambridge, UK; 1:500), COXVI (A6431, Molecular Probes, Eugene, OR, USA; 1:1000), ATUB (T9026, Sigma-Aldrich; 1:1000)] overnight at 4 ∘C. Protein bands were visualized by chemiluminescence using Immobilon ECL Ultra Western HRP Substrate (Merck, Millipore, Burlington, MA, USA) and the results were captured and analyzed with an iBright™ FL1500 Imaging System (Thermo Fisher Scientific).

2.8. Secretion of adipokines and cytokines by adipocytes

Adipokines and cytokines excretion to the media was assessed through ELISA for human ADIPONECTIN (EZHADP-61 K, Merck Life Science) and LEPTIN (DLP00, R&D Systems, MI, USA). In the case of inflammatory cytokines, Interleukin-6 (IL-6), interleukin-8 (IL-8), and chemokine (C-C motif) ligand 2 (CCL2; also called monocyte chemoattractant protein-1, MCP-1) were quantified using a multiplex analysis system (Lynco Research/Merck) and a Luminex 100ISv2 instrument (Thermo Fisher Scientific).

2.9. Cell respiration assays

SGBS preadipocytes were cultured and differentiated into mature adipocytes in 24-well Seahorse test plates (Agilent Technologies, Santa Clara, CA, USA). Briefly, SGBS adipocytes were washed and incubated for 1 h (37 °C, 5 % CO_2) in Agilent Seahorse XF Base Medium (Agilent Technologies) with 5 mM glucose, 2 mM glutamine and 0.5 mM pyruvate added. Oxygen consumption rates and extracellular acidification rates (ECAR) were measured using a Seahorse XF24-3 extracellular flux analyzer (Agilent Technologies). After the assay, media were collected and snap-frozen, cells were lysed in NaOH buffer, and total DNA quantification was performed and used to normalize the data.

2.10. Glycerol release to the culture media

The concentration of glycerol in cell culture supernatants was determined using a commercial kit following the manufacturer's instructions (MAK117, Sigma-Aldrich).

2.11. Statistical analyses

Statistical analyses and data imagining were accomplished with GraphPad Prism 9 Software (San Diego, CA, USA). One-way ANOVA and Dunnett's multiple comparison tests were performed to each set of data. Significance was showed in the text and was accepted at $P \leq 0.05$.

3. Results

3.1. DTG alone or in combination with TAF and TDF do not affect lipid accumulation but rather alter mRNA expression of adipogenic markers and adipokine release in human adipocytes

The impact of DTG, TAF, and TDF on the adipogenesis of human SGBS preadipocytes were experimented. SGBS preadipocytes were exposed to DTG (1 and 10 μM), TAF (0.033 μM), and TDF (0.4 μM) alone or in combination for 24 h, and cytotoxicity was assessed. No significant

Table 2
Effects of dolutegravir (DTG), tenofovir alafenamide (TAF), and tenofovir disoproxil fumarate (TDF) on lactate dehydrogenase activity in the culture media.

	-			TAF			TDF		
DTG μM	-	1	10	-	1	10	-	1	10
	6.1±0.3	5.2±0.3	6.0±0.8	5.6±0.6	5.9±0.8	5.8±0.8	5.9±0.4	5.8±0.8	5.9±0.5

Table 3
Effects of dolutegravir (DTG), tenofovir alafenamide (TAF), and tenofovir disoproxil fumarate (TDF) on SGBS pre-adipocyte proliferation.

	-			TAF			TDF		
DTG μM	-	1	10	-	1	10	-	1	10
	0.7±0.08	0.7±0.03	0.5±0.07**	0.7±0.04	0.7±0.06	0.5±0.06**	0.7±0.03	0.7±0.03	0.5±0.06**

cytotoxic effect was observed at the tested concentrations of DTG, TAF, or TDF (Table 2). The effects of these antiretroviral drugs on SGBS preadipocyte proliferation were also assessed. DTG at 10 μM alone or in combination with TAF or TDF resulted in a mild but significant decrease in cell proliferation, whereas TAF and TDF alone had no such effect (Table 3). The combination treatments did not yield a differential change in cell proliferation. Treatments were started on the day of differentiation induction and lasted throughout adipogenesis (up to 10 days). To study differentiating adipocytes, a phase-contrast microscope was used to obtain micrographs at day 10 after differentiation initiation (Fig. 1a); differences in morphological differentiation of adipocytes was established by quantifying the area occupied by lipid droplet-containing cells (i.e., differentiated adipocytes) in the images. No significant difference in lipid accumulation was observed under treatment with the studied mono- or cotreatments (Table 4). After 10 days of adipogenesis, the mRNA expression of selected gene markers was quantified (Fig. 1b). DTG at 1 μM had no apparent effect on the expression of adipogenesis-related marker genes, whereas 10 μM DTG significantly repressed the mRNA expression levels of the adipocyte identity markers, peroxisome proliferator-activated receptor-gamma (*PPARG*) and lipoprotein lipase (*LPL*), as well as solute carrier family 2, facilitated glucose transporter member 4 (*SLC2A4*, GLUT4), and the adipokine, adiponectin (*ADIPOQ*), but induced that of solute carrier family 2, facilitated glucose transporter member 1 (*SLC2A1*, GLUT1)0 and leptin (*LEP*). TAF or TDF did not have any significant effect on the expression levels of these genes alone or in combination with 1 μM DTG. However, the effects observed with 10 μM DTG alone were either maintained or rescued by the co-application of an NRTI. The DTG-triggered inductions of *LEP* and *SLC2A1* (GLUT1) were impaired by the addition of TDF and TAF, respectively, while the repression of *SLC2A4* (GLUT4) was reverted by both TDF and TAF. Regarding the secretion of adipokines to the culture medium, treatments with 1 μM DTG, TAF, and TDF alone showed no effect. However, consistent with our observations regarding *ADIPOQ* mRNA expression, 10 μM DTG repressed SGBS adipocytes adiponectin release to the culture medium. The addition of TAF or TDF did not alter this DTG-induced change in adipokine secretion. Unlike our mRNA results, however, the secretion of leptin was decreased in response to 10 μM DTG alone or combined with NRTI. In addition, no significant effect over adipokine secretion were observed in cotreatments.

