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Short communication

Ritonavir inhibition of calcium-activated neutral proteases

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Abstract

Calpains (EC 3.4.22.17) are intracellular calcium-activated cysteine proteases that mediate tissue injury following post-ischemic and post-traumatic stress. Both human HIV protease and calpains share a similar secondary structure, where the active site is flanked by hydrophobic regions. The present study demonstrates that ritonavir, a hydrophobic HIV protease inhibitor, also inhibits calpain activity. In PC12 cell extracts assayed for calpain at maximal activity (2 mM calcium), ritonavir exhibited competitive inhibition with a K_i of 11 ± 7.0 μM. Experiments with purified enzymes showed inhibition for both m- and μ-calpain isoforms (m-calpain, $K_i = 9.2 \pm 1.2$ μM; μ-calpain, $K_i = 5.9 \pm 1.4$ μM). Ritonavir also inhibited calcium-stimulated calpain activity in PC12 cells *in situ*. These results suggest that ritonavir or analogues of the drug should be investigated as cytoprotective agents in conditions where cell death or injury is mediated via calpain activation.

Keywords: Calpain; Enzyme inhibitors; Ritonavir; PC12 cell; HIV protease

1. Introduction

Calpains (EC 3.4.22.17) exist as heterodimers containing a unique heavy 80-kDa subunit and a common 30-kDa regulatory subunit. At least three unique gene products of the 80-kDa subunit can be identified in mammalian cells based on their calcium requirements for proteolytic activity: μ-calpain, requiring 5–50 μM calcium; m-calpain, requiring 150–1000 μM calcium; and p94, requiring intermediate concentrations of calcium for half-maximal activity [1,2].

Calpain inhibitors are neuroprotective in models of traumatic brain and myocardial injury [3–9]. Studies have indicated that some calpain inhibitors exhibited protective effects against neuronal damage both *in vitro* and *in vivo* [10–12]. These promising studies suggest that calpain inhibition may find therapeutic applications in the treatment of post-ischemic or post-traumatic tissue injury [13].

For both human HIV protease and calpains, the potency of inhibitory compounds is increased by hydrophobic groups flanking non-hydrolyzable pseudo-peptide bonds [14,15]. The active site in both proteases is also located in the terminal pocket of a groove flanked by hydrophobic domains [16,17]. While HIV protease is an aspartyl protease, and calpains are cysteine proteases, peptide aldehyde inhibitors of calpain were shown previously to also inhibit HIV protease [18]. Taken together, these findings suggested that ritonavir (Fig. 1), a hydrophobic HIV protease inhibitor, might also demonstrate activity as a calpain inhibitor.

The present study used PC12 cell extracts to characterize the mechanism of protease inhibition by ritonavir. The kinetics of inhibition were confirmed using purified m- and μ-calpain preparations. These results were extended to a whole cell assay, based on the ability to monitor calpain activation in live whole cells.

2. Materials and methods

2.1. PC12 whole cell preparation

Unless otherwise stated, all reagents were obtained from the Sigma Chemical Co. PC12 cells were cultured in RPMI medium (Gibco) containing 5% fetal bovine serum, 10% heat-inactivated horse serum, and 50 mg/L of gentamicin at 37°, 5% CO_2. Cells were isolated by centrifugation (200 *g* for 4 min at 25°) and suspended in Hanks' Balanced Salt Solution (HBSS). Enzymatic activity was measured as previously described for whole cell experiments [19].

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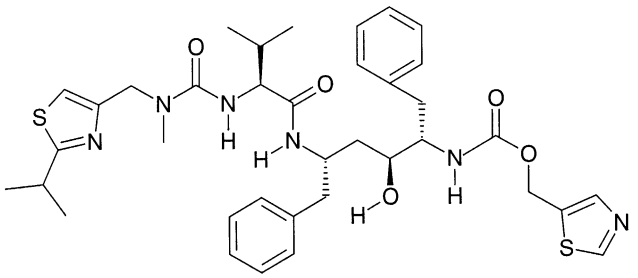

Fig. 1. Molecular structure of ritonavir. Hydrophobic planar groups are seen flanking non-hydrolyzable pseudo-peptide bonds.

2.2. PC12 extract preparation

PC12 cells were isolated as described above, and cell pellets were suspended in 1 mL of extract buffer, containing 30 mM Tris at pH 6.8, 15 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 100 μg/mL of phenylmethylsulfonyl fluoride, 0.1 μg/mL of 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 14.4 mM 2-mercaptoethanol, and 1% Triton X-100. After one cycle of freeze–thaw, the suspension was centrifuged at 14,000 *g* for 20 min at 4°, and aliquots of the supernatant were used in the experiments. The protein concentration of the supernatant used for the assays was approximately 4 mg/mL.

