Telmisartan reverses antiretroviral-induced adipocyte toxicity and insulin resistance in vitro

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Abstract
Background: Antiretroviral therapy in HIV-positive patients leads to insulin resistance which is central to the pathogenesis of various metabolic abnormalities and cardiovascular disease seen in this patient group. We have investigated the dose–response relationship of telmisartan, an antihypertensive, on adipocytes in vitro in order to determine whether it may have metabolic beneficial effects.
Methods: Using in vitro chronic toxicity models (3T3-F442A murine and primary human adipocytes), we evaluated the effects of different concentrations of telmisartan on adipocyte differentiation and adipogenic gene expression using lipid accumulation assays and real-time polymerase chain reaction, respectively. Adipokine secretion and expression of insulin signalling mediators were evaluated using enzyme-linked immunosorbent assays.
Results: Telmisartan partially reversed the deleterious effects of antiretrovirals on adipocyte lipid accumulation, expression of adipogenic regulators (peroxisome proliferator receptor-gamma and lipin 1), adipokine secretion and expression of the insulin signalling mediator pAktSer473. The metabolic effects of telmisartan followed a non-monotonic response with the maximal effect observed at 5 µM in the primary human adipocyte model.
Conclusion: Telmisartan has beneficial metabolic effects in adipocytes in vitro, but its potential to reduce antiretroviral-induced cardiometabolic disease in HIV-infected individuals needs to be evaluated in a well-designed adequately powered clinical trial.

Keywords
HIV, antiretroviral, insulin resistance, metabolic disease, telmisartan, adipocyte
either boosted atazanavir (ATV) or boosted darunavir or raltegravir showed a 1.9-fold increase in homeostatic model assessment–IR (HOMA-IR) within 4 weeks. Importantly, HIV-associated metabolic disease results in increased healthcare burden; a recent study in the United States identified the management of IR/diabetes to be the biggest contributor to the cost burden and resource use among all HIV-related adverse events studied.

Adipose tissue is an important determinant of IR and may therefore play a key role in cART-associated metabolic disease. Adipose tissue has also been shown to be a reservoir for HIV and a source of chronic inflammation. There is therefore a need for novel clinical interventions that can reduce cART-induced IR in HIV-positive individuals.

Clinical interventions to arrest or reverse cART-associated adipose-mediated IR are a potential strategy to reduce the incidence of T2DM and CVD in HIV-positive patients. To this end, insulin sensitisers such as thiazolidinediones and metformin have been trialled, but results from randomised clinical trials in HIV-positive patients have been disappointing and sometimes deleterious. There is therefore a need for novel clinical interventions that can reduce cART-induced IR in HIV-positive individuals.

Preliminary in vitro studies have suggested that telmisartan (TEL), an angiotensin II receptor blocker (ARB), reduces cART-induced adipose dysfunction by inhibition of the renin–angiotensin system (RAS). In addition to being an ARB, TEL is also a partial agonist at the peroxisome proliferator receptor-gamma (PPARγ) receptor, being an ARB, TEL is also a partial agonist at the peroxisome proliferator receptor-gamma (PPARγ) receptor.
Lipid accumulation assay. Lipid accumulation in differentiating adipocytes was assessed on day 10 (3T3-F442A cells) or day 12 (primary human adipocytes) of differentiation using Oil Red O (Sigma, Dorset, UK) as previously described. Lipid-bound dye was extracted using 70% isopropyl alcohol and staining was quantified at 520 nm. The drug-treated cells were compared against the vehicle-treated control (methanol).

RNA extraction and gene expression. Total RNA was isolated using the RNeasy kit (Qiagen, Manchester, UK). Total RNA was reverse transcribed using the TaqMan® reverse transcription kit (Life Technologies Ltd). Gene expression of PPARG and LPIN1 were assessed by real-time polymerase chain reaction (PCR) using TaqMan Assays-on-Demand gene expression assays on a 7900HT Fast Real-Time PCR System (Life Technologies Ltd).

Assessment of lipolysis. Free fatty acid concentration in the conditioned media of primary human adipocytes was determined using the Free Fatty Acid Quantification Assay Kit as per manufacturer’s instructions (Abcam). Briefly, palmitic acid standard (1 mmol/µL) was used to prepare the standard curve dilution; the reaction plates were prepared and incubated in the dark at room temperature for 30 min and the absorbance was measured using a microplate spectrophotometer (Beckman Coulter DTX880 Multimode Detector) at 595 nm.