3.2. TAF and TDF ameliorate the DTG-induced mRNA downregulation of mitochondrial OXPHOS components and respiration in human adipocytes but do not affect the DTG-induced repression of glycolytic and lipolytic activity

We extended our study to analyze the effects of antiretroviral drugs on mitochondrial and respiratory function in differentiating adipocytes. First, we assessed the mRNA and protein expression levels of a set of OXPHOS subunits encompassing distinct mitochondrial respiratory complexes and molecular actors related to mitochondrial brown/beige adipocyte thermogenesis. Overall, 1 μM DTG had mild effects on the mRNA expression levels of genes encoding mitochondrial respiration components and thermogenesis. However, treatment with 10 μM DTG markedly reduced the mRNA levels for OXPHOS protein subunits [succinate dehydrogenase complex iron sulfur subunit B (*SDHB*), cytochrome *c* oxidase subunit IV (*COXIV*), and ATP synthase-coupling factor 6 (*ATP5J*); Fig. 2a – OXPHOS] and thermogenesis markers [uncoupling protein 1 (*UCP1*), peroxisome PPARG coactivator 1-alpha (*PPARGC1A*); Fig. 2a – Thermogenesis]. Interestingly, combined treatment with TAF or TDF blunted most of the ability of 10 μM DTG to repress these mRNA expression levels (Fig. 2a). Consistent with these observations, 10 μM DTG also depleted the protein levels of OXPHOS subunits (Fig. 2b). However, the NRTI cotreatment-induced recoveries observed at the mRNA level were not observed for OXPHOS proteins (Fig. 2b). TDF appeared to repress protein expression levels of *SDHB* (complex II), ubiquinol-cytochrome *c* reductase core protein 1 (*UQCRC1*, complex III), and *ATP5J* (complex V) (Fig. 2a). The mRNA expression levels of thermogenesis-related genes, such as *UCP1*, *PPARGC1A*, and Iodothyronine deiodinase 2 (*DIO2*) were also downregulated in response to the highest dose of DTG, alone and in combination with TAF or TDF (Fig. 2a).

To functionally assess whether the observed alterations of mRNA and protein expression affected mitochondrial adipocyte function, we measured respiration in human adipocytes differentiated in the presence of antiretroviral drugs (Fig. 2c, left). Basal respiration was only mildly affected by 1 μM DTG, whereas 10 μM DTG significantly repressed oxygen consumption. TAF or TDF induced basal oxygen consumption when combined with 1 μM DTG and even alone in the case of TDF. TAF and TDF did not fully prevent 10 μM DTG from repressing respiration but did, however, moderate the extent of the decrease. In summary, the overall trends seen for the mitochondrial and thermogenesis markers under DTG treatment were consistent with its observed effects on respiratory performance.

We next measured how the drugs affected the extracellular acidification rate (ECAR), which is a surrogate indicator for the glycolytic activity of cells. Our results indicated that 10 μM DTG repressed glycolytic activity, whereas neither TAF nor TDF altered this parameter alone or in combination with DTG (Fig. 2C, middle).

We also quantified changes in the glycerol concentration, which is an indicator of lipolytic activity, in adipocyte cell cultures. The amount of glycerol released to the culture medium was significantly reduced in adipocytes treated with 10 μM DTG alone or in combination with NRTIs. Cotreatment with NRTIs do not alter the degree of decrease observed with DTG alone (Fig. 2c, right).

3.3. TAF, but not TDF, prevents DTG-induced inflammation but exacerbates cellular stress profile in differentiating human adipocytes

We next analyzed how antiretroviral drug treatment during adipocyte differentiation affected inflammation-related gene expression by

