Biochemical Pharmacology, Vol. 59, pp. 1063–1068, 2000.



ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00246-X

# Stimulation of Vitamin $A_1$ Acid Signaling by the HIV Protease Inhibitor Indinavir

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**ABSTRACT.** HIV protease inhibitors (PIs) are effective drugs for the treatment of AIDS. However, PI therapy is sometimes associated with side-effects including increased plasma lipids and altered body fat distribution, although fat redistribution may occur in some patients not treated with PIs. Overdosage with vitamin $A_1$ acid (all-*trans*-retinoic acid, ATRA) or its metabolites may cause similar changes in lipid metabolism. Moreover, the PI indinavir and retinoids have been associated with nail, skin, and hair defects, suggesting that indinavir and retinoids may exert their effects through similar molecular mechanisms. This hypothesis was tested by examining the effects of PIs on retinoid signaling *in vitro*. Mesenchymal stem cells (C3H10T1/2) were cultured in the presence of various PIs (amprenavir, indinavir, nelfinavir, ritonavir, and saquinavir) and synthetic retinoids, and the metabolic response was assessed by measuring the activity of a retinoid-regulated protein, alkaline phosphatase (ALP). Of the PIs tested, only indinavir stimulated ATRA-dependent ALP activity and altered stem cell morphology; the effects of indinavir occurred in the presence of ATRA, but not in its absence. Moreover, indinavir increased the effects of ATRA on lipid accumulation during fat cell differentiation. AGN 193109 (4-[[5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl]ethynyl]-benzoic acid), a retinoic acid receptor (RAR) antagonist, inhibited the synergistic effects of indinavir and ATRA, indicating that indinavir increases RAR signaling. However, indinavir did not potentiate ALP activity in the presence of the RAR agonist CH55 (3,5-di-*tert*-butylchalcone 4′-carboxylic acid). Unlike ATRA, CH55 does not bind to cytosolic retinoic acid binding protein (CRABP), suggesting that CRABP may regulate the effects of indinavir on RAR signaling. These observations support the proposal that altered retinoid signaling promotes some of the adverse reactions associated with indinavir therapy, such as altered lipid metabolism. BIOCHEM PHARMACOL **59**;9:1063–1068, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** adipogenesis; dyslipidemia; lipodystrophy; indinavir; retinoic acid; toxicity

HIV PIs§ are a new class of drugs used for treating AIDS. PIs interfere with the viral life cycle by preventing maturation of proteins required for the release of virions from infected cells. However, PI therapy may be associated with side-effects that include fat wasting, central adiposity, insulin resistance, and hyperlipidemia [1–10]. Although mortality decreases with PI therapy [11], cardiovascular complications may result from altered fat metabolism in individuals who receive therapy. Whereas changes in lipid metabolism are associated with PI therapy [1–10], fat redistribution and dyslipidemia are also observed in HIV-infected individuals in the absence of therapy or when treated with non-PI-containing regimens [7, 12–14]. Thus, it is likely that multiple metabolic syndromes exist resulting from HIV infection and various treatment regimens.

Retinoids, such as vitamin $A_1$ acid (ATRA), increase lipid and apolipoprotein secretion by the liver, resulting in increased cardiovascular and hepatic risk [15–17]. Additionally, retinoids alter cell growth and differentiation, accounting for their beneficial effects in treating cancer and dermatological problems [15]. In the liver, the fat-storing cells (also known as Ito or stellate cells) and not hepatocytes are the main storage site of retinoids in the body. *In vitro*, ATRA inhibits fat cell differentiation and stimulates expression of osteoblast genes, such as ALP [18, 19]. The pharmacological effects of retinoids are due, in part, to their ability to bind and transactivate heterodimers consisting of RARs and RXRs [15–22].