Estimation of adipokines. Adipokine (adiponectin, IL-6, resistin and TNF-α) concentrations in the conditioned media were determined on day 10 (3T3-F442A) or day 12 (primary human adipocytes) post differentiation using bead-based Milliplex Mouse Sandwich Multiplex ELISA kits (Merck Millipore) and Human Singleplex ELISA kits (Life Technologies Ltd), respectively. The ELISA kits used had the following detection limits: adiponectin, 5.2 pg/mL (murine) and 100 pg/mL (human); IL-6, 5.3 pg/mL (murine) and <1 pg/mL (human); TNF-α, 11.2 pg/mL (murine) and <2 pg/mL (human) and resistin, 6.1 pg/mL (murine) and 10 pg/mL (human).

Estimation of phosphorylated Akt content. Phosphorylated Akt (serine residue 473) as well as total Akt was quantified by a sandwich ELISA, as recommended by the manufacturer (Thermo Fisher Scientific). Briefly, the diluted lysates were applied to 96-well plates containing immobilised monoclonal antibodies specific for human Akt and incubated for 2 h at room temperature. A pS473 Akt standard and the samples were pipetted into the wells, followed by washing and incubation with a rabbit antibody (Cell Signalling, MA, USA) specific for AKT phosphorylated at serine 473. Following washing, a horseradish peroxidase-labelled anti-rabbit IgG was added and washed; a substrate solution [3,3′,5,5′-tetramethylbenzidine (TMB)] was added to produce colour and the absorbance was read at 450 nm.

Statistical analysis

Data are presented as mean ± standard deviation (SD) for at least three independent experiments to ensure reproducibility. Statistical significance was determined using the non-parametric Mann–Whitney U test (IBM SPSS Statistics, version 22). The threshold of significance was set at \( p < 0.05 \).

Results

ARVs cause adipocyte cytotoxicity

Cytotoxicity was observed with all ARVs during differentiation of preadipocytes and followed a similar trend in both 3T3-F442A and primary human cells. In differentiating 3T3-F442A adipocytes, the rank order for cytotoxicity was LPV (IC\(_{50}=14\) µM) > RTV (IC\(_{50}=48\) µM) > ATV.
In primary human adipocytes undergoing differentiation, the rank order for cytotoxicity was LPV (IC_{50} = 28 µM) > RTV (IC_{50} = 38 µM) > ATV (IC_{50} = 84 µM; Figure 1(b)).

(Initial concentration = 66 µM; Figure 1(a)). In primary human adipocytes undergoing differentiation, the rank order for cytotoxicity was LPV (IC_{50} = 28 µM) > RTV (IC_{50} = 38 µM) > ATV (IC_{50} = 84 µM; Figure 1(b)).

**TEL reverses ARV-induced inhibition in adipocyte lipid accumulation**

In 3T3-F442A adipocytes, a dose-dependent reduction in lipid accumulation was observed for LPV (at 20 µM, a reduction of 32% in mean absorbance, \( p < 0.01 \)) and RTV (at 20 µM, 44% reduction, \( p < 0.01 \)) but not for ATV (at 20 µM, 4% increase, non-significant (NS) in comparison with the vehicle-treated control, \( p < 0.01 \)) the ARV-induced inhibition of lipid accumulation partially in primary human adipocytes (Figure 2(b)). The effect shown by ROSI was stronger than that by TEL.

**TEL reverses ARV-induced downregulation of PPAR\(\gamma\) and LPIN1 gene expression**

**PPAR\(\gamma\).** In 3T3-F442A adipocytes, both LPV and RTV (75% and 73% downregulation, respectively, \( p < 0.01 \)), but not ATV, downregulated PPAR\(\gamma\) gene expression in comparison with the vehicle-treated control. This was partially but significantly reversed by 1 µM TEL (Figure 3(a)). A similar result was observed in primary human adipocytes (LPV and RTV with 78% and 80% downregulation, respectively) which was partially reversed by 5 µM TEL (LPV + TEL, \( p = 0.03 \); RTV + TEL, \( p = 0.01 \); Figure 3(b)).