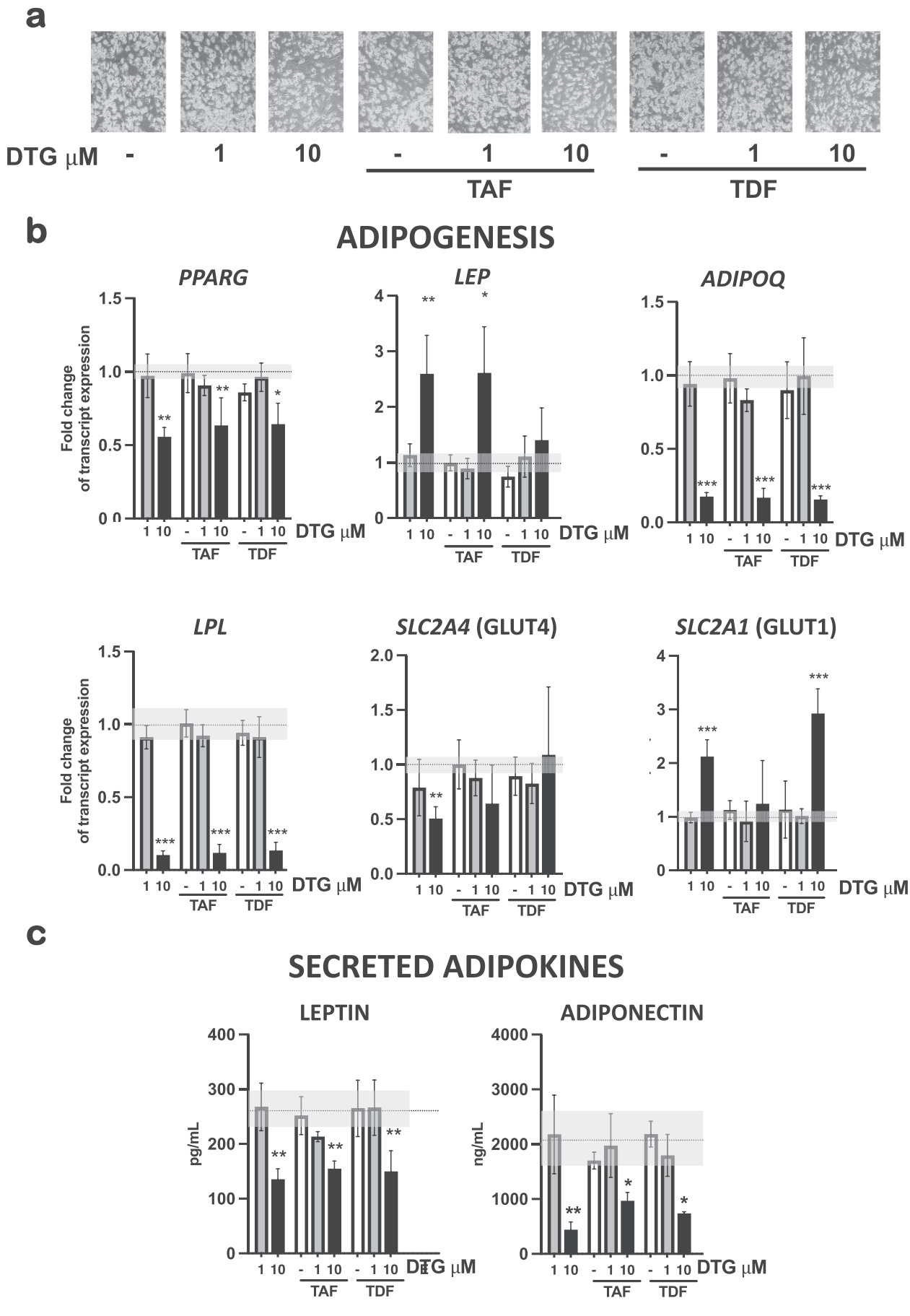

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Fig. 1. Effects of dolutegravir (DTG), tenofovir alafenamide (TAF), and tenofovir disoproxil fumarate (TDF) on adipogenic differentiation of human preadipocytes in culture. SGBS human preadipocytes were differentiated in culture in the presence of 1 or 10 μM of DTG with or without 0.033 μM of TAF or 0.4 μM of TDF. Treatment was initiated on day 0, when the differentiation cocktail (Quick Diff) was added to the cells and continued throughout the differentiation process (10 days). Representative micrographs of adipocyte cell cultures differentiating in the presence of the indicated concentrations of drugs are shown **(a)**. Data on the mRNA expression levels of genes related to adipogenic function and adipokines in SGBS human adipocytes differentiating in culture are presented **(b)**. The effects of the treatments on the releases of leptin and adiponectin to the cell culture medium of differentiating SGBS human adipocytes are shown **(c)**. Data in histograms are presented as means of fold mRNA expression ± SD from 3 independent cell culture experiments with each sample analyzed in duplicate and are expressed relative to values from untreated control cells (defined as 1, dotted line ± gray area). Statistical analyses were performed using one-way ANOVA and Dunnett's multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 for each drug treatment vs. control.

Table 4
Effects of dolutegravir (DTG), tenofovir alafenamide (TAF), and tenofovir disoproxil fumarate (TDF) on lipid accumulation in SGBS differentiated adipocytes.

	-			TAF			TDF		
DTG μM	-	1	10	-	1	10	-	1	10
	98.9±12.2	101±8.7	86±7.4	94.3±2.1	98.8±9.3	98.8±0.5	90.7±4.4	86±3.8	90.2±7.4

quantifying the mRNA expression levels of a set of proinflammatory cytokines (Fig. 3a). DTG at 1 μM showed little or no effect. However, DTG at the higher dose resulted in increased expression of proinflammatory cytokines [chemokine (C-C motif) ligand 2 (*CCL2*, MCP-1) and interleukin-6 (*IL-6*)]. Treatment with TAF or TDF alone showed mild or no effects. However, the addition of TAF, but not TDF, to the 10 μM DTG condition restored the basal expression levels of *CCL2* and *IL-6* in human adipocytes. Interleukin-8 (*IL-8*) was induced in all experiments involving the higher dose of DTG and with 1 μM DTG plus TDF (Fig. 3a).

