**Background & introduction :**

HIV protease inhibitors (PIs) have been associated with the HIV-associated lipodystrophy syndrome defined as a combination of fat tissue redistribution, dyslipidemia and insulin resistance (1-4). Due to the complexity and heterogeneity of the situations in vivo, studying adipose cells in culture offers the advantage to test the PIs independently of the NRTIs on well-known adipose cell functions, to provide insights into the molecular and pathophysiological mechanisms of action and to generate data to prevent or reverse the adverse effects. Adverse effects of PIs on adipose cell differentiation and insulin sensitivity have been documented in adipose cell lines (5-14).

Objectives:

- To demonstrate the differences between several PIs commonly prescribed in clinical practice, indinavir, nelfinavir and amprenavir, on adipose cell functions
- To approach their mechanisms of action
- To test the impact of rosiglitazone, a strong agonist of PPAR γ , on the adverse effect of PIs in view to provide a rationale for treatment.
- To discuss the clinical relevance of these experiments.

Adipose cell line

- 3T3-F442A cells are an adipose cell line engaged in the adipogenic program as preadipocytes.
- Stimulation of the confluent preadipocytes with insulin and SVF initiates the mitotic clonal expansion step and activates a cascade of transcriptional events that regulate expression of adipogenic genes.
- The early differentiation markers, C/EBP β and δ , activate PPAR γ and C/EBP α , the major transcription factors of adipogenesis, that cooperate with SREBP-1 to ensure and maintain full expression of the adipocyte phenotype. Together with differentiation the cells improve their sensitivity to insulin.

Using this cellular model we allowed us to investigate the PI effects step by step on the adipose cell differentiation program, response to insulin and survival.

Methods

3T3-F442A preadipocytes were cultured with PI for at 4-10 days. At confluency (day 0), cell differentiation was induced by insulin and SVF. After a 2-day step of clonal expansion (2-3 cell cycles), the differentiation program was achieved at day 6-8, as evidenced by the number of newly formed adipocytes (>90%) and triglyceride (TG) staining by oil red O.

The PI effects were examined step by step on :

- the preadipocytes growth and clonal expansion by counting the total cell number
- the adipose cell formation by counting the number of refringent cells and staining the cellular TG, and by the expression (Western Blot) of the transcription factors SREBP-1, PPAR γ and C/EBP α
- the cell survival by flow cytometry and degradation of the cell-death caspase substrate PARP
- the nuclear immunolocalization of SREBP-1 by confocal microscopy
- the nuclear distribution of lamin A/C and B, two proteins of the lamina network involved in a human genetic disease associated with lipodystrophy and severe insulin resistance (15-17).

Results:

