Background: The complex interplay between viral infection and virus-activated inflammatory pathways with protease inhibitors (PIs) contributes to the increased risk of developing atherosclerosis and coronary artery disease in HIV-infected patients. Leflunomide is an antirheumatic drug whose administration to HIV-1-infected persons effectively decreases T-cell turnover and activation. In this study we have investigated the effects of leflunomide on dyslipidaemia and lipodystrophy induced by ritonavir in rodents.

Methods: Mice were administered ritonavir (5 mg/kg/day) alone or in combination with leflunomide (40 mg/kg/day) for 12 days. Expression of nuclear receptor and lipidogenic genes was measured in liver and adipose tissues.

Results: Administration of the HIV PI ritonavir to mice increased plasma triacylglycerols, free fatty acids and cholesterol levels, and this effect was reverted by cotreatment with leflunomide. Ritonavir administration was associated with reduced epididymal fat/body weight ratio and increased liver content of triacylglycerols content. These effects were reverted by leflunomide. Histopathology analysis shows that exposure to ritonavir causes inflammation of epididymal fat as demonstrated by dense leukocytes infiltration as well as by increased levels of proinflammatory mediators and reduced expression and activity of peroxisome proliferator-activated receptor-γ (PPAR-γ). Leflunomide reduced epididymal fat inflammatory-metabolic alteration induced by ritonavir and restored PPAR-γ expression in the epididymal fat.

Conclusions: We have shown that the anti-inflammatory drug leflunomide protects against ritonavir-induced inflammation and dysmetabolism in adipose tissue and might be a promising strategy in the setting of HIV-infected patients at risk for HIV-induced dyslipidaemia.

Adipose tissue exerts two important functions in the regulation of lipid metabolism and insulin sensitivity: the storage of free fatty acids (FFA) in triacylglycerols into adipocytes and their disposal by lipolysis, as well as the production of adipokines and cytokines that promote either insulin sensitivity or its resistance in target tissues [1]. In HIV-infected persons treatment with highly active antiretroviral therapy (HAART) has been linked to the development of dyslipidaemia and lipodystrophy, a clinical condition characterized by an abnormal distribution of a body’s adipose tissue [2,3]. HAART regimens, especially those including protease inhibitors (PIs), cause dyslipidaemia that may be a causative factor of the increased risk of cardiovascular diseases recorded in HIV patients taking long-term HAART [4]. Metabolic features associated with the use of PIs include somatic changes (lipodystrophy), dyslipidaemia (~70% of patients), insulin resistance, type 2 diabetes mellitus (8–10% of patients), hypertension (≤75% of patients), lactic acidemia and non-alcoholic steatohepatitis [4–7]. Lipodystrophy has two clinical subsets, lipohypertrophy and lipatrophy, in which abnormal fat distribution associates with a greater risk of coronary heart disease and diabetes [4–7]. Lipatrophy is characterized by peripheral fat wasting with loss of subcutaneous tissue in the face, arms, legs and buttocks. Despite severe forms of lipoatrophy being mainly found in HIV-infected patients taking thymidine analogues, especially stavudine and zidovudine [8], PIs seem to be associated with a progressive, even
if less serious, lipoatrophy [9]. In vitro and in vivo studies suggest some PIs may act towards lipoatrophy [10], amplifying in synergy the nucleoside reverse transcriptase inhibitor’s (NRTIs) effects [11,12].

PIs exert a variety of metabolic effects. Ritonavir inhibits the activity of insulin-sensitive glucose transporter 4 leading to glucose intolerance and insulin resistance, a condition often associated with central adiposity and hypertriglyceridaemia [3]. In addition, PIs promote a proinflammatory phenotype in adipocytes and macrophages [13]. Thus, exposure of differentiating 3T3-F442A adipocytes to widely used PIs and NRTIs increases the expression and secretion of proinflammatory mediators, including interleukin (IL)-6, tumour necrosis factor (TNF)-α and IL-1β, and drives apoptosis of adipocytes. Consistent with these in vitro findings, markers of inflammation are usually higher in men with lipodystrophy compared with HIV-infected patients without lipodystrophy taking HAART. In addition, in HIV-positive patients with lipodystrophy, inflammatory mediators decrease in adipose tissue following HAART discontinuation [14], such as in serum samples after PI interruption [15], suggesting a mechanistic role for inflammatory mediators in promoting a lipoatrophic condition in PI-treated patients.