Regarding the release of proinflammatory cytokines to the culture medium, 1 μM DTG mildly reduced the releases of IL-6 and IL-8, whereas 10 μM DTG massively increased the releases of MCP-1 and IL-8 and mildly induced that of IL-6. TAF and TDF alone showed no effect on the releases of MCP-1, IL-6, and IL-8. However, the combination of TAF with 10 μM DTG reversed the ability of 10 μM DTG to induce the releases of these proinflammatory cytokines (Fig. 3b). We then assessed the mRNA expression levels of cellular stress markers (Fig. 4). DTG at its lower dose, TAF, and TDF did not alter markers of mitochondrial stress [mito stress; fibroblast growth factor 21 (*FGF21*) and growth differentiation factor 15 (*GDF15*)], endoplasmic reticulum stress [ER stress; DNA-damage inducible transcript 3 (*DDIT3*, CHOP-10)], hypoxia [hypoxia inducible factor 1 subunit alpha (*HIF1A*)] or fibrosis [collagen type III alpha 1 chain (*COL3A1*) and actin alpha 1 (*ACTA1*)]. TDF induced heat shock protein family A (*HSPA5*, BIP) and transforming growth factor beta 1 (*TGFB1*), which are marker genes for ER stress and fibrosis, respectively. Treatment of differentiating adipocytes with 10 μM DTG induced markers of mitochondrial and ER stress (*GDF15*, *DDIT3*) and fibrosis (*TGFB1*). Combination treatments with TAF or TDF maintained these effects and additionally increased the mRNA expression levels of *HIF1A* and *TGFB1*, which are markers of hypoxia and fibrosis, respectively (Fig. 4).

3.4. TAF and TDF ameliorate DTG-induced impairment of OXPHOS subunits and basal respiration in fully differentiated human adipocytes

To further explore the effects seen in adipocytes treated during differentiation, we expanded our study to assess the effects of the drugs on human adipocytes differentiated in the absence of these antiretrovirals (Fig. 5). Preadipocytes were cultured and differentiated in a standard manner (see M&M) until they acquired a full adipocyte morphology (usually after 14 days of the initiation of differentiation). The effects of 24-hour treatments with antiretroviral drugs alone and in combination were tested. Brown/beige adipocyte thermogenesis marker genes mRNA expression profiles appeared mainly unaffected. Both concentrations of DTG repressed the mRNA expression levels of OXPHOS protein subunits [NADH:ubiquinone oxidoreductase subunit B8 (*NDUFB8*), *UQCRC1*, mitochondrially encoded cytochrome *b* (*MTCYB*), and *ATP5J*]. TAF and TDF in combination with DTG recovered the mRNA expression level of *UQCRC1*, and the DTG-induced downregulation of *MTCYB* and *ATP5J* was rescued by TAF. The mRNA level of *SDHB* was induced by combined treatments of DTG with TAF or TDF (Fig. 5a).

Regarding effects on respiration, DTG at 1 μM and TDF alone showed no effect, while TAF alone induced basal respiration (Fig. 5a, left). DTG at 10 μM repressed oxygen consumption, and both TAF and TDF improved this effect. The glycolytic rate, as estimated by ECAR, was repressed by 10 μM DTG and the addition of TAF or TDF did not alter this repression (Fig. 5b, middle). Finally, the lipolytic rate appeared unaltered by these drugs within a 24-h treatment period (Fig. 5b, right).

4. Discussion

Despite growing awareness that the NRTI, TAF, contributes to the obesogenic response to current INSTI-containing DTG + TAF or TDF treatment patterns, quite few data has been reported on how TAF alone or in combination with DTG affects human adipocytes. A single recent study indicated that TAF antagonizes the profibrotic effects of DTG in a mouse adipocyte cell line [30]. With the aim of better understanding the additive effects of DTG + TAF or TDF on potential adipocyte dysfunction, we comprehensively analyzed the effects of DTG (INSTI) + TAF or TDF (NRTIs) on biological processes of human adipocytes that are thought to be associated with adipose tissue dysfunction (adipogenesis, mitochondrial respiration, inflammation, cellular stress, fibrosis, and hypoxia). Consistent with previous reports from our research group and others [25,26,29], DTG did not massively disturb adipocyte morphology acquisition, nor did the NRTIs, TAF and TDF, alone or combined with DTG. The reduction in preadipocyte proliferation elicited by the higher dose of DTG, alone and in combination with TAF and TDF, could impact overall tissue metabolism, as it has been previously proposed that a limited capacity of adipose tissue turn-over, -meaning proliferation and differentiation of new "healthy" adipocytes- might compromise overall adipose tissue performance [34]. Whether this effect is also occurring at the tissue level remains to be elucidated. Here, we confirmed the effects previously observed for DTG, where the highest dose resulted in repressed expression of key genes in adipocyte metabolism [29,30]. Neither TAF nor TDF alone exhibited a similar repression, but the combination of DTG with TAF or TDF resulted in recovery of expression of the insulin-sensitive glucose transporter GLUT4. A reciprocal effect was observed for the genes encoding leptin (LEP) and the glucose transporter GLUT1. They were induced by the highest dose of DTG, but DTG plus TAF resulted in normalization of the GLUT1 gene expression and DTG plus TDF recovered *LEP* expression. These effects on *LEP*, however, did not result in increased release of leptin to the adipocyte culture medium; on the contrary, DTG resulted in a reduced release that was maintained under cotreatment with TAF or TDF. This effect on leptin secretion was also observed in a recent report where leptin levels

