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HIV protease inhibitors block adipogenesis and increase lipolysis in vitro

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Abstract

AIDS therapies employing HIV protease inhibitors (PIs) are associated with changes in fat metabolism. However, the cellular mechanisms affected by PIs are not clear. Thus, the affects of PIs on adipocyte differentiation were examined in vitro using C3H10T1/2 stem cells. In these cells the PIs, nelfinavir, saquinavir, and ritonavir, reduced triglyceride accumulation, lipogenesis, and expression of the adipose markers, aP2 and LPL. Histological analysis revealed nelfinavir, saquinavir and ritonavir treatment decreased oil red O-staining of cytoplasmic fat droplets. Inhibition occurred in the presence of the RXR agonist LGD1069, indicating the inhibitory effects were not due to an absence of RXR ligand. Moreover, these three PIs increased acute lipolysis in adipocytes. In contrast, two HIV PIs, amprenavir and indinavir, had little effect on lipolysis, lipogenesis, or expression of aP2 and LPL. Although, saquinavir, inhibited ligand-binding to PPARγ with an IC_{50} of 12.7 ± 3.2 μM, none of the other PIs bound to the nuclear receptors RXRα or PPARγ, (IC_{50}s > 20 μM), suggesting that inhibition of adipogenesis is not due to antagonism of ligand binding to RXRα or PPARγ. Taken together, the results suggest that some, but not all, PIs block adipogenesis and stimulate fat catabolism in vitro and this may contribute to the effects of PIs on metabolism in the clinic.

Keywords: HIV; Protease inhibitors; Adipogenesis; Lipolysis; Lipodystrophy

1. Introduction

Highly active anti-retroviral therapy (HAART), a combination of three types of drugs comprising non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs), is used to control HIV replication and the develop-

Abbreviations: LPL, lipoprotein lipase; PI, HIV-protease inhibitor; PPAR γ, peroxisome proliferator activated receptor γ; RXRα, retinoid X receptor α.

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ment of AIDS (Lea and Faulds, 1996; Havlir and Lange, 1998; Jarvis and Faulds, 1998). From 1995 to 1997, HIV-related mortality decreased by 70% with HAART (Palella et al., 1998; Schooley, 1999). Unfortunately, antiretroviral therapy is associated with unusual side effects, of which subcutaneous fat wasting (lipoatrophy), abdominal obesity (lipomegaly), insulin resistance and hyperlipidemia have caused the most concern.

The syndrome of metabolic abnormalities associated with HAART are multifactoral and issues such as duration of infection, prior HAART treatment, age, sex, and environmental and genetic factors play an important role (Carr et al., 1998a; Walli et al., 1998). As the syndrome developed with increased use of the PIs, it was attributed to the introduction of the PIs into the treatment regimen (Carr et al., 1998a; Walli et al., 1998). However, recent studies and clinical trial results have demonstrated that lipodystrophy and dyslipidemia are observed in PI-naive patients and patients treated with NRTIs (Mallal et al., 1999; Saint-Marc et al., 1999). The NRTIs are also associated with adverse effects such as myopathy, neuropathy, pancreatitis, lactic acidemia and hepatic toxicity, possibly due to mitochondrial toxicity (Lewis and Dalakas, 1995; Brinkman et al., 1998, 1999). While the development of lipodystrophy has been associated with the duration of NRTI treatment, it may be accelerated by the use of PIs (Galli et al., 1999; Mallal et al., 1999). Thus, multiple drugs may contribute to the development of the syndrome.