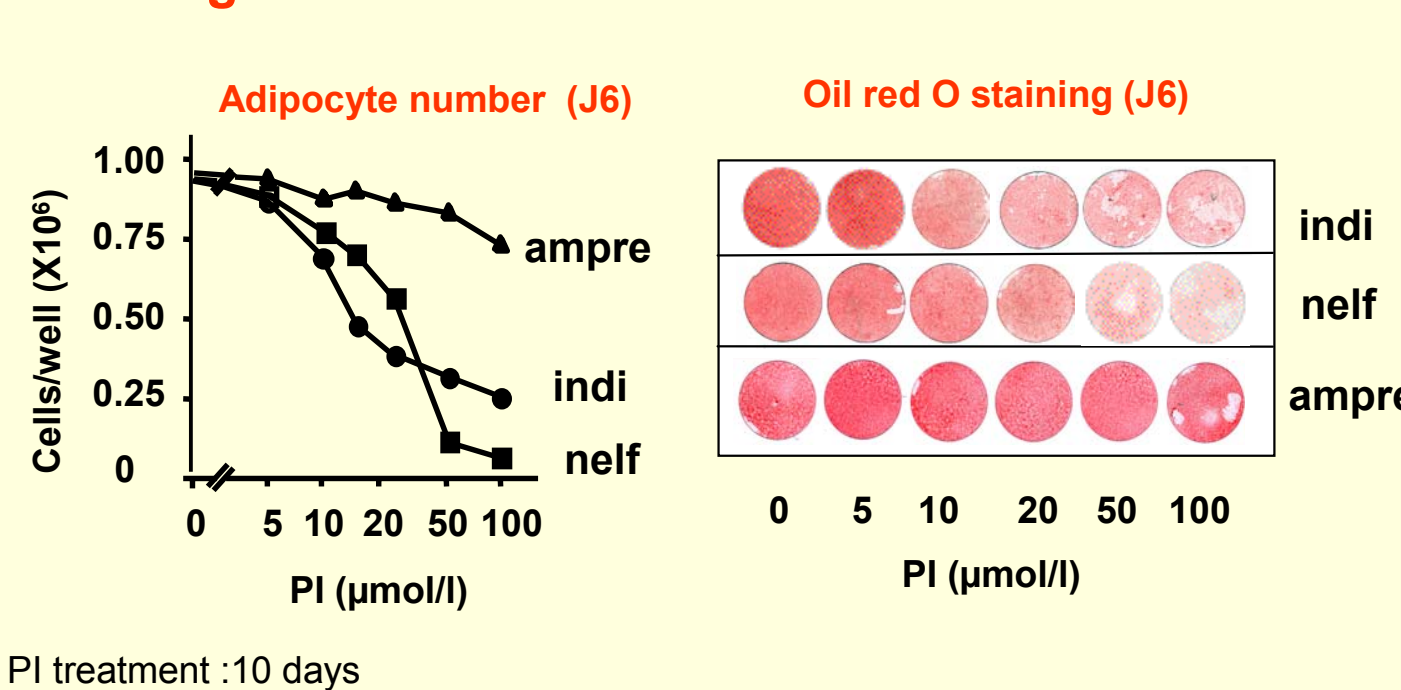
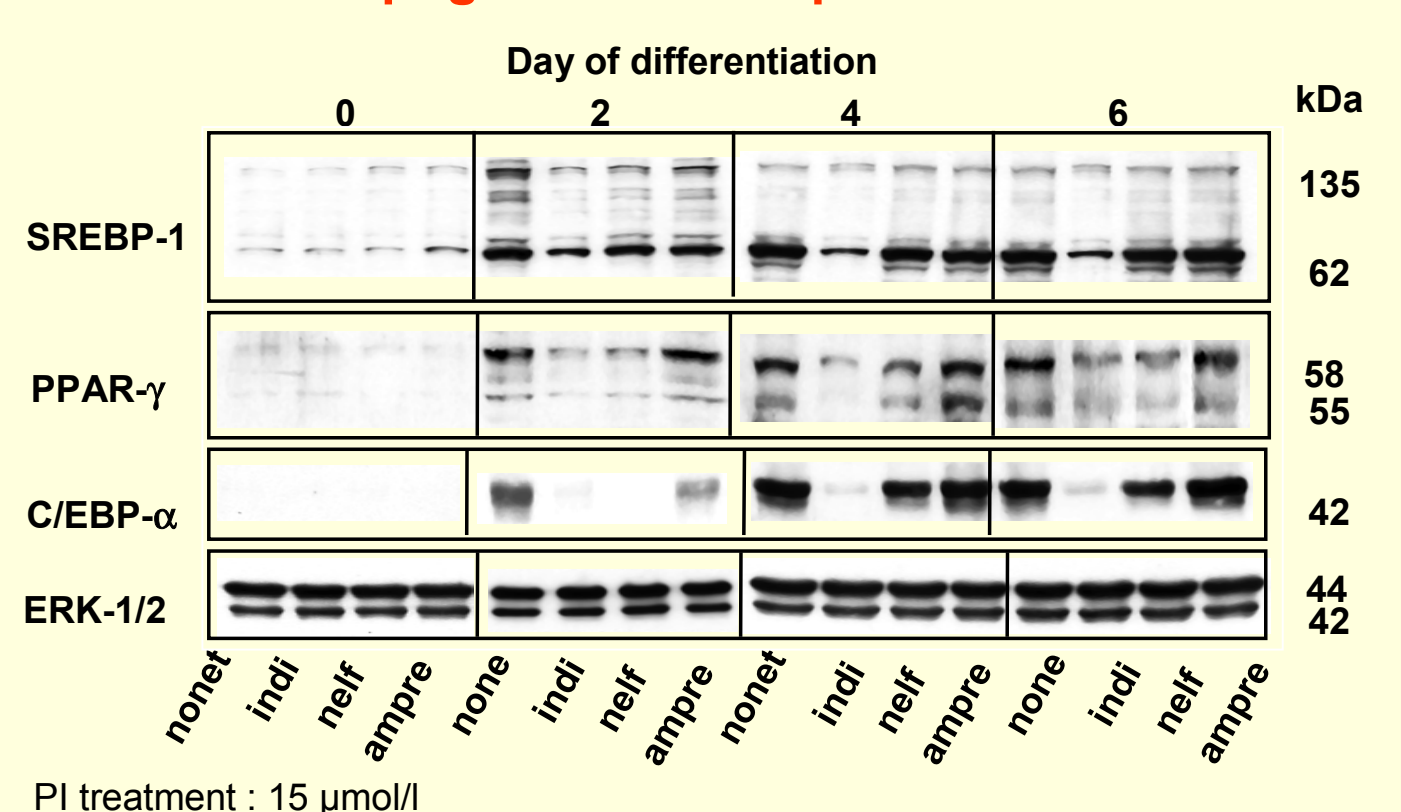
1- Preadipose cell proliferation and clonal expansion were not affected by a long-term treatment with the three drugs (Table 1).

