Efficacy of Novel Calpain Inhibitors in Preventing Renal Cell Death

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ABSTRACT
Inhibitors of calpains, calcium-activated neutral proteases, protect against cell death produced by anoxia and a variety of toxicants both in vitro and in vivo. The problems with known calpain inhibitors are a lack of specificity, low membrane permeability, and/or low potency. The goal of this study was to determine the effects of seven novel dipeptide and tripeptide calpain inhibitors on calpain activity and antimycin A-induced cell death in rat proximal tubule (RPT) suspensions. We chose the compounds based on their inhibitory constants for \( \mu \)-versus \( m \)-calpain, specificity of the inhibitors for calpain, and membrane permeability. Only three of the compounds inhibited calpain in RPT and were cytoprotective (Z-Leu-Phe-COOh, Z-Leu-Abu-CONH-CH\(_2\)-OH-Phe, and Z-Leu-Phe-CONH-Et). Interestingly, Z-Leu-Phe-COOEt, Z-Leu-Abu-CONH-CH\(_2\)-2-quinolinyl were neither cytoprotective actions of some compounds.

Calcium-activated neutral cysteine proteases (calpains) are present as two major and ubiquitous isoforms (Saido et al., 1994; Sorimachi et al., 1997). \( \mu \)-Calpain (calpain I) is activated and undergoes autolysis in vitro in the presence of micromolar concentrations of Ca\(^{2+}\) and \( m \)-calpain (calpain II) is activated by millimolar concentrations of Ca\(^{2+}\). Both isoforms consist of a large 80-kDa subunit and a small 30-kDa regulatory subunit. Each subunit contains a Ca\(^{2+}\)-binding domain (domain IV) near the C-terminus, whereas the catalytic site is in domain II of the 80-kDa subunit.

Calpains are thought to play a role in numerous physiological and pathological events because cytoskeletal, membrane, and regulatory proteins are intracellular substrates (Saido et al., 1994). For example, they are a mediator of physiological processes by their modification of signal transduction components (Suzuki and Ohno, 1990, Suzuki et al., 1992) and regulation of the cell cycle (Schollmeyer, 1988). Calpains also contribute to cell injury and death. For example, increased calpain activity and cytoprotection by calpain inhibitors have been observed during hypoxia in rat renal proximal tubules (Edelstein et al., 1996), cerebrocortical neurons (Wang et al., 1996), and in rat hepatocytes subjected to anoxia (Bronk and Gores, 1993).

Previous studies in our laboratory have shown that calpain inhibitors 1 (N-acetyl-Leu-Leu-norleucinal) and/or 2 (N-acetyl-Leu-Leu-methioninal) block cytosolic and membrane-associated calpain activity and cell death produced by the mitochondrial inhibitor antimycin A (Waters et al., 1997). These calpain inhibitors were effective also in blocking cell death produced by a diverse group of toxicants that included an alkylating quinone (bromohydroquinone), an oxidant (\( t \)-butylhydroperoxide), and a toxicant that forms a reactive electrophile (tetrafluoroethyl-L-cysteine) (Waters et al., 1997). In contrast to the active site-directed irreversible peptide calpain inhibitors (calpain inhibi-
tor 1 and 2), a calpain inhibitor [3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid, PD150606] has been described that inhibits calpains by blocking the binding of Ca²⁺ to the Ca²⁺-binding domain of calpains (Wang et al., 1996). PD150606 also blocked cell death resulting from exposure to the toxicants tetrafluorothyl-L-cysteine, bromohydroquinone, t-butylhydroperoxide, antimony A, and ionomycin (Waters et al., 1997).

Because calpain activity increases and calpain inhibitors are cytoprotective during cell injury and death, calpains appear to play a major role in the pathological events leading to cell injury and death.

A number of synthetic compounds have been designed to inhibit calpain activity. These include transition-state inhibitors, irreversible inhibitors, calmodulin antagonists, and polyamines. Numerous peptide aldehydes and ketones are transition-state inhibitors, including calpain inhibitors 1 and 2. Transition state inhibitors can block both serine and cysteine proteases and have an electronegative functional group next to the scissile peptide carbonyl group of the substrate. Transition state calpain inhibitors form a tetrahedral hemiketal, hemiacetal, or hemithioketal enzyme-inhibitor complex with the active site serine of serine proteases or the cysteine residue of μ-calpain and/or m-calpain (Li et al., 1993).

Recently, the chemical synthesis and characterization of a series of new peptide α-keto amide inhibitors of calpains have been reported (Li et al., 1993, 1996). These studies identified several compounds that were either more potent than other peptide calpain inhibitors or were more selective for μ- or m-calpain. The inhibitory constants of a number of these compounds to purified μ-calpain and m-calpain are illustrated in Table 1. Compound 1 is 22 to 38 times more potent for m-calpain than Compound 2 is 80 times more selective for several compounds that were either more potent than other calpains and/or m-calpain (Li et al., 1993).