**LPIN1.** Both LPV and RTV downregulated LPIN1 gene expression in both 3T3-F442A adipocytes (LPV, 64%; RTV, 78%, \( p < 0.01 \); Figure 3(c)) and primary human adipocytes (LPV, 68%; RTV, 63%, \( p < 0.01 \); Figure 3(d)). In both models, this was partially reversed by 1 (3T3-F442A) or 5 µM (primary human adipocytes) TEL (Figure 3(c) and (d)). ATV did not have any effect on LPIN1 expression.

**TEL reverses ARV-induced changes in adipokine secretion**

**Adiponectin.** In 3T3-F442A adipocytes, both LPV (4.0 ng/mL ± 0.4; \( p = 0.002 \)) and RTV (7.0 ± 1.0; \( p = 0.001 \)) but not ATV (14.2 ± 2.4) caused downregulation in secreted adiponectin protein in comparison to the vehicle-treated control (16.5 ± 2.0; Figure 4(a)). 1 µM TEL resulted in a significant but partial reversal of PI-induced downregulation of adiponectin (Figure 4(a)). A similar effect was also observed in primary human adipocytes (LPV: 278% increase; RTV: 316% increase; both \( p < 0.01 \); Figure 4(d)). In both in vitro models, coincubation with TEL partially reversed PI-induced upregulation of secreted IL-6 (Figure 4(c) and (d)).

**IL-6.** Both LPV and RTV but not ATV increased the secretion of IL-6 in 3T3-F442A adipocytes (LPV, 190 ng/mL ± 11.3; RTV, 243 ± 7.9; both \( p < 0.01 \); ATV: 55 ± 8.0, NS) in comparison to the vehicle-treated control (45 ± 7.1; Figure 4(c)). A similar effect was also observed in primary human adipocytes for these PIs (LPV: 278% increase; RTV: 316% increase; both \( p < 0.01 \); Figure 4(d)). In both in vitro models, coincubation with TEL partially reversed PI-induced upregulation of secreted IL-6 (Figure 4(c) and (d)).
TNF-α. LPV (3T3-F442A, 45 pg/mL ± 2.1; primary human adipocytes, 62 pg/mL ± 4.2, both p < 0.001) and RTV (3T3-F442A, 57 ± 5.0; primary human adipocytes, 78 ± 5.7, both p < 0.01) but not ATV (3T3-F442A, 30 ± 2.0; primary human adipocytes, 45 ± 5.0) upregulated secreted TNF-α in comparison to the vehicle-treated control (3T3-F442A, 32 ± 4.3, and primary human adipocytes, 40 ± 5.0; Figure 4(e) and (f)). Coincubation with either 1 (3T3-F442A) or 5 µM TEL (primary human adipocytes) or 10 µM ROSI (primary human adipocytes only) significantly reversed PI-induced upregulation of TNF-α.

Resistin. All three PIs downregulated resistin in both murine (LPV: 74% decrease; RTV: 73% decrease; ATV: 57% decrease; all in comparison to vehicle-treated control; p < 0.01; Figure 4(g)) and primary human adipocytes (LPV and RTV: 65% decrease; ATV: 48% decrease; all in comparison to vehicle-treated control; p < 0.01; Figure 4(h)). Both TEL and ROSI (in primary human adipocytes only) showed a trend to reverse the PI-induced downregulation of resistin, but this was not significant in either of these models (Figure 4(g) and (h)).

TEL reverses ARV-induced adipocyte lipolysis and inhibition of Akt phosphorylation in primary human adipocytes

Both LPV (90% increase; p < 0.03) and RTV (109% increase; p < 0.01) but not ATV (23%; p = NS) resulted in an increase in free fatty acid levels in the conditioned media in primary human adipocytes suggesting enhanced lipolysis by these drugs (Figure 5(a)). Coincubation with 5 µM TEL reduced PI-induced lipolysis although the effect was statistically NS. However, 10 µM ROSI showed a significant partial reversal of ARV-induced lipolysis.

