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Anti-apoptotic effect of HIV protease inhibitors via direct inhibition of calpain

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Abstract

Treatment with drugs designed to inhibit the HIV protease ameliorates immune functions in AIDS patients, reducing cell deletion by apoptosis even in the absence of inhibition of viral spread. This suggests that they interact with the intrinsic apoptotic signaling. We found that caspases, the main executioner of the apoptotic process, are not directly inhibited. In search for the mechanism responsible for their anti-apoptotic effect, we have found that indinavir and ritonavir are able to inhibit apoptosis only in those cell systems where apoptosis involves the activation of calpains. They directly inhibit a calpain-like activity expressed in lysates from apoptotic cells, to the same extent as commercially available calpain inhibitor 1. In *in vitro* assays with purified calpains, indinavir and ritonavir strongly inhibit m-calpain, and moderately μ-calpain. These results have great therapeutic implications, going beyond AIDS treatment, since many degenerative disorders involve abnormal calpain activation, indicating calpain as an ideal pharmacological target. Indinavir and ritonavir, potent m-calpain inhibitors, largely used since several years on humans without important negative side effects, may become powerful tools against those pathologies.

Keywords: HIV; Protease inhibitors; Indinavir; Apoptosis; Calpain; Caspases

1. Introduction

Apoptosis is the result of the coordinated involution of cellular structures, achieved through multiple independent pathways, whose choice depends on cell type and/or apoptotic stimulus, i.e. physiological vs. cell damaging. The physiological and stress-induced pathways evolve through independent set of events, carried on by protein–protein interactions and conformational changes, characterized by the formation of protein complexes (DISC and the apoptosome, respectively), which recruit and activate a set of cystein proteases, the family of caspases [1].

It is now clear that intracellular apoptotic signaling and cellular involution characterizing apoptosis are carried on by proteolytically activated machineries; in addition to caspases, other proteases such as calpains are implicated in apoptosis [2]. These are a set of Ca^{2+}-dependent proteases, present in two isoforms, milli (m) and micro (μ), with different Ca^{2+} requirements, implicated in a huge series of physiopathological situations, such as multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's diseases, cardiac and brain ischemia, eye cataract, muscular dystrophy [3]; calpains are involved in several models of apoptosis, including U937 [4], but unlike caspases, they are not absolutely required for apoptosis.

Progressive deterioration of immune functions, accompanied by massive loss of immune (especially CD4+) cells by apoptosis, characterizes progression of AIDS disease

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Abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; DISC, death-inducing signaling complex; D-MEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; EDTA, etylendiaminetetracetic acid; EGTA, ethylenglycolaminoethyltetracetic acid; PMN, polymorphonucleates; PBMC, peripheral blood mononucleated cells; PMC, puromycin; VP16, etoposide; DFU, delta fluorescence units; RFU, relative fluorescence units; IND, indinavir; CS, calpastatin peptide; CI1, calpain inhibitor 1.

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[5,6]. Treatments of AIDS patients was greatly improved by highly active antiretroviral therapy, cocktails of compounds including drugs (i.e. indinavir and ritonavir) designed to inhibit the HIV protease [7], leading to reduction of inflammatory parameters, of the incidence of opportunistic infections and neurological problems [8,9]. These compounds counteract T cell deletion [10,11], reduce apoptosis of T cells and neutrophils [12] in AIDS patients even in the absence of inhibition of viral spread, and increase *in vitro* cell viability by inhibiting apoptosis of infected and uninfected T cells [13,14], suggesting a direct, HIV-independent effect on apoptosis. The current view is that HIV protease inhibitors, though specifically designed to inhibit the processing of the gag-pol viral polyprotein catalyzed by the HIV cysteine protease, may directly affect the cell-intrinsic process of apoptosis. The central role of two sets of cysteine proteases, caspases and calpains [2] in apoptosis, led us to wonder whether the HIV-designed cysteine protease inhibitors may directly inhibit one or the other cysteine protease involved in apoptosis. Indeed, cross-reactivity of protease inhibitors is a well-known phenomenon [15]. Thus, to study the mechanism responsible for the direct anti-apoptotic effect of HIV protease inhibitors, we tested whether these compounds are able to inhibit apoptosis in a cell model that we have thoroughly studied in terms of apoptotic signaling [16,17], namely the pro-monocytic human cell line U937, in the absence of viral infection.