TABLE 1

<table>
<thead>
<tr>
<th>Number</th>
<th>Compound</th>
<th>Kᵢ (μM)</th>
<th>Calpain I</th>
<th>Calpain II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Z-Leu-Phe-COOH</td>
<td>0.0085</td>
<td>0.0057</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Z-Leu-Abu-CONH-CH₂-COOH-Ph</td>
<td>1.1</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Z-Leu-Abu-CONH-CH₂-morpholine (AK295)</td>
<td>0.042</td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Z-Leu-Phe-CONH-OEt</td>
<td>0.2</td>
<td>0.039</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Z-Leu-Abu-CONH-CH₂-COOH-C₆F₅</td>
<td>0.05</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Z-Leu-Phe-COOEt</td>
<td>1.8</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Z-Leu-Abu-CONH-CH₂-2-quinoiethyl</td>
<td>0.13</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N-Acetyl-Leu-Leu-norleucinal (CI 1)</td>
<td>0.19</td>
<td>0.22</td>
<td></td>
</tr>
</tbody>
</table>

clostrasto-lactacycin β-lactone (β-LAC), and MG-132 were obtained from Calbiochem (La Jolla, CA). All other chemicals or reagents were purchased from Sigma Chemical Co. (St. Louis, MO). All glassware was silanized and autoclaved before use. All media and buffers were sterilized by filtering before use.

**Preparation of RPT.** Rabbit RPT were isolated and purified from female New Zealand White rabbits (Myrtle’s Rattery, Thompson Station, TN) by the method of Rodeheaver et al. (1990) and Groves and Schnellmann (1996) and suspended in incubation buffer containing (mM): alanine, 1; dextrose, 5; heptanoate, 2; lactate, 4; malate, 5; NaCl, 115; NaHCO₃, 15; KCl, 5; NaH₂PO₄, 2; MgSO₄, 1; CaCl₂, 1; and HEPES, 10; (pH 7.4, 295 mOsM/kg). Animal studies were conducted with appropriate IACUC review and approval. RPT suspensions were preincubated for 15 min before any experimental manipulations.

**Calpain Assays.** Calpain activity was determined in intact RPT by measuring the release of the fluorescent product 7-amido-4-methyl coumarin (AMC) from the membrane permeant calpain substrate SLLVY-AMC (Sasaki et al., 1984; Wang et al., 1986; Waters et al., 1997). Briefly, RPT suspensions (0.25 mg of protein/ml of Krebs’ buffer) were incubated with the calpain inhibitors or diluent for 30 min in 24-well plates (Falcon) on an orbital shaker placed in a 37°C incubator. The membrane permeant calpain substrate Suc-Leu-Leu-Val-Tyr-AMC (0.05 mM) was added and fluorescence monitored each min (360 nm ex.; 430 nm em.) in a CytoFluor 2350 Fluorescence Plate Reader (Perceptive Biosystems, Bedford, MA). Calpain activity was determined by measuring the linear change in fluorescence between 7 and 17 min.

Calpain activity was determined also in RPT homogenates using a modification of the method of Edelstein et al. (1995). An aliquot of RPT was removed after incubation with inhibitor or diluent, centrifuged at 1000g for 2 min and the supernatant aspirated. The pellet was resuspended in imidazole buffer (63 mM imidazole, 10 mM 2-mercaptoethanol, 1 mM EDTA, and 10 mM EGTA, pH 7.3) and incubated in the presence of digitonin (10 mg/ml) for 10 min at 37°C to permeabilize the plasma membrane (Gores et al., 1989). An aliquot was removed and total calpain activity was determined. In 24-well plates, 0.25 ml of cell suspension was preincubated in imidazole buffer in the presence and absence of 3 mM CaCl₂ for 5 min on an orbital shaker placed in a 37°C incubator. The samples incubated in the presence of CaCl₂ were incubated in an imidazole-HCl buffer without EDTA and EGTA. Total volume in each well was 1 ml. Following preincubation, 50 μM SLLVY-AMC was added and fluorescence determined under linear conditions at 10, 20, and 30 min following substrate addition in the CytoFluor 2350. An AMC standard curve was included in each experiment and calpain activity determined as the time-dependent difference between the calcium-dependent fluorescence and the calcium-independent fluorescence. Calpain activity was normalized to cellular protein using the method of Lowry et al. (1951).