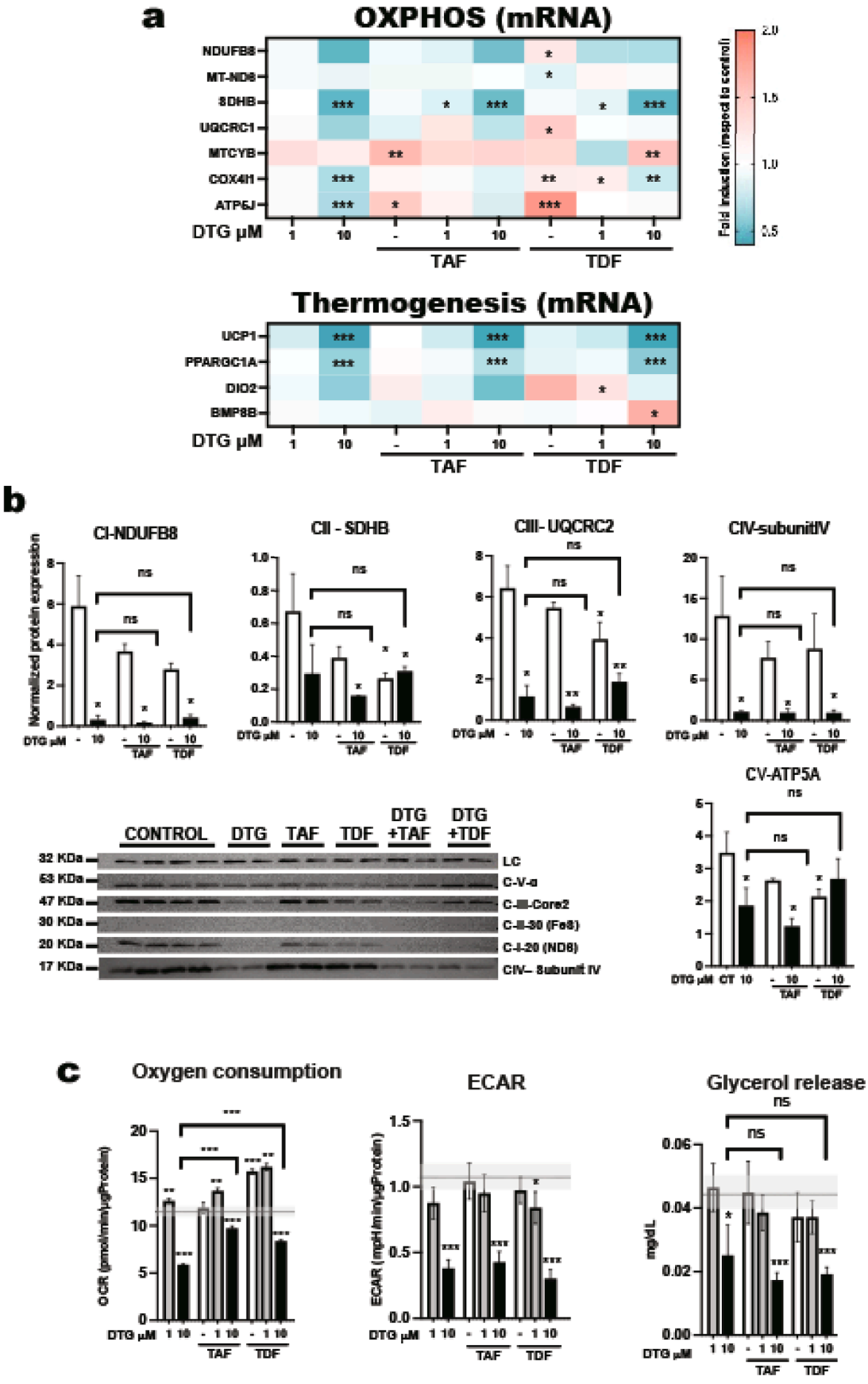

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Fig. 2. Effects of DTG, TAF, and TDF on respiration and thermogenesis markers of SGBS human preadipocytes in culture. SGBS human preadipocytes were differentiated in culture in the presence of the indicated concentrations of drugs alone or in combination. Heatmap represents transcript levels (mRNA) of genes related to oxidative phosphorylation (OXPHOS) in SGBS human adipocytes differentiating in culture and are presented as scaled means relative to values from untreated control cells (defined as 1) **(a)**. Bar plots of the normalized OXPHOS protein quantification (LC-ATUB) results and a representative immunoblot; the former are presented as means ± SD from 2 independent western blot membranes **(b)**. Human preadipocytes were differentiated in culture in the presence of the indicated concentrations of drugs alone or in combination. Heatmap represents mRNA levels of genes related to thermogenesis and mitochondrial biogenesis in human adipocytes differentiating in culture, presented as scaled means relative to values from untreated control cells (defined as 1) **(c)**. Oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and glycerol release to the culture medium of SGBS human adipocytes differentiating in culture treated with the drugs alone and in combination, shown as means ± SD **(d)**. Statistical analyses were performed using one-way ANOVA and Dunnett's multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 for each drug treatment vs. control.