Currently, five PIs are approved for AIDS therapy, including amprenavir (APV), indinavir (IDV), nelfinavir (NFV), ritonavir (RTV), and saquinavir (SQV). These inhibitors show multiple beneficial effects in the clinic, including reduced viral load and improved patient well being (reviewed in Flexner, 1998; Kaul et al., 1999). Although NRTIs may alter fat metabolism by inhibiting mitochondrial proliferation (Lewis and Dalakas, 1995; Brinkman et al., 1998, 1999), the molecular pathways affected by PIs are not well understood. Recently it was found that IDV increases retinoic acid signaling (Lenhard et al., 2000), possibly by displacing retinoic acid from a retinoic acid binding protein and activating the retinoic acid receptor (RAR). Activation of RAR-mediated gene transcription by IDV is proposed to prevent fat cell differentiation and cause hyperlipidemia and dry skin (Lenhard et al., 2000). Unlike IDV, other PIs (APV, NFV, RTV or SQV) do not affect retinoid signaling (Lenhard et al., 2000). Thus, it is unclear how the other PIs contribute to the changes in fat metabolism associated with HAART.

An alteration in the number and size of adipocytes controls adipose tissue mass and distribution, resulting in changes in lipid and carbohydrate metabolism (Geloen et al., 1989; Shimomura et al., 1998). Insulin increases adipocyte size (Krotkiewski and Bjorntorp, 1976) by stimulating lipogenesis and the activity of lipoprotein lipase (LPL) while inhibiting the activity of hormone-sensitive lipase (HSL) leading to increased triglyceride deposition within the fat cell. Adipocyte numbers are regulated by agents that affect adipogenesis or apoptosis (Martin et al., 1998). Adipogenesis is controlled, in part by two nuclear receptors, termed peroxisome proliferator activated receptor γ (PPARγ) and retinoid X receptor α (RXRα; Tontonoz et al., 1994; Rose et al., 1999), which form a heterodimer pair to affect gene transcription. Synthetic agonists for PPARγ and RXR include the thiazolidinediones (e.g. BRL49653) and rexinoids (e.g. LGD1069), respectively (Boehm et al., 1994; Lehmann et al., 1995). Interestingly, thiazolidinediones, rexinoids, and insulin stimulate adipogenesis in vitro (Kletzien et al., 1992; Tontonoz et al., 1994; Lehmann et al., 1995) and improve glucose utilization in vivo (Mukherjee et al., 1997; Lenhard et al., 1999b), suggesting adipocytes, in part, may mediate the anti-diabetic effects of these agents (Rose et al., 1999).

In this study we focused on understanding the effects of PIs on adipocyte metabolism in vitro. We report that SQV, RTV, and NFV inhibited adipogenesis of C3H10T1/2-stem cells in the presence of insulin and agonists for PPARγ and RXR. Furthermore, these protease inhibitors stimulated lipolysis and inhibited lipogenesis within mature adipocytes. To address whether the

PIs were altering PPAR/RXR signaling, we examined binding of the PIs to PPAR/RXR and expression levels of LPL and aP2, two genes regulated by PPAR/RXR. The data indicate that some protease inhibitors exert their biological effects, in part, through altering fat metabolism in adipocytes.

2. Materials and methods

C3H10T1/2 mesenchymal stem cells were grown in Dulbecco's modified Eagle medium-high glucose containing 10% fetal calf serum. Adipogenesis was induced by adding 1 μM BRL49653, 1 μM LGD1069, and 200 nM insulin to near confluent cells in 96-well microtiter plates. Various concentrations of protease inhibitors were added to the cells upon induction of adipogenesis. After 7 days in culture, lipogenesis, lipolysis, and triglyceride accumulation were measured as previously described (Lenhard et al., 1997). Histochemical staining of triglycerides was determined using oil red O (Novikoff et al., 1980). For Northern analysis, total RNA was isolated using the RNeasy Total RNA kit (Qiagen, Chatsworth, CA). RNA (10 μg/well) was electrophoresed in agarose gels and transferred to nitrocellulose. The blot was probed with mouse aP2 and LPL probes labeled via random-priming (Prime-It II Kit, Stratagene, La Jolla, CA) with [α^{32}P]dCTP. Autoradiographs were analyzed with a Biorad-Imaging System.