We have found that indinavir and ritonavir are able to inhibit apoptosis in those cell systems where apoptosis involves calpain activation, and that in *in vitro* assays, they inhibit purified m-calpain at high affinity, thus indicating that the anti-apoptotic effect of HIV protease inhibitors occurs via direct inhibition of calpain.

2. Materials and methods

2.1. Cell culture

(a) *Cell lines*: U937 (human pro-monocytes) and BL41 (human B lymphocytes) cells were cultured in RPMI 1640 medium supplemented with 10% FCS; SHSY5Y human neuroblastoma and B16F10 melanoma cells in D-MEM supplemented with 10% FCS. All cells are kept in a controlled atmosphere (5% CO_2) incubator at 37°. The experiments are performed on cells in the logarithmic phase of growth, in conditions of excellent viability, as assessed by trypan blue exclusion, 98%.

(b) *Primary cultures*: Polymorphonucleate (PMN) leukocytes were prepared from heparinized blood by Ficoll-Hypaque (Pharmacia Biotech.) density gradient centrifugation followed by dextran sedimentation. Contaminating erythrocytes were removed by a single hypotonic lysis in sterile distilled water for 30 s at room temperature. PMN were suspended in RPMI 1640 medium supplemented with 10% FCS, and adjusted to the desired concentration. The purity of PMN isolated was always greater than 95%, as determined by Giemsa staining.

Peripheral blood mononucleated cells (PBMC) were isolated from heparinized blood using the standard Ficoll-Hypaque (Pharmacia Biotech.) density separation method. After isolation, the PBMC were washed, counted, and suspended in RPMI 1640 supplemented with 20% FCS. Cells are kept in a controlled atmosphere (5% CO_2) incubator at 37°.

(c) *Induction and detection of apoptosis*: For cell lines, apoptosis was induced with the protein synthesis inhibitor puromycin (PMC, 10 μg/mL) or the topoisomerase II inhibitor etoposide (VP16, 100 μg/mL). Both compounds were kept throughout the experiment. Apoptosis is measured at 4-hr (U937), 6-hr (BL41) or 18-hr (all other cells) treatment, by analysis of nuclear vesiculation detectable by fluorescence microscopy on cells stained with the cell permeant DNA specific fluorescent dye Hoechst 33342; the fraction of cells with fragmented nuclei among the total cell population, is calculated for suspension cells as described [16]. For adherent cultures, the cells floating at the end of the treatment are taken apart; the remaining adherent cells tripsinized; floating and adherent cells are then pooled, stained with Hoechst 33342 and analyzed.

For primary cultures: ''Spontaneous'' apoptosis following explant was measured at 18 hr (PMN) or 48 hr (PBMC) postexplant.

(d) *Other treatments*: Indinavir was provided by Merck Sharp and Dohme; ritonavir was kindly provided by prof. M. Andreoni (University of Tor Vergata, Roma), CI1 (Calbiochem), zVAD caspase pan-inhibitor (Calbiochem), lactacystin (Calbiochem), calpastatin peptide (Calbiochem) were added 30 min prior to the apoptogenic treatment at 100 nM, unless otherwise specified.

2.2. Caspase and calpain activity

(a) Fluorigenic substrates for caspases 3, 8 and 6, and calpain were purchased from Calbiochem and used according to the manufacturer's instruction on cell lysates or purified enzymes.