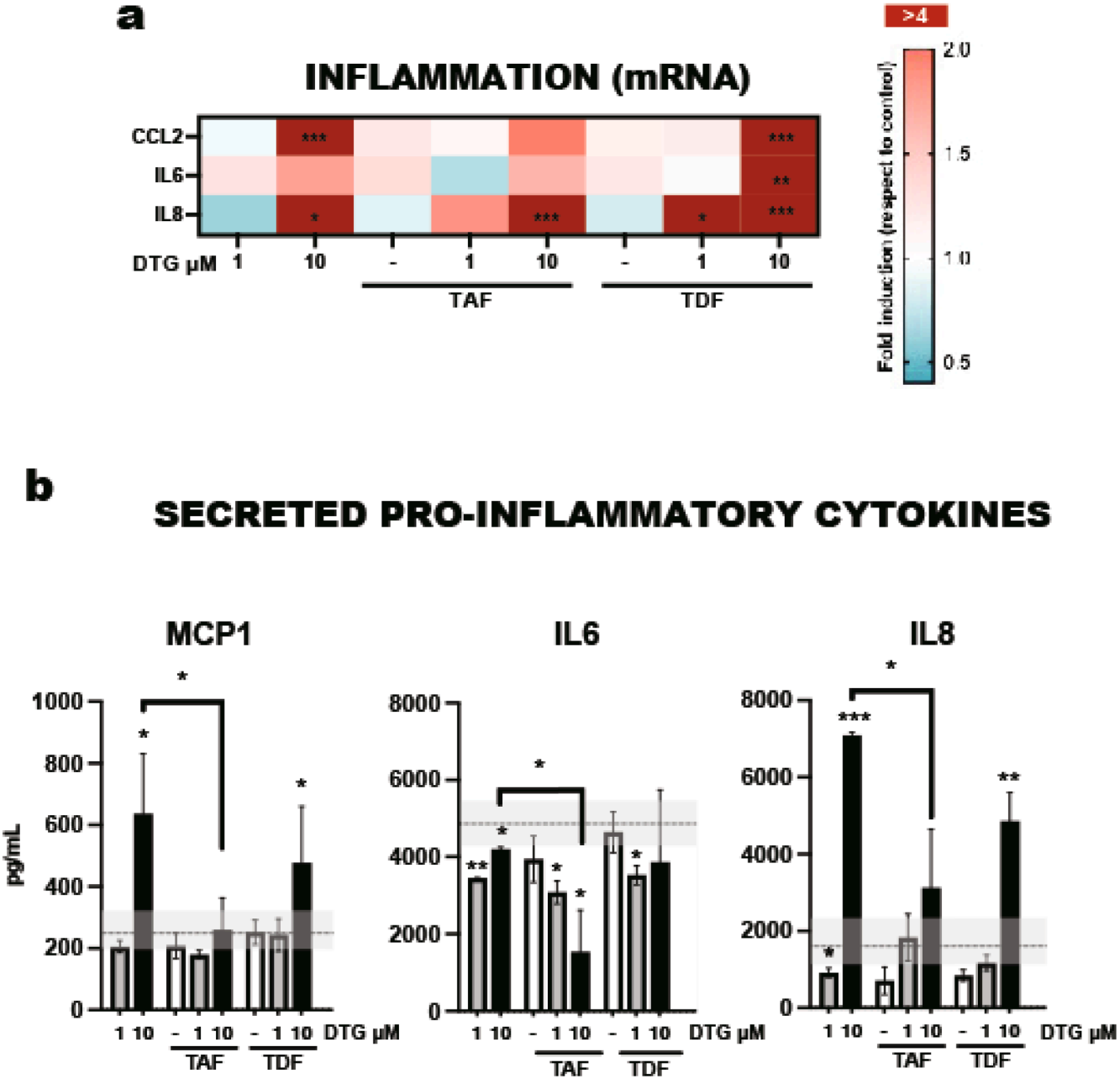

Fig. 3. Effects of DTG, TAF, and TDF on inflammation of SGBS human preadipocytes in culture. Heatmap represents mRNA levels of genes related to inflammation in SGBS human adipocytes differentiating in culture and are presented as scaled means relative to values from untreated control cells (defined as 1) in heatmaps **(a)**. The effects of the drugs added alone or in combination on the release of inflammatory cytokines to the cell culture medium of differentiating SGBS human adipocytes are shown **(b)**. Data in histograms are presented as means ± SD. Statistical analyses were performed using one-way ANOVA and Dunnett's multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 for each drug treatment vs. control.