Test compounds were assayed for binding to human PPARγ as previously described (Nichols et al., 1998). Ligand-binding to the human RXR ligand-binding domain was measured using a scintillation proximity assay similar to that described for PPARγ (Nichols et al., 1998). Briefly, biotinylated RXRα-was immobilized on streptavidin-modified scintillation proximity assay beads followed by incubation with 2.5 nM 9-*cis*-[^{3}H]-retinoic acid and various concentrations of PIs in 96 well polypropylene plates. The plates were incubated for 1 h at room temperature and bound radioactivity was determined in a Wallac 1450 Microbeta counter. The data was analyzed as previously described (Nichols et al., 1998).

3. Results

HIV protease inhibitors inhibit lipid accumulation in C3H10T1/2 cells. To assess the effects of HIV protease inhibitors on cellular lipid accumulation, C3H10T1/2 cells were cultured in the presence of various protease inhibitors under conditions permissive for adipogenesis (Lenhard et al., 1997; Paulik and Lenhard, 1997). Lipid accumulation was determined by histochemical-staining of fat droplets using oil-red O (Novikoff et al., 1980) and total triglyceride was measured using the glycerol phosphate oxidase-Trinder method (Kenakin et al., 1998). NFV, RTV, and SQV treatment decreased the number of cells and size of lipid droplets stained with oil-red O (Fig. 1) and total lipid accumulation (Fig. 2(A)). APV and IDV treatment had little effect on staining with oil-red O or lipid accumulation. Dose-response analysis revealed markedly different inhibition profiles for the various protease inhibitors on triglyceride accumulation (Fig. 2(B)). NFV and SQV were the most potent inhibitors of triglyceride accumulation, RTV was moderately effective, whereas APV and IDV had less effect on lipid accumulation (Table 1 and Fig. 2(A)).

Lipid accumulation during adipocyte differentiation involves the coordinated action of triglyceride synthesis (lipogenesis) and hydrolysis (lipolysis). It is important to note here that the hydrolysis of stored cellular triglycerides is primarily through hormone sensitive lipase (HSL). Thus, we wanted to determine which metabolic pathway protease inhibitor-treatment affected. To determine the effect of the protease inhibitors on lipolysis, we measured the accumulation of glycerol in the extracellular medium after culturing the cells for 7 days under conditions permissive for adipogenesis (Lenhard et al., 1997; Paulik and Lenhard, 1997). As shown in Fig. 2(C), NFV, RTV, and SQV stimulated lipolysis, whereas APV and IDV had little effect on lipolysis. Lipolysis was not inhibited by pretreatment with protein synthesis inhibitor, cycloheximide (data not shown). Next, we measured how these agents affected the conversion of [^{3}H]glucose into cellular lipids (i.e. lipogenesis; Lenhard et al., 1997) As shown in Fig. 2(D), NFV, RTV, and SQV were

the most effective compounds at inhibiting lipogenesis.

HIV protease inhibitors block expression of adipose-specific genes in C3H10T1/2 cells. Cellular triglyceride synthesis occurs after the action of LPL, which causes the hydrolysis of serum triglycerides and subsequent influx of non-esterified fatty acids into the cell. LPL contains a PPARγ/RXR recognition-site in its promoter and is one of the earliest genes induced during adipogenesis (Schoonjans et al., 1996). Similarly, the expression of aP2, an adipocyte specific fatty acid binding protein, is directly regulated by activation of the PPARγ/RXR heterodimer (Tontonoz et al., 1994). To characterize the effects of HIV protease inhibitors on differentiation at the molecular level, Northern blot analysis of LPL and aP2 was performed. As shown in Fig. 3, fat-specific mRNAs encoding LPL and aP2 were greatly reduced in cells treated with NFV, RTV, and SQV, whereas APV and IDV had no effect. Similarly, NFV and RTV treatment inhibited expression of adipsin/complement factor D, a protein that is expressed upon treatment of C3H10T1/2 cells with PPARγ agonists (Lehmann et al., 1995), whereas APV and IDV had no effect (data not shown).