Previous studies indicate that treatment with several PIs, including indinavir, nelfinavir, ritonavir, and saquinavir [2–9], or with retinoids [15–18], is associated with altered fat metabolism. Moreover, indinavir and retinoid therapies are associated with nail defects [23, 24] and hyperbilirubinemia [25, 26], suggesting that these effects may be mediated by related mechanisms. Consistent with this hypothesis, adverse skin and hair reactions have been reported in a patient treated with 13-*cis*-retinoic acid and antiretroviral therapy consisting of indinavir, ritonavir,

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§ *Abbreviations*: ALP, alkaline phosphatase; ATRA, all-*trans*-retinoic acid; CRABP, cytoplasmic retinoic acid binding protein; PI, protease inhibitor; PPAR, peroxisome proliferator activated receptor; RAR, retinoic acid receptor; and RXR, retinoid X receptor.

Received 26 July 1999; accepted 12 October 1999.

zidovudine, and lamivudine [27]. These adverse reactions were not observed in the absence of 13-*cis*-retinoic acid or antiretroviral therapy, indicating that retinoids may be incompatible with antiretroviral therapy [27]. Furthermore, indinavir therapy may alter hair growth [28], a side-effect also caused by retinoid therapy. The current study examines the effects of PIs (amprenavir, indinavir, nelfinavir, ritonavir, and saquinavir) on retinoid responses in the mesenchymal stem cell line C3H10T1/2 that differentiates into adipocytes. The results demonstrated that indinavir stimulated retinoid signaling *in vitro*, and suggested that adverse reactions associated with indinavir therapy may result from alterations in vitamin $A_1$ acid signaling.

## MATERIALS AND METHODS

The retinoids and PIs used in these studies were from the chemical stores of Glaxo Wellcome Inc. Measurements of ALP activity and histochemical staining in C3H10T1/2 clone-8 fibroblasts (American Type Culture Collection) were performed as described [19]. Cell culture and lipid accumulation assays were done following published procedures [29, 30]. In summary, C3H10T1/2 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Various concentrations of protease inhibitors or retinoids were added to the cells 1 day after they reached confluence. After 7 days, ALP activity and histochemical staining were measured using Sigma-Fast *para*-Nitrophenyl Phosphate Substrate and Alkaline Phosphatase-Leukocyte Staining assays, respectively, according to the manufacturer's specifications (Sigma). Alternatively, adipogenesis was induced by treating confluent cells for 7 days with 200 nM insulin, 200 nM BRL49653 (rosiglitazone), and various concentrations of protease inhibitors or retinoids. After 7 days in culture, cellular lipid was measured using the Sigma Diagnostic Glycerol-Triglyceride assay (i.e. Trinder reagent 337; Sigma).

## RESULTS

The goal of this study was to determine if PIs alter retinoid signaling. This hypothesis was tested by assaying the activity of a retinoid-responsive gene product, ALP, in C3H10T1/2 mesenchymal stem cells [19] exposed to PIs in the presence or absence of retinoids. Because ATRA stimulates ALP activity in these cells with an $EC_{50}$ of 650 nM, the stimulatory effects of PIs were assayed in the presence of low concentrations of ATRA (10–100 nM). Under these conditions, the assay has sufficient range and sensitivity to detect additive or synergistic effects of PIs and ATRA on ALP activity. Five protease inhibitors, amprenavir, indinavir, nelfinavir, ritonavir, and saquinavir, were tested in these cells. These PIs had little effect on ALP activity in the absence of ATRA (Fig. 1A). However, in the presence of 10 nM ATRA, indinavir ($EC_{50}$ = 1.9 ± 0.2 μM) stimulated ALP activity by greater than 3-fold. Moreover, adding 10 μM indinavir to the cells increased the potency of ATRA more than 10-fold (Fig. 1C). The other PIs tested did not stimulate ALP activity in the presence of ATRA (Fig. 1A), suggesting that the various PIs had pharmacologically distinct effects on retinoid signaling *in vitro*. Moreover, ritonavir appeared to attenuate the effect of ATRA on ALP activity (Fig. 1A). Although the mechanism for this attenuation is unknown, examination of the cells by light microscopy revealed that ritonavir, but not the other PIs, decreased the number of cells present in culture (data not shown). However, ritonavir did not cause cytosolic lactate dehydrogenase to be released into the medium, suggesting that ritonavir was not toxic to the cells.

