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Calpain inhibitors exhibit matrix metalloproteinase-2 inhibitory activity

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ABSTRACT

Matrix metalloproteinase (MMP)-2 is a zinc-dependent endopeptidase which, alongside its known extracellular actions, plays fundamental roles in oxidative stress-induced injury to the heart. Intracellular cleavage targets of MMP-2 selectively mediating this injury include the sarcomeric proteins troponin I, myosin light chain-1 and titin; some of these are also targeted by calpains. In myocardial ischemia and reperfusion injury, inhibitors of MMP-2 and some calpain inhibitors were shown to improve the recovery of contractile function. We hypothesized that the protective effects of calpain inhibitors may be due in part to their ability to inhibit MMP-2. Four calpain inhibitors (calpain inhibitor III, ALLM, ALLN, and PD-150606) were tested for their ability to inhibit MMP-2 in comparison to the selective MMP inhibitor ONO-4817. At 100 μM, all calpain inhibitors, except ALLM, showed significant inhibition of MMP-2 gelatinolytic activity. When assessed by the troponin I proteolysis assay, both ALLN and PD-150606, but neither ALLM nor calpain inhibitor III (at 20 μM), significantly inhibited MMP-2 activity. Using a fluorogenic MMP substrate peptide OmniMMP in a kinetic assay the rank order of IC_{50} values against MMP-2 were: PD-150606 < ALLN < calpain inhibitor III <<< ALLM. These experiments show that the calpain inhibitors PD-150606 and ALLN have significant additional pharmacological activity as MMP-2 inhibitors. This suggests that the protective effect of some calpain inhibitors is due in part to their ability to inhibit MMP activity.

1. Introduction

Matrix metalloproteinase (MMP)-2 is a member of the metzincin endopeptidase family that is capable of degrading the components of extracellular matrix. It was recognized, from the first description of their role in amphibian metamorphosis [1], that MMPs play an active role in remodeling the extracellular matrix that accompanies both physiological and pathological processes such as embryogenesis, wound healing, uterine involution, bone resorption, metastasis, arthritis, and heart failure [2]. Although most research has focused on the extracellular role of MMPs in long-term pathophysiological processes, it has more recently been recognized that MMPs also act on non-extracellular matrix substrates both outside [3] and inside [4,5] the cell. MMP-2 is also localized within cells as a result of the inefficient targeting of its signal sequence for the secretory pathway as well as the existence of a splice variant which lacks the signal sequence [6].

Many studies have shown that the activity of MMP-2 is enhanced in several cardiovascular diseases, including myocardial ischemia and reperfusion injury [7–9]. MMP-2 is expressed in the heart at substantial levels and its activity is increased upon oxidative stress injury [7,8,10]. It is well known that oxidative stress is an important mediator of acute myocardial contractile failure which occurs during ischemia-reperfusion injury [11] and involves an overproduction of reactive oxygen/nitrogen species, particularly peroxynitrite [12,13]. Peroxynitrite in turn activates intracellular MMP-2 via covalent modification of a critical cysteine residue in the propeptide domain [14]. Indeed, this intracellular activity of MMP-2 is responsible for the proteolysis of specific sarcomeric and cytoskeletal proteins including troponin I [15], myosin light chain-1 [16], α-actinin [17] and titin [9] resulting in acute myocardial contractile dysfunction.

Calcium overload is an additional mediator of ischemia and reperfusion injury [11]. During the early stages of reperfusion, there is an abrupt increase in intracellular calcium that induces myocardial contractile dysfunction by various mechanisms, one of which is activation of calpains which are calcium-dependent cytosolic proteases. Calpains belong to a family of non-lysosomal calcium-dependent cysteine proteases consisting of several isoenzymes, the best characterized of these being calpain-1 (μ-calpain),

Abbreviations: MMP-2, matrix metalloproteinase-2; TnI, troponin I.