Analysis of PI binding to RXR and PPARγ. Ligand activation of the RXR/PPARγ heterodimer results in inhibition of lipolysis (Lenhard et al., 1997), stimulation of lipogenesis (Lenhard et al., 1997; Paulik and Lenhard 1997), and increased aP2 (Tontonoz et al., 1994; Paulik and Lenhard, 1997), adipsin (Tontonoz et al., 1994) and LPL expression (Schoonjans et al., 1996). Since several PIs had opposite effects to RXR/PPARγ agonists in C3H10T1/2 cells, the PIs might be expected to antagonize ligand binding to RXR or PPARγ. Thus, we tested if the PIs could displace binding of radiolabeled [^{3}H] 9-*cis* retinoic acid and [^{3}H]BRL49653 from the ligand-binding domain of RXR and PPARγ, respectively (Table 1). None of the PIs inhibited ligand-binding to RXR at concentrations less than or equal to 20 μM. SQV inhibited ligand-binding to PPARγ with an IC_{50} of 12.7 ± 3.2 μM. APV, IDV, NFV, and RTV had little effect on ligand-binding to PPARγ at concentrations less than or equal to 20 μM.

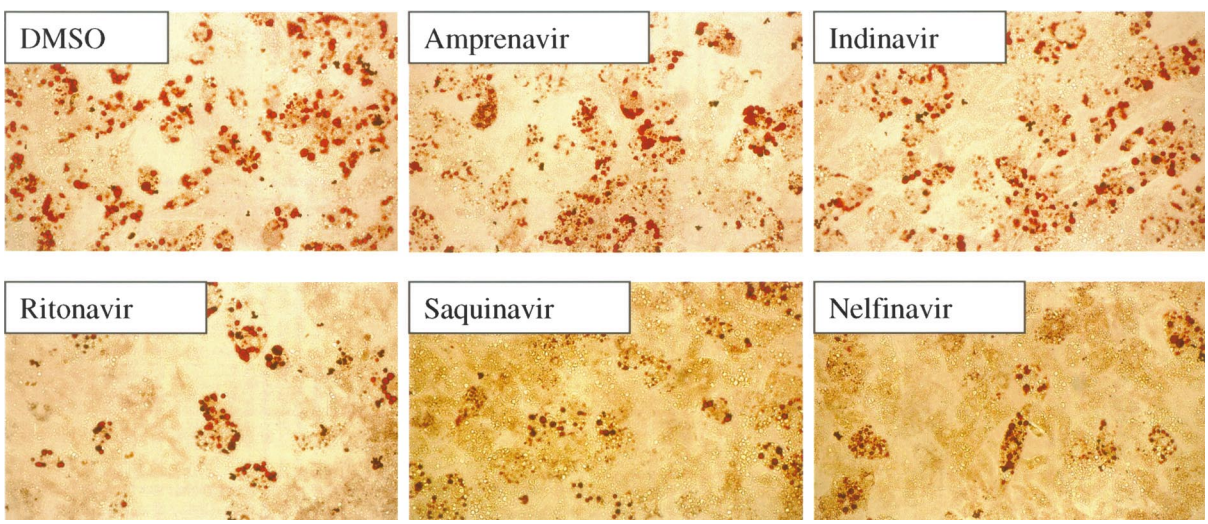

Fig. 1. C3H10T1/2 cells were treated for 7 days with 1 μM BRL49653, 200 nM insulin, 1 μM LGD1069 and 10 μM of the indicated protease inhibitors or 0.1% dimethylsulfoxide (DMSO), a vehicle control. The cells were stained with oil-red O to identify lipid droplet formation. Magnification is 85 × .