(b) *Preparation of lysates*: 10^7 cells were collected by centrifugation, washed two times in standard Ca^{2+}-free phosphate buffered saline; the pellet was resuspended in 0.1 mL of lysis buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 40 mM sucrose) and subjected to five cycles of freeze/thawing; the supernatant was then kept at −80°. Activity measurement: 10 μL lysates were added to 500 μL the assay buffer (100 mM HEPES, 10% sucrose, 10 mM dithiothreitol, 500 μM EDTA and adjust pH to 7.5 using 0.1 M NaOH or HCl) and brought to 30°; the substrate was then added at 200 μM.

(c) *Purified enzymes*: Caspase 3, μ- and m-calpain were purchased from Calbiochem.

One unit of the enzymes was added to the assay buffer (100 mM imidazole–HCl, pH 7.5, 5 mM cysteine, 50 mM $CaCl_2$) and brought to 30°; the substrate, at 200 μM, was then added.

(d) *Activity measurement*: The activity was measured in a LS-5 Perkin-Elmer spectrofluorimeter at 30° in continuous agitation; the activity was calculated by change in fluorescence (ΔFU) for each sample at 15 min as follows: ΔFU = (sample FU at 15 min − blank FU at 15 min) − (sample FU at time zero − blank FU at time zero). The different inhibitors were added 10 min prior to substrate addition in complete buffer.

2.3. Statistical analysis

Statistical analyses were performed using Student's *t*-test for unpaired data and P values <0.05 were considered significant. Data are presented as mean ± SD.

3. Results

3.1. Indinavir anti-apoptotic effect is not due to caspase inhibition

With the goal of investigating the effect of HIV protease inhibitors on a well-known model of apoptosis in the absence of HIV, we probed the effect of indinavir and ritonavir on apoptosis induced on U937 monocytic cells by two different stressing agents, puromycin and etoposide. We found that apoptosis is reduced by indinavir and ritonavir at concentrations equivalent, or lower, with respect to the plasma levels in the patients (Fig. 1A). The activity of caspase 3, the main executioner of apoptosis, and caspases 6 and 8, is reduced accordingly (Fig. 1B). To determine whether caspase inhibition might be the direct target of indinavir and ritonavir, we tested the compounds for their ability to inhibit caspase activity in a lysate from apoptotic U937 cells; no direct effect was detected on caspases 3, 6, and 8 (Fig. 1C). This shows that the decreased activity of these same caspases shown in Fig. 1B was a consequence and not a cause of the inhibition of apoptosis. This indicates that indinavir and ritonavir inhibit apoptosis at a step upstream to caspases activation.

3.2. Indinavir directly inhibit calpains

Searching for a direct target for indinavir and ritonavir anti-apoptotic effect among apoptosis-related proteases, we probed their effects on calpains, the other set of apoptosis-related cysteine proteases.

First, we analyzed calpain activity in lysates from healthy and apoptotic U937 cells by estimating the lysates' ability to cleave a commercially available fluorigenic substrate. These experiments evidentiated an apoptosis-associated calpain-like activity upon PMC treatment (Fig. 2A). Since other proteases may contribute to the calpain-like activity (i.e. proteasome), we determined the actual contribution of calpain to this activation, by evaluating the effect of the specific calpain inhibitor calpastatin-peptide. Calpastatin reduces, but not abolishes, the calpain-like activity (Fig. 2A, third bar). The residual activity is due, at least in part, to proteasome activation. Indeed, the specific proteasome inhibitor, lactacystin, is able by itself to reduce the activity detected by the assay (Fig. 2A, fourth bar). This indicates that (a) apoptosis enhances proteasome activity, and (b) the test also detects proteasome activity.

Next, we evaluated the effect of HIV protease inhibitors on the calpain activity of apoptotic lysates. In these assays, we observed that indinavir directly inhibited calpain activity, to an extent similar to that of CI1 (Fig. 2A). The different extent of inhibition exerted by CI1/indinavir with respect to the more specific calpastatin implicate that they inhibit a further, noncalpain activity. It is reported that CI1 also inhibits proteasome [18]. These data are consistent with the hypothesis that indinavir inhibits either proteasome or calpain, or both.