A significant reduction in the expression of pAktSer473 was observed with LPV (at 20 µM, 63% reduction, p < 0.01) and RTV (at 20 µM, 61% reduction; p < 0.01), but not with ATV, in comparison with the vehicle-treated controls (Figure 5(b)). Both LPV and RTV reduced pAktSer473 expression in a dose-dependent manner (see Supplementary Information). Coincubation with 5 µM TEL or 10 µM ROSI significantly reversed PI-induced downregulation of pAktSer473 (Figure 5(b)).
Figure 4. Adipokine secretion in differentiating 3T3-F442A and primary human adipocytes following incubation with PIs with or without TEL or ROSI: adiponectin: (a) 3T3-F442A and (b) primary human adipocytes; IL-6: (c) 3T3-F442A and (d) primary human adipocytes and TNF-α: (e) 3T3-F442A and (f) primary human adipocytes; resistin: (g) 3T3-F442A and (h) primary human adipocytes. ROSI was coincubated with LPV in the primary human adipocyte model only. Data are expressed as mean (n = 3) ± SD. (*p < 0.01, drug vs vehicle; †p < 0.01, drug vs drug + TEL or drug + ROSI; ‡p < 0.01, preadipocyte vs vehicle.).

Preadipo: preadipocyte; Veh: vehicle; RTV: ritonavir; LPV: lopinavir; ATV: atazanavir; TEL: telmisartan; ROSI: rosiglitazone.
Characterisation of optimal TEL dose to elicit metabolic effect

Using secreted adiponectin and PPARγ gene as the exemplar markers, we evaluated the concentration–response relationship of TEL in the presence of LPV (20 µM) in primary human adipocytes. TEL significantly reversed LPV-induced inhibition of adiponectin at 1, 5 and 10 µM concentrations; for PPARγ, the effect of TEL was observed at 5 and 10 µM only. Importantly, in both instances, the maximal response for TEL was observed at 5 µM with TEL showing a non-monotonic dose response (Figure 6(a) and (b)).

Discussion

We have confirmed the previously reported toxic effect of PIs on adipocytes and also identified how PIs affect novel adipokines such as LPIN1 and resistin. We also found that TEL results in a partial but significant reversal of ARV-induced adipocyte toxicity and, for the first time, characterised the concentration of TEL that elicits the maximal metabolic effect in vitro. We used 3T3-F442A cells as they are one of the most widely used in vitro models to study adipogenesis and are committed to differentiating into adipocytes.20 They have also been shown to develop a homogeneous population of mature adipocytes that are morphologically and biochemically similar to adipocytes in situ.20 The chronic drug treatment design enabled repeated drug exposure to the adipocytes over the entire period of adipocyte differentiation; this mimicked the in vivo situation where the long-term ARV drug treatment may result in cumulative adipocyte toxicity.21 LPV and RTV were toxic to adipocytes in both in vitro models, while ATV, a more lipid-friendly PI,22 was not, suggesting that the accumulation of certain ARVs over time may reduce cell viability in differentiating adipocyte populations in vivo and potentially deleteriously affect the fat cell turnover and thereby adipose tissue distribution.

Both LPV and RTV decreased lipid accumulation and messenger RNA (mRNA) expression of the adipogenic markers, PPARγ and LPIN1, consistent with their anti-adipogenic effects. By contrast, ATV did not show any
effect on any of the above markers of lipid metabolism even at a concentration of 20 µM (>4 times its Cmax value of 4.4 µM). The contrasting effects of ATV support clinical data,22 which show that ATV has very little effect on body fat distribution in HIV patients. This is the first study to report an effect of PIs on LPIN1, a gene that encodes a magnesium-ion-dependent phosphatidic acid phosphohydrolase enzyme involved in triglyceride synthesis23 and a key factor in the maturation and maintenance of adipocyte differentiation.24 LPIN1 is also a key transcriptional regulator of PPARγ and various genes involved in lipid metabolism.25 Interestingly, LPIN1 mutations cause different types of severe human lipodystrophy syndromes26 although our previous work has failed to identify any association between LPIN1 single-nucleotide polymorphisms and HIV lipodystrophy (HIVLD).27 The inhibitory effect of lipotoxic PIs on LPIN1 could potentially be one of the mechanisms involved in the transcriptional downregulation of PPARγ caused by these drugs.