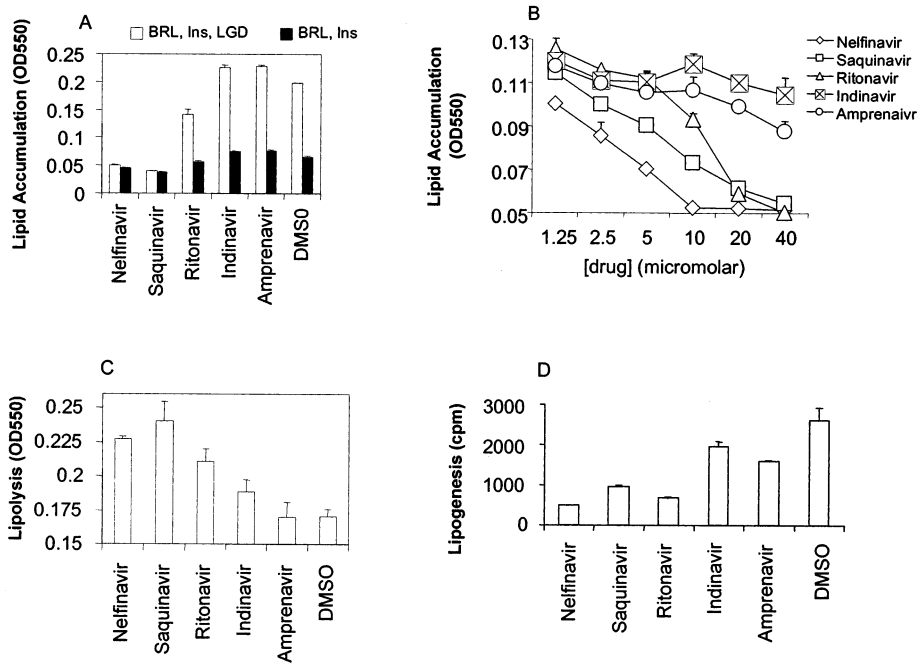

Fig. 2. Effect of HIV-protease inhibitors on lipolysis and triglyceride accumulation in cell culture. (A) Cell-associated triglyceride was measured after 7 days of incubation with the indicated protease inhibitor in the presence of 1 μM BRL49653 and 200 nM insulin in the absence (closed bars) or presence of 1 μM LGD1069 (open bars). (B) Dose response of the indicated protease inhibitors in the presence of 1 μM BRL49653, 200 nM insulin and 1 μM LGD1069 (C) C3H10T1/2 cells were cultured for 7 days in the presence of 1 μM BRL49653, 200 nM insulin, and 1 μM LGD1069. The effect of protease inhibitors on lipolysis was determined by measuring the accumulation of glycerol in the medium after 24 h treatment with 10 μM of the indicated compounds. (D) C3H10T1/2 cells were cultured for 7 days in the presence of BRL49653, insulin, and LGD1069. After the cells differentiated into adipocytes, the PIs (10 μM) were added to the cells for 48 h. Lipogenesis in the presence of the various PIs was determined by measuring the incorporation of [^{3}H]glucose into cellular lipid. In all experiments, the solvent used for the PIs, 0.1% DMSO, was included as a control. The mean and standard deviation from two to three replicates for each data point is given.

4. Discussion

The data presented here demonstrate that SQV, NFV, and RTV alter fat metabolism in a murine mesenchymal stem cell line, C3H10T1/2, that differentiates into adipocytes under permissive conditions. These PIs inhibited conversion of the stem cells into adipocytes, as well as inhibited lipogenesis and stimulated lipolysis of cellular triglycerides in mature adipocytes. These observations suggest that PIs may contribute to the peripheral fat wasting associated with HAART through a direct effect on adipocyte differentiation and metabolism.