To determine if indinavir and ritonavir directly inhibit calpains, we tested the compounds for their ability to inhibit purified calpains: this test would also evidentiate any eventual difference in the extent of inhibition of the two types of calpains, micro or milli, which are differently involved in physiological vs. pathological processes, respectively (see Section 4). The experiments showed that indinavir and ritonavir, unable to inhibit purified caspase 3 (as expected from the results shown in Fig. 1C), moderately inhibited μ-calpain, but exerted a strong inhibition on m-calpain, to the same extent as CI1 (Fig. 2B and C).

3.3. The anti-apoptotic effect of indinavir is due to calpain inhibition

So far, we have shown that indinavir and ritonavir directly inhibit m-calpain; and that calpain (and proteasome) are activated, in an indinavir-sensitive fashion, in U937 apoptosis. Now, we address the problem on whether calpain (and proteasome) activation in stress-induced apoptosis are just concomitant or exert a causative role in apoptosis. Thus, we assessed the possible role in apoptosis of calpains and proteasome, by inducing apoptosis in the presence of calpastatin peptide or lactacystin, respectively. As shown in Fig. 3A, calpastatin reduces apoptosis to an extent similar to indinavir or CI1, whereas lactacystin has no effect. This shows that proteasome activation is a downstream event in apoptosis, with no role in triggering the process in U937 (and in the melanoma cells B16F10, see legend to Fig. 3). Instead, calpain activation possesses an active role in triggering stress-induced apoptosis on U937.