**Cell Death Assay.** RPT suspensions (1 mg of protein/ml) were incubated in Erlenmeyer flasks under air/CO₂ (95%/5%) at 37°C in a shaking water bath. After 60 min of exposure to the calpain inhibitors and 30 min of exposure to 1μM antimycin A, RPT cell death was determined. RPT were separated from the surrounding buffer by rapid centrifugation through a layer of dibutylphthalate:diocetylphthalate (2:1). Release of lactate dehydrogenase (LDH) into the medium was used as a marker of cell death as previously described (Moran and Schnellmann, 1996). Percent protection is calculated using the formula 100 – 100(%LDH release in inhibitor-treated tubes – %LDH release in control tubes)/(%LDH release in anti-mycin A-treated tubes – %LDH release in control tubes)).

**Statistics.** The data are presented as means ± S.E. RPT suspensions isolated from one rabbit represented a separate experiment (n of 1). Data were analyzed by ANOVA and multiple means compared using Student-Newman-Keuls’ post hoc examination of multiple groups with P < .05.
To determine whether the calpain inhibitors decrease rabbit RPT basal calpain activity in a time-dependent manner, calpain activity was examined in the presence of compounds 1, 3, 4, and calpain inhibitor 1 (1 mM). With each compound, calpain activity decreased after 30 min of exposure and did not decrease further after 60 or 120 min of exposure (Fig. 1). Because maximal inhibition of calpain activity occurred after 30 min, we exposed RPT to the complete series of calpain inhibitors (1 mM) for 30 min and determined their efficacy in inhibiting calpain activity. Figure 2 illustrates that compounds 1, 2, and 4 decreased calpain activity 40 to 65% with calpain inhibitor 1 decreasing calpain activity 70%. In contrast, several calpain inhibitors were ineffective (compounds 3, 5, 6, and 7). In addition, no correlation was obtained between the inhibitory constants of these inhibitors to μ- or m-calpain and inhibition of total cellular calpain activity (Fig. 2). These results show that although all of these compounds are inhibitors of purified calpains, they exhibit marked differences in their ability to inhibit calpain in a cellular system.

The observation that none of the calpain inhibitors completely inhibited calpain activity raised the possibility that the hydrolysis of SLLVY-AMC in intact RPT may be mediated by other proteases. To verify that SLLVY-AMC was not being cleaved by serine proteases, proteasomes, or caspases, the compounds TLCK (serine protease inhibitor), DCI (serine protease inhibitor), MG132 (reversible proteasome inhibitor), β-LAC (irreversible proteasome inhibitor), and Z-VAD-FMK (pan caspase inhibitor) were tested for their ability to inhibit SLLVY-AMC hydrolysis. The concentrations of the inhibitors used were either the maximal nontoxic concentrations in RPT (TLCK, DCI) (Yang and Schnellmann, 1996) or have been shown to inhibit their respective enzymes in a number of cellular systems (Fearnhead et al., 1995; Zhu et al., 1995; Wiertz et al., 1996; Dick et al., 1996). None of the inhibitors had any effect on SLLVY-AMC hydrolysis (Fig. 3). These results suggest that SLLVY-AMC hydrolysis is not due to serine proteases, proteasomes, or caspases. We have previously shown that the cysteine protease inhibitors, E64d and leupeptin, and the aspartic acid protease inhibitor pepstatin A do not inhibit SLLVY-AMC hydrolysis in RPT (Waters et al., 1997).
itors in the whole cell calpain assay) and compounds 5 and 6 (ineffective calpain inhibitors in the whole cell calpain assay) were tested in a calpain assay using RPT homogenates and Ca\(^{2+}\)-dependent SLLVY-AMC hydrolysis. Qualitatively, the results were similar to those observed in Fig. 2 (Fig. 4). However, the degree of calpain inhibition produced by calpain inhibitor 1 and compound 1 was less than that observed with the intact RPT calpain assay.

To determine whether the calpain inhibitors were cytoprotective to RPT exposed to antimycin A, a concentration-response experiment was conducted using the compounds 1, 3, and 4. These compounds were chosen based on their effectiveness (compounds 1 and 4) or lack of effectiveness (compound 3) in inhibiting calpain activity in the intact RPT calpain assay. A 30-min preincubation period with compound 4 produced approximately 40% cytoprotection at 0.1 mM and was not more effective at higher concentrations (Fig. 5). Compound 1 produced approximately 30% cytoprotection at 0.1 mM and was more cytoprotective at higher concentrations (70% at 1 mM). Compound 3 was ineffective at all concentrations. Calpain inhibitor 1 was 55% cytoprotective at 1 mM (data not shown).

The degree of cytoprotection was determined also for the whole series of calpain inhibitors using equimolar concentrations (0.3 mM) and a 30-min pretreatment period. With the exception of compound 3, all compounds were greater than 60% cytoprotective against antimycin A-induced cell death (Fig. 6).

