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Ritonavir does not inhibit calpain in vitro ☆

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Abstract

Ritonavir, an inhibitor of HIV-1 protease, has been reported to also inhibit the Ca^{2+}-dependent cysteine protease, calpain. We have investigated these claims with an in vitro study of the effect of ritonavir on the m-calpain and μ-calpain isoforms. Ritonavir failed to block either autolytic or hydrolytic calpain activity, but remained fully capable of inhibiting the HIV-1 protease. Any calpain-related effects of ritonavir in cells must, therefore, arise by a mechanism other than direct inhibition of calpains.

Keywords: Calpain; Protease; Ritonavir; Inhibitor; Autolysis

Inhibitors of the HIV-1 aspartyl protease have shown remarkable success in the treatment of patients infected with HIV by inhibiting the viral enzyme required for maturation of the virus. Some of these compounds, such as ritonavir (10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester), have also been reported to have effects in non-HIV-related systems, showing: inhibition of tumor necrosis factor-α-mediated cell death in Jurkat cells [1]; anti-atherogenic properties on vascular smooth-muscle cells [2]; inhibition of brush-border microvilli assembly in *Escherichia coli* [3]; as well as having anti-tumorigenic properties [4,5]. Since these drugs have already been shown to be safe for humans, the potential for using these drugs in the treatment of conditions other than HIV infection has led to these effects being further studied.

Three groups have already reported that ritonavir could exert its non-HIV-protease-related effects by acting upon the calpain system [3,6,7]. Calpains, a family of intracellular Ca^{2+}-activated cysteine proteases, are involved in a number of important pathways such as cytoskeleton remodeling, cell motility, apoptosis, and transcription regulation [8]. They form a class of pharmacologically significant targets due to their involvement in cellular degenerative conditions such as ischemia [9], Alzheimer's disease [10], and Parkinson's disease [11]. The possibilities arising from the application of these inhibitors towards treating calpain-related pathologies are therefore far-reaching.

Investigations using purified recombinant and natural calpains in in vitro inhibition studies led to claims that the HIV protease inhibitors ritonavir and indinavir directly inhibit calpain activity [7], and that ritonavir shows a competitive mode of inhibition [6]. This supported the observation that ritonavir has an effect similar to known calpain inhibitors in brush-border assembly in *E. coli* [3] and that indinavir and ritonavir

☆ *Abbreviations:* HIV, human immunodeficiency virus; SLY-MCA, succinyl-leucine-tyrosine-aminomethyl coumarin; E-64, *trans*-epoxy-succinyl-L-leucylamido(4-guanidino)butane.

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demonstrate anti-apoptotic activity only in cell systems where the apoptotic pathway involved calpain activation [7]. Consequently, this has led to speculation that the cellular effects occurring upon exposure to ritonavir occur via inhibition of calpain-mediated pathways. Using enzyme from both recombinant and natural sources, we report here a re-evaluation of the effect of ritonavir on calpains in vitro, which refutes the direct inhibition of calpains by ritonavir.

Materials and methods

Materials. Porcine erythrocyte μ-calpain and calpeptin (calpain inhibitor I) were obtained from Calbiochem. Recombinant rat m-calpain was expressed and purified as previously described [12]. Recombinant HIV-1 protease (0.54 mg/mL) and its substrate DABCYL-γ-Abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS were purchased from Bachem Bioscience. Leupeptin, E-64, and SLY-MCA (succinyl-leucine-tyrosine-aminomethyl coumarin) were purchased from Sigma. Ritonavir was obtained from two different sources: one sample was purchased from Abbott Labs; the other sample was purified from the pharmaceutical product (a gift from Dr. D. Potter, Indiana University). Stock solutions of ritonavir (100 mM) were prepared in absolute ethanol and then diluted in aqueous buffer for a maximum of 1 h prior to use in an assay. Ethanol stocks were stored at −20 °C.

SLY-MCA hydrolysis assay. Assays were performed at room temperature in 50 mM HEPES–NaOH (pH 7.6), 0.2 M NaCl, and 10 mM DTT using 0.25 mM of the fluorogenic substrate SLY-MCA. Substrate hydrolysis was monitored at excitation and emission wavelengths of 360 and 460 nm, respectively, while continuously stirring. For μ-calpain, the reaction was initiated by the addition of 0.01–0.02 mg/mL enzyme, and ritonavir (10–300 μM) was added within the first 10 min. For m-calpain, ritonavir (10–300 μM) was present in solution prior to addition of the enzyme. Activity assays were performed at both saturating (1 mM $CaCl_2$) or non-saturating (240 μM for m-calpain; 35 μM for μ-calpain) Ca^{2+} concentrations.