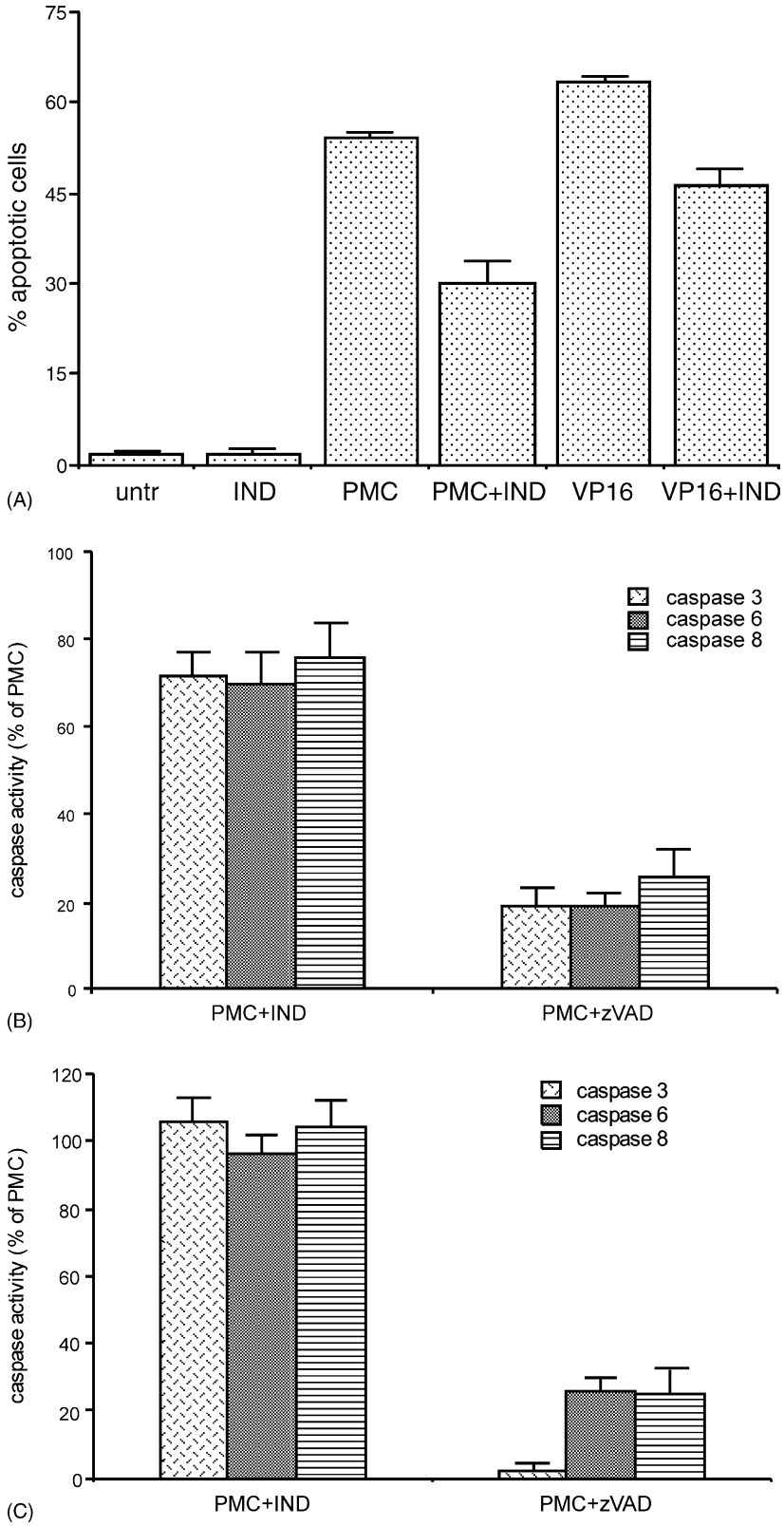
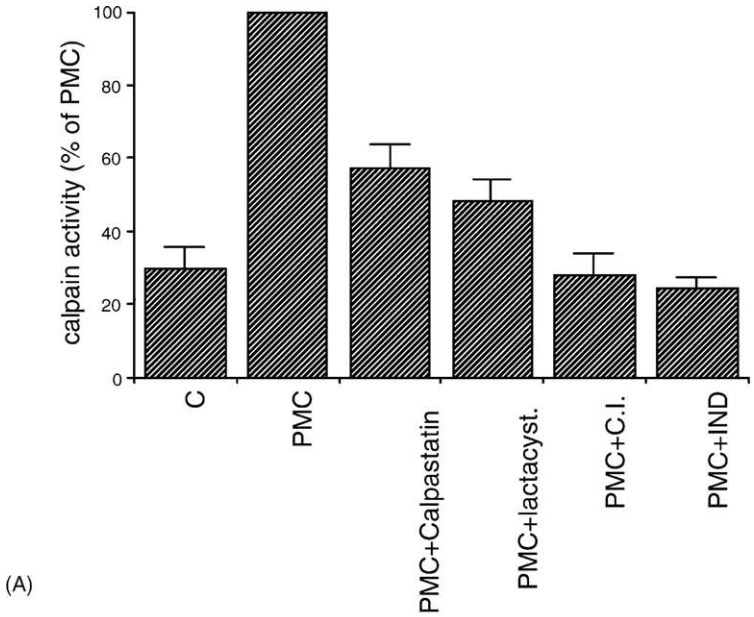
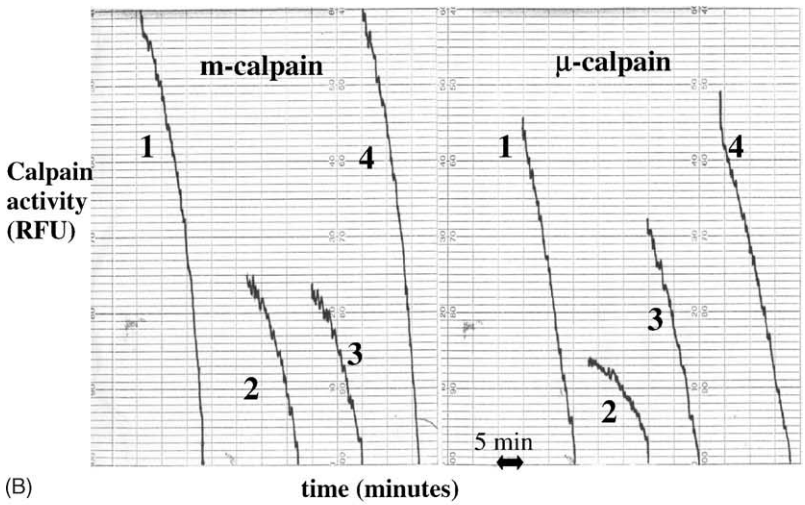