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calpain-2 (m-calpain) and calpain-3. The terms μ-calpain and m-calpain indicate the required calcium concentration (micromolar range for μ-calpain and millimolar range for m-calpain) to facilitate in vitro activity. Calpain-1 and -2 are heterodimers consisting of a large (82 kD) catalytic subunit and a small (28 kD) regulatory subunit. These calpains participate in various cellular processes including cytoskeletal remodeling, signal transduction, cell cycle and death [18,19]. For example, it was suggested that calpain-1 proteolyzes a wide variety of substrates including cardiac troponin I [20] and its activation seems to play an important role in myocardial ischemia and reperfusion injury, in a similar manner to the role played by MMP-2.

There is an apparent overlap in the substrates and/or biological actions of MMP-2 and calpains in various cellular pathways [21]. It may be that MMP-2 targets a similar subset of proteins as calpains, or that calpains have been misidentified as the proteases responsible for some intracellular proteolytic activity. Notably, much of the evidence for the substrate specificity of calpains in the heart is derived from experiments using pharmacological inhibitors [22–24]. In this regard, although some evidence supports troponin I as a calpain substrate, myocardial-specific overexpression of calpain-1 in transgenic mice did not alter troponin I levels in the heart [25]. In contrast, troponin I levels were reduced in hearts from transgenic mice with myocardial-specific overexpression of constitutively active MMP-2 [26]. These observations led us to hypothesize that the beneficial effects of calpain inhibitors may also involve direct inhibition of MMP-2 activity. Thus, we investigated the effects of four calpain inhibitors (Calpain inhibitor III (also known as MDL-28170), ALLN, ALLM and PD-150606; Fig. S1) on the activity of MMP-2 in comparison with the selective MMP inhibitor (ONO-4817). We demonstrated that certain calpain inhibitors (calpain inhibitor III, ALLN and PD-150606) are also effective inhibitors of MMP-2 at commonly employed concentrations.

2. Materials and methods

All reagents were of analytical grade and unless otherwise specified were purchased from Sigma–Aldrich (Oakville, ON). The catalytic domain of human recombinant MMP-2 (which lacks the C-terminus hemopexin domain) and OmniMMP® fluorogenic peptide substrate were obtained from Biomol (Plymouth Meeting, PA); calpain inhibitor III, ALLN, ALLM, PD-150606 and calpain-1 from human erythrocytes were from Calbiochem (San Diego, CA). ONO-4817 was a kind gift from Ono Pharmaceutical (Osaka, Japan).

2.1. Gelatin zymography

Measurement of gelatinolytic activity of 72 kDa MMP-2 (from conditioned HT1018 cell culture medium) by zymography was performed as previously described [27] with some modifications. Briefly, 10 μl aliquots of HT1080 medium were electrophoresed on an 8% polyacrylamide gel containing 2 mg/ml gelatin for 90 min (125 V, ambient temperature). After washing with Triton X-100 (2.5% v/v, 3 × 20 min), the gels were cut into individual strips and they were then incubated separately, overnight, at 37 °C in 10 ml zymography buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.6) in the absence or presence of different calpain inhibitors: calpain inhibitor III, ALLN, ALLM or PD-150606 (each at 100 μM). Further gel strips were incubated in parallel with the selective MMP inhibitor ONO-4817. This compound has a K_i in the nanomolar range for MMP-2 and shows negligible inhibitory activity up to 100 μM against several other proteases of different classes [28]. Gels were stained with 0.05% Coomassie blue and following destaining, gelatinolytic activities were observed as transparent bands against the blue-stained background.

2.2. Troponin I proteolysis assay

Two micrograms of recombinant human troponin I (TnI, a kind gift from Dr. James Potter, University of Miami) were incubated with either MMP-2 catalytic domain (10 ng) in 50 mM Tris–HCl buffer containing (5 mM $CaCl_2$, 150 mM NaCl, pH 7.4) or calpain-1 (33 ng) in 20 mM Tris–HCl containing (0.15 mM $CaCl_2$, 25 mM NaCl, 10 mM dithiothreitol, pH 7.5) at 37 °C for 30 or 60 min. In additional experiments MMP-2 or calpain-1 were incubated with the indicated calpain inhibitors (20 or 100 μM) or the MMP inhibitor ONO-4817 (20 or 100 μM) for 15 min at 37 °C before addition of TnI. The reaction mixtures (total volume 40 μL) were analyzed by 12% SDS–PAGE under reducing conditions and visualized by the Coomassie blue staining method. Band densities were measured using a densitometer (GS-800, BioRad) and ImageJ software. MMP-2 activity was expressed as the ratio of cleaved to full length TnI, calculated for each lane, and presented as a percentage change from the DMSO vehicle control.