LPV and RTV had a profound effect on the secretory characteristics of the adipocyte regardless of the model used. Our results on adiponectin, IL-6 and TNF-α further highlight how certain adipins on LPIN1 may interact with the adipokine network and regulate their transcription leading to adipocyte dysfunction and interference with insulin signalling. In addition, LPV and RTV but not ATV showed a significant reduction in the expression of phosphorylated form of Akt (Ser473); Akt is a serine/threonine kinase and a downstream target of phosphoinositide 3-kinase (PI3K) signalling, and phosphorylation of its serine residue at position 473 is an important step in the insulin signalling pathway.28

This study also explored the effects of PIs on resistin, an adipocyte-secreted protein which is implicated in IR. While higher circulating levels of resistin have been implicated in the development of IR29 and diabetes,30 its role in HIVLD is inconclusive. Some cross-sectional studies have reported an increase in circulating resistin levels in HIVLD patients,31 while other studies failed to find an association32 or even reported a reduction in resistin levels.33 We observed a significant reduction in the amount of resistin secreted by the adipocyte with all PIs including ATV although the ATV effect was comparatively less than those of other PIs. If resistin was involved in IR or adipocyte dysfunction, we would have expected its level to increase; our results suggest that secreted resistin might not be contributing directly to IR or adipocyte dysfunction in this ARV-treated cellular model. Previous clinical studies have reported a decrease in plasma resistin with TEL in diabetes patients;34 however, neither TEL nor ROSI had any effect on resistin in these in vitro models. It should be noted that in humans, resistin is primarily produced by cell populations other than adipocytes,35 including peripheral blood mononuclear cells, macrophages and bone marrow cells. This could potentially explain the discrepancy in resistin levels between clinical and in vitro studies.

TEL is widely used as an antihypertensive because of its ability to antagonise the effect of angiotensin II. However, TEL is highly lipophilic, and it has been suggested that its off-target effect on PPARγ could be beneficial in the treatment of metabolic disease and CVD.36–38 In this study, TEL was able to partially reverse the PI-induced inhibition in adipogenesis (lipid accumulation, expression of PPARγ and LPIN1), improve PI-induced reduction in adiponectin and expression of pAktSer473 (effect on insulin sensitivity), and reverse PI-induced upregulation in the secretion of proinflammatory markers, IL-6 and TNF-α. A previous in vitro study had shown that TEL improves adipocyte function following incubation with ARVs through blockade of the adipose RAS.14 It should be noted that PPARγ is also a modulator of adipocyte RAS and activation of PPARγ using full/partial agonists like ROSI or TEL could potentially counter the effects of RAS. By testing a wide range of concentrations of TEL (0.5–20 µM) on two exemplar markers, PPARγ gene and adiponectin protein, we observed TEL to show a non-monotonic response with the maximal effect observed at 5 µM in the primary human adipocyte model. This dose response shown by TEL here is different to that seen on blood pressure, which is linear and mediated by the angiotensin receptor, AT1R. It might very well be that both RAS and PPARγ play an independent role in the development of PI-induced adipocyte dysfunction; given that PPARγ full agonists such as ROSI suffer from serious adverse effects, ARBs such as TEL with dual activity on both PPARγ and RAS may offer an opportunity to reduce PI-induced toxicity.

**Limitations of the study**

This study has not investigated the effect of ARVs (with/without TEL) in mature adipocytes; of course, the adipocyte population in vivo is a mixture of differentiating and differentiated adipocytes, but we felt it was important to focus on differentiating adipocytes, as harmful effects here would ultimately affect the population of differentiated adipocytes. This study did not assess the effect of PI drug combinations as used in the clinic; relating the concentration–response relationships in vitro to the in vivo situation is challenging because of differences that can occur in protein binding and drug distribution. It should be noted that we have only used three replicates (biological replicates) for each experiment in this study, but there was a high degree of reproducibility within the experiments. Taken together, our findings support the beneficial metabolic effects observed with TEL and open up the intriguing possibility that TEL could be used to prevent the increase in IR that is seen in HIV-infected individuals treated with ARVs.
Conclusion

This study has shown that TEL has beneficial metabolic effects on adipocytes when given in combination with PIs and therefore has the potential to reverse adipocyte toxicity and IR mediated by PIs. The study also, for the first time, has characterised the dose response of TEL in human primary adipocytes. These in vitro findings now need to be validated in a clinical study which preferably not only evaluates, in a randomised fashion, the ability of TEL to reduce IR in vivo, but also identifies the optimal dose. This is currently being pursued in a phase IIb adaptive design clinical trial.39

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