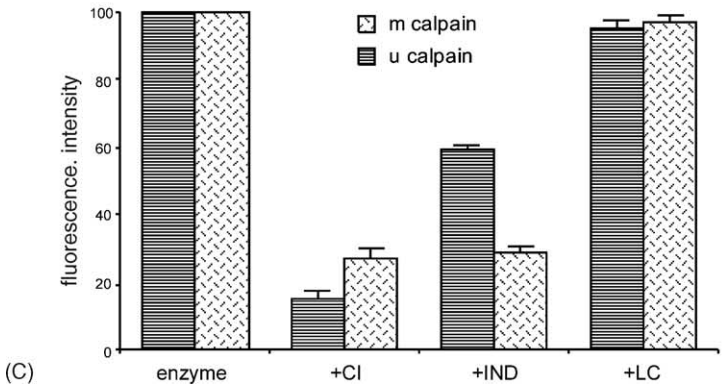
Fig. 1. Indinavir anti-apoptotic effect is not due to caspase inhibition. Panel A shows the decrease of PMC- and VP16-induced apoptosis by 100 nM IND on U937 cells (average of >3 independent experiments ± SD). Similar results were obtained with 100 nM ritonavir (not shown). Panel B shows caspase activity in U937 induced to apoptosis by PMC in the presence/absence of 100 nM IND or 100 nM of the caspase inhibitor zVAD (4-hr treatment). Caspase activity, shown as the percent of the activity found in cells induced to apoptosis by PMC without inhibitors, was measured as the digestion of commercially available fluorigenic substrate (see Section 2) and found to be as follows: caspase 3: 465 ± 45 μU/mL; caspase 6: 531 ± 38 μU/mL; caspase 8: 340 ± 88 μU/mL; average of >3 independent experiments ± SD). Similar results were obtained with lysates of U937 induced to apoptosis by VP16 (not shown). Panel C shows the effects that 100 nM IND or 100 nM zVAD, added *in vitro* to lysates of apoptotic U937, exert on caspases activity (average of >3 independent experiments ± SD).



(A)



(B)



(C)

Fig. 2. Indinavir directly inhibit calpains. Panel A shows the effects that 100 nM CS, 100 nM CI1, or 100 nM IND, added in vitro to lysates of apoptotic U937, exert on calpain activity; calpain activity, shown as the percent of the activity found in cells induced to apoptosis by PMC without inhibitors (495 ± 38 μU/mL) was measured as the digestion of commercially available fluorigenic substrate (see Section 2; average of >3 independent experiments ± SD). Also ritonavir was found to inhibit the apoptosis-dependent calpain activity, by about 60% (not shown). Panel B shows the fluorimetric profiles obtained by incubating 1 U purified m-calpain (left) or μ-calpain (right) with the calpain fluorigenic substrate (as described in Section 2) by themselves (lines 1) or in the presence of indinavir (lines 2) or CI1 (lines 3) or lactacystin as negative control (lines 4; RFU, relative fluorescence units). The quantification and statistical analysis of these results is shown in Panel C, where fluorescent intensity is given as percent of the fluorescence increase (ΔFU) of the samples without inhibitors (CI1, IND, LC). Ritonavir (100 nM) inhibited likewise m- and μ-calpains activity (not shown).