Leflunomide, an approved drug for the treatment of rheumatoid arthritis, exhibits anti-inflammatory, anti-proliferative and immunosuppressive effects. In a recent study, it was shown that leflunomide administered to HIV-infected persons who were not on antiretroviral therapy effectively reduced immune activation, suggesting a beneficial role for this agent in targeting immune activation in HIV-infected persons [16]. Based on these premises, we have designed a study to investigate whether leflunomide protects against the development of lipoatrophy in a model of dyslipidaemia induced in mice by administration of ritonavir [17–19]. The results of the present study highlights that anti-inflammatory drugs might protect against the development of lipoatrophy caused by PIs and paves the way to their testing in clinically relevant settings.

Methods

Animal and treatments

C57Bl6 mice were from Harlan Nossan (Udine, Italy). Mice, 9 weeks old, were housed under controlled temperatures (22°C) and photoperiods (12:12-h light/dark cycle), and allowed unrestricted access to standard mouse chow and tap water. The present protocol (authorization for this study was released on 25 January 2010, No. 11-2010-B, to SF) was approved by the Italian Health Minister and conforms to national guidelines for animal care. The mice were randomized into three groups: group 1 (n=5), vehicle (methylcellulose 1%); group 2 (n=7), intraperitoneally administration of 100 µl ritonavir (5 mg/kg/day) alone; and group 3 (n=7), received ritonavir (5 mg/kg/day) in combination with leflunomide (40 mg/kg/day by gavage). All drugs were administered for 12 days and prepared daily in sterile conditions. During the experiment mice were weighed once a week. At the end of the experiment, 12-h fasted animals received the latest administration of the specific agent and 4 h later were anaesthetized with sodium pentobarbital and then sacrificed. Blood liver and epididymal fat were collected. Serum content of total cholesterol, triglyceride, high-density lipoprotein, low-density lipoprotein and aspartate aminotransferase were measured by enzymatic assays (Wako Chemicals, Osaka, Japan). Liver and epididymal fat samples for RNA isolation or tissue lipid content analysis were immediately snap frozen in liquid nitrogen. Liver and adipose tissue samples for histology were fixed in formalin and embedded in paraffin. This protocol was repeated in two separate sets. Because results were highly homogenous, pooled data from the two experiments are presented.

Liver histology

Liver histopathology was assessed by scoring haematoxylin and eosin (H&E) and Oil Red O stained liver sections. Scoring for ballooning (0, none; 1, occasionally in zone III; 2, obvious in zone III; and 3, marked) and steatosis (based on Image J image analysis software of Oil Red O-stained slides) was performed in a blind fashion.

Liver triacylglycerols and cholesterol levels

Methods used for assessing liver triglyceride and cholesterol content are reported in Additional file 1.

Real-time PCR analysis

The expression of selected genes was quantified by performing quantitative real-time PCR. The primer and methods used for these protocols are described in Additional file 1.

Statistical analysis

All values are expressed as the mean ±SE of n mice per group. Comparisons of more than two groups were made with a one-way analysis of variance with post-hoc Tukey tests. Differences were considered statistically significant if P<0.05.

Results

Leflunomide protects against dyslipidaemia induced by ritonavir

We found that administration of ritonavir for 12 days in mice was adequate to increase plasma triacylglycerols,
Leflunomide protects against dyslipidaemia induced by ritonavir

Figure 1. Leflunomide attenuates dyslipidaemia induced by the HIV protease inhibitor ritonavir in mice

FFA, cholesterol and LDL levels, and reduce HDL levels (Figure 1; \( P < 0.05 \) versus control group; \( n = 10–14 \) per group). This effect was abrogated by cotreating mice with leflunomide (Figure 1; \( P < 0.05 \) versus ritonavir-treated group; \( n = 10–14 \) per group).

Leflunomide protects against liver steatosis induced by ritonavir

Administering mice with ritonavir had no effect on liver weight and liver weight/liver body weight ratio (Figure 2A; \( n = 10–14 \); \( P > 0.05 \)), but increased significantly the liver content of triacylglycerols (Figure 2B; \( n = 10–14 \); \( P < 0.05 \)). No difference was observed in cholesterol content (Figure 2C). Liver accumulation of triacylglycerols translated in the appearance of liver steatosis as demonstrated by the histopathology analysis shown in Figure 2D. Indeed, staining of liver sections with H&E demonstrates that exposure to ritonavir resulted in a significant increase in the hepatocyte ballooning score (Figure 2D). Administering mice with ritonavir in combination with leflunomide effectively attenuated the severity of the liver microsteatosis. The morphometric analysis of liver sections stained with Oil red-O, a measure of liver triacylglycerols content, also confirmed this pattern and corroborated biochemical analyses (Figure 2B; \( n = 10–14 \); \( P < 0.05 \)). To gain mechanistic insights on the effect exerted by ritonavir, we profiled the liver expression of genes involved in lipid and cholesterol homeostasis and found that treating mice with ritonavir caused a shift in the liver expression of sterol regulatory element-binding protein (SREBP)-1c [20,21] and its target genes, including fatty acid synthase (FAS), stearoyl-CoA desaturase 1, cluster of differentiation 36 (CD36) and fatty acid elongase (Figure 2E; \( n = 5 \); \( P < 0.05 \) versus control group). Of interest, while leflunomide failed to revert changes in
Figure 2. Leflunomide attenuates liver steatosis induced by the HIV protease inhibitor ritonavir