Casein hydrolysis assay. This fixed-point assay was performed as previously described [13] using 5 mM $CaCl_2$ or EDTA in Tris–HCl (pH 8.0) and 5 mg/mL casein, either with or without inhibitor. The reaction was initiated by addition of 6 μM m-calpain. Incubation time was for 30 min. Inhibitor concentrations of 5 μM (leupeptin, calpeptin), 25 μM (ritonavir), or 10 μM (E-64) were used.

Calpain autolysis assay. Autolysis of 0.5 mg/mL m-calpain was performed in 50 mM HEPES–NaOH (pH 7.6), 0.2 M NaCl, in the presence of 5 mM $CaCl_2$. Autolysis was initiated by addition of 20 μL of 1 mg/mL m-calpain to 20 μL of a solution containing buffer, calcium, and inhibitor, and was allowed to proceed for 15 min at room temperature. Final inhibitor concentrations of 25 μM (leupeptin, calpeptin, and ritonavir) or 50 μM (E-64) were used. Autolysis was terminated by addition of 15 μL of 3× SDS–PAGE sample buffer (200 mM Tris–HCl (pH 6.8), 6% (w/v) SDS, 30% glycerol, and 0.03% (w/v) bromophenol blue) and flash-freezing. Tris–Tricine SDS–PAGE analysis of the autolytic products was performed using a 10% polyacrylamide gel.

HIV-protease activity assay. Assays were performed in 50 mM NaOAc (pH 4.9), 200 mM NaCl, 5 mM DTT, and 10% glycerol using 20 μg/mL DABCYL-γ-Abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS substrate in the presence or absence of 50 μM ritonavir. Substrate stocks were prepared in DMSO at 1 mg/mL. The reaction was initiated by addition of the HIV-1 protease to a final concentration of 2.5 μg/mL. After incubation at 37 °C for 30 min, the reaction was quenched with the addition of 10% trifluoroacetic acid. The extent of substrate cleavage was quantified by a Microplate Fluorescent Reader (FL600, Bio-Tek) using excitation and emission wavelengths of 340 and 450 nm, respectively.

Results and discussion

In the presence of Ca^{2+}, the large 80 kDa subunit of m-calpain autolyzes to produce domain-sized fragments, particularly the 38 kDa catalytically inactive protease core encompassing domains I and II (Fig. 1, lane 6) [14]. This autolysis was severely attenuated by the presence of calpain active site-directed inhibitors calpeptin, E-64, and leupeptin (Fig. 1, lanes 1, 2, and 5, respectively). Ritonavir (25 μM) was completely ineffective at blocking autolysis (Fig. 1, lanes 3 and 4) which proceeded to the same extent that it did in the control in which inhibitor was omitted (Fig. 1, lane 6).

Ritonavir also showed insignificant inhibition of the hydrolysis of exogenous substrates. In a fixed-point casein hydrolysis assay, leupeptin (5 μM), calpeptin (5 μM), and E-64 (10 μM) showed greater than 85% inhibition of m-calpain activity, whereas ritonavir showed less than 1% inhibition (Fig. 2A) even at 2.5- to 5.0-fold higher concentration (25 μM). In a real-time assay of the hydrolysis of the fluorogenic substrate SLY-MCA by both recombinant m-calpain and purified erythrocyte μ-calpain, 84–100% of the initial enzyme activity was obtained when the assays were performed in high concentrations (300 μM) of ritonavir (Table 1). At least part of the apparent decrease in activity may be attributable to light scattering or to co-precipitation of the enzyme due to the presence of ritonavir at concentrations well above its solubility limit in aqueous buffers (that ranges from 1.4 μM [15] to ~9 μM [16]). The possibility that the compound affects protease activity by

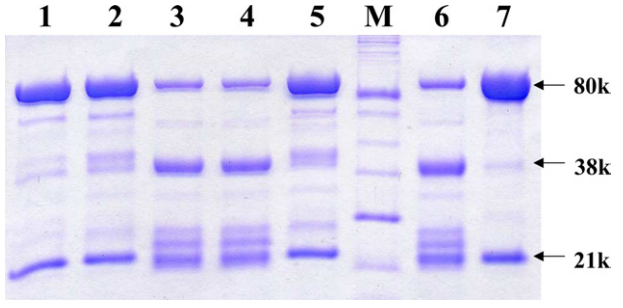

Fig. 1. The effect of ritonavir and various inhibitors on m-calpain autolysis. Autolysis of m-calpain was carried out in the presence of calpeptin (lane 1), E-64 (lane 2), ritonavir (lanes 3 and 4), leupeptin (lane 5) or ethanol (lane 6), the latter acting as a control lacking inhibitor. No autolysis is observed in the absence of Ca^{2+} (lane 7). Lane M contained molecular weight markers. The results in lanes 3 and 4 were obtained with ritonavir samples obtained from two different sources. The bands corresponding to the m-calpain heterodimer (80 and 21 k) as well as the major autolysis fragment (38 k) are shown by arrows.