**Discussion**

Calpains mediate cell injury and death produced by diverse insults in a variety of models (Bronk and Gores, 1993; Saido et al., 1994; Wang and Yuen, 1994; Bartus et al., 1994; Edelstein et al., 1995; Wang et al., 1996; Waters et al., 1997). However, identification of the specific roles of calpains in cell injury and death has been difficult due to the lack of potent and specific calpain inhibitors, the presence of multiple calpain isozymes, and unidentified key intracellular substrates. The current study focuses on the potency and efficacy of a series of new calpain inhibitors.

The calpain inhibitors tested had varying degrees of effectiveness in inhibiting calpain activity in a cellular system. Although all of the compounds tested have \(K_i\) values under 2 \(\mu M\) using purified calpains, only compounds 1, 2, and 4 (40–65%) and calpain inhibitor 1 decreased calpain activity (70%). The two most potent inhibitors of purified m-calpain (compounds 1 and 2) were effective in reducing basal calpain activity, and the least potent inhibitors of m-calpain (com-
pounds 6 and 7) were ineffective in reducing basal calpain activity. However, some compounds with high potency to purified m-calpain were ineffective in reducing basal calpain activity in RPT (compounds 3 and 5). A similar lack of correlation was observed between the potency of the calpain inhibitors to inhibit purified μ-calpain and their ability to inhibit calpain activity in RPT. Consequently, the inhibition of calpain activity by these compounds could not be associated with the specific inhibition of μ-calpain or m-calpain.

The inability of some of these compounds to inhibit RPT calpain activity may be due to their limited uptake into RPT. Limited uptake of calpain inhibitors has been noted previously (Li et al., 1993). For example, compound 4 is approximately 5-fold more potent than compound 1 and 3-fold more potent than compound 2 in a platelet permeability assay (Li et al., 1993). In addition, compound 3 is 10-fold more permeable than calpain inhibitor 1 in hypoxic hippocampal slices (Bartus et al., 1993). However, with the exception of compound 3, it is unlikely that the calpain inhibitors were not transported into RPT because all the remaining compounds were cytotoxic (see below).

Alternatively, the inability of the calpain inhibitors to inhibit calpain activity may be due to the lack of specificity of the cellular calpain assay. None of the compounds were able to completely inhibit cellular hydrolysis of SLLVY-AMC. To ensure that the substrate hydrolysis in the calpain assay was specific for calpains, inhibitors of serine proteases, proteasomes, and caspases were tested in the calpain assay but had no effect in reducing substrate hydrolysis. We have shown previously that the cysteine protease inhibitors, E64d and leupeptin, and the aspartic acid protease inhibitor pepstatin A does not inhibit SLLVY-AMC hydrolysis in RPT (Waters et al., 1997). Consequently, the hydrolysis of SLLVY-AMC is not due to many common proteases.

Results similar to those obtained with the cellular calpain assay were obtained with an assay that measured Ca\(^{2+}\)-dependent SLLVY-AMC hydrolysis in RPT homogenates. Thus, the hydrolysis of SLLVY-AMC is due primarily to calpain activity. The fraction of SLLVY-AMC hydrolysis not inhibited by the calpain inhibitors may reflect a calpain pool not readily accessible to the calpain inhibitors or to some other protease(s). With the exception of compound 3, all compounds were greater than 60% cytoprotective against antimycin A-induced cell death in RPT. Compounds 2, 6, and 7 exhibited the greatest degree of cytoprotection. No clear correlation was obtained between the inhibitory constants of μ- or m-calpain and cytoprotection. Compound 3 did not inhibit basal calpain activity nor was it cytoprotective. Lack of cell permeability may be the limiting factor for its effectiveness in RPT.

Compounds 1, 2, and 4 inhibited calpain activity and were also cytoprotective. In contrast, compounds 5, 6, and 7 were cytotoxic without inhibiting basal calpain activity. These results indicate that compounds 5, 6, and 7 may be cytoprotective through the inhibition of a protease(s) other than calpains. The effectiveness of these compounds in inhibiting other types of proteases has received limited attention, but considering their cytotoxic properties warrants further study.

In conclusion, we have tested an α-keto acid, an α-keto ester, and α-keto amides, as well as the aldehyde, calpain inhibitor 1, for both inhibition of basal calpain activity and prevention of antimycin A-induced cell death in RPT. The effectiveness of the inhibitors in inhibiting calpain activity varied markedly and was not associated with their potency in inhibiting purified μ- and m-calpains. In contrast, most of the inhibitors were cytoprotective, suggesting that calpains and other proteases may be involved in RPT cell death.

References
Edelstein CL, Wieder ED, Yaqoob MM, Gengaro PE, Burke TJ, Nemenoff RA and Schreiber 1995. “The fraction of SLLVY-AMC hydrolysis not inhibited by the calpain inhibitors were obtained with an assay that measured Ca\(^{2+}\) uptake.”

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