(A) Liver weight/liver bodyweight ratio, (B) liver triacylglycerols and (C) cholesterol (µg/mg protein) after 12 days of administration of ritonavir alone or combination with leflunomide (leflun). Treating mice with leflun protected against increased liver triacylglycerol accumulation caused by ritonavir. In panels A, B and C, data are mean ± se of 10–14 animals per group. (D) Histopathological analysis (haematoxylin and eosin staining) of livers obtained from mice administered with ritonavir alone or in combination with leflun (top). Oil-Red-O staining showing liver accumulation of triacylglycerols and moderate steatosis (bottom). Haematoxylin and eosin staining magnification 40×; Oil red O staining magnification 100×. Ballooning score and Oil-Red-O quantification are mean ± se of six sections for each liver examined (n=5 per group). (E) Real-time PCR analysis of liver expression of genes involved in lipid and cholesterol homeostasis. Effects of ritonavir alone or in combination with leflun in the expression of genes involved in triacylglycerols synthesis (sterol regulatory element-binding protein [SREBP]-1c, fatty acid synthase [FAS], stearoyl-CoA desaturase [SCD]-1, CD36 and fatty acid elongase [FAE]), as well as genes involved in cholesterol synthesis (SREBP-2, HMGCoA reductase and HMGCoA synthase). Data are mean ± se of five animals per group. *P<0.05 ritonavir versus control; †P<0.05 leflun versus ritonavir alone.
Leflunomide protects against dyslipidaemia induced by ritonavir

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FAS and stearoyl-CoA desaturase 1 mRNA expression, it reduced fatty acid elongase expression and CD36 expression (Figure 2E; \( n=5 \); \( P<0.05 \) versus ritonavir group). In addition, ritonavir induced the expression of HMGCoA synthase, a gene involved in cholesterol metabolism (Figure 2E; \( n=5 \); \( P<0.05 \) versus control group), whereas it had no effects on the expression of SREBP-2 and HMGCoA reductase. Leflunomide cotreatment reduced basal expression of HMGCoA reductase (Figure 2E; \( n=5 \); \( P<0.05 \) versus control and ritonavir groups).

Leflunomide antagonizes the epididymal fat lipoatrophy induced by ritonavir

Administration of ritonavir (5 mg/kg/day for 12 days) had no effect on mice body weight: in control mice (\( n=10 \)), body weight rose from 18.76 ± 0.45 g to 21.4 ± 0.42 g; in ritonavir-administered mice (\( n=14 \)), from 18.96 ± 0.31 g to 21.88 ± 0.3 g; and in ritonavir/leflunomide-administered mice (\( n=14 \)), from 18.68 ± 0.15 g to 21.45 ± 0.21 g. However, treatment with ritonavir resulted in a robust reduction in the epididymal fat (Figure 3A, \( n=10–14 \); \( P<0.05 \)). Histopathological analysis of epididymal fat isolated from ritonavir-treated mice revealed extensive leukocytes margination and diapedesis, and perivascular accumulation of leukocytes in the tissue (Figure 3E). Compared with naive mice, the epididymal fat depots from ritonavir-treated mice were also characterized by increased levels of proinflammatory mediators, including TNF-α, monocyte chemotactic protein (MCP)-1 (Figure 3B and 3C, \( n=5 \); \( P<0.05 \)) and CD68 (Figure 3D, \( n=5 \); \( P<0.05 \) versus control group), a transmembrane glycoprotein highly expressed by tissue macrophages that is known to participate in lectin-selectin-mediated cell adhesion and locomotion [22]. The lipoatrophy observed in the mice treated with ritonavir was reversed by coadministration of leflunomide (Figure 3A, \( n=10–14 \); \( P<0.05 \) versus ritonavir group). This effect was associated with a robust attenuation in leukocytes/macrophages infiltration in the epididymal fat, as indicated by histopathological analysis and the reduction in the expression of TNF-α, MCP-1 and CD68 (Figure 3B and 3E, \( n=5 \); \( P<0.05 \) versus ritonavir group). In addition, ritonavir induced FAS and CD36 gene expression and reduced the expression of leptin mRNA. These changes were left unaffected by leflunomide (Figure 4B, 4C and 4D; \( n=5 \); \( P<0.05 \)). Despite the fact that FAS and CD36 mRNAs as well as leptin gene expression are regulated by SREBP-1c [20,21], the expression of this regulatory factor was also unchanged (Figure 4A). However, this was not surprising because the mechanism by which HIV PIs cause SREBP accumulation in the nucleus is due to the inhibition of protein degradation rather than to a modulation of the gene expression [18]. Finally, leflunomide effectively restored the expression of peroxisome proliferator-activated receptor (PPAR)-γ mRNA. Indeed, while ritonavir administration reduced the expression of PPAR-γ and its target genes, including phosphoenoxypropane carboxykinase (PEPCK; although not significantly), perilipin A, lipoprotein lipase, long-chain fatty acid transport protein 1 and fatty acids binding protein-4, these changes were abrogated by cotreating ritonavir-administered mice with leflunomide (Figure 4E, 4F, 4G, 4H, 4I and 4J, \( n=5 \); \( P<0.05 \)) [23,24].