2.3. m-Calpain- and μ-calpain-purified enzyme preparation

Calpains were suspended at a concentration of 2.6 units/mL for m-calpain (Calbiochem-Novabiochem) and 0.53 units/mL for μ-calpain (Biovision Research) in a buffer solution containing 60 mM imidazole (pH 7.3), 5 mM L-cysteine (Fluka), and 2.5 mM 2-mercaptoethanol.

2.4. Calpain activity assays

Calpain activity in cell extracts was measured using the fluorescent calpain peptide substrate *N*-succinyl-Leu-Tyr 7-amido-4-methylcoumarin as previously described [19]. Fluorescence activity was monitored with a SpectraMAX Gemini microplate spectrophotometer (Molecular Devices) at 380 nm excitation and 480 nm emission. Four substrate concentrations (0, 80, 240 and 720 μM) and four ritonavir (Moravek Biochemicals, Inc.) concentrations (0, 1, 10 and 100 μM) were used for kinetic determinations. In assays using PC12 whole cells, the substrate concentration was held at 80 μM. The reaction was started by adding 10 μL of extract or cell suspension to the wells. In purified enzyme experiments, 5 μL of buffer containing enzyme (m-calpain, 2.6 units/mL; μ-calpain, 0.53 units/mL) was added to the wells. Total volume for assay was 150 μL.

For PC12 extract experiments, calpain specific activity was obtained by subtracting the rate obtained in the absence of calcium from the rate obtained in the presence of calcium. For purified enzyme experiments, no baseline fluorescence was observed in the absence of added calcium.

2.5. Kinetic analysis and statistics

Data consisting of relative fluorescence units (RFU) was obtained every 2 min for 1 hr. Data were analyzed based on initial rate, defined as the slope of the increase of the obtained RFU value up to a maximum time of 10 min. Data points from 7–9 separate experiments were used for the kinetic analyses. Each experimental point was determined as the mean of six replicates. Maximal rate was obtained in the presence of 2 mM calcium. Steady-state kinetic data were fit to a series of model equations describing competitive, uncompetitive, and noncompetitive inhibition. Nonlinear regression with an adaptive non-linear squares algorithm [20] was employed for the analyses utilizing NLREG software written by Phillip H. Sharrod. Model sufficiency was evaluated based on parameter convergence with a tolerance factor of 1×10^{-10}, the overall F-value for the regression, and the magnitude and sign of the parameters obtained after convergence.

To take into account differences in enzymatic activity between experiments, the kinetic parameters in experiments were estimated based on the ratio r:

$$r = \frac{v_i}{v_m} \tag{1}$$

where v_i is the velocity in the presence of inhibitor, and v_m is the velocity in the absence of inhibitor. We chose the following sets of kinetic equations based on the assumption that ritonavir was a substrate analogue and would therefore inhibit product formation [21]. This scheme is a modification of a previously described method useful in characterizing inhibitors following fluorescence-based studies of enzymatic activity [21].

For a competitive inhibitor, the initial reaction velocity from steady-state kinetics is:

$$v = \frac{V_{\max}}{[1 + (K_a/A) \times (1 + I/K_i)]} \tag{2}$$

where v = initial velocity; I = concentration of inhibitor; $K_i = (E)(I)/(EI)$; A = substrate concentration; and K_a = Michaelis–Menten constant. The rate in the absence of the inhibitor is:

$$v_m = \frac{V_{\max}}{(1 + K_a/A)}$$

We define a ratio (r) between the observed velocity in the presence of the inhibitor and the velocity in the absence of the inhibitor, where $0 < r \leq 1$:

$$r = \frac{v_i}{v_m} = \frac{V_{\max}/[1 + (K_a/A \times (1 + I/K_i))]}{V_{\max}/[1 + K_a/A]} \tag{3}$$

For competitive, uncompetitive, and noncompetitive inhibition, the kinetic equations therefore reduce to the following:

$$r = \frac{A + K_a}{A + K_a(1 + I/K_i)} \quad \{\text{Competitive inhibition}\} \quad (4)$$

$$r = \frac{A + K_a}{A(1 + I/K_i) + K_a} \quad \{\text{Uncompetitive inhibition}\} \quad (5)$$

$$r = \frac{A + K_a}{A(1 + I/K_{ii}) + K_{ia}(1 + I/K_i)} \quad \{\text{Noncompetitive inhibition}\} \quad (6)$$

where K_i is the dissociation constant for the enzyme–inhibitor complex, K_{ia} is the dissociation constant of the enzyme–substrate complex, and K_{ii} is the dissociation constant of the enzyme–substrate–inhibitor complex.