The finding that indinavir stimulated ALP activity in the presence but not the absence of ATRA suggests that indinavir may stimulate signaling through the RAR/RXR heterodimer. To substantiate this hypothesis, the ability of the RAR-selective antagonist AGN 193109 (4-[[5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl]ethynyl]-benzoic acid) [20] to prevent ALP induction by indinavir and ATRA was tested. Figure 1B shows that increasing concentrations of AGN 193109 ($IC_{50}$ = 1.4 ± 0.1 nM) progressively inhibited ALP activity in the presence of indinavir and ATRA. There was little effect in the absence of indinavir (data not shown) or in the presence of amprenavir, nelfinavir, ritonavir, and saquinavir (Fig. 1B). Thus, activation of RAR is necessary for indinavir to stimulate ALP activity.

In addition to the RAR/RXR heterodimer, ATRA can also bind to CRABP with an affinity of 0.4 to 2 nM [31]. The effect of indinavir could be at one or both of these sites of action. In contrast, CH55 (3,5-di-*tert*-butylchalcone 4′-carboxylic acid) is a selective RAR agonist that does not bind CRABP [21]. If indinavir interacts with CRABP, then indinavir should stimulate ALP activity in the presence of ATRA but not CH55. Concentration–response analysis showed that indinavir had no effect on the ability of CH55 to stimulate ALP activity (Fig. 1D). In contrast, indinavir increased the potency of ATRA by greater than 10-fold in this assay (Fig. 1C). These results suggest that some of the pharmacological effects of indinavir may be due to altered CRABP activity in mesenchymal stem cells.

Retinoids cause morphological changes in stem cells, indicating altered differentiation [18, 19]. Thus, the effects of indinavir on cell morphology were determined by histochemical methods. As shown in Fig. 2, ALP staining increased at the surface of cells treated with indinavir and ATRA (10 μM and 30 nM, respectively; Fig. 2D) compared with untreated cells or cells treated with either agent alone (Fig. 2, A-C). The data also revealed that treatment with indinavir and ATRA increased the number and alignment of elongated cells. Similar morphological and biochemical changes (i.e. increased ALP) occur during the conversion of these non-committed stem cells into osteoblasts [19], indicating that indinavir alters stem cell differentiation *in vitro*. This raises the intriguing hypothesis that altered stem cell differentiation may contribute to the body fat redistribution associated with indinavir therapy.