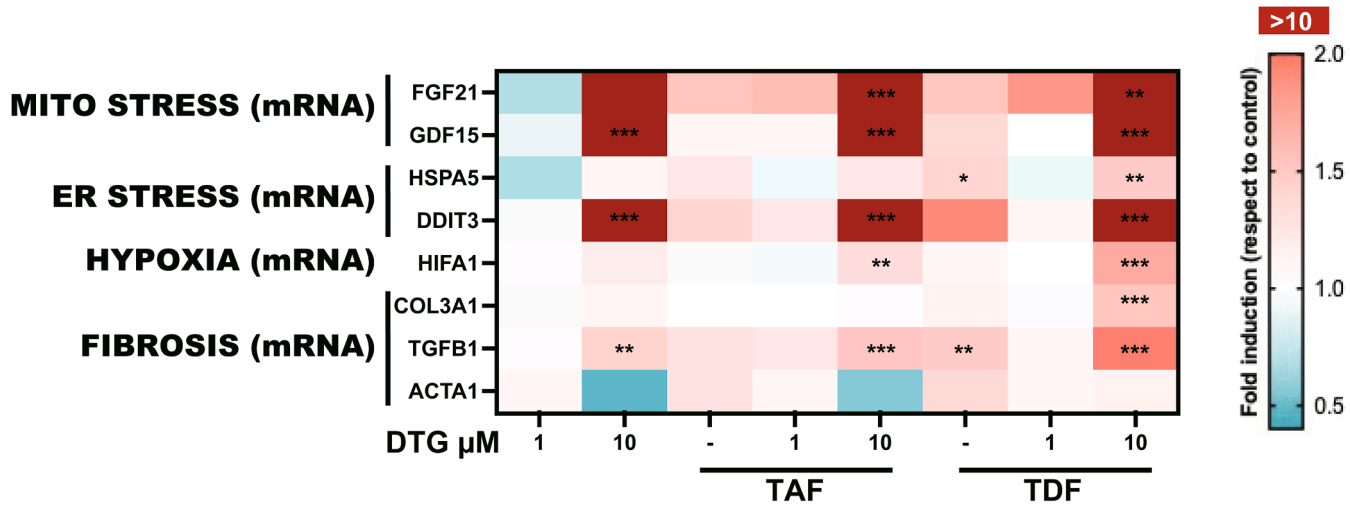

Fig. 4. Effects of DTG, TAF, and TDF on stress and fibrosis of human preadipocytes in culture. Heatmap represents mRNA levels of genes related to mitochondrial stress, endoplasmic reticulum stress, hypoxia, and fibrosis in SGBS human adipocytes. Data are presented as scaled means relative to values from untreated control cells (defined as 1) **(a)**. Statistical analyses were performed using one-way ANOVA and Dunnett's multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 for each drug treatment vs. control.

were decreased in PLWH under INSTI- containing cART [35]. Leptin secretion by adipocytes is known to involve both transcriptional and post-transcriptional processes [36]; it is possible that drugs exert distinct effects at these cellular regulatory levels thus resulting in a discordance between mRNA changes and actual leptin secretion.

Our current findings indicating reduced release of adiponectin by human adipocytes in response to the highest dose of DTG is consistent with the hypoadiponectinemia observed in patients under cART [26] and with our previous studies in human adipocytes [25]. These effects were maintained but not exacerbated by the addition of TAF or TDF, possibly suggesting that cART regimens containing DTG might be conditioning the hypoadiponectinemia observed after the treatment initiation.

Our findings indicating repressed expression of OXPHOS machinery and thermogenesis marker genes in response to DTG were also consistent with the reduced adipocyte respiration and lipolysis in DTG-treated adipocytes, and with the known correlation of adiponectin and overall mitochondrial function [37]. Interestingly, the reduced mRNA expression levels of OXPHOS and thermogenesis marker genes and the concomitant decreased cellular respiration caused by DTG were recovered under combined treatment with TAF or TDF. In line with this, respiration in human adipocytes was increased in the presence of TAF or TDF and the decreased oxygen consumption observed under DTG treatment was fully recovered by the co-application of TAF or TDF. Of notice, the recovery in OXPHOS mRNA levels and mitochondrial respiration elicited by TAF and TDF in DTG-treated cells was not observed for OXPHOS protein levels, which also suggests drug actions on the post-translational mechanisms of regulation of OXPHOS activity in cells [38].

Inflammation was also affected by DTG, which upregulated the mRNA expression of proinflammatory marker genes, and these effects were maintained or even exacerbated in the presence of TDF. However, TAF rescued the expression levels of proinflammatory markers under the highest dose of DTG. Interesting, although TAF and TDF had some ability to ameliorate the effects of DTG on adipogenesis and mitochondrial function, this was not seen for DTG-induced mitochondrial and endoplasmic reticulum stress, hypoxia, and fibrosis. In fact, TAF or TDF even exacerbated some of the DTG effects on these parameters. TAF and TDF were previously reported to have similar effects on fibrosis in a murine adipocyte cell model [30] and a previous study also reported that hypoxia and oxidative stress marker genes appear to be induced by DTG [28]. These observations are generally consistent with clinical parameters in patients and resemble observations from clinical trials in which inflammation and fibrosis are reportedly enhanced in PLWH under cART treatments containing DTG and TAF [26,39]. Even though data collected from patients and experimental models suggest that regimes containing TAF or TDF favor metabolic disturbances (possibly triggered by weight gain) [40], we observed that TAF or TDF alone failed to significantly affect adipogenesis, the mRNA expression of adiponectin and leptin, and the release of these factors to the culture media. These results suggest that DTG has a dominating effect on the mRNA expression and release of systemic metabolism-related adipokines. This is also in line with the effects observed on cell respiration, where DTG potently repressed basal cell respiration and glycolytic rate and TAF or TDF alone enhanced respiration.

In sum, we report here a set of significant effects of DTG on key markers of adipocyte metabolism, inflammation, fibrosis, and overall mitochondrial status, overall commented on the previous data [25,26,28,29]. We further show that TAF or TDF alone had mild effects on differentiating and differentiated adipocytes but exacerbated some of the DTG effects on cell stress, fibrosis, and hypoxia in human adipocytes. This could contribute to dysregulating adipocyte function and overall adipose tissue function in the long term. Some of our findings indicating distinct effects of TAF versus TDF in modulating the effects of DTG on adipocyte parameters may contribute to explain the fact that cART-naïve women that started DTG develop a higher weight gain when on a DTG + TAF regimen compared to DTG + TDF [11].