3. Results

When the kinetics of calpain activity inhibition by ritonavir were examined in PC12 cell extracts, the best model fit was found with the equation describing competitive inhibition, having an overall F-value of 118.48 with an associated $P < 0.00001$. The observed K_i for ritonavir was 11 ± 7.0 μM (mean ± SEM). Noncompetitive and uncompetitive inhibition models were rejected because the parameters either failed to converge or converged to negative values. Since only the competitive model was found to converge, it was not necessary to compare model fit taking into account the number of model parameters. With experiments employing cell extract, the observed kinetic parameters represent aggregate estimates since both m- and μ-calpain isoforms were present.

Enzymatic studies were performed to characterize the inhibitory properties of both purified isoforms of calpain. Ritonavir exhibited competitive inhibition with $K_i = 9.2 \pm 1.2$ μM (F-value = 353.81, $P < 0.00001$) against m-calpain. Ritonavir also exhibited competitive inhibition with $K_i = 5.9 \pm 1.4$ μM (F-value = 28.72, $P = 0.0002$) against μ-calpain. Results for both sets of experiments are shown in Table 1.

Substrate hydrolysis in the presence of ritonavir was examined in whole PC12 cells in the presence of 10 μM ionomycin and 1.4 mM calcium. Ritonavir inhibited calpain activity with increasing potency as the ritonavir concentration was increased from 0 to 100 μM, as shown in Fig. 2.

Table 1
Comparison of estimated K_i values

Experiments	K_i (μM)
PC12 cell extract	11 ± 7.0
Purified m-calpain	9.2 ± 1.2
Purified μ-calpain	5.9 ± 1.4

Values are means ± SEM, $N = 7–9$.

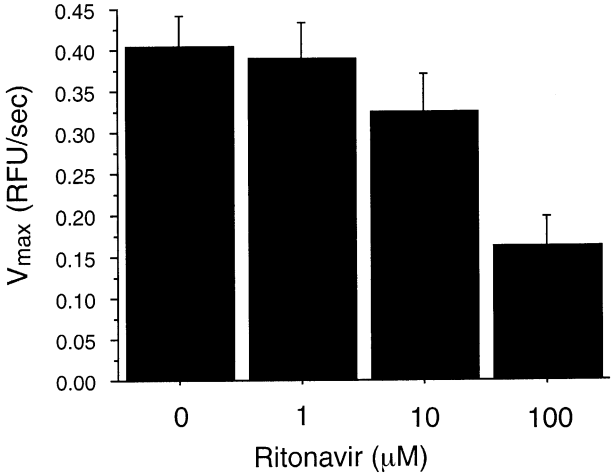

Fig. 2. Concentration–response curve of substrate hydrolysis inhibition by ritonavir. The height of the bars represents the means ± SEM of initial velocities, relative fluorescence units per second (RFU/sec), under conditions of different inhibitor concentrations. Initial velocity at 100 and 10 μM ritonavir was significantly different from control (0 μM) at $P \leq 0.05$, $N = 8$.

4. Discussion

The present study is the first to indicate that ritonavir, an HIV protease inhibitor, also inhibits calcium-activated protease activity. This raises the possibility that ritonavir might also exert cytoprotective effects in cells after diverse types of insults associated with increased intracellular Ca^{2+} where calpain activation has been shown to occur and cell survival could be increased by treatment with calpain inhibitors. For example, calpain inhibitors have already been shown to protect auditory sensory cells from hypoxia and neurotrophin withdrawal-induced apoptosis [22]. Other studies have exposed cytoprotective properties of calpain inhibitors in models of tissue damage caused by ischemic stroke [23].

Concentrations of ritonavir used in this study are readily achievable with oral dosing regimens in humans. Compounds such as PD150606, a calpain inhibitor, shown to decrease hypoxic/hypoglycemic and excitotoxin-mediated neuronal injury *in vitro*, have K_i constants of 0.21 to 0.37 μM [24]. While the K_i of ritonavir is an order of magnitude higher, plasma concentrations of ritonavir used for anti-retroviral therapy are well within range of the K_i. Human subjects administered ritonavir orally at doses of 600 mg twice per day achieved peak plasma concentrations ranging from 18.6 to 46 μM and trough concentrations from 10.4 to 17.5 μM [25].

The cytoprotective effects of ritonavir in this model system should prompt the investigation of its *in vivo* therapeutic efficacy in animal models. Many *in vivo* animal models of cerebral ischemia, shock trauma, and

neurodegenerative disorders are suitable for further investigation of its effects as a neuronal protective agent. These results point to promising new indications for ritonavir or its analogues as cytoprotective agents.

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