With the exception of SQV, which had low affinity for PPARγ, none of the other PIs bound to RXR or PPARγ, two essential receptors that regulate adipogenesis. Thus, the metabolic effects of PIs in vivo are unlikely to be the result of direct antagonism of ligand-binding to RXR or PPARγ. However, it is noteworthy that NFV, RTV, and

Table 1
IC_{50} of HIV-PIs (μM)

Protease inhibitor	Triglycerides	RXR	PPAR
Nelfinavir	8.7 ± 1.7	>20.0	>20.0
Saquinavir	9.7 ± 6.5	>20.0	12.7 ± 3.2
Ritonavir	17.0 ± 2.7	>20.0	>20.0
Indinavir	>20.0	>20.0	>20.0
Amprenavir	>20.0	>20.0	>20.0

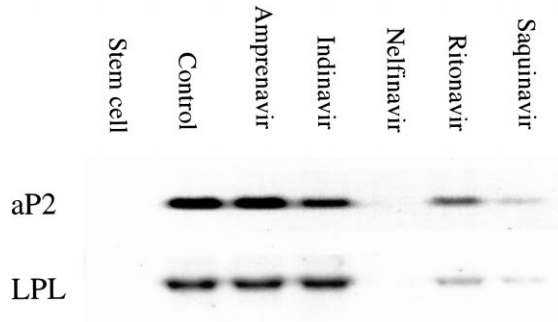

Fig. 3. C3H10T1/2 cells were maintained under conditions permissive for adipogenesis for 1 week in the absence or presence of the various PIs. Expression of aP2 and LPL was determined by Northern analysis.

SQV inhibited expression of gene products (e.g. LPL and aP2) under transcriptional control of the RXR/PPARγ heterodimer. This is consistent with the hypothesis that in some cases, PI induced lipodystrophy may result from impaired RXR/PPARγ signaling, causing reduced differentiation of peripheral adipocytes and storage of fat (Carr et al., 1998b). It is also possible that the PIs affect other pathways involved in adipogenesis, such as those regulated by glucocorticoids, cAMP, C/EBP or ADD1.

One hypothesis is that all PIs alter fat metabolism by affecting the same mechanism, such as inhibition of 9-*cis* retinoic acid synthesis that is needed for activation of RXR (Carr et al., 1998b). In contrast to this hypothesis, our data show that NFV, SQV, and RTV inhibit adipogenesis in the presence of the RXR agonist, LGD1069. Thus, while the mechanism by which these PIs hinder adipogenesis is not clear, the inhibitory effect of these PIs on adipogenesis can not be due to the lack of a ligand for RXR. Moreover, our data clearly indicate disparate activities among the PIs. Unlike the other PIs tested in this report, APV and IDV had little affect on adipogenesis. Although IDV and APV did not affect cultured fat cells using the conditions described in this report, the results do not rule out the possibility that these PIs affect a shared metabolic pathway in vivo. For example, PIs may interact with common proteins (proteases or cytochrome P450) in other tissues and this may account for some overlapping toxicities. Further studies are needed to determine how common and unique pathways are affected by PIs and how these contribute to the metabolic changes reported in the clinic. However, IDV is the only PI that enhances retinoic acid signaling in vitro and this can result in inhibition of adipogenesis (Lenhard et al., 2000). These observations are consistent with the hypothesis that the various PIs affect distinct molecular pathways, perhaps accounting for the different side effects observed for each PI.