Discussion

Lipodystrophy induced by HIV PIs is characterized by extensive inflammation of lipoatrophic subcutaneous
abdominal adipose tissue and accumulation of fat in the liver, which indicates a pathogenetic mechanism that share similarities with those reported in patients with metabolic syndrome and diabetes [25–31]. In the present study, we reported that rodents administered the HIV PI ritonavir develop severe lipoatrophy and inflammation of epididymal fat, as demonstrated by a dense infiltration of this tissue by leukocytes as well as by increased levels of CD68, TNF-α and MCP-1. Conversely, ritonavir administration associates with reduced expression of PPAR-γ in this tissue. PPAR-γ is required to maintain the adipocyte phenotype by directly binding to and transactivate response elements in a number of adipocyte-specific genes involved in uptake, cytosolic binding and activation of FFA for triacylglycerols storage [23,24]. A reduced expression of PPAR-γ might therefore be sufficient to explain the epididymal fat atrophy and dysfunction seen in this model. To support this hypothesis, we have shown that exposure to ritonavir reduced the levels of PEPCK and perilipin A, two canonical PPAR-γ target genes involved in triacylglycerols storage in
adipose tissue [23,24]. Indeed, during fasting conditions, the expression of PEPCK, an enzyme mediating glycerogenesis (a cellular pathway that increases FFA incorporation into triacylglycerols), is regulated by PPAR-γ [24]. Perilipin A is an adipocyte-specific protein associated with lipid droplet formation that acts as a protective coating factor against natural lipases, which breaks triacylglycerols into glycerol and FFA [32]. Perilipin A expression is increased in obese animals and humans, and polymorphisms in the human perilipin gene have been associated with an altered regulation of body weight and might have a genetic influence on obesity risk in humans [32]. Thus, down regulation of PEPCK and perilipin A in epididymal fat caused by ritonavir not only contributes to an increased cellular concentration of FFA, but it might be a driving factor involved in FFA release into the circulation [23,24].

In the present study, we have documented that ritonavir administration causes a severe atrophy of epididymal fat. Because epididymal fat is considered as a ‘central’ adipose tissue, our finding represent a variation of lipodystrophy observed in HIV-infected persons taking HAART, which is mostly characterized by increases in central fat depots. Nevertheless, despite the fact that lipodystrophy occurring in animal models is only partially characterized [33,34], there is evidence that a reduction in white adipose tissue and epididymal fat occurs in these models [35,36], possibly highlighting a different metabolic response of adipose tissue to ritonavir in mice compared with humans. Although this could be a limitation of the model, it has to be taken into consideration that in HAART-treated patients metabolic alterations develop after several years of treatment and therefore are only partially recapitulated by short-lasting experimental models.

In summary, we have provided evidence that administration of the HIV PI ritonavir to mice leads to a dysregulated balance of lipid handling by liver and epididymal fat. These changes appear to be mechanistically linked to the development of dyslipidaemia, whose pathogenetic mechanisms present similarities with inflammation-driven metabolic dysfunction seen in type 2 diabetes [37]. Despite the molecular pathogenesis of these changes being multifactorial, we have obtained evidence that ritonavir acts as a potent regulator of the expression and/or function of master genes, such as SREPB1c and PPAR-γ in these tissues. By extension it might be speculated that a reduction of PPAR-γ in adipose tissue might support an influx of inflammatory cells, contributing to the feature of ritonavir-induced lipodystrophic inflammation of epididymal fat observed in this study.