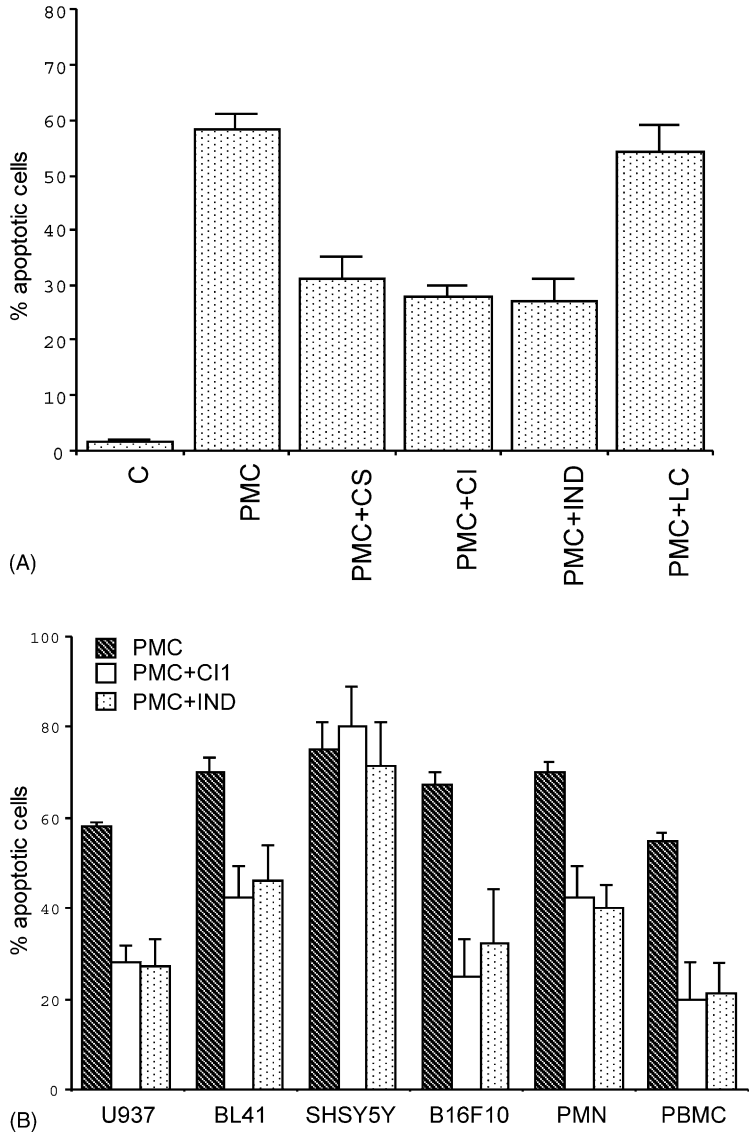


Fig. 3. The anti-apoptotic effect of indinavir is due to calpain inhibition. Panel A shows the effects of CS, CI1, IND and LC on the extent of apoptosis induced by puromycin in U937 cells (average of >3 independent experiments ± SD). In the melanoma cells B16F10, LC failed to protect from apoptosis, whereas CS had a protective effect (not shown). Panel B shows the effects of IND or CI1 on apoptosis induced in a panel of different cell systems by puromycin (cell lines) or by explant in culture (primary cells, see Section 2 cells; average of two–four independent experiments ± SD).

It is known that calpain are not universally required for apoptosis. Thus, we wondered whether indinavir and ritonavir are able to interfere with apoptosis also in those systems where calpains are not involved. We addressed this question by a parallel analysis of the anti-apoptotic effect of CI1, indinavir and ritonavir on a small panel of calpain-sensitive/insensitive cell types. It turned out that the anti retroviral drugs are effective against apoptosis only when calpain inhibitor is effective (Fig. 3B); in particular, in the neuroblastoma cell line SHSY5Y, known to undergo a calpain-independent apoptosis [19], indinavir and ritonavir are ineffective; in the sensitive cell lines, the extent of the protection exerted by the drugs is similar to that exerted by CI1. All this evidence suggests that the target of indinavir and ritonavir anti-apoptotic effect is indeed calpain.