2.3. OmniMMP kinetic assay

The hydrolysis of OmniMMP® fluorogenic substrate (25 μM, prepared in 0.4% v/v DMSO) by MMP-2 catalytic domain (0.5 nM in assay buffer consisting of 50 mM Tris, 10 mM $CaCl_2$, 0.05% Brij-35, 10 μM $ZnSO_4$, pH 7.6) was measured at 37 °C in the absence or presence of the calpain inhibitors or ONO-4817, using a plate reader-based protocol [27]. Assays were made in a total volume of 120 μl in black polystyrene half-area plates (Corning, NY) and contained MMP-2 (60 μl in assay buffer) and substrate (60 μl) or DMSO vehicle. Fluorescence associated with the (7-meth-oxycoumarin-4-yl)acetyl-tagged cleavage product was measured in five replicate wells every 30 s for 1 h (λ_{ex} 328 nm, λ_{em} 393 nm) in a SPECTRAmax Gemini XPS (Molecular Devices, Sunnyvale, CA) fluorescence plate reader. The initial rate of product formation in each well was determined by linear regression of fluorescence-time data ($r^2 > 0.98$) using SOFTmax Pro 4.8 software (Molecular Devices, Sunnyvale, CA). Enzymatic rates in the presence of inhibitors were expressed as a percentage of rates in the vehicle control. Inhibitor concentrations required to produce 50% inhibition of enzyme activity (IC_{50}) were determined by fitting a three-parameter logistic equation to initial rate data, using the non-linear regression facility of GraphPad Prism (version 5.0; GraphPad software, San Diego, CA).

3. Results

3.1. Calpain inhibitors, but not ONO-4817, inhibit calpain-1-induced TnI cleavage

Fig. 1 shows that TnI was completely hydrolyzed to a ~15 kD cleavage product when incubated with calpain-1 (37 °C, 60 min). As expected, all calpain inhibitors (100 μM) tested in this study abolished calpain-1-induced TnI proteolysis. In contrast, the same concentration of the selective MMP inhibitor ONO-4817 did not show any calpain inhibitory activity, verifying its selectivity towards MMPs.

3.2. Calpain inhibitors decrease the gelatinolytic activity of MMP-2

Initially we screened the MMP-2 inhibitory effects of calpain inhibitors, as well as the selective MMP inhibitor ONO-4817, using gelatin zymography. Used as a positive control, ONO-4817 (100 μM) showed complete inhibition of 72 kDa MMP-2 gelatinolytic activity (Fig. 2). MMP-2 activity was also significantly reduced by several of the calpain inhibitors (100 μM, each) including

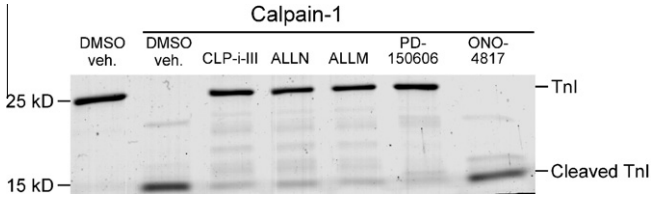

Fig. 1. Calpain inhibitors, but not the selective MMP inhibitor ONO-4817, inhibit calpain-1 activity. Coomassie blue stained SDS–PAGE gel following incubation of TnI with calpain-1 at 37 °C for 60 min. TnI is completely degraded by calpain-1 to a ~15 kD product. DMSO was used as a vehicle control. Calpain inhibitors (calpain inhibitor III, ALLN, ALLM or PD-150606, 100 μM each) efficiently inhibit calpain-1 activity whereas the selective MMP inhibitor ONO-4817 (100 μM), does not. CLP-i-III, calpain inhibitor III. Position of molecular weight markers is shown on the left.