2- Adipose cell conversion was inhibited by a clinically relevant concentration (15 μ mol/l) of indinavir (50-60%), nelfinavir (20-30%) but not amprenavir (5%). This was shown by counting and staining the cells with oil red O (Fig. 1, Table 1) and by the expression of the major markers of adipogenesis SREBP-1, PPAR γ and C/EBP α (Fig. 2).

3- Adipose cell survival was also affected by the PIs, indinavir being by two fold more effective than nelfinavir. Amprenavir did not promote cell apoptosis (Fig. 3, table 1).

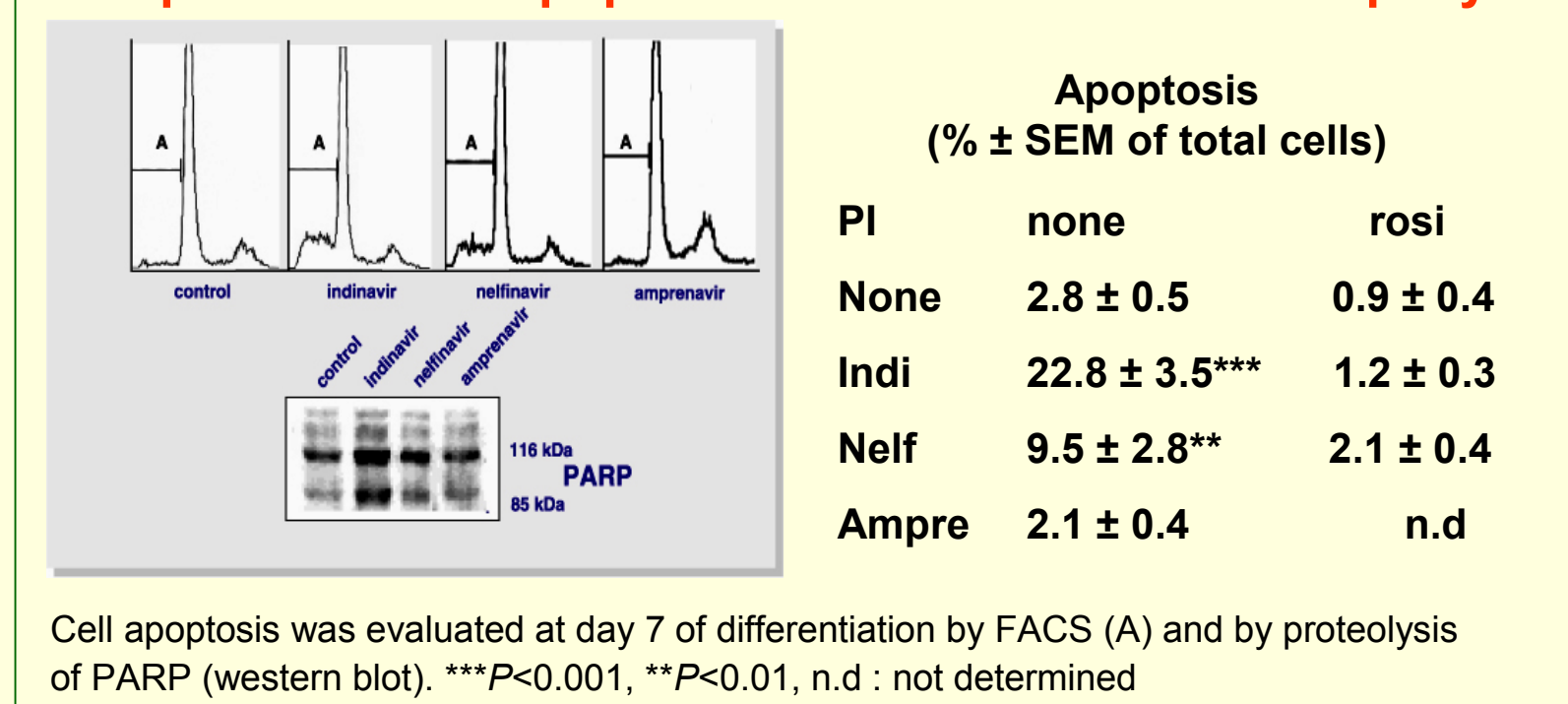
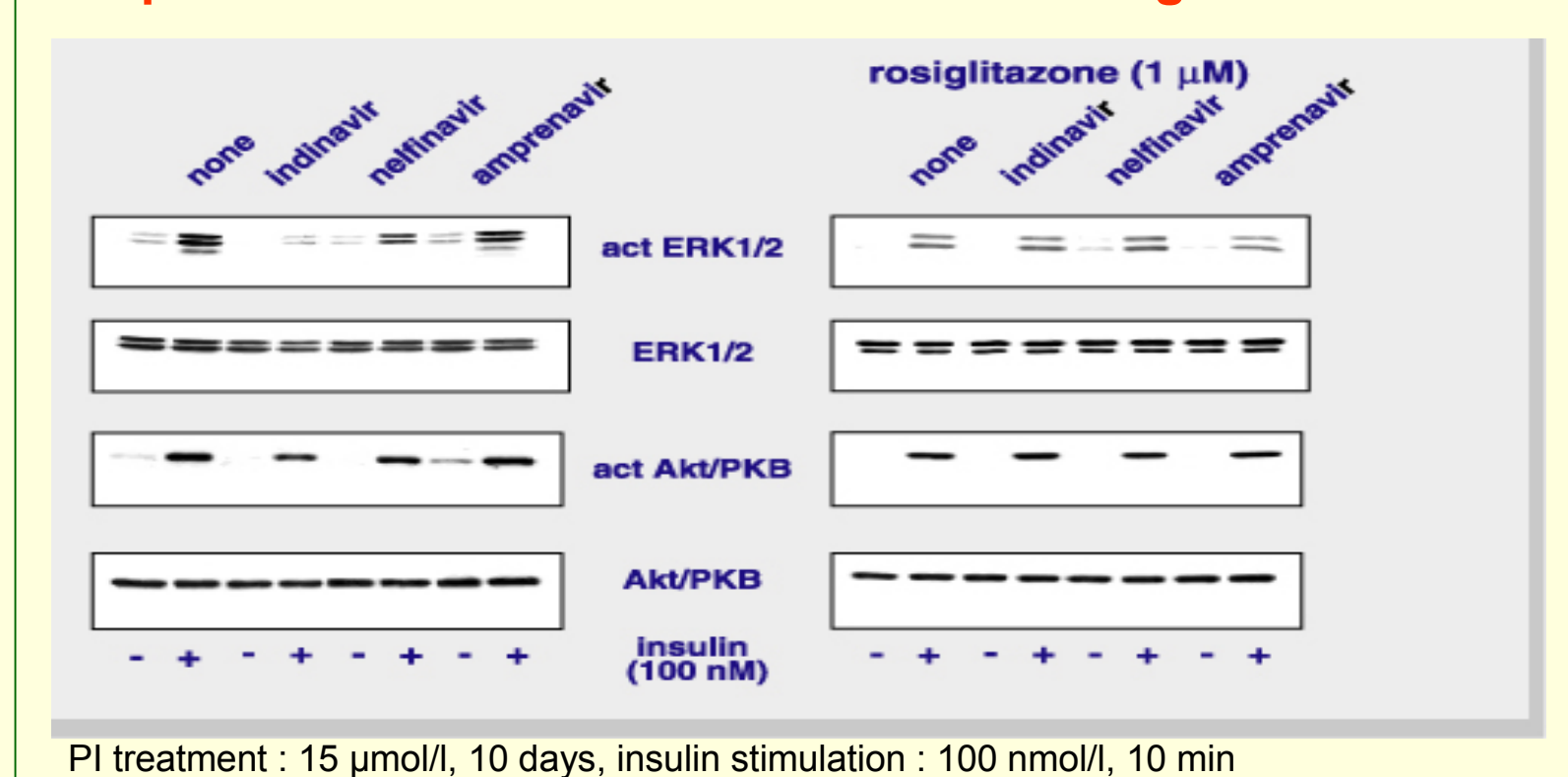
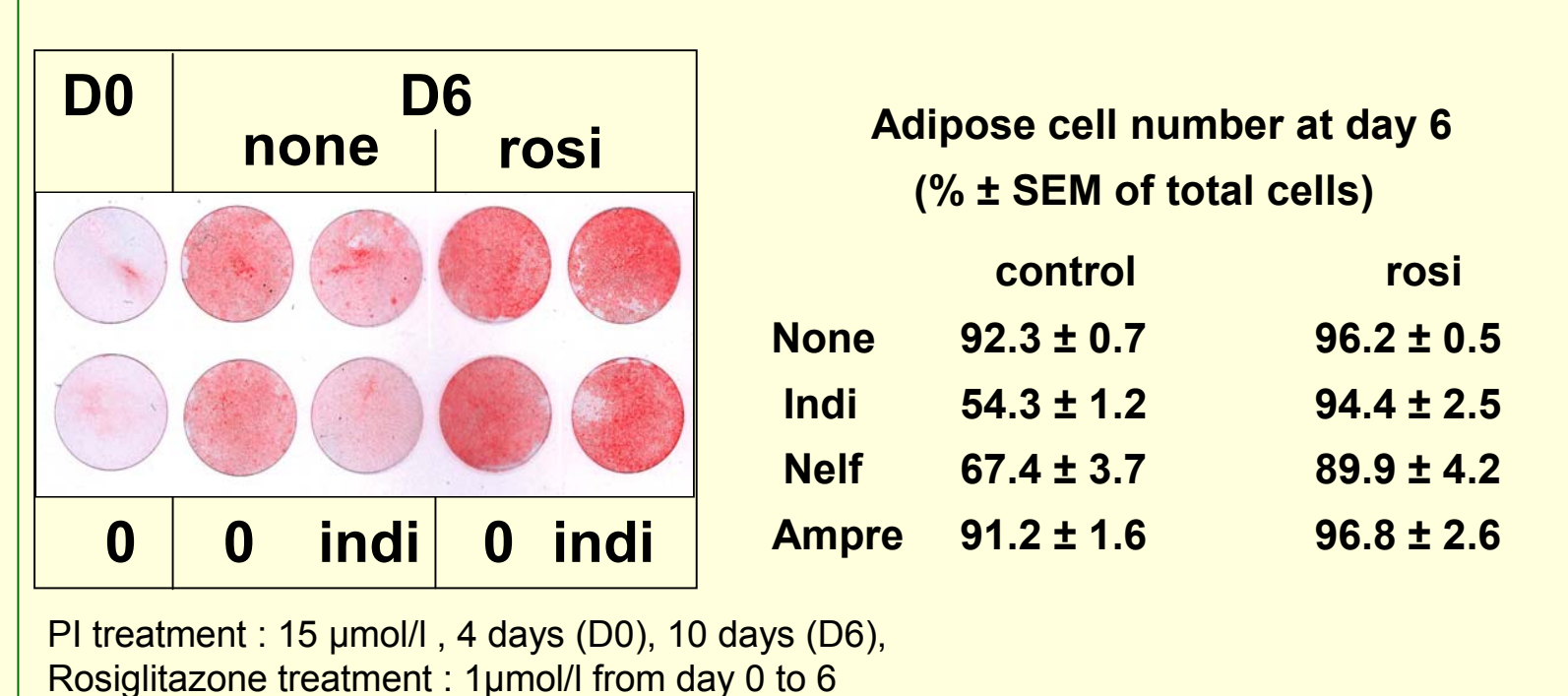
4- Indinavir, nelfinavir and amprenavir blunted MAP Kinase activation by insulin with IC₅₀ concentrations of 16, 25 and > 100 μ mol/l and Akt/PKB kinase (Fig. 4).