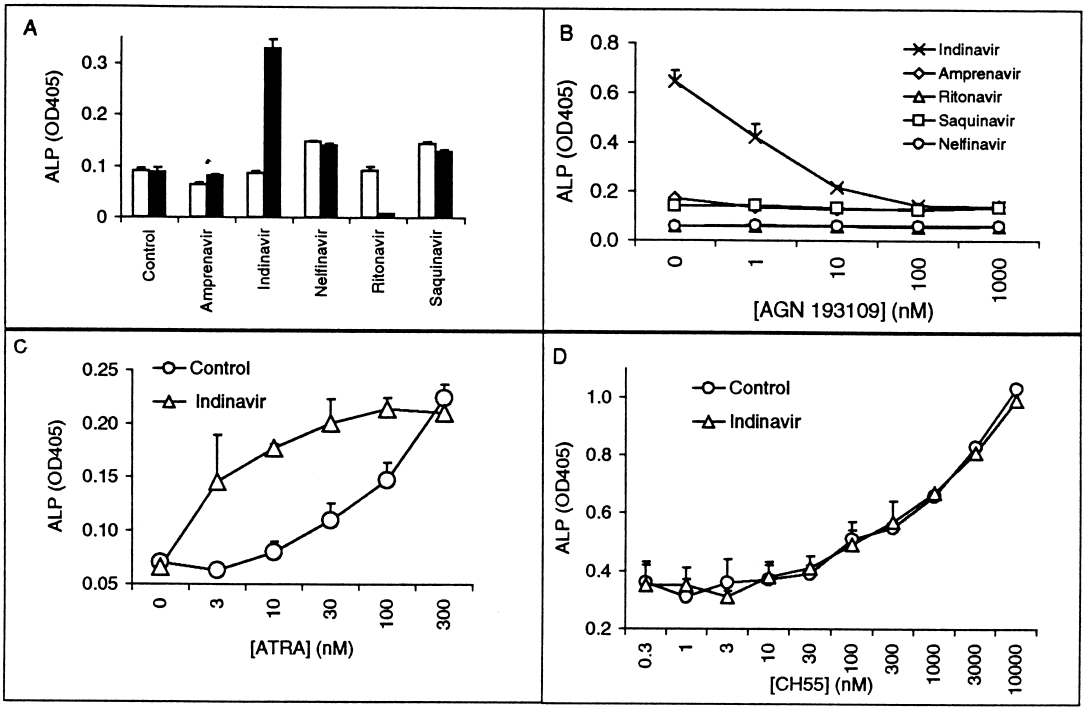


**FIG. 1. ALP activity in C3H10T1/2 stem cells. (A) Cells were incubated in the absence (open bars) or presence (closed bars) of 10 nM ATRA, 1 μM LG100268 (6-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cyclopropyl]nicotinic acid), and 20 μM of the indicated PIs. The RXR selective agonist LG100268 [22] was included to rule out any direct effect that ATRA or PIs may have on this signaling pathway (such as binding to RXR). (B) Cells were incubated in the presence of increasing concentrations of AGN 193109, 20 μM PIs, and 100 nM ATRA. (C) Cells were incubated in the absence (circles) or presence of 10 μM indinavir (triangles), 1 μM LG100268, and increasing concentrations of ATRA. (D) Cells were incubated in the absence (circles) or presence of 10 μM indinavir (triangles), 1 μM LG100268, and increasing concentrations of CH55. ALP activity was measured after 7 days in culture. The means and standard deviations are representative data (each point determined in triplicate) from two or more independent experiments.**

In previous studies, an inverse relationship was demonstrated between expression of the osteoblast protein ALP and fat cell differentiation [19]. This relationship results from the opposing effects on differentiation between agonists for RAR/RXR and PPARγ/RXR heterodimers. Thus, if indinavir increases RAR signaling, then indinavir should stimulate ALP activity and inhibit fat cell differentiation. To test this hypothesis, adipogenesis was induced in C3H10T1/2 cells by the addition of insulin and the PPARγ agonist BRL49653. At the same time, indinavir and various concentrations of ATRA were added to the cells, and the degree of fat cell differentiation was determined by measuring lipid accumulation [29, 30]. Figure 3A shows that indinavir increased the inhibitory potency of ATRA towards lipogenesis by more than 10-fold, suggesting that indinavir inhibits fat accumulation by stimulating RAR signaling. Indinavir did not inhibit lipogenesis in the presence of the RAR antagonist AGN 193109 (data not shown) or in the absence of ATRA (Fig. 3A), confirming that indinavir action is mediated by RAR. In the presence of 30 nM ATRA, the $IC_{50}$ for indinavir was 2.1 ± 0.6 μM in this assay. The inhibitory effect of indinavir on fat accumulation *in vitro* is consistent with clinical results showing that indinavir therapy is associated with lipodystrophy [8].

ATRA is selective for RAR at low nanomolar concentrations ($EC_{50}$ < 100 nM), but activates RXR at high concentrations (>200 nM) [21, 22]. Moreover, activation of the RAR/RXR heterodimer inhibits adipogenesis [18, 19], whereas activation of the PPARγ/RXR heterodimer stimulates adipogenesis [19, 29]. Therefore, the effects of indinavir on lipogenesis were determined at both low (25 nM) and high (400 nM) concentrations of ATRA in the presence of the PPARγ agonist BRL49653 and insulin. As discussed above, Fig. 3B shows that indinavir inhibited lipid accumulation in the presence of low concentrations of ATRA (25 nM), consistent with the observation that activation of RAR inhibits adipogenesis. In contrast, Fig. 3B also shows that indinavir stimulated fat accumulation in the presence of high concentrations of ATRA (400 nM). The RAR antagonist AGN 193109 did not inhibit fat accumulation in the presence of 400 nM ATRA and indinavir (Fig. 3B), indicating that this effect is not mediated by RAR and may be mediated by RXR. As expected, at saturating concentrations of ATRA (10 μM), indinavir had little effect on the cells (data not shown). The differential responsiveness of stem cells to low (25 nM) or high (400 nM) concentrations of ATRA supports the idea that retinoids have pharmacologically distinct effects on RAR and RXR [17, 20–22]. Amprenavir, nelfinavir,