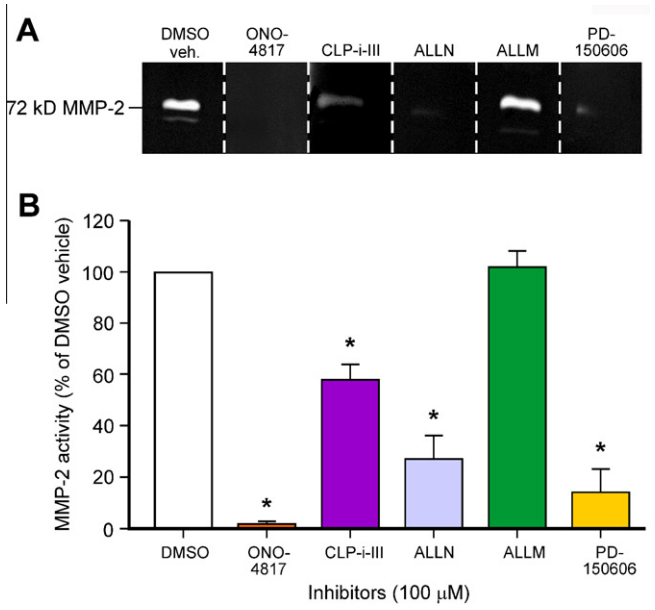

Fig. 2. Calpain inhibitors inhibit MMP-2 gelatinolytic activity. (A) Representative gelatin zymograms of 72 kD MMP-2 incubated at 37 °C overnight with calpain inhibitors (calpain inhibitor III, ALLN, ALLM or PD-150606, 100 μM each) or selective MMP inhibitor (ONO-4817, 100 μM). DMSO was included as a vehicle control. (B) Quantitative analysis of MMP-2 gelatinolytic activity (% of DMSO control) in the presence of calpain inhibitors or selective MMP inhibitor ONO-4817. n=3, $^{*}p < 0.05$, one-way ANOVA followed by Dunnett's multiple comparison post hoc test.

calpain inhibitor III (39%), ALLN (67%) or PD-150606 (84%) as compared with DMSO vehicle control (Fig. 2). In marked contrast, ALLM did not inhibit MMP-2 gelatinolytic activity.

3.3. Inhibition of MMP-2 catalytic activity by calpain inhibitors

As we have previously shown, troponin I is highly susceptible to proteolysis by MMP-2 [15]. MMP-2-induced TnI proteolysis was used as another means to further test the effects of calpain inhibitors on MMP-2 catalytic activity. The catalytic domain of MMP-2 hydrolyzed TnI to yield a major ~22 kD cleavage fragment when incubated for only 20 min at 37 °C (Fig. 3A). The ratio of cleaved to full length TnI was used as a measure of MMP-2 catalytic activity and was considered 100% in the absence of any inhibitors (DMSO vehicle control). All inhibitors were tested at 20 μM. ONO-4817 significantly inhibited MMP-2 activity by 97% in comparison to DMSO vehicle control (Fig. 3B). MMP-2 catalytic activity was significantly reduced by ALLN (43%) and PD-150606 (80%). In