5- Differentiation of preadipocytes with rosiglitazone, a potent agonist of PPAR γ , almost totally protected the adipose cells against the adverse effect of PIs on differentiation (Fig. 5), insulin sensitivity (Fig. 4) and survival (Fig. 3), thus providing a potential rationale for treatment.

Fig. 1 : Long-term effects of PIs on preadipocyte cell growth and differentiation**Fig. 2 : PIs inhibited the protein expression of the adipogenic transcription factors****Table 1 : PIs differently altered cell proliferation, differentiation and survival**

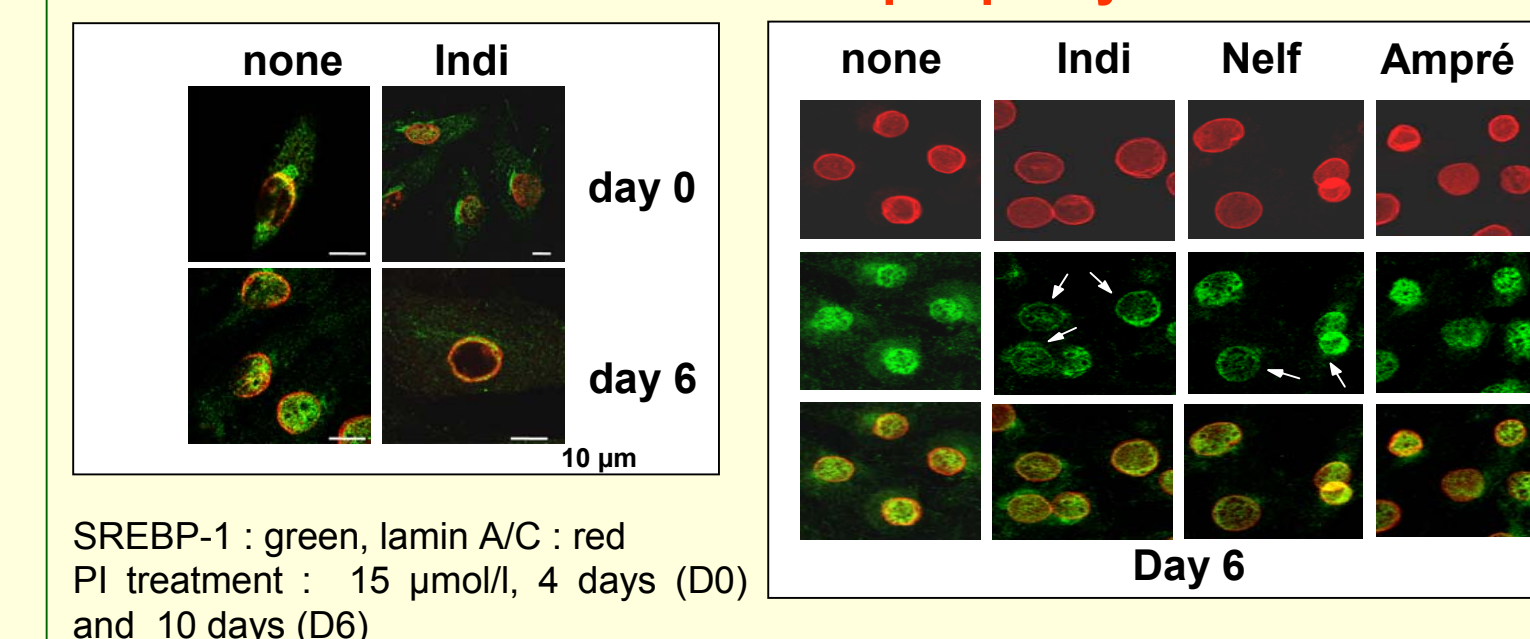
	control	indinavir	nelfinavir	amprenavir
Total cell number	100	92.6 ± 1.0	93.2 ± 3.8	99.4 ± 1.4
Adipocytes	92.5 ± 0.4	54.8 ± 1.2***	68.0 ± 3.0***	89.2 ± 0.4
Oil red O staining	100	35.0 ± 1.3***	40.6 ± 5.5**	92.0 ± 9.6
Apoptotic cells	2.8 ± 0.5	22.8 ± 3.5***	9.5 ± 2.8	2.1 ± 0.4