4. Discussion

In this study, we show that calpain activation is causative for triggering stress-induced apoptosis in U937 cells. In the frequently discussed problem of the position and role of calpain activation during apoptotic signaling, our results are in agreement with studies reporting a very early calpain activation (i.e. in thymocytes apoptosis [20]; the studies on proteolytical activation of pro-apoptotic proteins [21,22]). Indeed, our data implicate that calpain activation occurs upstream with respect to caspase activation, since indinavir, ritonavir, or CI1 reduce the apoptotic activation of caspases by inhibiting calpain, whereas none of the compounds can directly block caspases.

M- and μ-calpains are the products of different genes; they are similar for sequence, structure, substrate specificity and

mode of action, but differ for the concentration of Ca^{2+} required for activation, being in the micromolar or millimolar range, respectively [23]. For this reason, it is believed that they may play different roles in physiopathology. As far as apoptosis is concerned, it is known that calpains are able to cleave specific substrates important for apoptosis execution (i.e. fodrin digestion [21]) or commitment (proteolytical Bax [24] and caspase 3 [22] activation). This latest study addresses the question of which calpain is involved in apoptosis, and shows that m-calpain, but not μ-calpain, can proteolytically activate caspase 3. This is in line and reinforces our findings that indinavir and ritonavir strongly inhibit m-calpain rather than μ-calpain. This cause–effect relationship is further strengthened by our finding that indinavir and ritonavir inhibit apoptosis only in cells system where apoptosis involves calpain activation.

Recently published data [25] show that ritonavir preferentially inhibits μ-calpain over m-calpain ($K_i = 9.2$ μM on m-calpain vs. 5.9 μM on μ-calpain), thus apparently contradicting our results. However, the authors did not mention the concentration range used for calculating the K_is. In a dose–effect experiment, we observed that ritonavir exerts a quite complex mechanism of inhibition on both calpains (data not shown). Thus, we cannot exclude that different concentrations of ritonavir may differentially inhibit m- and μ-calpains.

The data shown in Fig. 2A are compatible with an inhibition of proteasome activity by indinavir in the lysates of apoptotic cells. This is in line with recent results showing that purified proteasome activity is inhibited by antiretroviral agents [26]; this inhibition may prevent apoptosis in those cell systems where proteasome has been shown to play a role in triggering apoptosis [27], even though it is now clear that in most instances proteasome inhibition is a trigger for, rather than a protection from, apoptosis [28]. Thus, our results suggest that the protease inhibitors contained in the cocktails of compounds included in the highly active antiretroviral therapy, block apoptosis through the inhibition of calpains.

The results reported in this study operate a merging between two lines of research carried out by different laboratories, i.e. the studies showing that many leukocytes (either cell lines or freshly explanted cells) undergo a calpain-dependent apoptosis [20,29] and the studies aimed at investigating the mechanism(s) through which inhibitors of HIV proteases affect the apoptotic process in HIV-infected and uninfected cells. In particular, they might provide a model for explaining the improvements of neurological disorders in AIDS patients treated with highly active antiretroviral therapy. Indeed, the specific literature has documented a strict involvement of calpains in the onset of neurodegenerative disorders. Alzheimer's, Parkinson's and Huntington's diseases, amyotrophic lateral sclerosis, multiple sclerosis, eye cataract, cardiac and brain ischemia, muscular dystrophy [30,31], all involve abnormal calpain activation, thus indicating calpain as an ideal pharmacological target. This suggests that our findings may have great therapeutic implications, going beyond AIDS treatment, in pathologic processes that involve abnormal calpain activation. So far, the clinical research on the therapeutic use of calpain inhibitors is slowed by the absence of nontoxic, cell permeant drugs. HIV protease inhibitors have been largely used since several years on AIDS patients; their side effects, though annoying, are not serious enough to discourage their use for severe pathologies such as AIDS. The severity of the calpain-dependent neurodegenerative disorders suggests that indinavir and ritonavir, and possibly other anti-HIV protease drugs possessing the ability of cross-inhibiting calpains, might become powerful tools against those pathologies.

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