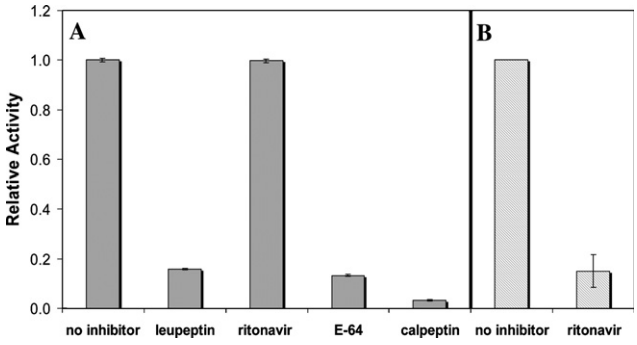

Fig. 2. Inhibition of m-calpain and HIV-1 protease. (A) The hydrolysis of casein by m-calpain assayed in the absence or presence of inhibitors. The data were corrected for controls containing EDTA and normalized to the activity of the sample to which only ethanol was added (no inhibitor). Error bars represent the standard deviation resulting from triplicate assays. (B) The HIV-1 protease was assayed in the presence or absence of 50 mM ritonavir. The error bar represents the standard deviation resulting from two assays containing ritonavir from two independent sources.

Table 1
The effect of ritonavir on calpain hydrolysis of SLY-MCA

Enzyme	[$CaCl_2$]	Ritonavir concentration		% initial activity
		0 μM	300 μM	
m-Calpain	1 mM	0.533	0.448	84
	240 μM	1.333	1.335	100
μ-Calpain	1 mM	0.255	0.234	92
	35 μM	0.077	0.067	87

Activity values reported are in fluorescence units per second. The values obtained using different calpain isoforms or [$CaCl_2$] used different detector settings on the instruments and are not directly comparable.

altering the Ca^{2+} requirements of the enzyme was excluded as no inhibition of SLY-MCA hydrolysis was observed by up to 300 μM ritonavir in non-saturating Ca^{2+} concentrations (240 μM for m-calpain, 35 μM for μ-calpain).

Therefore, the compound ritonavir, at concentrations up to and beyond its solubility limit, consistently showed no effect on the activity of either m- or μ-calpain from recombinant or natural sources, whereas the presence of the well-documented calpain inhibitors leupeptin, calpeptin, and E-64 all stopped the enzyme from working at similar or lower concentrations. The identity of the ritonavir compound was confirmed by the *m/z* of the parent peak and the fragmentation pattern obtained from electrospray mass spectrometry (not shown), and its intact functionality was verified by its ability to inhibit the HIV-1 protease (Fig. 2B). To ensure batch reliability, the ritonavir compound was obtained from two independent sources.

Inhibition by ritonavir of calpain activity in vitro was previously concluded independently by two groups [6,7]. However, two observations cast some doubt on the results of these studies. First, ritonavir has an extremely poor solubility in aqueous solutions, as well as a slow rate of dissolution [15], which limits its usefulness for in vitro studies [16]. This puts into question the reliability of studies reporting binding parameters for ritonavir at concentrations close to the maximum solubility noted above [6]. Moreover, m- and μ-calpain rapidly autolyze upon exposure to Ca^{2+} to form virtually inactive fragments [17,18]. Therefore, kinetic studies of calpains initiated by addition of substrate to an enzyme already exposed to Ca^{2+} are likely to show loss of activity due to autolysis that could easily be misinterpreted as an effect occurring from the presence of the presumed inhibitor [7]. In this case, the faster rate of autolysis of m-calpain relative to μ-calpain (unpublished observation) could explain its higher apparent susceptibility to ritonavir.

Reports of ritonavir and other related HIV-protease inhibitors affecting cellular pathways in non-HIV-related systems are numerous and may be explained without resorting to calpain inhibition. One possible target is the proteasome, where ritonavir has been shown to inhibit a chymotrypsin-like activity as well as enhance a tryptic activity [19,20]. Our results clearly show that the reported cellular effects resulting from the administration of ritonavir must occur by a mechanism other than by the direct inhibition of calpains.

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