apoptotic cells were expressed as % ± SEM of control. ***P < 0,001, **P < 0,01

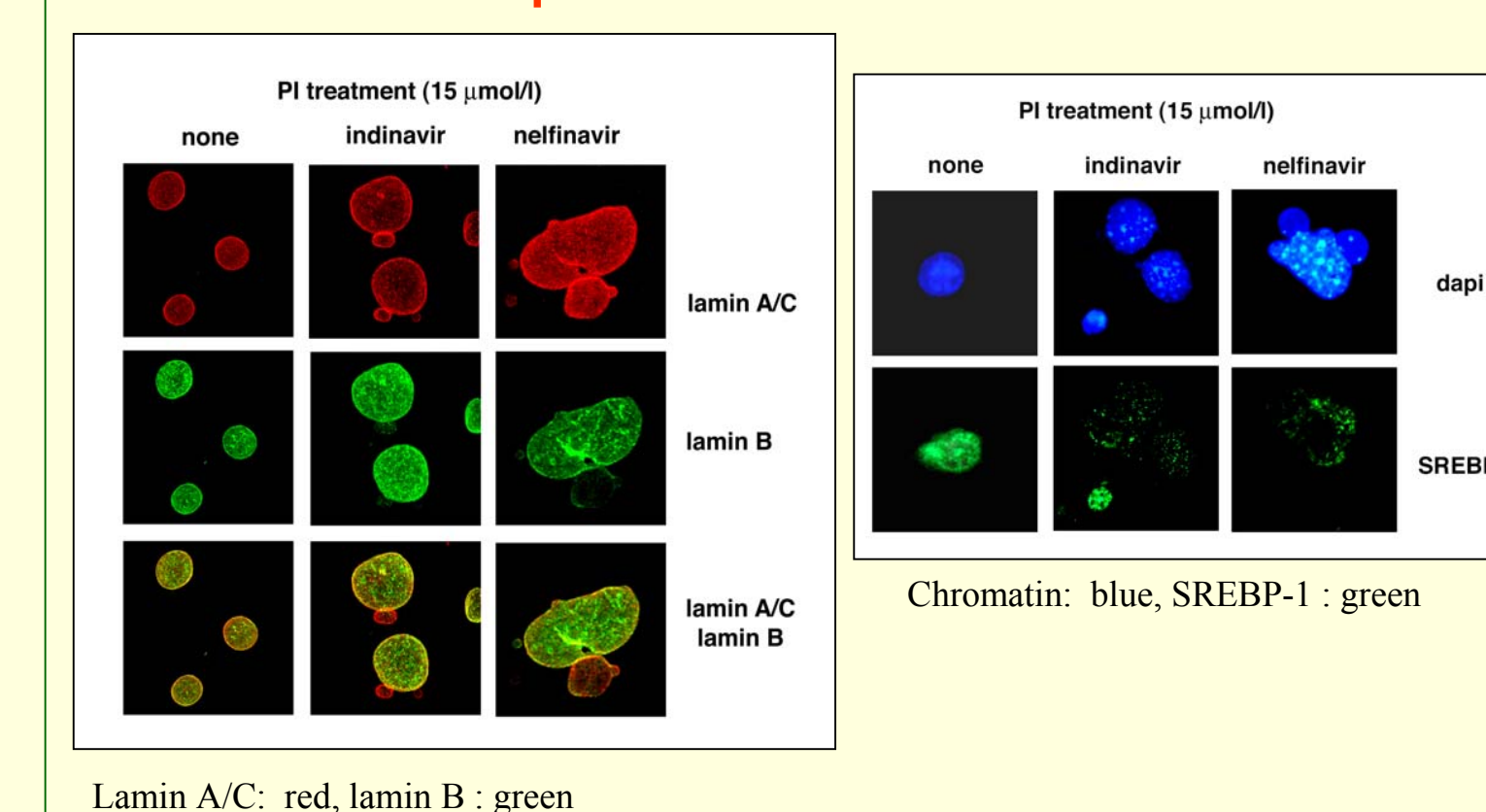
Fig. 3 : Indinavir and nelfinavir, but not amprenavir, promoted the apoptosis of the differentiated adipocytes**Fig. 4 : PIs exerted inhibitory effects on adipose cell response to insulin. Protective effect of rosiglitazone.****Fig. 5 : Rosiglitazone, protected the adipocytes against the adverse effect of PI on cell differentiation****Potential mechanism(s) of PI action**

1- Alteration of SREBP-1 intranuclear trafficking : by using SREBP-1 immunolabelling we observed that indinavir and nelfinavir induced the sequestration of SREBP-1 at the nuclear periphery in about 50 and 30 % of the cells. Amprenavir did not appreciably altered SREBP-1 nuclear entry (Fig. 6).

2- Presence of dysmorphic nuclei (Fig. 7): the indinavir and nelfinavir-treated adipocytes had about 10% of dysmorphic nuclei (left images) that could not accumulate SREBP-1 (right green images). Nuclear dysmorphism was evidenced by dapi-staining and by immuno-labelling lamin A/C and lamin B, two proteins of the lamina network involved in a genetic syndrome of lipodystrophy associated with severe insulin resistance (15-17).

Fig. 6 : PIs differently promoted the sequestration of SREBP-1 at the nuclear periphery

SREBP-1 : green, lamin A/C : red
PI treatment : 15 μ mol/l, 4 days (D0) and 10 days (D6)

Fig. 7 : Altered morphology of 10 % of adipose cell nuclei. The dysmorphic nuclei did not accumulate SREBP-1.