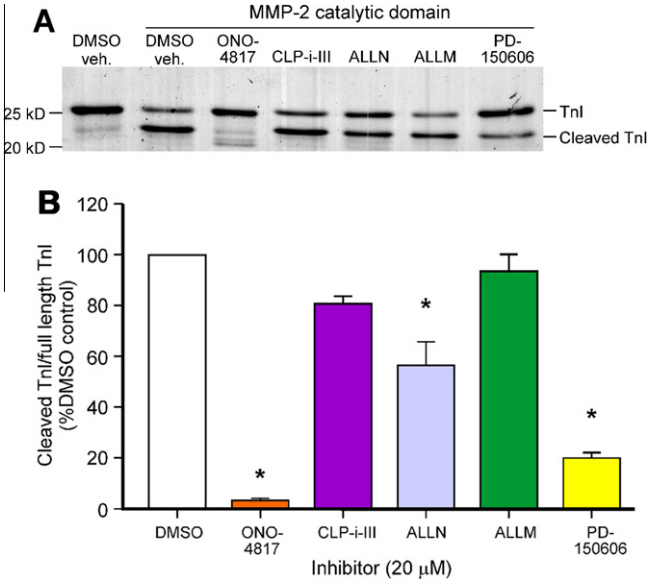

Fig. 3. Some calpain inhibitors exhibit MMP-2 inhibitory activity as assessed by TnI proteolysis assay. (A) Coomassie blue stained SDS–PAGE shows that the MMP-2 catalytic domain efficiently cleaves TnI in vitro. In contrast to calpain-1, MMP-2 cleavage results in a ~22 kD product. This proteolysis of TnI is significantly inhibited by the calpain inhibitors ALLN and PD-150606 but not by ALLM nor calpain inhibitor III. ONO-4817 (selective MMP inhibitor) was used as positive control. DMSO was included as a vehicle control. (B) Quantitative analysis of MMP-2 induced cleavage of TnI expressed as the ratio of cleaved TnI: full length TnI (as a % of DMSO control) in the presence of calpain inhibitors (calpain inhibitor III, ALLN, ALLM or PD-150606) or the selective MMP inhibitor (ONO-4817). All inhibitors were tested at 20 μM. n = 3, $^{*}p < 0.05$, one-way ANOVA followed by Dunnett's multiple comparison post hoc test. CLP-i-III, calpain inhibitor III. Position of molecular weight markers is shown on the left.

contrast, neither calpain inhibitor III nor ALLM significantly inhibited MMP-2 activity.

3.4. Determination of IC_{50} values for calpain and MMP inhibitors on MMP-2 activity using the OmniMMP assay

We then analyzed the MMP-2 inhibitory effects of calpain inhibitors, versus the selective MMP inhibitor ONO-4817, using the OmniMMP kinetic assay. ONO-4817 or the calpain inhibitors blocked MMP-2 activity in a concentration-dependent manner with the following rank order of potency: ONO-4817 >>> PD-150606 > ALLN > calpain inhibitor III >>> ALLM (Fig. 4). The IC_{50} values of ONO-4817, PD-150606 and ALLN were 0.25 nM, 9.3 μM and 21.9 μM respectively. The IC_{50} value of calpain inhibitor III was estimated to lie in the range 50–100 μM whereas ALLM was devoid of MMP-2 inhibitory activity.

4. Discussion

This study demonstrates for the first time that certain commonly utilized calpain inhibitors (calpain inhibitor III, ALLN and PD-150606) possess significant pharmacological activity as inhibitors of MMP-2 enzymatic activity. In fact, two calpain inhibitors (PD-150606 and ALLN) have similar potencies as MMP-2 inhibitors compared to those of some widely used MMP inhibitors such as doxycycline, minocycline and o-phenanthroline [27]. These data suggest that the biological effects of some calpain inhibitors may result from their inhibition of MMP-2 activity.

Among the calpain inhibitors tested in the current study, PD-150606 was the most potent MMP-2 inhibitor. In its action as a calpain inhibitor, PD-150606 does not bind to the catalytic thiol of