One important observation we made is that leflunomide, an anti-inflammatory agent used in the therapy of rheumatoid arthritis, antagonizes several features of ritonavir-induced inflammation and dysmetabolism. Of interest, leflunomide cotreatment effectively reduced circulating levels of triacylglycerols, FFA and cholesterol triggered by ritonavir. Furthermore, leflunomide administration effectively protected against epididymal fat atrophy caused by ritonavir. The molecular basis of anti-inflammatory effects of leflunomide in rheumatoid arthritis are multiple, including a robust inhibition of nuclear factor-κB signalling induced by a wide variety of inflammatory stimuli, TNF-α, \( H_2O_2 \), LPS, okadaic acid and ceramide [38]. Despite the fact that leflunomide had no effect on epididymal expression of SREBP-1c, FAS and CD36, it effectively counteracted the inflammatory activation of adipose tissue caused by ritonavir, restoring the expression of PPAR-γ and its target genes including PEPCK and perilipin A. Thus, the ability of leflunomide to restore triacylglycerols biosynthesis might support the beneficial effects of this agent on epididymal fat tissue.

TNF-α targets PPAR-γ by inhibiting the expression of PPAR-γ gene and also through suppression of its transcriptional activity. The latter can occur by promoting serine phosphorylation of key regulatory residues in the N-terminal domain of PPAR-γ. These phosphorylation events are mediated by JNK and extracellular signal-regulated kinase (ERK1/2) [39]. Therefore, epididymal fat atrophy could be the result of a paracrine loop between adipocytes and macrophages. Stimulation of TNF-α and chemokines production by ritonavir in adipocytes could lead to macrophages recruitment [14,40]. In turn, macrophage-secreted cytokines activate the proinflammatory pathways in adipocytes, resulting in a self-expanding loop that potently inhibits adipocyte differentiation and lipogenesis [41,42].

In addition to its potent PPAR-γ-restoring effect documented in this study, leflunomide has been shown to act on two additional nuclear receptors, that is, the pregnane-x-receptor (PXR) and the aryl hydrocarbon receptor [43,44]. Indeed, leflunomide has been reported to act as a PXR antagonist [43]. Interestingly, ritonavir is known to act as PXR agonist, while it inhibits the activity of CYP3A4 [45]. However, interaction on PXR does not provide an explanation to the observed effects because we have found that both ritonavir and leflunomide act as partial PXR agonist in vitro (see Additional file 2). Furthermore, feeding of PXR transgenic mice, which harbour a human PXR gene on a background of mouse PXR knockout, with ritonavir and leflunomide leads to a comparable activation of PXR while metabolic effects are similar to those observed in wild-type mice (see Additional file 3). Lastly, activation of aryl hydrocarbon receptor and PXR does not provide a mechanistic explanation for our results because activation of both receptors is known to cause liver steatosis [46,47].
In conclusion, we have provided evidence that leflunomide, an antirheumatic drug, attenuates dysmetabolic changes caused by the HIV PI ritonavir (See Figure 5). Because this agent has shown beneficial effects also in attenuating generalized immune activation, a key pathogenetic factor involved in the relentless CD4+ T-cell depletion and AIDS progression [48] in HIV-infected persons, our study support the notion that leflunomide might be an effective strategy in targeting HIV-associated immune activation and HIV PI induced metabolic syndrome. Finally, since ritonavir is mostly used as a booster for other HIV PIs rather than for its anti-protease activity, it appears that introduction of novel boosters devoid of metabolic activities is desired.

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Conceived and designed the experiments: AM, FrB, DF, and SF. Performed the experiments: AM, CD, SC, BR, and FlB. Analyzed the data: AM. Wrote the paper: AM FrB and SF.

Disclosure statement

The authors declare no competing interests.

Additional files

Additional file 1: Additional methods on assessing liver triacylglycerols and cholesterol levels, and on RT PCR analysis can be found at http://www.intmedpress.com/uploads/documents/AVT-11-OA-2152_Mencarelli_Add_file1.pdf

Additional file 2: Luciferase reporter assay performed in HepG2 transiently transfected with pSG5-PXR, pSG5-RXR, pCMV-bgal, and (cyp3a4)TKLUC vectors and stimulated rifaximin, ritonavir and leflunomide can be found at http://www.intmedpress.com/uploads/documents/AVT-11-OA-2152_Mencarelli_Add_file2.pdf

Additional file 3: Leflunomide attenuate dyslipidaemia induced by the HIV PI ritonavir in humanized (h)PXR mice can be found at http://www.intmedpress.com/
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