Declaration of Interest

The authors herein declare the following financial interests/personal relationships which may be considered as potential competing interests:

Dr. Pere Domingo reported receiving honoraria from Merck Sharp & Dohme, Gilead Sciences, ViiV Healthcare, Janssen, Cilag, Thera technologies, and Roche. All of the other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors guarantee that they have the authority to publish the work and that the manuscript and confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal.

CRediT authorship contribution statement

T. Quesada-López: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **R. Cereijo:** . **A. Blasco-Roset:** . **A. Mestres-Arenas:** . **P. Prieto:** . **J.C. Domingo:** . **F. Villarroya:**

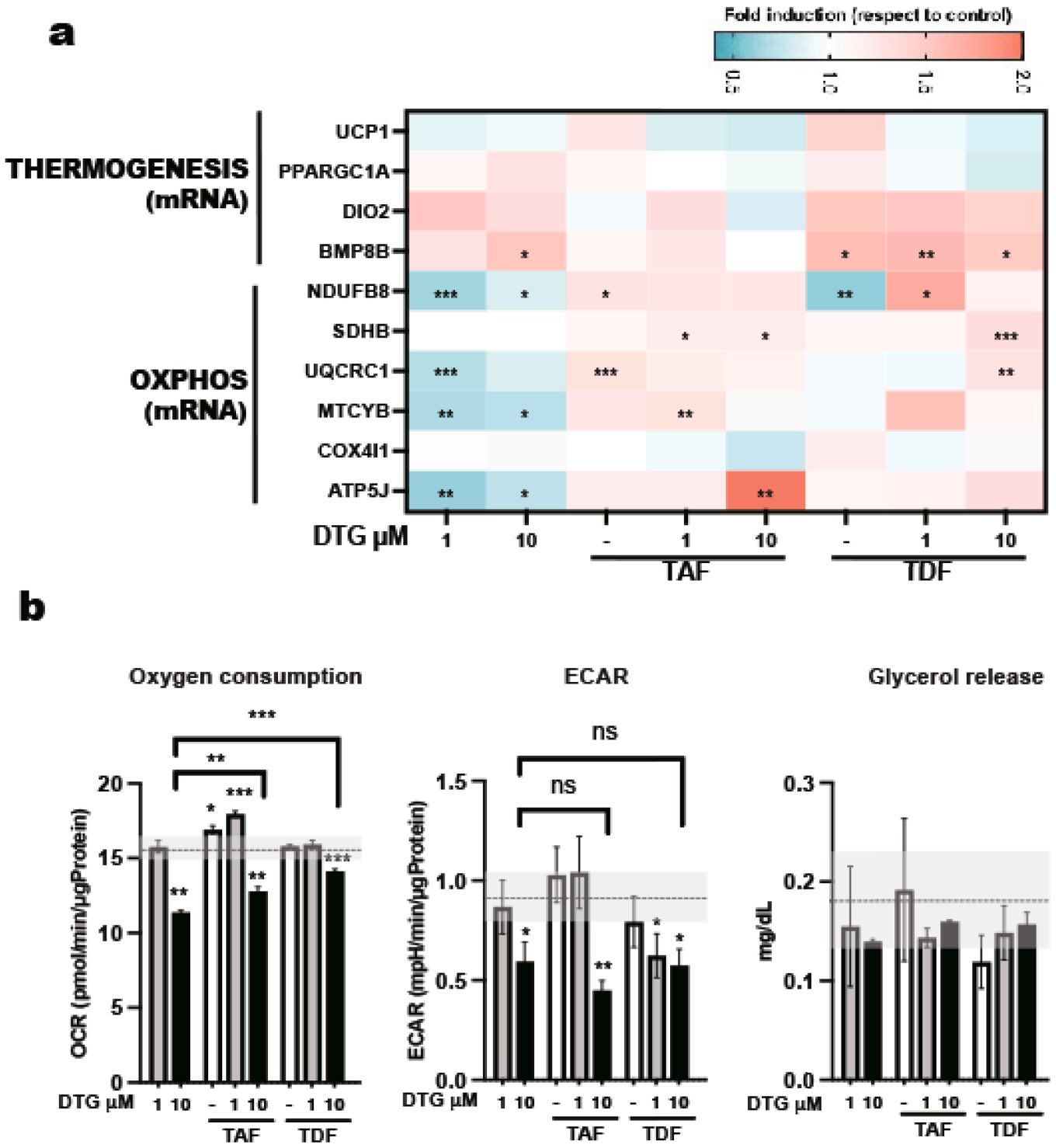

Fig. 5. Effects of DTG, TAF, and TDF on respiration and thermogenesis markers in fully differentiated human adipocytes in culture. Fully differentiated SGBS human adipocytes were treated for 24 h with 1 or 10 µM of dolutegravir with or without 0.033 µM of TAF or 0.4 µM of TDF. Heatmap represents mRNA levels of genes related to oxidative phosphorylation (OXPHOS), thermogenesis, and mitochondrial function in SGBS adipocytes. Data are presented as scaled means relative to values from untreated control cells (defined as 1) **(a)**. Oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and glycerol release to the medium, under treatment with drugs alone and in combination, are shown as means ± SD **(b)**. Statistical analyses were performed using one-way ANOVA and Dunnett's multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 for each drug treatment vs. control.

Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Resources. **P. Domingo:** Writing – review & editing, Resources. **M. Giralt:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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