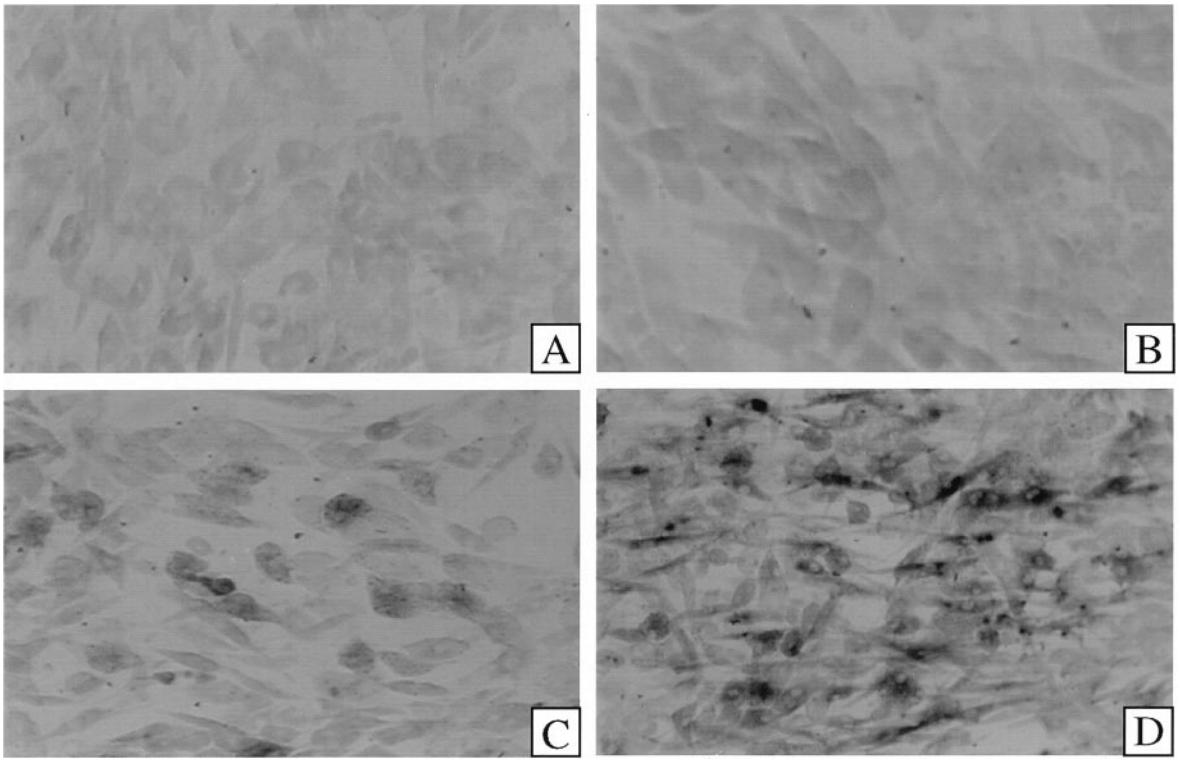


**FIG. 2. Histology of C3H10T1/2 stem cells. Confluent stem cells were incubated for 7 days in the presence of 1 μM LG100268 and in the absence (A) or presence of 10 μM indinavir (B), 30 nM ATRA (C), or 10 μM indinavir and 30 nM ATRA (D). Light microscopy images were recorded after histochemical staining for ALP. Representative data are given of cells stained from four different areas on slides. Original magnification, 300x**

ritonavir, and saquinavir did not stimulate the effects of retinoic acid on adipogenesis in these cells (data not shown). The *in vitro* effects of these PIs on adipocytes are described elsewhere [32].