Lamin A/C : red, lamin B : green
Chromatin : blue, SREBP-1 : green

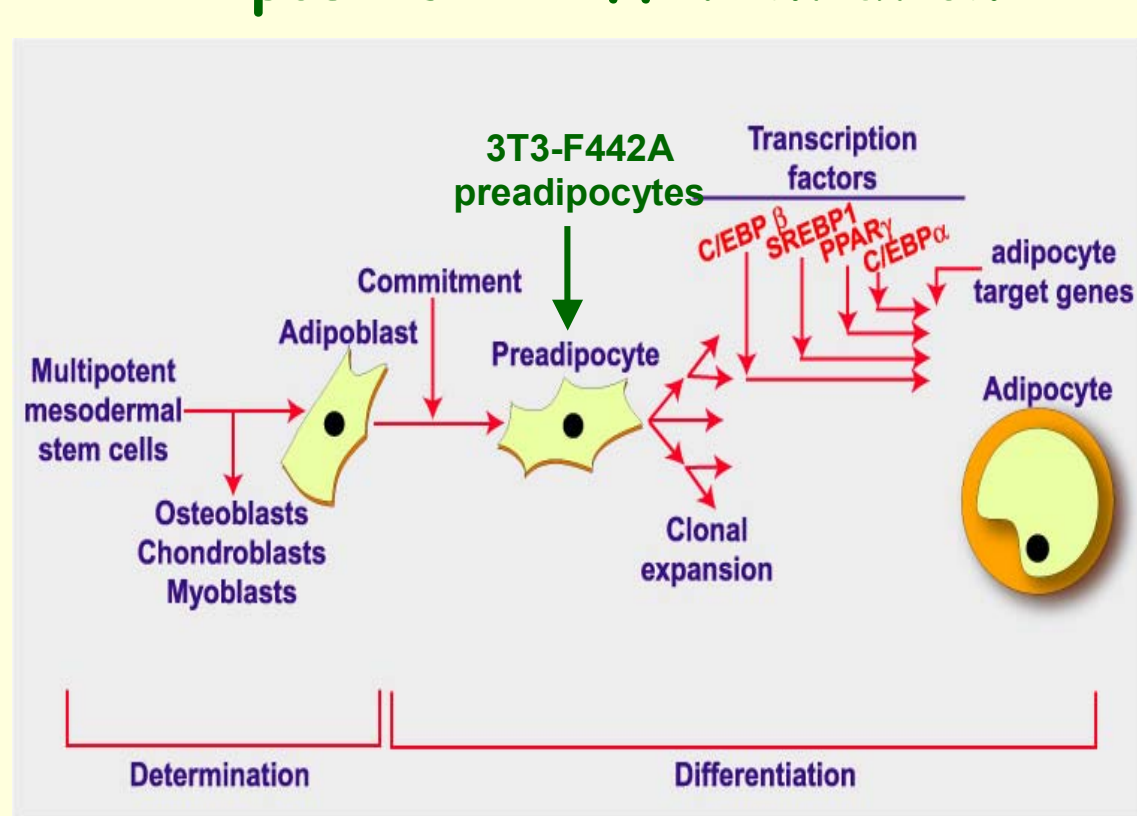
These findings suggested that PIs acted at a step that promoted disorders at the nuclear level and impeded SREBP-1 trafficking, thereby resulting in altered cell differentiation, resistance to insulin and programmed cell death. Whether this step is shared by the different processes, or whether PIs acted through a different mechanism on each of them remains to be determined. It is however important to evaluate whether the nuclear alterations found in PI-treated adipocytes were similar to those observed in LMNA-mutated patients' fibroblasts (17), to investigate the mechanism by which PI treatment can alter the major structural components of the lamina network and to establish the link between nuclear dysmorphism and SREBP-1 dysfunction.

In conclusion, we observed in our cell system that fat cell differentiation, metabolism and apoptosis were altered by a chronic exposure to indinavir, nelfinavir and amprenavir, although with notable differences according to the PI used. Although in vitro studies could not be directly extrapolated in vivo, the results suggest that PIs may be directly responsible for the loss and altered regeneration of the fat tissue. Further studies with this adipose cell model relevant for PI treatment are now in progress to assess the effects produced by treatments with PIs and/or NRTIs, to delineate the role played by SREBP-1 and the nuclear lamina and the mechanism of protection by thiazolidinediones.

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Adipose cell differentiation**The major transcription factors of adipose cell differentiation**