Using the 3T3-L1 murine adipocyte cell line, Zhang et al. (1999) have validated a portion of our earlier observations (Lenhard et al., 1999a) showing NFV and RTV inhibit lipid accumulation in vitro, yet contradict the results of Gagnon et al. (1998). Gagnon et al. (1998), show RTV and IDV stimulate adipogenesis as much as 10–40% in 3T3-L1 cells. Differences between the cell lines may account for disparity between these results. For example, 3T3-L1 cells are committed to undergo differentiation into adipocytes, whereas C3H10T1/2 cells are stem cells that are not committed to the adipocyte lineage, but have the capacity to do so when appropriately induced. Induction of adipogenesis in 3T3-L1 cells requires insulin, glucocorticoids, and a cAMP elevating agent (e.g. isobutyl methyl xanthine), whereas C3H10T1/2 cells require insulin and agonists for the RXR/PPARγ heterodimer. Moreover, 3T3-L1 cells express a phenotype similar to white adipose tissue (Rosen et al., 1979) whereas C3H10T1/2 cells express a phenotype similar to brown adipose tissue (Paulik and Lenhard, 1997). Recently, it was shown that 50 μM IDV and 10 μM SQV inhibit differentiation of primary human adipocytes (Wenworth et al., 2000). Moreover, IDV and SQV failed to inhibit PPARγ/RXR activity in human transfected cells (Wenworth et al., 2000), consistent with our observation that PIs do not bind to PPARγ/RXR. Taken together, these results suggest that the effects of PIs on fat metabolism may vary between the PIs, cell types and fat depots.

Consistent with this hypothesis, PI therapy has been shown, to cause a loss of fat from the face and limbs but an increase in fat in the back of the

neck and abdomen (Carr et al., 1998a; Lo et al., 1998; Miller et al., 1998; Walli et al., 1998). One possibility is that the decrease in subcutaneous fat results in lower leptin levels. As leptin levels decrease, a signal to deposit more fat is generated by the hypothalamus. As peripheral subcutaneous tissue is more responsive to PPARγ/RXR (Adams et al., 1997) and this is in some way blocked, by PIs, preferential deposition may occur in the viscera, which is less responsive to PPARγ/RXR; thus leading to abdominal obesity. These results point to important differences between various fat depots in the development of PI-associated metabolic changes.

Although the cause of PI-associated lipodystrophy is unknown, several groups have suggested hypocomplementemia may cause partial lipodystrophy (Sissons et al., 1976; Ipp et al., 1977; Levy et al., 1998). Both human and murine adipocytes express the complement components C3, factor B and factor D (adipsin; Peake et al., 1997). The combined activity of these components generates a protein, termed acylation-stimulating protein, which stimulates lipogenesis (Baldo et al., 1993). Since some of the PIs inhibited adipogenesis, lipogenesis, and adipsin expression in vitro, this raises the intriguing possibility that PI-associated lipodystrophy may, in part, result from decreased complement activity. If this hypothesis is correct, future clinical studies of PIs may include evaluation of their effects on hypocomplementemia.

A comparison of the maximum serum concentration (5–20 μM) for PIs in patients (Balani et al., 1996; Hsu et al., 1997; Pai and Nahata, 1999) to the IC_{50} value for altering fat metabolism reveals that the in vitro effects of PIs can occur at physiologically relevant serum concentrations. However, these in vitro studies do not include multiple treatments, which result in peak and valley effects on drug levels in the serum of patients. Likewise, these studies do not account for protein binding which may affect the activity of PIs. A number of other factors, such as drug–drug interactions may be involved in the metabolic abnormalities associated with HAART. Specifically, it has been observed that there is an acceleration of the syndrome when PI therapy is combined with NRTI therapy (Galli et al., 1999; Mallal et al., 1999; Saint-Marc et al., 1999). While our results suggest a potential mechanism by which some PIs affect fat metabolism, the data do not account for many factors, including pharmacokinetic parameters, active metabolites, environmental factors, and genetic predispositions, which may influence development of lipodystrophy in the clinic. These differences may explain the lack of correlation between the in vitro effects and clinical observations associated with the use of PIs. Thus, a direct comparison of our data to the human lipodystrophy syndrome should be done with caution.

In summary, our data indicate that some but not all PIs altered fat metabolism in vitro, suggesting that not all PIs affect the same molecular pathways. Since HAART is associated with metabolic complications in the clinic, perhaps PIs contribute to this syndrome by inhibiting adipocyte metabolism. If this hypothesis is correct, then identification of PIs having minimal affects on adipocyte metabolism should aid in the development of safer anti-HIV drugs.

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