## DISCUSSION

The molecular mechanism by which indinavir alters retinoic acid signaling is not clear. The observation that the catalytic site of the HIV protease shares 58% sequence similarity with CRABP led to the suggestion that PIs bind CRABP [10]. This hypothesis is consistent with the finding that indinavir increases ALP activity in the presence of ATRA and not CH55; unlike ATRA, CH55 does not bind CRABP [21]. Carr *et al.* [10] propose that PIs hinder CRABP-mediated synthesis of 9-*cis*-retinoic acid from ATRA, which results in decreased RXR activity and dyslipidemia. Standeven and colleagues [17] provide evidence that contradicts this hypothesis by showing that administration of 9-*cis*-retinoic acid to rodents causes dyslipidemia. Since there are no biochemical data supporting the concept that CRABP mediates the synthesis of 9-*cis*-retinoic acid from ATRA, an alternate proposal is that indinavir displaces ATRA from CRABP, and the displaced ATRA binds and activates the RAR/RXR heterodimer, resulting in RAR/RXR-mediated adverse reactions. This explanation is consistent with the observations that the RAR antagonist AGN 193109 prevents ATRA-induced dyslipidemia in rats [17], and inhibits the synergistic effects of indinavir and ATRA *in vitro*.

The observation that indinavir increased the potency of ATRA in ALP and adipogenesis assays and that this was blocked by a selective RAR antagonist suggests that indinavir activates the RAR/RXR heterodimer, perhaps by displacing ATRA from CRABP. However, it is also possible that other proteins that interact with retinoids may mediate the effects of indinavir. For example, naturally occurring retinoids bind to over a dozen proteins, including members of the RXR (α, β, γ), RAR (α, β, γ), CRABP (I, II), cellular retinol-binding protein (I, II), retinol-binding protein (I, II), cytochrome P450 (CYP26), and retinoic acid receptor-related orphan receptor/RZR (α, β, γ) protein families. The expression level of each of these proteins is likely to affect the potency and efficacy of indinavir and retinoic acid in various cell types (e.g. stem cells or adipocytes). Thus, it is important to realize that multiple factors can influence how indinavir affects retinoid signaling, and further studies are needed to understand the complex nature of these effects.

As described above, these studies show that indinavir, but not amprenavir, nelfinavir, ritonavir, or saquinavir, stimulated retinoid signaling *in vitro* (Fig. 1, A and B). This result is in apparent contradiction with the fact that indinavir, nelfinavir, ritonavir, and saquinavir may induce similar adverse reactions in AIDS patients [1–9]. One explanation for this discrepancy may be differences in

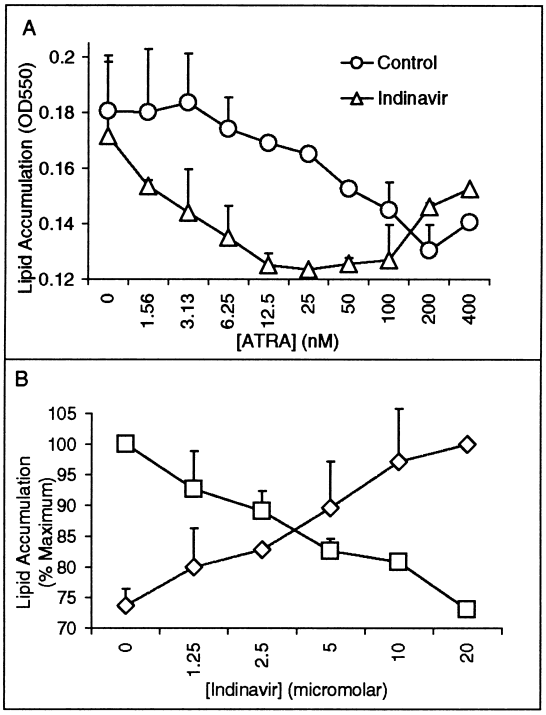


**FIG. 3. Accumulation of triglycerides in C3H10T1/2 adipocytes. Adipogenesis was stimulated in C3H10T1/2 stem cells by adding 1 μM BRL49653 and 1 μM insulin to the growth medium. (A) The cells were incubated in the absence (○) or presence of 10 μM indinavir (△) and increasing concentrations of ATRA. (B) Cells were incubated with increasing concentrations of indinavir in the presence of 25 nM ATRA (□) or 400 nM ATRA and 1 μM AGN 193109 (◇). The RAR antagonist AGN 193109 [20] was included to rule out any effect RAR may have on the RXR signaling pathway. The data in panel B are given as percent of the maximum lipid accumulation relative to untreated cells (i.e. 100% is the amount of fat accumulated in the absence of ATRA or indinavir). Triglyceride accumulation was measured after treating the cells for 7 days in culture [28, 29]. The means and standard deviations are representative data (each point determined in triplicate) from duplicate data sets.**