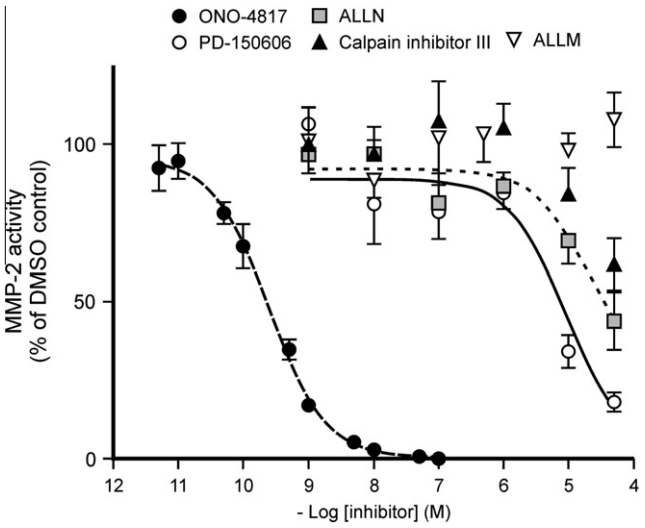

Fig. 4. Concentration-dependent inhibitory effects of calpain inhibitors compared to the selective MMP inhibitor (ONO-4817) on MMP-2 activity as measured by a fluorogenic substrate (OmniMMP) kinetic assay. Calpain or MMP inhibitors were incubated with 0.5 nM MMP-2 catalytic domain and 25 M OmniMMP fluorogenic substrate at 37 °C in 50 mM Tris buffer, pH 7.6, containing 10 mM $CaCl_2$ and 10 μM $ZnSO_4$ and the change in fluorescence was monitored. Sigmoidal curves were fitted with a three parameter logistic equation. Data represent means ± SEM of five replicate determinations.

calpain as do other calpain inhibitors, but rather targets the calcium binding domain of the small regulatory subunit [29]. It is worth noting that MMP-2 has a calcium binding subdomain within its catalytic domain and that calcium ions stabilize MMP-2 structure [30]. The potential interaction of PD-150606 with the calcium binding site of MMP-2 provides a possible explanation for its marked inhibitory effect on MMP-2 activity. On the other hand carboxylate and thiol groups, both present in the PD-150606 structure (Fig. S1), are reported to bind zinc ions [31]. Thus, PD-150606 may also chelate the catalytic zinc which is crucial for MMP-2 activity. Although hydroxamate compounds (e.g. ONO-4817, Fig. S1) are much stronger MMP inhibitors than carboxylate compounds, it was suggested that at lowered pH (to values seen during inflammatory or ischemic conditions) protonated carboxylates may become more potent MMP inhibitors [31]. Aside from chelation of the catalytic zinc, recently designed MMP inhibitors bind the S_1 pocket, a critical determinant of MMP inhibitor selectivity, and interact with amino acid residues (e.g. T, Y, F, and M) in the specificity loop, thereby interfering with enzyme activity [32]. PD-150606 contains an electron-rich aromatic ring that establishes hydrophobic stacking interactions with similar residues (Y, F and M) within the hydrophobic pocket on domain VI of the calpain small subunit [29]. Similar interactions may result in the observed MMP-2 inhibitory effect of PD-150606.

The cardio- [33], renal- [34] or neuro- [35,36] protective effects of PD-150606 against ischemia and reperfusion injury were previously shown when the compound was used in a concentration range of 25–100 μM. Although the IC_{50} of PD-150606 versus calpain-1 is around 0.2 μM [35], the protective effects were observed within a range of concentrations (25–100 μM) that markedly exceeded its IC_{50} versus MMP-2. It is now well established that MMP-2 plays an important role in mediating ischemia-reperfusion injury in the heart [7–9,15,16], kidney [37] and brain [38]. The protective effect of ALLN in various models of ischemia and reperfusion injury has been shown to occur at concentrations (20–100 μM) that here are shown to inhibit MMP-2 activity [39–41]. Interestingly, a recent study shows that although both ALLM and ALLN at 25 μM inhibit calpain activity in vitro to a similar extent, only ALLN protected against myocardial contractile dysfunction in rat hearts subjected to ischemia and reperfusion injury [41]. We previously observed that 50 μM ONO-4817, a selective MMP inhibitor which does not interfere with calpain activity up to 100 μM, functionally protected hearts against myocardial ischemia and reperfusion injury [9], and similar results have been obtained with other MMP inhibitors including o-phenanthroline, doxycycline or a neutralizing MMP-2 antibody [7]. Together, these observations strongly suggest that the protective effect of ALLN in myocardial ischemia and reperfusion injury [41] was most likely due to its ability to inhibit MMP-2 activity.

In conclusion, the MMP-2 inhibitory effect demonstrated by some calpain inhibitors shown here may reveal an alternative strategy for designing more selective MMP inhibitors. Furthermore, our work suggests that the interpretation of pharmacological results obtained using some calpain inhibitors must be reconsidered to include effects resulting from their ability to inhibit MMP-2 activity, which is found in almost every cell type.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.005.

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