metabolism of the different drugs in clinical studies; these *in vivo* metabolic processes might lead to effects that are not observable in cell culture experiments. Another possibility is that these drugs may affect different molecular pathways. For example, we observed recently that nelfinavir, saquinavir, and ritonavir stimulate lipolysis in fat cells, whereas indinavir and amprenavir have no effect on lipolysis [32]. Thus, nelfinavir, saquinavir, and ritonavir may alter fat metabolism by stimulating lipid turnover, whereas indinavir may alter lipid metabolism by stimulating retinoid signaling. Whereas the data presented here support the hypothesis that the various PIs affect distinct molecular targets, they do not rule out the possibility that the PIs alter similar metabolic pathways.

As fat redistribution and dyslipidemia are observed in AIDS patients in the absence of PI therapy [7, 12–14], it is likely that several factors contribute to the lipodystrophy syndrome or that several syndromes are caused by combination therapies and infection. However, PI therapy is associated with increased wasting of subcutaneous fat [3, 6, 8], fat deposition in the abdomen, breast, and over the cervical vertebrae [4, 7, 8], and dyslipidemia [2, 5, 6, 9]. Thus, in addition to infection and non-PI-containing regimens, some PIs may increase the susceptibility of an individual to changes in fat metabolism.

These observations indicate that there are important differences between the various fat depots in the development of lipodystrophy and central adiposity. One possibility is that indinavir may increase the incidence of subcutaneous fat wasting, which results in decreased secretion of cytokines such as leptin [6]. Decreased cytokine secretion may bring about secondary changes in metabolism (e.g. dyslipidemia) and lead to abdominal fat deposition. Alternatively, variations in the concentration of retinoids (ATRA and 9-*cis*-retinoic acid), retinoid binding proteins/receptors (CRABP and RAR/RXR), adipogenic receptors (PPARγ/RXR), and hormones (insulin) in the different fat depots may influence indinavir-induced body fat redistribution. Thus, conditions that activate RAR/RXR may increase fat wasting, and conditions that activate RXR/PPARγ may promote fat accumulation. This is consistent with the observation that indinavir inhibits or stimulates *in vitro* lipid accumulation in the presence of low or high concentrations of ATRA, respectively.

A comparison of the maximum serum concentration (4.7 μM) for indinavir in patients [33] to the $EC_{50}$ value (1.9 μM) for altering retinoid signaling *in vitro* reveals that the *in vitro* effects of indinavir occur at physiologically relevant concentrations. Although indinavir may cause adverse reactions in HIV patients by altering ATRA signaling, the data presented here do not account for many factors that influence development of adverse reactions in the clinic, such as pharmacokinetic parameters, active metabolites, diet, and genetic predisposition. Thus, a direct comparison of the effects observed in these *in vitro* data to the side-effects associated with indinavir therapy should be made with caution. Whereas the exact mechanism by which PIs alter body fat distribution remains unknown, the results presented here suggest an important role for retinoids in the development of adverse reactions associated with indinavir therapy.

In conclusion, the data presented here demonstrate that indinavir stimulated ATRA signaling *in vitro*. Since indinavir and retinoid therapy are associated with dyslipidemia, nail defects [23, 24], and hyperbilirubinemia [25, 26], perhaps indinavir promotes these adverse reactions by increasing RAR activity in AIDS patients. If this hypothesis is correct, then natural retinoids in the diet and synthetic retinoids in the clinic may be complicating risk factors associated with indinavir therapy. However, vitamin A levels are tightly regulated in humans, and it is unlikely that high levels would be achieved in most patients, with the exception of overdose. Nonetheless, continued studies on the effects of PIs on retinoid signaling both *in vitro* and *in vivo* should aid in the development of more effective and safer therapies for AIDS.

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